Carcinogenesis vol.20 no.4 pp.641–644, 1999

Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor

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Inducible nitric oxide synthase (iNOS) is overexpressed in colonic tumors of humans and also in rats treated with a colon carcinogen. iNOS appear to regulate cyclooxygenase-2 (COX-2) expression and production of proinflammatory prostaglandins, which are known to play a key role in colon tumor development. Experiments were designed to study the inhibitory effects of *S*,*S*′-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (PBIT) a selective iNOS-specific inhibitor, measured against formation of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF). Beginning at 5 weeks of age, male F344 rats were fed experimental diets containing 0 or 50 p.p.m. of PBIT, or 2000 p.p.m. of curcumin (non-specific iNOS inhibitor). One week later, rats were injected s.c. with AOM (15 mg/kg body wt, once weekly for 2 weeks). At 17 weeks of age, all rats were killed, colons were evaluated for ACF formation and colonic mucosa was assayed for isoforms of COX and NOS activities. Both COX and iNOS activities in colonic mucosa of the AOM-treated rats were significantly induced. Importantly, 50 p.p.m. PBIT suppressed AOM-induced colonic ACF formation to 58% ($P < 0.0001$) and crypt multiplicity containing four or more crypts per focus to 78% ($P < 0.0001$); it also suppressed AOM-induced iNOS activity. Curcumin inhibited colonic ACF formation by 45% ($P < 0.001$). These observations suggest that iNOS may play a key regulatory role in colon carcinogenesis. Developing iNOS-specific inhibitors may provide a selective and safe chemopreventive strategy for colon cancer treatment.

Introduction

Large bowel cancer is one of the leading causes of cancer deaths in both men and women in Western countries, including the USA (1). Epidemiological and experimental studies indicate that the risk of developing colon cancer may be due to combined actions of environmental factors and endogenous promoting agents (2). Recently, much attention has been given to endogenous factors, which appear to be directly responsible for tumor cell growth, spreading and invasion (progression and metastasis). Identifying such endogenous factors should lead not only to an understanding of the processes of tumor cell progression and metastasis but also provide new strategies for developing agents that specifically suppress these processes.

Nitric oxide (NO) is produced endogenously by a family of nitric oxide synthases (NOSs), with a wide range of physiological and pathophysiological actions (3,4). Only iNOS, a distinct, Ca^{+2}-independent isoform of NOS (130 kDa protein), can be expressed in response to pro-inflammatory agents; it produces sustained NO concentrations which are high when compared with the low levels produced by the Ca^{+2}-dependent neuronal and endothelial isoforms (3–5). Recent studies in our laboratory and by others suggest that iNOS may play a role in tumor development. Increased NOS expression and/or activity was reported in human gynecological (6), breast (7) and central nervous system (8) tumors. Also, nitrotyrosine accumulation in inflammed mucosa of patients with ulcerative colitis and gastritis, indicates production of NO and its involvement in the pathogenesis of these diseases (9). Recently Ambs *et al.* (10) have documented expression and activity of iNOS in human colon adenomas. Our results (11) and those of Takahashi *et al.* (12) have also demonstrated that azoxymethane (AOM)-induced colon tumors have increased expression and/or activity of iNOS by comparison to levels in adjacent colonic tissue. Importantly, iNOS have been shown to be involved in the regulation of cyclooxygenase-2 (COX-2), which plays a pivotal role in colon tumorigenesis (13). These observations clearly suggest that iNOS may enhance tumorigenesis. In the present study we tested the hypothesis that iNOS-selective inhibitors may be effective colon cancer chemopreventive drugs which offer several advantages over known agents.

Aberrant crypt foci (ACF) are recognized as early preneoplastic lesions. They have consistently been observed in experimentally induced colon carcinogenesis in laboratory animals (14). Pretlow *et al.* (15) have also shown that these lesions are present in the colonic mucosa of patients with colon cancer and have suggested that aberrant crypts are putative precursor lesions from which adenomas and carcinomas develop in the colon. ACF express mutations in the *apc* gene and *ras* oncogene that appear to be biomarkers of colon cancer development (16). The present study was designed to evaluate the inhibitory activity of *S*,*S*′-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (PBIT) (Figure 1), an iNOS-selective inhibitor, and of curcumin, a non-specific inhibitor, against AOM-induced ACF formation and on modulation of COX and NOS activities in the colon of male F344 rats. The major goal of this study was to determine whether PBIT compound is conceivably an effective chemopreventive agent in preclinical efficacy studies, so that it may eventually be considered for human clinical trials

