CHEMOPREVENTION OF ESOPHAGEAL CANCER:
INVESTIGATION OF INDUCIBLE NITRIC OXIDE SYNTHASE AS A
CHEMOPREVENTIVE TARGET IN N-NITROSOMETHYLBENZYLAMINE-
INDUCED ESOPHAGEAL TUMORIGENESIS

DISSERTATION

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the Degree Doctor of Philosophy in the Graduate
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Esophageal cancer is the third most common gastrointestinal malignancy and is the sixth most frequent cause of cancer death in the world. Estimates of cancer incidence in the United States for 2003 indicate that 13,900 citizens will be diagnosed with esophageal cancer. It is usually discovered at an advanced stage and becomes to fatal rapidly. One-year and 5-year survival rates for esophageal cancer are very low. Seventy-five percent of untreated patients with esophageal cancer die within 1 year of diagnosis and 5-year survival rates are only 5-10%. The N-nitrosomethylbenzylamine (NMBA)-induced rat model of esophageal cancer has been used extensively in our laboratory to investigate the mechanisms of tumor development in the esophagus and to evaluate the efficacy of potential chemopreventive agents including whole food and a variety of single agents. Our investigations provide experimental support for chemoprevention trials of esophageal cancer in humans.

Nitric oxide (NO) is a single molecular and a high level of NO is synthesized from L-arginine by inducible nitric oxide synthase (iNOS). Increased NO production appears to be associated with many disorders including cancer. Therefore, as shown in many studies, iNOS plays a very important role in carcinogenesis. To investigate the association between iNOS and tumor development in rat esophagus, we conducted a
bioassay in which rats were sacrificed at three-week intervals during and following exposure to NMBA. Real-time PCR and immunohistochemistry assays have been used to determine mRNA and protein expression of iNOS. The results of this study suggest that overexpression of iNOS is associated with tumor development in the rat esophagus. Based on our findings, we conducted a second bioassay to evaluate a selective iNOS inhibitor, PBIT, and black raspberries as chemopreventive agents targeting the function of iNOS. We observed a statistically significant reduction in tumor incidence and multiplicity in rats fed with PBIT or black raspberries when compared to rats fed with regular diet only. In summary, our data indicate that iNOS plays an important role in esophageal cancer and its inhibitor(s) might be potential chemopreventive agents in esophageal cancer in humans.
Dedicated to my parents Aijun Lu and Lunquan Chen, my husband, Wenxin, and my sister Wei. Their love and support have been invaluable throughout the years.
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CHAPTER 1

INTRODUCTION

1.1. Esophageal cancer

1.1.1. Epidemiology of esophageal cancer

It is estimated that about 1,334,100 new cancer cases will be diagnosed in the United States in 2003. About 556,500 Americans are expected to die of these cancers, more than 1,500 people a day. With respect to esophageal cancer, 13,900 United States citizens will be diagnosed with esophageal cancer in 2003 and 13,000 people will die of the disease (1).

Esophageal cancer is the third most common gastrointestinal malignancy (2) and is the sixth most frequent cause of cancer death in the world (3). The incidence rate of esophageal cancer varies dramatically in different geographical areas of the world (4). The differences in incidence from one country/region to another, and from one area to another within same country/region are significant. For example, in Utah, USA, the incidence rate of esophageal cancer is 0.4 per 100,000 women while in the Gonbad Region of Iran, it is 174 per 100,000 women (5). The highest incidence areas for the disease occur within the “Esophageal Cancer Belt” which stretches from eastern Turkey to Iraq, Iran, China, India, and certain regions of South Africa (6).
On average, males worldwide are twice as likely to develop esophageal cancer as females. In the Normandy region of France, however, the male/female ratio is 20/1. In France overall, and in Spain and Switzerland the male/female ratios are 11.9/1.0, 6.7/1.0, and 5.6/1.0, respectively (7). In contrast, in the Gonbad region of Iran, the male/female ratio is 0.85/1, and in Iceland, it is 0.84/1 (4). There is no evidence, however, to suggest that esophageal cancer is sex-linked. Epidemiological data suggest that alcohol consumption is a major cause of esophageal cancer in males, whereas women are more affected by poor diet.

A time trend has been observed in the incidence of esophageal cancer in some areas of the world, which is associated with alcohol, tobacco and dietary factors. In South Africa, esophageal cancer was uncommon in 1930. With increased use of alcohol and the smoking of tobacco from 1949 to 1958, the incidence of the disease increased three-fold when compared to 1930 (8). In contrast, in Switzerland, the incidence rate of esophageal cancer decreased from 1951 to 1984, with increased use of fruits and vegetables and decreased alcohol consumption (9). In the United Kingdom, alcohol consumption increased beginning in 1930 and the incidence rate of esophageal cancer increased in 1950 (10).

The majority of esophageal malignancies are classified as either squamous cell carcinomas (SCC) or esophageal adenocarcinomas (EAC) (11). SCC is the dominant type, accounting for 90% of esophageal cancer worldwide, while EAC accounts only for 5%. In addition, some rare types of esophageal cancer account for the remaining 5% and they include SCC with sarcomatous and adenoid cystic features, mucoepidermoid carcinomas, small cell carcinoma, and leiomyosarcoma (12). In the United States, there
has been a dramatic change in the occurrence of EAC in the past few decades. Before 1980, EAC comprised about 15% of esophageal cancer, but by 1994, nearly 60% of esophageal cancers were adenocarcinomas. The incidence rate of EAC increased 10% yearly from 1980 to 1994 (13). Although the precise etiology of EAC has not been determined, considerable data indicate that it results from chronic gastroesophageal reflux disease leading to the formation of a precancerous lesion termed Barrett’s esophagus (14). Barrett’s esophagus is characterized by a replacement of the squamous epithelium in the lower esophagus with a columnar epithelium (14).

Esophageal cancer was first recorded in China over 2000 years ago using the name “Ye Ge”, which means “hard of swallowing with blockage of the gullet and vomiting after eating”. It is also termed “Yi Guan” in many Traditional Chinese Medicine textbooks and is commonly seen in the elderly and rarely develops in young people (15). Esophageal cancer is the fourth leading cause of cancer death in China. Nearly all esophageal cancer in China is SCC. Linxian County in Henan Province has the highest incidence rate of esophageal cancer in the world, with annual age-adjusted mortality rates of up-to 169 per 100,000 individuals and cumulative death rates of 20% for females and 32% for males (16). A retrospective study of esophageal cancer from 1941 to 1968 in Linxian County revealed that the mortality from the disease during that period fluctuated from 100 to 150 per 100,000 people (17). During the same period, esophageal cancer mortality among Caucasian Americans was between 3.82 and 4.23 per 100,000 for men and 0.93 and 1.08 per 100,000 for women (18).
1.1.2. Etiology of esophageal cancer

1.1.2.1. Alcohol and tobacco

The first possible report of the association between alcohol consumption and esophageal cancer was in 1910, and referred to the consumption of absinth (19). In the 1920’s, as alcohol consumption increased, mortality from esophageal cancer increased by two-fold in barmen and waiters in England and Wales (10). It is now well established that alcohol consumption leads to the development of esophageal cancer, particularly among tobacco smokers (20). Alcohol and tobacco act synergistically in causing esophageal cancer, particularly SCC of the esophagus. In the United States, the risk of developing esophageal SCC in heavy drinkers is 10-fold for beer drinkers and 25-fold for whisky drinkers compared to smoking-matched non-drinkers (21). In Japan, the relative risk (RR) of esophageal cancer for the individual who smokes and drinks alcohol daily is 3 times higher than the individual who neither smokes nor drinks alcohol. In Japan, the RR was lowest for beer and increased with saki, whisky and shochu (22). Esophageal cancer is very prevalent among brewery employees, barman, and waiters, who were found to smoke tobacco and consume alcohol excessively (23). In contrast, in Linxian County in China, there is no association between alcohol and/or tobacco and the development of esophageal cancer (24). The etiological factors of the high occurrence of esophageal cancer in Linxian County are multiple and will be described below.

1.1.2.2. Hot Tea and Food

In Linxian County, consuming hot tea is a daily habit. The heat may damage the mucosal epithelium of the esophagus by direct thermal injury and facilitate
carcinogenesis (25). The Japanese consume a very hot tea-cooked rice gruel (chagayu), which may contribute to their high occurrence of esophageal cancer. Chronic thermal irritation, coupled with high levels of tannin in hot tea may cause esophageal cancer (26). Some epidemiological studies however, found no clear link between consumption of hot tea and the risk of developing esophageal cancer. One such study was a case-control study in Kazakh, where citizens drink a large amount of hot tea daily. No relationship was found between consumption of hot tea and esophageal cancer (27). In Linxian County, citizens consume corn meal or millet gruel at the temperature of 69°C, however, this may not be a problem since food at a temperature of 75°C causes only minor irritation to the esophagus (28). The possible role of hot tea and food consumption in the etiology of esophageal cancer still needs further investigation.

1.1.2.3. Molds and mycotoxins

Fungi are a group of plants without root, stem, leaf or chlorophyll and are comprised of mushroom, yeast and molds. Fungi infection in esophageal tissue may contribute to inflammation, hyperplasia, dysplasia and neoplastic lesions of the esophagus. In Linxian County, 155 biopsy samples were examined and various degrees of fungal infection were observed. Various degrees of fungal infection were found in hyperplastic epithelium (30%), and in carcinoma (50%) (29). Cornbread and millet are staple foods in Linxian County and their improper preservation leads to fungal infection. Common fungi, which contaminate food, include *Fusarium moniliforme* Sheldon, *Geotrichum candidum* Link, *Penicillium cyclopium* Westling, *Aspergillus Flavus* Link, and *P.chrysogenum* Thom (30). Some fungi such as *Fusarium*, *Geotrichum* and
Aspergillus decompose proteins, leading to an increase in the amount of amines in foodstuffs, and the formation of nitrosamines. Another source of nitrosamines in Chinese food are picked vegetables. When the leaves of Napa, soybeans and other vegetables are gathered in the autumn, they are chopped, pressed, and covered with water in a ceramic container. The vegetables are allowed to ferment for a couple of months, during which a layer of white mold grows on the leaves and vegetables. These vegetables are consumed without cooking. Pickled vegetables from Linxian have been found to contain a nitroso compound called Roussin red methyl ester (31). Roussin red may produce nitric oxide ions and react with secondary amines to form nitrosamines (32). Pickled vegetables have also been found to contain a high level of benzo[a]pyrene and other PAH carcinogens (33). Chinese in rural areas also have high exposure to PAH through the burning of coal in unvented dwellings for cooking and heating (34, 35). The PAH may contribute to the development of esophageal cancer in high-risk areas in China, although this has not been proven.

1.1.2.4. Dietary factors

Dietary deficiencies in certain micronutrients and protective substances have been shown to be associated with increased risk for esophageal SCC. The incidence of esophageal cancer is high in poor rural areas where dietary deficiencies are common (36). Esophageal cancer in Iran has been associated with dietary zinc deficiency (37). Zinc deficiency is usually related to low socioeconomic status since zinc is found in relatively expensive foods such as meat, fish and green leafy vegetables. The recommended daily allowance for zinc in the United States is 15mg (38). The optimum intake of dietary zinc,
however, remains to be determined. Riboflavin deficiency is also found in areas with a high incidence of esophageal cancer, and the disease is also associated with deficiencies in other vitamins (A, C, and E) and minerals (copper, magnesium and molybdenum) (39). In Linxian County, the daily intake of zinc, riboflavin, vitamins A, C and E, animal protein, fresh vegetables and fruit is very low. These deficiencies are undoubtedly very important for increased cancer risk.

1.1.2.5. Hereditary factors

Tylosis is a rare disease characterized by hyperkeratosis of the palms and soles, and is inherited in an autosomal dominant manner. Patients with tylosis have a very high risk for development of esophageal SCC (40). In a study of three generations of two families in Liverpool, England in 1958, 17 of 18 cases of esophageal cancer were associated with tylosis. There was only one case of esophageal cancer among 86 other members of these 2 families, who did not have tylosis (41). These and other data suggest that tylosis is linked to a high risk for development of esophageal cancer.

1.2. Nitrosamines and esophageal cancer

1.2.1. Discovery of nitrosamines

Nitrosamines are the most prevalent of all environmental chemical carcinogens. More than 300 nitrosamine compounds have been shown to induce cancer, including esophageal cancer, in more than 30 animal species (42). Nitrosamines exhibit significant organ specificity producing tumors in specific organs in which they are metabolized (43). The most common organ site for tumor induction in the rat by nitrosamines is the
esophagus however, nitrosamines do not induce tumors in the esophagus of hamsters (44). More than one-half of the 300 $N$-nitroso compounds are carcinogenic for the rat esophagus (45). The structure of nitrosamine compounds may be symmetric, asymmetric, cyclic, aromatic, or heterocyclic (Table 1.1). Organs in which nitrosamines induce cancer include esophagus, liver, kidney, lung, forestomach, nasal cavity, pharynx, and tongue (46).

1.2.2. Formation of nitrosamines

Nitrosamines are formed whenever nitrosatable amines and nitrites come together in an appropriate pH and environment. For example, in the acidic conditions of the stomach, nitrosamines can be easily formed endogenously by the reaction of primary and secondary amines and nitrites (47,48). A major source of nitrite is its use as a food preservative. Nitrite can also be formed from nitrate via bacterial reduction during food storage. Amines are present in food and can also be formed by bacteria during food storage. The most common amines in foodstuff are dimethylamine, diethylamine and pyrrolidine (49). Ingestion of precursors, such as amines, nitrate, and nitrite can result in nitrosamine production.

1.2.3. Exposure to nitrosamines

As discussed above, nitrosamines are widespread in our environment. They are present in food, drinking water, alcoholic beverages, tobacco, cosmetics, and in various materials produced by industry. To determine if an exposure level to nitrosamines is
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Table 1.1. *N*-nitrosamines carcinogenic to esophagus in the rat. Modified from Preussmann and Stewart, 1984.
sufficient to induce cancer, the level of the nitrosamines themselves as well as of their precursor amines, nitrites and nitrates should be taken into consideration. In Linxian, China, nitrosamines are found at high concentration in the food and drinking water. Pickled vegetables such as turnips, Napa and sweet potato leaves are fermented in water without adding salt. The nitrate in the water is converted to nitrite, which then reacts with amines in vegetables to form nitrosamines. In addition, high levels of nitrate and nitrite are found in the drinking water itself. The average concentration of nitrates in water samples from 495 wells in Linxian was 10.55 mg/liter, and of nitrites was 0.039 mg/liter. In the summer, the levels increased to 12.65 mg/liter and 0.052 mg/liter for nitrates and nitrites, respectively. A positive correlation was established therefore, between water levels of nitrate and nitrite and the incidence of esophageal cancer (50).

