



Review

New light on the anti-colitic actions of therapeutic aminosalicylates: the role of heme oxygenase

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Abstract:

Although a variety of pharmaceutical preparations of aminosalicylate are commonly used in the clinic for the control of inflammatory bowel disease, the mechanisms underlying their therapeutic actions remain unclear. Recent *in vivo* and *in vitro* studies have demonstrated that 5-aminosalicylic acid (5-ASA), regarded as the active moiety in aminosalicylate preparations such as sulfasalazine, can induce the heat shock protein, heme oxygenase-1 (HO-1) and up-regulate HO enzyme activity in the colon. As HO-1 can produce endogenous anti-oxidant and anti-inflammatory moieties such as bilirubin and carbon monoxide (CO), these findings suggest a novel mechanism of action for aminosalicylates, acting as anti-colitic agents through the up-regulation of HO-1 enzyme expression and activity.

Key words:

heme oxygenase-1, carbon monoxide, 5-aminosalicylic acid, mesalamine, inflammatory bowel disease, oxygen free radicals, antioxidants, aminosalicylates models of colitis

Introduction

The pioneering work of Sir John Vane identified the powerful inhibitory actions of aspirin and the non-steroid anti-inflammatory drugs on pro-inflammatory prostanoid biosynthesis [79], yet the full mechanistic profile of the salicylates themselves proved less easy to define [19, 20]. Many of the salicylates, including sodium salicylate, generally had very poor activity *in vitro*, with only very weak inhibitory properties on such systems as the cyclooxygenase enzymes, yet these agents clearly had anti-inflammatory actions *in vivo* [30]. Moreover, as with aspirin itself, its break-

down product salicylate, could reduce local prostanoid production at the site of inflammation [84]. Even today, this latter class of anti-inflammatory agent remains relatively poorly understood as regards their full pharmacological profile and underlying mechanisms. Indeed, salicylates may have yet-undefined properties that could further distinguish them from other classes of anti-inflammatory drug.

One widely used class of salicylates are the aminosalicylates, agents that are not generally utilized as anti-inflammatory drugs yet are the mainstay of the gastroenterologist for treatment of the inflammatory bowel diseases (IBD), ulcerative colitis and Crohn's

disease [27, 78]. This latter class may therefore have a unique profile of pharmacological properties that contribute to their effectiveness in colitis.

Aminosalicylates as anti-colitic drugs

Aminosalicylates are available as a range of commercial medicinal products formulated for either oral or colonic administration and have been used over many decades for the treatment of ulcerative colitis and Crohn's disease [57, 78]. 5-aminosalicylic acid (5-ASA) also known as mesalamine or mesalazine, is regarded as the active therapeutic moiety of the older but still widely used anti-colitic agent, sulfasalazine. Importantly, mesalamine use may also decrease the risk of colorectal cancer in patients with IBD [61, 73].

5-ASA has been pharmaceutically prepared as a range of clinically useful formulations that are enteric coated for controlled and delayed release, combined with an inert carrier or prepared as an azo-linked dimer for oral ingestion or prepared as enemas for rectal administration, procedures designed to yield high colonic levels [57, 64]. Indeed, even in the past few years, newer preparations of mesalamine that allow high sustained levels following daily oral dosing are still being developed, approved and launched [33, 35].

Previously defined mechanisms of action

The definitive mechanisms underlying the anti-colitic properties of the aminosalicylates have, however, still not been fully identified from the range of biochemical or pharmacological experimentation conducted over the past 25 years. Their complex biochemical and pharmacological profile include an ability to inhibit intestinal macrophage chemotaxis [52] and to reduce the secretion of antibodies from mononuclear cells [42]. Other work has demonstrated that 5-ASA can attenuate the release of pro-inflammatory cytokines [24] while earlier work has shown that aminosalicylates can inhibit the arachidonate lipoxygenase and cyclooxygenase pathways [28].

