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 (N MBA)-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-isothioureia (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 < 0.0001) 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 chemotherapeutic agents or nutrients 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 ± 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% corn starch, 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
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:H2O 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 each from groups 1–3 and 10 rats each from groups 4–6; and, at 25 weeks, 15 rats each from the groups 1–3 and 30 rats each from the groups 4–6, were euthanized by CO2 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 / 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 gels. A representative sample of the tumors indicated that all were papillomas. None of the tumors showed evidence of metastasis.

**Table 1 Experimental design for bioassay with PBIT**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Amount administered (ml)</th>
<th>Dose admin. (mg/kg body weight)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO + H2O0</td>
<td>25</td>
<td>0.2</td>
<td>0</td>
<td>Control AIN-76A</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>AIN-76A + PBIT (50 ppm)</td>
</tr>
<tr>
<td>3</td>
<td>NMBA'</td>
<td>50</td>
<td>0.25</td>
<td>0.25</td>
<td>AIN-76A + PBIT (50 ppm)</td>
</tr>
<tr>
<td>4</td>
<td>NMBA</td>
<td>50</td>
<td>0.25</td>
<td>0.25</td>
<td>AIN-76A + PBIT (100 ppm)</td>
</tr>
<tr>
<td>5</td>
<td>NMBA</td>
<td>50</td>
<td>0.25</td>
<td>0.25</td>
<td>AIN-76A + PBIT (100 ppm)</td>
</tr>
</tbody>
</table>

*PBIT, S,S’-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea; NMBA, N-nitrosomethylbenzylamine. DMSO + H2O, vehicle for NMBA. 0.25 mg/kg body weight NMBA injected s.c. three times per week for 5 weeks.

**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 Dunnett’s multiple comparison test to identify individual differences when the ANOVA was significant. Tumor incidence was compared using the χ2 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:H2O) -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...
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 NO inhibitors in animal and clinical studies including aminoguanidine (40), N^\text{\textsuperscript{\textregistered}}-nitro-L-arginine methyl ester (41), N-iminoethyl-L-ornithine (42), 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 elicited 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 L-acetoxycavicol 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
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.

REFERENCES

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