Peroxy nitrite and inflammatory bowel disease

Introduction
Idiopathic inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn’s disease, are chronic inflammatory disorders of the gastrointestinal tract which lead an unpredictable clinical course undergoing a succession of exacerbations and remissions of variable intensity. The highest incidence rates for IBD are found in developed countries of the northern hemisphere (UK, Scandinavia, Canada, USA) with the average annual incidence ranging from six to 13 new cases per year per 100 000 of the general population.1 For the patient, the consequences of IBD include rectal bleeding, diarrhoea, weight loss, and a reduced quality of life and may even result in death (mortality rate for UK, USA, Canada, Scandinavia ~3.9 per 10^5 per year (combined statistics from 1950 to 19942)).

Investigators working on the aetiology of IBD have proposed many factors which are thought to be involved in the initiation or exacerbation, or both, of the inflammatory process. Some of these factors include increased epithelial permeability, inappropriate neutrophil infiltration into the intestine, activation of mast cells, and increased concentrations of pro-inflammatory mediators (cytokines, leukotrienes, reactive oxygen metabolites (ROMs)). In addition, over the past 10 years, the overproduction of nitric oxide (NO) has received considerable attention as an important player in the pathogenesis of IBD.3–5 NO is a free radical produced from l-arginine via the enzyme nitric oxide synthase (NOS). Alone, NO is a weak free radical; however, it can react with superoxide (O_2^-) to produce peroxy nitrite (ONOO^-) a highly toxic reactive nitrogen intermediary. In 1990, Beckman and colleagues6 showed that peroxy nitrite is a relatively weak free radical and its cytotoxic effects are generally ascribed to its role as a precursor for hypochlorous acid and OH radical formation. Although the iron catalysed Haber-Weiss reaction has been suggested previously to explain the generation of OH from O_2^- (see A below) the formation of peroxy nitrite is another possible mechanism.

Role of superoxide in inflammatory bowel disease
A pathological role for the O_2^- radical in IBD is generally accepted.8 Reactive oxygen metabolites like O_2^- and hydrogen peroxide (H_2O_2) are byproducts of normal cellular metabolism produced by many enzyme systems (e.g. xanthine oxidase, NADPH oxidase) in the body. Antioxidant enzymes (superoxide dismutase (SOD), catalase, glutathione peroxidase) and free radical scavengers (tocopherol, ascorbate, uric acid) are contained in all tissues. Inappropriate oxidative reactions resulting in inflammation will develop when these antioxidants and scavengers are depleted/overloaded by high levels of ROMs. Like NO, O_2^- is a relatively weak free radical and its cytotoxic effect is generally ascribed to its role as a precursor for hypochlorous acid and OH radical formation. The Haber-Weiss reaction has been suggested previously to explain the generation of OH from O_2^- (see A below) but the formation of peroxy nitrite is another possible mechanism. Peroxy nitrous acid (B) is generated from H_2O_2 and chloride (Cl^-) via myeloperoxidase (MPO), an enzyme which is mainly found in granulocytes and is present in high amounts in intestinal tissue of patients with IBD.

\[ \text{Fe}^{3+} + \text{NO} + \text{O}_2^- \rightarrow \text{NO}_2 + \text{Fe}^{2+} + \text{O}_2 \] (A)

\[ \text{MPO} \]

\[ \text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \] (B)

It is well documented that these radicals are generated in high amounts in clinical biopsy samples9–11 and that neutrophils from patients with IBD are capable of generating increased levels of superoxide relative to controls.12 SOD and catalase (scavengers of O_2^- and H_2O_2 respectively) and glutathione are generally decreased in ulcerative colitis and Crohn’s disease15–17 suggesting depletion by high levels of ROMs. Experimental animal models of IBD have demonstrated similarly high levels of ROMs and a benefit of antioxidant therapy.18–20 For example, antioxidant therapy has improved the colonic damage and diarrhoea observed in the trinitrobenzene sulphonic acid (TNBS) model of colitis.19

Role of nitric oxide in inflammatory bowel disease
A pathological role for NO in IBD is less clear-cut. As stated previously NO is produced from l-arginine via NOS. NOS exists in three distinct isoforms: constitutively expressed, vascular endothelium (eNOS or NOS-3) and neuronal NOS (nNOS or NOS-1), and an inducible isoenzyme that operates independently of calcium (iNOS or NOS-2).20 iNOS expression is induced by cytokines and requires protein synthesis and is capable of high output production of NO at sites of inflammation. A role for NO in IBD has been suggested by various clinical studies which demonstrated the presence of increased levels of nitrite/nitrates in plasma and NO in rectal dialysates, as well as increased NOS activity in biopsy samples from patients with ulcerative colitis and Crohn’s disease.21–23 The latter was shown to be calcium independent suggesting increased iNOS activity. iNOS expression has since been demonstrated in numerous cell types in biopsy specimens from patients with ulcerative colitis or Crohn’s disease, including neutrophils, macrophages, epithelial cells, and endothelial cells.5–21 Although it is well documented that NO has homoeostatic regulatory functions in the intestine and has many anti-inflammatory mechanisms of action,21–23 studies using inhibitors of NOS in experimental models of colitis would suggest that inhibition of NO production will attenuate the intestinal inflammation.24–26 To explain this dichotomy, the prevailing view has been that the small amount of NO produced under normal or acute inflammatory conditions via constitutive NOS (cNOS or eNOS) may be an important endogenous inhibitor of inflammation; however, high levels of NO associated with chronic inflammatory conditions such as IBD (via iNOS) may be

