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The role of constitutive and inducible nitric oxide synthase in senna- and cascara-induced diarrhoea in the rat

Angelo A. Izzo, Lidia Sautebin, Laura Rombolà, Francesco Capasso *

Department of Experimental Pharmacology, University of Naples 'Federico II', via D. Montesano 49, 80131 Naples, Italy

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Abstract

The role of constitutive and inducible nitric oxide (NO) synthase in rats treated with senna and cascara was studied. Senna (60 mg/kg p.o.) and cascara (800 mg/kg p.o.) ex vivo significantly increased Ca^{2+}-dependent constitutive NO synthase activity in the rat colon. Induction of NO synthase (12% of the total NO synthase) was associated with cascara, but not senna, administration. Dexamethasone (0.03–0.3 mg/kg i.p.), which inhibits the expression of inducible NO synthase, significantly and dose-dependently reduced cascara- (but not senna-) induced diarrhoea and colonic fluid secretion. These findings suggest that senna probably exerts its laxative effect through stimulation of the constitutive isoform of NO synthase, while the inducible isoform of NO synthase also seems to be involved in the laxative effect of cascara.

Keywords: Anthraquinone; Intestinal secretion; Nitric oxide (NO); Glucocorticoid; Laxative; Natural drug

1. Introduction

Senna and cascara are laxative anthraquinone drugs containing glycosides as active ingredients (Gaginella, 1994). Being large polar molecules, the glycosides are poorly absorbed in the small intestine. As they appear in the large intestine, colonic bacteria liberate the aglycone which is then reduced to the active anthrol form (Van Os, 1976; Lemli and Lemmens, 1980).

Several mechanisms of action have been proposed to explain the laxative effect of anthraquinones. Mechanisms include inhibition of Na^+,K^+-ATPase (see Gaginella, 1994), stimulation of prostaglandin (Beubler and Kollar, 1988), histamine (Capasso et al., 1986) or serotonin (Beubler and Schirgi-Degen, 1993) biosynthesis and other non-specific metabolic effects on the epithelial cells (Verhaeren, 1980). However, despite the fact that numerous mechanisms of action have been proposed to explain the laxative effect of senna and cascara, their precise mechanism of action has not been clearly elucidated.

Recently it has been shown that the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) reduces senna- and cascara-induced diarrhoea and fluid secretion (Izzo et al., 1996). NO, an endogenous mediator synthesized by the enzyme NO synthase from the amino acid L-arginine, is now recognized as an important regulator of gut functions (Miller and Gaginella, 1995). NO synthase can exist as a constitutive (Ca^{2+}/calmodulin-dependent) and an inducible (Ca^{2+}/calmodulin-independent) isoform in many tissues, including the gastrointestinal tract (Moncada and Higgs, 1993; Boughton Smith et al., 1993; Nichols et al., 1993). The expression, but not the activity, of the inducible NO synthase is inhibited by glucocorticoids such as dexamethasone. This action is distinct from that of arginine analogues, such as L-NAME, which are inhibitors of both the constitutive and inducible NO synthases (Moncada and Higgs, 1993).

The aim of the present study was to evaluate the role of constitutive and inducible NO in senna- and cascara-induced intestinal fluid secretion and diarrhoea. For this purpose we examined the activity of the constitutive and the inducible NO synthase following senna or cascara administration. The effect of dexamethasone, which inhibits the expression of the inducible NO synthase, was also evaluated.

* Corresponding author. Tel.: (39-81) 748-6415; Fax: (39-81) 748-6403.

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2. Materials and methods

2.1. Animals

Male Wistar (Nossan) rats, weighing 160–180 g, were used after one week for adaptation to the housing conditions. Standard food (Morini) was withheld 20 h before the experiments but there was free access to drinking water.

2.2. Nitric oxide synthase activity

The animals were killed by CO_2 8 h after oral administration of senna 60 mg/kg or cascara 800 mg/kg. Full thickness segments of the colon were homogenized at 4°C in 4 vols. of 20 mM HEPES buffer (pH 7.2) containing 320 mM sucrose, 1 mM DL-dithiothreitol, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin and 10 μg/ml leupeptin. The homogenates were centrifuged at 10 000 × *g* for 30 min at 4°C. The supernatants, i.e. the cytosolic fractions containing NO synthase activity, were stored at −70°C until use. Protein concentration in the cytosolic fraction was measured spectrophotometrically using bovine serum albumin as standard (Bradford, 1976).

