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Time-dependent actions of nitric oxide synthase inhibition on colonic inflammation induced by trinitrobenzene sulphonic acid in rats

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Abstract

The time-dependent actions following pretreatment or delayed administration of the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) on colonic inflammation and inducible NO synthase activity following the intrarectal administration of trinitrobenzene sulphonic acid (TNBS) were evaluated in the rat. Intracolonic instillation of TNBS (30 mg in 0.25 ml of 50% ethanol) led to macroscopic injury, an increase of mucosal myeloperoxidase activity and the expression of the Ca^{2+}-independent inducible NO synthase over 8 days. The inflammatory response following TNBS reached maximum levels between 12 and 72 h and then it declined until 14 days. Oral administration of L-NAME (25 mg/kg per 24 h in the drinking water) 2 days before TNBS augmented macroscopic damage and increased colonic inducible NO synthase activity 6, 12, 24 and 72 h after TNBS administration. In contrast, when L-NAME was administered 6 h after TNBS instillation, at time of expression of inducible NO synthase, the macroscopic lesions were reduced, as well as the enhanced inducible NO synthase activity, determined, over 72 h. Delayed (6 h after TNBS) administration of L-NAME also attenuated the colonic myeloperoxidase activity provoked by TNBS, after 24 h. This activity was not affected by pretreatment (2 days before TNBS) with L-NAME. These findings indicate that the timing of administration of non-selective NO synthase inhibitors such as L-NAME, in models of colitis is critical to the eventual outcome. Thus, pretreatment with L-NAME, which will inhibit constitutive NO synthase, exacerbates the subsequent damage following challenge. In contrast, delayed administration of L-NAME at the time of inducible NO synthase expression, has a beneficial action on the colonic injury and inflammation. © 1997 Elsevier Science B.V.

Keywords: Nitric oxide (NO); N^G-nitro-L-arginine methyl ester (L-NAME); Colon; Inflammation; Nitric oxide (NO) synthase, constitutive; Nitric oxide (NO) synthase, inducible

1. Introduction

Under physiological and pathophysiological circumstances, nitric oxide (NO), formed from L-arginine by the Ca^{2+}-dependent constitutive NO synthase plays an important role in the maintenance of tissue integrity in the gastrointestinal tract (Whittle, 1994). However, under certain pathological conditions, such as following endotoxin and cytokine exposure, NO can also be formed following the induction of a distinct Ca^{2+} independent isoenzyme (Radomski et al., 1990; Busse and Mülsch, 1990). The expression of this inducible nitric oxide synthase can lead to the overproduction of NO under these conditions, which can provoke cytotoxic actions including the cellular injury to endothelial and epithelial cells (Palmer et al., 1992; Tepperman et al., 1993).

An increased generation of NO and the expression of the inducible NO synthase has been demonstrated in mucosal biopsies of patients with the inflammatory bowel disease ulcerative colitis (Boughton-Smith et al., 1993a; Middleton et al., 1993; Rachmilewitz et al., 1995). Moreover, in several animal models of inflammatory bowel disease, the increased production of NO and the detection of the inducible NO synthase has also been described (Miller et al., 1993a,b; Boughton-Smith et al., 1994; Seo et al., 1995; Rachmilewitz et al., 1995). Administration of NO synthase inhibitors and the anti-inflammatory corticosteroid dexamethasone, which can inhibit the induction of NO synthase, ameliorate experimental colonic inflammation (Miller et al., 1993a; Whittle et al., 1995). In contrast, the inhibition of constitutive NO synthase appears to be detrimental under challenge conditions, since early administration of NO synthase inhibitors exacerbates acute intestinal damage provoked by endotoxin (Hutcheson et al., 1990; Laszlo et al., 1994a).

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The timing of administration of non-selective NO synthase inhibitors thus appears to be crucial in determining their actions on tissue injury. In the present study, therefore the activity of inducible NO synthase in relation to formation of macroscopic lesions and intramucosal myeloperoxidase activity (as a measure of inflammatory cell infiltration) has been evaluated following the pretreatment, or the delayed administration of NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) in an experimental model of colitis induced by trinitrobenzene sulphonic acid (TNBS).

