

# Calpain inhibitor I reduces colon injury caused by dinitrobenzene sulphonic acid in the rat

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## Abstract

**Background and aims**—Inflammatory bowel disease is characterised by oxidative and nitrosative stress, leucocyte infiltration, upregulation of expression of intercellular adhesion molecule 1 (ICAM-1), and upregulation of P-selectin in the colon. The aim of the present study was to examine the effects of calpain inhibitor I in rats subjected to experimental colitis.

**Methods**—Colitis was induced in rats by intracolonic instillation of dinitrobenzene sulphonic acid (DNBS).

**Results**—Rats experienced haemorrhagic diarrhoea and weight loss. Four days after administration of DNBS, the mucosa of the colon exhibited large areas of necrosis. Neutrophil infiltration (determined by histology as well as by an increase in myeloperoxidase activity in the mucosa) was associated with upregulation of ICAM-1 and P-selectin as well as high tissue levels of malondialdehyde. Immunohistochemistry for nitrotyrosine and poly (ADP-ribose) polymerase (PARP) showed intense staining in the inflamed colon. Staining of sections of colon obtained from DNBS treated rats with an anti-cyclooxygenase 2 antibody showed diffuse staining of the inflamed tissue. Furthermore, expression of inducible nitric oxide synthase was found mainly in macrophages located within the inflamed colon of DNBS treated rats. Calpain inhibitor I (5 mg/kg daily intraperitoneally) significantly reduced the degree of haemorrhagic diarrhoea and weight loss caused by administration of DNBS. Calpain inhibitor I also caused a substantial reduction in (i) degree of colon injury, (ii) rise in myeloperoxidase activity (mucosa), (iii) increase in tissue levels of malondialdehyde, (iv) increase in staining (immunohistochemistry) for nitrotyrosine and PARP, as well as (v) upregulation of ICAM-1 and P-selectin caused by DNBS in the colon.

**Conclusion**—Calpain inhibitor I reduces the degree of colitis caused by DNBS. We propose that calpain inhibitor I may be useful in the treatment of inflammatory bowel disease.

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**Keywords:** calpain; calpain inhibitor I; cyclooxygenase; nitric oxide; inflammatory bowel disease; rat

dependent on intracellular calcium levels. To date, two isoforms of calpain have been identified: calpain I (or  $\mu$ -calpain) and calpain II (or  $m$ -calpain), which require low and high micromolar concentrations of calcium for their activation, respectively.<sup>1,2</sup> Following activation by calcium, calpain cleaves a specific subset of cellular proteins, including cytoskeletal proteins, membrane receptors, calmodulin binding proteins, G proteins, protein kinase C (and other enzymes involved in signal transduction), and many transcription factors, including nuclear factor  $\kappa$ B (NF- $\kappa$ B).<sup>3</sup> For example, calpain I inhibitor reduces the degradation of I $\kappa$ B (I $\kappa$ B $\alpha$  or I $\kappa$ B $\beta$ ) in the proteasome and hence prevents the translocation of NF- $\kappa$ B from the cytosol into the nucleus.<sup>4-7</sup> Thus calpain inhibitor I prevents the expression (for example, after exposure to endotoxin) of many NF- $\kappa$ B dependent genes, including those for the inducible isoforms of nitric oxide synthase (iNOS)<sup>8-10</sup> and cyclooxygenase (COX-2).<sup>11,12</sup>

Reactive oxygen species (ROS) and nitrogen species have been implicated as mediators of disruption of the intestinal barrier in inflammatory bowel diseases (IBDs) but their molecular targets and pathways have not been defined.<sup>13-19</sup> In addition to ROS, overproduction of nitric oxide (NO) due to expression of iNOS also plays an important role in various models of inflammation.<sup>20,21</sup> NO has been postulated to play a dual role in the gastrointestinal tract. Continuous release of NO from a constitutive NO synthase, which is located in intestinal epithelial and lamina propria cells, neuronal terminals, and endothelial cells, is involved in the physiological maintenance of motility, tone, permeability, and tissue blood flow.<sup>22</sup> On the other hand, overwhelming production of NO by iNOS have been postulated to have a pathological role in IBD. Numerous clinical reports have demonstrated elevated levels of nitrite in rectal dialysates and increased iNOS activity in colon biopsies of patients affected by Crohn's diseases or ulcerative colitis.<sup>16,15,22,23</sup> Animal models of IBD have provided evidence that colitis is associated with increased expression or activity of iNOS and that inhibitors of NOS activity have anti-inflammatory effects.<sup>24-28</sup> Recent data suggest that the damaging effects of

**Abbreviations used in this paper:** NF- $\kappa$ B, nuclear factor  $\kappa$ B; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; ROS, reactive oxygen species; IBD, inflammatory bowel disease; NO, nitric oxide; PARP, poly (ADP-ribose) polymerase; DNBS, dinitrobenzene sulphonic acid; PBS, phosphate buffered saline; ICAM-1, intercellular adhesion molecule 1; MPO, myeloperoxidase; PMN, polymorphonuclear leucocyte; MDA, malondialdehyde.

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The neutral protease calpain is one of the many intracellular proteins the activity of which is

NO in various forms of inflammation are mediated, at least in part, by peroxynitrite, a potent oxidant produced by the reaction of NO and superoxide anion.<sup>29–30</sup> Peroxynitrite is cytotoxic via a number of independent mechanisms, including (i) initiation of lipid peroxidation, (ii) inactivation of a variety of enzymes (most notably mitochondrial respiratory enzymes and membrane pumps),<sup>31</sup> and (iii) depletion of glutathione.<sup>32</sup> Moreover, peroxynitrite can also cause DNA damage<sup>33–34</sup> resulting in activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP; also known as poly(ADP-ribose) synthetase, PARS), depletion of NAD and ATP, and ultimately cell death.<sup>35</sup> Indeed, DNA from colon biopsy specimens of patients with ulcerative colitis has significantly increased levels of 8-hydroxyguanine, 2-hydroxyadenine, 8-hydroxyadenine, and 2,6-diamino-5-formamidopyrimidine.<sup>36–37</sup> Activation of PARP may also play an important role in various experimental models of inflammation, including colitis.<sup>38–42</sup> In this condition, pharmacological inhibition or gene targeted disruption of PARP appears to exert beneficial effects.<sup>43–47</sup>

The complex role of NO in the physiology and pathology and the cytotoxic potential of ROS in the gastrointestinal tract have directed research to the investigation of pharmacological tools to neutralise the cytotoxic effects of NO and/or ROS without interfering with the physiological roles of NO.<sup>48</sup>

There is evidence that inhibition of calpain I activity reduces the injury associated with ischaemia-reperfusion of the brain,<sup>49–51</sup> liver,<sup>52</sup> and heart.<sup>53–56</sup>

Here we investigate the effects of calpain inhibitor I on the inflammatory response (colitis) caused by intracolonic administration of 2,4,6-dinitrobenzene sulphonic acid (DNBS). In particular, we investigated the effects of calpain inhibitor I on the colon injury associated with DNBS induced colitis. In order to gain a better insight into the mechanism of action of calpain inhibitor I, we also investigated its effects on expression of iNOS, and COX-2 protein (immunohistochemistry) and activity, and peroxynitrite formation and activation of the nuclear enzyme PARP (by immunohistochemistry).

