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The mammary gland is unique in that it grows and develops throughout the lifetime of a reproductive female and ovarian steroids play a central role in this growth and development. Estrogens are responsible primarily for stimulating ductal and stromal proliferation. The effects of estrogens are due, in part, to their ability to induce the local synthesis of growth factors such as insulin like growth factor-I (IGF-I). In turn, IGF-I activates ER-a through a hormone independent mechanism, however, the precise mechanism as well as the signal transduction pathways involved in the cross talk between IGF-I and ER-a are poorly defined. In fact, the ras-MAPK and the PI3K-AKT pathways has been shown to mediate the effects of IGF-I on ER-a activity. The original proposal was to test the hypothesis that the downstream mediator of these pathways is p70/S6 kinase. However, early results showed that the effects of IGF-I were not mediated by p70/S6 kinase. The revised aim was to test the hypothesis that the effects of IGF-I were mediated through the activation of nitric oxide synthetase by Akt. Preliminary results show that nitrite and nitrates, the breakdown products of nitric oxide, activate ER-a suggesting that the effects of IGF-I are mediated, in part, by activation of nitric oxide synthetase.

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Table of Contents

Introduction

Breast cancer

Epidemiological studies show that endocrine status is an important underlying factor in the risk for breast cancer. Prominent risk factors related to endocrine status include age at menarche and menopause and age at first full-term pregnancy. This increase in risk is thought to reflect the larger lifetime number of normal menstrual cycles. Oral contraceptives and replacement estrogens account for a small increase in risk in specific subgroups of women. On the other hand, a decrease in risk is associated with bilateral ovariectomy and earlier age at menopause reflecting the decrease in levels of estrogen. The primacy of estrogens in the epidemiology of the disease is due to the hormonal control of breast cell proliferation.

In the mammary gland, estrogens are primarily responsible for stimulating ductal and stromal proliferation, whereas progestins stimulate alveolobular development. The actions of estrogens are mediated by two isoforms of the estrogen receptor, alpha and beta. Although the precise role of ER-α and ER-β in mediating the effects of estradiol in the breast are not well defined, it is thought that ER-α mediates the mitogenic actions of estradiol while ER-β mediates an antimitogenic effect. In addition to a role in mammary gland development, ER-α is overexpressed in mammary tumorigenesis. As a consequence of the overexpression of ER-α, the early stages of breast cancer are frequently characterized by hormonal control of its growth. However, as breast cancer progresses, tumors become hormone independent for growth. The mechanisms responsible for the acquisition of a hormone independent phenotype are not well understood but hormone independent activation of the estrogen receptor by growth factor signal transduction pathways is thought to play a role in the malignant progression of the disease. The revised aim of this proposal tested whether insulin like growth factor-I activation of ER-α is mediated, in part, by activation of NOS and by nitrite and/or nitrate.

Nitrites and Nitrates

Human exposure to nitrites and nitrates occurs through both exogenous and endogenous sources as a result of the diet, environment, medicinal drugs, and as the natural breakdown product of nitric oxide. In the environment, nitrites and nitrates are naturally occurring inorganic ions that are part of the nitrogen cycle. Microbial action in soil and water converts organic nitrogen into ammonia in decomposing wastes. Ammonia is then oxidized to nitrite and subsequently to nitrate. In addition to naturally occurring nitrates, environmental exposure to nitrites and nitrates occur as a result of nitrogen based fertilizers. In agricultural areas, fertilizers are o a major source of contamination of wells and shallow groundwater aquifers that provide drinking water. During the spring melt and drought conditions, public water systems using surface water also show increased nitrate levels. A recent US Geological Survey showed that greater than 8,000 wells in the nation are contaminated with nitrate levels above the EPA drinking water standard of 10 ppm. Diet is also an important source of naturally occurring and added nitrites and nitrates. The use of nitrates (saltpetre) ocurred around 1700 and nitrates are still used today as preservatives and color enhancing agents in meat products and canned vegetables. Nitrite and nitrate exposure also occurs from medications including quinone derivatives (antimalarials), bismuth subnitrite (antidiarrheal), ammonium nitrate (diuretic), amyl and sodium nitrites (antidotes for

cyanide and hydrogen sulfide poisoning), and nitroglycerin and isosorbide dinitrate/tetranitrates (vasodilators).

