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Chemoprevention of Colon Cancer by iNOS-Selective Inhibitors

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Abstract

Nitric oxide (NO) is a short-lived pleiotropic regulator and is required for numerous pathophysiological functions, including macrophage-mediated immunity and cancer. It is a highly reactive free radical produced from *L*-arginine by different isoforms of NO synthases (NOSs). Sustained induction of inducible NOS (iNOS) during chronic inflammatory conditions leads to the formation of reactive intermediates of NO, which are mutagenic and cause DNA damage or impairment of DNA repair, alter cell signaling, and promote proinflammatory and angiogenic properties of the cell, thus contributing to carcinogenesis. Besides its well-established role in inflammation, increased expression of iNOS has been observed in colorectal tumors and other cancers. NO-related signaling pathways involved in colon tumorigenesis seem to progress through stimulation of proinflammatory cytokines and via posttranslational protein modifications of important antiapoptotic molecules in the tumors. NO can stimulate and enhance tumor cell proliferation by promoting invasive, angiogenic, and migratory activities. In contrast, studies also suggest that high levels of NO may be protective against tumor growth by inducing tumor cell death. However, a number of in vitro studies and particularly experimental animal data support the notion that NO and its reactive metabolite peroxynitrite stimulate cyclooxygenase-2 activity, leading to generation of prostaglandins that enhance tumor growth. These prostaglandins further augment tumor promotion and invasive properties of tumor cells. Hence, selective inhibitors of iNOS and combination strategies to inhibit both iNOS and cyclooxygenase-2 may have a preventive role in colon cancer.

Keywords

nitric oxide; angiogenesis; chemoprevention; iNOS-selective inhibitors; colorectal cancer

I. ROLE OF INOS IN COLON CANCER

Inducible nitric oxide synthase (iNOS) is an enzyme dominantly expressed during inflammatory reactions. Synthesis of high amounts of nitric oxide (NO) by iNOS has been demonstrated in pathophysiological processes such as acute or chronic inflammation and tumorigenesis in both rodents and humans. The role of iNOS activity in these diseases is still not clear. Gochman et al¹ recently reported elevated levels of iNOS protein and nitro tyrosine expression in human colitis and colon cancer tissues compared with normal tissue. Current interpretations of the data suggest a dose-dependent relationship between NO expression and tumor response. NO is generally synthesized (picomolar to nanomolar range) by 3 main forms of NOS enzymes, of which iNOS is calcium-independent and expressed

upon induction.²⁻⁵ iNOS, once induced, is capable of generating a high output of NO compared with other NOS enzymes (100- to 1000-fold more). Specific targeting of tumor NOS enzymes using NOS inhibitors consistently has resulted in retarded tumor growth. iNOS expression was reported in a human colorectal adenocarcinoma cell line.⁶ The common observation of high expression of endogenous NO in tumors forms the basis for many studies supporting the hypothesis that NO has a protumorigenic function. Cells from both the primary tumor SW-480 and from lymph node SW-620 metastases were shown to express calcium-independent NOS activity. The protumorigenic and metastatic roles of NO were shown by Radomski et al⁶ who reported that addition of the iNOS inhibitor *N*-monomethyl-*L*-arginine increased the metastatic potential of SW-480 cells to the same level as that of SW-620 cells. Since then, a large amount of data has shown that NO has physiological and pathological functions in tumor biology (Table 1).

Increased activity of iNOS in the gut is considered proinflammatory; it is associated with mucosal lesions, ulcerations, intraluminal bleeding, and bowel dilatation and dysfunction.^{27,28} Increased expression of iNOS protein and messenger RNA was found in acute colitis induced by 2,4,6-trinitrobenzene sulfonic acid along with neutrophil infiltration, inflammatory edema, and tissue damage.²⁹ Furthermore, use of the selective iNOS inhibitor *N*-[3 amino methyl-benzyl] acetamide (1400W) in experimentally induced acute colitis in the rat reduced formation of edema, neutrophil infiltration, and macroscopic inflammatory damage.³⁰ A finding by Roberts et al³¹ revealed that iNOS is induced by luminal factors such as bacterial lipopolysaccharide and is expressed at the surface epithelium in noninflamed tissue. This suggests that iNOS expression in the human colon is dependent upon inflammatory conditions such as those occurring in ulcerative colitis. In addition, our study and several others have shown increased expression and activity of iNOS in human colon adenomas.³¹⁻³³ Studies of experimental models of colon cancer indicate that azoxymethane (AOM)-induced colon tumors have higher expression and activity of iNOS or both compared with levels found in adjacent, seemingly normal colonic tissue.^{7,34} We reported that use of an iNOS-selective inhibitor suppressed AOM-induced colon adenocarcinomas in rats.³⁴

