Ph.D. thesis

STUDY ON AN INFLAMMATORY BOWEL DISEASE COLITIS MODEL

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INTRODUCTION

The pathogenesis of the inflammatory bowel diseases (IBD), ulcerative colitis and Crohn's disease is still incompletely understood. It is likely that pro-inflammatory cytokine and chemokine release and derangement of immune responses, along with genetic factors, play a role in the inflammatory processes.

In addition to these factors, it has long been speculated that the local release of reactive oxygen species may also be involved, creating epithelial and vascular injury in the colon. It is therefore possible that endogenous protective antioxidant systems could be evoked in order to attenuate colonic tissue injury under such conditions.

One such system is the microsomal inducible enzyme, heme oxygenase-1 (HO-1), which converts heme into biliverdin, carbon monoxide (CO) and free ferrous iron, the biliverdin product beeing subsequently reduced to bilirubin. HO-1 can provide an endogenous protective anti-oxidant system related to the increase in biliverdin and bilirubin, which possess anti-oxidant and anti-complement activity and which can reduce lipid peroxidation.

Preparations of aminosalicylates, exemplified by 5-aminosalicylic acid (5-ASA), for oral and colonic administration have been used over many decades for the treatment of the inflammatory bowel diseases, ulcerative colitis and Crohn's disease. Despite its long term and wide-spread use, the mechanisms underlying the anti-colitic actions of 5-ASA have not been fully identified. However, an enduring concept is that at least part of the beneficial activity of 5-ASA reflects its actions as an anti-oxidant and free radical scavenger. Thus, the generation and release of local reactive oxygen species have long been considered to be involved in the vascular, epithelial and mucosal inflammatory injury in colitis and hence scavenging these moieties offers a potential mechanism of action of 5-ASA.

The present study investigates whether the beneficial effects in vivo could involve induction of heme oxygenase-1, known to provide endogenous anti-

oxidant and anti-inflammatory moieties which can modulate colonic inflammation. The therapeutic activity in colitis may also reflect additional pharmacological actions to up-regulate endogenous anti-oxidant and anti-inflammatory systems.

In our work we have studied a well-known colitis model to know more information about the defence mechanisms, mainly the HO enzyme system in the inflammatory processes.

AIMS

In our experiments we used a widespread colitis model, the 2,4,6 trinitrobenzene sulphonic acid (TNBS) model in rats.

In the first part of the study we examined

- the time dependence of HO activity, and HO-1 expression
- the effect of HO-1 inducers (heme, cadmium chloride), and a HO-1 inhibitor (zinc protoporphyrin-ZnPP) on the colonic inflammatory damage (lesion, myeloperoxidase (MPO) activity).

In the next part we studied the effect of 5-aminosalicylic acid (5-ASA), the active therapeutic moiety of a number of clinically used anti-colitic agents on

- the TNBS induced colonic inflammatory damage (lesion, colon weight, MPO activity, tumour necrosis factor-α (TNF-α) levels).
- the HO-1 enzyme expression and on the heme oxygenase activity.
- Finally we examined the effects of a heme oxygenase inhibitor, ZnPP on the effect of 5-ASA. We have measured the colonic inflammatory damage (lesion, MPO activity) as well as the HO activity.

METHODS

Induction of colitis and pharmacological treatments

Male Wistar rats (200-250 g) were randomised before commencement of the study, housed in groups and inspected and weighed every day. Food was withdrawn overnight for 12h prior to TNBS administration only, but the rats were allowed free access to drinking water. Under transient ether-induced anaesthesia, 2,4,6 trinitrobenzene sulphonic acid (TNBS; 30 mg or 10 mg in 250 µl 50 %-os ethanol, v/v) was administered intra-rectally through an 8 cm long soft plastic catheter. The distal colon was dissected, photographed, processed and stored appropriately for subsequent analyses at different time points.

