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Review

Reactive Nitrogen Species in Colon Carcinogenesis

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ABSTRACT

The role of reactive nitrogen species (RNS) in colon carcinogenesis is multifactorial and affects diverse processes, such as proliferation, apoptosis, differentiation, tumorigenesis, and metastases. This review describes the stages in colon carcinogenesis where nitric oxide (NO) and inducible NO synthase (NOS2) may influence the progression of a normal mucosa to overt metastatic cancer. Overexpression of NOS2 and an increase in the generation of NO and other RNS may lead to apoptosis resistance, DNA damage, mutation, up-regulation of COX-2, increased proliferation, an increase in oxidative stress and an increase in tumor vascularity and metastatic potential. Therefore, future goals are to establish mechanistically based biomarkers to assess individuals at risk for colon cancer and to implement chemopreventive and dietary strategies that reduce colon cancer risk. An understanding of NO signaling pathways in colon epithelial cells should provide the basis for novel biomarker development. Colon cancer prevention may be achieved effectively by chemically interfering with key components of the NO signaling pathways, changing dietary habits to reduce fat and increase antioxidant-containing vegetables, and dietary supplementation to increase DNA repair. Antiox. Redox Signal. 1, 449–467.

INTRODUCTION

NITRIC OXIDE (NO) is a noxious gas and, as a component of the exhaust from motorcars, is a cause of acid rain. Thus, it is surprising that normal cells produce such a molecule. However, this molecule functions in natural biologic processes such as vasodilation and neurotransmission, and is produced in a wide range of organisms (*e.g.*, slime molds, locusts, beetles, horseshoe crabs, mollusks, chickens, mice, rats, cows, and humans) (Nathan and Xie, 1994). Green *et al.* (1981) showed that nitrate, a by-product of NO metabolism, is synthesized by human cells mainly outside of the intestine and, therefore, was not of bacterial origin. Stuehr and Marletta (1985) first demonstrated NO synthesis by a mammalian cell. Palmer *et al.* (1987) showed that the release of NO accounted for the biologic activity of endothelium-dependent relaxation. Palmer *et al.* (1987) then showed that L-arginine is the physiological precursor for the formation of NO in endothelium-dependent relaxation. The enzyme that synthesizes NO from L-arginine is NO synthase (NOS). Three isoforms of NOS were found and named NOS1, NOS2, and NOS3, in their order of discovery (Forstermann *et al.*, 1995). NOS1 is a constitutive form of NOS first purified from cerebellum, and also referred to as nNOS (neuronal NOS). NOS2 is an inducible form of NOS first isolated from murine macrophages, and also referred to as iNOS. NOS3 is a constitutive form of NOS first iden-

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tified in endothelial cells, and also referred to as eNOS. It is now known that the "constitutive" isoforms, NOS1 and NOS3, are also subject to some control at the transcriptional and post-transcriptional levels (Forstermann *et al.*, 1998). Because NO activates guanylate cyclase, resulting in an increase in cGMP, it is an important mediator of signal-transduction pathways.

The enzymatic activity of NOS2 results in the formation of NO in micromolar concentrations. This accounts for some of the cytotoxic effects of macrophages on phagocytized microbes. In nonphagocytic cells, intracellular NO can react with superoxide anions, resulting in the accumulation of the destructive molecule, $ONOO^-$ (peroxynitrite). Reactive nitrogen species (RNS) such as $ONOO^-$ can have deleterious effects on mitochondria (Richter, 1998) and damage macromolecules, such as DNA (Wiseman and Halliwell, 1996), proteins, and lipids (Beckman and Koppenol, 1996). Because RNS influence signal transduction and can damage DNA, it is probable that they contribute to carcinogenesis (Ohshima and Bartsch, 1994, Jenkins *et al.*, 1995; Tamir and Tannenbaum, 1996; Wink and Mitchell, 1998).

RNS appear to have a role in colon carcinogenesis, and their production is stimulated by dietary factors (*e.g.*, bile acids) involved in tumor promotion. Recent work from our laboratory indicates that the bile salt, sodium deoxycholate (NaDOC), induces apoptosis in colonic epithelial cells (Payne *et al.*, 1995b), and that epithelial cells within the flat mucosa of patients with colon cancer exhibit resistance to NaDOC-induced apoptosis (Bernstein *et al.*, 1999a; Garewal *et al.*, 1996; Payne *et al.*, 1995a). Apoptosis resistance is now believed to be a predisposing factor in tumorigenesis, and creates a permissive environment for genomic instability and accumulation of gene mutations (Reed, 1999). We showed that NO protects colonic epithelial cells from NaDOC-induced apoptosis (Washo-Stultz *et al.*, 1999). The role of NO and its congeners in carcinogenesis is most likely multifactorial. The purpose of this overview is to describe some of the likely molecular mechanisms by which RNS contribute to colon carcinogenesis. We will focus on the role of RNS in the processes of proliferation, apoptosis, differentiation, tumorigenesis, and metastasis, summarize the effects of dietary factors related to the production of RNS, propose hypothesis-driven biomarker development to assess cancer risk, and indicate chemopreventive and dietary strategies likely to lower colon cancer risk.

DNA DAMAGE AND REPAIR

NO can interact with oxygen and superoxide to form RNS that directly or indirectly damage DNA and cause mutations, some of which might lead to cancer (Felley-Bosco, 1998; Wink and Mitchell, 1998). Direct DNA damage caused by RNS includes base deamination, adduct formation, and single-strand breaks. Indirect DNA damage occurs by the interaction of RNS with other molecules such as amines, thiols and lipids.

DNA base deamination reactions are a consequence of the reaction of NO with O_2 to form N_2O_3. N_2O_3 reacts with the primary amino group of different DNA bases leading to deamination of the bases. These reactions include the conversion of cytosine to uracil, guanine to xanthine and adenine to hypoxanthine (Felley-Bosco, 1998; Wink and Mitchell, 1998). The altered bases, if unrepaired, can give rise to mutation upon DNA replication. NO can react with superoxide anion (O_2^-) to generate $ONOO^-$ This reactive molecule produces altered DNA bases, such as 8-hydroxyguanine (Epe *et al.*, 1996; Kennedy, *et al.*, 1997) and 8-nitroguanine (Yermilov *et al.*, 1995), which can cause mutations if unrepaired. $ONOO^-$ can also cause DNA single-strand breaks (Szabo *et al.*, 1996). It appears that a single species generated by $ONOO^-$ that has a reactivity comparable to the hydroxyl radical, but neither peroxynitrite itself nor the hydroxyl radical, is responsible for the DNA damage observed, predominantly 8-hydroxyguanine and single-strand breaks (Epe *et al.*, 1996). DNA modifications may also be produced indirectly by RNS through the generation of *N*-nitrosoamines and DNA-reactive lipid peroxidation intermediates (Felley-Bosco, 1998). *N*-Nitrosoamines and products of lipid peroxidation, such as malondialdehyde, are mutagenic. The efficiency of each of these damaging pathways may depend on the antioxidant status of

the cell or the presence of free metals. Although RNS cause DNA damage at high concentrations, it is not clear whether the concentrations generally present in a cell are significantly harmful, and there is evidence that at low concentrations RNS protect against oxidative stress (Wink and Mitchell, 1998).