In Western countries, tobacco smoking and alcohol consumption are the major etiological factors for esophageal SCC. These products do contain nitrosamines. In 1968, nitrosamines in homemade alcoholic beverages were associated with a high incidence of esophageal cancer in Zambia (51). Northern France has the highest incidence of esophageal SCC in Europe, and both cider and apple brandies are popular beverages there. The beverages were found to contain high levels of certain nitrosamines (52).

Cigarette smoking is responsible for 35% of all cancer deaths worldwide (53). Smoking is responsible for 85% of all lung cancer in both men and women (54). In addition, it is the major etiological factor for cancers of the oral cavity, pharynx, larynx, and esophagus (55, 56). The role of tobacco in esophageal cancer is closely related to its content of nitrosamines. All forms of tobacco, e.g. smoking tobacco, fine-cut chewing tobacco, and ground tobacco, all contain high levels of certain nitrosamines. In the United
States, the maximum level of total nitrosamines in food is 10µg/kg, whereas the nitrosamine levels in snuff are 10,000 times higher (57). Although it’s not clear whether alcoholic beverages and tobacco play an equal role in esophageal tumorigenesis, it is certain that consumption of both has a marked synergistic effect on the occurrence of the disease (58).

Nitrosamines can be produced wherever amines and nitrites come together. Cosmetics are thus a source of nitrosamines since a large number of raw materials from which cosmetics are made contain amines and other nitrosamine precursors (59, 60, 61, 62). The nitrosamines detected in cosmetics include $N$-nitrosodiethanolamine, $N$-nitrosomethyl-n-dodecylamine, $N$-nitrosomethyl-n-tetradecylamine, and $N$-nitrosomethyl-n-octadecylamine (63). With reference to esophageal cancer, the asymmetric nitrosoamines, $N$-nitrosomethyl-n-dodecylamine and $N$-nitrosomethylbenzylamine could be important causes of the disease (45).

1.2.4. Carcinogenicity of nitrosamines

There are three major steps in the initiation of esophageal cancer from nitrosamines: metabolic activation of the nitrosamines, alkylation of DNA, and replication of the alkylated DNA (64, 65, 66, 67). Many nitrosamines are metabolized in the endoplasmic reticulum by cytochrome P450 enzymes CYP2E1, CYP2A6, and CYP2D6. These enzymes convert nitrosamines into reactive metabolites that bind to DNA and form DNA adducts which have been associated with cancer (68). The major DNA adducts to be identified from nitrosamine activation include 7-methylguanine, $O^6$-methylguanine, and $O^4$-ethylthymine (69). 7-Methylguanine is removed from DNA by
non-enzymatic depurination and by a very slow repair mechanism in rat liver (70). \( O^4 \)-Ethylthymine is formed after treatment with \( N \)-nitroso-\( n \)-diethylamine (NDEA) (71). Since \( O^4 \)-ethylthymine is not a prevalent adduct, it is usually not measured in tissue (71).

The \( O^6 \)-methylguanine adduct is more likely to lead to base mispairing during replication and is most closely associated with carcinogenesis. This adduct can be actively removed from DNA by \( O^6 \)-alkylguanine-DNA-alkyltransferase. This enzyme transfers the methyl group to a sulfhydyl group in a cysteine residue in the repair protein (72).

DNA adducts cause base mispairing during DNA replication and result in base substitutions, insertions, or deletions in the daughter strand. The alkylated bases therefore, play an important role in initiating cancer. Cancer occurs more easily if the cell replicates before the alkylated base has been removed from the DNA-by-DNA repair systems. In the esophagus, DNA alkylation occurs very rapidly, while repair is relatively slow. When determining the risk for developing esophageal cancer through exposure to nitrosamines therefore, at least three factors should be considered: the initial level of alkylation, the quality of DNA repair, and the timing of cell replication (73).

1.2.5. \textit{NMBA-induced esophageal tumorigenesis in the F344 rat}

\( N \)-Nitrosomethylbenzylamine (NMBA) is an asymmetrical nitrosamine that readily induces mutagenic, toxic, and carcinogenic effects in esophagus tissues (74,75,76). In Linxian China, the region with the highest incidence of esophageal cancer in the world, Roussin red methyl ester was identified in pickled vegetables. This ester can react with benzylamine to form NMBA (77). NMBA has received considerable attention since the first metabolism study of this compound was published in 1982 (78).
1.2.5.1. NMBA metabolism and $O^6$-methylguanine adducts

As indicated above, more than half of the 300 different $N$-nitro compounds are carcinogenic for the esophagus of rats (42). NMBA is the most potent esophageal carcinogen in the rat among all active nitrosamines (79). NMBA is highly species specific in that it produces cancer in the rat esophagus, but not in the esophagus of hamsters or mice (44). NMBA is metabolically activated to form benzaldehyde and a methyl carbonium ion, which methylates DNA to produce 7-methylguanine and $O^6$-methylguanine adducts depicted in Figure 1.1. (80, 81, 82). The $O^6$-methylguanine adduct has been studied the most extensively due to its accumulation and persistence in DNA and its ability to induce cancer (83, 84, 85). Several investigations have shown that $O^6$-methylguanine is associated with base mispairing and mutagenesis in vitro (86-90). In China, $O^6$-methylguanine has been detected in esophageal DNA of patients with esophageal cancer (91).

In previous studies in Dr. Stoner’s laboratory, the direct genotoxic effect of mispairing of $O^6$-methylguanine was examined and it was found that the H-ras gene and p53 gene in rat esophageal papillomas induced by NMBA have mutations consistent with $O^6$-methylguanine adduct formation (92, 93, 94, 95). Activation of these two genes, particularly the H-ras gene, is important for esophageal tumor development in rats (96, 97). In a twenty-seven week study, F344 rats were administered subcutaneous injections of NMBA at 0.5mg/kg b.w. once per week for 18 weeks and sacrificed at the end of week 27. A G:C$\rightarrow$A:T transition mutation at the second base in codon 12 of the Ha-ras gene was found in all papilloma DNA samples, and no mutations were detected in other ras genes (92). In another thirty-week study, lesions in NMBA-treated rat esophagus were
Figure 1.1. Methylene hydroxylation pathway of \( N \)-nitrosomethylbenzylamine.
examined at different stages of tumor development and it was found that the frequency of G:C→A:T mutations was an average of 4.3% and 57.1% in premalignant lesions and papillomas, respectively (95). Mutations in the p53 gene in rodent tumors tend to be less frequent than mutations in the ras gene (98, 99, 100). Thirty percent of esophageal papilloma DNA samples exhibited mutations in the p53 gene and 89% of these mutations were G:C→A:T transitions (95).

1.2.5.2. NMBA dose and tumor response

The esophageal tumor response to NMBA in rats varies with the dose and duration of carcinogen treatment (102). Administration of subcutaneous injections of NMBA at 0.25 mg/kg b. w. once per week for 15 weeks, resulted in the development of 2-4 papillomas per esophagus. When the dose was increased to 0.5 mg/kg b. w., however, 3-6 papillomas were produced per esophagus. When these same doses of NMBA were administered subcutaneously in only 5 weeks, the papilloma response at 25 weeks was reduced to lower than 1.5 and 3.0 tumors per esophagus at doses of 0.25 and 0.5 mg/kg b. w., respectively. The duration of NMBA treatment therefore, has a marked effect on its tumorigenic potential in the rat esophagus, and other studies support this conclusion (102). The reason(s) for this are unknown, however, fractionating the dose of NMBA over a longer period may lead to a “tumor promotional” effect.

1.2.5.3. Histopathology

The esophagus is a fixed muscular tube, approximately 25cm in length in the human and 7cm in the rat. It delivers food and liquid from the pharynx to the stomach.
The histology of the rat esophagus is discussed below in terms of the three broad layers of mucosa, submucosa, and muscularis externa.

The normal rat esophageal mucosa consists of a stratified squamous epithelium, lamina propria, and muscularis mucosa (103). The epithelium has a keratinized layer on the luminal surface that extends into the stomach through transition to a simple columnar epithelium. In humans however, the stratified squamous epithelium is not keratinized. The basal cell layer of the epithelium of the rat esophagus is approximately 1-2 cells thick and replicates every three to five days. During replication, one daughter cell becomes another basal cell with capabilities for additional replication and the other daughter cell undergoes squamous differentiation. In this process, the cell becomes a spinocyte, then a granulocyte and eventually it sheds into the lumen. The lamina propria of the rat esophagus consists of loose connective tissue and lymphatic nodules. The muscularis mucosa is the deepest layer and consists of a single layer of longitudinally oriented smooth muscle that thickens as it becomes closer to the stomach. Unlike in human esophagus, the rat submucosa does not contain tubuloalveolar glands scattered along the length of esophagus. The deeper layer of the rat esophagus, muscularis externa, is comprised of two muscle layers, inner circular and outer longitudinal, and one fibrous coat.

In NMBA-induced rat esophageal tumorigenesis, the epithelium undergoes a sequence of changes including hyperplasia, dysplasia, papilloma, and carcinoma (10, 65, 105). Hyperplasia, the earliest lesion, appears as a thickening of the basal cell layer (103). In hyperplastic epithelium, there is an increase in the number of basal cells, while the size and shape of the cells remains unchanged. When hyperplastic lesions progress to
dysplasia, the epithelium increases further in thickness and there is an accompanying increase in cell size and a progressive loss of cellular polarity. In addition, the nuclei of the basal cells are changed in both shape and size. Ultimately, dysplastic lesions can progress to papilloma, which consist of either sessile and pedunculated exophytic lesions. Sessile papillomas contain a confluent connective tissue core, which underlies more than 50% of the maximum diameter of the papilloma. In pedunculated papillomas, the tumor is connected to an intact basement membrane by a well-vascularized connective tissue stalk. Epithelial changes within papillomas consist of hyperplasia, hyperkeratosis, and local dysplastic lesions. The atypical or dysplastic lesions can ultimately invade the basement membrane and become squamous cell carcinomas (SCC). However, SCC may also arise from in situ dysplastic lesions without going through the papilloma stage. If the papillary cells invade into the underlying connective tissue, or if severe cytologic atypia is observed, the papillary lesions are considered malignant even though metastasis or invasion into adjacent organs is rare. Papillary neoplasms can frequently grow into the lumen of the esophagus and become obstructive, leading to premature death of the rats due to diminished food intake. In addition, large papillomas press against the trachea leading to respiratory insufficiency.
Figure 1.2: NMBA-induced lesions in the rat esophagus. (A) Normal esophageal epithelium; (B) epithelial hyperplasia; (C) low-grade dysplasia; (D) high-grade dysplasia; and (E) squamous cell papilloma.
1.2.5.4. Molecular biology

In NMBA-induced rat esophageal tumorigenesis, numerous molecular alterations have been found. As described above, preneoplastic lesions and papillomas from NMBA-treated esophagus contain G:C → A:T transition mutations (92). Mutations in the p53 gene have also been observed in esophageal papillomas induced by NMBA (101). mRNA expressions of transforming growth factor-α (TGF-α), and epidermal growth factor receptor (EGFR) are elevated in papillomas when compared to normal epithelium. The protein levels of TGF-α and EGFR are also upregulated in hyperplastic and dysplastic lesions as shown by immunohistochemistry (106). Transforming growth factor-β (TGF-β) has also been evaluated in this animal model (107). Overexpression of TGF-β mRNA was observed in papillomas at 45 weeks but not at 25 weeks, when compared to normal epithelium. This overexpression was not correlated with the protein level of TGF-β. A decreased protein level of TGF-β was revealed by immunohistochemistry. The discordant mRNA and protein expression may cause tumor cells to evade TGF-β controlled negative growth regulation, leading to tumor promotion and progression (107).

Changes in cell cycle regulators have also been investigated during rat esophageal tumorigenesis. Cyclin D1 and cyclin E are the cyclins that regulate the key G1 and G1/S transition points (108). A previous study found that Cyclin D1 mRNA levels were increased 2.8-fold in week 25 and 6.8-fold in week 45 in papillomas when compared to normal epithelium. mRNA levels for cyclin E were also elevated in papillomas by 6.2-fold and 6.8-fold in weeks 25 and 45, respectively. Overexpression of both cyclin D1 and E mRNA correlated with their protein levels (109). Expression of the cell adhesion
molecule, $\alpha 6\beta 4$ integrin, was markedly downregulated in NMBA-induced rat esophageal tumorigenesis. Similarly, changes were found in the membrane localization of E-cadherin and $\alpha$-catenin. The expression of p21, a cyclin-dependent kinase inhibitor, was reduced by 1.6-fold in preneoplastic lesions and 3.1-fold in papillomas (110, 111). Increased cyclooxygenase-1 and cyclooxygenase-2, and elevated prostaglandin E$_2$ were also observed in NMBA-induced preneoplastic lesions and tumors in the rat esophagus (112).

1.3. Nitric oxide and carcinogenesis

Nitric Oxide (NO), a lipid- and water-soluble radical gas, is an important bioactive agent and signaling molecule. In 1818, a large amount of nitrate was first detected in the urine of a febrile patient (113). However, there were no additional studies on nitric oxide until in 1981, when in a nitrite/nitrate balance study, Tannenbaum and his colleagues reported that the excretion of nitrogen oxides in human volunteers exceeded their intake (114). This discovery led to an explosion of investigations on nitric oxide. During the past decade, many reports have found that NO plays a very important role in physiological as well as pathological processes in the body, including inflammation and cancer (115-120).

