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NO to Breast: When, Why and Why Not?

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Abstract

Nitric oxide is a pleiotropic ancestral molecule, which elicits beneficial effect in many physiological settings but is also tenaciously expressed in numerous pathological conditions, particularly breast tumors. Nitric oxide is particularly harmful in adipogenic milieu of the breast, where it initiates and promotes tumorigenesis. Epidemiological studies have associated populations at a greater risk for developing breast cancer, predominantly estrogen receptor positive tumors, to express specific polymorphic forms of endothelial nitric oxide synthase, that produce sustained low levels of nitric oxide. Low sustained nitric oxide generates oxidative stress and inflammatory conditions at susceptible sites in the heterogeneous microenvironment of the breast, where it promotes cancer related events in specific cell types. Inflammatory conditions also stimulate inducible nitric oxide synthase expression, which dependent on the microenvironment, could promote or inhibit mammary tumors. In this review we re-examine the mechanisms by which nitric oxide promotes initiation and progression of breast cancer and address some of the controversies in the field.

Keywords

Breast cancer; oxidative stress; estrogen; angiogenesis; inflammation; nitric oxide; proliferation

INTRODUCTION

Nitric oxide (NO) is an active physiological signaling molecule, which is expressed either constitutively or can be induced to modulate the fate of some cell types through specific mechanisms [1–3]. While the physiological role of NO in specific tissues (cardiovascular and brain) is well appreciated, its role in some pathological conditions, particularly in breast cancer, are still perplexing and somewhat alarming [4–6]. The beneficial or harmful effects of NO is dependent on the concentration/duration of NO exposure and the levels of reactive

nitrogen species/reactive oxygen species (RNS/ROS) generated, together with the genetic make up of the targets and the surrounding microenvironment [7–12]. Though not free of controversies, a consensus is emerging that low and sustained level of NO generated from endothelial nitric oxide synthases (eNOS) or inducible nitric oxide synthase (iNOS) promotes breast cancer progression [13–15]. The first evidence that NOS is expressed in breast cancer was provided by Thomsen *et al.* [15]. These investigators demonstrated that breast cancer tissue had higher NO biosynthesis and NO synthase activity when compared with normal breast tissue; whereas in normal breast tissue, the NO biosynthetic capability was either low or not detectable. NOS activity was detected only in invasive tumor tissue. Furthermore, these investigators demonstrated that for invasive ductal carcinomas, NO biosynthesis correlated positively with tumor grade. NOS was detected predominantly with the tumor associated macrophages but was also expressed in vascular endothelial cells as well as the myoepithelial cells [15].

There are strong evidences that eNOS promotes initiation and progression of estrogen (E2)-induced sporadic ductal breast cancer (SDBC), which accounts for at least 70% of all known breast cancer in women [16, 17]. Compelling evidence, accumulated over many decades, has shown that estrogens are the major causative agent in the genesis and development of human sporadic ductal breast cancer [18–20]. Why and how low concentrations of NO promotes transformation of specific cell types in the adipose rich micro environment of the mammary gland remains poorly understood. In this review, we will re-examine the properties and pathways that enable NO in specific milieu of the mammary gland to promote breast cancer. We will also discuss the controversial role of iNOS in breast cancer together with evaluating the possibilities whether inhibition of eNOS or even iNOS in the breast would be beneficial in terms of controlling the disease.

NITRIC OXIDE SYNTHASES IN NORMAL MAMMARY GLANDS AND IN BREAST CANCER

Nitric Oxide Synthases in Normal Mammary Glands

In normal mammary gland, the NOSs are present not only in the endothelium of blood vessels but also in the epithelial and myoepithelial cells [21,22]. Though not directly proven, there has been some speculations that NO could have a functional role in the physiology of the mammary glands, particularly in post-pubertal growth and differentiation stages via expression of eNOS [23]. Beneficial role of NO has been demonstrated in rat mammary gland ductal epithelium, which secretes NO to control its own blood supply [24]. There has also been implication of functional roles of iNOS produced by the macrophages, which are an integral part of both normal mammary gland development and mammary tumors [25].

Association of NOS Expression with Progression of Breast Cancer

While the role of NO in normal mammary glands is still speculated, there is an astounding volume of studies that have strongly associated NO with mammary tumorigenesis [26–32]. NO formation has been elegantly demonstrated in both benign and malignant breast tumors using spin-trapping electron paramagnetic resonance (EPR) spectroscopy [26]. eNOS and iNOS are expressed in a large number of human breast tumors, where they correlate with

tumor grades [27, 28]. Active iNOS is higher in less differentiated breast carcinoma, where it is primarily in tumor infiltrating macrophages [29]. The expression of iNOS was positively correlated with angiogenesis (and high VEGF expression), microvascular density, tumor size and poor survival [29, 30]. Apart from strong association with tumor grade, iNOS expression has also been correlated with short disease free survival in human breast cancer patients [31]. In breast carcinoma eNOS expression has been strongly correlated with that of iNOS not only in surrounding stroma but also within carcinoma cells [32]. However the role of iNOS in metastasis of breast cancer cells to the bone remains inconclusive [33].

