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SCAVENGING EFFECT OF 5-AMINOSALICYLIC ACID ON NEUTROPHIL-DERIVED OXIDANTS

POSSIBLE CONTRIBUTION TO THE MECHANISM OF ACTION IN INFLAMMATORY BOWEL DISEASE

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Abstract—Inflammatory phagocytic leukocytes produce superoxide and hydrogen peroxide and secrete myeloperoxidase (MPO) into the extracellular medium. MPO catalyzes the oxidation of Cl^- by H_2O_2 to yield chlorinated oxidants (e.g. HOCl and NH_2Cl), which have been shown to induce pathologic changes in mucosal function. We examined the ability of 5-aminosalicylic acid (5-ASA), a drug used to treat inflammatory bowel disease (IBD), to inhibit oxidation of L-cysteine by NH_2Cl, HOCl and H_2O_2. NH_2Cl and HOCl were especially strong oxidants against L-cysteine. 5-ASA prevented L-cysteine oxidation by NH_2Cl and HOCl; an interaction associated with the formation of characteristic absorption spectra due to the oxidation of 5-ASA was observed. NH_2Cl and HOCl evoked characteristic increases in short-circuit current (I_{sc}), indicative of net electrolyte transport, when added to the serosal side of stripped rat colon mounted in Ussing chambers. Premixing of NH_2Cl with 5-ASA 10 min before addition to the tissue markedly reduced the secretory response to NH_2Cl. In contrast, 5-ASA immediately reduced the response to HOCl. The reduction in the functional response to NH_2Cl and HOCl by 5-ASA may contribute to its mechanism of action in the treatment of the symptoms of IBD.

Activated granulocytes secrete myeloperoxidase (MPO‡) into the extracellular medium where it catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid (HOCl) [1]. HOCl reacts rapidly with primary amines to yield chloramines (RNHCl) [2–5]. Because of the large influx of inflammatory neutrophils into the mucosa of patients with active inflammatory bowel disease (IBD), these stable leukocyte-derived oxidants are thought to induce pathologic changes in mucosal function related to IBD [6–9].

Although 5-aminosalicylic acid (5-ASA) is believed to be the active component of sulfasalazine [10, 11], its mode of action is still unclear. Various activities of 5-ASA have been reported [12]. One possibility is that 5-ASA acts as an antioxidant [13–16]. It also inhibits luminol-dependent chemiluminescence by reacting with HOCl [14]. Further, 5-ASA may attenuate the formation of proinflammatory leukotrienes by inhibiting 5-lipoxygenase [17–19], although the concentration required for this action is well above that determined to be present in the normal colonic mucosal interstitium [20, 21].

Sulfhydryl (SH) groups associated with cellular components are important moieties for maintaining proper protein and membrane structure and function. In addition they can provide protection against endogenous and exogenous oxidants. SH content correlates well with cell viability *in vitro* [22]. L-Cysteine is one of the important constituents of cell membrane proteins and a precursor of the sulfhydryl-containing molecule, glutathione (GSH). Using L-cysteine oxidation as a model of oxidant-induced injury *in vitro*, we assessed the ability of 5-ASA to inhibit oxidation of L-cysteine mediated by the various neutrophil-derived oxidants.

We previously reported that NH_2Cl and HOCl stimulated increases in intestinal electrolyte transport, short-circuit current (I_{sc}) *in vitro* [7, 9]; the response to 50 μM HOCl, but not 50 μM NH_2Cl was inhibited by pretreatment with 5-ASA. In the present study, we assessed the antioxidant properties of 5-ASA relative to its inhibitory effects against HOCl- and NH_2Cl-induced changes in I_{sc}.

MATERIALS AND METHODS

Preparation of oxidants. To 5 mL of 40 mM NH_4Cl in 10 mM phosphate buffer (pH 8.0) was added 4.8 mL of distilled water. An aliquot of NaOCl (200 μL) was added to the above amine solutions at 4° in 4 equal volumes, waiting 30 sec between each addition. The concentration of NH_2Cl was determined assuming a molar extinction coefficient of 42.9 at 242 nm [23].