## Methods

### ANIMALS

Male Sprague-Dawley rats (300–350 g; Charles River, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purpose (DM 116192) as well as with EEC regulations (OJ of ECL 358/1 12/18/1986)

### EXPERIMENTAL GROUPS

In the treated group of animals, calpain inhibitor I was given daily as an intraperitoneal bolus of 5 mg/kg (DNBS+Calp group). In the vehicle treated group of rats, vehicle (saline) was given instead of calpain inhibitor I (DNBS group). In

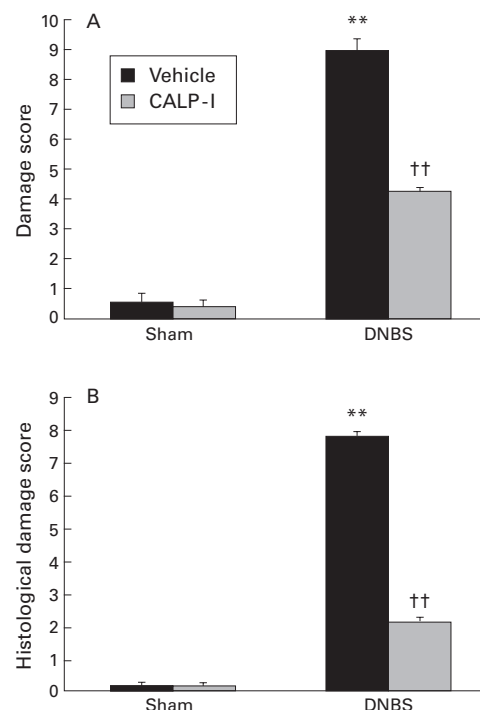


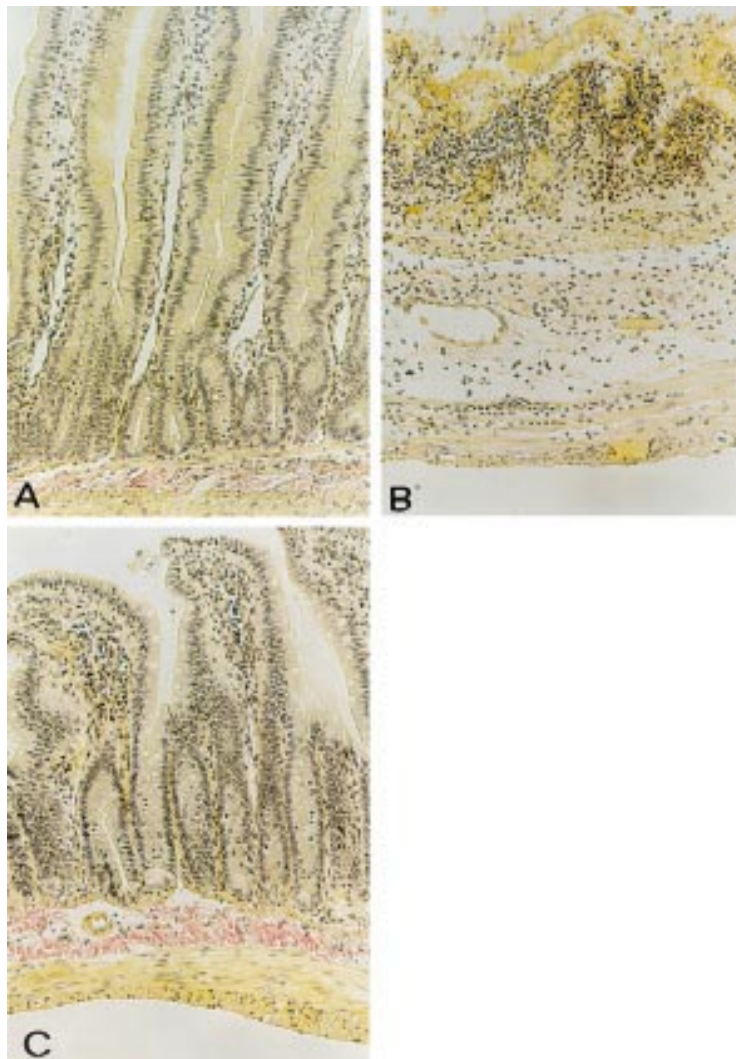
Figure 1 Effects of calpain inhibitor I (CALP-I) on the macroscopic damage score (A) and microscopic histological score (B). Colon damage was scored by two independent observers. Values are mean (SEM) of data obtained from 10 rats in each group. \*\* $p < 0.01$  v sham; †† $p < 0.01$  v dinitrobenzene sulphonic acid (DNBS).

separate groups of rats, surgery was performed in an identical manner as in the DNBS group except that saline was injected instead of DNBS (sham group). In an additional group of animals, sham surgery was combined with administration of calpain inhibitor I (dose as above) (sham+Calp).

### INDUCTION OF EXPERIMENTAL COLITIS

Colitis was induced using the technique of acid induced colon inflammation, as described previously.<sup>27</sup> In fasted rats lightly anaesthetised with isoflurane, a 3.5 F catheter was inserted into the colon via the anus until approximately the splenic flexure (8 cm from the anus). DNBS (25 mg/rat) was dissolved in 50% ethanol (total volume 0.8 ml). Thereafter the animals were kept for 15 minutes in a Trendelenburg position to avoid reflux. Seven animals (sham colitis) received an enema with vehicle alone (50% ethanol, 0.8 ml). After colitis and sham colitis induction, the animals were observed for three days. On day 4, the animals were weighed and anaesthetised with chloral hydrate (400 mg/kg intraperitoneally), and the abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, rinsed, weighed, and processed for histology and immunohistochemistry. In an additional experiment, colitis and sham colitis were induced in 28 rats (seven animals/group). Animals were monitored for evaluation of mortality for seven days.

