5-Aminosalicylic acid prevents oxidant mediated damage of glyceraldehyde-3-phosphate dehydrogenase in colon epithelial cells

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Abstract

Background—Reactive oxygen and nitrogen derived species produced by activated neutrophils have been implicated in the damage of mucosal proteins including the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the active inflammatory lesion in patients with inflammatory bowel disease (IBD). This study investigated the efficacy of currently used IBD therapeutics to prevent injury mediated by reactive oxygen and nitrogen derived species.

Methods—GAPDH activity of human colon epithelial cells was used as a sensitive indicator of injury produced by reactive oxygen and nitrogen derived species. HCT116 cells (10^6/ml phosphate buffered saline; 37°C) were incubated in the presence of 5-aminosalicylic acid (5-ASA), 6-mercaptopurine, methylprednisolone, or metronidazole before exposure to H₂O₂, HOCl, or NO in vitro. HCT116 cell GAPDH enzyme activity was determined by standard procedures. Cell free reactions between 5-ASA and HOCl were analysed by spectrophotometry and fluorimetry to characterise the mechanism of oxidant scavenging.

Results—GAPDH activity of HCT116 cells was inhibited by the oxidants tested: the concentration that produced 50% inhibition (IC₅₀) was 44.5 (2.1) µM for HOCl, 379.8 (21.3) µM for H₂O₂, and 685.8 (103.8) µM for NO (means (SEM)). 5-ASA was the only therapeutic compound tested to show efficacy (p<0.05) against HOCl mediated inhibition of enzyme activity; however, it was ineffective against H₂O₂ and NO mediated inhibition of GAPDH. Methylprednisolone, metronidazole, and the thiol-containing 6-mercaptopurine were ineffective against all oxidants. Studies at ratios of HOCl:5-ASA achievable in the mucosa showed direct scavenging to be the mechanism of protection of GAPDH activity. Mixing 5-ASA and HOCl before addition to the cells resulted in significantly greater protection of GAPDH activity than when HOCl was added to cells preincubated with 5-ASA. The addition of 5-ASA after HOCl exposure did not restore GAPDH activity.

Conclusions—Therapies based on 5-ASA may play a direct role in scavenging the potent neutrophil oxidant HOCl, thereby protecting mucosal GAPDH from oxida-
oxidants such as HOCl, chloramines, NO, and H$_2$O$_2$ react rapidly with thiols and may also contribute to the decrease in GSH levels found in the inflamed mucosa of IBD patients and in dextran sodium sulphate induced colitis in mice. The thiol group in 6-mercaptopurine may also offer protection against cellular oxidative injury by providing an alternative thiol target for inflammatory oxidants.

Oxidation of the active site thiol of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and subsequent inhibition of enzyme activity were consistent findings in colon epithelial cells purified from the inflamed mucosa of IBD patients and in dextran sodium sulphate induced colitis in mice. The thiol group in 6-mercaptopurine may also offer protection against cellular oxidative injury by providing an alternative thiol target for inflammatory oxidants.

In this paper we compare the efficacy of a range of currently used IBD therapeutic compounds (5-ASA, 6-mercaptopurine, methylprednisolone, and metronidazole) in the prevention of GAPDH inhibition caused by in vitro exposure to HOCl, NO, or H$_2$O$_2$.

**Materials and methods**

**TREATMENT OF HCT116 CELLS WITH THERAPEUTIC COMPOUNDS**

HCT116 cells were maintained in RPMI 1640 containing 10% fetal calf serum. Cell suspensions were washed into Hanks balanced salt solution (HBSS), pH 7.4, without phenol red or glucose and preincubated in a shaking water bath for 30 minutes at 37°C with 5-ASA (Fluka Chemie, Buchs, Switzerland), 6-mercaptopurine (Sigma, St Louis, Missouri, USA), methylprednisolone (Solu-Medrol; Upjohn Pty Ltd, Rydalmere, NSW, Australia), or metronidazole (Flagyl; May and Baker Pty Ltd, Sydney, NSW, Australia). The therapeutic compounds were added at a final concentration of 250 µM for the HOCl treated samples and at 2 mM for the H$_2$O$_2$ and NO treated samples such that the final molarity of the therapeutic compound was twice the maximum concentration of oxidant to be used.