2. Materials and methods

Male Wistar rats (180–220 g) were allowed free access to water and food during the experiments. Under transient ether anaesthesia, 2,4,6 trinitrobenzene sulphonic acid (TNBS; 30 mg in 0.25 ml of 50% ethanol) was instilled by an 8 cm long plastic catheter inserted rectally into the rat colon, as described previously (Morris et al., 1989). The animals were sacrificed by cervical dislocation 3, 6, 12, 24 and 72 h and 8 days after TNBS administration and the distal 8 cm portion of the colon was removed and, after photography, cut longitudinally into two segments for determination of inducible NO synthase and myeloperoxidase activity.

2.1. Macroscopic analysis

The extent of macroscopically-visible damage, involving regions of haemorrhagic necrosis was determined in a randomized manner from photographs on colour transparency film via computerized planimetry. The area of mucosal damage was calculated as the percentage of the total colonic segment area that showed macroscopically-visible damage.

2.2. Nitric oxide synthase activity

The activity of inducible NO synthase was assessed by measuring the conversion of ^{14}C-labelled L-arginine monohydrochloride to [^{14}C]citrulline by the method described previously (Boughton-Smith et al., 1993b). After the homogenisation of the 8 cm segment of colon tissue (Ultraturrax T25, 13 500 s^{-1}, 2×30 s) in ice-cold buffer (250 mg/ml containing 10 mM HEPES, 32 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 0.01 mg/ml soybean inhibitor, 0.01 mg/ml leupeptin, 2 μg/ml aprotinin) and centrifugation ($10\,000 \times g$, 20 min, 4°C), an aliquot of the supernatant was removed both for the assay of the enzyme and for the determination of the protein level. A 40 μl sample of the supernatant was incubated for 10 min at 37°C in 100 μl buffer containing 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM L-valine, 1 mM dithiothreitol, 1 mM L-citrulline, 15 nM L-arginine, 0.3 mM NADPH, 3 μM FAD, 3 μM FMN 3 μM tetrahydrobiopterin and 157 pM [^{14}C]L-arginine (110 000 dpm/ml) in the presence of 1 mM N^G-monomethyl-L-arginine (L-NMMA) or 1 mM EGTA. The reaction was stopped via the removal of the substrate L-arginine by the addition (0.5 ml) of the 1:1 suspension of Dowex (AG 50W-8) in water. Distilled water (850 μl) was added to the mixture and centrifuged ($10\,000 \times g$, 5 min). A sample of 970 μl of the supernatant was removed for estimation of the radiolabelled products by scintillation counting. The NO synthase activity inhibited by L-NMMA was taken as the total NO synthase activity whereas that inhibited by L-NMMA but not by EGTA was taken as inducible NO synthase activity. The constitutive NO synthase activity was calculated from the difference between the inducible NO synthase and the total activity. NO synthase activity was expressed as pmol/min per mg protein.

2.3. Myeloperoxidase activity

The myeloperoxidase activity was determined using the method described by Bradley (Bradley et al., 1982) with minor modifications. The 8 cm segment of the colon was homogenised in ice cold phosphate buffer (50 mM, pH 6) containing 0.5% hexadecyltrimethylammonium-bromide, freeze-thawed three times, centrifuged ($10\,000 \times g$, 15 min, 4°C) and assayed spectrophotometrically (500 nm). Then 30 μl of the supernatant was mixed with 710 μl phosphate buffer (50 mM, pH 6) containing 0.167 mg/ml O-adenosine dihydrochloride and 0.0005% hydrogen peroxide. myeloperoxidase activities were expressed as mU/g wet tissue.

2.4. Effects of L-NAME

In the first series of experiments, the NO synthase inhibitor, L-NAME was administered in the drinking water (100 μg/ml) 2 days prior the instillation with TNBS and continued to the end of the study.