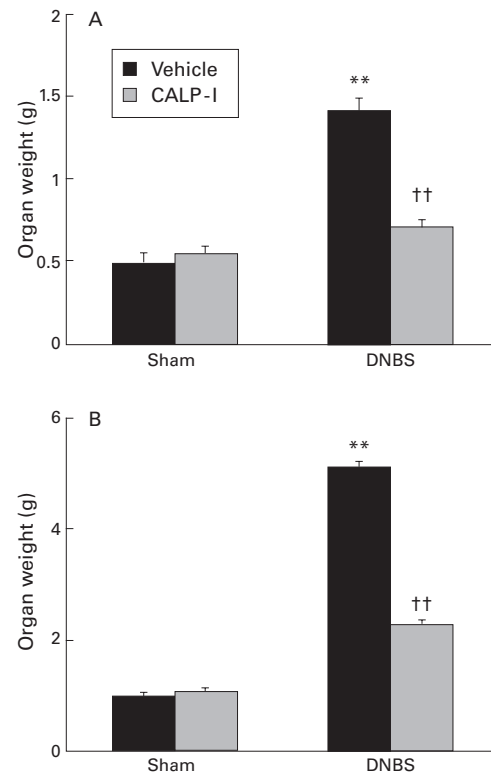
The macroscopic damage score was assessed according to Wallace and colleagues.<sup>57</sup>



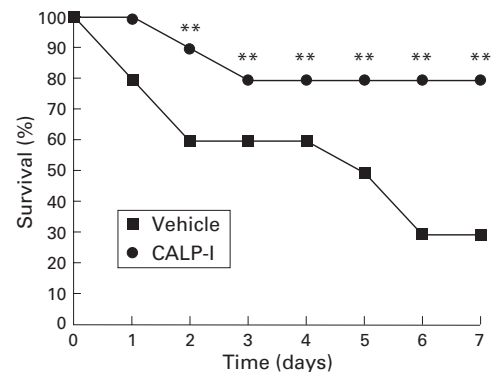
**Figure 2** Effects of calpain inhibitor I on colon injury. No histological modification was observed in the mucosa of sham operated rats (A). Mucosal injury was produced after dinitrobenzene sulphonic acid (DNBS) administration, characterised by the absence of epithelium and massive mucosal and submucosal infiltration with inflammatory cells (B). Treatment with calpain inhibitor I (C) corrected the disturbances in morphology associated with DNBS administration. Original magnification  $\times 120$ . The figure is representative of at least three experiments performed on different experimental days.

#### LIGHT MICROSCOPY

After fixation for one week at room temperature in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, New Jersey, USA). Thereafter, 7  $\mu\text{m}$  sections were deparaffinised with xylene, stained with haematoxylin-eosin and trichromic van Gieson's stain, and evaluated with a Dialux 22 Leitz (Wetzlar, Germany) microscope. Colon damage was scored by two independent observers, as described previously,<sup>58,59</sup> according to the following morphological criteria: 0, no damage; 1, localised hyperaemia without ulcers; 2, linear ulcers with no significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending  $>1$  cm along the length of the colon; and 6–8, one point is added for each centimetre of ulceration beyond an initial 2 cm.



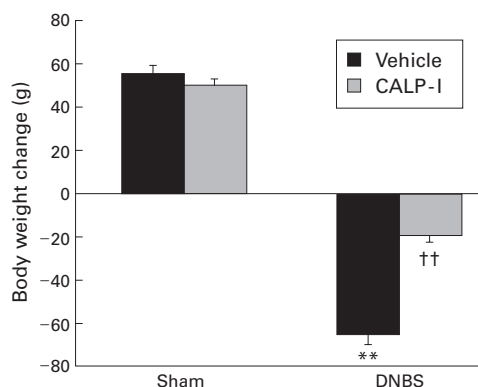
**Figure 3** Organ weight. A significant increase in weight in the spleen (A) and colon (B) was consistently seen four days after dinitrobenzene sulphonic acid (DNBS) injection. The weight of the organs was significantly reduced in rats who had been treated with calpain inhibitor I (CALP-I). Values are mean (SEM) of 10 rats in each group. \*\* $p < 0.01$  v sham; †† $p < 0.01$  v DNBS.



**Figure 4** Effect of calpain inhibitor I (CALP-1) treatment on dinitrobenzene sulphonic acid (DNBS) induced mortality. Survival is significantly improved in CALP-1 treated rats in comparison with the high mortality rate of the DNBS treated rats.  $n=10$  rats in each group. \*\* $p < 0.01$  v DNBS.

#### LOCALISATION OF NITROTYROSINE, PARP, P-SELECTIN, ICAM-1, iNOS, AND COX-2 BY IMMUNOFLUORESCENCE

Indirect immunofluorescence staining was performed on 7  $\mu\text{m}$  thick sections of unfixed colon of the rat. Sections were cut with a SLEE and London cryostat at  $-30$   $^{\circ}\text{C}$ , transferred onto clean glass slides, and dried overnight at room temperature. Sections were permeabilised with acetone at  $-20$   $^{\circ}\text{C}$  for 10 minutes and rehydrated in phosphate buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.2) at room temperature for 45 minutes. Sections



**Figure 5** Effect of calpain inhibitor I (CALP-I) treatment on changes in body weight four days after dinitrobenzene sulphonic acid (DNBS) intracolonic administration. Body weight was recorded immediately before DNBS administration and at the end of the experimental period. CALP-I treatment significantly prevented the loss in body weight. Values are mean (SEM) of 10 rats in each group. \*\* $p < 0.01$  v sham; †† $p < 0.01$  v DNBS.

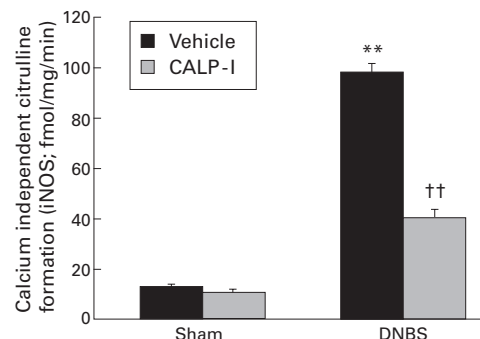
were coincubated overnight with: (1) antinitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v) or with anti-poly (ADP-ribose) goat polyclonal antibody rat (1:500 in PBS, v/v); (2) primary anti-iNOS antibody (1:500 in PBS, v/v) or anti-COX-2 antibody (1:500 in PBS, v/v); or (3) rabbit antihuman polyclonal antibody directed at P-selectin (CD62P) which reacts with rat and mouse antirat antibody directed at intercellular adhesion molecule 1 (ICAM-1) (CD54) (1:500 in PBS, v/v) (DBA, Milan, Italy). Sections were washed with PBS and co-incubated with secondary antibody (TRITC conjugated antigoat) and FITC conjugated antirabbit (Jackson, West Grove, Pennsylvania, USA) antibody (1:80 in PBS, v/v) for two hours at room temperature. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with a Nikon RCM8000 confocal microscope equipped with a 40 $\times$  oil objective.