Cell preparations were then exposed to various concentrations of HOCl (Merck, Melbourne, Vic, Australia), H$_2$O$_2$ (BDH Chemicals, Melbourne, Vic, Australia), or NO (diethylamine NONOate; Cayman Chemical Company, Ann Arbor, Michigan, USA). Stock solutions of oxidants in HBSS, pH 7.4, or phosphate buffered saline, pH 8.5, for diethyleamine NONOate, were added to 2 ml cell preparations in 100 µl aliquots. Final concentrations were 0, 31.25, and 125 µM HOCl and 0, 250, and 1000 µM for H$_2$O$_2$ and NO respectively. The range of oxidant concentrations was selected to produce about 20–90% inhibition of GAPDH enzyme activity. After the 30 minute incubation, cell preparations were washed twice in HBSS to remove unreacted oxidant and therapeutic compound, resuspended in 100 mM Tris/HCl with 0.5 mM EDTA, and assayed for GAPDH.

Cell viability, assessed by trypan blue exclusion after
treatment by each oxidant or drug combination, was always 95% or greater.

GAPDH ACTIVITY

GAPDH activity of cell lysates was determined as described previously\textsuperscript{22} and adapted for use in HCT116 cells.\textsuperscript{2} Cell lysates (100 µl) were added to a 1 ml reaction mixture containing 10 mM MgCl\textsubscript{2}, 200 µM NADH, 2 mM ATP, 5 U/l phosphoglycerate kinase in 100 mM Tris/HCl/EDTA, pH 8, and preincubated at 37°C for about 10 minutes. The reaction was initiated by the addition of 10 mM 3-phosphoglycerate. Enzyme activity was determined using a Varian Cary 1 UV/visible spectrophotometer to monitor the oxidation of NADH ($\lambda_{340} = 6.22 \times 10^3$ M\textsuperscript{−1} cm\textsuperscript{−1}) by the reverse reaction of GAPDH coupled with phosphoglycerate kinase.

REACTION OF 5-ASA WITH HOCI

5-ASA (250 µM) in HBSS was allowed to react with HOCI in a cell free system for 15 minutes at 37°C with constant agitation using the ratios of HOCI:5-ASA of 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, and 5:1. After 15 minutes of incubation, the reaction was stopped by the addition of excess taurine (6.25 mM, five times the maximum HOCI concentration) to scavenge any unreacted HOCI. The absorbance profiles of the reaction products of 5-ASA and HOCI were determined by scanning absorbance from 280 to 900 nm (Varian Cary 1 UV/visible spectrophotometer). The reaction of HOCI and 5-ASA was stopped after 15 minutes of incubation by the addition of 6.25 mM taurine. There was no significant absorbance between 280 and 900 nm of either 6.25 mM taurine or a mixture of 6.25 mM taurine and 1.25 mM HOCI (data not shown). The fluorescence of 250 µM 5-ASA in HBSS after the addition of HOCI (final concentration 250 µM) was continuously monitored for 30 minutes (excitation wavelength 340 nm, emission wavelength 500 nm; Hitachi 3000 fluorimeter, fitted with a magnetic stirrer). To analyse this reaction further, the fluorescence emission scans from 220 to 800 nm (excitation wavelength 340 nm) were determined for the reaction products of 5-ASA and HOCI.

STATISTICAL ANALYSIS

Paired Student’s t tests were used to assess the statistical significance of the data. Statistical significance was accepted when $p < 0.05$.

Results

EFFICACY OF THERAPEUTIC AGENTS TOWARD OXIDANT INDUCED INHIBITION OF GAPDH ACTIVITY

The oxidants H\textsubscript{2}O\textsubscript{2}, HOCI, and NO induced concentration dependent inhibition of GAPDH activity, with HOCI being about 10-fold more effective than H\textsubscript{2}O\textsubscript{2} and NO (figs 1 and 2, open circles), consistent with our earlier observations.\textsuperscript{2} The concentrations producing 50% inhibition of GAPDH ($IC_{50}$) were...
44.5 (2.1) µM for HOCl, 379.8 (21.3) µM for H2O2, and 685.8 (103.8) µM for NO (means (SEM)). To determine the relative oxidant scavenging capacity of each of the therapies, identical ratios of oxidant:therapy were used.