1.3.1. Biochemistry of nitric oxide

NO is synthesized from L-arginine by a family of nitric oxide synthases (NOS), which have three isoforms: nNOS, eNOS, and iNOS (121). All three isoforms of NOS catalyze the production of NO by the same biochemical pathway, which is composed of two sequential monooxygenase reactions. One molecule of L-arginine is oxidized at a
guanidine nitrogen to produce N⁰⁰-OH-L-arginine as an intermediate. N⁰⁰-OH-L-arginine is further oxidized to yield one molecule each of NO and L-citrulline. One and one-half molecules of NADPH and two molecules of dioxygen serve as cosubstrates and are converted to 1.5 molecules of NADP⁺ and two molecules of water as coproducts (122).

\[
(1) \text{L-arginine} + \text{O}_2 + \text{NADPH} \rightarrow \text{N}⁰⁰-\text{OH-L-arginine} + \text{NADP}^+ + \text{H}_2\text{O}
\]

\[
(2) \text{N}⁰⁰-\text{OH-L-arginine} + \text{O}_2 + 0.5 \text{NADPH} \rightarrow \text{L-citrulline} + \text{NO} + 0.5 \text{NADP}^+ + \text{H}_2\text{O}
\]

NO is a free radical with an unpaired electron, therefore, it can denote or accept an electron to become a nitrosium cation (NO⁺) or a nitroxyl anion (NO⁻), respectively (123). NO⁺ is involved in nitrosation reactions with nucleophilic groups; i.e. thiols, amides, carboxyls, hydroxyls, and aromatic rings. NO⁻ can undergo spontaneous dimerization to form dinitrogen oxide (N₂O) or promote the oxidation of sulphhydryl groups through reacting with thiols. NO reacts with oxygen to yield nitrogen dioxide (NO₂), with O₂⁻ to yield unstable higher oxide (N₂O₃), unstable peroxynitrite (ONOO⁻), or either moderately stable anions (NO₂⁻), or very stable anions (NO₃⁻).

Historically, NOS have been classified into two categories, constitutive (nNOS and eNOS) and inducible (iNOS) (121). In general, nNOS and eNOS are continuously present in neurons and endothelial cells, respectively. The “n” denotes its cloning from brain neurons, and is called NOS1; the “e” signifying its cloning from endothelial cells and is called NOS3. These two isoforms are regulated by calcium influx and require attendant activation of calmodulin to produce NO. They are calcium-dependent and produce only a low level of NO (124, 125). On the other hand, iNOS, an inducible isoform, also called NOS2, is calcium-and calmodulin-independent and generates a high
concentration of NO. In general, iNOS is quiescent in normal cells and must be induced by cytokines and/or bacterial endotoxins (126-129). However, iNOS has been detected in normal human large-airway epithelium. Since airway epithelium is repeatedly exposed to lipopolysaccharide (LPS), ozone, and other potentially inductive stimuli, it is difficult to determine whether iNOS is expressed constitutively or is continuously induced in this tissue.

1.3.2. Nitrosative stress and oxidative stress

Increased NO production appears to be associated with many disorders including cancer (115-120). NO can undergo numerous reactions including the formation of biologically reactive nitrogen oxide species (RNOS), which may be carcinogenic by chemically altering DNA as well as by enhancing the genotoxicity of other substances such as alkylating agents (130-132).

The chemical reactions of NO can be divided into two categories, direct and indirect (133, 134). Direct chemical reactions are those in which NO reacts with biological targets without any further modification. For example, the low level reaction of NO with the heme-containing protein, guanylate cyclase, as well as oxyhemoglobin and cytochrome P450 is responsible for the neuromodulatory effect of nNOS and the vasodilatory effect of eNOS. Indirect chemical reactions are those in which RNOS instead of NO reacts with biological targets. The RNOS are formed through the reaction of NO either with oxygen ($O_2$) or superoxide ($O_2^-$). These reactions require high local concentrations of NO and account for the biological effect of iNOS. Direct effects
therefore, are caused by NO produced at low concentrations for short periods of time and indirect effects are induced by high concentrations of NO for prolonged periods.

The indirect effects of NO are associated with pathophysiological conditions, and are responsible for the etiology of many diseases including cancer. Indirect chemical reactions are nitrosation and oxidation reactions that may result in nitrosative stress and oxidative stress. Nitrosation reactions are defined as donation of a nitrosonium cation (NO⁺) to nucleophiles such as thiols and amines by RNOS. The formation of nitrosonium adducts elicits nitrosative stress. Oxidative reactions mediate the removal of electrons from a substrate. The formation of oxidative lesions results in oxidative stress. These lesions include oxidation of nucleic acids resulting in DNA strand breaks, oxidation of lipids leading to lipid peroxidation, and oxidation of proteins which can result in blocking enzyme activity (135).

Due to its ability to elicit nitrosative and oxidative stress, NO produces genotoxic effects through three mechanisms. First, high levels of NO and RNOS can lead to the formation of nitrosamine compounds through nitrosation reactions with amines (136, 137). Nitrosative stress under conditions such as inflammation, in which high levels of iNOS are found in macrophages and neutrophils, can result in the formation of carcinogenic nitrosamines (138, 139). Second, the nitrosative chemistry of N₂O₃ can induce deamination of guanine, cytosine and adenine, which results in the conversion of cytosine to uracil, guanine to xanthine, methylcytosine to thymine, and adenine to hypoxanthine (140, 141). Deamination occurs predominately during DNA replication and transcription since single stranded DNA is more susceptible to nitrosative stress than double stranded DNA (142). Oxidative stress can lead to the formation of peroxynitrite,
which can alter DNA leading to carcinogenesis (143). Third, high levels of NO can inactivate DNA repair proteins resulting in indirect mutations in DNA. DNA repair proteins have been reported to be influenced by NO and/or RNOS including alkyl transferase, formamidopyrimidine-DNA glycosylase (Fpg protein), and DNA ligase (144-148). DNA alkyl transferase contains a thiol group and is involved in the repair of the DNA adducts: \(O^6\)-methylguanine and \(O^{4}\)-methylthymine (144, 145). Nitrosation of the thiol residue with RNOS inactivate this repair protein and inhibit its activity (130). Similarly, the Fpg protein contains a thiol residue, and has a high binding affinity to \(\text{N}_2\text{O}_3\) leading to degradation of its structure. This leads to inhibition of the repair of some lesions such as 2,6-diamino-4-hydroxy-5-\(N\)-methylformamidopyrimidine (Fapy) and 8-oxoguanine (147). DNA ligase is another DNA repair protein inhibited by RNOS, resulting in DNA single strand breaks after exposure to NO (141).

In addition to the formation of nitrosamines, deamination of DNA bases, and inactivation of DNA repair proteins, NO may also promote carcinogenesis by inactivating the tumor suppressor gene, p53. Frequent G:C→A:T mutations in p53 have been reported in lung adenocarcinomas, and both colon and head and neck cancers (149, 150), due to the concomitant loss of DNA-binding activity (151). Loss of binding activity is attributed to nitration of tyrosine residues in p53 in a high NO environment (152). Interestingly, a strong association between increased iNOS activity and p53 mutations has been observed in colon cancer, breast cancer and in Barrett’s esophagus (153, 154).
1.3.3. iNOS and carcinogenesis

iNOS is calcium-independent and produces a high level of NO, however, it is usually absent in resting cells. After stimulation by cytokines and bacterial cell wall products such as the interleukins, tumor necrosis factor alpha (TNFα) and endotoxin lipopolysaccharide (LPS), iNOS can become involved in carcinogenesis (155-159). The expression of iNOS is associated with the activation of transcriptional factors including NFκB, STST-1, and IRF-1 (160). Numerous experimental and clinical reports indicate that iNOS expression is upregulated in chronic inflammatory diseases as well as cancer. In addition, iNOS protein has been detected in both premalignant and malignant clinical biopsies from the stomach, colon, lung, esophagus, prostate, and duodenum (161-166). Moreover, iNOS expression is upregulated after cytokine stimulation in mammary carcinoma, melanoma, and in adenocarcinomas of colon (167-169). Increased iNOS activity has also been reported in human cancers including esophageal, colorectal, gastrointestinal, gynecological, pancreatic, breast, lung, head and neck, and central nervous system tumors (170-178).

Although iNOS plays an important role in carcinogenesis through producing high levels of NO, which elicits DNA damage, NO has no direct effect on cellular DNA. The formation of peroxynitrite (ONOO-) appears to be the key link between NO and tumor formation and/or progression (179). In inflammatory conditions, macrophages and neutrophils migrate to the site of inflammation and are stimulated to produce NO by iNOS through inflammatory cytokines released from adjacent mononuclear cells (180-183). Ultimately, this leads to the formation of peroxynitrite. Peroxynitrite production parallels the production of NO. The production of NO by iNOS in macrophages
therefore, leads to inflammation-mediated carcinogenesis by peroxynitrite (184-188). Peroxynitrite is a strong oxidant formed from an essentially irreversible reaction between NO and superoxide \((O_2^-)\) (189). It is a potent nitrating and oxidizing agent that produces oxidative damage to DNA, lipids, carbohydrates, and sulphhydryl groups in proteins. Peroxynitrite produces DNA strand breaks and modification of purine bases. For example, it reacts with deoxyguanosine leading to the formation of 8-oxo-dG, 8-nitroguanine, 2,2-diamino-4-[(2’-deoxy-β-D-erythro-penta(furanosyl))amino]-5-(2H)-oxazolone (oxazolone), 4-hydroxy-8-oxo-4,8-dihydro-2’-deoxyguanosine, and 4,5-nitrosooxy-2’-deoxyguanosine (190-194). 8-oxo-dG is thought to be associated with carcinogenesis since it produces G:C→A:T transversion mutations and results in multiple amino acid substitutions (195-199). Peroxynitrite is unstable and can rapidly form nitrosated tyrosine residues in proteins or tissues to form the stable end product, nitrotyrosine (200). Nitrotyrosine is a biomarker for assessing oxidative stress, and can be detected immunohistochemically using anti-nitrotyrosine antibodies. Significant amounts of nitrotyrosine have been observed in human cancers including esophageal and head and neck squamous cell carcinomas, and in adenocarcinomas of the stomach, colon, and ductal pancreas (201-204).

1.4. Cancer chemoprevention

Cancer chemoprevention refers to the use of either naturally occurring dietary constituents or synthetic agents (individual drugs) to prevent cancer initiation and progression (205, 206). The American Cancer Society estimates that 556,500 cancer deaths are expected to occur in the United States in 2003 and one third of them are
related to nutrition and other lifestyle factors, which could be prevented (1). Cancer chemoprevention is one strategy for cancer prevention that could be effective for many cancers. Esophageal cancer, for example, has very low five-year survival rate (< 10%): 75% of patients die within one year of initial diagnosis (207). Chemoprevention could be important for this disease since high-risk populations can be identified in specific geographical regions.

1.4.1. Animal efficacy bioassay

Animal model studies are crucial for determining organ specificity, and to generate dose-response, toxicity, and pharmaceutical data for specific chemopreventive agents. The most commonly used animals for this purpose are rats, mice, and hamsters (Table 1.2). Ideally chemopreventive agents should be non-toxic, highly effective, orally available and inexpensive (205). In efficacy bioassays, animals are administered with test agents before, concurrently, or following carcinogen exposure. A Maximum Tolerated Dose (MTD) is determined during the assay. The MTD is defined as the highest dose that does not cause ≥ 10% reduction in body weight in a 90-day subchronic study (208). Chemopreventive efficacy is based on the percentage reduction in tumor incidence, multiplicity and volume as well as the modulation of selected intermediate biomarkers.
<table>
<thead>
<tr>
<th>Organ Model</th>
<th>Species</th>
<th>Carcinogen</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>Rat</td>
<td>Nitrosamines</td>
<td>Squamous cell carcinoma, Papilloma</td>
</tr>
<tr>
<td>Colon</td>
<td>Rat</td>
<td>azoxymethane(AOM)</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td></td>
<td>Adenoma, Aberrant Crypt Foci (ACF)</td>
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<tr>
<td>Forestomach</td>
<td>Mouse</td>
<td>B(a)P</td>
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<tr>
<td>Intestine</td>
<td>Rat</td>
<td>azoxymethane(AOM)</td>
<td>Adenocarcinoma, Adenoma</td>
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<tr>
<td>Lung</td>
<td>Mouse</td>
<td>B(a)P, NNK</td>
<td>Adenoma</td>
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<td></td>
<td>Hamster</td>
<td>DEN</td>
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</tr>
<tr>
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<td>Mouse</td>
<td>UV radiation, B(a)P, B(a)P/TPA, DMBA, DMBA/TPA</td>
<td>Carcinoma, Papilloma</td>
</tr>
</tbody>
</table>

Table 1.2: Most commonly used carcinogen-induced animal models for chemopreventive efficacy bioassay. Modified from Bergan, 2001.
1.4.2. Chemoprevention agents

The scientific rational for chemoprevention is based largely upon carcinogenesis being a multistage process, composed of initiation, promotion and progression. Each of these stages has distinct biological characteristics (209). Initiation is a primary and essential step, in which a single cell becomes transformed as a result of irreversible mutations in certain genes. These mutations are induced by chemical carcinogens, viruses, radiation, such as UV light, errors in DNA replication and various unknown factors (210). Promotion is the second stage when the initiated cell undergoes clonal expansion to form a benign tumor, such as a papilloma or adenoma (211). Promotion agents can include components of the inflammatory response, hormones, and growth factors (212). Promotion is a reversible process. Tumor progression involves the conversion of benign tumors to malignant tumors, which have the ability to invade. Malignant tumors can acquire a resistance to chemotherapy and radiotherapy (213). Chemoprevention agents target the stages of initiation, promotion and progression, and are classified as either blocking (anti-initiation) agents or suppressing (anti-promotion/progression) agents (Table 1.3).