Materials and methods

Animals, diets, carcinogen and chemopreventive agents

AOM (CAS:25843–45–2) was purchased from Ash Stevens (Detroit, MI). PBIT was obtained from Cayman Chemicals (Ann Arbor, MI). Curcumin (Gene Print, Philadelphia, PA), a known inhibitor of colon carcinogenesis, was included in the current study as a positive control and non-specific iNOS

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PBIT, *S*,*S*′-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiorea; TLC, thin layer chromatography.

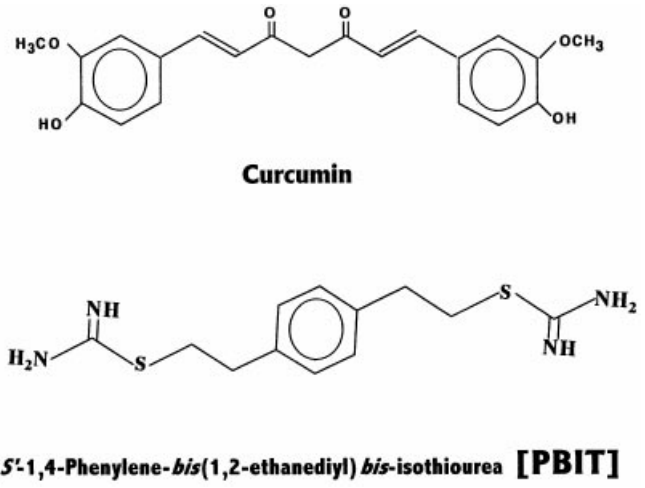

Fig. 1. Structure of PBIT and curcumin.

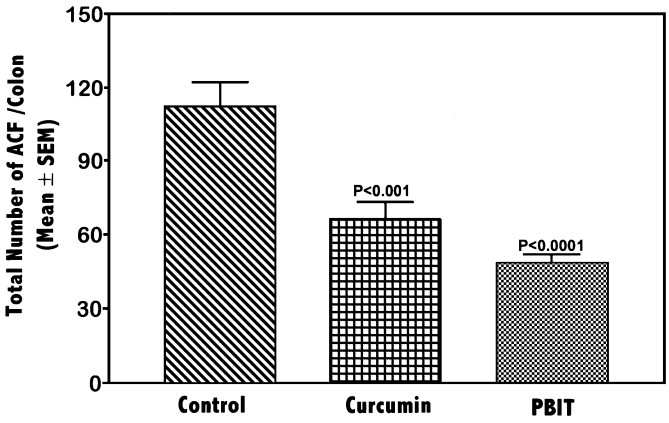

Fig. 2. Effect of PBIT and curcumin on mean number of AOM-induced colonic ACF in F344 rats.

inhibitor. The specificity of PBIT on iNOS inhibition has been established previously in cytokine-induced colorectal adenocarcinoma DLD cells (17). PBIT has been shown to be >500 times and ~25 times more selective as an inhibitor toward iNOS, when compared with human endothelial NOS and brain NOS synthetic activities, respectively (17).

Weanling male F344 rats were purchased from Charles River Breeding Laboratories (Kingston, NY). All ingredients of the semipurified diet were obtained from Dyets (Bethlehem, PA) and were stored at 4°C until the experimental diets were prepared. The rats were held in quarantine for 1 week with *ad libitum* feeding of modified AIN-76A semipurified control diet (18). They were randomly distributed into various dietary groups and were transferred to an animal holding room where they were housed in plastic cages, three rats to a cage, under controlled conditions of a 12 h light/12 h dark cycle, 50% relative humidity and 21°C room temperature. Experimental diets were prepared by mixing PBIT or curcumin with the modified AIN-76A control diet.

