Nitric oxide in gastrointestinal epithelial cell carcinogenesis: linking inflammation to oncogenesis

MEETA JAISWAL, NICHOLAS F. LARUSSO, AND GREGORY J. GORES
Center for Basic Research in Digestive Diseases, Division of Gastroenterology and Hepatology,
Mayo Clinic, Foundation, and Medical School, Rochester, Minnesota 55905

Jaiswal, Meeta, Nicholas F. LaRusso, and Gregory J. Gores. Nitric oxide in gastrointestinal epithelial cell carcinogenesis: linking inflammation to oncogenesis. Am J Physiol Gastrointest Liver Physiol 281: G626–G634, 2001.—Chronic inflammation of gastrointestinal tissues is a well-recognized risk factor for the development of epithelial cell-derived malignancies. Although the inflammatory mediators linking chronic inflammation to carcinogenesis are numerous, current information suggests that nitric oxide (NO) contributes to carcinogenesis during chronic inflammation. Inducible nitric oxide synthase (iNOS), expressed by both macrophages and epithelial cells during inflammation, generates the reactive molecule NO. In addition to causing DNA lesions, NO can directly interact with proteins by nitrosylation and nitrosation reactions. The consequences of protein damage by NO appear to be procarcinogenic. For example, NO inhibits DNA repair enzymes such as human 8-oxoguanosine DNA glycosylase 1 and blocks apoptosis via nitrosylation of caspases. These cellular events permit DNA damage to accumulate, which is required for the numerous mutations necessary for development of invasive cancer. NO also promotes cancer progression by functioning as an angiogenesis factor. Strategies to inhibit NO generation during chronic inflammation or to scavenge reactive nitrogen species may prove useful in decreasing the risk of cancer development in chronic inflammatory gastrointestinal diseases.

Apoptosis; cytokines; oxidative DNA damage; DNA repair; p53
including gastric cancer (86), colonic adenomas (100), Barrett's esophagus and associated adenocarcinomas (73), hepatocellular carcinoma (84), and cholangiocarcinoma (33, 40) (Fig. 2). These observations suggest that iNOS may play a fundamental role in the initiation and promotion/progression of cancers arising within a background of inflammation. Therefore, it is both timely and topical to review the role of iNOS-generated NO in carcinogenesis of gastrointestinal epithelial cells.

**NITRIC OXIDE SYNTHASES AND NITRIC OXIDE**

NO is a 30-kDa gaseous biological mediator synthesized in mammalian cells by a family of three NOS isoforms. The human genes for NOS are characterized as neuronal or nNOS (NOS1), endothelial or eNOS (NOS3), and iNOS (NOS2) (23). NOS catalyzes the conversion of the terminal guanidino nitrogen of the amino acid l-arginine to form NO plus citrulline in a complex reaction involving molecular oxygen and NADPH as cosubstrates with enzyme-bound heme, FAD, flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4) as cofactors (5, 110). The two constitutive isoforms of NOS, eNOS, and nNOS, are controlled by calcium fluxes and produce only nanomolar NO concentrations. In contrast, the dimeric iNOS protein is permanently active and capable of generating micromolar concentrations of NO on induction in the absence of changes in cellular calcium (68, 72). The iNOS gene is under transcriptional control of inflammatory cytokines and lipopolysaccharides (25, 39, 79). The human iNOS gene was first cloned from synergistic activation of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN-γ) in hepatocytes (24, 83). iNOS gene expression is regulated by the transcription factors nuclear factor (NF)-κB, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, and c-Jun NH2-terminal kinase (JNK)-activator protein-1 (AP-1) pathways (103). The enzyme is downregulated by steroids, transforming growth factor (TGF)-β, the heat shock response element, p53, and NO itself (111). The complexity of iNOS catalysis and its regulation is reflected in the broad range of biological and cell/tissue-specific physiological and pathophysiological functions of NO (6). Studies have shown very little correlation between iNOS mRNA expression and enzyme activity, suggesting a considerable degree of posttranscriptional regulation. iNOS induction by cytokines and NF-κB likely accounts for its ubiquitous expression and activity during inflammation.

