Suppression of Acute Experimental Colitis by a Highly Selective Inducible Nitric-Oxide Synthase Inhibitor, N-[3-(Aminomethyl)benzyl]acetamidine

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Received March 20, 2001; accepted May 28, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

High concentrations of nitric oxide (NO) produced by the inducible nitric-oxide synthase (iNOS) are associated with ulcerative inflammation and disease activity in colitis. Therefore, inhibition of iNOS serves as a novel experimental approach to treat gut inflammation. The aim of the present study was to investigate the effects of a novel highly selective iNOS inhibitor, N-[3-(aminomethyl)benzyl]acetamidine (1400W), as compared with a nonselective NOS inhibitor, N(G)-nitro-L-arginine-methyl-ester (L-NAME), in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced acute colitis in the rat. Increased expression of iNOS protein and mRNA was found in acute TNBS-induced colitis along with neutrophil infiltration, inflammatory edema, and tissue damage. In a 24-h model of acute colitis, subcutaneous injections of 1400W (5 or 10 mg/kg t.i.d.) produced a 56 and 95% reduction in inflammatory edema formation, a 68 and 63% reduction in neutrophil infiltration (measured as myeloperoxidase activity), and a 19 and 26% decrease in the size of mucosal lesions as compared with vehicle treatment. Administration of L-NAME (35 mg/kg) failed to produce any significant beneficial effects as compared with vehicle treatment in this experimental model of acute colitis. Treatment with 1400W, a highly selective inhibitor of iNOS, reduced formation of edema, neutrophil infiltration, and macroscopic inflammatory damage in experimentally induced acute colitis in the rat. In contrast, nonselective nitric-oxide synthase inhibition with L-NAME provided no benefit. These results support the idea that selective iNOS inhibitors have a promise in the treatment of colitis.

Inflammatory bowel disease (IBD) is characterized by inflammatory lesions and ulcerations which predominantly affect the colon and rectum. At present, the pharmacotherapy of IBD is based on aminosalicylates and glucocorticoids. However, these are far from perfect therapeutically, and despite aggressive medical care acute exacerbations or relapses of inflammation may occur. Thus, there is a strong need for improved therapies, which may be based on increased knowledge and understanding of the pathogenesis and etiology of colitis. In patients with IBD as well as in experimental models of colitis, excessive production of nitric oxide (NO) has been documented (Boughton-Smith et al., 1993; Kimura et al., 1997; Rachmilewitz et al., 1998; Kankuri et al., 1999).

NO is produced by two types of enzymes: constitutively expressed and inducible NO synthases (NOS) (Knowles, 1996). The former (cNOS), including neuronal and endothelial nitric oxide synthases (nNOS or NOS1, and eNOS or NOS3) is responsible for physiological NO production in various tissues. The inducible NOS (iNOS or NOS2) is expressed in response to proinflammatory stimuli in, for example, colon epithelial cells (Kolios et al., 1998; Salzman et al., 1998; Lähde et al., 2000). iNOS, in contrast to cNOS, is capable of producing high amounts of NO (Mayer and Andrew, 1998). In an inflammatory focus, NO may react with superoxide anion, resulting in oxidative tissue damage through production of peroxynitrite (Schmidt and Walter, 1994; Grisham et al., 1999), which is believed to mediate many of the destructive effects of NO in gut inflammation (Miller and Sandorval, 1999; McCafferty, 2000).

Increased iNOS activity in the gut is considered proinflammatory and is associated with mucosal lesions, ulcerations, intraluminal bleeding, and bowel dilatation and dysfunction (Guslandi, 1993; Miller and Sandorval, 1999). However, a basal production of NO seems to be crucial for the maintenance of epithelial integrity and resistance to injury (Lefer and Lefer, 1999).