RNS can also increase DNA damage by inhibiting DNA repair enzymes. RNS have a particularly high affinity for amino acids containing thiol residues, suggesting that enzymes having thiol residues critical to their function may be inhibited. The DNA repair enzymes, O^6-methylguanine-DNA-methyltransferase and formamidopyrimidine-DNA glycolyase contain thiol groups necessary for their function, and both are inhibited by RNS (Wink and Mitchell, 1998). On the other hand, the stress response protein metallothionein, which is rich in thiol residues, was found to protect against the cytotoxic and DNA-damaging effects of RNS (Schwarz *et al.*, 1995).

DNA ligase, an enzyme that seals single-strand nicks in DNA is also inhibited by RNS, possibly by deamination of a lysine at the active site of the enzyme (Wink and Mitchell, 1998). The increase in single-strand DNA breaks observed upon exposure to RNS may be due, at least in part, to failure of ligase to seal nicks formed naturally during replication and repair. The increase in single-strand breaks upon $ONOO^-$ exposure activates poly(ADP-ribose) polymerase (PARP), an enzyme that adds polymers of ADP-ribose to proteins in the region of single-strand breaks as part of the repair process for such damages (Szabo *et al.*, 1996). Since nicotinamide adenine dinucleotide (NAD^+) is the substrate for this enzyme and is the source of ADP ribose units of the ADP-ribose polymers, NAD^+ is depleted by exposure to $ONOO^-$ (Szabo *et al.*, 1996). NAD^+ depletion results in a decline of ATP production and reduced availability of energy for metabolism.

EFFECTS OF RNS ON ENZYME ACTIVITY AND TRANSCRIPTION FACTOR ACTIVATION

Covalent interactions of small diffusible ligands, such as NO and $ONOO^-$, with the centers of iron-sulfur and zinc-sulfur clusters, heme proteins, thiol groups, and tyrosine residues, can lead to either a decline or an increase in protein activities, depending on the enzyme and the particular RNS involved (Stamler, 1994; Billiar, 1995; Crow *et al.*, 1997; Ischiropoulos, 1998a; Ji *et al.*, 1999). In addition to the inhibitory effects of NO and $ONOO^-$ on the classic DNA repair enzymes described above, RNS can also inhibit complexes I and II of the mitochondrial electron transport chain, aconitase (tricarboxylic acid cycle enzyme), glyceraldeyde 3-phosphate dehydrogenase [major NADH (reduced form of NAD^+) producing enzyme and DNA repair enzyme], and caspases (Kim *et al.*, 1997a; Dimmeler *et al.*, 1997). Although aconitase was found to be inhibited by $ONOO^-$ and superoxide anion, it was not inhibited by NO, indicating a specificity of particular RNS for the inhibition of certain enzymes. On the other hand, NO can activate certain enzymes, including the prototypical enzyme, guanylate cyclase, inducible cyclooxygenase-2 (COX-2) (Salvemini *et al.*, 1993; Corbett *et al.*, 1993), redox-sensitive kinases (Lander *et al.*, 1996) and protein phosphatases, and $p21^{ras}$ (Lander *et al.*, 1993, 1995a,b, 1997; Mirza *et al.*, 1995). NO activates the redox-sensitive transcription factors, AP-1 and NF-κB (Stamler, 1994). The activation of AP-1 by NO is most probably mediated through the interaction of NO with cysteines critical to DNA binding. The activation and nuclear translocation of NF-κB by NO is probably mediated through the dissociation of the inhibitory proteins of the I-κB protein family from NF-κB dimers, which is a sulfhydryl-sensitive event (Schreck *et al.*, 1991). The activation of $p21^{ras}$ by NO has been shown to be an important signal transduction pathway, resulting in the downstream activation of NF-κB (Lander *et al.*, 1995b). The NO-$p21^{ras}$ interaction may also be involved in other signal transduction pathways that involve oxidative signaling through ras (Lander *et al.*, 1995a; Irani *et al.*, 1998; Deora *et al.*, 1998). In some instances, however, NO has been shown to inhibit the activation of NF-κB, most probably through its activation of protein phosphatases (Peng *et al.*, 1995). The dephosphorylation of I-κB will prevent its proteasomal degradation, resulting in

the maintenance of the masking of the nuclear localization signal sequence on the NF-κB subunits, and thus preventing NF-κB translocation to the nucleus. The positive and negative effect on the activity of different proteins can explain the modulation by RNS of diverse processes, such as proliferation, differentiation, apoptosis, tumorigenesis and metastases. These modulatory effects are discussed below.