1.4.3. Chemoprevention studies in NMBA-induced F344 rat tumor model

1.4.3.1. Ellagic acid, strawberries and black raspberries

Ellagic acid is a plant polyphenol present in high concentrations in many fruits and nuts (214). It has been shown to inhibit the bioactivation of procarcinogens through inhibition of cytochrome P450 enzymes, and to enhance the ability of the target tissues to detoxify the DNA-reactive metabolites of carcinogens (215). Cytochrome P450s 2E1
<table>
<thead>
<tr>
<th>Mechanism of Action</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibitors of Carcinogen Formation</strong></td>
<td></td>
</tr>
<tr>
<td>Prevention of nitrosamine formation</td>
<td>Ascorbic acid, alpha-tocopherol, caffeine acid, ferulic acid, proline, thioproline</td>
</tr>
<tr>
<td><strong>Blocking agents (anti-initiation)</strong></td>
<td></td>
</tr>
<tr>
<td>Inhibition of cytochrome P450</td>
<td>Isothiocyanates, ellagic acid, dithiocarbamates, diallyl sulfide</td>
</tr>
<tr>
<td>Induction of cytochrome P450</td>
<td>Indole-3-carbinol, beta-naphthoflavone</td>
</tr>
<tr>
<td>Induction of phase II enzymes</td>
<td>Isothiocyanates, dithiolethiones, polyphenols, selenium</td>
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<tr>
<td>Scavenging electrophiles</td>
<td>Ellagic acid, N-acetylcysteine, sodium thiosulfate, polyphenols, vitamin E</td>
</tr>
<tr>
<td>Induction of DNA repair</td>
<td>Vanillin, N-acetylcysteine, protease inhibitors</td>
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<tr>
<td><strong>Suppressing agents (anti-promotion/progression)</strong></td>
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<tr>
<td>Inhibition of polyamine metabolism</td>
<td>Difluoromethylornithine, polyphenols</td>
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<td>Inhibition of arachidonic acid metabolism</td>
<td>NSAIDs, polyphenols, N-acetylcysteine</td>
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<tr>
<td>Protease inhibition</td>
<td>Bowman-Birk protease inhibitor</td>
</tr>
<tr>
<td>Induction of differentiation</td>
<td>Retinoids, calcium, vitamin D</td>
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<td>Inhibition of oncogene expression</td>
<td>Lovastatin, limonene</td>
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<td>Inhibition of oncogene activity</td>
<td>NSAIDs, monoterpenes</td>
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<tr>
<td>Inhibition of protein kinase C</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>Inhibition of oxidative DNA damage</td>
<td>Selenium, epigallocatechin gallate</td>
</tr>
</tbody>
</table>

Table 1.3: Classification and examples of cancer chemoprevention agents based on mechanism. Modified from Morse and Stoner, 1993.
and 1A1, for example, are the principal enzymes involved in the bioactivation of procarcinogens such as NMBA and benzo(a)pyrene [B(a)P] (216). Ellagic acid decreased the activation of these two enzymes and inhibited carcinogenesis (215). In addition, in the Syrian hamster embryo (SHE) cell transformation assay, ellagic acid reduced B(a)P-induced cell transformation (217). Ellagic acid has also been shown to inhibit chemical-induced cancer in vivo. In a 27-week anti-initiation bioassay, ellagic acid at 0.4 and 4.0 g/kg diet, reduced the number of esophageal papillomas in F-344 rats by 21% and 55%, respectively. In the same study, ellagic acid reduced the number of preneoplastic lesions (218).

Strawberries and black raspberries are regularly consumed by humans and contain relatively high concentrations of ellagic acid and other compounds that exhibit chemopreventive effects (217, 219). In the NMBA-induced rat esophageal tumor model, 5% and 10% strawberries significantly reduced tumor multiplicity in a 30-week anti-initiation bioassay and a 25-week post-initiation bioassay, respectively (220). Similarly, black raspberries have been reported to reduce tumor incidence, multiplicity as well as cell proliferation at both the initiation and promotion/progression stages in rat esophagus (221). In addition, black raspberries are capable of inhibiting tumor development in the rat colon and in the hamster oral cavity (222, 223).

1.4.3.2. Isothiocyanates

Arylalkyl isothiocyanates are present in various vegetables. Most are potent blocking agents in that they inhibit tumor development at the initiation stage; however, they exhibit no effects when administered post-initiation (224). Several isothiocyanates
have been evaluated for their ability to inhibit NMBA-induced esophageal tumorigenesis including benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), 3-phenylpropyl isothiocyanate (PPITC), 4-phenylbutyl isothiocyanate (PBITC), and phenylhexyl isothiocyanate (PHITC) (225-229). The inhibitory effects of these isothiocyanates were correlated with the length of their side chains. BITC was less effective than either PEITC or PPITC. However, the longer chain isothiocyanate, PBITC, was less effective than either PEITC or PPITC, and PHITC was actually found to enhance tumor development in the rat esophagus (230). The inhibitory effects of the isothiocyanates on DNA methylation 24 hours after a single treatment with NMBA followed the order: PPITC > PEITC > PBITC > BITC. The enhancing effect of PHITC could be due to a cytotoxic effect in rat esophagus, which may lead to induction of cell proliferation (228, 231).

1.4.3.3. Resveratrol and 1’-acetoxychavicol acetate

Resveratrol (RV) is a naturally occurring polyphenol found in grapes, peanuts and mulberries (232). Many studies have suggested that it has potent cancer preventive and therapeutic effects (233). RV was found to significantly reduce tumor incidence, multiplicity and volume in an anti-initiation study in the rat esophagus using NMBA as the carcinogen (234). 1’-Acetoxychavicol acetate (ACA) is present in seeds and widely consumed by populations in Southeast Asia (235). It exhibits inhibitory effects in both the initiation and post-initiation stages in NMBA-induced rat esophageal tumorigenesis (236).
1.4.3.4. Piroxicam, sulindac, and blueberries

Numerous studies have suggested that nonsteroidal anti-inflammation drugs (NSAIDs), such as piroxicam and sulindac have cancer inhibitory potential (237-247). Neither piroxicam nor sulindac, however, possess significant inhibitory activity in the NMBA-induced rat esophageal tumor model (248, 249). This might be due to an inability of these compounds to modulate biochemical pathways involved in rat esophageal tumorigenesis. Piroxicam, however, was shown to reduce PGE\(_2\) levels in NMBA-treated rat esophagus, but this did not result in a significant reduction in the tumor response (112).

Freeze-dried blueberries were not effective in inhibiting NMBA tumorigenesis in the rat esophagus, even though they were added at 5 and 10% of the diet. The lack of inhibitory effects by blueberries might be due to their lower concentration of ellagic acid when compared to strawberries and black raspberries. In addition, their anthocyanin content is qualitatively and quantitatively different from both strawberries and black raspberries (249).

1.4.4. Combination study in chemoprevention

Treatment of animals with two or more chemopreventive agents in combination can lead to additive or synergistic effects in the tumor response. Combining two agents may permit efficacy at lower doses than either agent alone. Combination chemoprevention therefore, is an approach to improve efficacy and lessen toxicity. Positive results were reported when \(N\)-(4-hydroxyphenyl)retinamide (4-HPR) was used in conjunction with tamoxifen in the rat mammary gland (250, 251). Similarly,
difluoromethylornithine (DFMO) and piroxicam (252, 253) and L-\(N^6\)-(1-iminoethyl)lysine tetrazole-amide (SC-51) plus celecoxib exhibited synergistic or additive inhibitory effects in rat colon carcinogenesis (254).

Agents in combination may also be problematic when one agent down regulates a pathway while another agent stimulates a pathway that promotes carcinogenesis. For example, iNOS is a key link between NO and cancers, therefore, iNOS inhibitors potentially have cancer-inhibitory properties. iNOS inhibitors however, might also enhance cancer due to simultaneous stimulation of other signal transduction pathways (255). L-arginine is a precursor of both nitric oxide and the polyamines. Ornithine decarboxylase (ODC) is a key enzyme in the formation of the polyamines (putrescine, spermidine, and spermine). Several studies have shown that certain iNOS inhibitors can both inhibit iNOS activity and increase the biosynthesis of polyamines through stimulation of ODC activity (255). Thus, the administration of iNOS inhibitors combined with ODC inhibitors rather than iNOS inhibitors alone could be an approach to this problem.

1.5. NOS inhibitors

Numerous studies in animal models have provided direct evidence for the role of iNOS in tumorigenesis using iNOS inhibitors as chemopreventive agents. Most of them are L-arginine-based substrate analogs and directly bind to the iNOS active site thereby decreasing NO production. The oxidation of L-arginine by NOS led to the discovery of the first-generation L-arginine-based iNOS inhibitors (256-258). A large number of L-arginine analogs have been developed as NOS inhibitors in many animal and clinical
studies including $N^G$-methyl-L-arginine (L-NMMA), $N^G$-nitro-L-arginine methyl ester (L-NAME), $N$-iminoethyl-L-ornithine (L-NIO), $N^G$-nitro-L-arginine (L-NNA), $\beta$-(5-imino-2-pyrrolidine-carboxamido)-proamidine (Norformycin), and S,S$'$-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea (PBIT) (Figure 1.3) (259-263).

L-NAME is an analog of L-arginine methyl ester, which has a nitro group at the $N^G$ of the guanidine moiety (260). It is a potent NOS inhibitor that reduces NO production and tumor development in the rat colon (264). L-NMMA has a methyl group on the $N^G$ of the guanidine moiety and is a very selective iNOS inhibitor among the arginine analogs (259). Studies have demonstrated its anti-tumor and anti-metastatic effects in the mouse mammary adenocarcinoma model (265, 266). L-NNA has a nitro group at the $N^G$ of the guanidine moiety, while L-NIO has an iminoethyl group instead of an amine group (261, 262). Both compounds inhibit NO production in activated murine macrophages through their inhibitory effects on iNOS (267, 268). Noformycin is a natural product originally isolated from Nocardia formica that exhibits inhibitory activity against iNOS (262). PBIT is a selective iNOS inhibitor that suppresses iNOS and reduces the formation of aberrant crypt foci (ACF) in the azoxymethane model of rat colon carcinogenesis (263).

Other compounds, which exhibit inhibitory effects on iNOS include the non-steroidal anti-inflammatory drugs (NSAIDs). One such NSAID is ibuprofen, which reduces iNOS activity in rat alveolar macrophage cultures stimulated by LPS and IFN$\gamma$ (269). $(1S,5S,6R,7R)$-7-Chloro-3-imino-5-methyl-2-azabicyclo-heptane hydrochloride (ONO-1714), a novel cyclic amidine analogue, inhibits iNOS activity in humans (270). Several natural products also inhibit NOS activity and tumor development. For example,
1’-acetoxychavicol acetate (ACA), present in seeds and widely used as a spicy food in Southeast Asian countries, has been shown to reduce NO generation through inhibition of iNOS gene transcription (271). ACA also inhibits tumor development in the NMBA-induced esophageal rat tumor model (236).
Figure 1.3: Chemical structures of L-arginine and PBIT.
1.6. Proposed research

One objective of my research was to investigate the correlation between the expression of iNOS and the development of esophageal tumors in Fisher 344 rats treated with NMBA. Previous studies have shown that iNOS is up regulated in preneoplastic and neoplastic lesions in animals and humans. However, iNOS expression has not been investigated in the rat model of squamous cell carcinoma of the esophagus.

It was my hypothesis that overexpression of iNOS is an early event in NMBA-induced rat esophageal tumorigenesis. To test this hypothesis, the expression of iNOS mRNA during early esophageal tumor development was measured using Real-Time polymerase chain reaction (Real-Time PCR). Immunohistochemistry was also performed to examine expression of iNOS protein, and to determine its cellular localization during esophageal tumorigenesis.

Another objective of my research was to determine if the iNOS inhibitor, PBIT, and black raspberries would influence the occurrence of esophageal tumors in NMBA treated rats. The abilities of PBIT and black raspberries to inhibit tumor incidence, multiplicity, and volume were investigated as well as their abilities to inhibit iNOS expression and NO production.

In summary, the overall objective of my research was to explore the role of iNOS in NMBA-induced rat esophageal tumorigenesis and its feasibility as a target in the chemoprevention of esophageal cancer.
1.7 References


52. International Agency for Research on Cancer (1978) Tobacco habits other than smoking; betel-quid and Areca nut chewing; and some related nitrosamines.


and 8-hydroxy-2'-deoxyguanosine levels in the Fisher 344 rat. *Nutrition and Cancer, 40 (2)*, 125-133.


CHAPTER 2

INCREASED INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN N-NITROSOMETHYLBENZYLAMINE-INDUCED RAT ESOPHAGEAL TUMORIGENESIS

2.1. Abstract

Nitric oxide (NO), an important regulatory molecule for immune response and cytotoxicity, is endogenously generated from L-arginine by nitric oxide synthase (NOS). One mechanism for NO-induced cytotoxicity is through its interaction with superoxide to produce peroxynitrite, which causes DNA damage. Three distinct isoforms of NOS have been isolated and represent the products of three different genes. The inducible form, iNOS, is induced by certain cytokines, microbial products, or lipopolysaccharides. iNOS is a mediator of inflammation and a regulator of epithelial cell growth. Sustained induction of iNOS expression in chronic inflammation may be carcinogenic through NO-mediated DNA damage or hindrance to DNA repair. Upregulation of iNOS has been linked to epithelial tumorigenesis in various human and animal tissues. In the current investigation, normal and N-Nitrosomethylbenzylamine (NMBA)-induced preneoplastic and papillomatous lesions of the rat esophagus were characterized for expression of iNOS. F344 rats were injected subcutaneously with NMBA (0.5 mg/kg body weight) three times per week for 5 weeks. At 3, 6, 9, 12, 15, 18, 21, 24, 30 and 36 weeks following initiation of NMBA treatment, esophagi were collected from 12 untreated and
12 NMBA treated animals. Results of Real-time PCR and immunohistochemistry demonstrated a correlation between the upregulation of iNOS and neoplastic progression in the rat esophagus. The expression of iNOS mRNA in preneoplastic tissues and papillomas was significantly elevated when compared to normal tissues. Immunohistochemical analysis showed positive cytoplasmic staining of iNOS protein in preneoplastic tissues and papillomas when compared to normal tissues. Our data suggest that iNOS plays an important role in NMBA-induced esophageal tumorigenesis in rats.
2.2. Introduction

Esophageal cancer is the third most common gastrointestinal malignancy (1) and is the sixth most frequent cause of cancer death in the world (2). In the United States in 2003, it is estimated that 13,900 United States citizens will be diagnosed with esophageal cancer and 13,000 people will die of this malignancy (3). Numerous experimental and clinical reports indicate that iNOS expression is upregulated in the tissues of chronic inflammatory diseases as well as in esophageal cancer (4-8). In addition, iNOS protein has been detected in both premalignant and malignant clinical biopsies from the esophagus (9). Moreover, increased iNOS activity has also been reported in human cancers including breast, head and neck, and colon (10-12).

