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Nitric Oxide Synthase Inhibition Promotes Carcinogen-Induced Preneoplastic Changes in the Colon of Rats

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L-Arginine is metabolized either to polyamines through arginase and ornithine decarboxylase (ODC) activities or to citrulline and nitric oxide (NO, nitrogen monoxide) through the NO synthase (NOS) pathway. Polyamine levels and ODC activity are high in tumor cells. The aim of this study was to test whether N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, modulates colon carcinogenesis. Adult male Wistar rats were treated with azoxymethane (AOM, 15 mg/kg ip), a chemical carcinogen, once a week for 2 weeks. One week after the second injection the rats were randomly divided into two groups. One group ($n = 8$) received L-NAME (10 mg/kg body wt/day) in drinking water. The control group ($n = 8$) received tap water. After 5 weeks, the rats receiving L-NAME showed enhanced mean basal arterial blood pressure, decreased heart rate, and a significant decrease of the cGMP content in the colonic mucosa. In both groups, AOM induced the formation of colonic aberrant crypt foci (ACF). In L-NAME-treated rats, the number of ACF was higher than in controls by 47%. ODC activity was enhanced by 11-fold. *S*-Adenosylmethionine-decarboxylase activity and putrescine concentration were significantly increased in the colonic mucosa of L-NAME-treated rats. The data suggest that L-NAME promotes carcinogen-induced preneoplastic changes in the colon by inhibiting NOS activity and by stimulating polyamine biosynthesis.

Key Words: **colorectal cancer; nitric oxide; N^G-nitro-L-arginine methyl ester; ornithine decarboxylase; putrescine.**

In many cells L-arginine is a precursor of both the polyamines and nitric oxide (NO,[2] nitrogen monoxide). Arginase, ornithine decarboxylase (ODC), and *S*-adenosylmethionine decarboxylase (AdoMetDC) are key enzymes in the formation of the polyamines (putrescine, spermidine, and spermine) (1, 2). Tumor cells exhibit higher levels of ODC activity than the corresponding nontransformed cells (3–5). NO synthase (NOS) catalyzes the formation of NO and L-citrulline from L-arginine (6, 7).

Three isoforms of NOS have been identified: constitutive endothelial and neuronal NOS and inducible NOS (8). Constitutive endothelial NOS and neuronal NOS are calcium and calmodulin dependent. The endothelial NOS is expressed in endothelial, cerebellar, and myocardial cells, while neuronal NOS is expressed in renal, bronchial, skeletal muscle, intestinal, and pancreatic cells (9, 10). The expression of inducible NOS is calcium and calmoduline independent and is induced in smooth muscle cells, macrophages, leukocytes, and Kupfer and epithelial cells after stimulation by inflammatory cy-

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[2] Abbreviations used: NO, nitric oxide/nitrogen monoxide; ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; NOS, NO synthase; L-NAME, N^G-nitro-L-arginine methyl ester; AOM, azoxymethane; ACF, aberrant crypt foci.

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tokines and endotoxins (8, 10–12). NO is a free-radical gas which has diverse actions in a number of tissues (13, 14). NOS can be inhibited by a nonselective inhibitor such as N^G-nitro-L-arginine methyl ester (L-NAME) (15, 16).

In the present study, we used azoxymethane (AOM) for the rapid induction of preneoplastic lesions in the colonic mucosa of rats (17). L-NAME was given in drinking water at doses which inhibited NOS activity in the colonic mucosa.

Our results support the conclusion that L-NAME promotes the development of preneoplastic lesions in the colon of AOM-treated rats presumably by inhibiting NO synthase activity and by increasing the biosynthesis of polyamines through the stimulation of ODC activity.

MATERIALS AND METHODS

Animals and Experimental Design

The experiments were conducted according to the National Research Council Guide for Use and Care of Laboratory Animals with authorization (No. 00573) of the French Ministry of Agriculture.

Male Wistar rats weighing 200 to 250 g were fed *ad libitum* a standard diet (A04, UAR, Villemoisson/Orge, France) and allowed free access to water. The animals were injected ip with AOM (15 mg/kg body wt) once a week for 2 weeks. One week after the last injection, the animals were randomly divided into two groups. One group ($n = 8$) (AOM alone) was used as control. The second group (AOM + L-NAME) was given L-NAME (10 mg/kg body wt) in drinking water daily for 5 weeks.

