iNOS/COX-2 pathway interaction: a good molecular target for cancer treatment.

iNOS/COX-2 Pathway Interaction: A Good Molecular Target for Cancer Treatment

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Abstract: An increase in the expression and activity of both inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) has been shown in several types of human tumors. A large body of evidence has demonstrated that these two enzymes are involved in tumor progression through several molecular mechanisms, such as promotion of tumor cell proliferation, inhibition of apoptosis and stimulation of angiogenesis. iNOS and COX-2 share a number of similarities in terms of pathophysiological phenomena and are often co-expressed in cancer tissues. The product of iNOS, nitric oxide (NO), has been demonstrated to modulate COX-2 expression and prostaglandin production in both inflammatory and tumor experimental models. Cyclic GMP and peroxynitrite, the coupling product of NO and superoxide, appear as the most important pathways by which NO may regulate COX-2 expression. We have recently shown that both NO- and COX-2-related angiogenesis is mediated by an increase in VEGF production in colorectal cancer. We also provided evidence that NO can stimulate COX activity and that its pro-angiogenic effect is mainly mediated by COX-2-related PGE_2 production. The purpose of this review is to summarize experimental data on the molecular mechanisms underlying iNOS-COX-2 cross-talk and investigate the pathophysiological significance of this interaction in cancer. Given the availability of highly selective inhibitors of both iNOS and COX-2, dual inhibition of these enzymes appears as a promising therapeutic tool in the treatment of various types of human cancers by producing a possible synergistic anti-tumor effect.

Keywords: Nitric oxide synthase, cyclooxygenase-2, prostaglandins, cancer, angiogenesis.

INTRODUCTION

Nitric oxide (NO) and prostaglandins (PGs) are two of the best known mediators of the inflammatory process. NO is synthesized by a family of three NO synthase (NOS) isoenzymes that convert L-arginine into L-citrulline in the presence of molecular oxygen yielding free NO [1,2]. The endothelial NOS and the neuronal NOS are Ca^{2+} and calmodulin-dependent isoforms and are constitutively expressed in endothelial cells and neurons, respectively. They are responsible for low levels of NO production (pico molar to nano molar) for short periods. The third isoform, inducible NOS (iNOS), is Ca^{2+} independent and requires induction in response to cytokines and pro-inflammatory agents in essentially every cell type [3]. It can produce large quantities of NO (μM) over extended time (days to weeks) and induces the production of the second messenger cyclic GMP (cGMP) [4]. In inflamed tissue, iNOS is richly expressed by infiltrating and resident activated macrophages. The NO produced by activated macrophages may have important physiological benefits, such as antimicrobial and antiviral functions [5]. Inflammatory cytokines may also trigger iNOS expression by epithelial cells [6]. Chronic and sustained generation of epithelial cell NO can be associated with direct reactions between NO and cellular constituents and the generation of reactive nitrogen species with potentially detrimental consequences for the host [7].

Cyclooxygenase (COX) is the enzyme responsible for the conversion of arachidonic acid to PGs. There are two isoforms of COX: COX-1 and COX-2 [8]. COX-1 is expressed constitutively in most tissue and appears to be responsible for the production of PGs that control normal physiological functions, such as gastric cytoprotection, platelet aggregation and regulation of renal blood flow. COX-2 is undetectable in most normal tissues whereas it is rapidly induced by both growth factors and inflammatory stimuli resulting in enhanced synthesis of PGs, in particular PGE_2, in inflamed tissue [9,10]. In general, COX-2 expression under inductive stimuli follows the pattern of the so-called early genes with a rapid increase after 2-4 h and a gradual diminution after 24-48 h.

iNOS shares significant features with COX-2 in terms of tissue distribution, regulatory function and participation in pathophysiological phenomena. For example, their products, NO and PGE_2, are proven to provide a proliferative, survival, and angiogenic advantage for proliferating cells at inflammation sites. iNOS and COX-2 have been found to be frequently co-expressed within the same type of cells and under the same experimental circumstances, including inflammation [reviewed in ref. 11], coronary vasodilation [12], cervical ripening during pregnancy [13], cerebral ischemia [14] and endotoxin-induced septic shock [15]. Moreover, co-induction of COX-2 and NOS has been demonstrated in several cell lines after exposure to lipopolysaccharide (LPS) [16,17] and cytochines such as interleukin-1 (IL-1) [18], tumor necrosis factor-α (TNF-α) [19] and γ-interferon [17].