The roles of NO in pathogenesis have been implicated in tumor initiation, promotion, and progression. NO can cause oxidative damage to DNA, inhibition of DNA repair enzymes,³⁵ posttranslational modifications such as nitrosylation, or all three, potentially leading to tumor initiation and promotion/progression³⁶⁻³⁸ (Fig. 1). Sustained low levels of NO under chronic conditions lead to an accumulation of gene mutations, including those of the tumor suppressor gene p53,^{31,39-41} which contribute to malignant transformation (Fig. 1). Furthermore, modulation of the expression of oncogenes and the activity and inhibition of apoptosis by transcription factors lead to metastasis of tumor cells through capillary leakage, angiogenesis, and leukocyte adhesion and infiltration^{33,37,42} (Fig. 1). Overall, these reports suggest protumorigenic roles of NO in colon cancer.

II. REGULATION OF INOS BY INFLAMMATORY SIGNALING PATHWAYS

The functions of NO in tumor biology are dependent on its concentrations and on spatial and temporal constraints. A threshold concentration of NO is needed for it to activate key proteins involved in various signaling pathways. Several sources of NO exist in the tumor immune system. Macrophages are one of the predominant sources of iNOS⁴³ and play a significant role during bacterial infections (in the development of innate immunity), in the fight against tumors, and in coordinating healing of tissue damage and remodeling. NO is generated in different levels by macrophages, depending on the functional requirements. Thus, macrophages respond differently during the resolution of inflammation and at inflammatory sites.⁴⁴ They produce varied amounts of NO, depending on the type of

cytokine/interferon exposure; the *iNOS* gene is under transcriptional control by inflammatory cytokines (interleukin-1 β , interferon- γ , interleukin-6, and tumor necrosis factor- α) and lipopolysaccharides.⁴⁵ Several transcriptional factors are reported to regulate expression of the *iNOS* gene, including activating protein 1, nuclear factor- κ B, janus kinase signal transducer, and the STAT and janus kinase-activating protein 1 pathways.⁴⁶ Lower levels of NO (<50 nM) are associated with increased extracellular signal-regulated kinases phosphorylation; intermediate levels (>100 nM) are associated with stabilization of hypoxia-inducible factor 1 α ; and high levels (>300 nM) are associated with activation of the p53 pathway⁴⁷ (Fig. 1). Steady state or low levels of NO favor antiapoptotic and proliferative activity of cells, which leads to protumorigenic conditions⁴⁸ (Fig. 1). Sustained low levels of NO lead to the formation of reactive nitrogen species (RNS), and these are the conditions that play a vital role in tumorigenesis. At high levels (>400 nM), NO leads to apoptotic and antitumorigenic conditions⁴⁷⁻⁴⁹ (Fig. 1).

The consequences of the formation of RNS include protein modification, DNA base deamination, and the formation of *N*-nitrosamines, which are among the most potent mutagenic and carcinogenic compounds in humans. Nitrosylating species can convert cytosine, adenine, or guanine to uracil, hypoxanthine, and xanthine, respectively.⁵⁰ These modifications can lead to the formation of base pair mutations and transverse mutations. Furthermore, formation of hypoxanthine can cause DNA strand breaks.⁵⁰ Another major mechanism of toxicity induced by RNS involves nitrosylation of secondary aliphatic and aromatic amines and leads to the formation of carcinogenic *N*-nitrosamines.⁵¹ In addition to DNA and proteins, nitrite can cause structural modifications of a variety of endogenous and exogenous organic compounds, ranging from polyunsaturated fatty acids to estrogens, tocopherol, catecholamines, furans, retinoids, dietary phenols, and a range of xenobiotics. The toxicological sequences are increased further by NO-mediated deamination of purine and pyrimidine nucleotides, leading to mutagenesis and carcinogenesis.