NO is produced from L-arginine by the family of nitric oxide synthase (NOS) enzymes, forming the free radical NO and citrulline as byproducts (13). Increased NO production appears to be associated with many disorders including cancer (4-8). Three distinct isoforms of the NOS enzyme have been isolated and represent the products of three different genes: nNOS, eNOS, and iNOS (14). The inducible form, iNOS, which is not typically expressed in resting cells, must first be induced by certain cytokines, microbial products, or lipopolysaccharides (15-18). iNOS may be regulated differently in different cell types. The effect of agents used to modify NO formation may depend upon the nature of the stimuli that are being used to activate the particular cell type.

Genotoxicity of NO is due to its reaction with oxygen and superoxide to produce either direct or indirect DNA damage (19, 20). Direct damage includes DNA base deamination, peroxynitrite-induced adduct formation and single strand breaks in DNA. Indirect damage is caused by the interaction of NO reactive species with other molecules such as
amines, thiols and lipids. One mechanism for NO-induced cytotoxicity is through its reaction with superoxide to produce peroxynitrite, an oxidizing agent that initiates lipid peroxidation, sulfhydryl oxidation of proteins, and nitration of aromatic amino acids (21).

Central to the concept of inflammation and cancer is the hypothesis that chronic irritation of squamous or glandular epithelium results in migration of inflammatory cells to the injured site followed by a respiratory burst liberating superoxide anions, and subsequent radicals and reactive oxygen species (22-25). Evidence suggests that peroxynitrite is involved in carcinogenesis, which is strongly associated with high NO production, subsequently linked with induction of iNOS in the inflammatory cells (26-30). Therefore, increased expression of iNOS may contribute to tumor development and progression. The purpose of our studies is to explore the role of iNOS in NMBA-induced rat esophageal tumorigenesis and the feasibility of iNOS as a target in cancer chemoprevention.

2.3. Materials and methods

2.3.1. Animals

Five to six week-old male Fischer 344 rats (n = 240) were purchased from Harlan Sprague-Dawley. The rats were housed three per cage under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12-hour light/dark cycles). Purified AIN-76A diet and water were provided ad libitum. N-Nitrosomethylbenzylamine (NMBA), with a purity of >99%, was purchased from Ash Stevens, Inc. The rats were dosed with NMBA (0.5 mg/kg body weight) in 20% dimethyl sulfoxide (DMSO) 3 times per week for 5 weeks (Table 2.1). At 3, 6, 9, 12, 15, 18, 21, 24, 30 and 36 weeks following initiation of NMBA
treatment, esophagi were collected from 12 untreated and 12 NMBA-treated animals (Figure 2.1). Half of each esophagus was fixed in 10% neutral buffered formalin (NBF) for 4 hours, and then transferred to phosphate buffered saline (PBS). These tissues were then cut into three segments and embedded in paraffin. The other half of esophagus was stripped of submucosal and muscularis layers and frozen in liquid nitrogen. Papillomas in this half esophagus were cut off and frozen in liquid nitrogen separately.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. Rats</th>
<th>Amt. Administered (ml)</th>
<th>Dose Admin. (mg/kg b.w.)</th>
<th>Dosing Protocol (inj./wk × total wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO + H₂O³</td>
<td>120</td>
<td>0.2</td>
<td>0</td>
<td>3 × 5</td>
</tr>
<tr>
<td>2</td>
<td>NMBA</td>
<td>120</td>
<td>0.2</td>
<td>0.5</td>
<td>3 × 5</td>
</tr>
</tbody>
</table>

³DMSO + H₂O, vehicle for NMBA.

Table 2.1: Experimental design for iNOS expression bioassay.

![Figure 2.1: Experimental protocol for iNOS expression bioassay.](image)

Figure 2.1: Experimental protocol for iNOS expression bioassay.
2.3.2. RNA isolation, Semi-quantitative PCR, and Real-Time PCR analysis

Total cellular RNA was isolated from frozen half esophagus by using TRIzol Reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer’s instructions. All RNA samples were analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining of 0.5 µg of RNA resolved by electrophoresis on 1.2% agarose formaldehyde gels. The RNA was incubated at 60°C for 5 minutes and then chilled to 4°C immediately before being reverse transcribed. Reverse transcription (RT) of 2 µg of total RNA was performed for 60 minutes at 37°C in a volume of 20 µl containing 5.0 mM MgCl₂, 1 U/µl Rnasin RNA inhibitor, 100 pmole random primer, 1 mM each dATP, dGTP, dCTP, and dTTP, and 2.5 U/µl SuperScript reverse transcriptase (GIBCO, BRL, Gaithersburg, MD). The samples were incubated for 10 minutes at 25°C before RT and heated to 99°C for 5 minutes to terminate the RT reaction in a Perkin-Elmer DNA Thermocycler 9600 (Perkin-Elmer, Norwalk, CT). An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA. The cDNA was stored at -20°C until ready to use.

Semi-quantitative PCR was performed using a Perkin-Elmer GeneAmp PCR System 9600. The photographs of gels were shown by staining with ethidium bromide. Real-Time PCR amplifications were performed in a GeneAmp 5700 (Perkin-Elmer, Norwalk, CT) sequence detection system using the SYBR Green I PCR kit as recommended by the manufacturer. Reactions were performed in a reaction mixture consisting of a 50 µl volume solution containing 1x SYBR PCR buffer, 200 µM of each dATP, dCTP, dGTP, and 400 µM dUTP, 0.025 U/µl AmpliTaq Gold, 0.01 U/µl
AmpEraseUNG (uracil-N-glycosylase), 3 mM MgCl₂, and 900 nM forward and reverse primers and 10 ng of cDNA. The reactions were performed in MicroAmp 96-well plates capped with MicroAmp optical caps. The reactions were incubated at 50°C for 2 minutes to activate the uracil-N-glycosylase and to prevent the reamplification of carryover PCR products, and then 10 minutes at 95°C to activate the AmpliTaq Gold polymerase followed by 40 cycles of 15 seconds denaturation at 95°C, 30 seconds annealing at 60°C, and 30 seconds elongation at 72°C.

To exclude the contamination of unspecific PCR products such as primer dimer, melting curve analysis was applied to all final PCR products after the cycling protocol. The reactions without the RT reaction were performed for each sample in order to exclude genomic DNA contamination. To assess the reproducibility of SYBR Green assays, amplifications were carried out independently on different days. In a 96-well plate, each sample had 3 replicates.

The Real-Time PCR was amplified for iNOS and hypoxanthine-guanine phosphoribosyltransferase (HPRT). HPRT was used as an internal control. PCR primers for iNOS and HPRT were designed for Real-Time PCR using the Primer Express 1.0 Software program (Perkin-Elmer, Norwalk, CT). Primers used for the Real-Time PCR are listed in Table 2.2 All of the PCR primers were synthesized by GIBCO BRL, (Gaithersburg, MD).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>5’-GCT CGA GAT GTC ATG AAG GAG AT-3’</td>
<td>5’-AGC AGG TCA GCA AAG AAC TTA TAG C-3’</td>
</tr>
<tr>
<td>HPRT</td>
<td>5’-GCT CGA GAT GTC ATG AAG GAG AT-3’</td>
<td>5’-AGC AGG TCA GCA AAG AAC TTA TAG C-3’</td>
</tr>
</tbody>
</table>

Table 2.2: The nucleotide sequences of the primers used to assay gene expression by Real-time PCR.

2.3.3. Immunohistochemistry

NMBA-treated and untreated esophagi were preserved in 10% neutral buffered formalin for 4 hours and then stored in phosphate buffered saline (PBS) at 4°C. Formalin-fixed esophagi were cut into three equal segments and embedded in paraffin. Paraffin blocks were serially sectioned at 4 µm and mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). Slides were heated in a dry oven (55°C for 10 minutes), deparaffinized in histoclear, rehydrated in graded ethanol (100% to 70%) and rinsed in deionized water. Sections were then microwaved in 10 mM citric acid buffer (pH 6.0) at 70% power for 3 minutes for antigen retrieval. Endogenous peroxidase was quenched using 3% hydrogen peroxide for 20 minutes followed by rinsing with wash buffer. Nonspecific binding was blocked with casein for 15 minutes, goat serum for 20 minutes, avidin and biotin for 15 minutes. Then sections were incubated for 1 hour at room temperature with mouse monoclonal IgG for rat iNOS (1:100) (Santa Cruz Biotechnology, CA). Antibody incubation was followed by 20 minutes incubation with a mouse adsorbed link (goat anti-mouse biotinylated immunoglobulin) and strepavidin-horseradish peroxidase label. The sections were developed with diaminobenzidine (DAB)
chromogen and then counterstained with hematoxylin, dehydrated, and mounted. A slide containing rat spleen was utilized as positive control for iNOS. Negative control was established by replacing the primary antibody with PBS and normal serum. Reagents were supplied by BioGenex, Inc., San Ramon, CA. Slides stained with iNOS were viewed with a Nikon bright field microscope mounted with a high-resolution spot camera, which was interfaced with a computer containing a matrox frame grabber board and loaded with image analysis software (Simple PCI Imaging Systems by Compix, Inc., Cranberry Township, PA).

2.3.4. Data and statistical analysis

After a SYBR Green Real-Time PCR run, data acquisition and subsequent data analyses were done using the 5700 Sequence Detection System. The fluorescence of SYBR Green against the internal passive reference dye, ROX (ΔRn) is measured at the end of each cycle. A sample is considered positive when ΔRn exceeds the threshold value. The threshold value is set at the midpoint of ΔRn vs. cycle number plot. For all amplifications performed in this study, the threshold value of ΔRn was taken as 0.10. The endpoint used in the Real-Time PCR quantification, C_T, is defined as the PCR cycle number that cross an arbitrarily placed signal threshold. Gene expression and C_T values are related inversely. A sample containing higher level of the target cDNA will cross the threshold at an earlier cycle compared to a sample with lower level of cDNA. The fold change in iNOS cDNA relative to HPRT endogenous control was determined by:

“Fold change = 2^ΔΔC_T”, where ΔΔC_T = (C_{TINOS} - C_{TPRT})_{abnormal} - (C_{TINOS} - C_{TPRT})_{normal}.

This method was first described by K. Livak in PE Biosystems Sequence Detector User
Bulletin 2 (31). The expression of each housekeeping gene, like HPRT, was presented as $2^{-\Delta CT}$, where $\Delta CT (C_{TINOS} - C_{THPRT})$ and $(C_{TINOS} - C_{THPRT})_{normal}$ represents the 1 X expression of iNOS.

For further statistical analyses, the $C_T$ values were exported onto a Microsoft Excel Worksheet. Differences between groups were analyzed for statistical significance using One-way ANOVA following Dunnet’s multiple comparison tests. $P$-values were considered to be significant when less than 0.05.

2.4. Results

2.4.1. Semi-quantitative PCR and Real-Time PCR analysis

In this bioassay, we evaluated the effects of NMBA treatment on iNOS mRNA expression in the rat esophagus. In addition, we determined whether these effects were correlated with the function of time. All esophagi were classified into three different types: normal, preneoplasia, and papilloma. Esophagi collected from NMBA-untreated rats were classified as normal; esophagi from NMBA-treated rats after the papillomas were removed were classified as preneoplasia; papillomas were the lesions $\geq 0.5$ mm$^3$ and they were cut off from the esophagus and stored separately.

Total cellular RNA was isolated from these tissues and cDNAs were developed through RT reactions. Semi-quantitative PCR and Real-Time PCR assays were performed to determine iNOS mRNA in different types of tissues at different time points. Representative photographs of ethidium bromide stained gels are shown in Figure 2.2 and the $C_T$ values generated from Real-Time PCR are shown in Figure 2.3. As indicated in Table 2.3, in NMBA-induced lesions, the $C_T$s were lower when compared to normal
tissues and these differences were significant in preneoplastic lesions at weeks 9, 12, 15, and 21; and in papillomatous lesions at weeks 24 and 36. As described above, the lower level of C_T, the higher level of mRNA gene expression. According to the method of K. Livak et al, 2^{-\Delta\Delta C_T} was calculated as an example given in Table 2.4. After normalization to HPRT housekeeping gene expression for all samples, the expression of iNOS mRNA in preneoplastic tissues was elevated from 1.84 (week 3) - to 17.23 (week 24) - fold when compared to normal tissues; and, in papillomas, it was elevated from 7.01 (week 18) - to 114.7 (week 36) - fold relative to normal tissues.

Interestingly, iNOS mRNA expression was upregulated as early as week 3 (1.84-fold), and at week 9 (6.60-fold), the change was significant. After papillomas developed, iNOS mRNA was increased up to 42.94-fold at week 30 and 114.7-fold at week 36 when compared to normal tissues.