This evidence has led to the demonstration of a interrelationship between the activities of these two enzymes. Several studies have shown that NO can exert a stimulatory effect on COX-2 catalytic activity in various *in vitro* and *in*

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vivo systems [20-26]. However, the molecular mechanisms of this activation have not been elucidated yet. Salvemini *et al.* [20] have demonstrated that NO enhances COX-2 activity in the mouse macrophage cell line RAW264.7 through a mechanism independent of GMP, i.e. the downstream cyclic nucleotide effector of NO. Landino *et al.* [22] suggested that this effect is mediated by peroxynitrite ($OONO^-$), the coupling product of nitric oxide and superoxide (O_2^-), possibly through its interaction with the heme group of COX. Another possibility is that lipid peroxidation initiated by peroxynitrite liberates arachidonic acid from the cell membrane which in turn activates COX-2 [21]. On the other hand, an inhibitory effect of NO on COX activation has also been reported [27,28]. The different modulatory actions of NO on COX induction have been attributed to its different concentrations in cell microenvironment: large amounts of NO suppress PG production while low levels activate COX. However, even the cell type investigated and/or the state of activation of the cells are likely to influence the type of response of COX-2 to NO stimuli [18,29,30].

Interestingly, not only does NO modulate the activity of COX-2, but it has also been shown that NO can stimulate COX-2 protein and gene expression. The role of NO in inducing COX-2 expression has been assessed in a number of *in vitro* and *in vivo* experimental models of inflammation [reviewed in ref. 11 and 31]. NO has been reported to modulate the signal-transduction cascades leading to COX-2 expression through the cGMP-dependent stimulation of tyrosine phosphatase activity [32] or activation of the c-Jun N-terminal kinase (JNK) and the p38 mitogen-activated protein kinase (MAPK) [33]. A transcriptional regulation of COX-2 by NO has also been postulated. The human COX-2 promoter contains a number of sites for transcription factors that are potential targets for NO modulation [31]. Among these transcriptional factors, nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) appear important in the regulatory effects of NO on COX-2 gene expression [34-36].

Interestingly, the recent demonstration of the NO-mediated up-regulation of COX-2 in colonic epithelial cells and in cholangiocytes may provide insight into the link between chronic inflammatory disorders and carcinogenesis. Mei *et al.* [17] have demonstrated that exogenous NO increases the expression of COX-2 at both mRNA and protein levels in the mouse colonic epithelial cell line YAMC. They have also suggested that transcriptional activation of the COX-2 gene may be a result of the NO-mediated accumulation of free soluble β-catenin in the cytoplasm and the formation of β-catenin/T-cell factor-lymphocyte enhancing factor (TCF-LEF) DNA binding complex in the nucleus. The NO-mediated stimulation of COX-2 mRNA and protein expression through a p38 MAPK and JNK1/2-mediated pathway has also been demonstrated in the mouse cholangiocyte cell line 603B [33]. This study showed that the ability of iNOS to increase cholangiocyte growth is largely dependent on COX-2 induction and PGE_2 production. Collectively, these data suggest that chronic inflammatory disease such as ulcerative colitis or primary sclerosing cholangitis may predispose to colorectal cancer or cholangiocarcinoma, respectively, through the induction of both iNOS and COX-2.

A large body of evidence supports the hypothesis that products of iNOS and COX-2 pathways are involved in the regulation of several processes responsible for tumor growth. Both gene and protein overexpression of the two enzymes has been demonstrated in several experimental and human tumors [reviewed in ref. 7 and 37]. Fig. (**1**) summarizes the putative mechanisms underlying the carcinogenetic effect of iNOS and COX-2. iNOS activity has been associated with direct DNA damage [38,39], stimulation of angiogenesis [40-42], alteration of the apoptotic process [43,44], induction of oncogene expression [45,46] and activation of transcription factors [34-36]. COX-2 activity has been linked to stimulation of angiogenesis [47,48] and cell proliferation [49,50], inhibition of apoptosis [51,52] and immune surveillance [53] as well as activation of matrix metalloproteinases [54].

Notably, iNOS and COX-2 have frequently been found to be co-induced within the same tumor cells. This finding supports the hypothesis of a causal relationship between the activity of the two enzymes even in tumor cells. The purpose of this review was to present the experimental data that provide evidence of cross-talk between the iNOS and COX-2 pathways in some types of human tumors, the possible molecular mechanisms underlying the carcinogenetic effect of this interaction and the potential of iNOS and COX-2 as molecular targets for cancer prevention and treatment.