#### MYELOPEROXIDASE ACTIVITY

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leucocyte (PMN) accumulation, was determined as previously described.<sup>60</sup> Four days after intracolonic injection of DNBS the colon was removed and weighed. The colon was homogenised in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 minutes at 20 000  $g$  at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H<sub>2</sub>O<sub>2</sub>. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1  $\mu$ mol of peroxide/minute at 37°C and was expressed in milliunits per gram weight of wet tissue.

#### MALONDIALDEHYDE (MDA) MEASUREMENT

Levels of malondialdehyde (MDA) in the colon were determined as an indicator of lipid peroxidation.<sup>61</sup> Four days after intracolonic injection the colon was removed, weighed, and



**Figure 6** Effect of calpain inhibitor I (CALP-I) treatment on inducible nitric oxide synthase (iNOS) activity four days after dinitrobenzene sulphonic acid (DNBS) intracolonic administration. DNBS administration induced a significant increase in iNOS activity. CALP-I treatment significantly reduced iNOS activity. Values are mean (SEM) of 10 rats in each group. \*\* $p < 0.01$  v sham; †† $p < 0.01$  v DNBS.

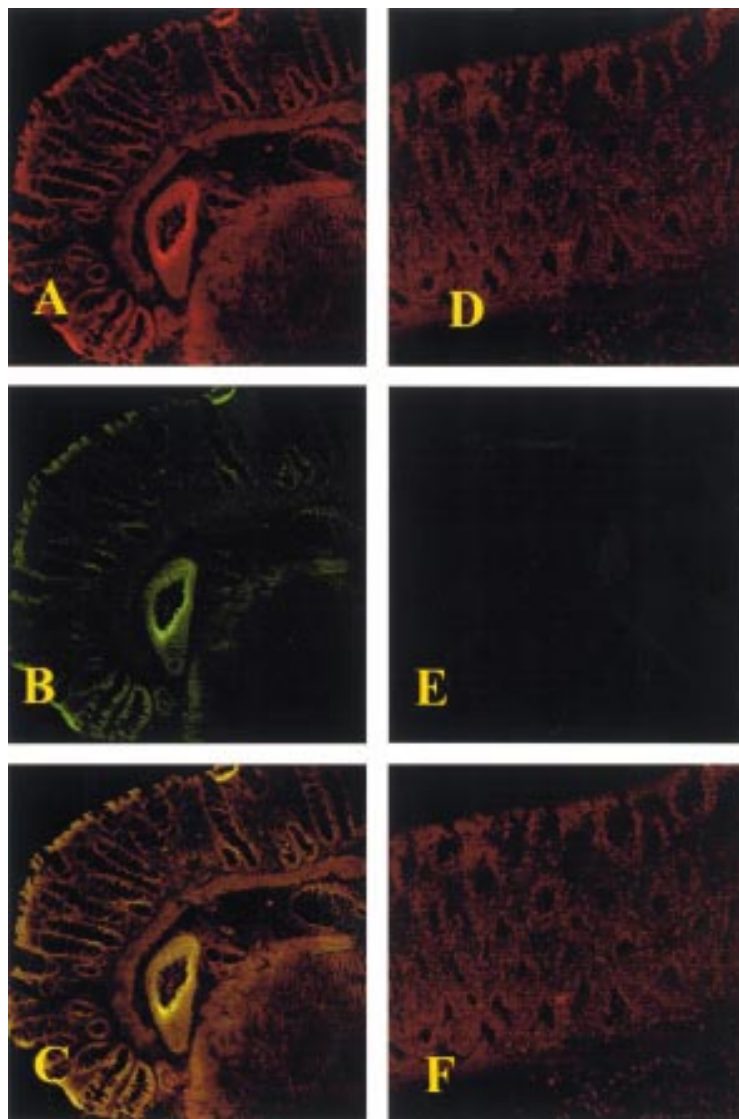
homogenised in 1.15% KCl solution. An aliquot (100  $\mu$ l) of the homogenate was added to a reaction mixture containing 200  $\mu$ l of 8.1% sodium dodecyl sulphate, 1500  $\mu$ l of 20% acetic acid (pH 3.5), 1500  $\mu$ l of 0.8% thiobarbituric acid, and 700  $\mu$ l of distilled water. Samples were boiled for one hour at 95°C and centrifuged at 3000  $g$  for 10 minutes. Absorbance of the supernatant was measured spectrophotometrically at 650 nm.

#### DETERMINATION OF NITRIC OXIDE SYNTHASE ACTIVITY

The calcium independent conversion of L-arginine to L-citrulline in the homogenates of the colon (obtained three days after DNBS treatment in the presence or absence of calpain inhibitor I) served as an indicator of iNOS activity.<sup>62</sup> Tissues were homogenised in buffer composed of 50 mM Tris HCl, 0.1 mM EDTA, and 1 mM phenylmethylsulphonyl fluoride (pH 7.4) on ice using a tissue homogeniser. Conversion of [<sup>3</sup>H]-L-arginine to [<sup>3</sup>H]-L-citrulline was measured in homogenates as described previously.<sup>63</sup> Briefly, homogenates (30  $\mu$ l) were incubated in the presence of [<sup>3</sup>H]-L-arginine (10  $\mu$ M, 5 kBq per tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5  $\mu$ M), and EGTA (2 mM) for 20 minutes at 22°C. Reactions were stopped by dilution with 0.5 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na<sup>+</sup> form) columns and the eluted [<sup>3</sup>H]-L-citrulline activity was measured by a Beckman scintillation counter.

#### MATERIALS

Biotin blocking kit, biotin conjugated goat antirabbit IgG, and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, California, USA). Primary antinitrotyrosine antibody was purchased from Upstate Biotech (Saranac Lake, New York, USA). Primary P-selectin (CD62P) and ICAM-1 (CD54) were purchased from Pharmingen (DBA, Milan, Italy). All other reagents and compounds used were purchased from Sigma Chemical Company (St Louis, Missouri, USA).