Role of NOS in Breast Cancer: Lessons from Animal Studies

Experiments in animal models have established that NOS play a critical role in breast tumor progression. Contributory role of NO in mammary tumor progression comes from murine mammary adenocarcinoma model, where spontaneous mammary tumors were formed in female C3H/HeJ mice that received subcutaneous implants of tumor cells that express NOS [34]. Treatment of these mice with L-NAME and N-methy-L-arginine, which are inhibitors of NOS, had antitumor and anti metastatic effect [34]. In another prominent animal model, expression of the Polyoma middle T-antigen (PyV-mT) under the control of the mouse mammary tumor virus (MMTV) long terminal-repeat enhancer/promoter induced mammary tumorigenesis in the transgenic mice [35]. In this MMTV-induced tumor model, latency of tumor formation was increased when the mice were crossed into an iNOS-deficient background [35]. Furthermore, tumors in PyV-mT/iNOS^{-/-} mice were more differentiated than the tumors that were found in PyV-mT mice [35]. Genetic modifiers have been shown to modulate mammary tumor latency in mouse models of breast cancer where FVB strain was susceptible while B6 was resistant to tumor induction [36]. A complex relationship where tumor and host derived NO differentially regulates breast carcinoma metastasis to the lungs has also been demonstrated [37].

NO promotes cancer related events like angiogenesis, proliferation, apoptosis, invasion and metastasis. Genetics, microenvironment and various mechanisms cooperate to enable low sustained levels of NO to promote initiation and progression of mammary tumors.

OXIDATIVE STRESS AND BREAST CANCER

Association of NO, Oxidative Stress and Breast Cancer in Epidemiological Studies

Chronic inflammation is strongly associated with the development and progression of breast cancer [38–40]. Prominent mediators of the inflammatory responses are cytokines, free radicals, prostaglandins and growth factors, which can induce genetic and epigenetic changes including point mutations in tumor suppressor genes, DNA methylation and post-translational modifications, causing alterations in critical pathways responsible for maintaining the normal cellular homeostasis and leading to the development and progression of cancer [41–43]. NO and free radical species derived from NO are key components in inflammation and oxidative stress-accelerated tumorigenesis [44,45]. Oxidative stress entails breaching oxidant defenses to an extent that can initiate tissue damage. A close relationship between expression of nitrotyrosine, eNOS and anti-oxidative enzymes was found in breast cancer samples studied, where cancer cells increase the expression of anti-oxidants to

counteract the oxidative effects of NO [46]. It has been shown that women who have high iron intake and higher levels of iron-generated oxidative stress are at a greater risk of breast cancer [47]. A strong association between oxidative stress, chronic inflammation and breast cancer comes from epidemiological studies where dietary antioxidants have been found to react with endogenous sources of pro-and anti oxidants to reduce breast cancer risk [48,49]. Dietary curcumin reduces iNOS induction by LPS in mammary glands and scavenge NO radicals, which might explain at least partly, its therapeutic properties in inflammation in breast cancer [49]. Curcumin also reduces the risk of breast cancer by down regulating inflammatory cytokines CXCL-1 and 2 in breast cancer cells via NF- κ B [50]. Changes in oxidative stress markers seem to be a function of duration of exposure to E2, which elicit pro inflammatory changes and oxidative stress in certain forms of breast cancer [51–53]. Vitamin C, α -naphthoflavone and anti-oxidant butylated hydroxyanisole has been found to prevent E2-induced mammary tumors and decrease oxidative stress in female ACI rats [54,55].

Level of Oxidant and Anti-Oxidant Enzymes in Breast Cancer

Further evidence that induction of oxidative stress compromises cellular anti-oxidative functions comes from a study which has found an association between multilocus genotypes across 10 genes encoding proteins in the anti-oxidant defense system and breast cancer risk [56]. Exogenous and endogenous modulators of oxidative stress could modify polymorphism of myeloperoxidase (MPO), which is an endogenous oxidant enzyme that generates reactive oxygen species, and breast cancer risk [57,58]. A complex interplay of genetic and non genetic factors in breast cancer becomes evident in a study where overweight women with high activity eNOS and MPO genotype are at a higher risk of acute toxicity from radiation therapy following lumpectomy for breast cancer [59]. There is also evidence that genetic polymorphism in the manganese superoxide dismutase (MnSOD) gene may be associated with an increased risk of male breast cancer [60]. MnSOD polymorphism was found to contribute to breast cancer risk in women with high alcohol consumption, but was not an overall risk factor for breast cancer in the premenopausal population examined [61]. Over expression of SOD could be a promising strategy to treat breast cancer by inhibiting heparinase and enhancing the efficacy of heparin [62]. Modifications in catalase genotype also suggest a role for oxidative stress in the association of hormone replacement therapy with postmenopausal breast cancer risk [63]. NO-induced oxidative stress in breast carcinoma can also up regulate anti-oxidative enzymes like manganese superoxide dismutase (MnSOD) and catalase [46]. Therefore, in breast cancer patients the antioxidant status (vitamin A, C, E, glutathione peroxidase, glutathione reductase, superoxide dismutases and catalases) was found markedly reduced, while the oxidant status (hydroperoxides, carbonyl proteins, conjugated dienes, triglycerides and cholesterol) was elevated.

