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Role of Neutrophil-Derived Oxidants in the Pathogenesis of Intestinal Inflammation

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Summary. There is a growing body of experimental data to suggest that the chronically inflamed intestine and/or colon may be subjected to considerable oxidative stress. The most probable sources of these oxidants are the phagocytic leukocytes since these cells are known to be present in large numbers in the inflamed mucosa and are known to produce significant amounts of reactive oxygen species in response to certain inflammatory stimuli. Because the colonic mucosa contains relatively small amounts of antioxidant enzymes (e.g. SOD, catalase, GSH peroxidase) it is possible that the gut mucosa may be overwhelmed during times of active inflammation which could result in intestinal injury. If reactive oxygen species play an important role in mediating mucosal injury in IBD then it should be possible to attenuate this injury by the use of antioxidants. One such drug is the sulfasalazine metabolite 5-ASA. It may not be coincidence that this potent antiinflammatory metabolite is a potent antioxidant that possesses multiple mechanisms of action including nitrogen, carbon and oxygen-centered free radical scavenging properties as well as the ability to decompose HOCl and scavenge hemoprotein-associated oxidants. In addition 5-ASA has the additional property of being able to chelate iron and render it poorly redox active. The reason that 5-ASA is so effective in vivo may be due to this multitude of antioxidant properties. This would also suggest that other, more potent antioxidants may prove beneficial in the treatment of IBD.

Key words: Granulocytes – Superoxide – Myeloperoxidase – Aminosalicylic acid

Intestinal Inflammation and Oxidative Stress

Active episodes of inflammatory bowel disease (IBD; Crohn's disease and ulcerative colitis) are characterized by the extravasation and infiltration of large numbers of neutrophilic polymorphonuclear leukocytes (neutrophils) into the mucosal interstitium (lamina propria) [1]. This enhanced inflammatory infiltrate is accompanied by extensive mucosal and/or transmural injury including increased vascular permeability, disruption of the extracellular matrix, edema, epithelial cell damage and erosions and ulcers [1]. Because the lamina propria provides structural support for the epithelium and villi, disruption and degradation of the interstitial matrix may contribute greatly to the pathogenesis of mucosal erosions and ulcers. The apparent association between neutrophil infiltration and mucosal injury has led to the proposal that neutrophils may play an important role in the pathophysiology of IBD [2]. Inflammatory neutrophils possess an arsenal of cytotoxic weapons that can be released into the surrounding environment where they may injure cells and tissue. One major metabolic pathway by which neutrophils may mediate mucosal injury is by the synthesis and release of reactive oxygen metabolites. There are several lines of indirect evidence which suggest that the chronically inflamed intestine or

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Abbreviations: SAZ = sulfasalazine; 5-ASA = 5-aminosalicylic acid; IBD = inflammatory bowel disease; SOD = superoxide dismutase

colon may be subjected to considerable oxidative stress and thus susceptible to oxidative injury: first, it is well known that phagocytes are activated by certain pro-inflammatory mediators such as leukotriene B4 (LTB_4) or platelet activating factor (PAF) to release large amounts of potentially cytotoxic reactive oxygen metabolites into the interstitial compartment [3, 4]. Enhanced synthesis of LTB_4 and PAF have been demonstrated in mucosal samples obtained from patients with active IBD [5, 6]. Second, there are several reports that have demonstrated that phagocytic leukocytes (monocytes, neutrophils, macrophages) obtained from patients with active IBD respond to various proinflammatory stimuli with enhanced reactive oxygen metabolism when compared to cells obtained from healthy volunteers [7–9]. Third, a preliminary report suggests, using low level chemiluminescence as an index of active oxygen generation, that oxy radical formation is enhanced in mucosa of animals with experimental colitis when compared to normal controls [10]. Finally, it is well known that certain drugs (e.g. 5-aminosalicylic acid) used clinically to attenuate the mucosal inflammation and injury associated with IBD are potent antioxidants and free radical scavengers [11–20]. This review discusses the pathways of oxidant generation by activated neutrophils and the mechanisms by which these oxidants may directly or indirectly injure the intestinal mucosa during times of active inflammation.