III. ROLE OF INOS IN IMMUNE FUNCTIONS

NO is a multifunctional molecule with important roles during infections. The signaling processes through which NO acts to regulate immune cells are extremely complex. In addition to macrophages, a large number of other immune system cells (dendritic cells, natural killer cells, mast cells, and phagocytic cells, including monocytes, microglia, Kupffer cells, eosinophils, and neutrophils) as well as other cells involved in immune reactions (such as endothelial cells, epithelial cells, vascular smooth muscle cells, fibroblasts, keratinocytes, chondrocytes, hepatocytes, mesangial cells, and Schwann cells) produce and respond to NO. NO can exert its effects on immune responses either directly or through its derivatives, which modify the structure of regulatory molecules and influence their functions. There is increasing evidence that NO is one of the most versatile mediators in the control of viral infections as well as in the pathogenesis of many human infectious and inflammatory diseases. In the immune system, the use of NO donors and NOS inhibitors and the analysis of NOS^{-/-} mice have provided evidence that NO governs a broad spectrum of processes.⁵²⁻⁵⁴ During inflammatory responses, leukocyte recruitment and adhesion are regulated by iNOS.^{55,56} In several disease models, the antimicrobial and host-protective functions of iNOS/NO are restricted to certain organs and stages of the infection. iNOS is more critical during the late stage of infections rather than the early phase.^{57,58} In some infections, the expression of iNOS clearly is associated with a more severe or even fatal disease outcome. Possible underlying mechanisms include NO-mediated cytotoxicity and tissue damage; inhibition of T-cell proliferation, induction of T-cell apoptosis, or both; generation of viral escape mutants; and direct positive effects on viral or microbial growth.⁵⁹⁻⁶² Infections not only induce iNOS but also cyclooxygenase (COX)-2 and various cytokines. Available data suggest a link between iNOS and COX-2 in inflammation.

III.A. The Interrelationship Between iNOS and COX-2 Pathways in Cancer

The role of COX-2 in colon carcinogenesis is well established.⁶³⁻⁶⁵ Tumor inflammatory processes enhance both iNOS and COX-2 at the site. The COX-2 metabolite PGE₂ enhances tumor cell proliferation and formation of high levels of vascular endothelial growth factor, resulting in angiogenesis.^{31,66} These data provide the basis for development of COX-2 inhibitors. Several in vitro and in vivo reports suggested an interplay between COX-2 and iNOS expression and activities.⁶⁷⁻⁷³ NO has been reported to stimulate COX activity in selected cell types by a heme-independent mechanism.⁷⁴⁻⁷⁶ Evidence suggests that NO produced by iNOS enhances the activity of COX-2.⁷⁷ This is revealed further in an in vivo iNOS-deficient mouse model that shows significant reduction in PGE₂ levels correlated with reduced levels of nitrate and nitrite in urinary and peritoneal macrophages.⁷⁸ We have shown increased expression of iNOS and COX-2 in AOM-induced colonic tumors, compared to colonic mucosa of F344 rats.^{8,79,80} Both NO and COX-2 have carcinogenic effects that are achieved either directly or by producing mediators that regulate cellular growth.^{81,82} The mechanism involved in this interrelationship between iNOS and COX-2 is yet to be resolved. A few studies suggest that the catalysis of NO/peroxynitrate requires COX-related arachidonic acid oxygenation.^{71,73,83} Regulation of the COX pathway by iNOS is very complex and requires a transcriptional pathway mediated by β -catenin/T-cell factor/lymphocyte enhancer factor.⁸⁴ These reports support the development of COX-2 and iNOS inhibitors in combination for the prevention of colon carcinogenesis.