Concentrations of H_2O_2 and HOCl were determined assuming molar extinction coefficients of 43.6

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‡ Abbreviations: 5-ASA, 5-aminosalicylic acid; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); IBD, inflammatory bowel disease; I_{sc}, short-circuit current; MPO, myeloperoxidase; PD, potential difference; R_T, tissue resistance; RNHCl, chloramines; and SH, sulfhydryl.

at 240 nm for H_2O_2 and 142 at 291 nm for HOCl (at pH 7.0) [23].

Analysis of sulfhydryl oxidation. SH groups were analyzed by the method of Ellman [24]. Briefly, 0.1 mL of L-cysteine solution was added to 0.9 mL of 50 μM oxidant solutions (NH_2Cl, H_2O_2 and HOCl) dissolved in 0.1 M phosphate buffer (pH 8.0) containing deferoxamine (0.1 mM). The oxidant solutions had been preincubated in the presence or absence of 500 μM 5-ASA for 60 min. An aliquot (7 μL) of 10 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) solution was added to 1 mL of the reaction mixture 5 min after addition of the L-cysteine solution. Absorbance at 412 nm was determined after the addition of DTNB. Since 5-ASA reacted with NH_2Cl or HOCl and developed a slight brown color, its absorbance at 412 nm was corrected for in the L-cysteine determination. The final concentrations were as follows: oxidants, 50 μM; L-cysteine, 100 μM; and 5-ASA, 500 μM. In separate experiments, three concentrations (5, 25 and 50 μM) of NH_2Cl or HOCl, which had been preincubated with 5-ASA for 5, 10, 45, 60 min or 30 sec, 1 min and 5 min, respectively, were added to the L-cysteine solution. L-Cysteine and DTNB were dissolved in 0.1 M sodium phosphate buffer, pH 8.0 and pH 7.0, respectively. In some experiments, DETAPAC (0.1 mM) was substituted for deferoxamine (0.1 mM).

Scanning wavelength measurements of 5-ASA. NH_2Cl, HOCl or H_2O_2 (50 μM) was added to 5-ASA solution (500 μM) in 0.1 M sodium phosphate buffer (pH 8.0) containing deferoxamine (0.1 mM). Absorbance was scanned from 600 to 200 nm after 60 min by a Beckman DU 70 spectrophotometer (Beckman Instrument Inc., Fullerton, CA). In separate experiments, the absorbance of 5-ASA reacted with NH_2Cl or HOCl was scanned every 12 min.

Ion transport studies. Male Sprague–Dawley rats (200–300 g, Charles River Breeding Laboratories, Wilmington, MA) were maintained on a standard laboratory diet and allowed free access to food and water before being killed by cervical dislocation. The distal colon was excised and placed immediately in oxygenated modified Krebs–Ringer buffer solution of the following composition (mM): NaCl, 120.2; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$; 25; and glucose 11.1. The tissue was stripped of its underlying longitudinal and circular muscle layers by blunt dissection; the resulting preparation consisted of only mucosa and submucosa. Adjacent tissues were then mounted as flat sheets on pins between two lucite half-chambers (World Precision Instruments, Inc., New Haven, CT) having an area of 0.64 cm^2 and bathed on both sides by 10 mL of buffer solution, circulated by gas lift and maintained at 37° by water-jacketed reservoirs. The solution was gassed continuously with 5% CO_2 in O_2 and maintained at pH 7.4.