Mechanisms of Neutrophil-Mediated Oxidant Production and Tissue Injury

Interaction of certain proinflammatory mediators (e.g. LTB_4, PAF, immune complexes, bacterial products) with specific receptors on the neutrophil plasma membrane results in the dramatic increase in oxygen (O_2) consumption due to the activation of the latent, plasma membrane-associated NADPH oxidase [21]. Activation of this multicomponent, flavoprotein results in the production and release of large amounts of the superoxide anion radical (O_2^-; Fig. 1):

$$NADPH + O_2 \rightarrow NADP^+ + O_2^-.$$

Superoxide is very unstable at neutral pH and will spontaneously (or enzymatically) dismutate to yield hydrogen peroxide (H_2O_2) and oxygen (O_2):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2.$$

Some investigators have proposed that neutrophil-derived O_2^- and H_2O_2 may interact with low molecular weight, redox-active iron (Fe) to yield the highly reactive hydroxyl radical ($\cdot OH$) via the superoxide-driven Fenton reaction:

$$O_2^- + Fe^{+3} \rightarrow O_2 + Fe^{+2},$$

$$H_2O_2 + Fe^{+2} \rightarrow OH^- + \cdot OH + Fe^{+3}.$$

However, recent data suggest that neutrophils produce very little (if any) $\cdot OH$ in vitro. The reasons for this lack of $\cdot OH$ production are two fold: First,

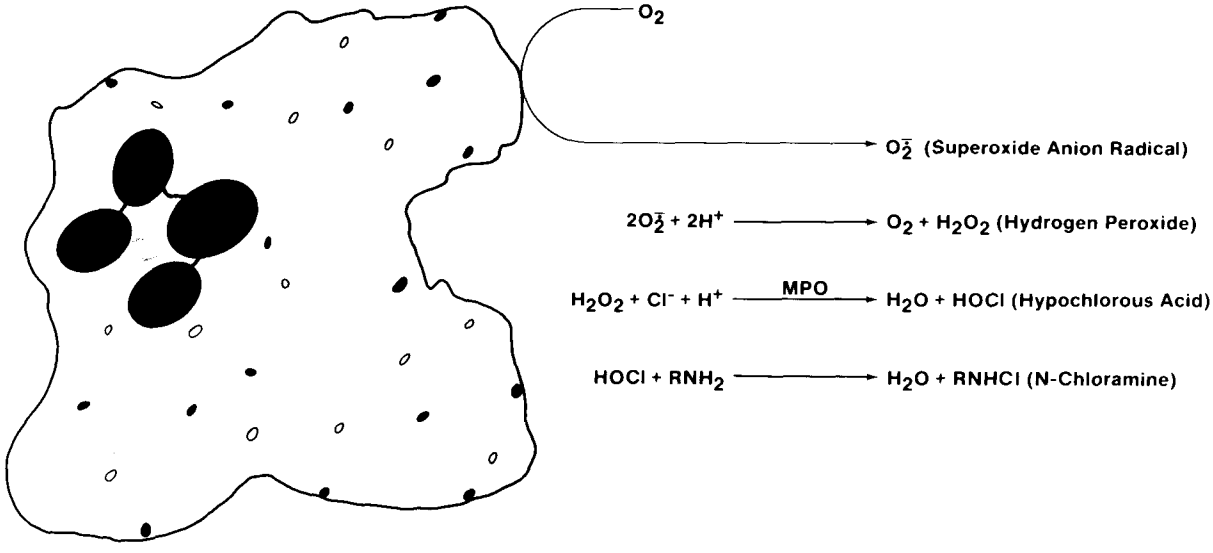

Fig. 1. Extracellular production of reactive oxygen metabolites by activated neutrophils. Activated neutrophils reduce molecular oxygen (O_2) to yield the superoxide anion radical (O_2^-) via the action of NADPH Oxidase. Superoxide spontaneously (or enzymatically) dismutates to yield hydrogen peroxide (H_2O_2) which will interact with extracellular myeloperoxidase (MPO) to yield the potent oxidizing agent hypochlorous acid (HOCl). HOCl will then interact with certain primary amines (RNH_2) to yield N-chlorinated derivatives or N-chloramines (RNHCl)