**iNOS EXPRESSION IN GASTROINTESTINAL EPITHELIA**

Almost every cell in the body has the capacity to express iNOS (78). In inflamed gastrointestinal tissue, iNOS is richly expressed by infiltrating and resident activated macrophages (15, 78). The NO generated by activated macrophages may have significant physiological benefits (82). For example, NO produced by macrophage iNOS has important antimicrobial functions (6, 119) and inhibits the growth of viruses (45), parasites (27), and gram-positive organisms (35) that invade the gastrointestinal tract. Although macrophage-derived NO appears to be important in innate immunity, the high diffusion efficiency of NO makes it potentially reach unintended targets (e.g., neighboring epithelial cells). Inflammatory cytokines will also trigger iNOS expression by epithelial cells (112). Indeed, iNOS has been identified in gastric metaplasia of the esophagus (73), mucosal cells of the stomach in chronic gastritis (66), hepatocytes in viral hepatitis (65, 84), cholangiocytes in primary sclerosing cholangitis (38), and colonocytes in inflammatory bowel disease (100). In contrast to macrophage-generated NO, epithelial cell NO has ready access to epithelial cell targets including those important in carcinogenesis. Thus the salutary effects and potentially detrimental conse-

![Fig. 2. Inducible nitric oxide synthase (iNOS) expression in response to inflammatory diseases in the gastrointestinal tract. There are emerging clinical data showing that chronic inflammatory conditions along the digestive tract, liver, and pancreas have increased iNOS expression in response to inflammatory diseases in the gastrointestinal tract.](http://ajpgi.physiology.org/ by 10.220.33.1 on June 24, 2017)
quences of NO generation in inflammation exist along a continuum related to the magnitude and chronicity of NO exposure. This continuum of iNOS activity and NO generation in immunoregulatory functions can be divided into three stages: 1) under physiological conditions, normal to low quantities of NO produced by constitutive and inducible NOS act as an intracellular activation signal; 2) when NO production is increased for longer periods of time via iNOS, NO functions as an autocrine or paracrine immunoregulatory molecule; and 3) chronic and sustained generation of NO can be associated with direct reactions between NO and cellular constituents and generation of reactive nitrogen species. It is this latter condition that has been postulated to play a pivotal role in carcinogenesis. This interplay is exemplified in *Helicobacter pylori* infection of the stomach, an infection well documented to be a risk factor for gastric cancer (66, 121). In this process, *H. pylori* elicits a host inflammatory response with iNOS generation of NO to potentially eradicate the organism. However, because of this bacterium’s ability to persist, the inflammatory response becomes chronic and predisposes to cancer with persistence of iNOS expression by the malignancy (86).

**NO AND CELLULAR DNA AND PROTEIN DAMAGE**

The toxicity of NO during chronic inflammation occurs by two chemical processes, 1) oxidation of NO with superoxide to form peroxynitrite (ONOO\(^{-}\)) and nitrosating species, namely, NO\(_3\) (nitrates), NO\(_2\) (nitrites), and N\(_2\)O\(_3\) (126), referred to as reactive nitrogen oxide species (RNOS), and 2) direct reactions between proteins and NO by nitrosylation events.