Electrical measurements were monitored with an automatic voltage clamp (TR100-F, JWT Engineering, Overland Park, KS). Direct connecting voltage and current passing electrodes (World Precision Instruments, Inc.) were utilized to measure transepithelial potential difference (PD) and short-circuit current (I_{sc}). Transepithelial PD was periodically measured and tissue resistance (R_T) was calculated from Ohm's law. I_{sc} was recorded continuously on a GOULD model 2800S recorder (Gould Inc., Cleveland, OH). Tissues were equilibrated under short-circuit conditions until I_{sc} had stabilized (usually 30–45 min). Oxidants and drugs were added to the serosal bathing medium. Equivalent amounts of vehicle had no effect on I_{sc}. Either theophylline (1 mM) or carbachol (100 μM) was added at the end of each experiment to confirm tissue viability. Tissues which did not respond (<40 $\mu A/cm^2$) to these agents were discarded.

To determine the scavenging ability of 5-ASA against NH_2Cl and HOCl in the Ussing chambers, different incubation protocols were utilized. Because we found in a preliminary study that NH_2Cl reacts slowly with 5-ASA, we sometimes premixed NH_2Cl with 5-ASA for various times prior to addition to the Ussing chambers. In contrast, HOCl reacts so rapidly, we added HOCl 15 min after addition of 5-ASA to the Ussing chambers.

Because HOCl rapidly reacts with NH_4^+ (NH_4Cl) to yield NH_2Cl [4, 5], we added HOCl to the Ussing chambers in the presence of NH_4^+ (NH_4Cl) to test whether 5-ASA attenuates the NH_2Cl produced from HOCl plus NH_4Cl in the Ussing chambers.

Materials. 5-ASA, L-cysteine, deferoxamine, H_2O_2, DTNB, and diethylenetriaminepentaacetic acid (DETAPAC) were purchased from the Sigma Chemical Co. (St. Louis, MO). NH_4Cl and NaOCl were obtained from the Aldrich Chemical Co. (Milwaukee, WI).

RESULTS

L-Cysteine oxidation and reversal by 5-ASA. NH_2Cl, HOCl and H_2O_2 oxidized L-cysteine (Fig. 1). Preincubation with 500 μM 5-ASA prevented the oxidation of L-cysteine by NH_2Cl and HOCl. The same results were obtained when DETAPAC (0.1 mM) was substituted for deferoxamine (data not shown).

Figure 2 shows the result of increasing the preincubation time of NH_2Cl with 500 μM 5-ASA on the oxidation of L-cysteine. three different concentrations of NH_2Cl (5, 25 and 50 μM) were utilized. NH_2Cl oxidized L-cysteine in a concentration-dependent manner. L-Cysteine was protected by 5-ASA pretreatment depending on preincubation time. Incubation of 5-ASA with NH_2Cl for 10 min inhibited the oxidation of L-cysteine by 50%. Figure 3 shows that incubation of 5-ASA with HOCl for 30 sec completely inhibited the oxidation of L-cysteine.

Changes in spectrophotometric patterns after incubation of 500 μM 5-ASA with 50 μM NH_2Cl at various times were noted (Fig. 4). 5-ASA displayed two prominent peaks (210 and 298 nm) and exhibited no absorbance above 390 nm. The absorbance on both sides of the second peak (298 nm) increased in a time-dependent manner, and new peaks emerged at 420 nm, 500 nm and possibly at 270–280 nm.

Figure 5 shows changes in the absorbance of 5-ASA in the presence of NH_2Cl, HOCl or H_2O_2.