NO and Oxidative Stress in Breast Cancer

The site of inflammation in a mammary gland, where multi step transformation process is initiated is a unique microenvironment comprising of cells that produce NO as well as those that are targeted and probably altered. NO, participates in diverse chemical reactions and multiple chemical pathways to cause damage to critical cellular macromolecules and induce

biological responses [64,65]. Some of the major determinants of the biological responses of NO depend on cells that produce it, such as the rate of NO production, rate of its diffusion, intracellular consumption, and auto oxidation to form reactive nitrogen species (RNS), such as nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), which rapidly decomposes to NO^+ and nitrite (NO_2^-). Since NO mediated specific chemical reactions and chemical pathways in the adipogenic rich microenvironment of a mammary gland are poorly understood, we will summarize some of the general reactions of NO that have been elucidated. NO^+ has been found to nitrosylate protein macromolecules through oxidation of thiols, heme, Fe-S clusters and other non heme iron prosthetic groups and also cause DNA base deamination [66–68]. NO also interacts with reactive oxygen species (ROS, O_2^-), which are generated by various enzymatic reactions and chemical processes that routinely occur in normal cells, to form peroxynitrite (ONOO^-) [69,70]. Inability of the cells and the microenvironment to quench the ROS (O_2^- , $\text{OH}^\cdot$) and RNS (NO_2 , N_2O_3 , ONOO^-) can induce stress, damage DNA and proteins, impair cellular functions and enhance inflammatory reactions. NO and NO-derived reactive nitrogen species together with oxidative stress, induce DNA damage (such as nitrosative deamination of nucleic acid bases, transition and/or transversion of nucleic acids, alkylation and DNA strand breakage) and inhibition of DNA repair enzymes (such as alkyl transferase and DNA ligase) through direct or indirect mechanisms. Gene mutation and/or transformation together with cell death of wild-type cells could contribute to clonal selection of adapted cells and the acquisition of apoptosis resistance, and thereby promote tumor progression.

ADIPOSE RICH MILIEU IN THE MAMMARY GLAND AND NO

Mammary gland is mostly white fat comprising of adipocytes, preadipocytes, macrophages, endothelial cells, fibroblasts and leukocytes. The multifarious cell types that compose the mammary fat also render it as an important mediator of inflammation where many inflammatory cytokines including tumor necrosis factor alpha ($\text{TNF-}\alpha$) and monocyte chemoattractant protein-1 (MCP-1) are secreted [71]. Adipose tissue is also an independent endocrine organ on its own, which secretes various hormones or adipokines, prominent being leptin and adiponectin [72,73]. Breast cancer is associated with lipid alteration and enhanced oxidative stress linked to high leptin levels in overweight patients [74–77]. In pathological situations excess of RNS and ROS target susceptible cells, which could undergo macromolecular damages at different levels initiating various cellular changes, which is in sharp contrast to normal mammary gland microenvironment, where RNS/ROS probably scavenge each other out partly due to multifaceted active cellular defense system. One study has reported signs of pro-inflammatory/genotoxic switch in the mammary fat adjacent to the mammary tumors where leptin, adiponectin, $\text{TNF-}\alpha$, IL-6 and lipid peroxidation was higher than other areas of the gland [78]. However, given the dynamic nature of homeostatic systems, increases in oxidative damage do not necessarily suggest that the cell is succumbing to oxidative stress. NO in specific microenvironments can also scavenge ROS, where it can exert protective action through reaction with alkoxyl and peroxy radical through lipid peroxidation [79].

Emergence of tumors only in selected sites in a mammary gland highly implicates the presence of heterogeneous microenvironments in the mammary glands. It is reasonable to

assume that there are susceptible sites in the mammary gland of high risk women where persistent inflammatory conditions could play a pivotal role in inducing cellular proliferation, cytotoxicity and tissue injury that initiate the process of transformation and tumorigenesis. There is a high probability that generation of low NO for prolonged period generates excessive reactive species, which damage the DNA or post-translationally modify proteins of nearby epithelial and stromal cells at these susceptible spots in high risk mammary glands [80]. This could stimulate infiltration of inflammatory cells, which further release RNS, ROS and cytokines to exacerbate the inflammatory reaction. eNOS, has also been found to regulate the expression of the pro inflammatory molecules like nuclear factor- κ B (NF- κ B) and cyclooxygenase-2 (COX-2), which can also modulate the expression of eNOS [81]. The inflammatory cytokines also induce iNOSs in the already complex site of inflammation in the mammary gland and breast tumors, which further damage DNA and modify protein structure/function.