Blood Pressure, Heart Rate, and Body Weight Measurements

Systolic blood pressure and heart rate were determined by indirect tail cuff plethysmography in conscious rats using the electrosphygmomanometer (PE 300, Narco-Biosystem, Houston, TX). The mean of five separate determinations was taken as the day's blood pressure and heart rate. Body weight was also determined.

cGMP Determination in the Colonic Mucosa

The colon was flushed with ice-cold 0.9% NaCl and was cut open. For cGMP determination, the mucosa of the first half of the colon was obtained by scraping, homogenized in 1 ml of HCl (0.1 N), and centrifuged at 10,000*g* for 10 min at 4°C. The supernatants were stored at −70°C until cGMP assays were performed (18, 19).

Assessment of Aberrant Crypts in the Colon

Determination of aberrant hyperproliferative crypts was performed on a segment of 5 cm in length corresponding to the distal part of the colon. The segment was cut open, pinned out flat, and fixed in 10% buffered formalin. The colon samples were stained with 0.2% methylene blue for 5 min, rinsed in Krebs–Ringer buffer, placed onto a glass slide, and examined microscopically using a low-power objective (×5) for assessment of the number of aberrant crypts (17, 20). All counts were made by a blinded observer (F.G.). The criteria for the identification of aberrant crypts were (a) an increased size, (b) a thicker epithelial cell lining, and (c) an increased pericryptal zone relative to normal crypts.

Enzymes of Polyamine Metabolism

Colonic mucosal samples were homogenized in 100 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, 0.5 μM leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. After centrifugation at 33,000*g* for 25 min at 4°C, the supernatants were collected and ODC and AdoMetDC assays were performed rapidly. ODC activity was evaluated by measuring the rate of $^{14}CO_2$ formation from L-[1-^{14}C]ornithine (55 mCi/mmol) (21) and AdoMetDC activity was determined by measuring the rate of $^{14}CO_2$ formed from *S*-adenosyl-L-[carboxyl-^{14}C]methionine (60 mCi/mmol) (22).

Determination of Polyamines

Colonic mucosal samples were homogenized in 10 parts (w/v) 0.2 M perchloric acid, and the homogenates were centrifuged at 3000*g* for 10 min after standing for 16 h at 2°C. The clear supernatants

TABLE I

Effect of L-NAME on Body Weight, Systolic Blood Pressure, Heart Rate, and cGMP Content in the Colonic Mucosa

Treatment	Body weight (g)	Systolic blood pressure (mmHg)	Heart rate (beats/min)	Mucosal cGMP (pmol/g)
AOM (8)	382 ± 8	129 ± 1	450 ± 11	6.1 ± 0.9
AOM + L-NAME (8)	375 ± 12	143 ± 4*	405 ± 10*	2.7 ± 0.5*

Note. AOM, azoxymethane; AOM + L-NAME, azoxymethane + N^G-nitro-L-arginine methyl ester, 10 mg/kg body wt/day. Data are reported as means ± SE. Number of animals is in parentheses.

* $P < 0.05$ (Student's t test).

were diluted with 0.2 M perchloric acid and 200-μl aliquots were applied on a reversed-phase column for separation. The polyamines (putrescine, spermidine, and spermine) were determined by separation of their ion pairs formed with *n*-octanesulfonic acid, reaction of the column effluent with *o*-phthalaldehyde/2-mercaptoethanol reagent, and monitoring of fluorescence intensity (23).

Chemicals

L-NAME and AOM were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). L-[1-^{14}C]Ornithine and [1-^{14}C]*S*-adenosylmethionine were purchased from Amersham Pharmacia Biotech (Saclay, France).

Statistics

Data are reported as means ± SE. Statistical differences between groups were evaluated by one-way ANOVA and specific differences were identified using Student's t test.

RESULTS

Blood Pressure, Heart Rate, and Body Weight

Before L-NAME administration, the mean systolic blood pressure was 125 ± 4 mmHg and heart rate was 460 ± 8 beats/min. After 5 weeks of treatment with L-NAME (Table I), the systolic blood pressure of control rats was 129 ± 1 mmHg. Administration of L-NAME caused an increase in the blood pressure to 143 ± 3 mmHg ($P < 0.05$). Heart rate was significantly ($P < 0.05$) decreased after administration of L-NAME. Body weight was not affected by L-NAME.

cGMP Content of the Colonic Mucosa

The cGMP content of the colonic mucosa was estimated as a measure of NOS inhibition (Table I). Compared with the control group, mucosal cGMP content of the colon decreased by 56% in the L-NAME group ($P < 0.05$).