These data led us to hypothesize that a highly selective

ABBREVIATIONS: IBD, inflammatory bowel disease; NO, nitric oxide; iNOS, cNOS, eNOS, and nNOS, inducible, constitutive, endothelial, and neuronal nitric-oxide synthase; TNBS, 2,4,6-trinitrobenzenesulfonic acid; 1400W, N-[3-(aminomethyl)benzyl]acetamidine; L-NAME, N(G)-nitro-L-arginine-methyl-ester; MPO, myeloperoxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
iNOS inhibitor would possess anti-inflammatory effects in colitis. Studies on the effects of the partially selective iNOS inhibitors aminoguanidine, mercaptoethylguanidine, and N'-iminooethyl-L-lysine have been published (Zingarelli et al., 1998; Nakamura et al., 1999; Armstrong et al., 2000; Blanchard et al., 2001; Dikopoulos et al., 2001), with rather variable outcome from protection to exacerbation. However, these agents have only a modest degree of selectivity for iNOS over cNOSs, particularly versus nNOS. Moreover, interpretation of those results is further complicated by the fact that mercaptoethylguanidine and aminoguanidine have other actions beyond inhibition of NOS, e.g. as radical and peroxynitrite scavengers (Szabo et al., 1997; Wray et al., 1998; Alderton et al., 2001). Thus experiments with these agents, while interesting, cannot be readily interpreted in terms of whether highly selective inhibition of iNOS would be of therapeutic benefit in colitis. Recently, a highly selective inhibitor (N-[3-(aminomethyl)benzyl]acetamide; 1400W) of iNOS versus eNOS and nNOS became available (Garvey et al., 1997). In the present study we have tested the hypothesis that selective inhibition of iNOS would provide anti-inflammatory effects in colitis by investigating the effects of this highly selective agent in the TNBS-induced acute colitis in the rat.

Materials and Methods

Description of Experimental Protocol. To study the time course of iNOS expression in acute TNBS-induced inflammation, 36 male Wistar rats (220–286 g) were divided into six groups (n = 6 in each). Colitis was induced as described earlier by rectal intraluminal administration of 120 mg/kg 2,4,6-trinitrobenzenesulfonic acid (TNBS; Fluka Chemie AG, Buchs, Switzerland) in 50% ethanol (Morris et al., 1989; Kankuri et al., 1999). At 0, 6, 12, 24, 48, and 72 h following TNBS, a group of rats (n = 6) was sacrificed and samples were collected (see below).

To study the effects of NOS inhibitors, 28 male Wistar rats (278–355 g) were divided into four groups. Two groups received 1400W (5 mg/kg, n = 8; and 10 mg/kg, n = 7), one group received N(G)-nitro-L-arginine-methyl-ester (L-NAME; 35 mg/kg, n = 5), and one group received saline as vehicle treatment (n = 8). Colitis was induced with rectally administered TNBS (120 mg/kg) as described previously (Morris et al., 1989; Kankuri et al., 1999), and the animals were sacrificed 24 h after TNBS. In addition, a baseline control group (no drug treatment, no TNBS; n = 6) was included in the study. 1400W was administered subcutaneously three times at 8-h intervals, with the first injection just before TNBS, and then 8 and 16 h after TNBS. L-NAME and vehicle were given as single subcutaneous injections just before TNBS. The dosing schedule of 1400W was based on experiments in rats in which pronounced effects of 1400W (5–10 mg/kg) were observed on iNOS activity as assessed by endotoxin-induced increases in plasma nitrite (Hamilton and Warner, 1998) or by endotoxin-provoked vascular leakage (Garvey et al., 1997; Laszlo and Whittle, 1997). The dosing schedule of L-NAME was based on previous reports on its pharmacological actions (Pfeiffer and Qiu, 1995; Ceier and De Mey, 2000; Halac et al., 2000), its effects on NOS activity in vivo (Salter et al., 1995), and on its pharmacokinetics in the rat (Pfeiffer et al., 1996).

At the end of the experiment, the rats were sacrificed by decapitation under light halothane anesthesia, and the descending colon was cut at the pubic symphysis and seven centimeters proximally, weighed, and photographed. Three 0.5-cm long samples were cut from the distal part of this sample. Mucosa was scraped off from the remaining part of the sample for myeloperoxidase measurement.

The use of experimental animals and the study protocol was approved by the Ethics committee at the Institute of Biomedicine, University of Helsinki, Finland.