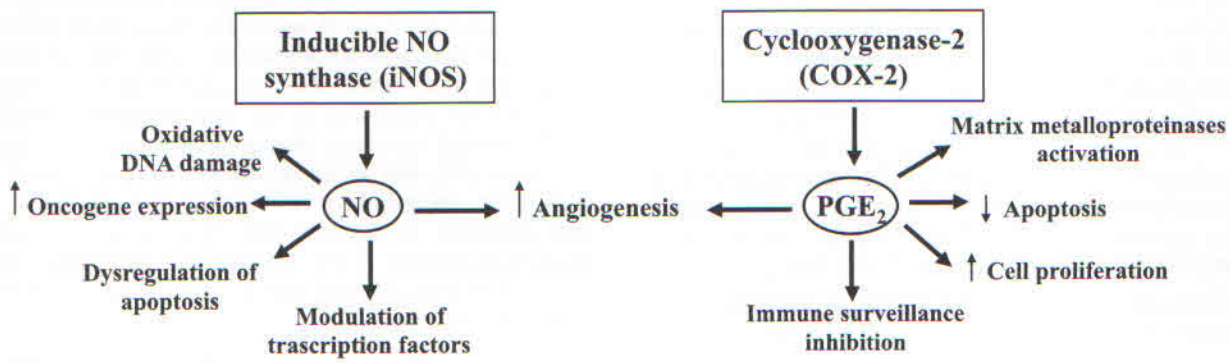

Fig. (1). Role of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in carcinogenesis. NO and PGE_2 have been implicated in modulating several events involved in malignant transformation and cancer promotion/progression. A common as well as fundamental mechanism underlying the tumorigenic effect of NO and PGE_2 is stimulation of angiogenesis.

INOS AND COX-2 INTERACTION IN PREMALIGNANCIES AND MALIGNANCIES

Colorectal Cancer

The possible independent role of either iNOS or COX-2 enzymes in colorectal tumor development and the possible underlying molecular carcinogenetic mechanisms have been extensively investigated in colorectal cancer [reviewed in ref. 55 and 56]. Recently, Rao *et al.* [57] have investigated the chemopreventive efficacy of the selective iNOS inhibitor SC-51, administered alone or in combination with the COX-2 selective inhibitor celecoxib, against azoxymethane (AOM)-induced formation of aberrant crypt foci (ACF) in rat colonic mucosa. They found that both iNOS and COX-2 activities are selectively inhibited in colonic mucosa by iNOS inhibitors after AOM treatment. Moreover, they showed that the administration of SC-51 plus celecoxib was more effective in inhibiting AOM-induced ACF formation than was administration of these agents individually. Collectively, these data suggest that suppression of iNOS activity may lead to the down regulation of COX-2 activity in colonic mucosa. Furthermore, it is likely that iNOS/COX-2 interaction is involved in early stages of colon carcinogenesis.

Overexpression of iNOS and COX-2 has been also correlated with advanced stages of disease in humans. However, only a few data exist regarding the possible cooperative effect of iNOS and COX-2 in promoting colorectal cancer progression. Bing *et al.* [58] have compared iNOS activity, PGI_2 and thromboxane production and COX-2 immunohistochemical expression in 11 colorectal cancer specimens and in the corresponding normal colonic mucosa. They found that the increase in iNOS activity and prostanoid production in tumors parallels the increase in COX-2 expression and suggests a possible co-regulation between iNOS and COX-2 in this type of tumor.

We have recently shown that iNOS and COX-2 are co-expressed within the same colorectal cancer cells and that iNOS activity is significantly correlated with PGE_2 production in human tumor samples [59]. These data strongly indicate an interaction between iNOS and COX-2 activities in this type of tumor. *In vitro* data on the stimulatory effect of both endogenous and exogenous NO on COX-2 activity in the HCT116 and HT29 colon cancer cell lines confirmed our hypothesis. We demonstrated a co-induction of iNOS and COX-2 activities in response to epidermal growth factor (EGF) or LPS treatment in the iNOS positive/COX-2 negative HCT116 cells. The selective inhibition of iNOS activity by 1400W significantly reduced both LPS- and EGF-induced PGE_2 production. The stimulated production of PGE_2 was also inhibited by the COX-2 inhibitor celecoxib. This finding suggests that COX-2 is the main source of the endogenous NO-stimulated increase in PGE_2 production after LPS or EGF treatment. The administration of the NO donor sodium nitroprusside to the iNOS negative/COX-2 positive HT29 cells determined an increase in PGE_2 production that was reversed by celecoxib. Therefore, even exogenous NO seems to be correlated with increased COX-2 activity in colon cancer cells.