5-ASA AND 6-MERCAPTOPURININE
The presence of low concentrations of 5-ASA protected HCT116 cells against HOCl-induced inhibition of GAPDH activity (p<0.05), whereas 5-ASA did not prevent inhibition of GAPDH activity by the myeloperoxidase substrate, H2O2, or the NO synthase product, NO, at comparable drug:oxidant ratios (fig 1A). The addition of 5-ASA after the addition of HOCl was also ineffective. At the same drug:oxidant ratios, the exposure of HCT116 cells to oxidants in the presence of the thiol-containing drug 6-mercaptopurine did not result in significant (p>0.05) protection of GAPDH activity, compared with control untreated cells exposed to the same concentrations of oxidant (fig 1B). Accordingly, there was no significant difference in the IC50 values of GAPDH enzyme activity (not shown), except for 5-ASA/HOCl when the IC50 value increased from 44.5 (2.1) to 536.8 (83.2) µM (p<0.05).

METHYLPREDNISOLONE AND METRONIDAZOLE
Methylprednisolone and metronidazole added before exposure of HCT116 cells (10^6/ml) did not prevent the inhibition of GAPDH activity induced by HOCl, NO, or H2O2 (fig 2). Neither compound caused a significant difference in the IC50 values of GAPDH activity (not shown).

CONCENTRATION DEPENDENCE OF 5-ASA PROTECTION OF CELLULAR GAPDH FROM OXIDANT MEDIATED INHIBITION
To investigate the mechanism of GAPDH protection further, HCT116 cells were incubated with increasing ratios of 5-ASA:HOCl according to the following protocol: 5-ASA alone, 5-ASA after exposure to 125 µM HOCl, 5-ASA before exposure to 125 µM HOCl, or the spent reaction resulting from the premixing of 5-ASA with 125 µM HOCl. Data represent mean (SEM) (n = 3).

400
300
200
100
0
GAPDH enzyme activity (mIU/n06 cells)
0.1
1.0
2.0
3.0
100
80
60
40
20
0
Fluorescence (% max)
0
5:1
5-ASA:HOCI
3:1
2:1
1:1
0:1
0.1
0.25
0.5
1.0
Absorbance
Wavelength (nm)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
5-ASA:HOCI; 37°C. Absorbance traces of 5-ASA:HOCI were as follows: A, 1:0; B, 1:0.25; C, 1:0.5; D, 1:0.75; E, 1:1; F, 1:2; G, 1:3; H, 1:4; I, 1:5. Insert, 5-ASA fluorescence.

REACTION OF 5-ASA WITH HOCl
In a cell free system, reaction of 5-ASA:HOCl ratios up to 1:1 caused no change to the maximum 5-ASA UV-visible absorbance at 330 nm. There was, however, an HOCl dependent increase in absorbance between 350 and 650 nm that resulted in a yellow-brown reaction product, with the greatest increase at 375 nm which intensified up to a ratio of 1:3 (fig 4).
Analysis of 5-ASA fluorescence spectra at the same 5-ASA:HOCl ratios showed that 5-ASA was consumed (fluorescence decay) rapidly on the addition of HOCl (not shown), consistent with a previous report. Coincident with the maximum formation of the yellow-brown reaction product was near complete loss of fluorescence after reaction of 1:3 5-ASA:HOCl (fig 4, insert). The marked decrease in absorption characteristics at >3:1 HOCl:5-ASA ratios indicates that each molecule of 5-ASA is able to scavenge/interact with at least three molecules of HOCl before losing potency and apparently undergoing decomposition reactions (fig 4, traces H and I). HOCl is also able to mediate the decomposition of the structurally similar salicylate and its hydroxylated derivatives with loss of fluorescence.

**Discussion**

5-ASA, the active principle in many standard therapies used in the treatment of IBD, was found to be an effective scavenger of HOCl and prevented oxidation and inhibition of GAPDH in colon epithelial HCT116 cells. Little scavenging effect was observed, however, with the relatively weaker oxidants, NO and H2O2. The other therapeutic compounds used in IBD, methotrexate, chloroquine, and the thiol-containing 6-mercaptopurine, when used at clinically relevant concentrations, did not protect GAPDH activity in HCT116 cells exposed to the oxidants HOCl, NO, and H2O2. The mechanism by which 5-ASA protects appears to involve a direct reaction between 5-ASA and HOCl, as 5-ASA added after HOCl treatment of cells was unable to reverse GAPDH inhibition. Furthermore, premixing of 5-ASA and HOCl before their addition to colon epithelial cells quenched the capacity of HOCl to injure GAPDH.