Molecular studies have more recently identified actions on the nuclear factor, NF κ B [17, 68]. Such ac-

tions, if elicited in the *in vivo* setting would be anticipated to reduce the biosynthesis of a range of pro-inflammatory mediators, including cytokines such as TNF- α . Other work has shown aminosalicylates to affect the peroxisome proliferator-activated receptor- γ (PPAR- γ), a nuclear receptor [60].

Along with all of these potentially beneficial pharmacological, biochemical and molecular activities, one enduring concept is that at least part of the therapeutic activity of the aminosalicylates reflects their actions as antioxidants and free radical scavengers [3, 5, 59, 65, 66].

Antioxidant activity of aminosalicylates

In addition to other key pathological factors including cytokine production, oxidative stress has long been considered to play a role in the tissue damage in colitis. Thus, the generation and release of local reactive oxygen species could be involved in the vascular, epithelial and mucosal inflammatory injury that underlies in colitis [16, 26, 46, 47, 56]. A defect in mucosal antioxidant defences is a potential etiological factor. Indeed, an imbalance between the local production of reactive oxygen species and the endogenous antioxidant defence mechanisms of the colon may be involved in the initiation and the aggravation of the local inflammatory process.

Aminosalicylates, notably 5-ASA, can exert antioxidant actions *in vitro*, and moreover, the findings from clinical studies on the nature of the mesalamine metabolites formed indicate that it exerts antioxidant effects *in vivo* following therapeutic dosing in colitic patients [2]. The direct antioxidant and radical scavenging properties of 5-ASA, which are attributed to its phenolic chemical structure, can be demonstrated in cell-free systems and in inflamed colonic tissue *in vitro*, but the concentrations required for such activity are often in the millimolar range [3, 5, 65, 66]. Although high, these concentrations can be achieved in the colonic lumen after therapeutic dosing of 5-ASA preparations and antioxidant effects are indeed observed following treatment with 5-ASA *in vivo* [2].

Studies in a rat model of colitis have also demonstrated that 5-ASA administration can alter oxidative parameters [23, 59]. However, it is also possible that such anti-oxidant properties of 5-ASA *in vivo* and in-

deed the therapeutic activity of the aminosalicylate preparations in patients with colitis may also reflect additional pharmacological actions to up-regulate endogenous anti-oxidant and anti-inflammatory systems.

HO-1 as an endogenous antioxidant system

One such endogenous system is the microsomal inducible enzyme, heme oxygenase-1 (HO-1; EC 1.14.99.3), which converts heme into biliverdin and produces carbon monoxide (CO) and free ferrous iron, the biliverdin product being subsequently reduced to bilirubin [1, 43, 44, 62, 74]. This 32 kDa heat shock protein (also termed hsp 32) can be expressed in numerous cell types following exposure to a number of different stimuli including heme or its Fe^{3+} oxidation product, hemin, nitric oxide, reactive oxygen species and heavy metals [44, 75] as illustrated in Figure 1. This induction of HO-1 can be rapid, a characteristic shared with other heat-shock proteins [67].

HO-1 can provide an endogenous protective antioxidant system related to the increase formation of biliverdin and bilirubin. Indeed, bilirubin has been considered as acting as an anti-oxidant of physiological importance [41, 43, 51, 69, 72].

Role of HO-1 in inflammation

Early work established that HO-1 was expressed at inflammatory sites and agents that could induce HO-1 could exert anti-inflammatory actions, while inhibition of HO-1 induction could exacerbate the inflammatory process [86, 87].

The mechanisms by which up-regulation of the heme oxygenase system can reduce inflammatory injury are likely to involve the end-products of the enzymatic reactions. Thus, the heme-oxygenase products, biliverdin and bilirubin (Fig. 1) as a consequence of their anti-oxidant properties can reduce lipid peroxidation and also have anti-complement activity [44, 51, 62]. In addition, these products of HO-1 activity

can attenuate mesenteric neutrophil rolling, adhesion and migration in acute inflammation [22].