We also found that PGE_2 levels in tumor samples were significantly correlated with the degree of tumor angiogenesis assessed as intratumor microvessel density and vascular endothelial growth factor (VEGF) expression, whereas the products of the iNOS pathway did not appear to be correlated with any of the angiogenic markers that we had investigated. These findings suggest a more direct involvement of COX-2 activity than that of iNOS in the induction of tumor angiogenesis. Even this hypothesis was confirmed by our *in vitro* data. Co-induction of iNOS and COX-2 activities after LPS- or EGF-treatment had different effects on VEGF production in the HCT116 and HT29 cell lines. We did not find any increase in VEGF production in the iNOS positive/COX-2 negative HCT116 cells whereas the iNOS negative/COX-2 positive HT29 cells showed a marked increase in VEGF levels. Moreover, the LPS- and EGF-stimulated production of VEGF in the HT29 cells was reversed by celecoxib. This finding suggests that the putative pro-angiogenic effect of NO in colorectal cancer is not direct but mainly mediated by COX-2 activity and thus, PGE_2 production. This interaction is likely to produce a cooperative effect in stimulating VEGF-mediated tumor angiogenesis.

The ability of NO to induce COX-2 in colorectal cancer has also been demonstrated by Liu *et al.* [60]. They found that the NO donor, S-nitrosoglutathione increases both COX-2 protein expression and PGE_2 production in a dose- and time-dependent manner in the HCA7, HT29 and HCT116 human colon cancer cells. Recently, the same author [61] has proposed an elegant model that elucidates the molecular mechanisms of NO-mediated induction of COX-2 in both non-transformed murine colonic epithelial cells and human colorectal cancer cells. NO treatment is known to cause the activation of matrix metalloproteinases which leads to the degradation of E-cadherin. This effect contributes to the cytosolic accumulation of β-catenin and nuclear formation of the transcription complex between β-catenin and TCF/LEF. The authors found that NO through the above mentioned β-catenin pathway stimulates the expression of the transcription factor polyoma enhancer activator 3 (PEA3) and its binding with DNA. PEA3 has been shown to strongly stimulate COX-2 promoter activity and thus, to increase the transcription of COX-2 gene.

Altogether, these data clearly demonstrate a pivotal role of NO in stimulating COX-2 expression and activity in human colorectal cancer. It is likely that one of the most important mechanisms underlying the carcinogenetic effect of this cross-talk is PGE_2-mediated stimulation of VEGF synthesis and thus, tumor angiogenesis.

Esophageal and Gastric Cancer

iNOS and COX-2 have been demonstrated to be involved in Barrett's metaplasia, i.e. columnar-lined esophagus arising in response to chronic reflux esophagitis, and associated esophageal adenocarcinoma. Wilson *et al.* [62,63] examined endoscopic mucosal biopsies obtained from Barrett's esophagus and control gastric body tissues in the same patients. Surgical resection samples from adenocarcinomas arising in Barrett's mucosa and adjacent normal esophagus were also studied. An increase in mRNA expression of iNOS and COX-2 in 76% and 80% of Barrett's tissues was found and it was significantly correlated with the expression of transforming growth factor-α. Up-regulation of

both iNOS and COX-2 at the mRNA and protein levels was also found in esophageal adenocarcinomas arising in Barrett's mucosa when compared with normal adjacent esophagus. These findings support the hypothesis that iNOS and COX-2 are involved early in Barrett's-associated neoplastic progression. It is likely that up-regulation of the two enzymes may be related to exposure of Barrett's epithelium to both the acid or acid plus bile salts as a consequence of duodenogastroesophageal reflux.

Van der Woude *et al.* [64] provide some insights into the possible molecular mechanisms underlying the involvement of iNOS and COX-2 in Barrett's metaplasia-dysplasia-carcinoma sequence. They found that iNOS is highly expressed in Barrett's epithelium with intestinal metaplasia and in 50% of samples containing dysplasia, but not in Barrett's esophagus-associated adenocarcinoma. COX-2 immunostaining was negative in intestinal metaplasia and dysplasia whereas it was present in most Barrett's esophagus-associated adenocarcinomas. They also evaluated the expression of some proteins involved in the apoptotic process, namely Bcl-2, Bax, Bcl-xl. They demonstrated that the apoptotic balance in the transformation from intestinal metaplasia to adenocarcinoma switches to an antiapoptotic phenotype because of increased Bcl-xl expression and decreased Bax expression. The authors concluded that pharmacologic inhibition of COX-2 activity is unlikely to be effective in preventing Barrett's esophagus adenocarcinoma. Moreover, no clear correlation can be established between iNOS expression and activation of pro-apoptotic and anti-apoptotic genes.