Abbreviations used in this review: ASA, aminosalicylic acid; COX, cyclooxygenase; IBD, inflammatory bowel disease; IL, interleukin; MEG, mercaptoethylguanidine; MPO, myeloperoxidase; NO, nitric oxide; NOS, nitric oxide synthase; NT, nitrotyrosine; ROM, reactive oxygen metabolites; SOD, superoxide dismutase; TNBS, trinitrobenzene sulphonic acid.
detrimental to intestinal integrity. Our understanding of the role of NO in ulcerative colitis and Crohn’s disease is further embroiled by a number of other inhibitor studies which have shown little or no effect, or even an exacerbation in experimentally induced inflammation. One possible explanation for this is the relative lack of specificity of the inhibitors used in these studies which have effects on both the constitutively expressed and the inducible isoform of NOS. Yet, when the iNOS gene is genetically deleted from mice using recombinant DNA technology, experimentally induced colitis is exacerbated acutely. In TNBS induced inflammation, the lack of iNOS induction made little difference to the developing chronic inflammation in one study and improved the mortality rate and some inflammatory indexes in the surviving mice in another. Furthermore, a chronic colitis which develops spontaneously in interleukin 10 (IL-10) deficient mice, developed at the same rate and intensity in mice which were doubly deficient in IL-10 and iNOS genes. In addition to this, in a clinical condition (collagenous colitis) which is never associated with obvious macroscopic ulcers, even higher levels of NO than in ulcerative colitis are found. These studies suggest that NO concentrations alone cannot dictate pathological inflammation in the intestine and makes the generation of peroxynitrite in vivo an exciting prospect, one which could explain the deleterious potential of both O₂⁻ and NO in IBD.

Peroxynitrite

Peroxynitrite has a half life of 1.9 seconds at pH 7.4 and exists in equilibrium with peroxynitrous acid (equation (1) below). The reactivity of peroxynitrite comes from two possible intermediary products in the degradation pathway of peroxynitrous acid to nitrate (NO₃⁻): (a) peroxynitrous acid may generate an excited isomer, defined as ONOO* in equation (2), which acts as a ‘OH-like oxidising species; or (b) homolysis of peroxynitrous acid produces the ‘OH and nitrogen dioxide (NO₂) radicals and these free radicals are the oxidants (equation (3)) (see Pryor and Squidder for review).

\[
\begin{align*}
O_2^- + NO &\rightarrow ONOO^- + H^+ \equiv ONOOH \\
ONOO^- + H^+ &\equiv ONOOH \equiv ONOOH^- \rightarrow NO_3^- + H^+ \\
ONOO^- + H^+ &\equiv ONOOH \equiv [NO+NO^-] \rightarrow NO_3^-+ H^+ (3)
\end{align*}
\]

Thus, through the generation of these potent oxidising intermediaries, peroxynitrite can oxidise a variety of molecules (sulphhydrils, thiols, ascorbate) and trigger cytotoxic processes including lipid peroxidation and DNA damage. The latter can lead to increased permeability in epithelial cells via activation of poly(ADP)-ribose synthetase. In addition to oxidation reactions, peroxynitrite can nitrate tyrosine in vitro to produce 3-nitrotyrosine (3-NT). This reaction requires the addition of nitronium (NO₂⁺) to tyrosine residues, a reaction which cannot be produced by NO itself. As peroxynitrite is difficult to measure in vivo owing to its high reactivity, 3-NT formation is used as the standard method for measuring peroxynitrite formation. Detection of 3-NT is most often achieved by antibody immunostaining of tissues although HPLC or GC based spectrophotometry have also been described.