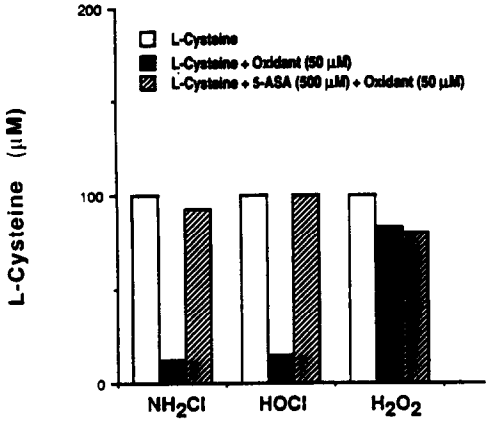

Fig. 1. Representative experiment showing the effect of 5-ASA on the oxidation of L-cysteine. 5-ASA (500 μM) and oxidant (50 μM) (NH_2Cl, HOCl or H_2O_2) were incubated for 60 min at room temperature before addition of 100 μM L-cysteine. The L-cysteine concentration was determined after 5 min spectrophotometrically at 412 nm using DTNB. Since 5-ASA reacted with NH_2Cl or HOCl developed a slight brown color gradually, the absorbance at 412 nm was corrected for in the L-cysteine determination. NH_2Cl, HOCl or H_2O_2 (50 μM) reduced L-cysteine by 90, 90 and 15% respectively. The same results were obtained when DETAPAC (0.1 mM) was substituted for deferoxamine (0.1 mM). Values are means for triplicate samples which did not vary by more than ±10%.

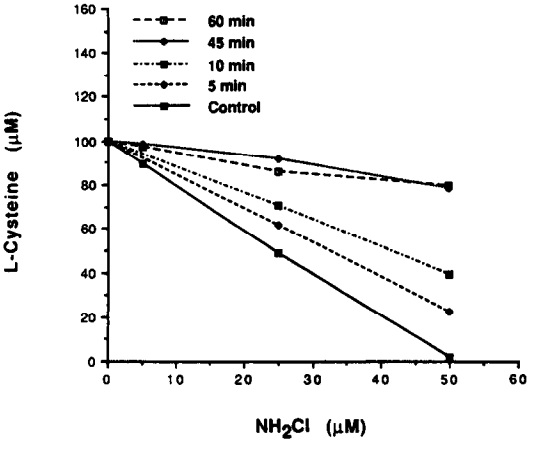

Fig. 2. NH_2Cl-induced time-related changes in L-cysteine concentration. 5-Aminosalicylic acid (500 μM) and NH_2Cl (50 μM) were incubated for 5, 10, 45 and 60 min before addition of 100 μM L-cysteine. The L-cysteine concentration was determined after 5 min spectrophotometrically at 412 nm using DTNB. Since 5-ASA reacted with NH_2Cl developed a slight brown color gradually, its absorbance at 412 nm was corrected for in the L-cysteine determination. One mole NH_2Cl reacted with two moles L-cysteine. The scavenging effect of 5-ASA for NH_2Cl increased with preincubation time. Control: L-cysteine (100 μM) + NH_2Cl. Data are from a representative experiment; values are means of duplicate samples and did not vary by more than ± 10%.

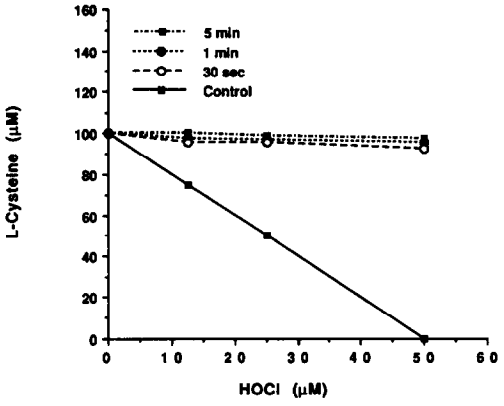

Fig. 3. HOCl-induced time-related changes in L-cysteine concentration. 5-ASA (500 μM) and HOCl (50 μM) were incubated for 30 sec, 1 min or 5 min before addition of 100 μM L-cysteine. The L-cysteine concentration was determined after 5 min as described in Materials and Methods. The scavenging effect of 5-ASA for HOCl was completed within 30 sec. Control: L-cysteine (100 μM) + HOCl. Values are means of duplicate samples and did not vary by more than 10%.