We are limited in our knowledge of the cell types that produces eNOS, the amount of NO produced, the production and accumulation of RNS/ROS that is specific to the adipogenic microenvironment, the cell types that are targeted by RNS/ROS and the type of modifications that are induced to promote transformation. It is not known whether sustained low levels of eNOS in mammary tumors are produced by excessive proliferation of eNOS expressing cells from the normal mammary gland or whether there are mesenchymal stem cells that were present embedded in the stroma of the mammary glands. If we are to increase our understanding of breast cancer as a disease, we should closely examine the initial events at the site of inflammation.

NO AND ANGIOGENESIS IN THE PROGRESSION OF BREAST CANCER

Perhaps, one of the most prominent and well understood pro carcinogenic effects of eNOS in breast cancer progression is its ability to promote angiogenesis, which is a key step in solid tumor progression [81–88]. Transformed mammary cells proliferate to increase in mass, and become dependent on establishment of vascular supply or angiogenesis, which is a conventional mechanism in which a new blood supply grows from an existing vessel. Angiogenesis in mammary tumors can be stimulated by inflammation, which induces proliferation and morphogenesis of vascular endothelial cells in response to a large number of cytokines or angiogenic molecules produced by tumor and host cells. In female C3H/HeJ mice it has been elegantly demonstrated that NO is a key mediator of C3L5 mammary tumor induced angiogenesis, and the antitumor effects of L-NAME are partly mediated by reducing tumor angiogenesis [81,82]. *In vivo* studies have also shown that caveolin-1 (a molecule that binds to and inhibits eNOS) and caveratin, which is a cell-permeable peptide derived from caveolin, impairs NO-dependent tumor blood flow and results in a dramatic tumor growth delay [83,84]. Some of the key angiogenic molecules are vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factor (bFGF), transforming growth factor alpha and beta (TGF- α , - β) and tumor necrosis factor- α (TNF- α). Tumor growth enhancing effects of VEGF are associated with increased NOS activity and inhibition of apoptosis in human breast carcinoma xenografts [85]. High grade nitrotyrosine staining, a biomarker for peroxynitrate formation from NO, was increased in more than 50% of invasive breast carcinomas analyzed and correlated with VEGF-C levels and lymph node

metastasis [86]. More direct role of eNOS in VEGF-induced angiogenesis and vascular permeability is demonstrated in a mice model where VEGF increases angiogenesis in both iNOS^{+/+} and iNOS^{-/-} mice but not in eNOS^{-/-} mice [87]. Mechanistic insights have been gained from cell cultures studies *in vitro* where VEGF, via a positive feedback loop, increases eNOS –phosphorylation and activity via PI-3 kinase/Akt pathway, along with induction of calcium flux and recruitment of heat shock protein 90 [88]. PGE₂ increases endothelial cell sprouting (the first step in neo-angiogenesis) through the NO/cyclic guanosine 3', 5'-monophosphate (cGMP) pathway [88]. The importance of VEGF in angiogenesis is amplified by the presence of various polymorphisms in its gene, where a strong association of VEGF-2578A and -1498C genotypes along with eNOS -786 TT and eNOS 894 GG genotype, is a significant predictor of breast cancer status after adjusting for non-genetic risk factors [89,90].

NO also participates in new vessel formation, though the mechanisms remain poorly understood. Recently it was demonstrated that NO stimulated the recruitment of bone-marrow-derived cells at the site of ischemic and/or neoplastic tissues that participated in new vessel formation [91]. Defects in angiogenesis in ischemic tissues of eNOS^{-/-} mice could be rescued by injection of stem/progenitor cells that were derived from wild-type mice [92]. However, this failed to correct angiogenic defects if ischemic insults were applied after the bone-marrow reconstitution, which indicates a defect in the mobilization of stem/progenitor cells from the bone marrow in eNOS^{-/-} mice [93]. VEGF-induced mobilization of endothelial progenitor cells, 5-fluorouracil-induced MMP9 activation and soluble KIT ligand production were all shown to be defective in eNOS^{-/-} mice [94]. However, baseline and ischemia-induced mobilization of endothelial progenitor cells was unaffected. The contribution of NO to tumor angiogenesis through bone-marrow-derived-cell recruitment has yet to be shown. Further studies are needed to clearly elucidate the role of NO in tumor angiogenesis through this mechanism.