NO synthase activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline, according to Salter et al. (1991). Briefly, an aliquot of the cytosolic fraction (100 μg of protein) was preincubated for 5 min at 37°C in 50 mM potassium phosphate buffer pH 7.2 containing 60 mM L-valine, 120 μM NADPH, 1.2 mM L-citrulline, 1.2 mM $MgCl_2$ and 0.24 mM $CaCl_2$. Samples were then incubated for 10 min at 37°C with L-[U-^{14}C]arginine (150 000 dpm) and 20 μM L-arginine. The reaction was stopped by the addition of 1.0 ml of a mixture of H_2O/Dowex-50W (1:1, v/v) (200–400, 8% cross-linked, Na^+ form). The Na^+ form of Dowex-50W was prepared by washing four times the H^+ form of resin with 1 M NaOH and then with bi-distilled water until the pH was less than 7.5. The resin was settled by centrifugation (11 000 × *g* for 3 min) in a microfuge (Beckman, Microfuge 11) and an aliquot of the supernatant was taken for scintillation counting (4 ml Pico-Aqua; Packard 1500). The activity of Ca^{2+}-dependent NO synthase was determined from the difference between the [U-^{14}C]citrulline produced by control samples and samples containg 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA); the activity of Ca^{2+}-independent enzyme was determined from the difference between the [U-^{14}C]citrulline produced by samples containg 1 mM EGTA and samples containing 1 mM EGTA plus 1 mM N^G-monomethyl-L-arginine (L-NMMA). The activity of both isoforms was expressed as pmol/min/mg of protein.

2.3. Laxative (diarrhoea) test

The rats were treated intraperitoneally with dexamethasone (0.03–0.3 mg/kg i.p.) 2 h before senna (60 mg/kg p.o.) or cascara (800 mg/kg p.o.) administration. The doses of senna and cascara were selected from our previous work (Izzo et al., 1996). 8 h after laxative administration, the rat cages were inspected (by an observer unaware of the treatment) for the presence of characteristic diarrhoeal droppings; their absence was recorded as a positive result, indicating protection from diarrhoea.

2.4. Fluid secretion

6.5 h after senna (60 mg/kg p.o.) or cascara (800 mg/kg p.o.) administration, the rats were anaesthetized with urethane (1.3 mg/kg i.p.) and the entire colon was rinsed with warm saline solution to remove the contents. 30 min later the colon was ligated and filled with 2.5 ml of Tyrode solution. After 60 min the rats were killed by CO_2 and the colon was removed. Net water transport was calculated from the volume of the fluid content minus the 2.5 ml of the solution used to fill the colon (Gaginella et al., 1994; Izzo et al., 1994; Mascolo et al., 1994a). Dexamethasone 0.03–0.3 mg/kg i.p. was given 2 h before laxative administration.

2.5. Chemicals

L-[U-^{14}C]Arginine hydrochloride (specific activity 304 mCi/μM) was obtained from Amersham (Amersham, UK). Dexamethasone 21-acetate, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), N^G-monomethyl-L-arginine (L-NMMA) and other reagents for NO synthase activity were purchased from Sigma (Milan, Italy). Senna pod extract (*Cassia angustifolia*) containing 45% sennoside B and cascara cortex extract (*Rhamnus purshiana*) containing 20% cascaroside A were a gift from Indena (Settala, Italy). Drugs were dissolved in distilled water.

2.6. Statistics

The χ^2-test was used to determine the significance of differences between groups with or without diarrhoea. One-way analysis of variance (ANOVA) followed by Duncan's new multiple-range test and Student's *t*-test were used for intestinal fluid volume and NO synthase activity data, respectively. A P value less than 0.05 was considered significant.

3. Results

3.1. Nitric oxide synthase activity

NO synthase activity, that was abolished by incubation in vitro with L-NMMA (1 mM), was detected in the supernatants of colonic homogenates of control rats, and was 0.506 ± 0.07 pmol/min per g tissue. This activity was abolished by incubation with EGTA (1 mM, Fig. 1).