**Figure 7** Immunohistochemical localisation for cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) in the colon. Immunohistochemical staining for COX (A) and iNOS (B) showed positive staining localised in the injured area from dinitrobenzene sulphonic acid (DNBS) treated rats. The intensity of the observed staining for COX (D) and iNOS (E) was significantly reduced in the colon from calpain inhibitor I treated rats. (C) and (F) represent the staining combination of (A-B) and (D-E), respectively. Original magnification  $\times 145$ . The figure is representative of at least three experiments performed on different experimental days.

#### DATA ANALYSIS

All values in the figures and text are expressed as mean (SEM) of  $n$  observations. For the in vivo studies,  $n$  represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analysed by one way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A  $p$  value  $< 0.05$  was considered significant

#### Results

##### EFFECTS OF CALPAIN INHIBITOR I ON THE DEGREE OF COLITIS (HISTOLOGY)

Four days after intracolonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. Macroscopic inspection of the caecum, colon, and rectum showed the

presence of mucosal congestion, erosion, and haemorrhagic ulcerations (see fig 1A). The histopathological features included transmural necrosis and oedema, and diffuse leucocyte cellular infiltrate in the submucosa (fig 1B, 2B). Inflammatory changes of the intestinal tract were associated with an increase in the weight of the colon (fig 3B). Treatment of rats with calpain inhibitor I significantly attenuated the extent and severity of the histological signs of colon injury (figs 1, 2C, 3B). A significant increase in the weight of the spleen, an indicator of inflammation, was also noted in vehicle treated rats who had received DNBS (fig 3A). No significant increase in weight of either the colon or spleen was observed in DNBS rats who had been treated with calpain inhibitor I (fig 3). Survival of the animals was monitored for seven days. DNBS rats who received vehicle developed severe haemorrhagic diarrhoea, and 40% and 80% of these animals died within two and six days, respectively, after DNBS administration. In contrast, only 20% of rats who had been treated with calpain inhibitor I had haemorrhagic diarrhoea and died (fig 4). The surviving rats appeared to be healthy and showed mild diarrhoea.

##### EFFECTS OF CALPAIN INHIBITOR I ON CHANGES IN BODY WEIGHT

In vehicle treated rats, severe colitis caused by DNBS was associated with a significant loss in body weight (fig 5). Treatment of DNBS rats with calpain inhibitor I significantly reduced the loss in body weight.

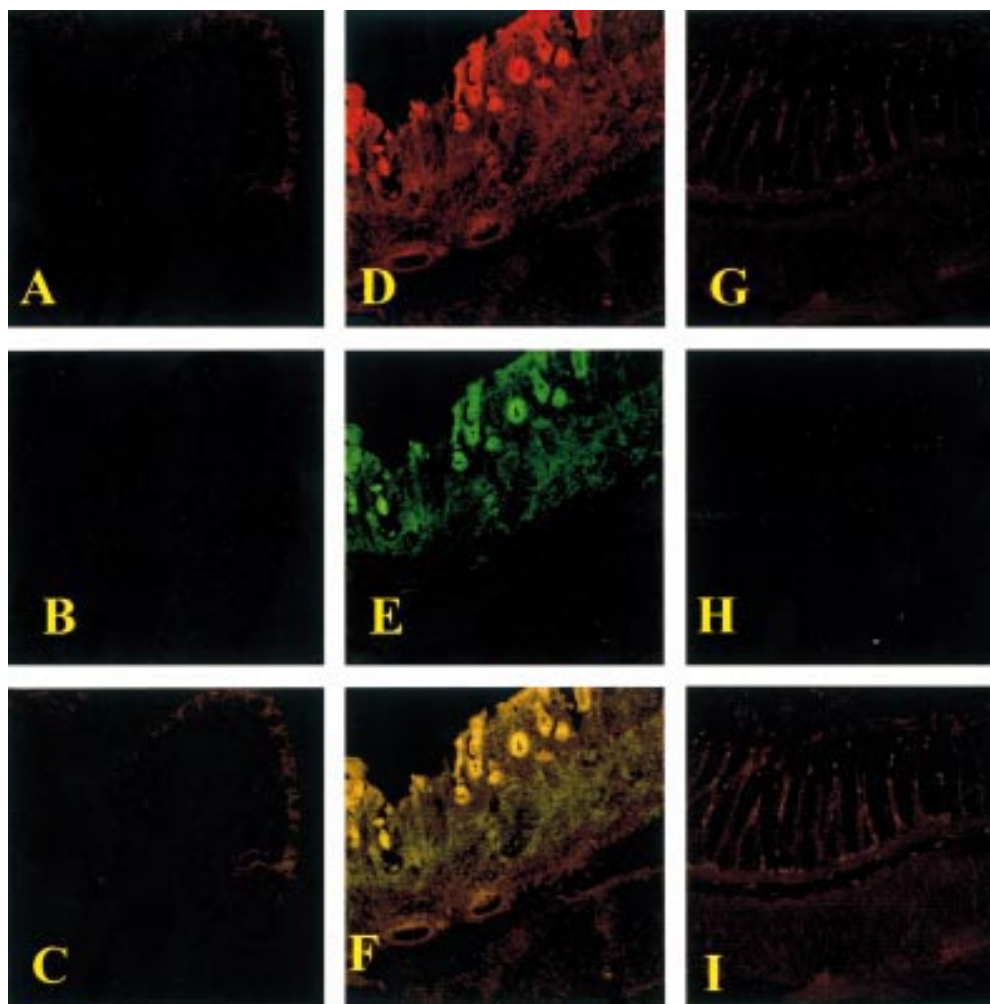
##### EFFECT OF CALPAIN INHIBITOR I ON NITRIC OXIDE PRODUCTION AND COX-2 EXPRESSION

In the colons obtained from animals subjected to DNBS induced colitis, a significant increase in iNOS activity was detected at four days (fig 6). iNOS activity was significantly ( $p < 0.01$ ) lower in DNBS rats treated with calpain inhibitor I (fig 6). Four days after DNBS treatment, colon sections were obtained to determine immunohistological staining for iNOS and COX-2. While there was negligible staining in intestinal sections from control animals (data not shown), immunohistochemical analysis, using specific anti-iNOS and anti-COX-2 antibodies, revealed positive staining primarily localised in the infiltrated inflammatory cells and in disrupted epithelial cells (fig 7A, B respectively). As demonstrated in fig 7C, positive staining for iNOS and COX-2 protein was colocalised in inflammatory cells and in disrupted epithelial cells. Calpain inhibitor I reduced the degree of immunostaining for iNOS and COX-2 in the colon of DNBS treated rats (fig 7D-F).