APOPTOSIS

Apoptosis, a controlled form of cell death (Kerr *et al.*, 1972), and proliferation are two factors that determine normal tissue kinetics and tumor growth. Defects in apoptosis are associated with carcinogenesis (Butler *et al.*, 1999; Reed, 1999), including that of the colon (Bernstein *et al.*, 1999a; Bedi *et al.*, 1995; Payne *et al.*, 1995a; Garewal *et al.*, 1996). A defective ability to undergo apoptosis (apoptosis resistance) can allow cells with DNA damage to replicate, thus increasing the probability of carcinogenic mutation. Therefore, an understanding of the mechanisms of apoptosis resistance is critical to chemoprevention or preventative dietary strategies. The generation of extracellular NO and $ONOO^-$ have been reported to induce apoptosis in numerous cell types (Ho *et al.*, 1996; Brune *et al.*, 1998b; Ischiropoulos, 1998b). The induction of apoptosis by RNS may be caused by DNA damage, inhibition of respiration, or alterations in signal-transduction pathways (Brune *et al.*, 1998a). On the other hand, RNS can also prevent apoptosis (Lipton *et al.*, 1993; Park *et al.*, 1996; Wink *et al.*, 1996; Shen *et al.*, 1998). The mechanisms by which RNS inhibit apoptosis are, like the induction of apoptosis, probably multifactorial in nature (Payne *et al.*, 1995a; Dragovich *et al.*, 1998). There are multiple mechanisms by which NO could protect cells against apoptosis (Brune *et al.*, 1998b). These potential mechanisms include: (1) the activation of soluble guanylate cyclase resulting in an increase in cGMP levels (Bredt and Snyder, 1994), (2) an inhibition of caspases (Dimmeler *et al.*, 1997; Kim *et al.*, 1997a; Fiorucci *et al.*, 1999), the ICE (interleukin converting enzyme)-like proteases necessary for carrying out the execution phase of apoptosis, (3) the scavenging of O_2^- (Squadrito and Pryor, 1995), a potent ROS, (4) the activation of COX-2 (Salvemini *et al.*, 1993), an enzyme responsible for the generation of mitogenic eicosanoids and increased cell survival, (5) *S*-nitrosylation of key proteins on the apoptosis pathway, such as protein kinase C (Gopalakrishna *et al.*, 1993; Martinez *et al.*, 1998) and caspase-3 (Mannick *et al.*, 1999), (6) ras activation (Gomez *et al.*, 1997), and (7) possible interference with tyrosine phosphorylation through the formation of nitrotyrosine (Crow *et al.*, 1996). The increase in COX-2 activity has been shown to increase the level of bcl-2, a known anti-apoptotic protein (Reed, 1999), through the production of prostaglandin E2 (PGE2) (Sheng *et al.*, 1998). In addition to COX-2 (von Knethen and Brune, 1997b) and bcl-2 (Genaro *et al.*, 1995; Melkova *et al.*, 1997), other proteins that protect against NO-induced cell death include hsp-70 (Kim *et al.*, 1997b), heme oxygenase (Motterlini *et al.*, 1996; Bishop *et al.*, 1999; Foresti *et al.*, 1999), ferritin (Juckett *et al.*, 1996), metallothionein (Schwarz *et al.*, 1995), Cu,Zn-superoxide dismutase (Yabuki *et al.*, 1999), and catalase (Yabuki *et al.*, 1999).

Recent evidence from our laboratory indicates that NO protects HT-29 colonic epithelial cells from apoptosis induced by the bile salt, NaDOC (Washo-Stultz *et al.*, 1999). We have ruled out the scavenging of O_2^- anion by NO as one of the mechanisms by which NO contributes to apoptosis resistance in this *in vitro* cell culture system (Washo-Stultz *et al.*, 1999). Because NOS2 is known to be upregulated in adenomas (Ambs *et al.*, 1998a), it is possible that an increase in intracellular concentrations of NO may be one mechanism by which cells become apoptosis resistant. Cells that overexpress NO may be clonally selected for their survival advantage, leading to a field of cells with reduced ability to undergo apoptosis. A biochemical and molecular evaluation of altered NOS gene expression in cell lines selected for apoptosis resistance *in vitro*, and in the normal-appearing flat mucosa of patients exhibiting apoptosis resistance (Bernstein *et al.*, 1999a) should prove rewarding.

DIFFERENTIATION

NO triggers growth arrest and differentiation of diverse cell types (*e.g.*, U-937 monoblasts, freshly isolated acute nonlymphocytic

leukemic cells, HL-60 myeloid cells, brown adipocytes, PC12 neuronal cells, N103, SK-N-SH and SK-N-MC neuroblastoma cells, osteoblasts, and keratinocytes). The mechanism by which NO affects colonic cell differentiation is, however, not known. Blachier *et al.* (1995, 1996) showed that there is an intimate relationship between the polyamine and NO signaling pathways. Sodium nitroprusside, an NO donor, inhibits cellular putrescine synthesis (Blachier *et al.*, 1996) by inhibiting ornithine decarboxylase (ODC), an enzyme involved in cellular proliferation. It is not known if NO induces growth arrest of colonic cells, a process necessary for differentiation. Recent work from our laboratory indicates that NF-κB, a redox-sensitive transcription factor, and NOS2, a known target gene for NF-κB transcriptional regulation, are highly expressed in differentiated cells at the surface epithelium and upper portion of normal colonic crypts (unpublished data). We also found decreased expression of both NF-κB (p65 protein) and NOS2 at the surface epithelium of the normal-appearing flat mucosa of patients with colon cancer, and increased expression in the proliferative regions of the crypts (unpublished data), suggesting that abnormal expression of these proteins may be involved in the etiology of colon cancer. These findings of abnormality were seen at some distance from the neoplasms, and were associated with a decrease of staining with the lectin, *Dolichos biflorus* agglutinin (a marker of mucous cell differentiation) and apoptosis resistance. These aberrancies may represent "field defects" in signaling by NF-κB and NOS2 in the normal processes of proliferation, differentiation, and apoptosis. An understanding of the role of NO in colon carcinogenesis will benefit from *in vitro* differentiation models where levels of NO can be experimentally manipulated, and from genetically engineered NOS2-deficient mice.

BIOSYNTHETIC PATHWAYS ASSOCIATED WITH ARGININE METABOLISM AND RELATIONSHIP TO APOPTOSIS AND TUMORIGENESIS

NO is produced as a consequence of arginine metabolism, via NOS. NOS is expressed in the colonic epithelium in humans (Moochhala *et al.*, 1996). Although controversial, recent evidence indicates that the calcium-independent NOS2 is increased in both colorectal adenomas and carcinomas (Ambs *et al.*, 1998a). NOS2 is apparently upregulated in gastrointestinal neoplasia along with other enzymes associated with the inflammatory response, including the cyclooxygenases (Wilson *et al.*, 1998). Studies in experimental models of colon carcinogenesis and in humans indicate that COX-2 is also elevated in intestinal neoplasia (Smalley and DuBois, 1997). Pharmacologic and genetic manipulation of these enzyme activities suggests that both NOS2 and COX-2 facilitate the growth of neoplastic intestinal cells, and their inhibition suppresses intestinal tumorigenesis.

COX-2 and NOS2 exert stimulatory effects on each other in some tissues. Bile acids, which are associated with carcinogenesis in both the colon and the esophagus, induce COX-2 in human esophageal adenocarcinoma cells via a mechanism involving the AP-1 transcriptional activation complex (Zhang *et al.*, 1998). In human platelets, inhibition of COX-2 activity suppresses NOS expression (Chen *et al.*, 1997). In a macrophage-derived cell line, NOS-mediated NO production stimulates cyclooxygenase activities (Salvemini *et al.*, 1993). It is unknown whether these mechanisms are operational in gastrointestinal tissues. As discussed earlier, in the presence of molecular oxygen, NO is converted to $ONOO^-$ which can cause DNA damage or nitration of proteins at tyrosine residues. These reactions have been implicated in apoptosis and carcinogenesis.