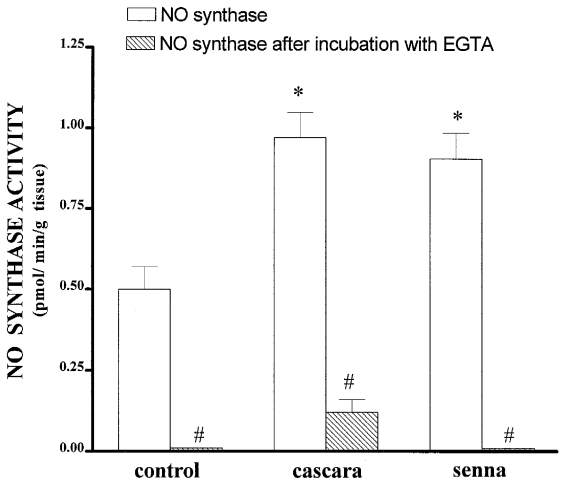

Fig. 1. Nitric oxide (NO) synthase activity in rat colonic tissue 8 h after oral administration of senna (60 mg/kg) or cascara (800 mg/kg). NO synthase activity, determined as the conversion of radiolabelled L-arginine to citrulline (pmol min/g/tissue), that is abolished in vitro by N^G-monomethyl-L-arginine (1 mM), in supernatant of colonic homogenates incubated in the absence or presence of EGTA (1 mM), is expressed as the mean values ± S.E.M. from 12–21 experiments for each experimental group. A significant increase in total NO synthase (compared to control) is given as * $P < 0.01$ and significant inhibition of this activity by incubation with EGTA is shown by $^{\#}$ $P < 0.01$.

Eight hours after senna (60 mg/kg) or cascara (800 mg/kg) administration, a significant increase of total NO synthase activity (abolished in vitro by L-NMMA 1 mM) was detected (Fig. 1). The NO synthase activity in the supernatant from the colon of senna-treated rats was abolished by incubation with EGTA (1 mM) whereas it was only partially reduced (88%) in cascara-treated rats (Fig. 1). The cascara-induced increase in Ca^{2+}-independent NO synthase activity was reduced by 98.2% ($n = 6$, $P < 0.01$) following dexamethasone (1 mg/kg) treatment.

In cascara-treated animals, Ca^{2+}-dependent NO synthase (0.86 ± 0.04 pmol/min per g) was significantly ($P < 0.01$) elevated compared to the control (0.506 ± 0.07 pmol/min per g).

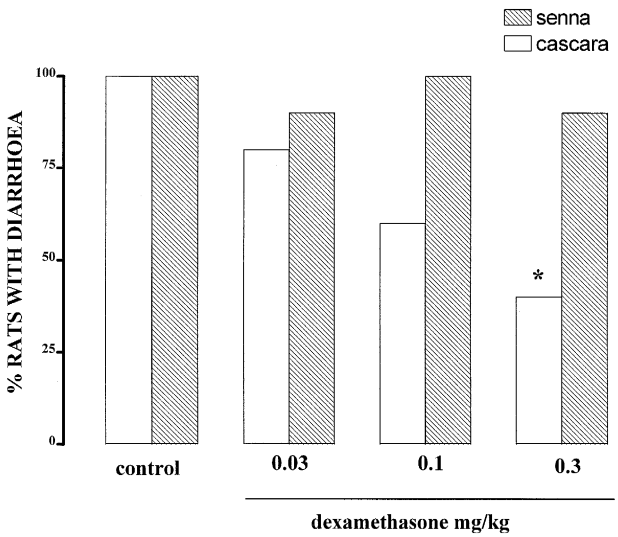

Fig. 2. Effect of graded doses of dexamethasone (0.03–0.3 mg/kg i.p., 2 h before laxative administration) on the percentage of rats (out of 10) with diarrhoea 8 h after oral senna 60 mg/kg and cascara 800 mg/kg. * $P < 0.05$ vs. control.