In a second series of studies, the administration of L-NAME (100 μg ml^{-1}) was commenced, 6 h after TNBS challenge. The animals received a 100 μg oral dose of L-NAME by gavage tube and then they received L-NAME (100 μg/ml) in the drinking water until the end of the experiment.

Daily water consumption was monitored for each cage containing 5 uniformly treated rats. L-NAME was added to the drinking water at a concentration of 100 μg/ml, corresponding to a dose of 25 mg/kg. This dose, route and method of L-NAME administration has been shown to inhibit NO synthase activity in the rat (Gardiner et al., 1990).

2.5. Materials

Trinitrobenzene sulphonic acid and [^{14}C]L-arginine monohydrochloride were obtained from Fluka (Fluka

Chemie AG, Buchs, Switzerland) and Amersham (Amersham France, Les Ulis, France), respectively. All other compounds were purchased from Sigma (Sigma Chimie, St Quentin Fallavier, France).

2.6. Statistics

Results are expressed as mean ± S.E.M. from n rats per experimental group. For statistical comparisons, the two-tailed Student's t-test and the analysis of variance with the Bonferoni test were used, where appropriate. $P < 0.05$ was taken as significant.

3. Results

3.1. Macroscopic damage, myeloperoxidase and nitric oxide synthase activity in TNBS colitis

Challenge with TNBS (30 mg in 0.25 ml of 50% ethanol) alone provoked macroscopic haemorrhagic necrosis of the colon, 6 h after its administration, reaching the maximum level between 6 and 72 h that involved 59 ± 4% and 54 ± 5% of the total colonic segment, respectively ($n = 10$ and 12; $P < 0.001$) and then declining over 8 days (Fig. 1).

Constitutive Ca^{2+}-dependent NO synthase activity in colon was 317 ± 29 pmol/min per mg protein ($n = 10$) in basal and was not modified over 72 h following TNBS administration: 333 ± 31 ($n = 10$), 266 ± 26 ($n = 7$), 265 ± 21, 193 ± 18 ($n = 8$) and 161 ± 16 ($n = 8$) pmol/min per mg protein at 3, 6, 12, 24 and 72 h, respectively. There was no detectable Ca^{2+}-independent inducible NO synthase activity in the rat colon prior to TNBS administration. Following TNBS challenge, colonic inducible NO synthase activity began to increase at 6 h, reaching its maximal level between 12 and 24 h (90 ± 9 pmol/min per mg protein at 24 h; $n = 9$; $P < 0.001$). The activity remained significantly increased until the end of the experiment as shown in Fig. 2.

After TNBS administration, colonic myeloperoxidase activity increased significantly from the control value (81 ± 7 mU/g tissue; $n = 5$), being increased by 15 fold at 24 h. This activity remained significantly elevated at 8 days in comparison with the control level, as shown in Fig. 3.

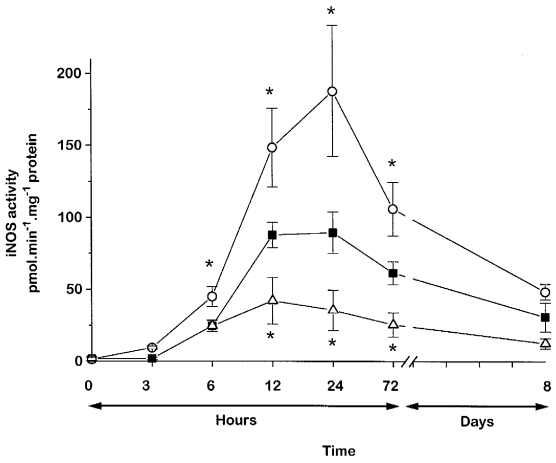

Fig. 2. Inducible nitric oxide activity following challenge with trinitrobenzene sulphonic acid (TNBS; 30 mg in 0.25 ml of 50% ethanol). Rats were treated with N^G-nitro-L-arginine methyl ester (L-NAME) added in a dose of 25 mg/kg per 24 h to drinking water commencing 48 h prior to challenge (○), or commencing 6 h following challenge (△) or in the control group, with saline (■). Data, shown as the Ca^{2+}-independent activity (pmol/min per mg protein), are means ± S.E. of 7–10 experiments, where * denotes significant difference from control ($P < 0.05$).