Production of NO is only one consequence of arginine metabolism in the colon. Arginine is a key component of the urea cycle, and urea cycle enzymes are expressed in both the small intestine and colon of rodents and humans. Arginase, which converts arginine to ornithine, is found in human colon cancer-derived cells (Selamnia *et al.*, 1998). Ornithine transcarbamylase (OTC) activity, which mediates the synthesis of citrulline from ornithine, is reduced in both the intestines and liver of OTC-deficient mice (Qureshi *et al.*, 1985). A major role of arginase in the liver and kidney is the reduction of excess levels of protein-derived ammonia via the production of urea, which is excreted (Lardner and O'Donovan, 1998).

Arginase inhibits apoptosis in some model systems (Esch *et al.*, 1998).

Ornithine is the substrate for ODC, the first enzyme in polyamine synthesis in mammals. ODC activity is regulated at the transcriptional and post-transcriptional level by several oncogenes and tumor suppressor genes implicated in colon carcinogenesis, and inhibitors of ODC enzyme activity suppress colon carcinogenesis in rodent models (Meyskens and Gerner, 1999).

The relationship between NO production, the urea cycle and the polyamine pathway are shown in Fig. 1. It is noteworthy that the expression of gene products acting to decrease tissue arginine levels, including NOS2 and possibly COX-2 through NO production and ODC in polyamine synthesis, are increased in neoplastic colonic tissues. Independent investigations have shown that arginine-deficient diets increase hepatocarcinogenesis (Vasudevan *et al.*, 1994), while arginine supplementation decreases aberrant crypt foci (ACF) formation (Wargovich *et al.*, 1996) in the colon of carcinogen-treated rodents.

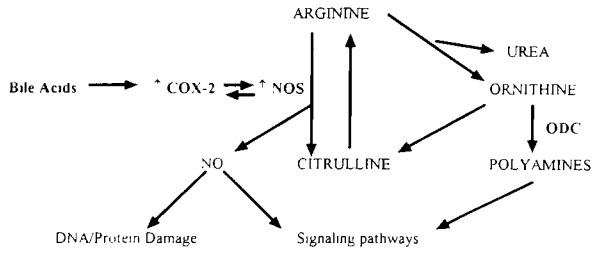

FIG. 1. Relationship between NO production, the urea cycle, and polyamines. See text for an explanation of the effect of bile acids on enzyme activity, cross-talk between NOS and COX-2, and the role of arginine in NO production, involvement in the urea cycle, and the generation of polyamines.

TUMORIGENESIS AND METASTASIS

The role of RNS in colon carcinogenesis is probably multifactorial. Unpublished work from our laboratory and reports of others (Ambs *et al.*, 1998a, 1999) have shown that NOS activity is increased in human colon adenomas, a precursor lesion to colon cancer (Bond, 1995; Boutron *et al.*, 1995). Since NO contributes to apoptosis resistance in colonic epithelial cells (Washo-Stultz *et al.*, 1999), cells that continuously produce excess NO may exhibit a survival phenotype that leads to clonal expansion and the development of adenomas. The work of Hunt *et al.* (1998) supports this apoptosis resistance hypothesis in response to oxidative stress. They demonstrated that a stable oxidative stress-resistant phenotype (involving antioxidant gene amplification) emerges after chronic exposure of cells to oxidative stress. Continuous oxidative stress to cells (*e.g.*, generation of micromolar quantities of NO by NOS2; Forstermann *et al.*, 1995) after cellular transformation could also contribute to tumor growth. This is supported by the work of Jenkins *et al.* (1995), who showed an increase in tumor growth rate when nude mice were injected with human colonic cells transfected with NOS2. This increase in tumor growth rate may also result from secreted NO (Nathan, 1992), which can signal an increase in angiogenesis (Ambs *et al.*, 1998a), a process known to contribute to tumor growth. Increased NO levels activate COX-2 activity, thereby increasing PGE2 levels, a basic regulator of angiogenesis (Chiarugi *et al.*, 1998). Analyses of colon microvasculature have revealed that adenomas have a lower capillary density than primary and metastatic tumors (Takahashi *et al.*, 1995), indicating a greater need for oxygen as tumors increase in size and/or metastasize to oxygen-poor microenvironments. The effect of NO on the growth of normal and tumor cells may, therefore, be very different. NO appears to have a growth inhibitory and differentiating effect on normal epithelial cells, but stimulates tumorigenesis and increases metastatic potential of tumor cells (Edwards *et al.*, 1996).

Some interesting relationships between p53 expression and NOS2 have recently emerged that may affect tumorigenesis. The normal p53 protein, in accordance with its role as a tumor suppressor gene, may serve to down-regulate NOS2 gene expression (Chiarugi *et al.*, 1998; Ambs *et al.*, 1998b). Mutation of P53, which appears as a late event in colon carcinogensis, increases NOS2 gene expression in some adenomas and adenocarcinomas over levels found in the flat colonic mucosa (Ambs *et al.*, 1999). An apparent feedback loop between wild-type p53 and NO (Forrester *et al.*, 1996) indicates that p53

down-regulates NOS2 activity, but increased NO also regulates p53 function. NO can, therefore promote colon carcinogenesis at an early stage prior to the appearance of mutant p53, possibly through a conformational change in wild-type p53 protein (Calmels *et al.*, 1997), resulting in functional impairment.

The involvement of NO in the early stages of colon carcinogenesis is strongly indicated by the recent findings of Rao *et al.* (1999). Using the azoxymethane (AOM)-rat model of colon carcinogenesis, Rao *et al.* (1999) found that both NOS2 and COX-2 activities were significantly induced by AOM, as was the formation of ACF. The use of a selective NOS2-specific inhibitor, *S,S'*-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea (PBIT), significantly suppressed crypt multiplicity in ACF and concomitantly suppressed AOM-induced NOS2 activity. It is probable that the up-regulation of NOS2 activity in the flat colonic mucosa can also activate $p21^{ras}$ (see previous section). This NO-$p21^{ras}$ interaction could mimic the upstream effects of ras mutations known to occur during the early to intermediate stages of colon carcinogenesis (Vogelstein *et al.*, 1988; Fearon and Vogelstein, 1990; Rusti and Podolsky, 1992; Jackson et al., 1996). In summary, there are multiple stages in colon carcinogenesis where NO can serve to promote colon carcinogenesis.