Myeloperoxidase (MPO) Activity Measurement. MPO activity in the distal colon mucosa was measured as previously described (Pfeiffer and Qiu, 1995; Kankuri et al., 1999). Briefly, the colonic mucosa was homogenized in ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium-bromide. The homogenate was sonicated, underwent three freeze-thaw cycles, and was centrifuged for 15 min at 40,000 g at 4°C. O-Dianisidine-H2O2 buffer was added to an aliquot of the supernatant, and the change in absorbance (λ = 450 nm) was measured for 2 min. One MPO activity unit corresponds to 1 μmol of H2O2 degraded in 1 min. To rule out nonspecific inhibition of MPO by the studied NOS inhibitors, the MPO assay with the drug was performed on rat peripheral blood leukocytes. Blood from healthy rats was collected into heparinized tubes. After centrifugation, an aliquot of white blood cells was collected. Samples were sonicated and underwent three freeze-thaw cycles followed by ultracentrifugation. The studied drugs were added (final concentration of 1 mM) to the sample, and MPO activity was measured as described.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (PCR). Colon samples (approximately 30 mg) were frozen and homogenized. Cells were lysed and purified using QIAshredder (Qiagen Inc., Santa Clarita, CA). Thereafter RNA was extracted using the RNeasy kit for isolation of total RNA (Qiagen Inc.). Synthesis of cDNA from mRNA and subsequent amplification of cDNA by PCR were performed with GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit (PerkinElmer; Roche Molecular Systems Inc., Branchburg, NJ). First strand cDNA was synthesized using sequence-specific downstream primer for rat iNOS or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as reference. The upstream primer was added into the reaction mixture at the beginning of PCR amplification. PCR conditions were denaturation, annealing, and extension at 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, respectively, and 25 cycles for both iNOS and GAPDH. The Mg2+ concentration in the reaction mixture was 1.5 mM. Primers for amplification of iNOS were 5’-CAGCTGTATGTTGACTCCATCGAC-3’ (sense) and 5’-AGATGACCTCATGCCAGTAATGAGCTG-3’ (antisense), resulting a 346-bp product (Nunokawa et al., 1993). Primers for amplification of GAPDH were 5’-CGGTGTCACCGGATTGCTGCACAG-3’ (sense) and 5’-AGCCTTTCTCATGTTGTAAGAC-3’ (antisense), resulting a 306-bp product. Products were analyzed on 1.5% agarose gel containing ethidium bromide and then visualized in ultraviolet light.

Western Blot Analysis. Colon samples were weighed, and 6 ml/mg ice-cold extraction buffer (10 mM Tris base, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethyl sulfonyl fluoride, 2 mM Na-orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM Na-pyrophosphate, 10 mM n-octyl-β-D-glucopyranoside) was added. Samples were homogenized using an ULTRA TURRAX T25 homogenizer (Janke & Kunkel GmbH, Staufen, Germany). After extraction, samples were centrifuged and the resulting supernatant boiled for 5 min in sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 5% 2-mercaptoethanol) and stored at −20°C until analyzed. An aliquot of supernatant was used to determine protein by the Coomassie blue method (Bradford, 1976). Protein samples (20 μg) were separated by SDS-polyacrylamide gel electrophoresis on 8% polyacrylamide gels and transferred to nitrocellulose membrane. iNOS protein was detected and identified by Western blotting using rabbit polyclonal antibody (M-19) obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Measurement of the Damaged Area from Photographs of Inflamed Colon. Photographs taken from colon samples were scanned using CanoScan 2700F film scanner (Canon Inc., Tokyo, Japan). Analysis of pictures was done using Corel Photo Paint version 7.373 (CorelDraw version 7.0 Corel Corporation, Ottawa, Canada). To gain best contrast, the scanned pictures were split into
hue-saturation-brightness channels, from which the latter was used for further evaluation. Damaged area was defined as that with a densitometric value below 126 (hemorrhagic, red); likewise, densitometric values of 126 and above (healthy, pale) designated the healthy area. The damaged area divided by the total area of the colon sample was used to score inflammatory damage.