myeloperoxidase (and catalase) will consume most of the H_2O_2 produced leaving little H_2O_2 to interact with O_2^- and iron [22]. Second, there is normally very little low molecular weight iron available in vivo with most of the metal chelated to proteins such as transferrin, lactoferrin and ferritin [23]. In addition to these reactive oxygen metabolites, the activated neutrophil secretes the green hemoprotein myeloperoxidase (MPO) into the extracellular medium. Myeloperoxidase catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid (HOCl; the active ingredient in Chlorox^R bleach; Fig. 1) [24]:

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O.$$

It is generally accepted that the myeloperoxidase-H_2O_2-Cl^- system is the most potent cytotoxic system of the neutrophil. HOCl is approximately 100–1000 times more toxic than either O_2^- or H_2O_2. HOCl is a nonspecific oxidizing and chlorinating agent that reacts rapidly with a variety of biological compounds including sulfhydryls, DNA, pyridine nucleotides, aliphatic and aromatic amino acids, and nitrogen-containing compounds [25]. Interestingly, HOCl does not appear to have the ability to peroxidize polyunsaturated lipids. HOCl also reacts very rapidly with primary amines (RNH_2) to yield derivatives that contain the nitrogen-chlorine bond (N-chloramines; RNHCl) [26–28]:

$$HOCl + RNH_2 \rightarrow RNHCl + H_2O.$$

RNHCl possesses the two oxidizing equivalents of H_2O_2 and HOCl however they may be more or less toxic than HOCl, depending on their lipophilicities (membrane permeabilities) [26]. Two classes of neutrophil-derived RNHCl have been identified: one class may be represented by taurine monochloramine (TauNHCl), the reaction product derived from the interaction between HOCl and taurine [28]:

$$HOCl + SO_3^-\text{-}CH_2\text{-}CH_2\text{-}NH_2 \rightarrow SO_3^-\text{-}CH_2\text{-}CH_2\text{-}NHCl + H_2O.$$

Because the sulfonic acid residue is negatively charged at physiological pH, TauNHCl is very hydrophilic (membrane impermeable), making it long-lived and relatively nontoxic. Intracellular (neutrophil) concentrations of taurine have been estimated to be approximately 26 mM [28] and we have proposed that taurine may function to trap excess HOCl, thereby preventing nonspecific cytotoxicity to surrounding cells and tissues. Although TauNHCl is a potent oxidizing agent which is capable of oxidizing certain compounds in free solution, it cannot gain access into the intracellular compartment of most cells and thus cannot mediate cytolysis [28]. A second class RNHCl are lipophilic in nature and are quite cytotoxic. These RNHCl are very short-lived owing to their ability to rapidly penetrate the membrane lipid bilayer and react with intracellular components. An example of this class of oxidants is monochloramine (NH_2Cl), the reaction product generated from the interaction between HOCl and ammonia (NH_3) [27]:

$$NH_3 + HOCl \rightarrow NH_2Cl + H_2O.$$

NH_2Cl has been shown to be produced by neutrophils and is significantly more toxic than HOCl toward bacteria and certain eukaryotic cells [28]. Other examples of cytotoxic RNHCl are the mono and poly-chlorinated derivatives of histamine and the polyamine putrescine [29].