GENERATION OF REACTIVE NITROGEN/OXYGEN SPECIES FROM INFLAMMATORY CELLS AND COLON CANCER RISK

Inflammatory bowel disease, which includes ulcerative colitis and Crohn's disease, has long been recognized as a risk factor in the development of colon cancer (Jain and Peppercorn, 1997). The chronic inflammation is marked by mucosal infiltration with macrophages, lymphocytes and neutrophils, resulting in the production of hydrogen peroxide (H_2O_2), O_2^-, NO, and $ONOO^-$. Some of these oxidants are known to modulate the expression of a variety of different adhesion molecules, cytokines, and enzymes through the activation of the redox-sensitive transcription factors, NF-κB and AP-1 (Jourd'heuil *et al.*, 1997). NO has been shown to be elevated in the rectal dialysates of patients with ulcerative colitis (Roediger *et al.*, 1986). During inflammation, NOS2 and nitrotyrosine (a footprint for the formation of RNS, including $ONOO^-$; Halliwell, 1997) are localized to both the inflammatory cells found in the lamina propria and the colonic epithelial cells, indicating protein damage through nitration (Singer *et al.*, 1996). Dijkstra *et al.* (1998), using immunohistochemical co-localization techniques, showed that nitrotyrosine was not localized to the epithelial cells that exhibited high NOS2 expression, but instead was detected only on the inflammatory cells at some distance from the NOS2-producing epithelial cells. This finding indicates that protein nitration (a measure of RNS-induced protein alterations) was not present in the epithelial cells that exhibited high expression of NOS2. In fact, epithelial-generated NO is believed to have a protective function (Alican and Kubes, 1996; McCafferty *et al.*, 1997). In an acetic acid-induced colitis mouse model, which has an injury phase and a healing phase, McCafferty *et al.* (1997) showed a seven-fold increase in damage score in genetically engineered NOS2-deficient mice compared with wild-type mice. In this model, NO played a critical role in resolving inflammation. There are several mechanisms by which epithelial-generated NO can protect against inflammation. NO can down-regulate adhesion molecule expression and leukocyte-endothelial cell interactions. This down-regulation is probably the result of NO inhibition of NF-κB (Peng *et al.*, 1995), a transcription factor for a number of genes involved in the inflammatory process, including interleukin-6 (IL-6) and tumor necrosis factor (TNF). Epithelial NO also appears to protect against inflammation by forming an oxidative barrier to bacterial invasion at the site of mucosal injury (Alican and Kubes, 1996).

COX-2 has been shown to be upregulated in the nonneoplastic colonic epithelial cells of patients with inflammatory bowel disease (Singer *et al.*, 1998). This elevation in COX-2 enzyme activity can result in an increase of mitogenic eicosanoids that contribute to cellular proliferation. NO and $ONOO^-$ (Landino *et al.*, 1996) can activate and increase the expression of COX-2. Therefore, the long-term effect of ep-

ithelial NO production during chronic inflammation could be deleterious. Because we have shown that NO protects colonic epithelial cells against apoptosis (Washo-Stultz *et al.*, 1999), NO-overexpressing cells could undergo clonal selection because of their survival advantage (Lala and Orucevic, 1998), resulting in a population of cells that are apoptosis resistant, a risk factor in carcinogenesis (Reed, 1999). These findings would be consistent with the hypothesis of Israel (1996) that the cancer phenotype is a survival phenotype. Israel (1996) proposed that tumor progression results from an integrated survival response to cellular stress conserved from unicellular organisms rather than a series of random mutations. In addition, RNS generated by inflammatory cells can have detrimental effects on epithelial cells by causing DNA damage. Apoptosis-resistant NO-overexpressing cells, because of their survival advantage, could generate fields of defective cells subject to increased DNA damage by RNS, genomic instability, increased DNA mutation rate and increased risk for colon cancer.

DIET AND BACTERIAL INFECTION CAN CAUSE GENERATION OF ROS IN THE COLON

Diets high in fat result in elevated levels of secondary bile acids in the fecal water of the colon. Deoxycholic acid (the major bile acid in the colon) occurs in fecal water at concentrations as high as 0.73 m*M* in individuals consuming a high-fat diet (Stadler *et al.*, 1988). Bile acids are implicated in the intracellular formation of NO and its subsequent products, nitrates, nitrites, and nitrotyrosine (Casellas *et al.*, 1996; Washo-Stultz *et al.*, 1999). Casellas *et al.* (1996) showed that bile acids at 3 m*M* stimulate production of nitrates and nitrites in the colon and in biopsy samples of colonic mucosa from individuals with irritable bowel syndrome. This nitrate/nitrite production was blocked by L-NAME (*N*-nitro-L-arginine methyl ester), an inhibitor of NOS. It was not determined in this study if bile acids stimulate production of RNS in colonic epithelial cells specifically, or if RNS are generated from macrophages within the lamina propria of the mucosal tissue. We recently showed that NaDOC at 0.5 m*M*, a physiological concentration of bile salts present in the fecal water of individuals on a high-fat diet, induces the formation of high levels of nitrotyrosine residues in proteins associated with the plasma membrane of colonic epithelial cells (Washo-Stultz *et al.*, 1999). These results indicate that a high-fat diet, and its corollary, a high physiologic level of bile acids, generates RNS in colonic epithelial cells, a cytotoxic effect that is independent of that generated by immune cells. Also, bile acids may generate ROS through activation of the transcription factor AP-1 and COX-2 (a target gene of AP-1), activation of the transcription factor NF-κB (Payne *et al.*, 1998) and NOS2 (a target gene of NF-κB), or through an effect on mitochondria (Krahenbuhl *et al.*, 1994). It is probable that bile acids cause release of calcium from mitochondria or the endoplasmic reticulum through membrane perturbation.