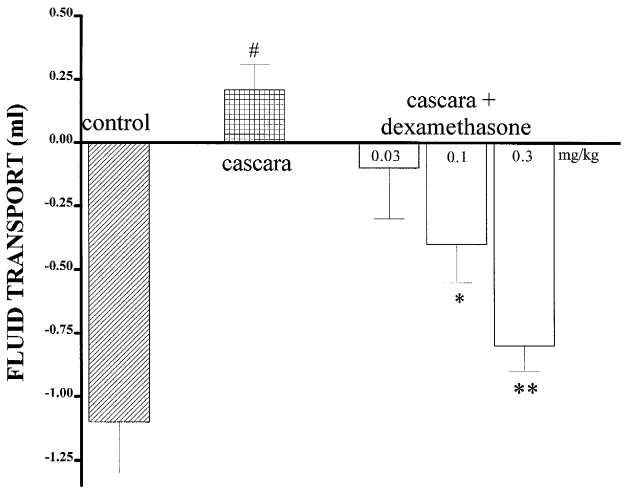

Fig. 3. Effect of dexamethasone (0.03–0.3 mg/kg i.p., 2 h before laxative administration) on water flux induced by cascara (800 mg/kg p.o.). A negative value represents net absorption and a positive value net secretion. Each bar represents the mean ± S.E.M. from 7–9 experiments. $^{\#}$ $P < 0.01$ vs. control, * $P < 0.05$ and ** $P < 0.01$ vs. cascara.

3.2. Diarrhoea

Eight hours after senna or cascara administration all rats produced evident diarrhoea (Fig. 2). Dexamethasone (0.03–0.3 mg/kg) dose dependently prevented cascara-induced diarrhoea (Fig. 2). A significant difference ($P < 0.05$, 60% protection) was achieved with the 0.3 mg/kg

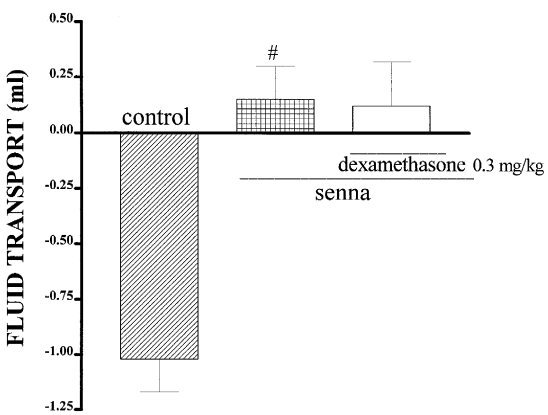

Fig. 4. Effect of dexamethasone (0.3 mg/kg i.p., 2 h before laxative administration) on water flux induced by senna (60 mg/kg p.o.). A negative value represents net absorption and a positive value net secretion. Each bar represents the mean ± S.E.M. from 7–9 experiments. $^{\#}$ $P < 0.01$ vs. control.

dose. Dexamethasone, at the same doses, failed to modify significantly senna-induced diarrhoea (Fig. 2).

3.3. Fluid secretion

Under control conditions there was absorption of water to the extent of 1.06 ± 0.15 ml. Pretreatment of the rats with senna or cascara reversed intestinal water absorption to net secretion (0.15 ± 0.15 ml for senna and 0.21 ± 0.10 ml for cascara, Figs. 1 and 2).

Dexamethasone (0.03–0.3 mg/kg) did not affect water movement in control rats (data not shown), but dose-dependently reversed the accumulation of luminal fluid evoked by cascara (Fig. 3). The effect of senna on fluid secretion was not significantly modified by dexamethasone (0.3 mg/kg, Fig. 4).

4. Discussion

We have shown that possible mediators for the laxative effect of senna and cascara include NO, which stimulates intestinal secretion in vitro (MacNaughton, 1993; Tamai and Gaginella, 1993; Wilson et al., 1993) and in vivo (Gaginella et al., 1994; Izzo et al., 1994; Miller et al., 1993a). We have recently shown that L-NAME, a reversible inhibitor of NO synthase, reduces cascara- and senna-induced colonic fluid secretion and diarrhoea (Izzo et al., 1996). In addition, the present results indicate that both senna and cascara, at doses that produced diarrhoea in the rat, increase NO synthase activity in the rat colon.