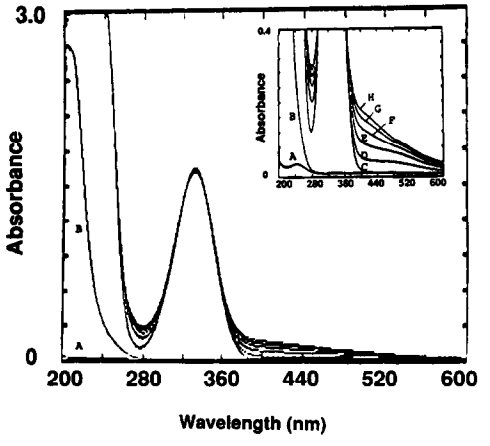

Fig. 4. Changes in absorbance of 5-ASA with 50 μM NH_2Cl. Changes in absorbance were determined by scanning the wavelength every 12 min. (A) 50 μM NH_2Cl; (B) 0.1 mM deferoxamine; (C) 5-ASA alone; (D) 12 min after mixture of 5-ASA and NH_2Cl; (E) 24 min; (F) 36 min; (G) 48 min; and (H) 60 min.

The changes in absorbance of 5-ASA caused by HOCl were similar and slightly more prominent than those for NH_2Cl. 5-ASA did not react with H_2O_2.

Effect of 5-ASA preincubation on NH_2Cl- *and* HOCl-*induced increases in* I_{sc}. NH_2Cl evoked a biphasic response having an initial peak at 2–4 min (peak I), followed by a second peak (peak II) at 15–30 min upon serosal addition to the bathing solution (Fig. 6A). In regard to the two different incubation protocols we used, 5-ASA (500 μM), when added to the colon 15 min prior to the addition of NH_2Cl (50 μM), did not inhibit the I_{sc} response

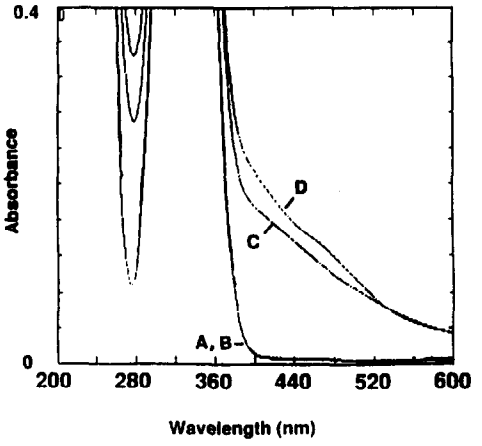

Fig. 5. Changes in absorbance of 5-ASA by NH_2Cl, HOCl and H_2O_2. 5-ASA (500 μM) was mixed with 50 μM NH_2Cl, HOCl and H_2O_2. The wavelengths were scanned from 600 to 200 nm after 60 min. (A) + buffer; (B) + H_2O_2; (C) + NH_2Cl; and (D) + HOCl. Line A was not distinguishable from line B.

to NH_2Cl (Fig. 6B). On the other hand, when NH_2Cl (200 μM) was premixed with 5-ASA (2 mM) for 1, 5 or 10 min in test tubes, and then aliquots of this mixture added to the serosal side of the tissue (final concentrations of NH_2Cl and 5-ASA were 50 and 500 μM respectively), a longer period between premixing and addition was required to inhibit the response (Fig. 6, C, D and E).

HOCl (50 μM) produced a monophasic response having a peak at 15–30 min (Fig. 7A). 5-ASA (500 μM) added to the colon 15 min prior to addition of HOCl 50 μM) completely inhibited the I_{sc} response (Fig. 7B), in contrast with NH_2Cl (Fig. 6B). 5-ASA (500 μM) plus NH_4^+ (NH_4Cl) (50 μM) added to the colon 15 min prior to addition of HOCl (50 μM) inhibited the I_{sc} response by 80 ± 6% (N = 4) (Fig. 7C).