Intraepithelial *Escherichia coli* has been found to be associated with macroscopically normal colonic tissue in 87% of patients with adenoma and carcinoma of the colon, but not within the tissue of individuals with no colonic neoplasia (Swidsinski *et al.*, 1998). However, a cause-and-effect relationship for this association has not been demonstrated. It is of interest that enteroinvasive bacteria activate NF-κB (Savkovic *et al.*, 1997) and NOS2 (Witthoft *et al.*, 1998) with resulting NO production in human colonic epithelial cells. After infection with enteroinvasive *E. coli*, *Salmonella dublin*, or *Shigella flexnerii*, human colonic epithelial cells (Caco-2 and HT-29) rapidly upregulate NOS2 mRNA and protein expression as well as NO production. An acute increase in NO through the activity of increased NOS activity can affect calcium release from mitochondria (Richter, 1998). The increase in cytosolic calcium levels can increase epithelial cell apoptosis through the activation of endonucleases, proteases, or phospholipases. Human colonic cells have indeed been reported to undergo apoptosis following infection with *Salmonella* or enteroinvasive *E. coli* (Kim *et al.*, 1998). The interaction of bacteria with epithelial cells could, therefore, allow for the selec-

tion of apoptosis-resistant cells (*e.g.*, after cycles of bacterial-induced apoptosis or through long-term activation of NF-κB or NOS2), leading to genomic instability and increased tumorigenesis. We have recently shown that *E. coli* respond to bile acids by activating the promoters of genes associated with DNA damage and oxidative stress (Bernstein *et al.*, 1999b), similar to the responses of human cells (Bernstein *et al.*, 1999c). The intracellular localization of *E. coli* within colonic epithelial cells may simply be a defensive strategy on the part of *E. coli* to evade the harmful effects of high bile acid concentrations in the gut. The effect of the sustained presence of intraepithelial *E. coli* on tumorigenesis is not known.

HYPOTHESIS-DRIVEN BIOMARKER DEVELOPMENT TO ESTABLISH COLON CANCER RISK

The need to develop and validate biomarkers or surrogate end points for cancer risk is now well recognized. Also referred to as intermediate end points, biomarkers can be described as measurable parameters that precede the formation of a malignancy and, if displayed, result in increased cancer risk for that individual. The disease process by which cancer develops is carcinogenesis, with malignant, invasive cancer being the end result. Biomarkers would be useful in identifying subjects that have been proceeding along the carcinogenesis pathway, thereby justifying more intense cancer screening efforts in them. Furthermore, modulation of these biomarkers would be useful end points in clinical efforts aimed at reducing cancer risk. Biomarker development is especially important, therefore, for cancer prevention efforts. Although a common disease, cancer is still an unusual event in the general population. Therefore, prevention studies with cancer as an end point are generally logistically impractical to conduct because of the large numbers of subjects required and the long follow-up needed. Biomarker end points would be much more practical. For these reasons, biomarker development is the target of intensive research efforts.

The above points are particularly relevant to the colon and colon cancer. From the screening standpoint, it has now been established that colonoscopy and removal of colon polyps markedly reduces the risk of colon cancer (Selby *et al.*, 1992; Winawer *et al.*, 1993). However, conducting screening colonoscopies in every adult subject is not feasible. Nevertheless, if high-risk individuals could be identified by biomarkers, then it would be possible to target them for colonoscopic screening. Additionally, modulation of biomarkers would serve as end points in testing chemopreventive agents that may prove useful in inhibiting colon carcinogenesis. Clearly, to be useful, these biomarkers must be measurable in easily obtained tissue samples (*e.g.*, blood, rectal biopsies, *etc.*). Recognizing the significance of biomarkers in colon carcinogenesis, a large number have been proposed, ranging from proliferation characteristics, enzymatic activities, and histologic characteristics to genetic alterations. Nevertheless, no biomarker has yet been fully validated and there is need for additional work in this area, especially that which is based on novel concepts and mechanistic pathways.

Putative biomarkers are much more likely to be eventually useful if they are based on testable hypotheses and mechanisms (Hursting *et al.*, 1999). As the role of RNS, together with other redox mechanisms, in colon carcinogenesis is elucidated, it will lead to novel hypotheses and mechanism-based concepts for testing as potential biomarkers in the human, clinical setting. Studies comparing colonic mucosa from high risk subjects [*e.g.*, those known to have cancer or precancerous lesions (large or villous polyps)] with control subjects for abnormalities in NO metabolic or signaling pathways, redox-related transcription factors or enzymes, and proteins involved in the apoptotic signaling pathways, selected on the basis of laboratory leads, could prove to be a useful strategy for new biomarker development. One of our group's goals is to conduct such investigations with the hope of developing a group of unique, new candidates for intermediate end points or biomarkers for colon cancer (Garewal *et al.*, 1996; Bernstein *et al.*, 1999a).

MECHANISM-BASED CANCER CHEMOPREVENTION STRATEGIES INVOLVING ROS

The biochemical pathways described in Fig. 1 are the targets for several chemoprevention strategies in colon carcinogenesis. Inhibitors of COX-2 inhibit both chemically-induced and genetic forms of experimental colon carcinogenesis (Oshima *et al.*, 1996; Kawamori *et al.*, 1998). Ursodeoxycholic acid (UrsoDOC), an inhibitor of carcinogen-induced colon carcinogenesis (Earnest *et al.*, 1994), is a potent inhibitor of NOS (Invernizzi *et al.*, 1997). Rao *et al.* (1999) further showed that a selective NOS2-specific inhibitor, PBIT, inhibited formation of ACF, regarded as early preneoplastic lesions in colon carcinogenesis (McLellan *et al.*, 1991). Difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC, inhibits colon carcinogenesis in experimental animal models and is currently under evaluation in humans with elevated risk for developing colon cancer (Meyskens and Gerner, 1999). Studies previously mentioned above show that arginine supplementation suppresses ACF in the colons of carcinogen-treated rats (Wargovich *et al.*, 1996), while arginine-deficient diets cause an increase in hepatocarcinogenesis in rodents (Vasudevan *et al.*, 1994). Together, these results suggest the hypothesis that some nonsteroidal anti-inflammatory drugs (NSAIDs), UrsoDOC and DFMO, all potent inhibitors of experimental colon carcinogenesis, are exerting part of their chemopreventive action by affecting aspects of arginine metabolism.

One consequence of this hypothesis is that combinations of chemopreventive agents might be suggested, or new points of intervention might be identified. For example, combinations of DFMO and either an NSAID or UrsoDOC might be expected to suppress arginine catabolism, thereby increasing colonic arginine concentrations more than either agent alone. Thus, the combination might be more potent in suppressing colon carcinogenesis. Both of these predictions are currently under evaluation by our group.

A second consequence of this hypothesis is that previously unknown mechanism(s) for specific chemopreventive agents might be suggested. For example, dietary selenium has been implicated in the prevention of certain cancers, including colon cancer (Clark *et al.*, 1996). Specific forms of selenium are known to display this anti-carcinogenic activity and protect against peroxynitrite (Sies *et al.*, 1998; Arteel *et al.*, 1999). Although a role in the detoxification of ROS for some selenium compounds has been reported, this mechanism of action in cancer chemoprevention is controversial (Sies *et al.*, 1997). Selenomethionine and selenocystine, for example, suppress DNA damage (Roussyn *et al.*, 1996) and protein modification as a result of $ONOO^-$ formation (Briviba *et al.*, 1996). Manipulation of arginine metabolic pathways can be performed to determine potential role(s) of RNS in the preventive activity of agents like selenomethionine in experimental colon carcinogenesis.