NO synthase can exist as a constitutive Ca^{2+}-dependent enzyme and an inducible Ca^{2+}-independent isoform. NO synthase can be induced by several drugs affecting intestinal secretion, including diphenylmethane laxatives (Gaginella et al., 1994), bile salts (Mascolo et al., 1994a) or endotoxin (Boughton Smith et al., 1993). We have shown that senna and cascara are able to increase the constitutive Ca^{2+}-dependent isoform of NO synthase, while only cascara is able to express the Ca^{2+}-independent inducible isoform of NO synthase. These results suggest that NO release arises from the activation of the constitutive isoform of NO synthase in the colon of rats treated with senna, while in cascara-treated animals NO is also derived from the inducible isoform of NO synthase. The potential sources for the NO released by senna and cascara include epithelial cells (Tepperman et al., 1993), the myenteric and/or submucosal neurons (Nichols et al., 1993), smooth muscle cells (Grider et al., 1992) and endothelial cells of the microvasculature (Palmer et al., 1988).

The response to cascara, but not senna, of diarrhoea and colonic fluid secretion was prevented, in a dose-dependent fashion, by dexamethasone, an inhibitor of inducible NO synthase in vivo and in vitro (Moncada and Higgs, 1993). Glucocorticoids such as dexamethasone seem the most efficient tools to distinguish between the constitutive and inducible isoform of NO synthase. Aminoguanidine, first described as a selective inhibitor of inducible NO synthase (Corbett et al., 1992; Hasan et al., 1993; Misko et al., 1993), has recently been shown to inhibit both the constitutive and the inducible isoform of NO synthase in the digestive tract (Laszlo et al., 1995). Although dexamethasone intrinsically enhances colonic electrolyte absorption (Binder, 1978) it is unlikely that its action is independent from the inhibition of inducible NO synthase. This is because the highest dose used was less than 1/10 of the dose that stimulates absorption in the rat colon (Binder, 1978; Sandle and McGlone, 1987). It seems probable, therefore, that inducible NO synthase could contribute to the laxative action of cascara, while it does not seem to play a role in senna-stimulated fluid secretion and diarrhoea. Because induction of NO synthase is expected when the intestinal mucosa is injured by chemicals or toxin (Boughton Smith et al., 1993; Miller et al., 1993b), the lack of induction of NO synthase in senna-treated animals is not surprising, since this laxative is well tolerated in the rat (Mascolo et al., 1992; Capasso et al., 1993; Izzo, 1996a).

The role of NO in mediating intestinal secretion seems to be uncertain, but probably depends upon whether the conditions under study are physiological or pathophysiological. In fact it has been demonstrated that L-NAME reversed rat jejunal fluid absorption in vivo (Schirgi-Degen and Beubler, 1995) and produced a secretagogue effect in isolated sheets of mouse ileum (Rao et al., 1994), suggesting that, physiologically, NO may promote fluid absorption. However, in some pathophysiological states, such as the diarrhoea associated with laxative administration (Izzo et al., 1994; Mascolo et al., 1994a; Gaginella et al., 1994) or trinitrobenzene sulphonic acid-induced colitis (Miller et al., 1993a), NO may be produced at a higher concentration capable of evoking net secretion.

Senna and cascara-induced diarrhoea might also result from changes in intestinal transit (Gaginella, 1994). Anthraquinone derivatives are reported to relax intestinal smooth muscle (Gaginella and Bass, 1978) and NO can relax intestinal circular muscles (Boeckxstaens et al., 1991). We can speculate that antraquinones could produce relaxation of the circular smooth muscle and thus produce an increase in the luminal diameter, thus promoting an increase in intestinal transit (expecially if the faecal contents are fluid, as is the case in diarrhoea). In addition it has been demonstrated that NO modulates motility changes produced by laxatives both in vitro (Izzo et al., 1993) and in vivo (Gaginella et al., 1994; Izzo et al., 1994; Mascolo et al., 1994a,b; Izzo, 1996b).

In conclusion, our results give direct evidence that NO may play an important role in senna- and cascara-induced diarrhoea and intestinal fluid secretion. NO arises from the activation of the constitutive isoform of NO synthase in senna-treated animals, while in cascara-treated animals

there is a probable contribution of both the constitutive and inducible isoform of nitric oxide synthase.

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