The central role of PMNs in mucosal injury stems from the characteristic extravasation and infiltration of large numbers of cells into the mucosa. Contemporary studies using PMN depletion strategies and a deeper understanding of the role cytokines play in the recruitment of inflammatory cells from the peripheral circulation confirm this role in tissue injury. PMNs possess a variety of mechanisms capable of initiating such injury including the generation of potent oxidants such as HOCl. We have reported recently that the glycolytic enzyme GAPDH became oxidized and lost activity in the inflamed mucosa of patients with IBD, a result that could be mimicked by exposing cells to oxidants in vitro. While we were unable to source the oxidant(s) responsible for this oxidation in vivo, the efficacy of HOCl compared with other oxidants, the irreversible nature of the oxidation, and the pattern of protein thiol oxidation in patient samples was representative of HOCl/chloramine rather than H2O2/NO. In animal models of inflammation, inflamed tissue contains upwards of 107 PMNs/g tissue, sufficient to yield about 0.6 mM HOCl/h. While defences against O2−/H2O2 and the hydroxyl radical (·OH) are part of normal homeostasis with regeneration of GSH and membrane antioxidants by metabolic reducing equivalents, the primary defence against HOCl would involve irreversible reaction with and depletion of GSH and protein thiols. The inability to readily regenerate thiols oxidised by HOCl would compromise other cellular defences to O2−/H2O2 or ·OH for example, an end result that appears to be present in the actively inflamed mucosa of IBD patients and in mice with dextran sulphate induced colitis.

The mechanisms of action of 5-ASA in the inflamed mucosa in vivo have been difficult to elucidate because 5-ASA inhibits multiple inflammatory processes in vitro ranging from the inhibition of cyclo-oxygenase and lipoxygenase pathways to scavenging of inflammatory cell derived oxidants. While the inhibitory activities of 5-ASA were observed at high concentrations (6–10 mM), the mucosal concentrations of 5-ASA were found to be very much lower (5–400 µM), comparable with the IC50 for the efficacy of 5-ASA scavenging of oxidants, supporting the scavenging mechanism. Scavenging of HOCl was observed here at 5-ASA:HOCl ratios expected in vivo and comparable with those that provided protection of essential thiols on α1-antiprotease inhibitor from oxidation by HOCl. HOCl promoted oxidation of the amino group of 5-ASA to form a short lived chloramine that appeared to spontaneously decompose to 5-nitrososalicylic acid and other species. The formation of a short lived chloramine intermediate may limit oxidative injury, as in previous in vitro studies, we have shown that chloramine T was 8–10-fold less reactive than HOCl in mediating oxidative inhibition of colon epithelial cell GAPDH.

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Direct alternative substrate substitution of the aminosalicylate with decomposition of the myeloperoxidase compound I protein free radical species has been shown to prevent oxidation of chloride anions and formation of HOCl. Thus 5-ASA may scavenge HOCl directly or indirectly, thereby protecting colon epithelial and lamina propria cells from injury.

The role of NO in causing tissue injury in the inflamed IBD mucosa remains unclear. Certainly, the evidence that NO, and peroxynitrite, are reactive toward thiol including GAPDH, the immunohistochemical evidence of increased expression of inducible NO synthase, and the detection of nitrotyrosine in inflamed lesions is compelling. However, the variable efficacy of inducible NO synthase inhibitors as anti-inflammatory agents and our observation that in vivo oxidation of GAPDH in IBD patient resembles in vitro oxidation by HOCl or a chloramine and appears distinct from oxidation by either NO or H2O2 challenges the acceptance of the role of NO in tissue injury. While 5-ASA failed to prevent the loss of GAPDH activity caused by NO, it can protect against NO oxidation of an amino amine in a cell free system. However, when the amino group is in the 4 position or 5-ASA, little NO scavenging was observed. Taken together, these findings suggest that, while considerable evidence implicates NO in the pathogenesis of mucosal inflammation in IBD, the scavenging of NO does not appear to be a significant mode of action of 5-ASA.

The results presented here show clearly that 5-ASA scavenges HOCl and is able to prevent GAPDH oxidation and inhibition in a cellular system at 5-ASA:HOCl ratios achievable in vivo. This supports the case for developing new HOCl scavenging compounds directed at conditions involving HOCl-mediated injury such as IBD and complement existing therapies used to modulate immune responsiveness.

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