##### EFFECT OF CALPAIN INHIBITOR I ON

##### NITROTYROSINE FORMATION AND PARP ACTIVITY

To determine localisation of “peroxynitrite formation” and/or other nitrogen derivatives produced during colitis, nitrotyrosine, a specific marker of nitrosative stress,<sup>29</sup> was measured by immunohistochemical analysis in the distal colon. Four days after DNBS treatment, sections of colon were obtained to



**Figure 8** Immunohistochemical localisation of nitrotyrosine and poly (ADP-ribose) polymerase (PARP) in the colon. No positive staining for nitrotyrosine (A) or PARP (B) was found in colon sections from sham operated rats. Immunohistochemical staining for nitrotyrosine (D) and PARP (E) showed positive staining localised in the injured area from a dinitrobenzene sulphonic acid (DNBS) treated rat. The intensity of the staining for nitrotyrosine (G) and PARP (H) was significantly reduced in the ileum from calpain inhibitor I treated rats. (C), (F), and (I) represent the staining combination of (A-B), (D-E), and (G-H), respectively. Original magnification  $\times 145$ . The figure is representative of at least three experiments performed on different experimental days.

determine immunohistological staining for PARP. Sections of colon from sham administered rats did not stain for nitrotyrosine or PARP (fig 8A, 8B). Colon sections obtained from vehicle treated DNBS rats exhibited positive staining for nitrotyrosine and PARP (fig 8D, E) which was colocalised in inflammatory cells and in disrupted epithelial cells. Calpain inhibitor I reduced the degree of immunostaining for nitrotyrosine and PARP in the colon of DNBS treated rats (fig 8G–I).

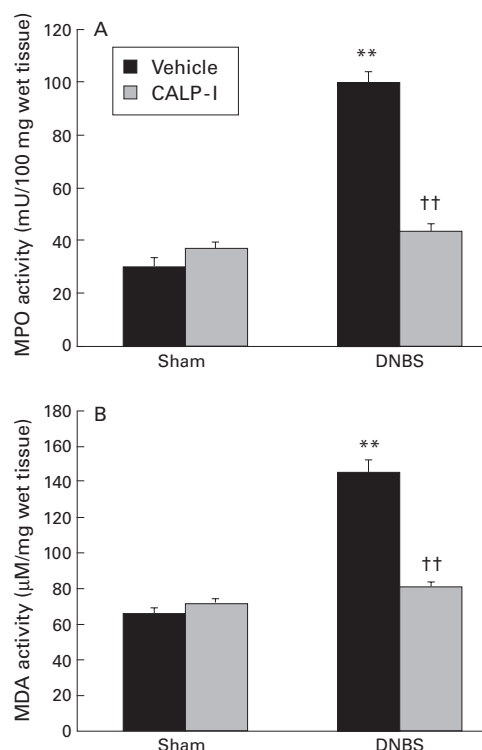
#### EFFECT OF CALPAIN INHIBITOR I ON MYELOPEROXIDASE ACTIVITY AND LIPID PEROXIDATION IN THE COLON

Colitis caused by DNBS was also characterised by an increase in MPO activity, an indicator of infiltration (accumulation) of the colon with PMNs (fig 9A). This finding is consistent with light microscopy findings that showed that the colon of vehicle treated DNBS rats contained a large number of PMNs. Infiltration of leucocytes into the mucosa has been suggested to contribute significantly to tissue necrosis and mucosal dysfunction associated with colitis as

activated PMNs release large amounts of free radicals. The increase in MPO activity in the colon correlated positively with the increase in tissue levels of MDA, indicating an increase in lipid peroxidation (fig 9B). Treatment of DNBS rats with calpain inhibitor I, however, significantly reduced both the degree of PMN infiltration (determined as an increase in MPO activity) and the associated lipid peroxidation (increase in tissue MDA levels) (fig 9).

#### EFFECT OF CALPAIN INHIBITOR I ON P-SELECTIN AND ICAM-1 EXPRESSION

To elucidate the effects of calpain inhibitor I on neutrophil accumulation in inflamed colon, we evaluated intestinal expression of ICAM-1 and P-selectin. Tissue sections obtained from sham operated rats stained with an anti-ICAM-1 antibody showed specific staining along the vessels (see arrows), demonstrating that ICAM-1 is expressed constitutively in endothelial cells (fig 10A). After DNBS administration, staining intensity substantially increased in vessels (see arrows) of the lamina propria. Immunohistochemical staining for



**Figure 9** Effect of calpain inhibitor I (CALP-1) on neutrophil infiltration and lipid peroxidation. Myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) levels (B) in the colon from dinitrobenzene sulphonic acid (DNBS) treated rats. MPO activity and MDA levels were significantly increased in DNBS treated rats in comparison with sham controls. CALP-1 treated rats showed a significant reduction in MPO activity and MDA levels. Values are mean (SEM) of 10 rats in each group. \*\* $p < 0.01$  v sham; †† $p < 0.01$  v DNBS.

ICAM-1 was also present in epithelial cells of injured colon and in infiltrated inflammatory cells in damaged tissues from DNBS treated rats (see small arrows in fig 10D). Sections from calpain inhibitor I treated rats did not reveal upregulation of constitutive ICAM-1, which was expressed in the endothelium along the vascular wall (fig 10G). No positive staining was observed in tissue sections obtained from sham operated rats that were stained with an antibody against P-selectin (fig 10B). In contrast, tissue sections of the colon obtained from DNBS treated rats showed positive staining for P-selectin localised in the vascular endothelium (see arrows in fig 10E). Tissue sections obtained from calpain inhibitor I treated rats, however, exhibited no staining for P-selectin (fig 10H). As can be seen in fig 10E, positive staining for P-selectin and ICAM-1 was (co-) localised in the endothelium.

### Discussion

IBD is a multifactorial disorder of unknown aetiology. There is however good evidence that enhanced formation of reactive oxygen or nitrogen species contributes to the pathophysiology of IBD. For example, monocytes from patients with Crohn's disease<sup>64</sup> and PMNs from patients with ulcerative colitis<sup>65</sup> have an increased capacity to generate free oxygen radicals. Furthermore, advanced stages of

bowel inflammation in humans<sup>16 17 22 23</sup> and animals<sup>24-28</sup> are associated with enhanced (local) formation of NO by iNOS. We have demonstrated for the first time that calpain inhibitor I attenuates: (i) colon injury (histology), (ii) infiltration of the colon with PMNs (histology and MPO activity), and (iii) degree of lipid peroxidation in the colon caused by DNBS in the rat. These findings support the view that calpain inhibitor I exerts potent anti-inflammatory effects and that this agent may be useful in the therapy of IBD.