Another important product of HO-1 activity, carbon monoxide (Fig. 1), also has anti-inflammatory properties, including down-regulating the expression of pro-inflammatory cytokines and affecting inflammatory cell function [6, 22, 25, 37, 48, 53, 54, 88]. Pharmacological studies using carbon monoxide-releasing molecules such as CORM-2 have shown a range of anti-inflammatory actions including modulating leukocyte-endothelial cell interactions [6, 14, 36, 45, 77].

Expression of HO-1 in colitis

The colonic expression of HO-1 and its enzyme activity has been demonstrated to be up-regulated in a range of models of colitis including that provoked by hapten, trinitrobenzene sulfonic acid (TNBS) in the rat [18, 39, 49, 81, 82] or mouse [34] and by dextran sulfate in the mouse [8, 49, 55]. Likewise, markers for HO-1 can be detected in samples of human colitic tissue [7, 55, 70]. The presence of HO-1 in such inflamed tissue may reflect a stress-gene related defence mechanism against the inflammatory insult and oxidative stress.

The mechanisms involved in promoting or causing the induction of HO-1 in the colitis model or clinical disease are not fully clear. However, it is feasible that

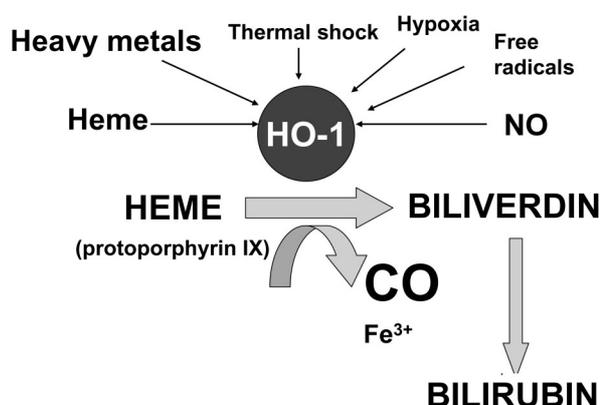


Fig. 1. The pathway of heme-oxygenase-1 (HO-1), an inducible heat shock protein. This diagram shows the primary compounds that induce enzymatic conversion of heme into the primary bio-active products, biliverdin, bilirubin and carbon monoxide (CO)

reactive oxygen species may themselves contribute as these moieties are established inducers of HO-1 [44]. Oxidative stress is thought to play a role in Crohn's disease and ulcerative colitis [40, 46], reactive oxygen species being formed in inflamed human colon [26, 31] and hence may also be involved in the colonic HO-1 expression observed in colitic patients [7, 55, 70]. The over-production of reactive oxygen moieties at the inflammatory site may overwhelm the usual endogenous antioxidant systems [40, 58] and HO-1 induction as an early onset cellular defensive response may thus help to re-address this balance and reduce the oxygen stress and minimize the resulting tissue disruption.

In addition to these reactive species, it is well established that the inducible nitric oxide synthase, iNOS is expressed in inflamed colonic tissue from colitic patients and from animal models of colitis [10, 38, 83]. Nitric oxide (NO) is another powerful stimulus of HO-1 induction, and the iNOS enzyme is capable of generating substantial levels of NO. Thus, the induction of HO-1 may act to offset the known deleterious actions of NO that results from a combination of NO and superoxide radicals to form peroxynitrite. Indeed, such a self-regulating action on these cytotoxic products resulting from the interactions of NO with oxygen species may help explain the somewhat equivocal pharmacological findings on the role of iNOS in gut inflammation and colitis [see 83 for review].

The inflammatory cell types that exhibit HO-1 expression include both neutrophils and macrophages, but other cells may also be involved. Thus, in a rat model of colitis, the early induction of colonic HO-1 within 90 minutes as well as the continued expression at 10 days, compared to the much slower time course of the changes in the neutrophil marker, myeloperoxidase, could argue against neutrophils being the sole source of HO-1 production in this model [32, 81]. Studies in the lung suggested that lipopolysaccharide-mediated HO-1 induction takes place both in inflammatory cells infiltrating the mucosa and the epithelial cells [12].