Aberrant Colonic Crypts and Crypt Foci

As shown in Fig. 1, all rats injected with AOM developed abnormal, hyperplastic colonic crypts, regardless of treatment. However, administration of L-NAME in the drinking water resulted in a 47% increase of the number of aberrant colonic crypts, when compared to AOM-treated controls.

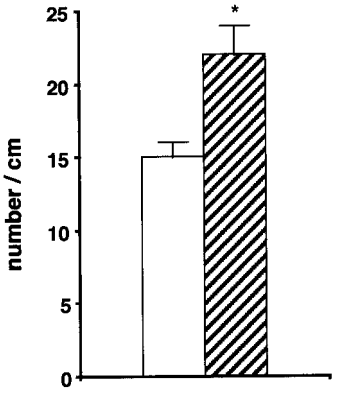

FIG. 1. Effect of L-NAME on the formation of aberrant crypt foci in the distal colon of azoxymethane (AOM)-treated controls (open column) and of rats receiving AOM and N^G-nitro-L-arginine methyl ester, 10 mg/kg body wt/day, in drinking water for 5 weeks (hatched column). Values are means ± SE of eight animals per group. *$P < 0.05$ (Student's t test).

TABLE II

Effect of L-NAME on Ornithine Decarboxylase (ODC) and *S*-Adenosyl-Methionine Decarboxylase (AdoMetDC) Activities in the Rat Colon

Treatment	ODC	AdoMetDC
	(pmol/mg protein/h)	
AOM (8)	1 ± 0.1	8 ± 0.4
AOM + L-NAME (8)	11 ± 3*	12 ± 0.7*

Note. AOM, azoxymethane; AOM + L-NAME, azoxymethane + N^G-nitro-L-arginine methyl ester, 10 mg/kg body wt/day. Data are reported as means ± SE. Number of animals is in parentheses.
* $P < 0.05$ (Student's *t* test).

Activity of Enzymes of Polyamine Metabolism

The activities of the two rate-limiting enzymes of polyamine synthesis, ODC and AdoMetDC, were measured in the colonic mucosa (Table II). Treatment with L-NAME increased markedly ODC activity from 1 ± 0.1 to 11 ± 3 pmol/mg protein/h ($P < 0.01$). The activity of AdoMetDC was also significantly ($P < 0.05$) increased by treatment with L-NAME.

Determination of Polyamines

Colonic polyamines (putrescine, spermidine, and spermine) were measured in AOM controls and AOM + L-NAME-treated rats (Fig. 2). The amount of putrescine was enhanced by 25% after L-NAME treatment (240 ± 22 pmol/mg protein vs 300 ± 12 pmol/mg protein, $P < 0.05$). The amounts of spermidine and spermine were not affected.

DISCUSSION

Administration of L-NAME, an inhibitor of NO formation, induced systemic hypertension and reduced heart rate in AOM-treated rats when given chronically in tap water for 5 weeks. These findings, which are consistent with previous reports (24, 25), confirm that under our experimental conditions administration of L-NAME inhibited NOS activity. NO is known to activate intracellular guanylate cyclase, leading to the accumulation of cGMP in various tissues (13). Cyclic GMP mediates the activation of protein kinases and phosphorylases, leading to vascular smooth muscle relaxation accounting for vasorelaxation (26, 27). In association with the observed systemic hypertension, the decrease of the cGMP content in the colonic mucosa is evidence for a decreased NO production in the colon of rats receiving L-NAME.

NO has recently been implicated in the development of established tumors, and indirect and direct evidence has been provided for the stimulation of tumor progression by NO. In a rat colon adenocarcinoma model, L-NAME reduced NO production and tumor growth (28). Similarly, in a mouse mammary adenocarcinoma model, antitumor effects of L-NAME have been described (29).

The reports suggesting a facilitatory role of NO on tumor growth and metastasis are numerous and contrast with those reported for murine K1735 melanoma cells, in which the level of inducible NOS expression was inversely correlated with their ability to form metastasis (30). The data may be explained by NO-mediated tumor cell apoptosis. However, such a mechanism is ineffective in many established tumors due to the clonal selection of NO-resistant cells. Mutation or loss of the tumor suppressor gene p53 may be one of the genetic mechanisms involved in the development of NO resistance (31).