What then are the mechanism(s) by which calpain inhibitor I inhibits the inflammation caused by injection of DNBS? There is good evidence that production of ROS such as hydrogen peroxide, superoxide, and hydroxyl radicals at the site of inflammation contributes to tissue damage.<sup>29 30 35-42</sup> Inhibitors of NOS activity reduce colon injury in experimental models of IBD suggesting a role for NO in the pathophysiology associated with this model of inflammation.<sup>48 66</sup> Activation of the transcription factor NF- $\kappa$ B plays an important role in expression of iNOS.<sup>67-70</sup> Our study demonstrated that calpain inhibitor I attenuated expression of iNOS in the colon of DNBS treated rats (figs 6, 7D). Therefore, we propose that the reduction by calpain inhibitor I of expression of iNOS may contribute to the anti-inflammatory effects of this agent. Enhanced formation of NO (by iNOS) in the presence of superoxide anions leads to generation of the oxidant peroxynitrite. We demonstrated that calpain inhibitor I attenuated nitration of proteins (determined by nitrotyrosine formation by immunohistochemistry) in the colon of rats with DNBS induced colitis (fig 8G). Nitrotyrosine formation, together with its detection by immunostaining, was initially proposed as a relatively specific marker for detection of the endogenous formation "footprint" of peroxynitrite.<sup>71</sup> There is however recent evidence that certain other reactions can also induce tyrosine nitration; for example, reaction of nitrite with hypochlorous acid and MPO with hydrogen peroxide can lead to the formation of nitrotyrosine.<sup>72</sup> Increased nitrotyrosine staining is therefore considered an indication of "increased nitrosative stress" rather than a specific marker of generation of peroxynitrite. Nevertheless, our results are consistent with the hypothesis that prevention of expression of iNOS by calpain inhibitor I in turn results in reduced formation of NO and subsequently peroxynitrite in the colon of DNBS treated rats.

In common with iNOS, expression of COX-2 is also mediated by transcription factor NF- $\kappa$ B. We have reported here that calpain inhibitor I also attenuated expression of COX-2 protein in inflammatory cells located in the colon of DNBS treated rats. There is good evidence in this and other models of inflammation that enhanced formation of prostanoids following induction of COX-2 contributes to the pathophysiology of local inflammation<sup>73 74</sup> and also that selective inhibitors of COX-2 exert potent anti-inflammatory effects.<sup>75-77</sup> We have demonstrated that expression of COX-2 protein was increased in the colon of DNBS

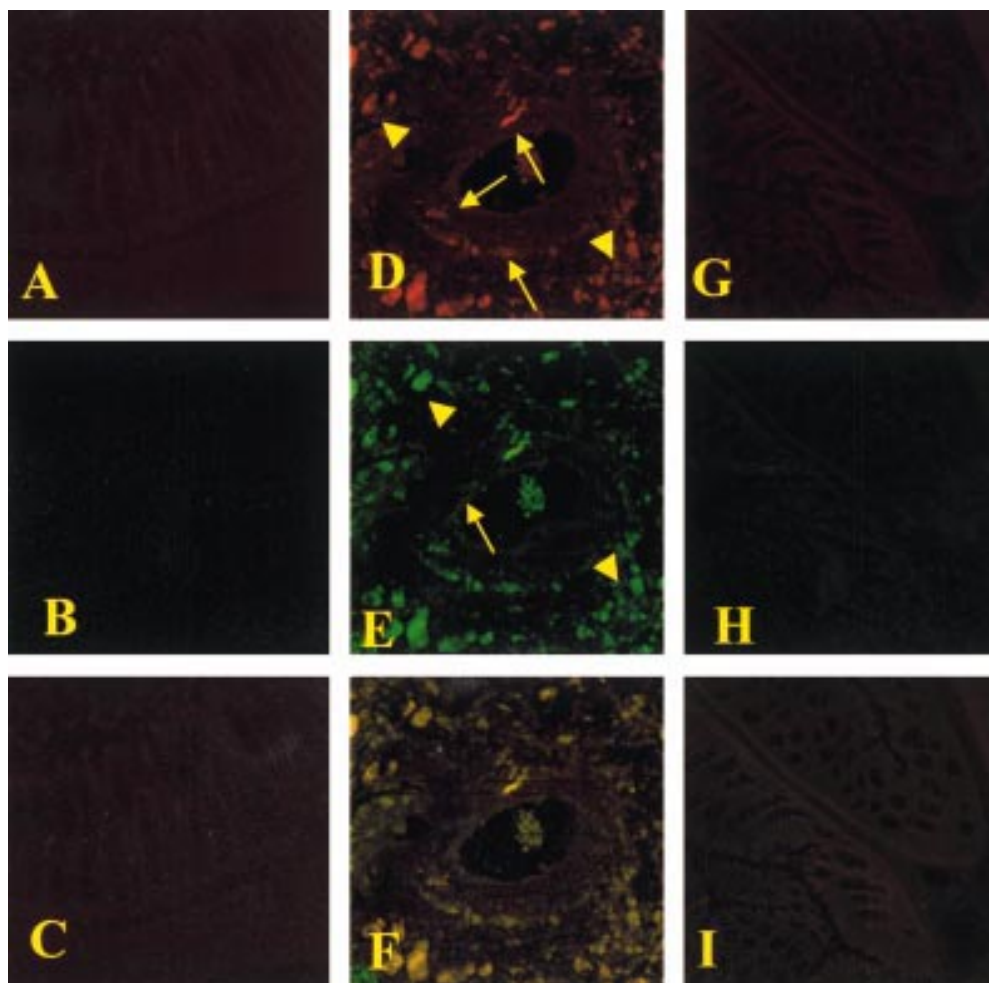


Figure 10 Immunohistochemical localisation of P-selectin in the colon. Staining of colon sections obtained from sham operated rats with anti-intercellular adhesion molecule 1 (ICAM-1) antibody showed specific staining along vessels, demonstrating that ICAM-1 is constitutively expressed (A). Ileum sections from sham operated rats revealed no positive staining for P-selectin (B). Sections obtained from dinitrobenzene sulphonic acid (DNBS) treated rats showed intense positive staining for ICAM-1 (D) and P-selectin (E) on endothelial cells. The degree of endothelial staining for ICAM-1 (G) and P-selectin (H) was markedly reduced in tissue sections obtained from calpain inhibitor I treated rats. (C), (F), and (I) represent the staining combination of (A-B), (D-E), and (G-H) respectively. Original magnification  $\times 145$ . The figure is representative of at least three experiments performed on different experimental days.