Evidence for peroxynitrite in inflammatory bowel disease

The formation of 3-NT using immunohistochemical techniques has been found both in clinical biopsy samples from patients with IBD and in experimentally induced animal models. Most experimental animal studies on the role of peroxynitrite in IBD have focused on inhibiting the inducible isoform of NOS in an attempt to attenuate NO production. In 1996, Southan and colleagues described a novel class of NOS inhibitors which had increased selectivity for iNOS over both eNOS and nNOS (EC₅₀ 11.5 µM v 110 µM, iNOS v eNOS respectively). These drugs belong to a series of aminooalkylisothioureas, compounds which are thought to exert their potent inhibitory effects through transguanidine rearrangement to mercaptoalkylguanidines such as mercaptoethylguanidine (MEG). MEG was shown to be almost 10-fold more potent than aminoguanidine (another “selective” inhibitor), at inhibiting the inducible isoform of NOS. This group later showed that MEG and related compounds could also act as scavengers of peroxynitrite. MEG was shown to inhibit peroxynitrite induced DNA single strand breaks, suppress mitochondrial respiration and prevent nitration of 4-hydroxyphenylacetic acid. Therefore, MEG could potentially reduce peroxynitrite formation, yet maintain the physiological effects of NO derived from eNOS. In fact, using the TNBS induced model of experimentally induced colitis in rats, Zingarelli and colleagues recently reported that administration of MEG significantly attenuated the clinical signs (diarrhoea and weight loss) of colitis as well as macroscopic and histological damage scores, granulocyte infiltration, iNOS immunoreactivity, and 3-NT formation. Previously, Miller and colleagues showed that aminoguanidine decreased the formation of NO and 3-NT formation and reduced inflammatory indexes in TNBS induced ileitis. Although compounds such as MEG may be of benefit in intestinal inflammation owing to their scavenging properties, it is impossible to compare the effectiveness of the two compounds between studies and a direct comparison has not yet been carried out in the literature.

As with aminoguanidine and other “selective” iNOS inhibitors MEG’s actions are not limited to this enzyme system. The effects of aminooalkylisothioureas (MEG-like compounds) as protective agents against radiation induced damage have been known since the late 1950s and may be explained in part by an oxyradical scavenging effect, and an effect which in itself may also reduce peroxynitrite formation. MEG has been shown to be a direct inhibitor of both cyclooxygenase 1 (COX-1) and COX-2 activity and this may contribute to its beneficial effects observed in inflammatory conditions. MEG also depletes noradrenaline (norepinephrine) stores when given for 3–4 days in vivo by a direct effect on dopamine β-hydroxylase, thereby preventing noradrenaline synthesis. More recently, MEG has been shown to inhibit ADP induced human platelet aggregation by activating guanylate cyclase.

Although peroxynitrite seems to be closely related to iNOS expression in some studies, in other studies this is not the case. Using immunohistochemistry to localise 3-NT formation in the rat ileum after TNBS administration, Miller et al showed staining primarily in the villi epithelium cells and neurons in the vicinity of iNOS expression. In Zingarelli’s recent study a reduction in the nitrosative and oxidative stress induced by TNBS administration was noted in mice genetically deficient in iNOS. These studies would suggest that increased NO from iNOS results in, and is required for, increased formation of peroxynitrite. However, in the same experimental model in the colon, iNOS staining was localised to infiltrating inflammatory cells, whereas 3-NT staining was found throughout the inflamed tissue. In agreement with this are findings by Miamamba and Sharkey who recently reported that the formation of 3-NT in the TNBS induced model of colitis was distinct from that of iNOS. In clinical studies, 3-NT was detected in epithelial cells and in the lamina propria in patients with ulcerative colitis, Crohn’s disease, and diverticulitis. Although Singer et al
showed intense iNOS immunoreactivity associated with 3-NT expression in epithelial cells, Dijkstra et al could not visualise 3-NT formation in the epithelium despite intense iNOS staining. In this latter study 3-NT staining was observed at the surface of inflammatory cells producing ROMs, some distance from the iNOS positive epithelium. These data would suggest that NO from iNOS does not necessarily determine the location of peroxynitrite formation and that NO from other NOS isoforms, as well as the site of ROMs, will also be important.

Direct administration of peroxynitrite in a bolus injection to the rectal mucosa of rats will induce oedema, mucosal ulceration, and severe histological damage. It is unlikely, however, that peroxynitrite would occur in a bolus form in vivo. Although many cell types are capable of producing both O$_2^-$ and NO, the relative flux of production has been proposed as an important factor in peroxynitrite formation. For example, Sea and coworkers measured iNOS activity and SOD protein concentrations in TNBS induced colitis in rats. They reported a reduction in SOD activity and an increase in iNOS activity at a time of severe inflammation and suggested that this might increase peroxynitrite formation in IBD. However, in 1996, Miles and colleagues showed that peroxynitrite formation, as indicated by oxidation of dihydrodihomadamine, was increased (in the absence of redox active iron) but only if NO$^-$ and O$_2^-$ were produced in equal amounts. If either O$_2^-$ or NO$^-$ were generated in excess, a decrease in peroxynitrite activity was observed. In the presence of redox active iron, NO$^-$ production could enhance or inhibit O$_2^-$, dependent oxidation depending on the relative fluxes of NO$^-$ and O$_2^-$.

