Chemopreventive Effects of a Selective Nitric Oxide Synthase Inhibitor on Carcinogen-Induced Rat Esophageal Tumorigenesis

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ABSTRACT

The inducible nitric oxide synthase (iNOS) generates a high concentration of nitric oxide (NO) in tissues. Increased NO production is associated with many disorders including esophageal cancer. Previous studies in our laboratory demonstrated an association between increased iNOS expression and the development of *N*-nitrosomethylbenzylamine (NMBA)-induced tumors in the rat esophagus. On the basis of these observations, we initiated a bioassay to evaluate the ability of *S,S'*-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (PBIT), a selective iNOS inhibitor, to prevent the progression of esophageal tumors in rats preinitiated with NMBA. Rats were given s.c. injections of NMBA (0.25 mg/kg body weight) three times per week for 5 weeks. One week later, they were fed a synthetic diet containing either 50 or 100 ppm PBIT until the end of the bioassay (25 weeks). PBIT reduced the incidence of esophageal cancer from 96% in NMBA-treated rats to 83% and 77% ($P < 0.05$) in rats treated with 50 and 100 ppm PBIT, respectively. Tumor multiplicity was reduced from 3.64 ± 0.42 tumors per esophagus in NMBA-treated rats to 1.79 ± 0.25 ($P < 0.001$) and 1.50 ± 0.24 ($P < 0.0001$) in rats treated with 50 and 100 ppm PBIT, respectively. PBIT reduced the production of NO in NMBA-induced preneoplastic and papillomatous esophageal lesions when compared with comparable lesions in rats treated with NMBA only. iNOS mRNA expression was not modulated by PBIT. These observations suggest that iNOS plays a role in tumor development and that its selective inhibitor, PBIT, significantly inhibits esophageal tumor progression presumably through reducing the production of NO.

INTRODUCTION

It is estimated that 14,250 United States citizens will be diagnosed with esophageal cancer in 2004, and 13,300 people will die of the disease (1). Esophageal cancer is the third most common gastrointestinal malignancy (2) and the sixth most frequent cause of cancer death in the world (3). It has a very low 5-year survival rate ($< 10\%$): 75% of patients die within 1 year of initial diagnosis (4). The American Cancer Society estimates that one third of cancer deaths are related to nutrition and other lifestyle factors, and these deaths may be preventable (1). One strategy for cancer prevention is chemoprevention, which is defined as the use of either naturally occurring dietary constituents or synthetic agents to prevent cancer initiation and progression (5). Chemoprevention could be an important strategy for esophageal cancer prevention, because high-risk populations for this disease can be identified (6).

Nitric oxide (NO) is a small endogenous biological mediator that has received considerable research activity during the last several years. NO has many physiological and pathophysiological actions (7–12). It is synthesized from L-arginine by a family of NO synthases (NOS; Ref. 13). Historically, NOS have been classified into two categories, constitutive (neuron-produced NOS and endothelial cell-produced NOS) and inducible (iNOS; Ref. 13). The two constitutive isoforms are regulated by calcium influx and require activation by calmodulin to produce NO. They are calcium-dependent and produce only a low level of NO (8, 11). In contrast, iNOS, the inducible isoform, is calcium- and calmodulin-independent and generates a high concentration of NO. Increased NO production appears to be associated with many disorders including cancer (9, 12, 14–17). Numerous experimental and clinical reports indicate that iNOS mRNA expression is up-regulated in chronic inflammatory diseases (18–20) as well as in cancer (21–23). iNOS protein has been detected in both premalignant and malignant clinical biopsies from the human stomach, colon, lung, esophagus, and prostate; and, increased iNOS activity was observed in human esophagus, colorectal, breast, lung, head and neck, and central nervous system tumors (18, 19, 23–29).