HO-1 expression has been demonstrated in the human intestinal epithelial cell lines Caco-2 and DLD-1, following exposure to heme and heavy metals [80]. A chalcone derivative has also been shown to induce HO-1 in the human epithelial intestinal cell line, HT-29 [39] as did the immunosuppressive fungal metabolite, gliotoxin [34]. In tissue obtained from colitic patients, expression of HO-1 could be observed in sur-

face and crypt epithelium, as well as in lamina propria inflammatory cells [7, 55]. In other work, there was an increase in the expression of both HO-1 mRNA and protein in the colonic mucosa of patients with ulcerative colitis, with mononuclear cells in the mucosa and submucosa staining for the presence of HO-1 [71].

Modulation of colitis by HO-1

A role for endogenous HO-1 in modulating colitis has been proposed based on the findings from pharmacological studies using heme oxygenase inhibitors. The agents used in these studies are metal derivatives of protoporphyrins, considered to inhibit heme oxygenase in a relatively selective manner [4]. Thus, tin protoporphyrin, in doses that reduced HO-1 activity, substantially augmented the colonic damage following intracolonic challenge with TNBS in the rat [81, 82]. The TNBS-provoked model of colitis has been used in large of studies in many laboratories to identify novel pathological processes involved in colitis, as well as for the evaluation of the pharmacological potential of novel anti-colitic agents [11, 85]. These findings on the potential endogenous role of HO-1 were confirmed using another heme oxygenase enzyme inhibitor, zinc protoporphyrin, which likewise augmented the extent of TNBS-provoked rat colitis as measured over a 10 day period [81].

This proposal of this protective role of HO-1 has been strengthened by pharmacological findings with agents that can induce HO-1 in models of colitis. Thus, HO-1 induction by cobalt protoporphyrin reduced the colonic inflammation caused by dextran sulfate in mice [8, 55], as well as the colitis seen in a Th1 cell-mediated murine model following IL-10 gene deletion [29]. Studies with both heme and the heavy metal cadmium chloride to induce HO-1 likewise attenuated the extent of inflammation in TNBS-induced rat colitis, as measured by tissue injury and inflammatory biomarkers [81]. Other agents that have been shown to induce HO-1 in the colon such as the synthetic somatostatin peptide, octreotide and the immunosuppressive gliotoxin can also attenuate TNBS-induced rat colitis [18, 34].

The mechanisms underlying the beneficial action of HO-1 are considered to reflect the overall pharmacological profile of actions of its products, particu-

larly the anti-oxidant, biliverdin and also carbon monoxide [9]. HO-1 activity may also evoke an increase in extracellular superoxide dismutase activity, which would hence contribute to the anti-oxidant events following HO-1 induction [76]. The administration of biliverdin can attenuate dextran-induced colitis in mice, through a process considered to involve HO-1 [8]. Although carbon monoxide alone did not alter the colitis in that latter study, other work has found that daily administration of carbon monoxide affected the inflammation in a IL-10 gene-deleted model of colitis [29]. Such actions could involve local vasodilatation or the ability of carbon monoxide to modulate adhesion molecules [54], as observed in the small bowel in transplantation studies. Such work also suggested that these actions of carbon monoxide could act in concert with the profile of properties of biliverdin [50]. It is feasible that these end-products of HO-1 activity may also provide distinct, but interactive, bio-protective processes that ameliorate the colitis.

HO-1 induction by 5-ASA

In early *in vitro* studies using the human intestinal epithelial cells, Caco-2 and DLD-1, the expression of the mRNA for HO-1 was shown to be stimulated by low concentrations of the aminosalicylates, 5-ASA or sulfasalazine in the micromolar range [13].

To study the possibility that the aminosalicylates could exert their beneficial properties *in vivo* through induction or activation of HO-1, the effects of the compound administered directly into the rat colon *in vivo* on the HO system were determined under control conditions. Moreover, the actions of 5-ASA on colonic heme oxygenase enzyme activity and HO-1 protein expression were evaluated in a model of colitis provoked by colonic instillation of TNBS [32].