Experimental procedure

Beginning at 5 weeks of age, groups of rats (18 rats/group) were fed the modified AIN-76A (control) or experimental diets containing 50 p.p.m. PBIT or 2000 p.p.m. curcumin. At 7 weeks of age, all animals except the vehicle-treated rats received AOM by s.c. injection once weekly for 2 weeks at a dose rate of 15 mg/kg body wt/week. Animals intended for vehicle treatment were given an equal volume of normal saline. Five days after the second AOM-treatment, rats intended for the analysis of Ca^{+2}-dependent and independent NOS and COX-1 and COX-2 activities were killed; their colonic mucosae were scraped, quickly frozen under liquid nitrogen and stored at –70°C for further analysis. The remaining rats continued on control and experimental diets until the termination of the study when they were 17 weeks of age. All rats were killed by CO_2 euthanasia. The colons were removed (12/group), flushed with Krebs Ringer solution, opened from cecum to anus and fixed flat between two pieces of filter paper in 10% buffered formalin for ACF analysis.

ACF analysis

After a minimum of 24 h in buffered formalin, the opened colons were cut into 2 cm segments, starting at the anus; for the next 5–10 min they were placed in a Petri dish containing 0.2% methylene blue in Krebs Ringer solution. They were then placed, mucosal side up, on a microscope slide and observed through a light microscope. ACF were recorded according to standard procedures that are being used routinely in our laboratory (19). Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from lamina to basal surface of cells and the easily discernible pericryptal zone. The parameters assessed were occurrence and multiplicity of aberrant crypts. Multiplicity was determined as the number of crypts in each focus and categorized as containing up to three, four or more aberrant crypts/focus. All colons were scored by one observer without knowing the identity of agents under study; scores were checked at random by a second observer.

Assay of NOS and iNOS activity

Conversion of L-arginine to L-citrulline was measured by a modification of an earlier described method (10). The assay was carried out by adding 100 µg sample protein to 150 µl of assay buffer (50 mM HEPES, 1 mM DTT, 1 mM $MgCl_2$, 5 mg/l pepstatin A, 0.1 mM PMSF and 3 mg/l aprotinin, pH 7.4) containing 70 µM arginine, 250 000 d.p.m. L-[^{3}H]arginine, 2 mM NADPH, 5 µM tetrahydro biopterin, 5 µM flavin adenine dinucleotide and 0.5 mM $CaCl_2$ for determining total NOS activity, or 1 mM EGTA (without calcium) was added to determine the Ca^{+2}-independent iNOS activity. After 30 min at 37°C, the enzymatic reaction was stopped with 100 µl of 1 M trichloroacetic acid. Then samples were adjusted to pH 4.6 by adding 500 µl of 20 mM HEPES and they were loaded onto a Dowex AG 50W-X8 resin column. L-[^{3}H]citrulline was eluted and separated on TLC. Radioactivity was counted by a BioScan Radiomatic detector. Results are expressed as pmol L-[^{3}H]citrulline released/mg protein/min.

COX-1 and COX-2 activity

Activities in colonic samples were assayed by using a slight modification of previously published methods (19). Briefly, 150 µl of the reaction mixture containing 12 µM [^{14}C]arachidonic acid (420 000 d.p.m.), 1 mM epinephrine, 1 mM glutathione in 50 mM of phosphate buffer (pH 7.4) and 20–30 µg of microsomal protein. To determine COX-1 activity, proteins were preincubated with 25 µM of celecoxib, a COX-2 selective inhibitor. To assess COX-2 activity, proteins were preincubated with 100 µM of aspirin to block the activity of COX-1. After incubation at 37°C for 15 min, the reaction was terminated by adding 40 µl of 0.2 M HCl. The COX-mediated metabolites of arachidonic acid were extracted with ethyl acetate (3×0.5 ml). The combined extracts were evaporated to dryness under nitrogen, redissolved in chloroform and subjected to thin layer chromatography on precoated TLC plastic plates (silica G 60, 20×20 cm, layer thickness 150 µm). The TLC plates were developed with a solvent system containing chloroform/methanol/acetic acid/water (100/15/1.25/1, v/v/v/v) and exposed in an iodide chamber for 5 min to visualize the standards. The metabolites of [^{14}C]AA corresponding to PGE_2, $PGF_{2\alpha}$, PGD_2, 6-Keto $PGF_{1\alpha}$ and TXB_2 were detected by their comigration (Rf-values) with authentic standards. The area of each metabolite was determined in a Bioscan System 200 image scanning counter (Bioscan, Washington, DC) equipped with β-detector.