N-nitrosomethylbenzylamine (NMBA)-induced tumors in the rat esophagus have been used as a model for esophageal squamous cell carcinoma in humans. Our laboratory and others have used the rat model for investigations of molecular events involved in the development of esophageal squamous cell carcinoma and for the evaluation of potential chemopreventive agents (30). Previous studies in our laboratory revealed that iNOS mRNA is significantly elevated in NMBA-induced preneoplastic esophageal lesions and in papillomas when compared with normal rat esophagus (31). *S,S'*-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (PBIT) is a selective inhibitor of iNOS; its specificity for iNOS was established in cytokine-induced colorectal adenocarcinoma DLD cells in which it was found to selectively inhibit iNOS but not endothelial cell-produced NOS or neuron-produced NOS (32, 33). In a study by Rao *et al.* (34), PBIT suppressed azoxymethane-induced aberrant crypt foci formation, crypt multiplicity, and iNOS activity in the rat colon.

In the present study, the effect of PBIT on NMBA-induced tumorigenesis in the rat esophagus was evaluated. The ability of PBIT to inhibit postinitiation events of tumorigenesis was determined by dietary administration of the compound to rats that had been pretreated with NMBA. The identification of chemopreventive agents that inhibit tumor progression in the esophagus of rats that have been preinitiated with NMBA has proven to be difficult (35); it was gratifying that PBIT was found to be effective.

MATERIALS AND METHODS

Chemicals and Reagent Kits. NMBA was obtained from Ash Stevens (Detroit, MI) and determined to be >98% pure by high-performance liquid chromatography. DMSO was purchased from Sigma Chemical Company (St. Louis, MO). PBIT and the Nitrate/Nitrite Colorimetric Assay kits were obtained from Cayman Chemical Company (Ann Arbor, MI). The QuantiTect SYBR Green reverse transcription-PCR (RT-PCR) kit was purchased from Qiagen Inc. (Valencia, CA).

Animals and Diet. Male Fisher 344 rats, 4–5 weeks old, were obtained from Harlan Sprague Dawley (Indianapolis, IN). The animals were housed 3 per cage under standard conditions (20 ± 2°C; $50 \pm 10\%$ relative humidity; 12 h light/dark cycles). Beginning 2 weeks after acclimation to the animal facility, the rats were placed on a modified AIN-76A synthetic diet (Dyets Inc., Bethlehem, PA) containing 20% casein, 0.3% D, L-methionine, 52% cornstarch, 13% dextrose, 5% cellulose, 5% corn oil, 3.5% American Institute of Nutrition salt mixture, 1% American Institute of Nutrition vitamin mixture, and 0.2% choline bitartrate. The synthetic diet and water were available *ad libitum*. Hygienic conditions were maintained by twice-weekly cage changes and routine cleaning of the animal rooms.

Chemoprevention Assay. Two hundred and twenty five rats were randomized into six experimental groups (Table 1) at the time they were placed on AIN-76A diet (2 weeks after initial housing in the animal facility) and treated

Table 1 *Experimental design for bioassay with PBIT[a]*

Group	Treatment	No. of rats	Amount administered (ml)	Dose admin. (mg/kg body weight)	Diet
1	DMSO + H_2O[b]	25	0.2	0	Control AIN-76A
2	None	25	0	0	AIN-76A + PBIT (50 ppm)
3	None	25	0	0	AIN-76A + PBIT (100 ppm)
4	NMBA[c]	50	0.2	0.25	Control AIN-76A
5	NMBA	50	0.2	0.25	AIN-76A + PBIT (50 ppm)
6	NMBA	50	0.2	0.25	AIN-76A + PBIT (100 ppm)

[a] PBIT, S,S′-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea; NMBA, *N*-nitrosomethylbenzylamine.
[b] DMSO + H_2O, vehicle for NMBA.
[c] 0.25 mg/kg body weight NMBA injected s.c. three times per week for 5 weeks.