DIETARY PREVENTION STRATEGIES

The main membrane antioxidant, vitamin E, consists largely of two forms, α-tocopherol and γ-tocopherol. Whereas α-tocopherol acts as an antioxidant, γ-tocopherol is required to remove $ONOO^-$-derived nitrating species effectively (Christen *et al.*, 1997). Apparently, γ-tocopherol acts as a trap *in vivo* for membrane-soluble electrophilic nitrogen oxides by forming stable carbon-centered adducts through the nucleophilic 5-position, which is blocked in α-tocopherol (Christen *et al.*, 1997). Large doses of dietary α-tocopherol displace γ-tocopherol in various tissues. Thus, vitamin E supplementation of the diet also may be useful in protecting against damage caused by reactive nitrogen species, but the amounts of α-tocopherol and γ-tocopherol should be in appropriate balance.

A polyphenolic compound, chlorogenic acid (CGA), is an ester formed between caffeic acid and quinic acid. CGA, found in many plant species, can scavenge reactive species of oxygen and nitrogen faster than most other antioxidants (Kono *et al.*, 1997). CGA specifically reacts with $ONOO^-$. CGA can inhibit DNA damage caused by $ONOO^-$, probably by acting as a scavenger of $ONOO^-$ and preventing it from reaching the DNA (Grace *et al.*, 1998). The products of the CGA (and of the caffeic

acid) reaction with free radicals are rapidly broken down to molecules which, unlike those of other antioxidant/free radical reactants, are unable to generate further free radicals (Kono *et al.*, 1997). As a dietary strategy, consuming plant material high in CGA should offer protection from excessive reactive oxygen and nitrogen species.

Another dietary strategy for reducing the deleterious effects of RNS is to inhibit their endogenous production. Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) stimulate NOS2 expression. However, Chan *et al.* (1995) showed that three dietary plant compounds, at micromolar levels, cause a 50% inhibition of nitrite production, otherwise induced by LPS and IFN-γ, in mouse peritoneal cells. These compounds were epigallocatechin gallate (EGCG) from green tea, carnosol from rosemary, and curcumin, a nonspecific NOS2 inhibitor (Rao *et al.*, 1999) from the spice turmeric.

Nutritional protective formulae have also been obtained by fermenting oatmeal in vitamin and mineral supplemented media with *Lactobacillus plantarum* strains 299 and 299V (Bengmark, 1998). Upon entering the colon, *L. plantarum* utilizes mannose-specific adhesins to compete with Gram-negative pathogens for receptor sites at mucosal cell surfaces, thereby initially reducing the load of pathogenic bacteria. In addition, one of the main actions of *L. plantarum* is to produce extracellular NO. The extracellular NO acts as an antibiotic to control pathogenic bacterial populations, amoebae, and other parasites. Extracellular NO also stimulates mucus exocytosis by human colonic goblet cells by both a cGMP-dependent and cGMP-independent pathway (Branka *et al.*, 1997). Exocytotic mucin, composed of 85% carbohydrate and 15% protein, forms a protective barrier on the lumen surface, and this mucin is, itself, effective in scavenging toxic oxygen metabolites (Hiraishi *et al.*, 1993).

One aspect of the diet that could potentially affect colon carcinogenesis is dietary vitamin deficiency, in particular niacin (also referred to as nicotinic acid) and niacinamide. Nicotinic acid and nicotinamide can increase intracellular levels of NAD^+, which is used as a substrate for PARP, an enzyme involved in DNA repair. Since ROS and RNS can damage DNA (see above), an important process that can help prevent mutations and the development of cancer is the adequate repair of DNA. We have reported that PARP is protective against apoptosis induced by the bile acid, NaDOC (Payne *et al.*, 1998), which can generate ROS and RNS and can cause oxidative DNA damage. PARP activity can deplete cellular reserves of NAD^+, thus it is essential to have an adequate supply of intracellular NAD^+. DNA damage caused by inflammation (*e.g.*, ulcerative colitis, Crohn's disease, and other inflammatory bowel diseases) or through the increase in ROS and RNS caused by bile acids may impose a further drain on the available NAD^+ levels, compromising adequate DNA repair. In fact, the clinical signs of pellagra have been reported to appear in some patients with Crohn's disease, which was alleviated by dietary niacin supplementation (Zaki and Millard, 1995). A biochemical assessment of erythrocyte NAD^+ levels, as a measure of niacin status, may be an effective biomarker of cancer risk, with low levels potentially predisposing affected individuals to cancer (Fu *et al.*, 1989; Jacobson and Jacobson, 1993; Jacobson, 1993). Therefore, dietary supplementation with niacin or niacinamide may allow for more effective repair of DNA and reduce cancer risk in patients with inflammatory bowel disease and in patients on a high-fat/low-fiber diet.

In addition to the ability of niacin and niacinamide to protect against DNA damage by increasing NAD^+ levels for adequate PARP activity, these NAD^+ precursors have been shown to modulate gene expression. Of particular interest is the ability of nicotinamide to suppress NOS2 mRNA expression and subsequent NO formation (Fujimura *et al.*, 1997). These findings indicate that nicotinamide can prevent DNA damage caused by the RNS, in addition to contributing to DNA repair through the generation of NAD^+. Recent work from our group indicates that both niacin and niacinamide can up-regulate the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase (G6PD) (Yan *et al.*, 1999). Both of these glycolytic enzymes have been shown to protect against oxidative stress through the generation of NADH and NADPH (NAD phosphate, re-

duced form), respectively. In addition, GAPDH, in its monomer configuration, functions as a uracil DNA glycosylase and helps repair DNA damage. To date, no clinical trials of niacin or niacinamide supplementation have been performed to address the effect of increased intracellular NAD^+ levels on colon cancer prevention. Because niacinamide has been used successfully over a 7-year period to prevent juvenile diabetes with no toxic side effects (Elliott *et al.*, 1996), future colon cancer prevention trials using niacinamide as a dietary supplement would appear to be worthwhile.

SUMMARY

Figure 2 is a schematic that summarizes the effects of NO and other RNS on signaling pathways in colonic epithelial cells and indicates where chemopreventive agents and dietary components can interact with these pathways.