Together, by performing Real-Time PCR assay, we found that iNOS mRNA expression was significantly upregulated in NMBA-induced preneoplastic and papillomatous lesions when compared to normal tissues; and, that increased iNOS expression is an early event in NMBA-induced rat esophageal tumorigenesis. Our data suggest that there is a correlation between overexpression of iNOS mRNA and neoplastic progression in the rat esophagus.
Figure 2.2: Representative photographs of ethidium bromide stained gels by Semi-quantitative PCR showing DNA fragments for iNOS and HPRT, respectively. (A) week 3 and 6; (B) week 24; and (C) week 36.
Figure 2.3: Representative $C_T$ for iNOS mRNA expression in the rat esophagus by Real-time PCR at weeks, 3, 6, 24, and 36, as indicated by Mean ± SEM.
<table>
<thead>
<tr>
<th>Time (wk)</th>
<th>$C_T$ (normal)$^a$</th>
<th>$C_T$ (preneoplasia)$^a$</th>
<th>$C_T$ (papilloma)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>29.47 ± 0.41</td>
<td>28.73 ± 0.28</td>
<td>NA$^b$</td>
</tr>
<tr>
<td>6</td>
<td>28.46 ± 1.25</td>
<td>25.68 ± 0.74</td>
<td>NA$^b$</td>
</tr>
<tr>
<td>9</td>
<td>27.64 ± 0.17</td>
<td>25.13 ± 0.29$^{d,**}$</td>
<td>NA$^b$</td>
</tr>
<tr>
<td>12</td>
<td>28.55 ± 0.48</td>
<td>25.79 ± 0.26$^{d,**}$</td>
<td>NA$^b$</td>
</tr>
<tr>
<td>15</td>
<td>28.20 ± 0.49</td>
<td>25.48 ± 0.33$^{d,*}$</td>
<td>25.09 ± 0.08</td>
</tr>
<tr>
<td>18</td>
<td>30.91 ± 0.56</td>
<td>29.44 ± 0.70</td>
<td>28.01 ± 0.00$^c$</td>
</tr>
<tr>
<td>21</td>
<td>28.66 ± 0.49</td>
<td>25.77 ± 0.25$^{d,*}$</td>
<td>25.20 ± 0.00$^c$</td>
</tr>
<tr>
<td>24</td>
<td>34.94 ± 1.84</td>
<td>30.21 ± 0.95</td>
<td>27.96 ± 0.06$^{d,**}$</td>
</tr>
<tr>
<td>30</td>
<td>35.13 ± 2.01</td>
<td>31.43 ± 0.14</td>
<td>30.43 ± 1.06</td>
</tr>
<tr>
<td>36</td>
<td>34.32 ± 1.58</td>
<td>30.62 ± 0.73</td>
<td>27.83 ± 1.20$^{d,*}$</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SEM (n = 4-12).

$^b$ NA: not applicable, no papillomas were observed.

$^c$ n = 1.

$^d$ Values in vertical columns are significantly different from normal tissues by unpaired $t$-test; $^* p < 0.05$; $^{**} p < 0.01-0.0001$.

Table 2.3: $C_T$ for iNOS mRNA expression in rat esophagus during or after NMBA treatment (0.5 mg/kg b.w., 3 times per week, for 5 weeks).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Average $C_T$</th>
<th>$\Delta C_T$ iNOS</th>
<th>$\Delta C_T$ HPRT</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>38.88</td>
<td>27.26</td>
<td>11.62</td>
<td>0</td>
</tr>
<tr>
<td>Preneoplasia</td>
<td>33.24</td>
<td>25.45</td>
<td>7.79</td>
<td>$^{-3.83}$</td>
</tr>
<tr>
<td>Papilloma</td>
<td>31.06</td>
<td>24.09</td>
<td>6.79</td>
<td>$^{-4.65}$</td>
</tr>
</tbody>
</table>

Table 2.4: An example of fold change calculation using $2^{-\Delta C_T}$ method in Real-Time PCR. A rat was randomly selected from NMBA-treated group at week 24.
<table>
<thead>
<tr>
<th>Time (wk)</th>
<th>Number of Rats (N)</th>
<th>Fold Change (Mean ± SEM)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>8</td>
<td>1.84 ± 0.30</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>8.97 ± 6.07</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>6.60 ± 1.45</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>7.50 ± 1.22</td>
</tr>
<tr>
<td>15</td>
<td>12</td>
<td>7.80 ± 1.75</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>4.09 ± 2.23</td>
</tr>
<tr>
<td>21</td>
<td>7</td>
<td>8.01 ± 1.24</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>17.23 ± 7.17</td>
</tr>
<tr>
<td>30</td>
<td>12</td>
<td>12.46 ± 1.23</td>
</tr>
<tr>
<td>36</td>
<td>8</td>
<td>8.54 ± 3.32</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fold change compared to normal.

Table 2.5: Fold change for iNOS mRNA expression in preneoplastic tissues in rat esophagus when compared to normal tissue.

---

**Figure 2.4:** Fold change for iNOS mRNA expression in preneoplastic tissues in rat esophagus when compared to normal tissue.
<table>
<thead>
<tr>
<th>Time (wk)</th>
<th>Number of Rats (N)</th>
<th>Fold Change (Mean ± SEM) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>3</td>
<td>8.62 ± 0.45</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>7.01 ± 0.00</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>10.93 ± 0.00</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>38.66 ± 18.00</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>42.94 ± 30.57</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>114.7 ± 73.22</td>
</tr>
</tbody>
</table>

a Fold change compared to normal (fold = 1).

Table 2.6: Fold change for iNOS mRNA expression in papillomataous lesions in rat esophagus when compared to normal tissue.

Figure 2.5: Fold change for iNOS mRNA expression in papillomatous lesions in rat esophagus when compared to normal tissue.
2.4.2. Immunohistochemical analysis

To determine whether iNOS protein is expressed by NMBA-induced preneoplasia and papilloma, we performed immunohistochemistry for iNOS in tissue specimens from both NMBA-untreated and NMBA-treated groups. iNOS staining was localized in the cytoplasm of the epithelial cells. As indicated in Figure 2.6, weak iNOS immunoreactivity was observed in the basal layers of non-neoplastic esophageal stratified squamous epithelium. Moderate iNOS positive staining was observed in the thickened basal layer of esophageal mucosal hyperplasia and dysplasia. Strong iNOS immunostaining was diffused in all cells in esophageal papillomas.

Together, immunohistochemical analysis showed positive cytoplasmic staining of iNOS protein in preneoplastic tissues and papillomas when compared to normal tissues. Our data suggest that increased iNOS protein is associated with NMBA-induced carcinogenesis in rats.
Figure 2.6: Immunohistochemical analysis of iNOS expression in normal epithelium (A); NMBA-induced preneoplastic epithelium (B and C); and NMBA-induced papilloma (D). In normal epithelium, Inos staining was very weak. In preneoplastic and papilloma lesions, positive iNOS staining was frequent and strong in the basal and suprabasal layers.
2.5. Discussion

Esophageal cancer is the third most common gastrointestinal malignancy (1) and is the sixth most frequent cause of cancer death in the world (2). Epidemiology studies show that exposure to nitrosamines is one of the risk factors for esophageal cancer development (32). As indicated previously, more than half of the 300 different N-nitro compounds are carcinogenic for the rat esophagus (33). N-Nitrosomethylbenzylamine (NMBA), an asymmetrical nitrosamine, is the most potent esophageal carcinogen in the rat among all active nitrosamines (34). Fisher 344 rats are predisposed to NMBA-induced esophageal tumorigenesis and are considered to be a good model for preclinical studies on esophageal cancer.

NO is often generated in inflammatory conditions due to the induction of NOS in epithelial cells by inflammatory cytokines released from adjacent mononuclear cells (22-25). Increased NO production appears to be associated with many disorders including cancer (35-40). Because it is induced, iNOS is distinguished from endothelial NOS and neuronal NOS (41). iNOS is capable of generating relatively large amounts of NO compared to endothelial NOS and neuronal NOS (42). Therefore, the fundamental information on the effect of iNOS is scientifically important. The major purpose of this study was therefore to exam the gene expression and histological distribution of iNOS in rat esophageal tumorigenesis after NMBA treatment by Real-Time PCR and immunohistochemistry assays.

Our results directly demonstrate the following: (a) the expression of iNOS mRNA in preneoplastic tissues and papillomas was significantly elevated when compared to normal tissues; (b) immunohistochemical analysis showed positive cytoplasmic staining
of iNOS protein in preneoplastic tissues and papillomas relative to normal tissues. Overexpression of iNOS has been observed in several human premalignancies and malignancies in which chronic inflammation is a predisposing factor, including colon cancer (43, 44), liver cancer (45, 46), Barrett’s esophagus (47, 48), and breast cancer (49). The outcome of this study is of great interest because it demonstrates for the first time the expression of iNOS in esophageal papilloma. The increased iNOS mRNA expression observed in this study is likely due to the induction of gene expression. Immunostaining for iNOS indicates that the iNOS protein expressed in preneoplasia and papilloma and its positive staining intensity is correlated with the stages of tumor progression. This observation is consistent with recent reports that iNOS protein has been detected in both premalignant and malignant clinical biopsies from the stomach, colon, lung, esophagus, prostate, and duodenum (50-55). It is also noteworthy that our study demonstrates for the first time that upregulation of iNOS expression is an early event in esophageal carcinogenesis. Forster et al. (56) reported that iNOS was induced in human brain 1-2 days after stroke. This report supports the idea that our results are relevant to clinical situations.

iNOS is calcium-independent and produces a high level of NO, however, it is usually absent in resting cells. After stimulation by cytokines and bacterial cell wall products such as the interleukins, tumor necrosis factor alpha (TNFα) and endotoxin lipopolysaccharide (LPS), iNOS can be induced and then become involved in carcinogenesis (4-8). Moreover, the expression of iNOS is associated with the activation of transcriptional factors including NFκB, STST-1, and IRF-1 (57). Initially, the major regulation of iNOS was believed to occur at the level of transcription, but subsequent
studies have shown that iNOS regulation can occur at the transcriptional, post-transcriptional, translational, and post-translational levels (58). We suggest that NMBA treatment might enhance a signaling pathway, which results in the induction of iNOS gene expression. This may explain the increased mRNA and protein levels of iNOS in the present study.

In conclusion, our data suggest that iNOS expression is strongly associated with tumor development in rat esophagus after NMBA treatment. Furthermore, the overexpression of iNOS is an early event in esophageal tumor development. The information obtained in this study would appear helpful in identifying the chemopreventive time window during which an iNOS inhibitor may have chemopreventive potential in the rat esophagus.
2.6. References


3.1. Abstract

Nitric Oxide (NO), a lipid- and water- soluble radical gas, is an important bioactive agent and signaling molecule. NO is synthesized from L-arginine by a family of nitric oxide synthases (NOS), which have three isoforms: nNOS, eNOS, and iNOS. iNOS, the inducible isoform, is calcium-independent and generates a high concentration of NO. Increased NO production appears to be associated with many disorders including esophageal cancer. Previous studies in our laboratory demonstrated an association between increased iNOS expression and the development of NMBA-induced tumors in the rat esophagus. Based upon these observations, we initiated a bioassay to evaluate the abilities of PBIT, a selective iNOS inhibitor, and 5% freeze-dried black raspberries, previously shown in our laboratory to inhibit NMBA tumorigenesis, to prevent tumor progression in the rat esophagus. Rats were given s.c. injections of NMBA (0.25mg/kg b. w.) three times per week for 5 weeks. One week later, the rats were fed a synthetic diet containing either 50 or 100 ppm PBIT or 5% freeze-dried black raspberries until the end of the bioassay (25 weeks). At week 25, 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. Five percent black raspberries reduced the cancer incidence to 90%. Tumor multiplicity was reduced from 3.64 ± 0.42 esophageal 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. Five percent black raspberries also caused a significant reduction in tumor multiplicity [2.00 ± 0.23 tumors/rat (p< 0.05)] when compared to the rats treated with NMBA only (3.64 ± 0.42 tumors per rat). Both 50 ppm and 100 ppm PBIT reduced the production of NO in preneoplastic and papillomatous lesions when compared to the production of NO in esophagus treated with NMBA only. Similarly, five percent black raspberries suppressed NO production in both preneoplastic and papillomatous lesions. Neither PBIT (50 ppm or 100 ppm) nor 5% black raspberries were found to down regulate iNOS mRNA expression. These observations suggest that iNOS plays a role in tumor development in the rat esophagus, and its selective inhibitor, PBIT, significantly inhibits esophageal tumor progression. Moreover, this study demonstrates for the first time that freeze-dried black raspberries influence NO production in the esophagus. The exact mechanism(s) remain to be elucidated.
3.2. Introduction

It is estimated that about 1,334,100 new cancer cases will be diagnosed in the United States in 2003. About 556,500 Americans are expected to die of these cancers, more than 1,500 people a day. With respect to esophageal cancer, it is estimated that 13,900 United States citizens will be diagnosed with esophageal cancer in 2003 and 13,000 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). The American Cancer Society estimates that one third of cancer deaths are related to nutrition and other lifestyle factors, which could be prevented (1). Cancer chemoprevention is one strategy for cancer prevention that could be effective for many cancers. Esophageal cancer, for example, has very low five-year survival rate (< 10%): 75% of patients die within one year of initial diagnosis (4). Chemoprevention could be very important in this disease since high-risk populations can be identified in specific geographical regions.

Nitric oxide (NO) is a one of the smallest endogenous biological mediators that has received considerable research attention during the last several years. NO has a wide range of physiological and pathophysiological actions (5-10). It is synthesized from L-arginine by a family of nitric oxide synthases (NOS) (11). Historically, NOS have been classified into two categories, constitutive (nNOS and eNOS) and inducible (iNOS) (11). In general, nNOS and eNOS are continuously present in neurons and endothelial cells, respectively. These two isoforms are regulated by calcium influx and require attendant activation of calmodulin to produce NO. They are calcium-dependent and produce only a low level of NO (6, 12). iNOS, an 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 (7, 10, 13-16). Numerous experimental and clinical reports indicate that iNOS expression is upregulated in chronic inflammatory diseases as well as cancer (17-19). In addition, iNOS protein has been detected in both premalignant and malignant clinical biopsies from the stomach, colon, lung, esophagus, prostate, and duodenum (20-25). Increased iNOS activity has also been reported in human cancers including esophageal, colorectal, gastro-intestinal, gynecological, pancreatic, breast, lung, head and neck, and central nervous system tumors (26-34).

\[\textit{N-}\text{Nitrosomethylbenzylamine (NMBA)-induced tumors in the rat esophagus is a model of human esophageal squamous cell carcinoma (ESCC). This model is utilized for investigations of molecular alterations involved in the development of ESCC and for the evaluation of potential chemopreventive agents (35). Previous studies in our laboratory showed that iNOS mRNA is significantly elevated in NMBA-induced preneoplastic esophageal lesions and in papillomas when compared to normal rat esophagus (Chen, unpublished).}\]

\[\textit{S,S'-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea (PBIT) is an iNOS-selective inhibitor. Its specificity for iNOS was established in cytokine-induced colorectal adenocarcinoma DLD cells in which it was found to be selective as an inhibitor of iNOS when compared to eNOS and nNOS (36). In a study reported by Rao and Kawamori et al. (37), PBIT suppressed AOM-induced colonic ACF formation, crypt multiplicity, and iNOS activity.}\]

\[\text{Epidemiological evidence suggests an inverse relationship between consumption of fruit and vegetables and the occurrence of several types of cancer (38, 39). Dietary}\]
deficiencies in certain micronutrients and protective substances have been shown to be associated with increased risk for esophageal SCC. The incidence of esophageal cancer is high in poor rural areas where dietary deficiencies are common (40). In Linxian County, China, for example, the daily intake of fresh vegetables and fruit is very low. These deficiencies are undoubtedly very important for increased cancer risk (41). In recent years, our laboratory has taken a food-based approach to the prevention of esophageal, colon and oral cancer utilizing freeze-dried berries (42-44). We found that black raspberries produced a significant reduction in esophageal tumor incidence and multiplicity when administered before, during, and after NMBA treatment in rats (42). The berries reduced the formation of $O^6$-methylguanine adducts through modulating the metabolism of NMBA, and also reduced the rate of esophageal cell proliferation in NMBA exposed rats (42). In addition, dietary supplementation with black raspberries was shown to suppress azoxymethane (AOM)-induced aberrant crypt foci in the rat colon (43) and 7,12-dimethylbenz(a)anthracene (DMBA)-induced oral cancer in the hamster cheek pouch (44).