NO can damage DNA through a variety of mechanisms that may be cell specific (107). Nitrosation of purines and pyrimidines results in hydrolytic deamination of cytosine to uracil and guanine to xanthine (81, 109). GC → AT and GC → CG transitions (replacement of purine by pyrimidine base or vice versa) are major mutations observed in naked DNA and human cells exposed to NO (127). Sequence data of gene mutations from patients with hepatocellular carcinoma and gastric carcinogenesis reveal the same GC → AT transitions (12, 121). N\(_2\)O\(_3\) is a powerful electrophilic nitrosating agent that causes the formation of carcinogens—N\(^-\)nitroso compounds, DNA strand breaks, and cross-linking of DNA (109). Peroxynitrite can also react directly with DNA and lead to mutations and DNA strand breaks (106). Besides deamination, NO and/or peroxynitrite can cause DNA base oxidation measured as conversion of guanine to 8-nitroguanine and 8-oxoguanine (8-oxoG). *H. pylori* infections in humans have been associated with an increased release of 8-oxoguanine in urine and the formation of nitrosytrozine in gastric antral biopsies (66, 129). Acute hepatic injury and/or cell necrosis was accompanied by significant induction of iNOS in the liver and other organs with increased nitrosamine formation and release in human urine (92). In fact, increased endogenous formation of nitrate and nitrosamines is recognized as a risk factor for cholangiocarcinoma in humans infested with *Opisthorchis viverrini* (94, 124) and for hepatocellular carcinoma in cirrhosis patients (61).

NO can also directly nitrosylate susceptible thiol residues on proteins, resulting in the loss of their catalytic activity (87). In many proteins, this is a reversible reaction, but in proteins containing zinc, copper, or iron, the thiol nitrosylation results in irreversible ejection of the metal and denaturation of the protein (58). The reaction of NO with iron-sulfur centers may also perpetuate radical reactions. NO and peroxynitrite react rapidly with iron-sulfur centers, and the resultant free iron may then generate hydroxyl free radical-induced damage via Fenton chemistry (125). Peroxynitrite and NO can oxidize protein and nonprotein thiols, protein sulfides, lipids, and low-density lipoproteins (14, 85). 3-Nitrotyrosine is a stable marker of oxidative protein damage by RNOS (93). The presence of nitrotyrosine has been identified by immunohistochemistry in relation to gut tissue subjected to chronic inflammation with increased iNOS expression such as *H. pylori* gastritis (66), primary sclerosing cholangitis (38), cholangiocarcinoma (40), pancreatic cancer (122), and esophageal squamous cell cancer (46). Thus there is ample evidence for the presence of reactive oxygen species and NO-mediated DNA and protein damage in gastrointestinal epithelia during chronic inflammation.

**NO AND DNA REPAIR PROTEINS**

Although NO and reactive oxygen species can directly damage DNA, mammalian cells have rich and complex mechanisms to repair the genome. Several DNA repair processes are now recognized including base excision repair (BER), nucleotide excision repair, transcription-coupled repair, double strand break repair, and mismatch repair (Table 1; Refs. 20, 97, and 130). Accumulation of DNA damage during inflammation, therefore, represents the net result of both direct damage and ineffective repair. Unfortunately, DNA repair enzymes also appear to be protein targets of NO and reactive oxygen species (102, 128). Thus iNOS-generated NO may give the cell a double hit by both damaging the DNA and inhibiting its repair processes. This effect of NO and its by-products may make NO one of the pivotal mediators linking inflammation to carcinogenesis.

Several key DNA repair enzymes are inhibited by NO-mediated nitrosylation of active site cysteine. The DNA repair enzymes with vulnerable active site cysteine residues include 6-O-alkyl DNA transferase (56), foraminidopirimidine glycosylase (54), xerodermia pigmentosum A protein (76), and DNA ligases with active site lysine residues (60). We recently demonstrated (40) that cytokine-mediated induction of iNOS with NO production is associated with diminished global DNA repair capacity in cholangiocarcinoma cell lines. Although DNA oxidative lesions, the predominant mechanism of DNA damage in inflammation, can be excised from DNA by multiple pathways, BER is quantita-
In addition to repairing damaged DNA, the organism has another safeguard against cellular transformation. Programmed cell death by a process known as apoptosis (34, 104). DNA damage that cannot be repaired is a potent inductor of apoptosis (53). For example, DNA-damaging drugs, γ-radiation, and ultraviolet light all trigger apoptosis by inducing DNA damage (8). In these circumstances apoptosis is mediated by activation of caspases, a family of cysteine proteases (42).