Studies on the unchallenged rat colon following daily intracolonic administration of 5-ASA demonstrated a significant increase in HO-1 protein expression as well as in heme oxygenase enzyme activity [32]. Such direct actions of 5-ASA on colonic HO-1 in the absence of invading inflammatory cells and detectible tissue injury suggest another class of HO-1 inducers, in addition to the known range of compounds including the substrate, heme, and the more classical

agents such as heavy metals, NO donors and free radicals themselves.

The processes by which the induction of this heat shock protein, HO-1, is brought about are not yet known. 5-ASA is an agent not considered to damage the colon or to cause any cellular injury in either ex-

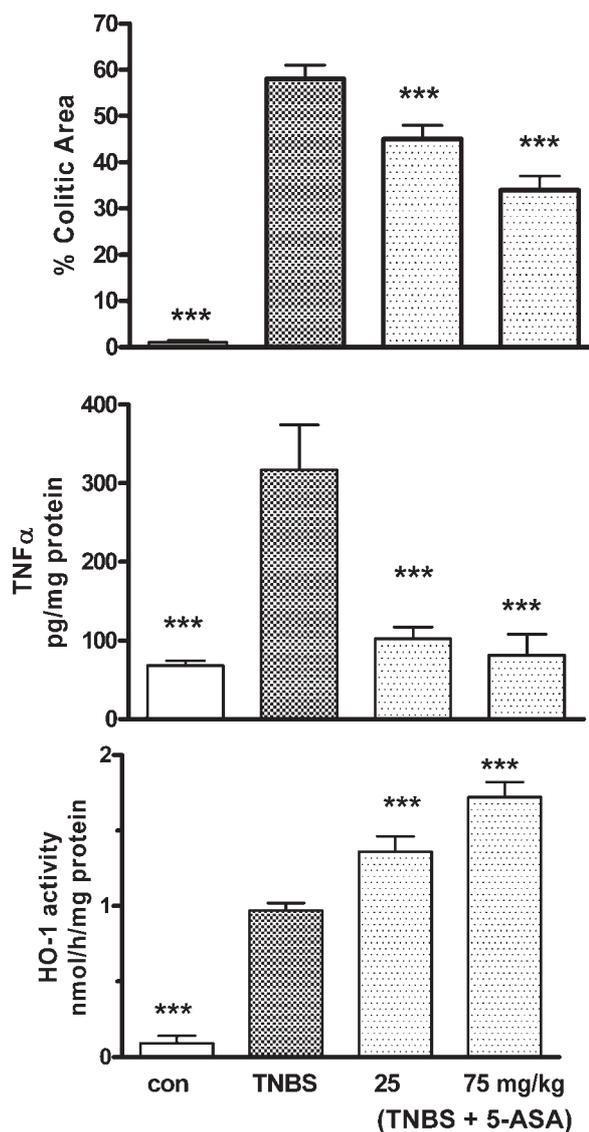


Fig. 2. Dose-dependent effects of intracolonic administration of 5-ASA (25 and 75 mg/kg per day; 48 h) on the area of macroscopic colonic inflammatory lesions (upper panel; expressed as % of the total colonic area), the expression of TNF- α in colon tissue (middle panel; expressed as pg/mg protein) and HO-1 activity in colon tissue (lower panel; expressed as nmol/h/mg protein) in a rat model of TNBS-induced colitis. Results are shown as the mean \pm SEM ($n = 6-8$ in each group). *** $p < 0.001$ denotes significance compared to the TNBS-alone group, con = control. This graph has been adapted from Horvath et al. [32]

perimental models or in its therapeutic use over the past years. Recent work has suggested that 5-ASA can act as a agonist at the nuclear receptor, PPAR- γ [60] and hence it is relevant that the known PPAR- γ agonist, rosiglitazone can also modulate TNBS colitis [63]. However, the possible involvement of PPAR- γ receptor activation in HO-1 mRNA or protein expression in this system with 5-ASA is still unknown.