This group suggested that NO$^-$ could react with a peroxynitrous acid intermediary and effectively reduce its oxidising capacity. The same group extend this work to suggest that as the relative cellular concentration of superoxide is in the order of 1000 times lower than nitric oxide, the amount of peroxynitrite produced is limited in a site specific fashion to areas of high superoxide anion generation. The study by Dijkstra and colleagues in 1998 showing 3-NT formation at the site of ROM formation and not at a site of high NO$^-$ output would support this view.

One situation where it has been suggested that NO$^-$ and O$_2^-$ production are ideally suited for peroxynitrite production is when L-arginine levels are depleted, such as in chronic inflammation where iNOS is upregulated and NO$^-$ is produced in excess. Xia and colleagues have reported that both nNOS and iNOS are capable of producing O$_2^-$ induced cytotoxicity in cells depleted of L-arginine. This superoxide production could be blocked by L-arginine supplementation and L-NAME, a NOS inhibitor, but not its inactive enantiomer. They hypothesised that O$_2^-$ concentrations were increased and NO$^-$ concentrations lowered while being produced in the same locale (by the same enzyme) providing appropriate amounts to generate peroxynitrite which induced the damage. Indeed peroxynitrite concentrations were increased in L-arginine depleted cells as measured by the formation 3-NT and a NOS inhibitor could inhibit this formation. This would suggest that L-arginine supplementation would be beneficial in intestinal inflammation and may explain in part the benefit observed in neonates with necrotising enterocolitis given a diet high in L-arginine. However, two recent studies would suggest that experimentally induced colitis is exacerbated by L-arginine supplementation.

Recently, the practice of detecting 3-NT formation as a specific indicator of peroxynitrite has been questioned. Firstly, Pfieffer and Mayer demonstrated that tyrosine was nitrated most efficiently with an NO$^-$ donor alone in the absence of O$_2^-$ formation. When O$_2^-$ was generated with NO$^-$ at equal rates or higher, little or no tyrosine nitration could be detected by HPLC. Their results suggested that nitrogen dioxide (NO$_2^-$) was the nitrating agent and not peroxynitrite. Furthermore, using human polymorphonuclear leucocytes, Eisrich and coworkers showed that in the presence of hypochlorous acid or MPO, 3-NT could be formed from nitrite (NO$_2^-$), via the formation of nitryl chloride (NO$_2Cl$) and nitrogen dioxide (NO$_2^-$). As previously stated hypochlorous acid is produced from H$_2$O$_2$ using Cl$^-$ and MPO. Their results indicate that 3-NT formation in vivo can result from an action of several reactive nitrogen species not just peroxynitrite and sheds some doubt on the evidence for peroxynitrite formation per se in IBD.

Finally, if peroxynitrite is formed in vivo, could drugs used clinically for IBD affect its production? Sandoval et al have recently reported that 5-aminosalicylic acid (5-ASA) is a potent scavenger of peroxynitrite and attenuated peroxynitrite induced apoptosis in a human intestinal epithelial cell line. This may not be surprising as 5-ASA has previously been described as a scavenger of the superoxide radical. Nevertheless, although the mechanism of action of 5-ASA is multifarious, some of its therapeutic effects may be owing to its ability to scavenge peroxynitrite or prevent its formation.

Conclusion

In summary, the formation of peroxynitrite in vivo would potentially make this molecule a vital regulatory step in the physiology and pathophysiology of both NO$^-$ and O$_2^-$; balancing a potent oxidant effect with a detoxification pathway for the superoxide radical. The biological formation, activity and decomposition of peroxynitrite is dependent on the chemical environment (concentration of proteins, thiols, sulphhydrils, redox active iron), the ratio of NO$^-$ to O$_2^-$ production and the cellular source(s) of NO$^-$ and O$_2^-$.

The exact role of peroxynitrite in IBD cannot be deciphered at present as it is one of many radicals produced in intestinal inflammation and it is impossible to scavenge one without affecting a myriad of others. The problem we are faced with is whether free radicals can induce damage in intestinal inflammation but rather distinguishing between them. Peroxynitrite, not unlike most radicals, is elusive because of its rapid reactivity. Therefore its detection is based on indirect evidence of the production of 3-NT formation, the end product of a number of different biochemical reactions involving reactive nitrogen metabolites which occur in vivo. What is clear, however, is that reactive nitrogen metabolites are produced in experimental models of IBD as well as in clinical biopsy samples and these metabolites (peroxynitrite included) have the potential to cause damage in the intestine in a similar manner to ROMs. To date “peroxynitrite scavengers” do much more than simply scavenge reactive nitrogen metabolites and therefore the exact contribution of these metabolites to inflammation in IBD cannot be truly elucidated.

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