Caspase activation occurs by one of two broad mechanisms, death receptors and/or mitochondrial cytochrome c release (26, 67). The death receptor pathway is initiated by caspase-8, whereas the mitochondrial pathway is dependent on caspase-9. Both death receptor- and mitochondria-initiated apoptotic cascades involve the downstream caspases-3, -6, and -7, which execute the apoptotic program (Fig. 4) (118). Although either or both pathways can be disrupted in established malignancies, dysregulation of the mitochondrial pathway appears to be very important in cancer development and therapy (47). In this pathway, apoptotic signals are initiated by the release of cytochrome c from mitochondria (34, 63). By inhibiting caspase activity through S-nitrosylation of the active site cysteine, NO inhibits apoptosis in hepatocytes (48), endothelial cells (10), and several tumor cell lines (9, 63). Indeed, NO has been shown to directly nitrosylate caspases-3, -8, and -9, resulting in their inactivation (49, 50). Studies have shown that prevention of NO protein damage induced by intestinal ischemia-reperfusion can protect against hypoxia-induced apoptosis in HT-29 colon carcinoma cells by inhibiting cytochrome c release from the mitochondria (34, 63, 64). In addition to the NOS isoforms described above, mitochondria are also capable of generating NO, although the mechanism responsible remains obscure (26). It is possible that this mitochondria-derived NO is capable of inhibiting apoptosis at

Table 1. DNA repair pathways

<table>
<thead>
<tr>
<th>DNA Repair Pathway</th>
<th>Description</th>
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<tbody>
<tr>
<td>Nucleotide excision repair</td>
<td>Executed by a complex process including up to 30 enzymes. Removes nucleotide</td>
</tr>
<tr>
<td>Base excision repair</td>
<td>Executed by substrate-specific repair enzymes. Removes single and multiple</td>
</tr>
<tr>
<td>Mismatch repair</td>
<td>Executed by specific mismatch base excision enzymes. Removes and replaces</td>
</tr>
<tr>
<td>Transcription-coupled repair</td>
<td>Executed by transcription-coupled repair enzymes and nucleotide excision</td>
</tr>
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The cells maintain genetic stability by removing damaged bases and adducts on DNA through a complex system of repair pathways. There are 4 major pathways to repair damaged DNA. Substrate-specific repair enzymes or enzymes involved in complex repair pathways may be susceptible to nitrosylation by nitric oxide.

Specifically the most important (41, 55). 8-oxoG, the most abundant oxidative DNA lesion, is excised in humans by 8-oxodeoxyguanosine DNA glycosylase 1 (hOgg1) (51, 95, 131). This glycosylase contains critical thiol moieties in its active sites (91, 108). Our group has demonstrated that the hOgg1 repair enzyme is inhibited by NO and peroxynitrite generation because of formation of S-nitrosylation at their active site cysteine residues and ejection/loss of zinc (38, 40). This is a good example of how iNOS overexpression due to inflammatory cytokines can be mechanistically linked to accumulation of DNA damage resulting from inhibition of a specific important DNA repair enzyme (Fig. 3).

Fig. 3. NO inhibits hOgg1 base excision repair activity. NO causes oxidative DNA damage [e.g., 8-oxoguanine (8-oxoG)] and potentiates the accumulation of the damage by inhibiting the repair activity of its key repair enzyme, human 8-oxodeoxyguanosine DNA glycosylase 1 (hOgg1). Unrepaired 8-oxoG causes GC to TA transitions and is mutagenic.

Fig. 4. Inhibition of caspase activation by NO prevents apoptosis. NO blocks both the death receptor and mitochondrial pathways by nitrosylation of caspase-8 and caspase-9 and -3, respectively. The resultant failure of apoptosis allows the survival of genetically altered cells and is thought to play a pivotal role in carcinogenesis. Bid, BH3-interacting domain death agonist.
the level of this organelle in addition to caspase nitrosoylation. Thus NO may inhibit apoptosis at several steps, further allowing the DNA-damaged cell to survive.