immediately as follows: rats in group 1 were injected s.c. with 0.2 ml of a solution of 20% DMSO in water, the solvent for NMBA, three times per week for 5 weeks (Fig. 1). Animals in groups 2 and 3 were given either 50 ppm PBIT or 100 ppm PBIT in the diet (chemopreventive agent controls) for the duration of the bioassay. Rats in groups 4–6 were injected s.c. with 0.2 ml of NMBA (0.25 mg/kg body weight) in 20% DMSO:H_2O three times per week for 5 weeks. Three days after the final NMBA treatment, all of the rats in groups 5 and 6 were given AIN-76A diet containing either 50 or 100 ppm PBIT for the duration of the bioassay. Diets containing PBIT were prepared fresh weekly and stored at 4°C. To insure its homogeneity in the diet, PBIT was mixed in the diet for 25 min with a Hobart mixer (Troy, OH). On a weekly basis, the experimental diets and control diet were placed in glass feeding jars and fed to the rats. Food consumption and body weight data were recorded weekly. At 9 and 15 weeks, 5 rats from each of groups 1–3 and 10 rats from each of groups 4–6; and, at 25 weeks, 15 rats from each of groups 1–3 and 30 rats from each of groups 4–6, were euthanized by CO_2 asphyxiation and subjected to gross necropsy. The esophagus of each rat was excised, opened longitudinally, and lesions (tumors) ≥ 0.5 mm in a single dimension were counted, mapped, and measured. Tumor volume was calculated using the formula for a prolate spheroid: length × width × height × $\pi/6$. The esophagus was then cut longitudinally into two parts. The epithelium was stripped of the submucosal and muscularis layers and frozen in liquid nitrogen; tumors were removed and stored separately in liquid nitrogen.

Real-Time RT-PCR Analysis. Total cellular RNA was isolated from esophagi that were frozen in liquid nitrogen using TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Each sample was extracted twice. All of the RNA samples were analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining of 1 μg of RNA resolved by electrophoresis on 1.2% agarose formaldehyde gels. One-Step Real-Time RT-PCR was performed in a GeneAmp 5700 sequence detection system (Perkin-Elmer Corp., Norwalk, CT) using the QuantiTect SYBR Green RT-PCR kit as recommended by the manufacturer. Each reaction contained 200 ng of total cellular RNA, 25 μl of the 2× QuantiTect SYBR Green RT-PCR Master Mix, 0.5 μl of the QuantiTect RT Mix, 5 μM forward and reverse primer, and water to 50 μl reaction volume. The reactions were performed in MicroAmp 96-well plates capped with MicroAmp optical caps. Reverse transcription was first performed at 50°C for 30 min. HotStar TaqDNA Polymerase was then activated at 95°C for 15 min followed by 40 cycles of 15 s at 94°C (denaturation), 30 s at 60°C (annealing), and 30 s at 72°C (extension). The expression of iNOS mRNA was normalized against expression of the housekeeping gene, hypoxanthine-guanine phosphoribosyl-transferase (*HPRT*). Primers for iNOS and *HPRT* were designed according to published sequences with Primer Express Software V 2.0 (Applied Biosystems, Foster City, CA). Base sequences were as follows: iNOS sense 5′-AGCGGCTCCATGACTCTCA-3′ and antisense 5′-TGCACCCAAACAC-CAAGGT-3′; and *HPRT* sense 5′-GCTCGAGATGTCATGAAGGAGAT-3′ and antisense 5′-AGCAGGTCAGCAAAGAACTTATAGC-3′. The sample distribution in the 96-well optical plates was three wells for each individual RNA sample for iNOS expression, three wells of the same samples for *HPRT* expression, and two wells for the control reactions. One control contained RNA template with all of the reagents except QuantiTect RT Mix to confirm that there was no genomic contamination. The other control contained all of the reagents without the RNA template to confirm that the reaction mix displayed no signal. All of the SYBR Green PCR data were collected using the SDS Sequence Detector Software (PE Applied Biosystems).