III.B. Chemoprevention by iNOS Inhibitors

Numerous reports suggest a contributing role of NO in cancers. It has been assumed that the continued production of NO through iNOS leads to neoplastic transformation, which is a vital step in carcinogenesis. Mice with mutations in both adenomatous polyposis coli (Apc) and iNOS showed fewer adenomatous polyps in the small and large intestines compared with mice with the mutation in *Apc* alone.⁸⁵ Also, iNOS^{-/-} mice showed a decreased incidence of gastric carcinogenesis induced by helicobacter.⁸⁶ These studies suggest the use of iNOS as a valid target in colon carcinogenesis. Hence, several iNOS inhibitors have been tested for the prevention of cancers. We tested *S,S'*-1,4-phenylene-*bis*(1,2-ethanediyl)-*bis*-isothiourea (PBIT) and *N*6-iminoethyl-lysine tetrazoleamide (NILT) and found them to be effective inhibitors in vivo for the development of colonic aberrant crypt foci in colon cancer.^{8,87} (Table 2). Dietary administration of *L*-NG-Nitroarginine Methyl Ester (*L*-NAME) for 11 weeks inhibited the development of aberrant crypt foci in rats.⁹² An interesting study was reported by Jenkins et al⁹³ in nude mice injected with human colon DLD-1 tumor cells overexpressing iNOS. These tumors grew faster than those injected with wild-type cells, and there was more vascularization. In another study, administration of 1400W [*N*-3-((amino methyl) benzyl)acetamide], an iNOS-selective inhibitor, to nude mice suppressed tumor growth.⁹¹ Curcumin was reported to inhibit iNOS gene expression in mouse liver.⁹⁴ We have shown that administration of curcumin inhibits the formation of chemically induced colon adenocarcinoma, at least in part, by suppressing iNOS expression and activities.⁷⁹ Also, trolox, ebselen, and Mn(III)tetrakis (4-benzoic acid) porphyrin chloride, a superoxide dismutase mimetic, have been used successfully to scavenge peroxynitrite in a murine model.⁹⁵⁻⁹⁷ Progressive development of increasingly severe inflammation, hyperplasia, dysplasia, and cancer associated with increased expression of iNOS in infected animals was prevented by the iNOS inhibitor *N*-methyl-arginine administered in drinking water.⁹⁸ These studies support the role of iNOS in colon cancer and suggest that administration of iNOS inhibitors is beneficial in the chemoprevention of colon cancer.

III.C. Combination Strategies to Inhibit Both iNOS and COX-2

Preclinical evaluations of iNOS inhibitors for chemopreventive effects have been demonstrated in both chemically induced and genetic models of colon cancer. Furthermore,

we observed a synergistic effect of an iNOS-selective inhibitor with a COX-2 selective inhibitor with regard to modulating AOM-induced colon carcinogenesis and the downregulation of COX activities in F344 rats.^{8,9} We have shown chemopreventive effects of PBIT in combination with the selective COX-2 inhibitor celecoxib in an intestinal tumor model of *Min* mice.⁹ We also have shown the inhibitory effects of the iNOS-selective inhibitors PBIT and NILT in combination with celecoxib in AOM-induced colonic carcinogenesis (using tumors as the end point) in male F344 rats^{89,90} (Table 2). In one of these studies, 4 weeks after AOM treatment groups of rats were fed experimental diets containing 0, 50, or 100 ppm PBIT; 250 or 500 ppm celecoxib; or the combination of 50 ppm PBIT plus 250 ppm celecoxib. After 48 weeks, rats were killed to evaluate the chemopreventive efficacy of PBIT and its low-dose combination with celecoxib. A dose-dependent decrease of total colonic adenocarcinomas and particularly greater suppression of invasive colon cancers were observed with dietary PBIT. Significant inhibition of the incidences of adenocarcinomas was observed with a low dose (34.6%; $P < 0.01$) and a high dose of PBIT (43%; $P < 0.002$). Celecoxib at both 250- and 500-ppm dose levels significantly suppressed the incidence of colon adenocarcinoma (55–57%; $P < 0.002$ –0.0001). It is important to note that low-dose combinations of 50 ppm of PBIT and 250 ppm of celecoxib significantly suppressed the incidence of colon adenocarcinoma by 70% and its multiplicity by 83% ($P < 0.0001$)⁸⁹ (Table 2). In the other preclinical study, all rats received AOM subcutaneously at a dose of 15 mg/kg body weight once weekly for 2 weeks, starting at 7 weeks of age. Four weeks after the AOM treatment, groups of rats were fed experimental diets containing either 0, 100, or 200 ppm of NILT; 250 and 500 ppm of celecoxib; or a combination of 100 ppm NILT and 250 ppm celecoxib. Rats were killed 48 weeks after AOM treatment and the colonic tumors were evaluated for the chemopreventive efficacy of each agent and combination. Both NILT and celecoxib significantly suppressed over all colon adenocarcinoma incidence and multiplicity. It is important to note that 100 ppm and 200 ppm of the iNOS inhibitor suppressed invasive adenocarcinomas multiplicities by 52% and 67%, respectively ($P < 0.007$). The low-dose combination of 100 ppm NILT plus 250 ppm celecoxib suppressed colon adenocarcinoma incidence by 70% and multiplicity by 77%. These results suggest that iNOS inhibitors particularly inhibit invasive cancers⁹⁰ (Table 2).