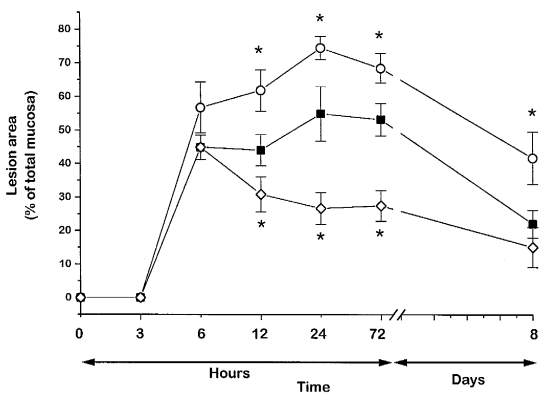

Fig. 1. Percentage of colonic mucosa damaged following challenge with trinitrobenzene sulphonic acid (TNBS; 30 mg in 0.25 ml of 50% ethanol). Rats were treated with N^G-nitro-L-arginine methyl ester (L-NAME) added in a dose of 25 mg/kg per 24 h to drinking water commencing 48 h prior to challenge (○), or commencing 6 h following challenge (◇) or in the control group, with saline (■). Data are shown as the mean ± S.E. of 6–15 experiments, where * indicates significant difference from control ($P < 0.05$).

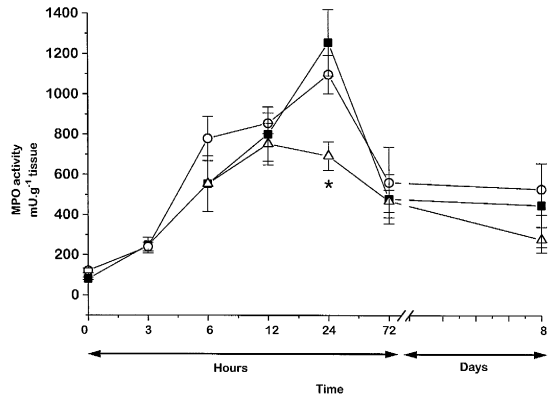

Fig. 3. Myeloperoxidase activity following challenge with trinitrobenzene sulphonic acid (TNBS; 30 mg in 0.25 ml of 50% ethanol). Rats were treated with N^G-nitro-L-arginine methyl ester (L-NAME) added in a dose of 25 mg/kg per 24 h to drinking water commencing 48 h previous to challenge (○), or commencing 6 h following challenge (△) or in the control group, with saline (■). Data shown as mU/g wet tissue, are means ± S.E. of 5–10 experiments, where * denotes significant difference from control ($P < 0.05$).

3.2. Effects of L-NAME pretreatment

The volume of drinking water consumed was not different between the groups treated by L-NAME (62 ± 20 ml) or not treated (64 ± 15 ml) and mean dose of L-NAME absorbed in overall was 27 ± 4 mg/kg per 24 h. Administration of L-NAME (25 mg/kg per 24 h) in the drinking water (commenced 2 days prior TNBS challenge) significantly augmented the colonic macroscopic mucosal injury determined from 12 h to 8 days after TNBS administration. The extent of mucosal damage reached a maximal area of $74 \pm 3\%$ of the total colonic segment after 72 h ($n = 15$, $P < 0.05$ as compared to the control challenged by TNBS) as shown in Fig. 1. Pretreatment with L-NAME, also appeared to cause an increase in induction of NO synthase provoked by TNBS administration during the experimental period, with a maximum elevation in inducible NO synthase activity of 209% as compared to the control (188 ± 45 pmol/min per mg protein, $n = 8$, versus 90 ± 14 pmol/min per mg protein, $n = 10$) (Fig. 2).