Nitrate/Nitrite Assay. NO production *in vivo* was determined by measuring nitrate and nitrite in the esophagus tissue after complete conversion of nitrate into nitrite by nitrate reductase (36). Total nitrite therefore represented reduced nitrate

and endogenous nitrite and was measured colorimetrically by the formation of a purple diazo dye through reaction of nitrite with sulfanilamide and *N*-(1-naphthyl)ethylenediamine using a nitrate/nitrite assay kit (Cayman Chemical). The accumulation of nitrate/nitrite was taken as an index of iNOS activity (37, 38). Esophagus tissue was weighed and homogenized in PBS (5 ml PBS/g) and centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was used for the nitrate/nitrite assay. An aliquot (500 μl) of supernatant was added to a M_r 30,000 molecular weight cutoff filter and ultrafiltered at 5,000 × *g* for 3 h. Briefly, 80 μl samples were pipetted into a 96-well optical plate, and then incubated with 10 μl of nitrate reductase and 10 μl of enzyme cofactor for 3 h. After incubation, Griess reagents [sulfanilamide and *N*-(1-naphthyl)ethylenediamine] were added to the wells, and the absorbance was measured at a wavelength of 550 nm. Standards of known concentrations of sodium nitrate in serial dilutions (0–35 μM) were used as positive controls to create a standard curve. Standards and samples were subjected to identical treatment. The final nitrite concentration was the sum of the nitrite plus the reduced nitrate and was reported in μM. Samples were assayed in triplicate, and each sample repeated twice.

Statistical Analysis. Body weight, food consumption, and tumor incidence, multiplicity, and volume data were determined for all of the control and experimental rats. Differences between groups were analyzed for statistical significance using one-way ANOVA followed by Dunnet's multiple comparison test to identify individual differences when the ANOVA was significant. Tumor incidence was compared using the χ^2 test. Comparisons of the incidence of esophageal tumors in rats treated with NMBA or a combination of NMBA and PBIT were made using the Kruskal-Wallis test. Software used in this study was GraphPad Prism 4.0. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Mean body weight and daily food consumption among control and treated rats were not significantly different during the bioassay (data not shown). Administration of 50 ppm or 100 ppm PBIT, therefore, did not influence food intake or body weight gain in either control or NMBA-treated rats. Esophageal tumors were counted, mapped, and measured immediately after euthanization. Histopathological examination of a representative sample of the tumors indicated that all were papillomas. None of the vehicle (DMSO:H_2O) -treated rats (group 1), or the rats treated with either 50 ppm or 100 ppm PBIT (groups 2 and 3) developed tumors. At week 9 of the bioassay, PBIT had no effect on either the incidence or multiplicity of NMBA-induced esophageal tumors. At week 15, PBIT had no significant effect on tumor incidence; however, tumor multiplicity was reduced significantly ($P < 0.05$) in rats given NMBA +100 ppm PBIT compared with rats given NMBA only (data not shown). At 25 weeks, PBIT reduced the incidence of esophageal tumors from 96% in
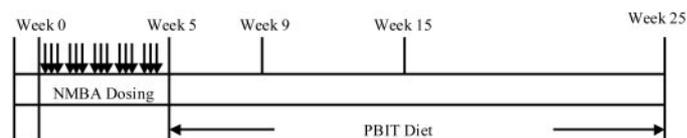

Fig. 1. Experimental protocol for S,S′-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (*PBIT*) bioassay. Rats were treated with *N*-nitrosomethylbenzylamine (*NMBA*; 0.25 mg/kg body weight) three times per week for 5 weeks. PBIT was administered after NMBA treatment and for the duration of the bioassay.

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Table 2 *Effect of PBIT[a] on postinitiation events of NMBA-induced tumorigenesis in the rat esophagus at 25 weeks*

Group	NMBA	Diet	No. of rats	Tumor incidence (%)	Tumor multiplicity mean ± SE	Tumor volume (mm³)[b] mean ± SE
1	-	AIN-76A	10	0	0	0
2	-	50 ppm PBIT	15	0	0	0
3	-	100 ppm PBIT	15	0	0	0
4	+	AIN-76A	28	96.4	3.64 ± 0.42	5.68 ± 0.86
5	+	50 ppm PBIT	29	82.8	1.79 ± 0.25[c]	4.97 ± 0.89
6	+	100 ppm PBIT	30	76.7[d]	1.50 ± 0.24[e]	4.57 ± 0.77