The mechanisms by which myeloperoxidase-derived HOCl and RNHCl damage cells and tissue remain speculative. These oxidants may mediate toxicity directly via sulfhydryl oxidation, hemoprotein bleaching, protein and amino acid degradation and inactivation of essential metabolic cofactors (e.g. NADH) and DNA [25]. In addition, MPO-generated oxidants may degrade and depolymerize important components of the extracellular matrix such as hyaluronic acid and collagen [30]. We found that luminal perfusion of the distal ileum in the rat with H_2O_2, HOCl or NH_2Cl produced a dose-dependent increase in mucosal permeability as measured by the blood-to-lumen clearance of 51-CrEDTA [31]. Perfusion with 0.1 mM, 0.5 mM and 1.0 mM oxidant produced a 2 ± 1, 5 ± 2, and 11 ± 5 fold increase in mucosal permeability for H_2O_2, a 2 ± 1, 8 ± 3 and 36 ± 12-fold increase for HOCl and a 3 ± 1, 11 ± 2 and 30 ± 7-fold increase for NH_2Cl. Taurine monochloramine was ineffective in enhancing mucosal permeability. The ability of these oxidants to increase mucosal permeability did not necessarily correlate with their cytotoxic potentials. We found that NH_2Cl and HOCl were cytotoxic to cultured intestinal epithelial cells in vitro whereas hydrogen peroxide was not toxic suggesting that its ability to enhance mucosal permeability in vivo involves noncytotoxic mechanisms (Fig. 2). In addition to disrupting the intestinal mucosal barrier we have also demonstrated that *nontoxic* concentrations of HOCl, NH_2Cl and H_2O_2 enhance significantly colonic Cl^- secretion suggesting that these oxidants may play a role in the pathogenesis of inflammation-induced diarrhea (Fig. 3). Recent work suggests that both prostaglandins and the enteric nervous system play important roles in oxidant-mediated Cl^- secretion [32].

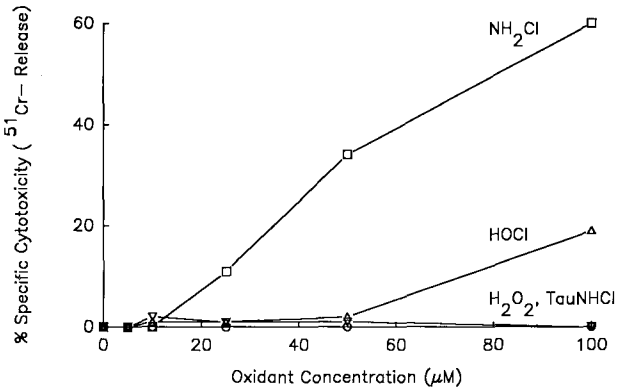

Fig. 2. Cytotoxicity of neutrophil-derived oxidants incubated with cultured intestinal epithelial cells (IEC-18) for 60 min at 37° C. Each data point represents the mean from triplicate determinations from at least four different experiments and did not vary by more than ±5%. Data derived from reference 31

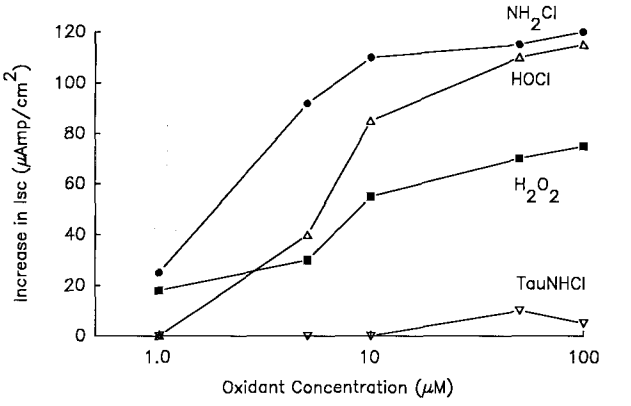

Fig. 3. Oxidant-induced increases in colonic chloride secretion as measured by increases in short-circuit current (Isc). Mucosal/submucosal preparations of rat colon were mounted in Ussing chambers, oxidants added to the serosal side and increases in Isc were recorded. Virtually all of the increase in Isc was due to increases in undirectional transport of Cl^-. Data derived from reference 32

In addition, we have found that these oxidants produced a concentration-depedent biphasic response with electrically-stimulated ileal smooth muscle strips that is characterized by a transient enhancement of neurally stimulated contraction followed by marked inhibition [33]. These data suggest that neutrophil-derived oxidants may play an important role in the pathogenesis of the depressed intestinal contractilitly observed in patients with IBD. Furthermore, Thomas and coworkers have demonstrated that sublethal concentrations of neutrophil-derived oxidants may be potent mutagens in vitro suggesting a possible link between chronic inflammation and neoplastic transformation [34].