In regard to gastric adenocarcinoma, Son *et al.* [65] investigated iNOS and COX-2 gene up-regulation in 23 tumor samples obtained from patients who underwent gastrectomy. They found that COX-2 and iNOS mRNA were significantly higher in gastric cancer tissues than in adjacent normal gastric mucosa. There was also a significant correlation between the level of iNOS and COX-2 mRNA in tumor samples. No significant association was found between mRNA levels and peritumoral gastric inflammation and status of *Helicobacter pylori* infection.

Rajnakova *et al.* [66] demonstrated a strong immunohistochemical expression of both iNOS and COX-2 in their 55 human gastric adenocarcinoma samples. This expression was significantly higher in large and advanced tumors than in small and early stage ones. In the same tumors, the immunohistochemical accumulation of p53, which is an indicator for a loss of p53 tumor suppressor function, was found to correlate with iNOS and COX-2 expression. The authors concluded that tumor-associated production of NO and PGs may provide a selective growth advantage to tumor cells with mutant p53.

Van der Woude *et al.* [67] evaluated the expression of iNOS and COX-2 according to Lauren's gastric cancer classification, i.e. diffuse and intestinal adenocarcinoma types. It is known that gastric carcinomas of the diffuse type are associated with a poor prognosis compared with tumors of the intestinal type. Although all the tumor samples showed a high expression of both the enzymes, no significant difference in the expression of either iNOS or COX-2 was found between the two types of tumor. The expression of Fas, Bcl-xl, Bcl-2, Bax, active caspase 3 and Ki-67 was investigated in the same tumor samples but no significant correlation was found between these apoptosis-related proteins and iNOS/COX-2 expression.

The pathogenesis of gastric lymphomas from mucosa-associated lymphoid tissue (MALT) has been demonstrated to be linked to chronic infection with *H. pylori*. It has also been shown that both iNOS and COX-2 are potentially involved in *H. pylori*-induced gastric mucosa alterations and development of this type of lymphoma [68]. Li *et al.* [69] demonstrated a high positive immunostaining rate for iNOS and COX-2 in their 32 gastric MALT lymphomas. In the same cases, COX-2 expression was significantly correlated with iNOS expression, tumor cell proliferative activity (assessed by Ki-67 labeling index) and p53 accumulation status. These findings suggest that iNOS and COX-2 may play a synergistic role in the evolution of *H. pylori*-associated gastritis to gastric MALT lymphoma

Head and Neck Cancer

The possible interaction between iNOS and COX-2 pathways in head and neck squamous cancer (HNSC) was first investigated by Gallo *et al.* [70]. They found a significant correlation between NO and PGE_2 production as well as between iNOS and COX-2 mRNA/protein expression in human HNSC samples. Moreover, both iNOS and COX-2 expression was significantly correlated with lymph node metastases and the degree of tumor angiogenesis evaluated as microvessel density. Their *in vitro* experiments on the A-431 and SCC-9 HNSC cells have shown that both endogenous (after iNOS induction by LPS and EGF) and exogenous NO increases PGE_2 production through the direct up-regulation of COX-2 mRNA and protein expression. These effects are likely to be mediated by a cGMP-dependent pathway given that they are reversed by blocking guanylate cyclase.

These findings have been confirmed by Park *et al.* [71]. In particular, these authors showed that the exposure of several HNSC cell lines to the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) increases COX-2 expression, resulting in increased PGE_2 synthesis. The up-regulation of COX-2 by NO has been demonstrated to be mediated *via de novo* synthesis of mRNA by using experiments involving a transcription inhibitor and by a COX-2 promoter activity assay. In addition, iNOS inhibitors have been found to downregulate the NO-mediated overexpression of COX-2. The same induction of COX-2 has been obtained by adding exogenous cGMP, thereby suggesting that NO-stimulated COX-2 up-regulation in HNSC cells is mediated by the activation of guanylate cyclase. Interestingly, it has also been shown that NO has no observable effect on COX-2 in cancer cell lines with very low levels of COX-2 expression while COX-2 protein was increased by NO and inhibited by iNOS inhibitors in cell lines with strong or moderate constitutive COX-2 expression. Therefore, the authors hypothesized that variations in increased COX-2 expression by NO may depend on whether signalling pathways inducing its expression are already active in cancer cells at the basal level.