**NO, p53, AND TUMORIGENESIS**

NO and reactive oxygen species generated during chronic inflammation may initiate and enhance carcinogenesis in humans by the biochemical interactions discussed above. Infection by bacteria (*H. pylori*), parasites (liver fluke helminthes), or viruses (hepatitis B and C) and chronic tissue inflammation are recognized risk factors in the etiology and development of cancers. The process of carcinogenesis is a multistep process involving inactivation of tumor suppressor genes and activation of oncogenes by either DNA mutations or deletions. The DNA repair molecule p53 plays an important role in the cellular response to DNA damage. As mentioned above, the NO-mediated deamination of DNA causes GC → AT transitions. NO-induced DNA damage results in upregulation of p53, an important *trans*-repressor of iNOS expression in vivo, and attenuates NO production by a regulatory negative feedback mechanism (3, 19). The p53 gene in many human cancers, including liver cancer from hepatitis B (32) and *H. pylori*-associated gastric cancer (121), has GC → AT transitions. These transitions occur mainly at CpG islands of the p53 gene and occur in a number of human cancers, including colon, gastric, and hepatocellular cancers (1, 28). NO production via iNOS may directly induce these GC → AT mutations in p53, providing another molecular link between chronic inflammation and cancer development. For example, a relationship between p53 mutations and iNOS expression has been established in human colorectal cancer and several human carcinoma cell lines (2, 37). Not only may NO contribute to the development of p53 mutations, but the loss of repressor activity of the mutated p53 would increase iNOS, resulting in enhanced cellular NO generation, further DNA damage, and the development of additional transforming mutations (Fig. 5).

**NO AND ANGIOGENESIS**

Because neovascularization is necessary for tumor growth and immunohistochemical studies have indicated that iNOS is overexpressed in solid tumors (22), production of NO may promote tumor growth by stimulating angiogenesis (43, 74), increasing vascular permeability, and suppressing the immune response (57). Angiogenesis is a complex, multistage process, regulation of which requires release of vascular endothelial growth factor (VEGF), angiogenin, adhesion molecules, and basic fibroblast growth factors delivered by the tumor cells themselves, the extracellular matrix, or incorporated macrophages (18). Initial studies suggest that NO plays a central role in the angiogenic cascade by demonstrating that VEGF released as a purified protein or produced by tumor cells requires a functional NO/cGMP pathway within the endothelial compartment to promote neovascular growth (22, 75, 135). Another mechanism by which NO may promote tumor growth is by modulating the production of proangiogenic factors and angiostatin that facilitate tumor growth by increasing vascular permeability, and by concomitant inhibition of apoptosis from enhanced Bcl-2 protein production (120). The use of iNOS inhibitors as antiangiogenesis agents is now supported by several reports in the literature (reviewed in Ref. 116).

**NO, CHRONIC INFLAMMATION, AND THERAPEUTIC INTERVENTION**

The use of oxygen by aerobic organisms invariably results in the formation of reactive oxygen species. Cells counterbalance prooxidants with antioxidants. For example singlet oxygen and superoxide are dismutated by Mn superoxide dismutase in the mitochondria and Cu/Zn superoxide dismutase in the cytosol, and H$_2$O$_2$ is detoxified by glutathione peroxidase, a selenium enzyme, and by catalases. Besides these defenses, the cell has other proteins like thioredoxin, glutaredoxin, and thioltransferase that participate in maintaining the equilibrium between prooxidant and antioxidant. In addition, many small molecules such as *α*-tocopherol, ascorbate, albumin, uric acid, and glutathione act as scavengers of reactive oxygen species. Genetic stability is also maintained by the activity of efficient DNA repair enzymes. However, during chronic inflammation, the cell is bombarded with the accelerated and sustained production of oxygen free radicals and ions that saturate the antioxidant capacity of the cell, inhibit DNA repair function and programmed cell death, and are potentially carcinogenic. The recognition of the multifaceted role of NO in modulating mutagenesis in chronic inflammatory diseases suggests several therapeutic approaches (36, 77).