NO stimulation of tumor progression has been shown to be the result of several mechanisms: promotion of tumor cell invasiveness (32), tumor cell

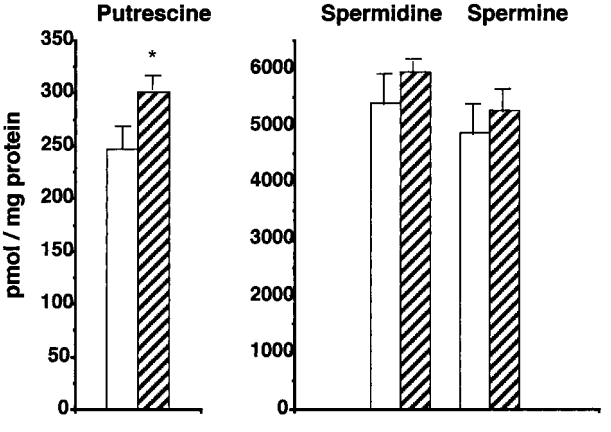

FIG. 2. Polyamine content in the colonic mucosa of azoxymethane (AOM)-treated controls (open column) and of rats receiving AOM and N^G-nitro-L-arginine methyl ester, 10 mg/kg body wt/day, in drinking water for 5 weeks (hatched column). Values are means ± SE of eight animals per group. *$P < 0.05$ (Student's *t* test).

migration (33), and tumor-induced angiogenesis (34, 35). In addition, it is known that human colon adenocarcinoma cells which constitutively express NO moderately exhibit a higher degree of vascularity after transplantation in nude mice (36). High NOS activity has also been associated with angiogenesis in colonic adenomas (37), and the angiogenesis-stimulating role of NO has been well established in wound healing (38), tissue ischemia (39), and VEGF-induced angiogenesis (40).

However, NO has also been suggested to act as an endogenous antiangionic mediator (41) since NO donors (e.g., sodium nitroprusside) caused inhibition of angiogenesis in the chorioallantoic membrane of chick embryos, whereas L-NAME was shown under these circumstances to stimulate angiogenesis. These contradictory results may be explained by the fact that the model (chick chorioallantoic membrane of chick embryos) does not allow one to distinguish between vasculogenesis (blood vessel formation from embryonic cells) and angiogenesis (blood vessel formation from preexisting vasculature).

As was shown above, the progression of established tumors seems generally strongly correlated with elevated NO production by NOS. However, this is not in contradiction with our present findings, which imply only an early preneoplastic state of colon carcinogenesis. During this stage, abnormal cells may be eliminated by NO-mediated apoptosis. Thus, NOS inhibition with L-NAME will favor mucosal hyper- and dysplasia leading to colonic preneoplastic lesions. This hypothesis is substantiated by the observation that allelic and p53 point mutations, which are associated with late colonic tumor development, rarely occur in preneoplastic lesions, i.e., in aberrant crypt foci (42).

We selected AOM as inducer of colon carcinogenesis since AOM is a potent and specific inductor of tumorigenesis in the colon of rats (43). The area of the colon in which multiple precancerous lesions and tumors are observed is the same as in humans (20). All animals examined after treatment with AOM presented multiple preneoplastic aberrant crypt foci (ACF) in the distal half of the colon. Administration of L-NAME, starting 1 week after the last AOM injection, increased the number of ACF in the colon. Concomitantly, ODC and AdoMetDC activities in the colon were increased. Numerous tumor promoters have been shown to induce ODC and AdoMetDC (5, 7, 44), which are key regulatory enzymes in polyamine synthesis.

Elevated levels of polyamines appear to be associated with tumor development (44). Low dietary levels of polyamines are able to inhibit the neoplastic process in the colon of rats treated with a chemical carcinogen (45). In contrast, high levels of dietary polyamines promote neoplastic growth (46). The formation of various tumors can be prevented in animal models by inhibiting ODC activity with 2-(difluoromethyl)-ornithine, a selective irreversible inhibitor of ODC (47). It is evident that during the early stages of colon carcinogenesis, an increased polyamine synthesis rate promotes cell proliferation.

Since L-arginine is a common precursor of both the polyamines and NO, an increased rate of polyamine synthesis may decrease the amount of L-arginine available for NO formation. In addition, there is some evidence that polyamines inhibit NOS activity directly (48). Since NO and polyamines have opposite effects on the regulation of colonic carcinogenesis and since they derive from the same precursor, a common regulatory switch that regulates the relative rates of NO and polyamine synthesis may be hypothesized.

It was reported that NO donors markedly suppress the activity of ODC in a transformed kidney proximal tubule cell line (49). ODC inactivation was reversed in the presence of a selective NOS inhibitor. These data suggest that ODC expression is mediated by NO and that increased intracellular levels of NO suppress ODC activity.

Our data suggest that blocking the physiological NO synthase pathway may promote preneoplastic changes in the colon by decreasing NO release and by inducing ODC and polyamine synthesis.

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