NO INCREASES PROLIFERATION OF BREAST CANCER CELLS

Sustained low NO in a mammary gland microenvironment appears to be favorably positioned to promote mammary tumor progression by increasing cell proliferation. We have demonstrated that sustained low NO concentrations increase the proliferation of numerous breast cancer cell lines by modulating a number of key cellular targets and pathways [95]. To simulate *in vivo* situations where cells are exposed to sustained NO, we use DETA-NONOate, which is one of the longest acting NO donor available [95]. One of the critical cellular targets of low NO in a number of breast cancer cell lines was RAS, a potent oncogene, whose activation elevated the signaling through PI-3 Kinase/Akt and Raf/MEK/ERK1/2 cascades [95]. Deregulation of proliferation pathways and protein synthesis has been strongly implicated in the pathogenesis of breast cancer and activated ERK and Akt, which are highly up regulated, have been identified as the main drivers of these tumors [96,97]. Some of the downstream targets of low NO were found to be mammalian target of rapamycin (mTOR) and p70S6kinase, which are the critical regulators of the translational machinery [95]. We also observed that treatment of breast cancer cells with low concentrations of NO increased thymidine uptake, which is a direct indicator of DNA synthesis [98]. In breast cancer cells low concentrations of NO increased proliferation,

protein and DNA synthesis and also enhanced cell cycle progression from G1/S phase by increasing the levels of cyclin D1 and ornithine decarboxylase (ODC), both of which are up regulated in mammary tumors (Fig. 1) [95,99,100]. While in our studies the effects of low NO were cGMP independent, there are reports that NO in MCF-7 cells increased the activity of extra-cellular signal regulated kinase (ERK) through cGMP dependent mechanisms [101]. These concentrations of NO was also found to activate Akt and stabilize hypoxia inducible factor- α (HIF- α), which is up regulated in breast cancer [7]. Therefore low concentration of NO, through multi cellular targets can increase proliferation by both cGMP independent and dependent mechanisms. In MCF-7 cells, eNOS has been demonstrated to inhibit apoptosis, increase survival capability and proliferation of cells [102]. Another pro-cancerous effect of eNOS mediated RNS and ROS can impact on apoptosis pathway at key points prominent among them being JNK, p38 MAP kinase [103]. NO-induced S-nitrosylation of caspases, can produce apoptosis-resistant cells and facilitate further accumulation of mutations and subsequent clonal selection [104].

POLYMORPHISMS OF NITRIC OXIDE SYNTHASE GENE IN BREAST CANCER

Recent studies on the possible association between polymorphisms of the eNOS gene and susceptibility of women to breast have produced contradictory results [105]. This would therefore depend on the amount of NO produced and whether the NO leads to growth of tumor by a direct action or by increasing angiogenesis or by decreasing tumor growth by apoptosis of tumor cells. Strong indications that eNOS could play a major role in breast cancer progression come from epidemiological studies, which have identified populations that express specific polymorphisms in the eNOS gene to be at a greater risk for developing breast cancer [105–110]. The functional implication of expressing polymorphic forms of eNOS is evident because it is associated with an increased cleavage rate of eNOS protein, which results in reduced NO concentrations [105]. eNOS gene (NOS3), located on chromosome 7q36 in humans, exhibit genetic variants of three clinically relevant polymorphisms, which are T (–786) C in the promoter, the VNTR in intron 4 and Glu298Asp variant in exon 7 [106,107]. Studies found that Asp-298 and C (–786) variants were more common in whites while VNTR 4a variant was significantly higher among the black populations [106,108]. In addition to race specific polymorphic forms of eNOS, specific eNOS polymorphisms have also been reported among various ethnic populations. A case control trial in Caucasian women showed an increased risk for breast cancer in women with a mutant genotype of the exon 7 eNOS polymorphism, while no statistical effect was noted in intron 4 polymorphism [105]. Exon 7 eNOS polymorphism could have lethal consequences because it is inherited [105]. Although more studies have reported positive correlations between eNOS polymorphism and breast cancer risks, there are a few reports where this correlation was not found. In particular was a study conducted among Spanish populations where no association between eNOS Glu298Asp and breast cancer risk was found [109,110]. A positive association has also been found between eNOS polymorphism and reduced vascular invasion and metastatic spread in breast cancer [89,90].

A strong positive correlation between expression of eNOS and the presence of estrogen receptors (ER) in human breast tumors has been consistently found in many studies [111]. Further studies also find a positive correlation between ER+ breast tumors expressing specific eNOS-786T>C polymorphisms, which expressed lower levels of eNOS, to breast cancer recurrence and death. There has also been a very interesting observation where breast cancer survivors expressing eNOS 786CT/TT polymorphism suffer from hot flashes, which highly correlates with increased angiogenesis [112].