In humans, nitrite and nitrate are natural breakdown products of nitric oxide. Nitric oxide plays an important role in host defense and in homeostatic and developmental functions as a result of its ability to act as either a generator of reactive oxygen molecules or as a signaling molecule. Nitric oxide is synthesized by a family of nitric oxide synthases (NOS) that include neuronal, endothelial, and inducible NOS. The expression of the inducible isoform of NOS is increased in response to cytokines such as TNF-alpha, INF-gamma, and IL-1 and 2 while the activity of endothelial and neuronal NOS is regulated by calcium-calmodulin. In addition, the activity of endothelial NOS, the form of NOS found in breast cancer cells, is increased upon phosphorylation by Akt, a serine/threonine kinase that mediates the cellular response to growth factors such as epidermal growth factor (EGF) and insulin like growth factor I (IGF-I), two growth factors critical to breast cancer development. Nitric oxide synthases catalyze the reaction of molecular oxygen with L-arginine to form nitric oxide and citrulline. Nitric oxide is highly reactive and has a biological half-life of seconds to minutes depending on its concentration and chemical environment. The major oxidative breakdown products of nitric oxide are nitrite and nitrate. In the cell, nitrates are also reduced to nitrite by nitrate reductase.

In addition to neurotransmission, vasodilation, and inflammation, there is evidence that nitric oxide and/or its metabolites have endocrine like effects. In older women, intermittent use of nitrates is associated with increased bone mineral density. In the neuronal NOS knock out mouse, more aggressive and inappropriate sexual behavior is observed. In several breast cancer animal models, nitric oxide promotes tumor progression. There is also evidence linking nitric oxide to the human disease. In breast tumors, the expression of nitric oxide synthase is associated with increased proliferation, tumor grade, and expression of progesterone receptor and nitric oxide is associated with invasive disease. Breast cancer patients also have elevated serum levels of nitrite and nitrate. Recent studies have also shown that hormone related cancers are elevated among farmers. Based on preliminary results and published studies, this proposal will test the hypothesis that nitrites and nitrates play a role in the etiology and progression of breast cancer, in part, due to their ability to activate ER-α.

Estrogen receptor: alpha and beta

The estrogen receptors are divided into domains, termed A through F. Region A/B is the variable N-terminal region of the receptor which has a modulatory effect on the transactivation of transcription through a domain referred to as activation function 1 (AF-1). The central domain, region C, is a short well conserved cysteine rich region which contains the DNA binding domain and a weak constitutive dimerization domain. Region E is the hormone binding domain and contains 12 alpha-helices (H1-H12) folded into a three layered antiparallel alpha-helical sandwich. Upon ligand binding, a conformational change is induced resulting in the repositioning of helix H12 and the formation of the activation function 2 (AF-2) domain. AF-2 is the binding site for steroid receptor coactivators. The hormone binding domain also functions to inhibit the DNA binding domain of the receptor. In the absence of hormone, the ER is sequestered in an inactive, repressed complex with molecular chaperones such as HSP90. Interestingly, NOS has been shown to bind to HSP90

and preliminary studies in our laboratory show that NOS also binds to the N-terminus of ER-α suggesting that NOS may be part of the inactive complex. It is possible that activation of NOS in close proximity to receptor results in the production of nitrite or nitrate that subsequently bind to and actiavate the receptor. A similar model has been proposed for the activation of ER-α by other anions.

In addition to estradiol, growth factors, specifically EGF and IGF-I, activate ER-α in the absence of hormone. Although growth factors have been clearly shown to activate ER-α, the signal transduction pathways involved in the cross talk are poorly defined. In fact, the ras-MAPK and the PI3K-AKT pathways have been shown to mediate the effects of growth factors on ER-α activity. Phosphorylation of the N terminus appears to be important in hormone dependent as well as in hormone independent activation of ER-α. The N terminus of ER-α contains several conserved serine residues, S104, S106, S118, and S167, within the AF-1 region that are targets for phosphorylation. Although phosphorylation is thought to play an important role in receptor activity, phosphorylation does not always correlate with transcriptional activity of the receptor suggesting a more complex mechanism for hormone independent activation of the receptor. In support of a more complex mechanism, several studies demonstrate that ligand independent activation of ER-α is not universal but is dependent on the cell type, signaling pathway, and the concentration of growth factor. High concentrations of EGF activate ER-α in the absence of estradiol and potentate the effects of the hormone, while low concentrations of EGF actually inhibit hormone activation of the receptor providing additional evidence in support of a complex mechanism of cross talk between growth factors and ER-α. This proposal tested the hypothesis that nitrite/nitrate, synthesized in response to activation of growth factor receptors, interacts with the hormone binding domain of ER-α to activate the receptor suggesting a role for nitrites and nitrates in the progression of breast cancer.