Recently, Gallo *et al.* [72] have reported data about the possibility of a close regulation of iNOS and COX-2 activities by the tumor suppressor gene p53. They found that

their tumor samples expressing a mutated p53 protein released the highest levels of nitrite/nitrate and prostaglandins, suggesting a key role of p53 mutation in the up-regulation of both iNOS and COX-2. This hypothesis has been confirmed by *in vitro* studies: restoration of wild-type p53 in the A431 cancer line results in down-regulation of both iNOS and COX-2 mRNA and protein expression as well as of their products. These data suggest that iNOS and COX-2 are p53 target genes subjected to p53 repression and might have important implications for the potential use of p53 gene therapy in head and neck squamous cancer.

Pancreatic Cancer

Inflammation has been identified as a significant factor in the development of pancreatic cancer. Both hereditary and sporadic forms of chronic pancreatitis may be associated with increased cancer risk [73]. iNOS and COX-2 have been demonstrated to be overexpressed in both chronic pancreatitis and pancreatic cancer, thereby providing a possible link between inflammation and tumor development. An immunohistochemical co-expression of iNOS and COX-2 has been shown in pancreatic adenocarcinoma by Kong *et al.* [74]. COX-2 overexpression positively correlated with high Ki-67 expression, i.e., tumor proliferation, while high iNOS expression was significantly associated with a high apoptotic index. Therefore, the activities of the two enzymes have been found to counteract each other. Although the authors did not explain this finding, they suggested that COX-2 up-regulation in pancreatic cancer might be an antagonistic pathway of an iNOS-induced apoptotic system and that NO-related apoptosis might be a result of various tumorigenic effects of NO, such as DNA damage, p53 mutation and oxidation by nitrotyrosin. In this study, no correlation was found between iNOS/COX-2 expression and either patient prognosis or degree of tumor angiogenesis.

Recently, Franco *et al.* [75] investigated the expression of iNOS and COX-2 protein expression in pancreatic cancer by Western blot analysis. They found a marked expression of both iNOS and COX-2 in tumor samples when compared with paired normal pancreatic tissue. Moreover, co-expression of the two enzymes was present in all the tumor samples. A moderate expression of COX-2 was also found in the surrounding non-neoplastic tissue, suggesting the involvement of this enzyme in early tumor development *via* chronic inflammation.

Contrasting results have been recently found by Kasper *et al.* [76]: they detected iNOS and COX-2 immunoreactivity in only 52.5% and 37.5%, respectively, of their 40 cases of pancreatic carcinomas. Moreover, they did not find any correlation between iNOS and COX-2 expression as well as between the expression of the two enzymes and degree of tumor angiogenesis assessed as microvessel density.

Taking together, these findings suggest that the role of NO/PGE_2 interaction and the underlying tumorigenic mechanisms in pancreatic cancer are not completely defined.

Other Types of Tumors

The possible opposite role of iNOS/COX-2 in ovarian tumors and tumor-associated macrophages was investigated by Klimp *et al.* [77]. They found an overexpression of both iNOS and COX-2 not only in malignant tumors (adenocarcinomas) but also in borderline and benign ovarian tumors (cystadenomas), demonstrating the involvement of both the enzymes in the progression of ovarian tumorigenesis. On the contrary, only a small proportion of the tumor-associated macrophages were found to express iNOS and COX-2, i.e. were in an activated state against tumor cell proliferation. The authors suggested that ovarian tumors can release mediators that can suppress iNOS/COX-2

Table 1. Interaction Between Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) and its Possible Tumorigenic Effect(s) in Premalignant and Malignant Conditions

Premalignancy and/or malignancy	Methods of iNOS and COX-2 investigation	Tumorigenic effect(s)	Ref.
Colonic aberrant crypt foci	Enzymatic activities	Not investigated	[57]
Colorectal cancer and cell lines	Enzymatic activities, mRNA and protein expression, immunostaining	Stimulation of angiogenesis	[58-60]
Barrett's esophagus and esophageal adenocarcinoma	mRNA and protein expression, immunostaining	Inhibition of apoptosis (?)	[62, 64]
Gastric cancer	mRNA expression, immunostaining	Association with p53 mutation	[65-67]
Gastric MALT lymphoma	Immunostaining	Increase in cell proliferation Association with p53 mutation	[69]
Head and neck cancer and cell lines	mRNA and protein expression, enzymatic activities, immunostaining	Stimulation of angiogenesis Association with p53 mutation	[70-72]
Pancreatic cancer	Protein expression, immunostaining	Increase in cell proliferation (?)	[74,75]
Ovarian adenocarcinoma	Immunostaining	Not investigated	[77,78]
Hepatocellular carcinoma	Immunostaining	Stimulation of angiogenesis	[79]
Lung carcinoma	Immunostaining	Stimulation of angiogenesis	[80]
Lymphocytic thyroiditis and thyroid tumors	Protein expression, immunostaining	Not investigated	[81]
Astrocytic gliomas	Immunostaining	Stimulation of angiogenesis	[82]

expression in tumor-associated macrophages and thus, their tumoricidal capacity.