In addition to these direct effects, neutrophil-derived oxidants may damage the epithelium and mucosal interstitium *indirectly* by altering the protease/antiprotease balance that normally exists within the intestinal interstitium. For example, activated neutrophils release a variety of proteolytic enzymes including elastase, collagenase and gelatinase. Each of these proteases is capable of attacking the key components of extracellular matrix (collagens, fibronectin, proteoglycans) and the epithelial membrane. The ability of these proteases to play a significant role in mediating tissue injury has been questioned in the past because the interstitium is continuously bathed in extracellular fluid (lymph) which contains powerful protease inhibitors (e.g. α1 antiprotease and α2 macroglobulin) that irreversibly bind to neutrophil-derived proteases and consequently render them inactive. Although metalloproteases (collagenase, gelatinase) are generally not as susceptible to inactivation by these antiproteases as is elastase, their role as potential mediators of tissue injury has also been questioned because they are synthesized by the neutrophil in a latent, inactive form. However, recent work suggests that oxidants, proteases and antiproteases interact to disrupt the normally protective environment of the interstitium and promote interstitial and cell damage via the oxidative activation of the neutrophil-derived proteolytic system [24]. Several groups of investigators have shown that MPO-generated HOCl inactivates the α_1-protease inhibitor (and $\alpha_2$macroglobulin) associated with extracellular fluid (plasma, lymph) thus allowing for uncontrolled proteolysis by elastase (reviewed in [24]). Apparently, HOCl (or RNHCl) oxidize essential methionine residues adjacent and distal to the active site of the protein to yield methionine sulfoxide derivatives. These oxidized proteins are incapable of binding and inhibiting elastase. In addition, Weiss and coworkers have demonstrated the extracellular MPO system (HOCl) activates the latent collagenase and gelatinase secreted by neutrophils [24]. These data suggest that oxidative inactivation of important protease inhibitors coupled to the oxidant-mediated activation of latent proteases creates an environment for the neutrophil that would allow elastase, collagenase and gelatinase to mediate degradation of the mucosal interstitial matrix as well as injuring epithelial cells. Taken together these observations suggest that HOCl may be cytotoxic directly by virtue of its potent oxidizing and chlorinating activity and/or it may mediate cytotoxicity indirectly by oxidatively activating latent proteases (collagenase, gelatinase) released by neutrophils and inactivating certain protease inhibitors (α-1 antiproteases, α_2-macroglobulin) normally present in intestinal interstitial fluid (lymph; Fig. 4).

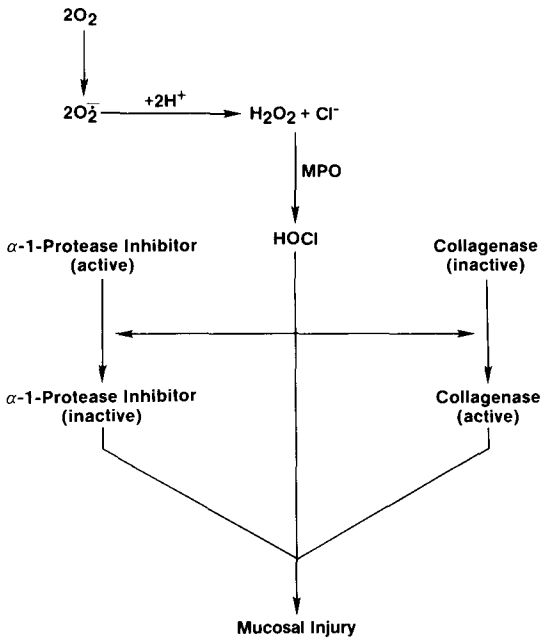

Fig. 4. Proposed interaction among neutrophil-derived oxidants, proteases and protease inhibitors. Activated neutrophils release large amounts of the superoxide anion radical (O_2^-) which spontaneously or enzymatically dismutates to form hydrogen peroxide (H_2O_2). In addition, activated neutrophils secrete myeloperoxidase (MPO) into the extracellular medium where it catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid (HOCl). HOCl or lipophilic N-chloramines may injure the mucosa directly by oxidative mechanisms or they may mediate mucosal injury indirectly by inactivating certain proteases inhibitors (α_1 protease inhibitor) and activating specific neutrophil-derived proteases (collagenase)