eNOS VS. iNOS IN BREAST CANCER

eNOS and Estrogen-Induced Breast Cancer

Exposure to E2 is a risk factor for sporadic ductal breast cancer, which accounts for 70% of all breast cancers [113–115]. Epidemiologic studies have established that eNOS expressing breast cancers were predominantly positive for estrogen and progesterone receptors (ER, PR) and positively correlated with aggressiveness and poor survival of the patients [16,17,107,111]. Further evidence that E2 could regulate NOS expression comes from human breast cancer cell lines, which on acquiring E2 independence express reduced levels of eNOS and iNOS [116]. E2 induction of eNOS was demonstrated in MCF-7 breast cancer cell line, which was mediated by phosphorylation of glycogen synthase 3 beta [117]. Prolonged E2 exposure induces eNOS through a genomic pathway, which ensures long-term NO availability (Fig. 3) [118,119]. Non genomic pathways by which E2 increased both eNOS expression and its activation have been demonstrated by studies in endothelial cells where ER- α was localized to caveolae in the membrane, where they were coupled with eNOS to increase its activity [120]. The downstream signaling includes activation of tyrosine kinase-MAPK and Akt/protein kinase B signaling, stimulation of heat shock protein 90 binding to eNOS, and perturbation of the local calcium environment, leading to eNOS phosphorylation and calmodulin-mediated eNOS stimulation [121]. While in ER+ MCF-7 cells, eNOS expression can be stimulated by E2, iNOS could only be induced by a combination of cytokines [122]. Progesterone, which plays a critical role in E2-induced initiation and progression of mammary tumors, can enhance cytokine stimulated iNOS expression in MCF-7 cells [123].

Sole utilization of MCF-7 cell line to study the characteristics of ER+ phenotype, has severely limited our understanding of the possible mechanisms by which E2 and eNOS promotes SDBC in human. ACI rat model is a relevant biological system where prolonged exposure of near physiological concentrations of E2 induces mammary tumors which share many pertinent histopathologic and molecular features with human SDBC [124,125]. We have found increased expression of eNOS but not iNOS in atypical ductal hyperplasia (ADH) ductal carcinoma in situ (DCIS), which are histologically defined structures in E2-treated ACI rats (unpublished results, Pervin *et al.*). As we have mentioned earlier in this review, E2 induced oxidative stress to promote mammary tumors in ACI rats and treatment with antioxidants like butylated hydroxyanisole, α -naphthoflavone or vitamin C reduced the incidence of mammary tumors, increased tumor latency and decreased oxidative stress *in vivo* [51–55]. Since the initial events in E2-mediated transformation is poorly understood, studies in E2-treated ACI rats will enable us to better understand the mechanisms by which

E2 and eNOS induce oxidative stress and transformation of specific cell types in the adipose milieu of the mammary glands.

iNOS Inhibit Breast Cancer Growth in Certain Conditions

In this dismal picture of NO and its role in breast cancer, there are many other studies that show that iNOS can retard breast tumor growth by inducing cytotoxicity and apoptosis [126–134]. In contrast to eNOS, which is present constitutively, iNOS is transcriptionally up regulated in macrophages and endothelial cells in response to cytokines such as interferons (IFN- α , - β and - γ), IL-1 α and β , bacterial lipopolysaccharide (LPS) and hypoxia [126]. The iNOS gene expression depends upon the activation of transcription factors as nuclear factor kappa-B (NF- κ B), Janus kinase-signal transducer and activator of transcription (JAK/STAT), and c-Jun NH2-terminal kinase (JNK), while it is down regulated by steroids, transforming growth factor- β (TGF- β), IL-10, p53 and NO itself [127]. A number of studies find iNOS levels in breast tumors to be inversely correlated with tumor stage, grade and progression but positively associated with apoptosis and patient survival [128,129]. Strong support of the anti tumor effect of iNOS comes from *in vivo* studies where induction of iNOS in p53 positive tumors reduced its growth [130]. Induction of iNOS has been found to inhibit tumor angiogenesis along with attenuating endothelial and vascular smooth cell proliferation [131]. In several mouse models it has been shown that transduction of iNOS in tumor cells inhibits tumor growth and metastasis [132–134]. Furthermore, studies show that among the breast carcinoma cells that metastasizes to lung; those clones that expressed iNOS were less tumorigenic when compared to the clones that did not express high levels of NO [135,136]. Studies from our group and others have elucidated some of the mechanisms by which high concentrations of NO induces cytostasis and apoptosis in human breast cancer cells.

We have found that high NO induces a G1 cell cycle arrest in highly undifferentiated MDA-MB-231 by down regulation of cyclin D1 protein and hypophosphorylation of retinoblastoma protein [8]. NO-mediated down regulation of cyclin D1, a short half-life protein, was translational, which reinforces the importance of the translational machinery to maintain oncogenesis. NO targets the translational machinery by inhibiting the initiation steps of protein synthesis in a variety of cell types by phosphorylating and inactivating eukaryotic initiation factor 2- α (eIF2- α) (Fig. 2) [137]. Therapeutic drugs to specifically target translational machinery are also being evaluated. We have reported that high NO can induce cytostasis in a large number of breast cancer cell lines via HRI (heme regulated inhibitor) and protein kinase R (PKR) mediated suppression of the translational machinery [137]. High concentration of NO induces MKP-1 mediated inactivation of ERK1/2 and AKT, which mediate proliferation and survival signals in a panel of human breast cancer cell lines [138]. In addition to cytostasis, high concentrations of NO can induce apoptosis by promoting Bax migration and integration into the mitochondria, which initiates events like cytochrome c release and activation of caspases-3,-9 [139].