In addition to these effects on the colon under these control conditions, significant effects were also observed in the colitis model. Thus, intracolonic daily doses of 5-ASA were found to be effective in modulating the inflammatory response (Fig. 2). A significant reduction of macroscopic lesion area and its severity in terms of a macroscopic score, as well as an index of edema, was observed, along with a reduction in colonic myeloperoxidase levels, an index of neutrophilic infiltration [32]. The increased colonic levels of the inflammatory biomarker, TNF- α following TNBS challenge was likewise significantly inhibited by 5-ASA treatment (Fig. 2).

As with the up-regulation of HO activity by daily subcutaneous administration of heme, which had been shown to reduce the extent of colonic injury in this model over a 2–3 day period [81], daily intracolonic administration of 5-ASA in anti-colitic doses increased HO-1 activity in the inflamed colon [32]. The higher dose of 5-ASA used caused a two-fold increase in heme oxygenase enzyme activity in the colon and a significant increase in the colonic expression of HO-1 protein [32]. This indicated that the increased heme oxygenase enzyme activity was probably a reflection of the production of new protein rather than simply a local co-factor or facilitatory action of 5-ASA on existing enzyme. Whether elevated HO-1 mRNA levels or transcription could also contribute to the overall process by which the up-regulation of heme oxygenase activity is elicited is currently not known.

To probe further the mechanism of action of 5-ASA in this colitis model, the effects of the heme oxygenase enzyme inhibitor zinc protoporphyrin, was evaluated on the responses to intracolonic 5-ASA. This compound abolished the elevation by 5-ASA of the HO-1 activity on the inflamed colon. Moreover, this heme oxygenase inhibitor reversed the anti-colitic effects of 5-ASA in the rat model, as shown by the increased colonic area of injury and the myeloperoxidase levels [32].

Conclusions

These recent findings thus suggest that 5-ASA can exert its colonic anti-inflammatory effects, at least in part, through the up-regulation of colonic HO-1 activity *in vivo*. It has been shown that HO-1 mRNA or protein expression can be detected in human intestinal cells lines, in human colitic tissue and in the invading inflammatory cells, while increased colonic luminal carbon monoxide can be detected in colitic patients [7, 13, 15, 55, 70, 71, 80]. This latter induction of HO-1 under challenged conditions may be indicative of a response by this early-response gene to provide a stress-driven protective process that limits the extent of tissue injury. Such a pathophysiological defensive action appears to be mimicked or enhanced by the administration of the aminosalicylates.

Further work will establish whether the recent findings *in vivo* in the rat colon following treatment with 5-ASA [32] can be translated into an understanding of the therapeutic effects of the various 5-ASA preparations in colitic patients. Some support for this concept comes from our earlier *in vitro* studies which have demonstrated that, the expression of the mRNA for HO-1 is stimulated by micromolar concentrations of 5-ASA and sulfasalazine in human intestinal epithelial cell lines [13]. Indeed, the concentrations required for such effects on HO-1 appear to be far lower than many of the previously reported actions *in vitro*, including effects of the nuclear factor NF κ B.

These current findings thus point to the need for further investigation into the mechanisms of action underlying the ability of 5-ASA to up-regulate the HO-1 system. It would appear worthwhile to evaluate the structure-activity relationship of other aminosalicylates and structurally related compounds for such promotor actions on mRNA, HO-1 protein and enzyme activity on colonic tissue through non-toxic processes. It may also be of some interest to explore the actions of aminosalicylates in other tissues including the vasculature, where HO-1 is also considered to play a key role in the provision of protective and vasoactive products [21, 77]. All such studies could yield additional pharmacological and molecular targets for the rational design of novel aminosalicylates for the effective treatment of inflammatory bowel disease and possibly, for the concomitant lowering the risk of colorectal cancer associated with this disease.

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