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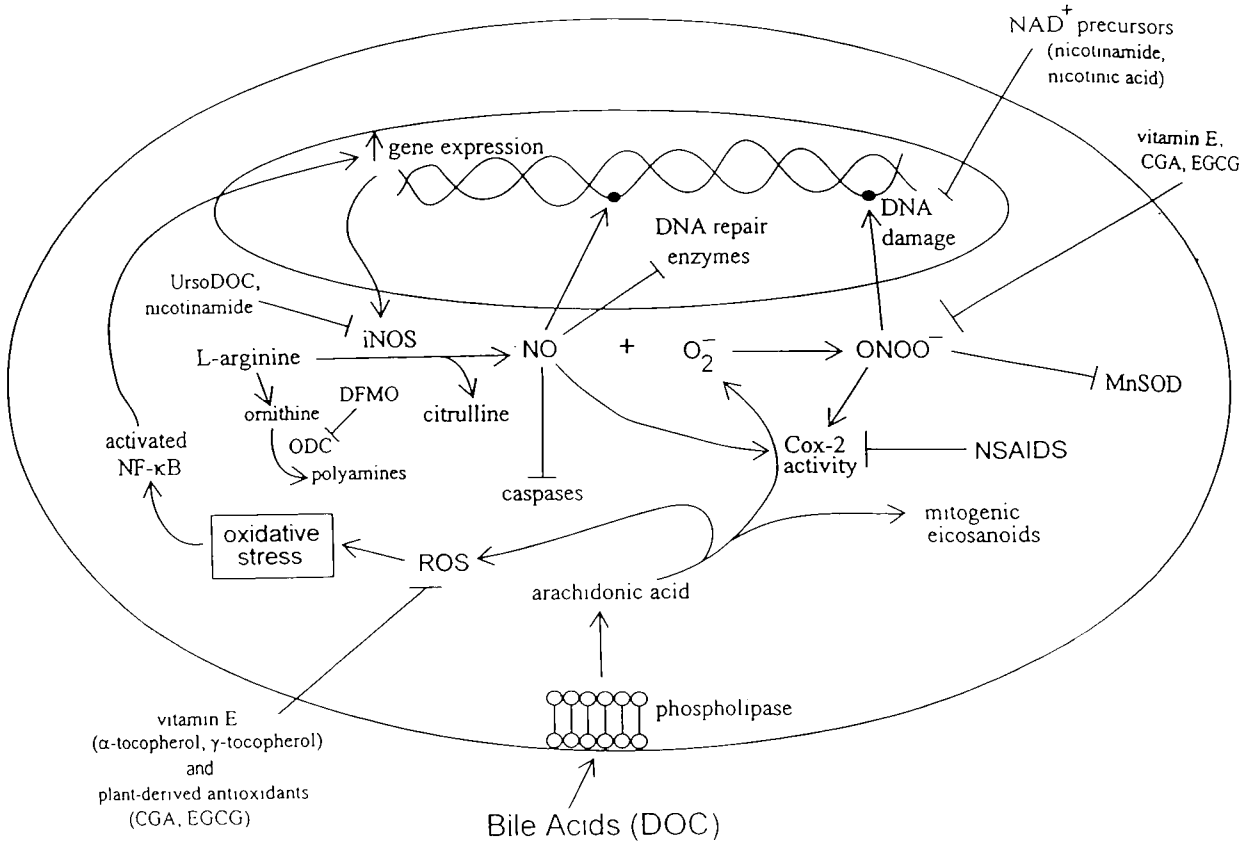

FIG. 2. Schematic illustrating the possible cellular effects of RNS on colon carcinogenesis. Bile acids interact with cellular membranes, activate phospholipases, and release arachidonic acid. The arachidonic acid serves as a substrate in the cyclooxygenase/lipooxygenase pathway with the release of ROS. The ROS activate NF-κB, which translocates to the nucleus and induces specific gene expression. One target gene of NF-κB transcriptional activation is NOS2 (iNOS). The resulting generation of NO can protect cells against apoptosis, possibly through the inhibition of caspases. The activity of NOS2 produces micromolar quantities of NO, which can directly damage DNA. NO reacts with superoxide O_2^- to produce $ONOO^-$, which also damages DNA. In addition to causing DNA damage, NO inhibits DNA repair enzymes, and $ONOO^-$ inhibits the activity of manganese superoxide dismutase (MnSOD). These are enzymes involved in the protection of cells against damage. Both NO and $ONOO^-$ activate COX-2, an enzyme that is elevated in colon cancer. The production of mitogenic eicosanoids by the cyclooxygenase pathway then contributes to cellular proliferation. The detrimental consequences of increased NO to colon carcinogenesis are, therefore, threefold: (1) an increase in apoptosis resistance, (2) an increase in DNA damage, and (3) an increase in COX-2 activity. Three possible sites for chemoprevention are indicated in the schematic: UrsoDoc and nicotinamide to inhibit NOS2 activity, NSAIDs to inhibit COX-2 activity, and DFMO to inhibit ODC. NAD^+ precursors (*e.g.*, niacin and niacinamide), vitamin E, CGA, or EGCG as dietary supplements can serve to increase DNA repair and lower oxidative stress.

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ABBREVIATIONS

ACF, Aberrant crypt foci; AOM, azoxymethane; CGA, chlorogenic acid; COX-2, cyclooxygenase-2; DFMO, difluoromethylornithine; *E. coli, Escherichia coli*; EGCG, epigallocatechin gallate; eNOS, endothelial NOS; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; ICE, interleukin coverting enzyme; IFN-γ; interferon-γ; IL-6, interleukin; iNOS, inducible NOS; L-NAME, *N*-Nitro-L-arginine methyl ester; *L. plantarum, Lactobacillus plantarum;* LPS, lipopolysaccharide; MnSOD, manganese superoxide dismutase; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of NAD; NaDOC, sodium deoxycholate; NADPH, NAD phosphate, reduced form; nNOS, neuronal NOS; NO, nitric oxide; NOS, NO synthase; NSAID, nonsteroidal anti-inflammatory drug; O_2^-; superoxide anion; ODC, ornithine decarboxylase; $ONOO^-$, peroxynitrite; OTC, ornithine transcarbamylase; PARP, poly(ADP-ribose) polymerase; PBIT, *S'*-S'-1,4-phenylene-*bis* (1,2-ethanediyl)*bis*-isothiourea); PGE2, prostaglandin E2; RNS, reactive nitrogen species; ROS, reactive oxygen species; TNF, tumor necrosis factor; UrsoDOC, ursodeoxycholate.

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