In the present study, we have evaluated the effect of PBIT and freeze-dried black raspberries on NMBA-induced tumorigenesis in the rat esophagus. The ability of PBIT and black raspberries to inhibit post-initiation events of tumorigenesis was determined by dietary administration of PBIT to rats that had been pretreated with NMBA. Identifying agents that inhibit tumorigenesis in the rat esophagus has proven to be a difficult task, and it is gratifying that both PBIT and black raspberries were found to be effective.
3.3 Materials and Methods

3.3.1. Materials

NMBA was obtained from Ash Stevens (Detroit, MI) and determined to be >98% pure by high-performance liquid chromatography (hplc). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Company (St. Louis, MO). Modified AIN-76A synthetic diet was purchased from Dyets, Inc. (Bethlehem, PA), and was stored at 4°C prior to preparation of experimental diets. Black raspberries of the Jewel variety were purchased from the Dale Stokes Fruit Farm (Wilmington, OH) and shipped frozen to Van Drunen Farms (Momence, IL) for freeze-drying as described by Stoner et al. (45). Certain components of the berries were measured by Covance Laboratories (Madison, WI) and the results are presented in Table 3.1. PBIT and the Nitrate/Nitrite Colorimetric Assay Kits were obtained from Cayman Chemical Company (Ann Arbor, MI). The QuantiTect SYBR Green RT PCR Kit was purchased from QIAGEN Inc. (Valencia, CA).
Table 3.1: Composition of black raspberries used in the bioassay.

<table>
<thead>
<tr>
<th>Components</th>
<th>Black raspberries$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minerals</strong></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>175</td>
</tr>
<tr>
<td>Copper</td>
<td>0.827</td>
</tr>
<tr>
<td>Iron</td>
<td>4.64</td>
</tr>
<tr>
<td>Magnesium</td>
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</tr>
<tr>
<td>Manganese</td>
<td>6.06</td>
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<tr>
<td>Phosphorus</td>
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<td>Potassium</td>
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</tr>
<tr>
<td>Sodium</td>
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</tr>
<tr>
<td>Zinc</td>
<td>2.34</td>
</tr>
<tr>
<td>Selenium</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0.0765</td>
</tr>
<tr>
<td>A from carotene</td>
<td>915</td>
</tr>
<tr>
<td>A by HPLC</td>
<td>&lt; 100</td>
</tr>
<tr>
<td>A, total</td>
<td>915</td>
</tr>
<tr>
<td>E (natural)</td>
<td>15.2</td>
</tr>
<tr>
<td>C</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Sterols</strong></td>
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</tr>
<tr>
<td>Cholesterol</td>
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</tr>
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<td>Campesterol</td>
<td>5.9</td>
</tr>
<tr>
<td>Stigmasterol</td>
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</tr>
<tr>
<td>β-sitosterol</td>
<td>88.8</td>
</tr>
<tr>
<td><strong>Phenolics</strong></td>
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</tr>
<tr>
<td>Ellagic Acid</td>
<td>200</td>
</tr>
<tr>
<td>Ferulic Acid</td>
<td>21</td>
</tr>
<tr>
<td>P-Coumeric Acid</td>
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</tr>
<tr>
<td><strong>Carotenoids</strong></td>
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<tr>
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</tr>
<tr>
<td>Lutein</td>
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</tr>
<tr>
<td>Zeaxanthin</td>
<td>0.106</td>
</tr>
<tr>
<td>β-carotene</td>
<td>0.549</td>
</tr>
</tbody>
</table>

$^a$Concentration is reported in mg/100g, except Vitamin A and E are reported in IU/100g.
3.3.2. 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 hour light/dark cycles), and fed modified AIN-76A diet containing 20% casein, 0.3% D, L- methionine, 52% cornstarch, 13% dextrose, 5% cellulose, 5% corn oil, 3.5% AIN salt mixture, 1% AIN vitamin mixture and 0.2% choline bitartrate. Black raspberries were mixed into modified AIN-76A diet (with the concentration of cornstarch adjusted to maintain an isocaloric diet) weekly and stored at 4°C until fed to the rats. Food and water were available ad libitum. Hygienic conditions were maintained by twice weekly cage changes and routine cleaning of the animal rooms.

3.3.3. Chemoprevention Study

Following a two-week acclimation period, 300 rats were randomized into eight experimental groups (Table 3.1) and placed on AIN-76A diet. Rats in Groups 5-8 were treated with NMBA at a dose of 0.25 mg/kg body weight by s.c. injection three times per week for five weeks. Rats in Group 1 were dosed with 20% DMSO in water, the solvent for NMBA, as a vehicle control and those in Groups 2, 3, and 4 were dosed with either 50 ppm PBIT or 100 ppm PBIT, or 5% black raspberries. Three days following the final NMBA treatment, rats received AIN-76A diet containing either 50 or 100 ppm PBIT, or 5% freeze-dried black raspberries. Diets containing either PBIT or black raspberries were prepared fresh each week and stored at 4°C. PBIT and black raspberries were added to AIN-76A diet and mixed for 25 minutes 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 animals. Animals were maintained on the diets for the duration of the bioassay. Food consumption and body weight data were recorded weekly. At 9 and 15 weeks, 5 rats from Groups 1-4 and 10 rats from Groups 5-8, and at 25 weeks, 15 rats from Groups 1-4 and 30 rats from Groups 5-8, were euthanized by CO₂ asphyxiation and subjected to gross necropsy. The esophagus of each rat was excised, opened longitudinally, and tumor ≥ 0.5 mm in a single dimension were counted, mapped, and measured. Tumor volume was calculated using the formula for a prolate spheroid: length x width x height x π /6. The esophagus was then cut longitudinally into two parts. The epithelium from one part was stripped of the submucosal and muscularis layers and frozen in liquid nitrogen; tumors on this part were removed and stored separately in liquid nitrogen. The other portion of the esophagus was fixed in 10% neutral-buffered formalin for four hours and then transferred to phosphate buffered saline (PBS).
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Amt. Administered (ml)</th>
<th>Dose Admin. (mg/kg b.w.)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>DMSO + H₂O</td>
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<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>None</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>AIN-76A + PBIT (100 ppm)</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>AIN-76A + 5% BRB</td>
</tr>
<tr>
<td>5</td>
<td>NMBA</td>
<td>50</td>
<td>0.2</td>
<td>0.25</td>
<td>Control AIN-76A</td>
</tr>
<tr>
<td>6</td>
<td>NMBA</td>
<td>50</td>
<td>0.2</td>
<td>0.25</td>
<td>AIN-76A + PBIT (50 ppm)</td>
</tr>
<tr>
<td>7</td>
<td>NMBA</td>
<td>50</td>
<td>0.2</td>
<td>0.25</td>
<td>AIN-76A + PBIT (100 ppm)</td>
</tr>
<tr>
<td>8</td>
<td>NMBA</td>
<td>50</td>
<td>0.2</td>
<td>0.25</td>
<td>AIN-76A + 5% BRB</td>
</tr>
</tbody>
</table>

Table 3.2: Experimental design for bioassay with PBIT and black raspberries.
Figure 3.1: Experimental protocol for PBIT and black raspberry bioassay. Rats were treated with NMBA (0.25 mg/kg b.w.) three times per week for 5 weeks. PBIT and black raspberries were administered following NMBA treatment and for the duration of the bioassay.
3.3.4. Real-Time PCR analysis

Total cellular RNA was isolated from the esophagus portion frozen in liquid nitrogen using TRIzol Reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer’s instructions. Each sample was extracted twice. All RNA samples were analyzed for integrity of 18S and 28S rRNA by ethidium bromide staining of 0.5 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, 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 2X 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 (RT) was first performed at 50°C for 30 minutes. Then HotStar Taq DNA Polymerase was activated at 95°C for 15 minutes followed by 40 cycles of 15 seconds at 94°C (denaturation), 30 seconds at 60°C (annealing), and 30 seconds at 72°C (extension). The expression of iNOS mRNA was normalized against expression of the housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (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’-AGC GGC TCC ATG ACT CTC A-3’ and antisense 5’-TGC ACC CAA ACA CCA AGG T-3’; HPRT sense 5’-GCT CGA GAT GTC ATG AAG GAG AT-3’ and antisense 5’-AGC AGG TCA GCA AAG AAC TTA TAG C-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 the reagents except QuantiTect RT Mix to confirm that there was no genomic contamination. The other control contained all reagents without the RNA template to confirm that the reaction mix displayed no signal. After the final PCR cycle, all reactions were heat denatured over a 35°C gradient at 0.03°C/sec from 60 to 95°C. All SYBR Green PCR data were collected using the SDS sequence detector software (PE, Applied Biosystems, Foster City, CA).

3.3.5. 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 (46). 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, Ann Arbor, MI). The accumulation of nitrate/nitrite was taken as an index of iNOS activity (47-50). Esophagus tissue was weighed and homogenized in PBS (5 ml PBS/g) and centrifuged at 10, 000 x g for 20 minutes at 4°C. The supernatant was used for the nitrate/nitrite assay. An aliquot (500 µl) of supernatant was added to a 30kDa molecular weight cut-off filter and ultrafiltered at 5000 x g for three hours. 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 three hours. After incubation, Griess reagents 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.

3.3.6. Statistical analysis

Body weight, food consumption, and tumor incidence, multiplicity, and volume data were determined for all control and experimental rats. Differences between groups were analyzed for statistical significance using one-way ANOVA followed by Dunnet’s multiple comparison tests to identify individual differences when the ANOVA was significant. Tumor incidence was compared using the Chi-square test. Comparisons of the incidence of esophageal tumors in rats treated with NMBA or a combination of NMBA and PBIT or black raspberries 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.
3.4. Results

3.4.1. Body weight and food consumption

Mean body weight and daily food consumption among control and treated rats were not significantly different during the bioassay (Figure 3.2). Therefore, administration of 50 ppm, 100 ppm PBIT or 5% black raspberries did not influence food intake or body weight gain in control and treated rats. At necropsy, multiple organs were examined grossly and no abnormalities were observed.

3.4.2. Tumor data

Tumors were counted, mapped, and measured immediately following euthanization. None of the vehicle (DMSO) treated rats developed esophageal tumors. In addition, rats treated with PBIT (50 ppm and 100 ppm) or 5% black raspberries only (Group 2, 3, and 4) had no tumors. At 9 weeks of the bioassay, PBIT and 5% black raspberries had no effects on the occurrence of NMBA-induced esophageal tumors (Tables 3.3 and 3.4). At 15 weeks however, PBIT reduced the incidence of esophageal tumor from 70% in rats treated with NMBA alone (Group 5) to 60% in rats treated with NMBA + 100 ppm PBIT (Group 7). Tumor multiplicity was reduced from 1.90 ± 0.53 in NMBA-treated rats (Group 5) to 0.70 ± 0.21 in rats given NMBA + 100 ppm PBIT. At 25 weeks, PBIT reduced the incidence of esophageal tumor from 96% in NMBA-treated rats to 83% and 77% (p<0.05) in rats treated with 50 and 100 ppm PBIT, respectively. Five percent black raspberries reduced the incidence of esophageal tumor to 90%. Tumor multiplicity was reduced from 3.64 ± 0.42 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. Five percent black raspberries caused a significant reduction in tumor multiplicity from $3.64 \pm 0.42$ in rats treated with NMBA only to $2.00 \pm 0.23$ ($p<0.05$).

Tumors $\geq 0.5$ mm in a single dimension were measured. Tumor volume was calculated using the formula for a prolate spheroid: $\text{length} \times \text{width} \times \text{height} \times \pi / 6$. At weeks 9 and 15, PBIT (50 ppm and 100 ppm) and 5% black raspberries had no effect on tumor volume. At 25 weeks, the tumor volume was reduced in rats fed either 50 and 100 ppm PBIT or 5% black raspberries to $4.97 \pm 0.89$ mm$^3$, $4.57 \pm 0.77$ mm$^3$, and $4.75 \pm 1.11$ mm$^3$, respectively, compared with tumor volume ($5.68 \pm 0.86$ mm$^3$) in rats fed the control diet (Tables 3.3 and 3.4).
Figure 3.2: Effects of PBIT and black raspberries on mean body weight gain (A) and food consumption (B) in the 25 week bioassay. Group symbols are as follows: vehicle control (■); 50 ppm PBIT control (Δ); 100 ppm PBIT control (●); 5% black raspberry control (□); NMBA control (♦); NMBA + 50 ppm PBIT (○); NMBA + 100 ppm PBIT (▲); NMBA + 5% black raspberries (◊).
<table>
<thead>
<tr>
<th>Group</th>
<th>NMBA</th>
<th>Diet</th>
<th>No. of Rats</th>
<th>Tumor Incidence (%)</th>
<th>Tumor Multiplicity Mean ± S.E.</th>
<th>Tumor Volume (mm³) Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>1</td>
<td>-</td>
<td>AIN-76A</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>50 ppm PBIT</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>100 ppm PBIT</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>AIN-76A</td>
<td>10</td>
<td>10%</td>
<td>0.10 ± 0.1</td>
<td>1.05 ± 0.00</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>50 ppm PBIT</td>
<td>10</td>
<td>30%</td>
<td>0.30 ± 0.15</td>
<td>6.63 ± 4.53</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>100 ppm PBIT</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Week 15</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>AIN-76A</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>50 ppm PBIT</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>100 ppm PBIT</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>AIN-76A</td>
<td>10</td>
<td>70%</td>
<td>1.90 ± 0.53</td>
<td>1.94 ± 0.79</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>50 ppm PBIT</td>
<td>10</td>
<td>70%</td>
<td>1.40 ± 0.43</td>
<td>8.17 ± 4.65</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>100 ppm PBIT</td>
<td>10</td>
<td>60%</td>
<td>0.70 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.76 ± 1.15</td>
</tr>
<tr>
<td><strong>Week 25</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>AIN-76A</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>50 ppm PBIT</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>100 ppm PBIT</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>AIN-76A</td>
<td>28</td>
<td>96.43%</td>
<td>3.64 ± 0.42</td>
<td>5.68 ± 0.86</td>
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<tr>
<td>6</td>
<td>+</td>
<td>50 ppm PBIT</td>
<td>29</td>
<td>82.76%</td>
<td>1.79 ± 0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.97 ± 0.89</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>100 ppm PBIT</td>
<td>30</td>
<td>76.67%</td>
<td>1.50 ± 0.24&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.57 ± 0.77</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly lower than Group 5 as determined by χ² test (P < 0.05).
<sup>b</sup> Significantly lower than Group 5 as determined by analysis of variance (P < 0.05).
<sup>c</sup> Significantly lower than Group 5 as determined by analysis of variance (P < 0.001).
<sup>d</sup> Significantly lower than Group 5 as determined by analysis of variance (P < 0.0001).
<sup>e</sup> Tumor volume calculated as length x width x depth x π/6 assuming a prolate spheroid shape.