Statistical analysis

All results were expressed as means ± SEM and were analyzed by one-tailed Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

General observation

The body weights of rats treated with vehicle or AOM and fed the control or experimental diets were comparable throughout the study period (data not shown). In vehicle-treated animals, feeding of experimental diets containing PBIT and curcumin did not produce any gross changes in the liver, kidney, intestine and lungs.

Rats that were treated with saline and fed the control or experimental diets showed no evidence of ACF formation in the colon (data not shown). In rats fed the control diet, AOM treatment induced, on average, ~114 ACF/colon and 23 foci that contained multiple (four or more) aberrant crypts/focus (Figures 2 and 3). ACF were predominantly observed in the

Table I. Effect of curcumin and PBIT on AOM-induced colonic mucosal NOS, iNOS, COX-1 and COX-2 activities in male F344 rats

	NOS activity (pmol [^{3}H]citrulline/mg protein/min)		COX activity (pmol [^{14}C]AA metabolized/mg protein/min)	
Experimental group	NOS	iNOS	COX-1	COX-2
Vehicle-treated				
Control	8.8 ± 1.6[a]	≤0.1	6.9 ± 1.1	0.3 ± 0.15
AOM-treated				
Control	10.3 ± 1.8	1.7 ± 0.4[b,**]	13.2 ± 2.3[b,**]	2.1 ± 0.4[b,**]
Curcumin	7.8 ± 1.5	1.0 ± 0.2[c,*]	8.3 ± 1.4	1.5 ± 0.3
PBIT	7.5 ± 1.6	0.5 ± 0.2[c,**]	10.4 ± 1.7	0.8 ± 0.2[c,**]

[a]Mean ± SEM (n = 6–8).
[b]Values in vertical columns are significantly different from vehicle control group by Student's t-test; *$P < 0.05$; **$P < 0.01$–0.001.
[c]Values in vertical columns are significantly different from carcinogen control group by Student's t-test; **$P < 0.01$–0.001.

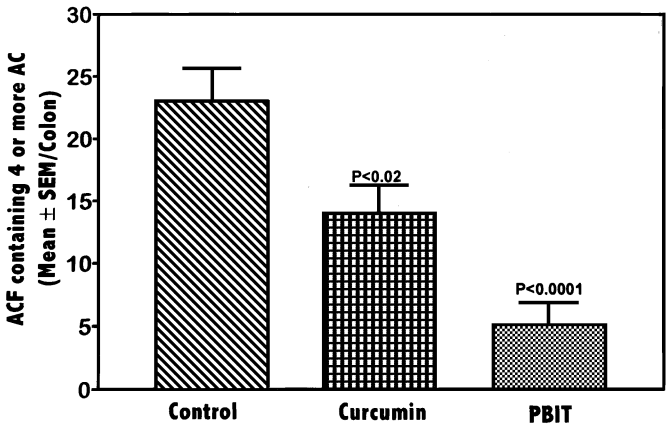

Fig. 3. Effect of PBIT and curcumin on AOM-induced colonic aberrant crypt multiplicity (four or more) in F344 rats.

distal colons. Efficacy endpoints used in this study were inhibition of total occurrence of ACF as well as reduction of number of multicrypt clusters (four or more) of aberrant crypts. In the present study, curcumin, a non-specific iNOS inhibitor, which inhibits colon carcinogenesis in animals was also found to be an effective inhibitor of total ACF/colon (>40%) and of multicrypt clusters containing four or more crypts/focus (46%, $P < 0.001$). Administration of 50 p.p.m. of PBIT significantly suppressed the total number of ACF/colon (58%, $P < 0.0001$) as compared with control diet. Importantly, PBIT significantly inhibited (78%, $P < 0.0001$) aberrant crypt multiplicities (four or more) per focus.

Table I summarizes the colonic mucosal activities of NOS and COX isoforms. Administration of AOM significantly increased iNOS activity ($P < 0.001$) in the colonic mucosa but had minimal effect on the Ca^{+2}-dependent NOS activity ($P > 0.05$). While AOM-induced both isoforms of COX activities ($P < 0.01$–0.002), importantly, we noticed 4- to 5-fold less induction of COX-2 synthetic activity than of COX-1 activity. Curcumin inhibited AOM-induced colonic mucosal iNOS by ~40%, but it had only a moderate effect on NOS, COX-1 and COX-2 activities. Interestingly, administration of PBIT produced significant and specific suppression ($P < 0.001$) of only the inducible isoforms of NOS and COX activities in colonic mucosa.