DISCUSSION

The oxidizing abilities of NH_2Cl and HOCl against L-cysteine were found to be greater than those of

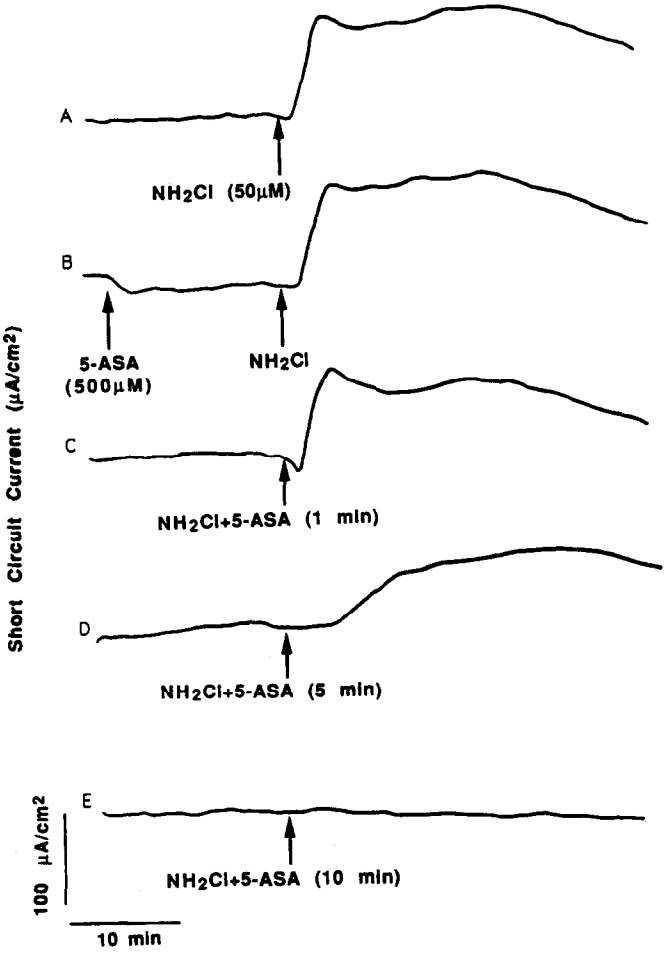

Fig. 6. Effect of 5-ASA on NH_2Cl-induced I_{sc}. (A) A typical representation of the effect of 50 μM NH_2Cl on I_{sc}. (B) 5-ASA (500 μM) was added to the colon 15 min prior to the addition of 50 μM NH_2Cl. An aliquot (2.5 mL) of the reaction mixture containing NH_2Cl (200 μM) and 5-ASA (2 mM), premixed for 1 min (C), 5 min (D) or 10 min (E), was added to the serosal side of the Ussing chamber which contained 7.4 mL of Ringer's solution. Final bath concentrations were 50 μM NH_2Cl and 500 μM 5-ASA. 5-ASA (500 μM) slightly reduced basal I_{sc} by 14 ± 4 $\mu A/cm^2$ (N = 15) when added alone.