iNOS Loses its Killing Edge in Breast Cancer

Although induction of iNOS in human cells requires multiple cytokines and/or endotoxins that include IL-1 α , TNF- α , IFN- γ and LPS, the level of iNOS expression in human cells is

lower than in its rodent homologues [140,141]. In some tissues, iNOS mRNA expression is not associated with iNOS enzyme and NO production [142]. The variations in the effect of iNOS in breast tumors could be explained due to the inability of various biological systems and especially tumor microenvironments to sustain high NO concentrations [143]. Failure to produce sufficiently high NO concentration could be partially attributed to transcriptional inhibition of iNOS expression by TGF- β 1, which is over expressed in breast cancer [144]. Another prominent factor that may limit iNOS production appears to be arginase over expression, which is a common phenomenon in breast cancer [145]. The mean arginase activity in breast cancer tissues as well as serum arginase activity in patients with breast carcinoma has been reported to be significantly higher compared to those of normal healthy women [146]. After surgery this activity, decreased abruptly during the first week and normalized within 15–30 days; whereas in benign disease of the breast, the arginase activity was found to be normal [147]. NOS and arginine compete for arginine to form either NO, which contributes to the tumoricidal activity or polyamine production, that promote growth of tumor cells. The K_m for L-arginine is 2–20 mM for mammalian arginase and 1–20 μ M for various NOS isoenzymes depending on the source of the enzyme [148,149]. The V_{max} of arginase at physiological pH is more than 1000 times that of the NOS enzyme which indicates that both enzymes, arginase and NOS, can use arginine at comparable rates at low concentrations of arginine [150]. However, over-expression of arginase appears to favor the synthesis of polyamines while limiting the production of NO in breast tumors.

We and others have reported that while breast cancer cells are susceptible to NO-induced cytostasis/apoptosis, they are also dependent on polyamines for survival and proliferation [151–153]. MDA-MB-468 and BT-474 cells express very high levels of arginase and the expression patterns of arginase in these cells were found to be inversely proportional to the expression levels of NOS. ZR-75-30 with high levels of NOS corresponding to significantly low levels of arginase [152]. It has also been found that N-Hydroxy L-arginine (NOHA) is an intermediate in NO biosynthetic pathway and is a potent inhibitor of arginase. NOHA was found to arrest high arginase expressing breast cancer cells at S phase of the cell cycle and reduce spermine content [153]. Inhibition of arginase also induced apoptosis by activating caspase-8 mediated truncation of pro-apoptotic molecule BID [154]. Inhibition of iNOS by N-bromotaurine, which is produced by eosinophil-peroxidase-expressing infiltrating eosinophils, can lower the amount of NO generated in breast cancer [155]. Also in microvascularized tumors, NO is rapidly consumed by red blood cells (RBC) through S-nitrosylation of RBC glutathione and hemoglobin and by oxidation to nitrate by RBC oxy-hemoglobin.

NO-BASED THERAPY FOR BREAST CANCER

It is clear that NO plays a central role in the process of accelerated and sustained production of ROS/RNS, oxidative stress, inflammation and angiogenesis, which ultimately favor initiation and progression of breast cancer. Thus, developing selective inhibitors of NOS and NO-releasing agents may lead to important strategies for chemoprevention of breast cancer. There have been some approaches to inhibit NOS enzymatic activity by drugs or RNA interference or using scavengers of NO in other forms of cancers [156]. A number of dietary products like omega-3-fatty acids, Tanshinone IIA, which is a major compound extracted

from a herbal medicine and anti-inflammatory flavonoids have shown promising activity in reducing the risk of breast cancer by inhibiting NOS and the production of NO [157–159]. In breast cancer *in vivo* models, curcumin has been shown to inhibit cancer development through a mechanism that scavenges NO and inhibits iNOS expression by inhibiting NF- κ B activation [49,50]. It has also been shown that supplementation of diet with compounds like lycopene and melatonin reduced the levels of serum NO, provided anti-oxidant defense and exerted strong chemo preventive activity against DMBA-induced mammary tumors [160]. Apart from dietary components, which can mediate reduction of NO levels and breast cancer risk; 1400W, a potent compound, which is a selective inhibitor of iNOS over eNOS and has been found to significantly reduce growth of murine mammary tumors [11].

Apart from the proven benefits of inhibiting NOS and NO production to inhibit breast cancer, there has been confusing reports where some NO releasing compounds mediate anti invasive effects in the treatments against metastatic breast cancer [161]. Nitric oxide–donating nonsteroidal anti-inflammatory drugs (NO-NSAIDS) represent an emerging class of compounds, which have been demonstrated to have chemopreventive properties in pre-clinical models including breast cancer cell lines and animal tumor models, but not in humans [162]. A more complex therapeutic mechanism is that of tamoxifen, which is known to reduce E2-induced breast tumors, stimulates mitochondrial NOS (mtNOS) activity, and hampers mitochondrial respiration to induce apoptosis [163].