Discussion

The present study demonstrates that administration of the iNOS-specific inhibitor, PBIT, significantly suppresses AOM-induced colonic ACF formation in rats and that PBIT selectively suppresses the carcinogen-induced colonic mucosal inducible NOS and COX activities. The effect of PBIT on the suppression of inducible isoforms of NOS and COX is selective and highly pronounced. In the present study it is not clear how this selective iNOS inhibitor, PBIT, produces modulatory effects on the AOM-induced COX-2 activity. Based on the extensive studies by Garvey *et al*. (20) PBIT possesses selective inhibitory activity against the iNOS, when compared with endothelial nitric oxide synthase (eNOS) or neuronal nitric oxide synthase (nNOS) and the nature of this inhibition is competitive. Although we have not tested possible effect of PBIT on the expression levels of iNOS in AOM-induced colonic extracts, it is unlikely that this agent influences iNOS expression. Also PBIT, or structurally similar agents, competes with the substrate (L-arginine) active site, because of its structural similarity to guanidine, that it binds rapidly in the guanidine portion of the substrate site (20). Previously, it had been shown that expression of inducible isoforms of NOS and COX was increased in human colorectal tumors as well as in carcinogen-induced tumors in laboratory rats (10–13). The precise pathobiological functions of both iNOS and COX in colorectal carcinogenesis are more difficult to specify. Recent reports suggest that iNOS may contribute to tumor development or acceleration of the progression stage. Apart from its endogenous carcinogenic activity, NO is an endothelial growth factor and specifically mediates the tumor vascularization to regulate blood flow (21). Importantly, only iNOS produces sustained NO concentrations in the micromolar range and this inducible isoform is specifically associated with neoplastic tissue provided as a selective target for the development of selective agents for neoplastic growth suppression. The inhibition of colon carcinogenesis by the iNOS selective inhibitor PBIT underscores that iNOS plays a role in tumorigenesis.

It is likely that iNOS is associated with the modulation of COX-2 activity in colo-rectal cancer. NO enhances the activity and expression of COX-2 in a variety of cell types (22). Increased COX-2 expression and activity in colorectal tumors has been reported in humans and rodents (23). Overexpression of COX-2 may increase the production of several mitogenic eicosanoids, which are involved in antiapoptotic activity in tumor epithelium. On the basis of this hypothesis, several selective COX-2 inhibitors were developed and tested in colon carcinogenesis. Recently, we have shown that celecoxib, a COX-2 inhibitor, suppresses AOM-induced colonic ACF (24) and development of adenocarcinoma (25) in male rats. In this study we have shown that carcinogen-inducible isoforms of NOS and COX are selectively suppressed in colonic mucosa by PBIT, indicating a possible association with early events

in colon carcinogenesis. Further, these results confirm that the suppression of iNOS activity by PBIT may lead to the down-regulation of COX-2 activity and formation of proinflammatory eicosanoids.

There is currently an intense effort to develop selective inhibitors of inducible isoform enzymes, such as iNOS and COX-2, while sparing the desirable functions of their constitutive isoforms. Because of the involvement of iNOS and COX-2 (NO or prostaglandins) in the pathogenesis of colon cancer, selective enzyme inhibitors are promising chemopreventive agents (26). COX-2-selective inhibitors are definitely holding advantages over COX-1 inhibitors. But recent observations in COX-2 deficient mice suggest that this isoform plays an important constitutive role in renal development, ovulation and uterine implantation (27,28). Reduction of the activity of this enzyme can lead to severe renal pathology and multiple reproductive failures (27,28). The constitutive and induced expression of COX-2 in the kidney suggests that recently developed (developing) specific inhibitors of this enzyme, while gastroprotective, may still cause renal toxicity. On the basis of the data presented here we believe that iNOS-specific inhibitors provide advantages over the COX-2 inhibitors as probably safer and more effective chemopreventive agents against colon cancer.

Acknowledgements

The authors would like to thank Ms Barbara Simi for technical assistance and the staff of the Research Animal Facility for expert handling of bioassays. They thank Ms Laura Nast for preparing and Ms Ilse Hoffmann for editing this manuscript. This work was supported in part by USPHS grant CA17613.

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Received August 24, 1998; revised November 19, 1998; accepted December 4, 1998