Raspollini *et al.* [78] evaluated iNOS and COX-2 expression in 78 stage III G3 cases of ovarian cancer. They found that immunohistochemical positivity for the two enzymes is associated with a poor prognosis after surgical and chemotherapeutic treatments. Moreover, this study showed that both iNOS and COX-2 negative ovarian carcinomas are correlated with complete clinical response to first-line chemotherapy.

Co-expression of iNOS and COX-2 has been shown in 100 hepatitis C virus-positive (HCV) hepatocellular carcinoma samples obtained from patients who underwent curative hepatectomy [79]. Although only COX-2 was found to significantly correlate with intratumor microvessel density, combined negative tumor expression of both iNOS and COX-2 provided a significant advantage to patient survival. The authors concluded that iNOS and COX-2 overexpression might be caused by a secondary effect of cytokines produced in response to HCV infection or by the direct activation of the HCV core protein. Moreover, the impact of iNOS and COX-2 on prognosis of patients with HCV-positive hepatocellular carcinoma might be attributable to modulation of angiogenesis by COX-2.

Marrogi *et al.* [80] demonstrated that expression of iNOS and COX-2 significantly correlated with both VEGF expression and microvessel density in non-small cell lung cancer. These findings suggest that stimulation of angiogenesis is one of the most important mechanisms involved in iNOS- and COX-2 mediated carcinogenesis in this type of lung tumor.

The possible involvement of iNOS and COX-2 in the pathogenesis of lymphocytic thyroiditis and thyroid tumors has been investigated by Nose *et al.* [81]. They found a stepwise increase in immunoreactive expression of both iNOS and COX-2 in epithelial cells from lymphocytic

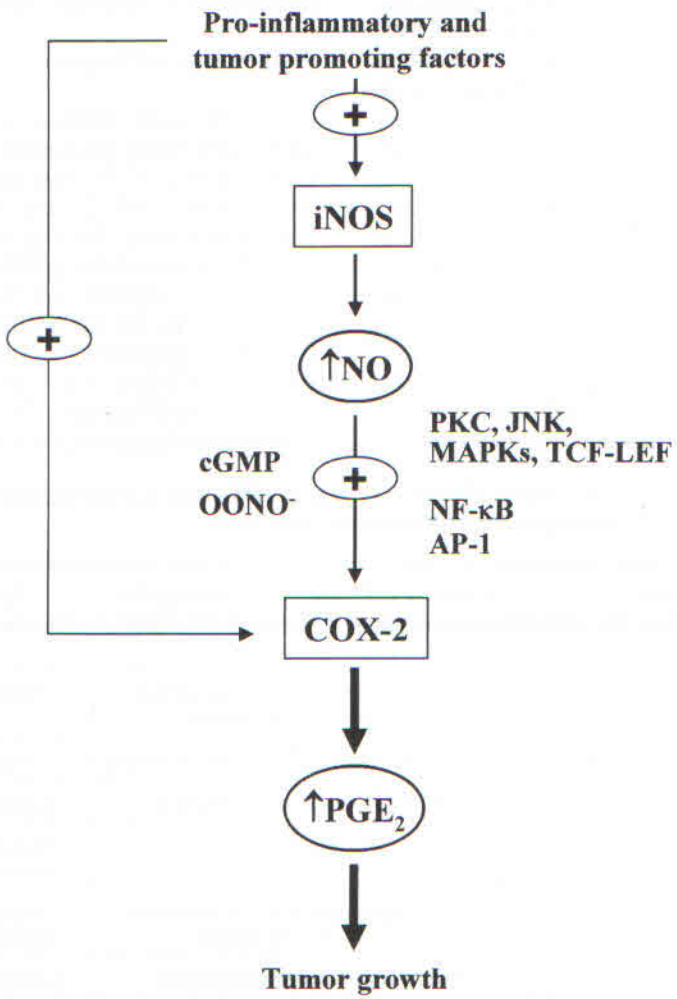

Fig. (2). Proposed model of nitric oxide (NO) signalling in cyclooxygenase-2 (COX-2) activation and enhancement of tumor growth. Pro-inflammatory and tumor promoting agents stimulate the activation of both inducible nitric oxide synthase (iNOS) and COX-2. iNOS produces NO that enhances both activity and expression (*via* a transductional and transcriptional regulation) of COX-2. COX-2 activation leads to the production of large amounts of tumor-promoting prostaglandin E_2 (PGE_2). Therefore, dual inhibition of iNOS and COX-2 is ideal strategy for cancer chemoprevention and therapy. AP-1, activator protein-1; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PKC, protein kinase C; TCF-LEF, T-cell factor-lymphocyte enhancing factor. + denotes stimulation; ↑ denotes increase.