We had proposed, that one potential method of targeted delivery of high concentration of NO to breast cancer tumor would be to tag a slow releasing NO donor to tamoxifen [164]. Such targeted delivery of NO to the breast would potentiate the effects of radiation on tumor cells in addition to its tumoricidal effect, as NO is known for its ability to increase the sensitivity of resistant hypoxic cells to radiation [165].

On a promising note, studies have revealed that low concentration of NO-mimetics can attenuate hypoxia-induced drug resistance in tumor cells *in vitro*. Polymer delivery of a plasmid encoding for iNOS with expression restricted to the radiation field by radiation responsive promoters have been effective against murine tumors [166,167].

In one of our studies, we report that while some benefits of reducing NO in specific cancer types including breast is being appreciated, it is very perplexing when administration of NO to specific clinical settings can also elicit anti tumor effects [164].

CONCLUSIONS

It still remains poorly understood why and when in the mammary gland setting, NO suddenly or gradually becomes an accomplice of a vicious life threatening process such as mammary tumorigenesis. It is also ill understood why some women will succumb to the disease, while in others increased oxidative damage by their continued existence leads to adaptations that are reached as a consequence of exposure to ROS. We are still treading with extreme caution in the field of NO biology and breast tumors because lot more remains to be known. Although there have been numerous attempts to snuffle the deleterious effect of eNOS in the mammary glands by inducing endogenous iNOS or exogenously delivering

high amount of NO, extreme caution needs to be exercised. Technically we are not yet equipped with efficient systems that could deliver the killing concentrations of NO to the mammary tumors after which it should adequately quench residual low NO and prevent it from initiating unwanted effects. Although this simple molecule in mammary gland setting has created dark clouds and also bright lights, we will surely in near future grip these images in a meaningful way that could provide therapeutic benefit to control mammary tumors.

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ABBREVIATIONS

NO	Nitric oxide
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
cGMP	Cyclic guanosine3',5'-monophosphate
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
VEGF	Vascular endothelial growth factor
MMTV	Mouse mammary tumor virus
SOD	Superoxide dismutase
COX2	Cyclooxygenase-2
MMP9	Matrix metalloprotease 9
MCP-1	Monocyte chemoattractant protein-1
TNF-α	Tumor necrosis factor-alpha
TGF-β	Transforming growth factor-beta
bFGF	Basic fibroblast growth factor
NF-κB	Nuclear factor kappa-beta
ERK	Extra-cellular signal regulated kinase
ODC	Ornithine decarboxylase
JNK	Jun N-terminal kinase
E2	Estrogen
ER	Estrogen receptor
PR	Progesterone receptor
IFN-γ	Interferon-gamma

NOHA	N-Hydroxy L-Arginine
NSAID	Non steroidal anti-inflammatory drugs
eIF2-α	Eukaryotic initiation factor 2-alpha
HRI	Heme regulated inhibitor
PKR	Protein kinase R
DBMA	7,12-dimethylbenz(a)anthracene

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Fig. (1). Low physiological concentrations of NO induce tumor cell proliferation

Low NO activates Ras/Raf/MEK/ERK and PI3 Kinase/Akt pathways leading to the activation of mammalian target of rapamycin (mTOR), which promotes cell cycle progression and tumor cell proliferation via induction of cyclin D1 and ornithine decarboxylase (ODC). Eukaryotic translational initiation factor eIF4E and p70s6 kinase are direct down-stream targets of mTOR.

Fig. (2). Inhibition of tumor cell proliferation by high patho-physiological concentrations of NO High concentrations of NO induce either cytosclerosis or apoptosis in breast cancer cells via two distinct mechanisms. Protein kinase R (PKR) and heme-regulated inhibitor (HRI) are induced by high NO, leading to the phosphorylation of eukaryotic transcription factor EIF2- α . This results in phosphorylation of retinoblastoma protein (Rb) and down-regulation of cyclin D1 leading to cell cycle arrest and cytosclerosis. On the other hand, high concentration of NO can also unregulated MAP Kinase phosphatase (MKP-1), which dephosphorylates ERK and Akt and promotes bax integration to the mitochondria to release cytochrome C from the mitochondria to the cytosol. Caspase3/9 gets activated which results in induction of apoptosis.

Fig. (3). Estrogen induction of breast tumor

Estrogen can activate eNOS through genomic pathway as well as non-genomic pathway via phosphorylation of eNOS. This leads to sustained low NO production, which upregulates reactive oxygen species (ROS)/reactive nitrogen species (RNS) to induce oxidative stress and inflammation. Sustained low NO can also promote tumor cell angiogenesis by up-regulating vascular endothelial growth factor (VEGF) and increase in tumor cell proliferation via up-regulation of cyclin D1.