Heme (30 µmol/kg), zinc protoporphyrin (ZnPP; 50 µmol/kg), or cadmium-chloride ($CdCl_2$; 2 mg/kg) were administered subcutaneously (s.c.), 18 h before TNBS (30mg) challenge, again at time of TNBS instillation and then once daily for 1 to 10 days. Heme and ZnPP were dissolved in sodium hydroxide (0,1 N) and sodium chloride (0,9 %) and pH adjusted to pH 7.4, with the concentration of the stock solutions being 0,5 mg/ml. Cadmium chloride was dissolved in distilled water.

5-Aminosalicylic acid (5-ASA; 8.3, 25, 75 mg/kg) prepared freshly in 1% carboxymethylcellulose (CMC), was administered once daily in a volume of 0,25 ml into the colon, 24 h and 3 h before TNBS challenge and 24 h after TNBS challenge. In studies on the effects of heme oxygenase inhibitor, zinc protoporphyrin (ZnPP) was administered at a dose of 50 µmol/kg, s.c. 18 h before TNBS challenge, again at time of TNBS instillation and 24 h after TNBS challenge.

Macroscopic and enzymatic assessment of the severity of colitis

Measurement of macroscopic colonic inflammatory damage

In all experimentel groups, the distal 8 cm portion of the colon (measured from the rectum) was removed, opened longitudinally and gently rinsed with ice-cold phosphate buffer (pH 7.4), blotted, weighed (Scaltec, Germany) and photographed (Samsung, Digimax 340, digital camera). The extent of macroscopically apparent inflammation, ulceration and tissue disruption was determined in a randomised manner from the colour images via computerised planimetry (Scion Image B4.02 version; Scion Corp.). The area of macroscopically visible mucosal involvement was calculated and expressed as the percentage of the total colonic segment area under study.

In all experimental groups, immediately after photography, the 8 cm long segment of the distal colon were cut longitudinally into 8 cm long strips. These strips included both the macroscopically inflamed and non-inflamed areas and were used to determine myeloperoxidase (MPO) and heme oxygenase (HO) enzyme activity and HO-1 protein expression.

Myeloperoxidase activity

MPO levels were measured as an index of neutrophilic infiltration. MPO was expressed as mU mg/protein.

Tumour necrosis factor-α levels

The TNF-α levels were determined with quantitative TNF-α solid-phase Enzyme Linked ImmunoSorbent Assay (ELISA). The samples were measured spectrophotometrically at 450nm and were diluted with the buffer included in the assay kit (Hycult Biotechnology d.v. 5405 Uden, The Netherlands). The TNF-α values were expressed as pg/mg protein.

Western blotting for HO-1 expression

Heme oxygenase-1 expression was measured by Western blotting technics. Results were determined by densitometry of the Western blots.

Heme oxygenase activity

Heme oxygenase activity was assessed by measuring bilirubin formation. One unit of heme oxygenase activity was defined as the amount of bilirubin (nmol) produced per hour per mg protein.

Protein determination

We have used Bradford's method. Protein level was expressed as mg protein/ml.

Statistical analysis

For statistical comparisons, the two-tailed Student's *t*-test and the analysis of variance with the Bonferroni test were used.

RESULTS AND DISCUSSION

In the present study, transitory increase in HO-1 expression and heme oxygenase activity was observed in animals challenged with 50% ethanol, the vehicle used for the TNBS challenge. Such ethanol –induced actions may help explain the early biphasic response of the HO-1 enzyme activity following TNBS challenge. Moreover, these findings with ethanol could also suggest that HO-1 induction could be a general stress-driven pathway induced by challenge with a range of injurious substances although this will need to be explored further.

Daily administration of the endogenous substrate of heme oxygenase and the known HO-1 inducer, heme, caused a substantial induction in HO-1 and heme oxygenase enzyme activity. This was accompained by a significant decrease in the macroscopically apparent mucosal damage in the colon, and a decrease in colonic MPO activity. Similar colonic protective actions to heme in the TNBS model were observed with the heavy metal, cadmium chloride in the current work. Whether the observed effect of heme could reflect a patho-physiological feed-back mechanism involving these endogenous agents, along with reactive oxygen species, to promote HO-1 activity and hence to offset the inflammatory process, is not yet known.