thyroiditis and follicular adenoma to papillary carcinomas, well-differentiated and poorly differentiated types, and follicular carcinomas. Moreover, the levels of the two enzymes were significantly correlated in all cases of thyroid disease. These findings suggest that iNOS and COX-2 pathway interaction may be involved in the early phases of thyroid tumorigenesis and provide a possible link between inflammation and carcinogenesis.

Angiogenesis plays a key role in the development of astrocytic gliomas. Hara *et al.* [82] examined the immunohistochemical expression of COX-2, iNOS and VEGF in 51 high-grade astrocytomas including 31 glioblastomas (grade IV) and 20 anaplastic astrocytomas (grade III), 49 low-grade astrocytomas (grade II) and 43 reactive astrogliosis specimens. A stepwise increase of COX-2, iNOS and VEGF expression was found from astrogliosis, through low-grade to high-grade astrocytoma. Moreover, COX-2 expression was significantly correlated with iNOS, VEGF and intratumor microvessel density, whereas iNOS expression was weakly associated with degree of angiogenesis. The authors concluded that iNOS/COX-2 interaction may contribute to astrocytic tumorigenesis by promoting new vessel formation.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

As summarized in Table (**1**), iNOS and COX-2 pathways and their interaction seem to be potentially involved in the majority of human solid tumors. Although the nature of this cross-talk is complex, most evidence points to a stimulatory effect of NO on both COX-2 activity and expression. From the majority of the above reported studies, it can be inferred that iNOS and COX-2 products may represent a common final pathway controlling various tumorigenic mechanisms. Among these, stimulation of tumor angiogenesis appears to be the most frequently involved. However, the products of the iNOS pathway did not appear to directly correlate with specific angiogenic markers, such as intratumor microvessel density or VEGF expression. This observation suggests a more direct link of COX-2 than iNOS in the induction of angiogenesis and the possibility that the potential proangiogenic role of NO is mainly mediated by inducing COX-2 activity and PGE_2 production. As a general mechanism, it might be hypothesized that the ability of iNOS to promote cancer cell growth is largely dependent on COX-2 induction. Fig. (**2**) summarizes a possible model of signalling pathways for iNOS-mediated COX-2 induction in tumor cells.

The selective inhibition of COX-2 in cancer has become one of the most actively investigated areas in molecular-targeted anti-tumor therapy. However, the majority of studies are limited to the development of selective inhibitors of COX-2 activity. It is likely that the blocking of COX-2 expression in cancer cells by inhibiting up-stream stimulating factors, such as NO, may offer a more effective therapeutic solution than only neutralizing the activity of existing COX-2 enzyme. Consistent with this concept, combination treatment with iNOS and COX-2 inhibitors may provide either a cooperative or a synergistic anti-tumor effect.

Notably, both iNOS and COX-2 have been shown to be expressed in inflammatory disease (i.e. Barrett's esophagus, *H. pylori* gastritis, pancreatitis, ulcerative colitis, primary sclerosing cholangitis) and in the cancers arising from these diseases. The interrelationship between these two enzymes may provide a mechanistic link between hyper-inflammatory states and cancer susceptibility. In this perspective, iNOS and COX-2 inhibition would appear to be the most proximal and optimal target for chemoprevention of inflammation-related tumors.

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ABBREVIATIONS

ACF	=	Aberrant crypt foci
AOM	=	Azoxymethane
AP-1	=	Activator protein-1
cGMP	=	Cyclic GMP
COX	=	Cyclooxygenase
EGF	=	Epidermal growth factor
HCV	=	Hepatitis C virus
HNSC	=	Head and neck squamous cancer
IL	=	Interleukin
JNK	=	Jun N-terminal kinase
LPS	=	Lipopolysaccharide
MALT	=	Mucosa-associated lymphoid tissue
MAPK	=	Mitogen-activated protein kinase
NF-κB	=	Nuclear factor-κB
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
PEA3	=	Polyoma enhancer activator 3
PG	=	Prostaglandin
TCF-LEF	=	T-cell factor-lymphocyte enhancing factor
TNF	=	Tumor necrosis factor
VEGF	=	Vascular endothelial growth factor

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