Body

To determine whether nitrite and/or nitrate have estrogen like activity, their ability to mimic the effects of estradiol in the estrogen responsive breast cancer cell line, MCF-7, was tested. The MCF-7 cells were treated with either 10^{-9} M estradiol, 10^{-6} M nitrite, or 10^{-6} M nitrate in the presence or absence of the antiestrogen ICI 182,780 (5×10^{-7} M). Following treatment, cells were counted at day four and progesterone receptor expression was measured at 24 hours. Preliminary results demonstrate that, similar to estradiol, nitrite and nitrate increased cell growth by approximately 9.5-fold (figure 1) and induced the expression of progesterone receptor by approximately 2.5-fold (figure 2). The effects on cell growth and gene expression were blocked by the antiestrogen, ICI 182,780, suggesting the effects of nitrite and nitrate are mediated by ER-α. To determine whether these compounds activate ER-α, a transient cotransfection assay was employed. Wild type ER-α and an estrogen response element (ERE)-CAT reporter construct were transiently cotransfected into CHO cells and the cells were treated with estradiol, nitrite, or nitrate for 24 hours (figure 3). As expected, estradiol stimulated CAT activity by approximately 2.5-fold. Nitrite and nitrate increased CAT activity by approximately 2.5-fold and the increase was blocked by the antiestrogen providing additional evidence that these compounds activate ER-α. To determine whether nitrite binds to ER-α, the effect of the compound on hormone binding to purified recombinant ER-α was measured using a single dose ligand binding assay. As shown in figure 4, nitrite blocked the binding of estradiol to ER-α with a Ki of approximately 10^{-10} M suggesting a high affinity interaction of nitrite with the receptor.

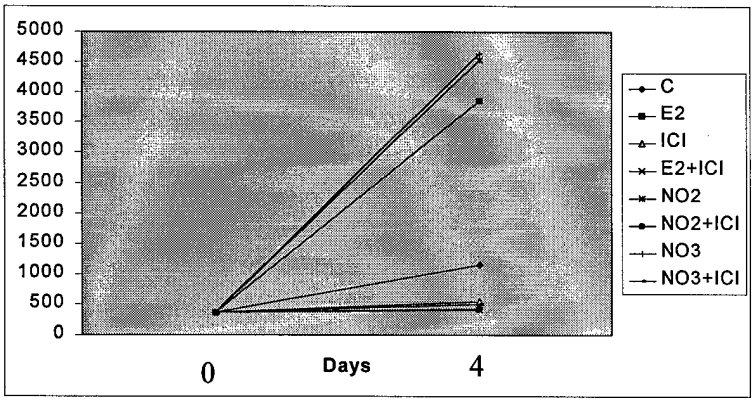

Figure 1. Effects of nitrite and nitrate on the growth of MCF-7 cells. MCF-7 cells were plated and grown in IMEM supplemented with 5% FCS. When the cells reached 40% confluence, the medium was changed to phenol red-free medium supplemented with 5% CCS for 2 days. Cells were treated with either 10^{-9} M estradiol, 10^{-6} M nitrite, or 10^{-6} M nitrate in the presence or absence of the antiestrogen ICI 182,780 (5×10^{-7} M). Following treatment cells were counted at day four. The results are the mean value of two experiments done in triplicate.

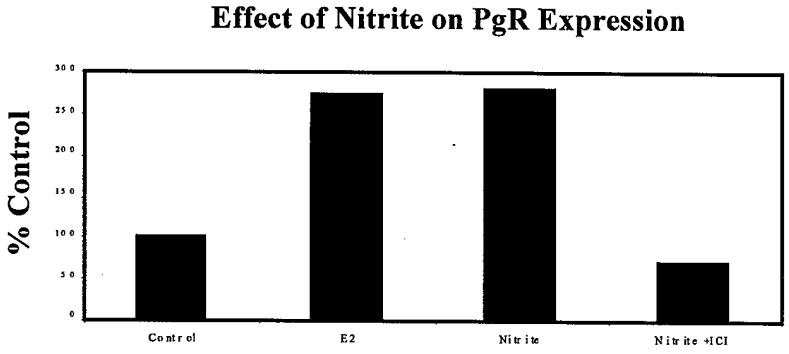

Figure 2. Effect of nitrite and nitrate on progesterone receptor mRNA.