In contrast to the effects of HO-1 induction, pharmacological approaches to reduce HO-1 activity by the administration of zinc protoporphyrin, a non-physiological metalloporphyrin, have shown a substantial aggravation of the colonic inflammation, with colonic mucosal damage being greater than 80% of the area studied. This treatment also provoked a concomitant increase in MPO activity. This inhibition of heme oxygenase activity and concurrent detrimental effects of ZnPP were seen over the full 10 days treatment period. These findings would suggest that the pathophysiological induction of HO-1 in colitis does

indeed play a role in modulating the severity of the colonic inflammation over both acute and chronic periods.

Intracolonic daily doses of 5-ASA of 8,3 to 75 mg/kg, commencing 24 h prior to challenge with TNBS were found to be effective. To see better effect we decreased the dose of TNBS. Dose of TNBS of 10 mg caused significant macroscopic damage, but the animals showed more sensitivity to the 5-ASA treatment. A significant reduction of macroscopic lesion area and its severity in terms of a macroscopic score, as well as a fall in the elevated colon weight as an index of oedema, was observed with all doses of 5-ASA. In addition, a reduction in colonic MPO levels, an index of neutrophilic infiltration, as well as the inflammatory biomarker, TNF-α was achieved at the two higher dose levels of 5-ASA investigated.

Intracolonic administration of 5-ASA in anti-colitic doses caused a dose-dependent increase in heme oxygenase activity in the inflamed colon, with the higher dose of 5-ASA causing an approximately two-fold increase in heme oxygenase activity. This dose of 5-ASA likewise caused a significant increase in the colonic expression of HO-1 protein in the TNBS-challenged rats, indicating 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, although this awaits direct evaluation.

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. Such direct actions of 5-ASA on the colonic heme oxygenase int he absence of invading inflammatory cells and detectible tissue injury suggest another class of inducers, in addition to the known range compounds including the substrate, heme, and the more classical agents such as heavy metals, nitric oxide donors and free radicals themselves. The processes by with such induction of this heat shock protein is brought about are not yet known.

Administration of ZnPP, in doses that inhibited HO-1 under these conditions, prevented the therapeutic effect of 5-ASA, as indicated by the abolition of the reduction in damage area and the colonic MPO levels. Although non-specific actions of this heme oxygenase inhibitor on colonic injury or on the action of 5-ASA cannot yet be excluded, these findings support the suggestion that the beneficial property of 5-ASA in this model involves the activity of HO-1.

SUMMARY

Our present study has confirmed that the endogenous anti-oxidant and anti-inflammatory enzyme, HO-1 is induced in the TNBS model of colitis in rats. This expression of HO-1 was further enhanced by the administration of heme, and cadmium chloride, which attenuated the extent of colonic damage and inflammation. In contrast, down regulation of HO-1 activity with a known inhibitor of such activity, zinc protoporphyrin, evoked an increase in inflammation and colonic damage.

Our results thus suggest that 5-ASA could exert its colonic anti-inflammatory effect, at least in part, through the up-regulation of colonic HO-1 activity. However, whether these present findings *in vivo* in the rat colon following treatment with 5-ASA can be translated into an understanding of the therapeutic effects of the various 5-ASA preparations in colitic patients awaits further study. However, earlier preliminary *in vitro* studies have demonstrated in human colonic epithelial cells that HO-1 mRNA expression is stimulated by micromolar concentrations of 5-ASA.

These current findings thus prompt for further investigation into the mechanisms of action underlying the ability of 5-ASA to affect the HO-1 system. Such studies could thus yield additional pharmacological and molecular targets for rational improvement on the classical aminosalicylates for the therapy of colitis.

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