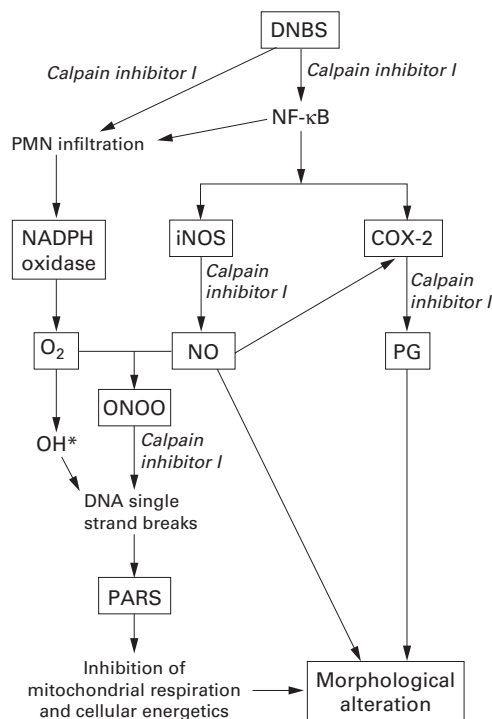
treated rats. Thus we propose that the observed anti-inflammatory effects of calpain inhibitor I in experimental colitis are, at least in part, due to prevention of expression of COX-2. Indeed, selective inhibitors of COX-2 activity exert potent anti-inflammatory effects in various models of inflammation.<sup>75-77</sup>

ROS and peroxynitrite produce cellular injury and necrosis via several mechanisms, including peroxidation of membrane lipids, protein denaturation, and DNA damage. ROS produce strand breaks in DNA which trigger energy consuming DNA repair mechanisms and activate the nuclear enzyme PARP. Activation of PARP results in depletion of its substrate NAD and also in a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed "the PARP or PARS suicide hypothesis".<sup>35</sup> There is recent evidence that activation of PARP may also play an important role in inflammation.<sup>38-42</sup> We have demonstrated in this study that the increase in PARP activity

caused by DNBS in the colon was attenuated by pretreatment of rats with calpain inhibitor I (fig 8H). Thus it is possible that prevention by calpain inhibitor I of activation of PARP contributes to the anti-inflammatory effects of this agent in experimental colitis.

There is little information on the role of calpain(s) in "classical models" of inflammation. Ischaemia and reperfusion of tissues as well as trauma lead to local or (even) systemic inflammatory responses. Interestingly, both ischaemia-reperfusion as well as tissue trauma cause activation of calpain.<sup>78-80</sup> We have recently discovered that haemorrhage and resuscitation (but not haemorrhage alone) leads to a significant increase in calpain activity (of the heart) which is abolished by the dose of calpain inhibitor I used here (McDonald, Cuzzocrea, Thiernemann, unpublished). Thus it is possible that prevention of calpain activity contributes to the beneficial effects of calpain inhibitor I, the colitis caused by DNBS in the rat. There is good evidence that inhibition of calpain I activity also reduces the injury associated with ischaemia-reperfusion of the brain,<sup>81-84</sup> liver,<sup>85-86</sup>





**Figure 11** Proposed scheme of some of the delayed inflammatory pathways involving nitric oxide ( $\text{NO}^*$ ), hydroxyl radical ( $\text{OH}^*$ ), and peroxynitrite ( $\text{ONOO}$ ) in dinitrobenzene sulphonic acid (DNBS) induced colitis, and potential sites of the anti-inflammatory actions of calpain inhibitor I. DNBS triggers expression of inducible nitric oxide ( $\text{NO}$ ) synthase (iNOS), at least in part, via activation of nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ).  $\text{NO}$  in turn combines with superoxide to form  $\text{ONOO}^-$ . Hydroxyl radical (produced from superoxide via the iron catalysed Haber-Weiss reaction) and  $\text{ONOO}^-$  or peroxynitrous acid ( $\text{ONOOH}$ ) induce cellular injury. Part of the injury is related to the development of DNA single strand breakage, with consequent activation of poly (ADP-ribose) polymerase (PARP), leading to cellular dysfunction. Expression of the inducible isoform of cyclooxygenase (COX-2) is also dependent on activation of NF- $\kappa\text{B}$ . In addition,  $\text{NO}$  can directly increase the catalytic activity of COX-2, leading to enhanced production of proinflammatory prostaglandin metabolites. We propose that the anti-inflammatory effects of calpain inhibitor I may include (1) inhibition of activation of NF- $\kappa\text{B}$  and prevention of expression of iNOS and COX-2, (2) inhibition of  $\text{ONOO}$  formation, and (3) prevention of activation of PARP (see discussion for further explanations).

and heart.<sup>78-79</sup> The mechanism by which inhibitors of calpain activity protect tissues/organs against reperfusion injury and/or the associated inflammation is not entirely clear. Calpain acts on several substrates causing proteolytic modifications of proteins which results in changes in their biochemical and morphological parameters. Activation of calpain results in proteolysis of several cellular proteins, mostly associated with the cellular membrane, including cytoskeletal proteins (for example, spectrin, fodrin, and microtubule associated proteins), membrane proteins (for example, growth factor receptors, adhesion molecules, and ion transporters), enzymes (kinases, phosphatases, and phospholipases), as well as cytokines and transcription factors. Although many of these are implicated in the pathophysiology of ischaemia-reperfusion injury, the exact role of calpain activation in inflammation is still unclear and warrants further investigation.

In conclusion, our study demonstrated that the degree of colitis caused by injection of DNBS was substantially reduced by treatment of rats with calpain inhibitor I. Hence we report for the first time that calpain inhibitor I exerts potent anti-inflammatory effects in vivo. The mechanisms of the anti-inflammatory effect of calpain inhibitor I are not entirely clear. Calpain inhibitor I inhibits activation of NF- $\kappa\text{B}$  (positive feedback; fig 11), which mediates expression of iNOS and COX-2 protein and activity, and ultimately the degree of peroxynitrite formation and tissue injury. In addition, calpain inhibitor I inhibits the formation of P-selectin and ICAM-1, which in turn may contribute to recruitment of PMNs. Our findings suggest that calpain inhibitor I treatment may be useful in conditions associated with local or systemic inflammation, including IBD.

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