[a] PBIT, *S,S′*-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea; NMBA, *N*-nitrosomethylbenzylamine.
[b] Tumor volume calculated as length × width × depth × $\pi/6$ assuming a prolate spheroid shape.
[c] Significantly lower than group 5 as determined by ANOVA ($P < 0.001$).
[d] Significantly lower than group 5 as determined by χ^2 test ($P < 0.05$).
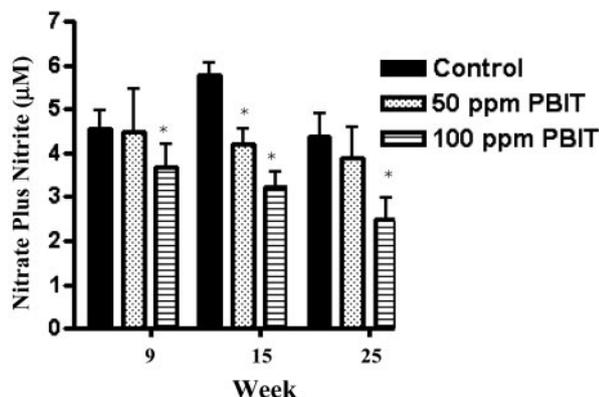[e] Significantly lower than group 5 as determined by ANOVA ($P < 0.0001$).

Fig. 2. Effect of *S,S′*-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (PBIT) on nitrate and nitrite production in preneoplastic tissues in rat esophagus. The values are expressed as mean; *bars*, ±SE. ∗, $P < 0.05$ as determined by Student's *t* test when compared with the control diet group.

NMBA-treated rats to 83% and 77% ($P < 0.05$) in rats treated with 50 and 100 ppm PBIT, respectively. Tumor multiplicity was reduced from 3.64 ± 0.42 tumors per rat in NMBA treated rats to 1.79 ± 0.25 ($P < 0.001$) and 1.50 ± 0.24 ($P < 0.0001$) tumors per rat in rats treated with 50 and 100 ppm PBIT, respectively. Tumors ≥0.5 mm in a single dimension were measured. At weeks 9 and 15, PBIT had no effect on tumor volume. At 25 weeks, the tumor volume was reduced in rats fed either 50 or 100 ppm PBIT to an average of 4.97 ± 0.89 mm³ and 4.57 ± 0.77 mm³, respectively, compared with 5.68 ± 0.86 mm³ in rats fed the control diet (Table 2).

One-Step Real-Time RT-PCR was performed to investigate whether the inhibition of esophageal tumor development by PBIT is associated with inhibitory effects on iNOS mRNA expression. Our data indicated that neither 50 ppm nor 100 ppm PBIT had any effect on iNOS mRNA expression in NMBA-treated rats (data not shown).

We also determined whether local NO production was decreased by PBIT through modulation of iNOS activity. Standards of known concentrations of sodium nitrate in serial dilutions (0–35 μM) were used as positive controls to create a standard curve. The total concentration of nitrate and nitrite in the esophagus was calculated using the slope and y intercept of the standard curve. The results indicated that both 50 ppm and 100 ppm PBIT decreased the concentration of total nitrate and nitrite in both preneoplastic lesions (hyperplasia and dysplasia; Fig. 2) and in papillomas (Fig. 3) when compared with comparable lesions from the esophagi of rats treated with NMBA only.

DISCUSSION

The present study demonstrates that administration of the selective iNOS inhibitor PBIT significantly suppressed NMBA-induced rat esophageal tumor development. Moreover, PBIT decreased the concentration of nitrate and nitrite, an index of NO production, in NMBA-treated esophageal tissues.