Table 3.3: Effect of PBIT on post-initiation events of NMBA-induced tumorigenesis in the rat esophagus.
<table>
<thead>
<tr>
<th>Group</th>
<th>NMBA</th>
<th>Diet</th>
<th>No. of Rats</th>
<th>Tumor Incidence (%)</th>
<th>Tumor Multiplicity Mean ± S.E.</th>
<th>Tumor Volume (mm³) Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Week 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>AIN-76A</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>5% BRB</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>AIN-76A</td>
<td>10</td>
<td>10%</td>
<td>0.10 ± 0.1</td>
<td>1.05 ± 0.00</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>5% BRB</td>
<td>10</td>
<td>20%</td>
<td>0.20 ± 0.13</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Week 15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>AIN-76A</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>5% BRB</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>AIN-76A</td>
<td>10</td>
<td>70%</td>
<td>1.90 ± 0.53</td>
<td>1.94 ± 0.79</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>5% BRB</td>
<td>10</td>
<td>70%</td>
<td>1.20 ± 0.36</td>
<td>6.06 ± 3.30</td>
</tr>
<tr>
<td>Week 25</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>AIN-76A</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>5% BRB</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>AIN-76A</td>
<td>28</td>
<td>96.43%</td>
<td>3.64 ± 0.42</td>
<td>5.68 ± 0.86</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>5% BRB</td>
<td>29</td>
<td>89.66%</td>
<td>2.00 ± 0.23</td>
<td>4.75 ± 1.11</td>
</tr>
</tbody>
</table>

*BRB: black raspberries.

b Significantly lower than Group 5 as determined by analysis of variance (P < 0.05).

c Tumor volume calculated as length x width x depth x π/6 assuming a prolate spheroid shape.

Table 3.4: Effect of 5% black raspberries on post-initiation events of NMBA-induced tumorigenesis in the rat esophagus.
3.4.3. iNOS mRNA expression

We further performed One-Step Real-Time RT-PCR to investigate whether the inhibition of esophageal tumor development by PBIT is associated with inhibitory effects on iNOS mRNA expression. Our data indicate that neither PBIT (50 ppm or 100 ppm) nor 5% black raspberries had an effect on iNOS mRNA expression (Table 3.5).

3.4.4. Determination of nitrate/nitrite

We also investigated that whether local NO production was decreased by PBIT and black raspberries 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 and papillomatous lesions when compared to esophageal lesions from rats treated with N MBA only. Five percent black raspberries also suppressed nitrate and nitrite production when compared to rats treated with N MBA only (Figure 3.3).
<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Tissue Type</th>
<th>Preneoplasia</th>
<th>Papilloma</th>
</tr>
</thead>
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<tr>
<td><strong>Week 9</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIN-76A</td>
<td></td>
<td>24.97 ± 0.42a</td>
<td>NAb</td>
</tr>
<tr>
<td>50 ppm PBIT</td>
<td></td>
<td>24.67 ± 1.04</td>
<td>NA</td>
</tr>
<tr>
<td>100 ppm PBIT</td>
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<td>25.49 ± 1.80</td>
<td>NA</td>
</tr>
<tr>
<td>5% BRB</td>
<td></td>
<td>24.73 ± 2.62</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Week 15</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIN-76A</td>
<td></td>
<td>24.47 ± 0.87</td>
<td>21.38 ± 0.66</td>
</tr>
<tr>
<td>50 ppm PBIT</td>
<td></td>
<td>24.77 ± 1.91</td>
<td>22.25 ± 2.10</td>
</tr>
<tr>
<td>100 ppm PBIT</td>
<td></td>
<td>24.7 ± 2.55</td>
<td>21.75 ± 2.96</td>
</tr>
<tr>
<td>5% BRB</td>
<td></td>
<td>22.51 ± 1.82</td>
<td>23.25 ± 0.75</td>
</tr>
<tr>
<td><strong>Week 25</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIN-76A</td>
<td></td>
<td>24.65 ± 1.96</td>
<td>20.38 ± 1.73</td>
</tr>
<tr>
<td>50 ppm PBIT</td>
<td></td>
<td>26.41 ± 1.54</td>
<td>20.03 ± 1.11</td>
</tr>
<tr>
<td>100 ppm PBIT</td>
<td></td>
<td>23.52 ± 1.67</td>
<td>21.08 ± 0.53</td>
</tr>
<tr>
<td>5% BRB</td>
<td></td>
<td>23.83 ± 2.04</td>
<td>21.23 ± 1.92</td>
</tr>
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</table>

*a Mean ± SEM.
bNA: not applicable, no papillomas were observed.

Table 3.5: Real-Time PCR analysis of iNOS mRNA expression at weeks 9, weeks 15, and weeks 25. No significant differences were observed between NMBA treated alone group and NMBA + PBIT or 5% black raspberries group.
Figure 3.3: Effect of PBIT and 5% black raspberries on nitrate and nitrite production in rat esophagus. The values are expressed as Mean ± SE. *, p < 0.05; **, p < 0.01; ***, p < 0.0001 as determined by Student’s t-test when compared with the control diet group.
3.5. Discussion

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

NO is a free radical with an unpaired electron; therefore, it can denote or accept an electron to become a nitrosonium cation (NO$^+$) or a nitroxyl anion (NO$^-$), respectively (51). Increased NO production appears to be associated with many disorders including cancer (52-57). iNOS is calcium-independent and produces a high level of NO however, it is usually absent in resting cells. After stimulation by cytokines and bacterial cell wall products such as the interleukins, tumor necrosis factor alpha (TNF$\alpha$), and endotoxin lipopolysaccharide (LPS), iNOS can be induced and then become involved in carcinogenesis (58-62).

Numerous studies in animal models have provided direct evidence for the role of iNOS in tumorigenesis using iNOS inhibitors as chemopreventive agents. Most inhibitors are L-arginine-based substrate analogs and bind directly to the iNOS active site thereby, decreasing NO production and preventing tumor development. Therefore, these inhibitors are not likely to influence iNOS gene expression. A large number of L-arginine analogs have been developed as NOS inhibitors in animal and clinical studies including $N^G$-methyl-L-arginine (L-NMMA), $N^G$-nitro-L-arginine methyl ester (L-NAME), $N$-iminoethyl-L-ornithine (L-NIO), $N^G$-nitro-L-arginine (L-NNA), and $\beta$-(5-imino-2-pyrrolidine-carboxamido)-proamidine (Norformycin) (63-66). PBIT, for example, has a structure similarity to guanidine, therefore, it competitively binds in the guanidine portion
of the L-arginine active site. PBIT produces a selective inhibitory effect against iNOS relative to eNOS and nNOS (37).

The present study demonstrated for the first time that freeze-dried black raspberries suppress the production of NO in the rat esophagus. The mechanism(s) are under investigation. Our data indicate the following: (1) PBIT has chemopreventive potential against N MBA-induced rat esophageal tumorigenesis possibly, through the inhibition of NO production; (2) Agents such as PBIT may be useful in diseases associated with an ongoing local or systemic inflammatory response in which an enhanced formation of NO by iNOS contributes to pathogenesis or pathophysiology; (3) Black raspberries exhibits inhibitory effects on NO production; (4) Our results suggest that selective iNOS inhibitors, and black raspberries, could be promising candidates for chemopreventive agents against esophageal cancer in humans.
3.6. References


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201-227.

Journal 6, 3051-3064.


CHAPTER 4

DISCUSSION AND SUMMARY

Esophageal cancer is the third most common gastrointestinal malignancy and is the sixth most frequent cause of cancer death in the world. Epidemiological studies suggest that increased risk for developing esophageal cancer has been associated with alcohol consumption, cigarette smoking, hot tea and food consumption, nutritional deficiencies and ingestion of moldy foods and pickled vegetables, which are frequently contaminated with $N$-nitrosamines. Nitrosamines are the most prevalent of all environmental chemical carcinogens. More than 300 nitrosamine compounds have been shown to induce cancer, including esophageal cancer. Nitrosamines exhibit significant organ specificity producing tumors in specific organs in which they are metabolized. The most common organ site for tumor induction in the rat by nitrosamines is the esophagus, and more than one-half of the 300 $N$-nitroso compounds are carcinogenic for the rat esophagus. Among all active nitrosamines, $N$-nitrosomethylbenzylamine (NMBA) is the most potent esophageal carcinogen in the rat and has been shown to induce squamous cell carcinomas in the rat esophagus regardless of the route of administration. NMBA has therefore been used to investigate the etiology, molecular alterations, and chemoprevention of esophageal cancer.
Nitric Oxide (NO) is an important bioactive agent and signaling molecule. Increased NO production is associated with many disorders including cancer. iNOS, an inducible isoform of nitric oxide synthase, plays an important role in carcinogenesis through producing high levels of NO. Numerous experimental and clinical reports indicate that iNOS expression and activity is upregulated in the tissues of human cancers including esophageal cancer.

Our laboratory has evaluated numerous molecular alternations in NMBA-induced rat esophageal tumorigenesis, and the chemopreventive potential of food-derived and synthetic single compounds in this animal model. In order to investigate the correlation between iNOS and tumor development, we conducted two independent studies as described in Chapters 2 and 3. The study reported in Chapter 2 was to characterize iNOS expression in esophageal tumor development and provide a mechanistic rational for the evaluation of an iNOS inhibitor in NMBA-induced rat esophageal tumorigenesis. Chapter 3 describes a chemopreventive bioassay to determine the ability of a selective iNOS inhibitor, PBIT, to inhibit NMBA-induced esophageal tumorigenesis in the rat.

As reported in Chapter 2, normal and N-Nitrosomethylbenzylamine (NMBA)-induced preneoplastic and papillomatous lesions of the rat esophagus were characterized for expression of iNOS. F344 rats were injected subcutaneously with NMBA (0.5 mg/kg body weight) three times per week for 5 weeks. At each of 3, 6, 9, 12, 15, 18, 21, 24, 30 and 36 weeks following initiation of NMBA treatment, esophagi were collected from 12 untreated and 12 NMBA treated animals. The expression of iNOS mRNA in preneoplastic tissues and papillomas was markedly elevated when compared to normal tissues. Immunohistochemical analysis showed more intense cytoplasmic staining of
iNOS protein in preneoplastic tissues and in papillomas when compared to normal tissues. Our data suggest that iNOS expression is strongly associated with tumor development in rat esophagus after NMBA treatment. It is also very important that our study demonstrates, for the first time, that upregulation of iNOS expression is an early event in esophageal tumor development. This information would appear helpful in identifying the chemopreventive time window in which an iNOS inhibitor would be effective. In addition, a selective iNOS inhibitor may have chemopreventive potential against esophageal cancer.

To investigate the potential inhibitory effects of an iNOS inhibitor, as described in Chapter 3, we evaluated the ability of PBIT, a selective iNOS inhibitor and of 5% freeze-dried black raspberries, a fruit which has been shown to inhibit the development of chemically induced cancer of the rodent esophagus, colon, and oral cavity. Rats were given s.c. injections of NMBA (0.25mg/kg b. w.) three times per week for 5 weeks. One week later, the rats were fed a synthetic diet containing either 50 or 100 ppm PBIT or 5% black raspberries until the end of the bioassay. At week 25, PBIT reduced the incidence of esophageal tumor from 96% in NMBA-treated rats to 83% and 77% (p<0.05) in rats treated with 50 and 100 ppm PBIT, respectively. Five percent black raspberries reduced the tumor incidence to 90%. Tumor multiplicity was reduced significantly by all treatments when compared to the group treated with NMBA only. The total concentration of nitrate and nitrite, as an index of NO production, was also determined. PBIT (50 ppm and 100 ppm) and 5% freeze-dried black raspberries decreased the concentration of total nitrate and nitrite in preneoplastic in papillomatous lesions relative to rats treated with NMBA only. PBIT is a L-arginine analog that inhibits L-arginine binding to iNOS, thus,
decreasing iNOS substrate and NO production. Black raspberries would appear to exhibit similar effects. Further studies are required to fully elucidate the mechanism(s) of inhibition of esophageal tumorigenesis by black raspberries.

In conclusion, our data suggest that iNOS plays an important role in NMBA-induced rat esophageal tumorigenesis. We have evaluated the overexpression of iNOS in early and late stages of tumor development and determined that PBIT, a selective iNOS inhibitor, is significantly effective in reducing tumor development in an animal model of esophageal cancer. The tumor inhibitory effect of freeze-dried black raspberries was also confirmed and one mechanism appears to be through inhibition of NO production.
LIST OF REFERENCES


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