Drugs. 1400W was supplied by Dr. Richard G. Knowles (Glaxo-SmithKline Research, Hertfordshire, UK). L-NAME was from Sigma Chemical Inc. (St. Louis, MO).

Statistical Analysis. The results are expressed as mean ± S.E.M. Statistical analysis was carried out using analysis of variance followed by Bonferroni multiple comparisons test. Differences at \( p \) values of <0.05 were considered significant.

Results

TNBS induced an ulcerative inflammation of the distal colon characterized by edema formation and leukocyte infiltration. To define a relevant time point for the study with 1400W, we characterized the time courses of iNOS expression and macroscopic mucosal damage of the distal colon. iNOS protein was present in distal colon samples taken 6 and 12 h after TNBS and declined thereafter (Fig. 1a). Accordingly, iNOS mRNA was found at 6 to 12 h after induction of colitis (Fig. 1b).

MPO activity was measured as a marker of neutrophil granulocyte infiltration into the colon mucosa. MPO activity attained its maximum levels at 12 to 24 h and declined thereafter (Fig. 2a). Inflammatory edema was quantitated as the weight of the distal colon (g/cm²) and peaked at 24 h after TNBS (Fig. 2b). The macroscopic mucosal damage of the distal colon increased in a progressive fashion up to 24 h and remained elevated for the 72-h follow-up period (Fig. 2c).

To test our hypothesis on the effects of iNOS inhibition in colitis, the 24-h time point was chosen for the further studies. A selective iNOS inhibitor, 1400W, decreased MPO activity and edema formation, and it reduced the damaged area of the distal, inflamed colon in doses previously shown to inhibit endotoxin-induced NO production in the rat (Hamilton and Warner, 1998).

Treatment with 1400W inhibited TNBS-induced neutrophil granulocyte infiltration into the inflamed colon (measured as mucosal MPO activity) by 68% (5 mg/kg) or 63% (10 mg/kg) (Fig. 3a). In contrast, treatment with L-NAME failed to reduce mucosal MPO activity.

Treatment with 1400W reduced the formation of inflammatory edema by 56% (5 mg/kg) to 95% (10 mg/kg) in the distal colon [as assessed by weight/total area of colon sample (g/cm²)] (Fig. 3b). Treatment with 1400W also produced a 19% (5 mg/kg) to 26% (10 mg/kg) decrease in the area of damaged mucosa in the distal colon (Fig. 3c). L-NAME treatment failed to reduce inflammatory edema or the area of damaged mucosa.

To rule out a direct effect of 1400W on MPO activity, experiments with rat peripheral blood neutrophils were carried out. Neither 1400W nor L-NAME (in 1 mM concentrations) had any effect on MPO activity in vitro (data not shown).

Discussion

The present results showed that treatment with 1400W, a highly selective inhibitor of iNOS, reduced neutrophil inflam...
time courses of MPO activity and tissue damage suggest that 1400W inhibits neutrophil infiltration and neutrophil-mediated mucosal injury in the gut.

The role of neutrophil granulocytes has been studied previously in TNBS-induced colitis. Palmen et al. (1995) showed that in acute TNBS colitis in the rat, treatment with monoclonal antibodies against leukocyte adhesion molecules reduced inflammatory injury, MPO activity, and leukocyte infiltration. Furthermore, treatment of TNBS-induced rat colitis with anti-neutrophil antiserum attenuated colonic edema formation even though this treatment was ineffective in acetic acid or phorbol-myristate acetate-induced colitis (Buell and Berin, 1994). These results are in concert with our present data, showing neutrophil dependence of the TNBS-induced acute inflammatory cascade. The activation state of the neutrophil granulocytes was not studied by us, nor has it been studied by any of the cited authors. Further studies are warranted to study the role and activation of neutrophils in inflammatory bowel disease.

TNBS induces a severe ulcerative inflammation in the rat colon. In this model of colitis, therapeutic effects of such potent anti-inflammatory drugs as dexamethasone or methyprednisolone on ulcers and mucosal lesions have been difficult to demonstrate (Anthony et al., 1997; Fries et al., 1998). In the present study, 1400W (10 mg/kg) reduced the area of mucosal lesions by 26% in addition to its more pronounced effects on inflammatory edema and neutrophil infiltration, indicating a significant protective action.