Pretreatment with L-NAME did not increase further, the elevation of myeloperoxidase activity induced by TNBS installation in the rat colon (Fig. 3).

3.3. Effects of delayed administration of L-NAME

The delay of administration of L-NAME (25 mg/kg per 24 h in the drinking water), at 6 h after TNBS challenge, significantly attenuated the macroscopic colonic injury 12, 24 and 72 h after TNBS challenge, with a maximal reduction by 63% (from 74 to 27%), as shown in Fig. 1. L-NAME administration also led to a significant reduction in the activity of inducible NO synthase in the colon at 12 to 72 h (Fig. 2). The inducible NO synthase activity was reduced by a maximum of 50% as compared to the control (45 ± 14 pmol/min per mg protein, $n = 8$, versus 90 ± 14 pmol/min per mg protein, $n = 10$) at 24 h after TNBS administration (Fig. 2).

The delayed administration of L-NAME attenuated the TNBS-induced elevation of the colonic myeloperoxidase activity, reducing the activity at 24 h by 45% (from 1097 ± 95 mU/g tissue, $n = 6$, to 692 ± 71 mU/g tissue as compared to the control group, $n = 9$) (Fig. 3).

4. Discussion

In the present time–response study, intrarectal administration of the hapten TNBS dissolved in 50% of ethanol provoked colonic macroscopic damage and an elevation of mucosal myeloperoxidase activity, as a measure of polymorphonuclear cell-related inflammation, within 6 h (Morris et al., 1989; Boughton-Smith et al., 1994; Pfeiffer and Qiu, 1995; Palmen et al., 1995). The inflammatory response reached its maximal level after 24 h, which then subsequently subsided over the ensuing two weeks. These observations are in agreement with previous findings, where TNBS-induced colonic injury showed a similar time-course of macroscopic lesions and mucosal myeloperoxidase activity (Morris et al., 1989; Yamada et al., 1992).

Expression of the calcium-independent inducible NO synthase following TNBS administration has also been detected. The inducible NO synthase activity was detectable 6 h after TNBS challenge and the increase was associated with the enhancement of mucosal myeloperoxidase activity and macroscopic lesions of the colon. This expression of inducible NO synthase activity in the rat colon has been previously observed 1 day following TNBS installation (Boughton-Smith et al., 1994). Such findings could indicate the involvement of inducible NO synthase in generation of colonic inflammation, as also described in other models of intestinal inflammation (Boughton-Smith et al., 1993b; Whittle et al., 1995).

In the current investigation, the inhibition of the NO synthase by pretreatement with L-NAME, commenced 2 days prior to challenge, augmented the colonic injury provoked by TNBS administration. Similar results have been described following continuous subcutaneous administration of L-NAME at a dose of 1–40 mg/kg per day in the 5 days preceding TNBS challenge (Pfeiffer and Qiu, 1995). Such pretreatment with L-NAME would lead to a inhibition of constitutive NO, known to be protective in the intestinal mucosa (Laszlo et al., 1994b; Lopez-Belmonte and Whittle, 1995). Pretreatment with L-NAME did not, however, affect the increased colonic myeloperoxidase caused by TNBS challenge. Similar findings on a lack of action on myeloperoxidase has been reported with subcutaneous infusion of L-NAME (Pfeiffer and Qiu, 1995). Thus, whereas inhibition of constitutive NO synthase is known to augment leukocyte adhesion to the microvasculature in naive rats (Kubes et al., 1991; Lopez-Belmonte and Whittle, 1995), further leukocyte infiltration does not appear following inhibition by L-NAME pretreatment, as least as estimated by myeloperoxidase in the inflamed colon. This finding suggests that leukocyte infiltration may not contribute to the mechanism of enhanced colonic injury. It is possible that pro-inflammatory mediators, such as platelet-activating factor, thromboxanes and leukotrienes could be participate in the increased colonic injury. The role of such mediators has been described in colon following concurrent acute administration of endotoxin and L-NAME (Laszlo et al., 1994a, Laszlo et al., 1994b). Moreover, the production of these pro-inflammatory products has been detected in the mucosa in TNBS-induced colitis (Morteau et al., 1993; Boughton-Smith et al., 1994).