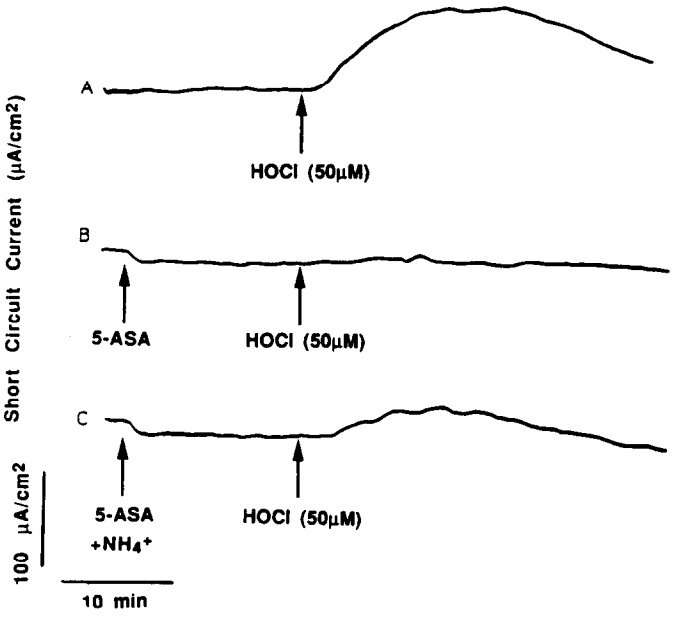

Fig. 7. Effect of 5-ASA on HOCl-induced I_{sc}. (A) A typical representation of the effect of 50 μM HOCl on I_{sc}. (B) 5-ASA (500 μM) added to the colon 15 min prior to the addition of 50 μM HOCl. (C) 5-ASA (500 μM) plus NH_4^+ (50 μM) added to the colon 15 min prior to the addition of 50 μM HOCl. NH_4Cl (50 μM) had no effect on basal I_{sc} when added alone.

H_2O_2. Preincubation with 5-ASA prevented the oxidation of L-cysteine by NH_2Cl and HOCl, suggesting that HOCl and NH_2Cl were potent enough oxidants to react with 5-ASA. This is borne out by the fact that NH_2Cl and HOCl oxidized 5-ASA to a chromogen with characteristic absorbances at 420 and 500 nm. The observation that longer preincubation times of 5-ASA were required for NH_2Cl to inhibit the L-cysteine oxidation than for HOCl (Figs. 2 and 3) can be explained on the basis of their oxidizing and chlorinating potentials, which are HOCl > NH_2Cl [2, 23]. The marginal effect of H_2O_2 is probably due to its weak oxidizing action [25]. DETAPAC (0.1 mM) did not inhibit the oxidation of L-cysteine caused by these oxidants (NH_2Cl, HOCl, H_2O_2), suggesting that iron-catalyzed oxidation of L-cysteine does not appear to be involved.

Although NH_2Cl and HOCl (50 μM) evoked immediate increases in I_{sc} across the rat colon (Figs. 6A and 7A), the inhibitory pattern for 5-ASA against these two oxidants was different. The I_{sc} response to 50 μM NH_2Cl was not inhibited by 15-min prior addition of 500 μM 5-ASA to the colon (Fig. 6B). When 5-ASA and NH_2Cl were premixed together for different times and then added to the colon in the Ussing chambers, the I_{sc} response to NH_2Cl was attenuated (Fig. 6, C, D, and E). The data indicate that 5-ASA reacted with NH_2Cl directly and diminished its oxidizing ability; the reaction was dependent on premixing time. This observation corroborates our data demonstrating that the NH_2Cl effect was less in the Ussing chamber after premixing with 5-ASA. This effect was time and dose dependent.

5-ASA added to the colon 15 min prior to addition of HOCl (50 μM) completely inhibited the I_{sc} response (Fig. 7B). This observation is explained by the rapid reaction of 5-ASA to reduce the oxidizing ability of HOCl against L-cysteine (Fig. 3). Once NH_2Cl was produced from HOCl in the presence of NH_4^+, however, 5-ASA could not inhibit the increase in I_{sc} (Fig. 7C), because the reaction of 5-ASA with NH_2Cl was slower than with HOCl.

Changes in the absorbance spectrum of 5-ASA detected the appearance of a new reaction metabolite(s) after incubation with HOCl and NH_2Cl. These data are consistent with the order of oxidizing and chlorinating potentials. In addition to oxidation reactions, 5-ASA reacts with hydroxyl radicals to generate several metabolites, whose chemical structures have not been identified [26, 27]. 5-ASA reacts with HOCl to produce a characteristic absorbance spectra (λ_{max} = 450 nm) [15], similar to our observation. Furthermore, the fluorescence of 5-ASA excited at 340 nm was reported lost by the reaction with equimolar concentrations of HOCl [16]. 5-ASA fluorescence disappeared after several minutes in the presence of NH_2Cl (data not shown).

We have demonstrated that the functional responses to NH_2Cl and HOCl were reduced in the presence of 5-ASA. Chemical reduction of NH_2Cl and HOCl by 5-ASA *in vivo* may attenuate their detrimental effects in inflamed tissue and thus contribute to the efficacy of 5-ASA in the treatment of IBD.

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