In the doses used in the present study, 1400W serves as a highly selective iNOS versus eNOS and nNOS inhibitor (Garvey et al., 1997) and L-NAME as a nonselective iNOS inhibitor (Salter et al., 1995; Pfeiffer et al., 1996). Treatment with 1400W at effective doses on iNOS has been shown to have no effect on basal systemic blood pressure or in exacerbating early effects of endotoxin on vascular leakage (cNOS effects), whereas treatment with L-NAME caused a significant increase in blood pressure and exacerbation of early vascular leak (Garvey et al., 1997; Laszlo and Whittle, 1997; Wray et al., 1998). Increased iNOS expression is associated with inflammation, mucosal lesions, and ulcerations in the gut (Laszlo and Whittle, 1997). Thus, the beneficial effects seen in this experimental model of colitis are probably mediated through inhibition of iNOS. In contrast, inhibition of cNOS has been reported to produce detrimental effects on gut mucosa (Laszlo and Whittle, 1997). Therefore, the lack of effect of the nonselective NOS inhibitor L-NAME in the present study may be due to combined detrimental inhibition of cNOS activity and beneficial iNOS inhibition (Whittle, 1997).

Our results are further supported by the earlier finding of Pfeiffer and Qiu (1995), who reported that treatment with a nonselective NOS inhibitor, L-NAME, did not produce any beneficial effects in TNBS-induced colitis. Studies on the effects of partially selective iNOS inhibitors aminoguanidine, mercaptoprothylguanidine, and N-iminoethyl-L-lysine have been carried out in colitis models (Zingarelli et al., 1998; Nakamura et al., 1999; Armstrong et al., 2000; Binion et al., 2000; Blanchard et al., 2001; Dikopoulos et al., 2001), with rather variable outcomes from protection to exacerbation. However, these agents have only a modest degree of selectivity for iNOS over cNOSs, particularly versus nNOS. For example, aminoguanidine is only approximately 5-fold selec-

**Fig. 3.** Effects of 1400W (5 mg/kg, n = 8; 10 mg/kg, n = 7) and L-NAME (35 mg/kg, n = 5) treatments on neutrophil granulocyte infiltration (measured as myeloperoxidase activity) into distal colon mucosa (a), edema formation (measured as weight of the distal colon) (b), and macroscopic damage of the distal colon (c). Data are expressed as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 as compared with the TNBS control group (n = 8). †p < 0.05, ††p < 0.01 as compared with the L-NAME-treated group. n = 6 for the untreated, healthy group of rats.
tive for iNOS versus nNOS (Alderton et al., 2001). Thus, in the studies with these agents, cNOSs are likely to be inhibited to variable degree in addition to inhibition of iNOS, with the consequent equivocal or deleterious outcome. These results are therefore not in conflict with the present results, in which highly selective inhibition of iNOS was beneficial and nonselective inhibition was not. Interestingly, iNOS-deficient mice have been shown to develop a more severe acute TNBS colitis than wild-type mice (McCafferty et al., 1999). Further studies will be required to determine whether this difference is a consequence of the complete absence of iNOS in these gene deletion mice (unlikely to be achieved with a pharmacological agent), compensatory changes in other pathways occurring during development of these mice, or a species difference between rats and mice.

In conclusion, the present study shows inhibition of inflammatory edema, neutrophil infiltration, and reduction of the size of mucosal lesions in experimentally induced severe acute colitis in the rat by the highly selective iNOS inhibitor, 1400W. This agent has also been shown to be a potent inhibitor of endotoxin-induced vascular leakage in rat intestine (Garvey et al., 1997; Laszlo and Whittle, 1997), and a potent inhibitor of iNOS in lipopolysaccharide-treated human colonic epithelial cells (Lahde et al., 2000). These results support the idea that selective iNOS inhibitors may have therapeutic potential in the treatment of acute colitis.

References


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