Role of Antioxidants in Protecting the Inflamed Intestine

Normally, most cells and tissue are protected from the injurious effects of reactive oxygen metabolites by the action of certain antioxidant enzymes such as superoxide dismutase (SOD), catalase and GSH peroxidase. However, it has recently been determined that the human colon contains relatively small amounts of these antioxidant enzymes when compared to tissue such as the liver ([35]; Table 1). These data suggest that the oxidant defenses of the colon may be overwhelmed during times of chronic inflammation and thus susceptible to oxidative tissue injury. If granulocyte-derived oxidants play some role in the pathogenesis of inflammation-induced mucosal injury, then one would predict that the administration of antioxidants may prove beneficial in the treatment of IBD. Indeed, a preliminary clinical report by Emerit et al. suggests that the intramuscular administration of SOD to patients with severe Crohn's disease leads to significant improvement as measured by decreases in the Crohn's disease activity index, endoscopic documentation of mucosal healing and by the shortening of duration of relapses [36]. Another drug that has been used for many years and has been shown to have potent anti-inflammatory and antioxidant activity is 5-aminosalicylic acid (5-ASA), the pharmacologically-active moiety of sulfasalazine. Recent studies have demonstrated that 5-ASA has potent superoxide dismutase (SOD)-like activity as measured by its ability to decompose the superoxide anion radical [16, 37]. We have found that 5-ASA only very sluggishly reacts with H_2O_2 and thus is unlikely to participate in the decomposition of this oxidant in vivo. Although 5-ASA has been shown to scavenge the $OH^\cdot$ its role as a selective $OH^\cdot$ scavenger is doubtful since the pharmacologically inactive metabolites N-acetyl-5-ASA and sulfapyridine are also potent scavegers of $OH^\cdot$ [37]. We have also found that 5-ASA but not SAZ, NASA nor SP is effective in inhibiting lipid peroxidation initiated by organic peroxyl radicals generated from the thermal decomposition of the free radical initiator 2,2′-azobis(amidinopropane)dihydrochloride (A-N=N-A) [37]:

$$\text{A-N=N-A} \rightarrow \text{A}^\cdot + \text{N}_2 + \text{A}^\cdot$$
$$\text{A}^\cdot + \text{O}_2 \rightarrow \text{AOO}^\cdot$$
$$\text{AOO}^\cdot + \text{LH} \rightarrow \text{AOOH} + \text{L}^\cdot$$
$$\text{L}^\cdot + \text{O}_2 \rightarrow \text{LOO}^\cdot$$
$$\text{LOO}^\cdot + \text{LH} \rightarrow \text{LOOH} + \text{L}^\cdot$$

where A·, AOO·, and AOOH represent the alkyl radical, peroxyl radical and hydroperoxide of the free radical initiator, respectively whereas LH, $L^\cdot$, $LOO^\cdot$, and LOOH represent polyunsaturated lipid, lipid alkyl radical, lipid hydroperoxyl radical and the hydroperoxide respectively. Because lipid peroxidation is not initiated by the superoxide-dependent, Fe-catalyzed formation of $OH^\cdot$ in this system, the inhibitory effect of 5-ASA is due solely to its ability to scavenge the peroxyl free radicals. These

Table 1. Antioxidant enzyme activities in different regions of the human colon

	Catalase	Superoxide Dismutase	GSH Peroxidase
Mucosa	11±3	4±0.3	15±0.8
Submucosa	11±5	4±0.4	9±2.3*
Muscularis/Serosa	8±4	2±0.2*	8±3*
Liver	269±69*	46±6*	37±2*