Numerous studies in animal models have provided direct evidence for the role of iNOS in tumorigenesis using iNOS inhibitors as chemopreventive agents (34, 39). Most inhibitors are L-arginine-based substrate analogs that bind directly to the iNOS active site, thereby decreasing NO production and preventing tumor development. Many L-arginine analogs have been developed as NOS inhibitors in animal and clinical studies including aminoguanidine (40), N^G-nitro-L-arginine methyl ester (41), *N*-iminoethyl-L-ornithine (42), β-(5-imino-2-pyrrolidine-carboxamido)-propamidine (Noformycin; Ref. 43), and PBIT (34). PBIT has a structural similarity to guanidine and it competitively binds in the guanidine portion of the L-arginine active site of iNOS (32). Because previous data from our laboratory demonstrated a several-fold overexpression of iNOS mRNA and protein in NMBA-induced preneoplastic lesions and papillomas of the rat esophagus, it seemed appropriate to evaluate an iNOS inhibitor such as PBIT for preventative effects. Results from the present study indicated that PBIT elicits inhibitory effects on tumor development in the esophagus of rats pretreated with NMBA, and these effects correlate with reduced NO production as indicated by the lowered levels of total nitrate and nitrite in esophageal lesions and papillomas. Because iNOS inhibitors such as PBIT do not influence the synthesis of iNOS, it was not surprising that PBIT had no effect on iNOS mRNA expression in NMBA-treated rat esophagus.

Other compounds that exhibit inhibitory effects on iNOS include the nonsteroidal anti-inflammatory drugs. One such nonsteroidal anti-inflammatory drug is ibuprofen, which reduces iNOS activity in rat alveolar macrophage cultures stimulated by lipopolysaccharide and IFN-γ (44). Some natural products including resveratrol (45), carnosol (46), and 1′-acetoxychavicol acetate (47), have been shown to inhibit iNOS gene expression and to reduce its activity. The mechanism(s) for their ability to elicit dual inhibitory effects have not been determined; however, the
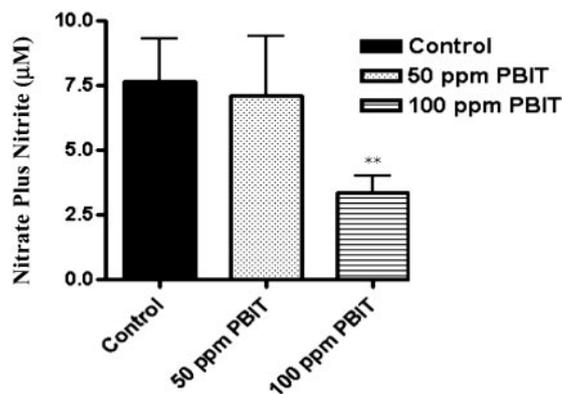

Fig. 3. Effect of *S,S′*-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (*PBIT*) on nitrate and nitrite production in papillomas in rat esophagus. The values are expressed as mean; *bars*, ±SE. ∗∗, $P < 0.0001$ as determined by Student's *t* test when compared with the control diet group.

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influence of these natural products on iNOS gene expression may be due, at least in part, to inhibition of nuclear factor κB activation (48).

Our laboratory has evaluated the ability of several chemopreventive agents to inhibit esophageal tumor progression in rats preinitiated with NMBA. These agents include ellagic acid, sulindac, calcium, phenethyl isothiocyanate (49), piroxicam (50), perillyl alcohol (35), and both freeze-dried strawberries (51) and black raspberries (52). Ellagic acid produced a moderate reduction in tumor incidence but not multiplicity. Other compounds produced either no inhibitory effects (calcium, phenethyl isothiocyanate, piroxicam, and sulindac) or they enhanced tumor development (perillyl alcohol). The mechanism(s) of inhibition of esophageal tumor development by freeze-dried strawberries and black raspberries when provided in the diet postinitiation are not fully known. However, black raspberries have been shown to reduce the growth rate of preneoplastic esophageal cells (52). 1'-Acetoxychavicol acetate was shown to inhibit NMBA-induced tumor development in the rat esophagus through its inhibitory effects on cell proliferation (53).

The results of the present study are potentially important because PBIT is the first chemopreventive agent found to be effective in inhibiting NMBA-induced rat esophageal tumorigenesis when administered in the diet postinitiation. Because iNOS is also overexpressed in both squamous cell carcinomas and adenocarcinomas of the human esophagus (22, 23), selective iNOS inhibitors might also exhibit chemopreventive effects on the development of esophageal cancer in humans.

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