MCF-7 cells were grown as described in figure 1. At 80% confluence, the medium was changed to phenol red-free IMEM and 5% CCS. Cells were grown in this medium for 2 days and then treated for 24 hours with 10^{-9} M estradiol, 10^{-6} M nitrite or 10^{-6} M nitrate in the presence or absence of $5x10^{-7}$ M ICI 182,780. PgR mRNA was determined by an RNase protection assay. Results are expressed as a percentage of untreated cells.

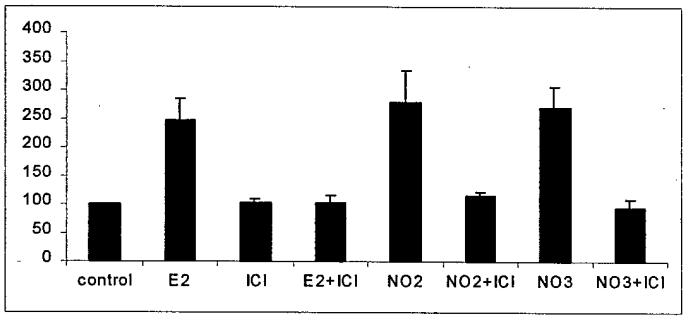

Figure 3. Effect of nitrite and nitrate on ER-α activity

Wild type ER-α was transiently transfected with an estrogen response element-CAT construct into CHO cells. The transfected cells were treated for 24 hours with 10^{-9} M estradiol, 10^{-6} M nitrite or 10^{-6} M nitrate in the presence or absence of $5x10^{-7}$ M ICI 182,780. CAT activity was measured and normalized to β-galactosidase. Results are expressed as percent of untreated control cells (mean of 3 experiments).

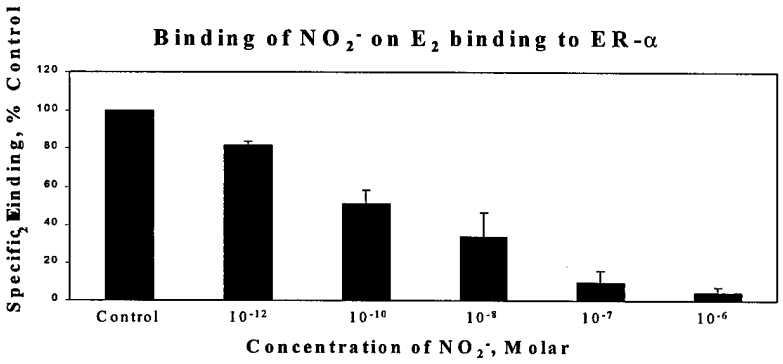

Figure 4. Effect of nitrite on estradiol binding to ER-α

Human recombinant ER-α (4×10^{-9} M) was preincubated for 1 hour at 4^0C with 10^{-6} M nitrite and then treated with 10^{-8} M [^{3}H]estradiol in the presence or absence of a 200-fold molar excess of DES for 2 hours at 37°C. The amount of specific binding was determined and expressed as a percentage of untreated cells. Results represent the mean value of 3 experiments +/- S.D.

Key Research Accomplishments

1. Nitrite and nitrate induce the growth of hormone dependent breast cancer cells in the absence of estrogen.

2. Nitrite induces the expression of the estrogen regulated gene, progesterone receptor, through an estrogen receptor dependent mechanism .

3. Nitrite and nitrate activatet estrogen receptor alpha in transient transfection assays.

4. Nitrite competes with estradiol for binding to the estrogen receptor.

Reportable Outcomes

1. Two grants have been submitted based on the work supported by this award.

2. An abstract was presented based on the results of this work.

Conclusions

1. The results suggest that nitric oxide synthetase is a downstream mediator of the effects of IGF-I on estrogen receptor activity.

2. The results also suggest that low dose exposure due to environmental or occupational exposure activate the estrogen receptor and may increase the risk of breast cancer.

3. Nitric oxide synthetase may be an important target for prevention and treatment strategies.