All data are corrected for contributions made by blood contamination of the tissue and are expressed as Units per mg protein except for GSH Peroxidase which is expressed as mUnits per mg protein. Data represent the mean±SEM for duplicate samples from $n=6$ individual specimens, except for liver which represents the mean±SEM from $n=3$ individual specimens. * represents $P<0.05$ compared to mucosa

data agree with and extend the findings of Ahnfelt-Ronne and Nielson who have demonstrated potent scavenging of a nitrogen-centered free radical by 5-ASA but not by SAZ or SP [17]. Another property of 5-ASA that may contribute to its antioxidant activity is its ability to chelate Fe. It is known that any compound capable of binding Fe and rendering it poorly redox active would be very effective in inhibiting the formation of secondarily-derived free radicals. We have recently demonstrated that 5-ASA inhibits the ferrous sulfate-mediated degradation of deoxyribose by chelating Fe and preventing its interaction with H_2O_2 to yield the hydroxyl radical [38]. N-acetylated 5-ASA and sulfasalazine were only modestly effective whereas sulfapyridine was inactive suggesting a relatively selective effect by the therapeutically active metabolite. This iron-binding property of 5-ASA may play an important role during chronic inflammation in that neutrophil-derived superoxide could reduce ferritin-associated Fe^{+3} to Fe^{+2} causing its release into the intestinal interstitium where it could participate in cytotoxic redox reactions.

MPO-catalyzed oxidation of Cl^- by H_2O_2 to yield HOCl represents another significant pathway of oxidant production in inflamed tissue. Aruoma and coworkers have shown that 5-ASA was selective in its ability to protect α-1-protease inhibitor against inactivation by HOCl suggesting that some of the beneficial effects of 5-ASA may be due to its ability to selectively interact with and decompose HOCl in the presence of other biological compounds [15]. We have found that 5-ASA and 4-ASA are very effective in inhibiting MPO-catalyzed reactions [39]. Apparently 5-ASA (and 4-ASA) act as alternative substrates for MPO which preferentially oxidizes these compounds instead of the substrate. It is quite possible that this is the reason why Ahnfelt-Ronne et al. detected significant levels of oxidation products of 5-ASA in SAZ-treated patients with active IBD [19]. It has also been suggested that the interstitial hemoglobin (Hb) released during intestinal bleeding may mediate some of the mucosal injury by interacting with phagocyte-derived H_2O_2 to generate ferryl (Fe^{+4}) hemoglobin [40]. Ferryl Hb is a potent, hemoprotein-associated oxidant which is capable of initiating lipid peroxidation and degrading carbohydrates. We have found that 5-ASA and to a lesser extent N-acetyl-5-ASA but not SAZ nor SP inhibited Hb-catalyzed lipid peroxidation by scavenging ferryl Hb [40].

These same mechanisms may explain the results of Hoult and Page who demonstrated that relatively small amounts of 5-ASA actually enhanced the synthesis of certain prostaglandins (PGs) by mucosal biopsies obtained from rat and human colons [41]. It is known that prostaglandin synthetase contains two enzymatic activities including cyclooxygenase and hemoprotein peroxidase activities. During the enzymatic reaction there is a progressive inhibition of the enzyme due to the oxidative inactivation of the hemoprotein peroxidase. Addition of certain antioxidants (e.g. phenolic compounds) inhibit this inactivation process, prolong prostaglandin production and thus increases the net synthesis of PGs. Apparently the lipid hydroperoxide generated by cyclooxygenase (PGG2) combines with the hemoprotein peroxidase to generate a hemoprotein associated free radical. In the absence of an exogenous electron donating substrate (antioxidant) the hemoprotein-localized free radical eventually oxidizes certain amino acid residues proximal to the active site which ultimately results in the inactivation of the enzyme. It is intriguing to speculate that 5-ASA, by virtue of its antioxidant activity, may protect the mucosa by enhancing the formation of protective PGs such as prostacyclin and/or PGE derivatives. Indeed, recent reports suggest that certain PG E analogs are very effective in protecting the colonic mucosa from the injurious effects of a variety of noxious agents [42, 43].

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