IV. CONCLUSIONS

In recent years, NO has been found to play a much more diverse role in infection-related immunity and vascular biology as well as a more important role in tumor biology than initially was thought. The concept that NO is playing both positive and negative roles in tumor biology is still undergoing extensive scrutiny. However, available literature better supports its positive role in colon carcinogenesis. Levels of NO and the site of its production by iNOS contribute to tissue damage and inflammation and are evidently important in colon cancer. It is now clear that iNOS is detrimental in inflammatory disease processes and that it helps to counteract immune reactions toward tumor cells, protects tumor cells against host immunity, and functions as an intra- and intercellular signaling molecule, shaping immune responses protective to the tumor. In addition, iNOS in high concentrations is known to participate in important immunological processes such as apoptosis, cell adhesion, autoimmunity, and perhaps antimicrobial defense. The demonstration of iNOS expression by macrophages and other cell types in tissues derived from patients with a wide variety of infectious, autoimmune, and degenerative diseases and colon tumors supports the important and pleiotropic functions of iNOS in human systems. Its interrelation with COX-2 is well defined so that the possible benefit of inhibiting both iNOS and COX-2 to inhibit colon cancer is well supported. Hence, the combination of iNOS inhibitors and COX-2 inhibitors is one valid approach toward the chemoprevention of colon carcinogenesis. However,

because the regulation, expression, and function of the iNOS isoforms are so complex, one needs to be cautious in developing NO-based chemopreventive agents and therapeutics.

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ABBREVIATIONS

AOM	azoxymethane
COX	cyclooxygenase
iNOS	inducible nitric oxide synthase
NILT	N ⁶ -iminoethyl-lysine tetrazoleamide
NO	nitric oxide
PBIT	<i>S,S'</i> -1,4-phenylene- <i>bis</i> (1,2-ethanediy1) <i>bis</i> -isothiurea
RNS	reactive nitrogen species

FIGURE 1.

Activities of nitric oxide (NO) initiating various cellular pathways at different levels of concentrations. COX, cyclooxygenase; IDO, indoleamine 2,3-dioxygenase; LOX, lipoxygenase; MMP, matrix metalloproteinase; ODC, ornithine decarboxylase.

TABLE 1

iNOS Expression in Preneoplastic Lesions and Adenocarcinomas of Various Cancers

Organ	Lesions	Expression of iNOS	References
	ACFs	+	
Colon	Adenoma	++	7-9
	Adenocarcinoma	++++	
Breast	Invasive lesions	+	10, 11
	Tumors	+++	
Prostate	High-grade PIN	++	12-16
	Cancer	+++	
Bladder	Dysplastic lesions	+	17-20
	Carcinoma	+++	
	Preneoplastic	?	
Skin	Papillomatous Lesions	+	21
		+++	
Esophageal	Barrett's	+	22
	Adenocarcinoma	+++	
Melanoma	Malignant	+++	23-25
Head and neck	Leukoplakia	+	26
	Carcinoma	+++	

ACF, aberrant crypt foci; iNOS, inducible nitric oxide synthase; PIN, prostatic intraepithelial neoplasia

TABLE 2

Effect of Various iNOS Inhibitors on ACFs and Colon Tumor Growth

Colon Cancer	iNOS Inhibitor	Inhibition (%)	References
Rat AOM-induced ACF	PBIT	78 (>4 crypts foci)	60
	PBIT-Se		88
	AG1 (high dose)		69
	SC51 (high dose)		69
	SC51+celecoxib (low-dose combination)		69
Rat AOM-induced adenocarcinoma	PBIT	Incidence: 43	89
		Multiplicity: 60	
		Incidence: 70	89
	PBIT+celecoxib	Multiplicity: 83	
		Incidence: 40	89
		Multiplicity: 46	
	NILT adenocarcinoma	Incidence: 40	90
		Multiplicity: 60	90
Mouse (Apc ^{Min/+} -iNOS ^{-/-})	AG	Invasive adenocarcinoma	Multiplicity: 67
		Multiplicity: 77	90
		Incidence: 70	90
	PBIT	NILT+celecoxib adenocarcinomas	Multiplicity: 7
		Incidence: 29	85
Mouse (Apc ^{Min/+}) high-fat diet		Multiplicity: 7	9
Mouse (xenograft with human colon tumor expressing iNOS)	1400W	>80	
Mouse (intratumoral macrophages expressing iNOS)	1400W	Promoting effect	91
		No effect	91