iNOS and its associated mediators have been targeted for therapeutic intervention. The current poten-
tial strategies for intervention include 1) scavengers of NO and peroxynitrite, 2) repression of iNOS expression by drugs, antisense technology, and p53, and 3) inhibition of iNOS protein and its cofactors. Carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (132) and PTIO (29) are potent scavengers for NO. Trolox (96), ebsealen (4) and Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), a superoxide dismutase mimetic, (17) have been used successfully to scavenge for peroxynitrite. The efficacy of these scavengers in a malignant murine model has been gratifying (30, 123). Curcumin (from Curcuma longa), a naturally occurring polyphenolic phytochemical, is in preclinical trial for cancer chemoprevention. Curcumin inhibits activation of free radical-activated transcription factors such as NF-κB and AP-1 and reduces the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-8. In addition to inhibiting cytokine-mediated activation of iNOS, curcumin has been shown to inhibit iNOS gene expression in mouse liver (11). Inhibition of gene expression by hybridization of antisense oligonucleotides to the transcripts also has therapeutic potential (13). An antisense oligodeoxynucleotide to iNOS mRNA designed to inhibit translation of iNOS was found to partially inhibit nitrite production in cytokine-stimulated cells (115). Selective inhibitors of iNOS that blocked iNOS dimerization and NO production both in vitro and in vivo were identified in an encoded combinatorial chemical library. These inhibitors were >1000-fold more potent than substrate-based iNOS inhibitors such as 1400W (70). Irreversible inhibition of iNOS was achieved by using flavoprotein iodonium inhibitors that prevented the binding of cofactors NADPH, FMN, and FAD (21). BH₄, another essential cofactor of iNOS, can be inhibited by N-acetylsertotonin and dicumarol (101). iNOS isoform-selective substrate-based arginine analog inhibitors can be exploited for therapeutic purposes because arginine binding domains for the isoforms are different. Several general analogs of arginine such as N⁶-monomethyl-L-arginine (L-NMMMA) acetate and L-N⁶-(1-iminoethyl) lysine (L-NIL) are effective nonspecific inhibitors of iNOS. 1400W, a selective inhibitor of iNOS, inhibits the growth of solid murine tumors expressing iNOS (117). Aminoguanidine and mercaptoethanlyguanidine are inhibitors of iNOS and have anti-inflammatory properties because they protect against peroxynitride-induced oxidative damage (105). Chemopreventive approaches may include inhibition of iNOS expression with tetracycline and minocycline (133, 134). Preclinical animal studies involving iNOS inhibitors have been effective in chronic colitis (88, 90). Expression of p53 in cancer cells lacking this tumor suppressor gene can lead to cell apoptosis or cycle arrest and may also inhibit the angiogenesis required for tumor growth by decreasing iNOS expression. Studies have initiated the use of adenoviral vectors for effective locoregional delivery and transient overexpression of p53 and consequently effective p53 gene therapy for cancer (80).

SUMMARY

The current literature suggests that NO is involved in many of the pathophysiological processes linking inflammation to cancer development and progression in the gastrointestinal tract. NO, generated by iNOS in these disease states, is a reactive biomolecule. When the generation of NO exceeds the antioxidant capacity of the cell, NO and its reactive nitrogen oxide species are capable of damaging DNA and proteins. In particular, NO-mediated nitrosylation of DNA repair proteins inhibits their enzymatic activity, potentiating accumulation of DNA damage. NO can also nitrosylate and inactivate the effector proteins of apoptosis, especially caspases. Inhibition of caspases results in dysregulation of apoptosis, further promoting malignant transformation. Because it is associated with VEGF formation, NO can also be considered an angiogenic factor, thereby promoting cancer progression. These concepts provide a model to test the utility of iNOS inhibitors and NO scavengers as chemopreventive agents to minimize the risk of human cancers in these premalignant diseases. We encourage these studies and await their results.

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REFERENCES