By contrast, the delayed administration of L-NAME during the expression of inducible NO synthase at 6 h after challenge attenuated the area of colonic damage and reduced the myeloperoxidase activity following TNBS challenge. In another model of colitis induced by sulfhydryl blocker (iodacetamide), oral administration of L-NAME likewise reduced the colonic damage and myeloperoxidase

activity when administered after challenge (Rachmilewitz et al., 1995). Such findings further implicate the involvement of inducible NO synthase in the colitis provoked by different harmful agents. Furthermore, administration of NO synthase inhibitors at the time of the known expression of inducible NO synthase has been previously demonstrated to reduce the vascular permeability, as an important feature of inflammatory response, in endotoxaemic rats and in indomethacin-induced jejunal damage (Boughton-Smith et al., 1993b; Laszlo et al., 1994a; Whittle et al., 1995).

Administration of L-NAME in rat provoke vasoconstriction and ischemia which could contribute to damage in pretreated animal. However, we anticipate that this vascular effects would be observed in both the early and late stages following challenge. Such an effect would be unlikely to explain the divergent effect of L-NAME when administered at different time.

The delayed administration of L-NAME led to a decrease in the inducible NO synthase enzyme activity measured at 12 until 72 h. By contrast, L-NAME administration, commenced 2 days before TNBS challenge, led to an early detectable expression of inducible NO synthase some 3 h after challenge. Furthermore, the subsequent inducible NO synthase activity was significantly elevated as compared to the group which received TNBS alone. This finding suggests that early inhibition of constitutive NO production may accelerated the subsequent induction of inducible NO synthase. Some support for such a concept comes from a recent study showing a enhanced inducible NO synthase gene mRNA induction and promoter activation by treatment with N^G-monomethyl-L-arginine in inflammatory cells exposed to lipopolysaccharides and IFN-γ interferon (Weisz et al., 1996). Moreover, by promoting acute tissue injury, L-NAME may enhance the production of local pro-inflammatory mediators and the generation of cytokines such as interleukin-1 and hence may accelerate the subsequent expression of the inducible NO synthase (Busse and Mülsch, 1990; Moncada et al., 1991; Casini-Raggi et al., 1995; Moncada and Higgs, 1995).

NO has been shown to be cytotoxic and in combination with the superoxide radical, leads to the subsequent formation of the moieties, peroxynitrite and the hydroxyl radicals, which are highly injurious to cells (Beckman et al., 1990; Ischiropoulos et al., 1995). Therefore, such reactive oxygen metabolites could be promoted by TNBS (Grisham and Yamada, 1991). Following TNBS administration, high concentration of NO may be produce by macrophages and neutrophils, known to express the inducible enzyme (Marletta et al., 1988; McCall et al., 1989). However, the possible source of inducible NO synthase could also be the vascular endothelium, vascular smooth muscle and intestinal epithelial cells (Busse and Mülsch, 1990; Radomski et al., 1990; Palmer et al., 1992; Tepperman et al., 1993).

These findings suggest that during the development of chronic colonic inflammation, a local overproduction of NO by the inducible NO synthase in inflammed tissue, is involved in the injury. Our present findings indicate that the timing of administration of non-selective NO synthase inhibitors, such as L-NAME, in models of colitis is critical to the eventual outcome. Thus, pretreatment with L-NAME, which inhibits the protective constitutive NO synthase, exacerbates the subsequent damage following challenge, whereas delay of its administration, until the time of expression of the inducible NO synthase, has a beneficial action on colonic injury and inflammation. Such studies would predict that selective inhibitors of inducible NO synthase may exert protective actions in such models, regardless of the time of their administration and hence may be of therapeutic benefit in inflammatory bowel diseases.

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