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The Role of Redox Signaling in the Molecular Mechanism of Tamoxifen Resistance in Breast Cancer

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THE ROLE OF REDOX SIGNALING IN THE MOLECULAR MECHANISM OF TAMOXIFEN RESISTANCE IN BREAST CANCER.

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by

Nana Aisha Garba

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To: Interim Dean Michele Ciccazzo
Robert Stempel College of Public Health and Social Work

This dissertation, written by Nana Aisha Garba, and entitled The Role of Redox Signaling in the Molecular Mechanism of Tamoxifen Resistance in Breast Cancer, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2012

DEDICATION

To my mother, Fatima Omonego Garba and My Son

Jayden Dangtiem Dama, whom I pray will exceed all of my accomplishments, and regard this dissertation as evidence that the sky is his limit.

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I gratefully acknowledge the support and encouragement of my family and friends over the years. My journey would have been more uphill without every single one of you. I wish to thank my major professor, Dr. Deodutta Roy and members of my committee Dr. Quentin Felty, Dr. Jai Parkash, and Dr. Ophelia Weeks for their invaluable advice and time commitment. I am also grateful to the University Graduate School for providing me with the dissertation year fellowship which enabled me to complete my dissertation in a timely manner.

ABSTRACT OF THE DISSERTATION

THE ROLE OF REDOX SIGNALING IN THE MOLECULAR MECHANISM OF TAMOXIFEN RESISTANCE IN BREAST CANCER.

by

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Professor Deodutta Roy, Major Professor

The emergence of tamoxifen or aromatase inhibitor resistance is a major problem in the treatment of breast cancer. The molecular signaling mechanism of antiestrogen resistance is not clear. Understanding the mechanisms by which resistance to these agents arise could have major clinical implications for preventing or circumventing it. Therefore, in this dissertation we have investigated the molecular mechanisms underlying antiestrogen resistance by studying the contributions of reactive oxygen species (ROS)-induced redox signaling pathways in antiestrogen resistant breast cancer cells. Our hypothesis is that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment of cells as a result of oxidative stress. The hypothesis of this dissertation was tested in an *in vitro* 2-D cell culture model employing state of the art biochemical and molecular techniques, including gene overexpression, immunoprecipitation, Western blotting, confocal imaging, ChIP, Real-Time RT-PCR, and anchorage-independent cell growth assays. We observed that tamoxifen (TAM) acts like both an oxidant and an antioxidant. Exposure of tamoxifen resistant LCC2 cell to TAM or 17 beta-estradiol (E2) induced the formation of reactive

oxidant species (ROS). The formation of E2-induced ROS was inhibited by co-treatment with TAM, similar to cells pretreated with antioxidants. In LCC2 cells, treatments with either E2 or TAM were capable of inducing cell proliferation which was then inhibited by biological and chemical antioxidants. Exposure of LCC2 cells to tamoxifen resulted in a decrease in p27 expression. The LCC2 cells exposed to TAM showed an increase in p27 phosphorylation on T157 and T187. Conversely, antioxidant treatment showed an increase in p27 expression and a decrease in p27 phosphorylation on T157 and T187 in TAM exposed cells which were similar to the effects of Fulvestrant. In line with previous studies, we showed an increase in the binding of cyclin E–Cdk2 and in the level of p27 in TAM exposed cells that overexpressed biological antioxidants. Together these findings highly suggest that lowering the oxidant state of antiestrogen resistant LCC2 cells, increases LCC2 susceptibility to tamoxifen via the cyclin dependent kinase inhibitor p27.

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LIST OF ACRONYMS

AE	Anti-estrogen
AI	Aromatase Inhibitor
AP-1	Activated protein 1
ARE	Antioxidant response element
ATP	Adenosine Triphosphate
Cdk	Cyclin-dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CIP/KIP	Cdk inhibiting and kinase inhibiting proteins
CREB	cAMP response element-binding
Cys	Cysteine
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUOX1	Dual oxidase 1
E2	17β-estradiol
EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen response element
ERK	Extracellular signaling kinase
ER+	Estrogen receptor positive

FOXO	Forkhead box class O
GPCR	G-protein-coupled receptors
GSSG	Glutathione disulfide
GSH	Glutathione
GPx	Glutathione Peroxidase
HBV	Hepatitis B virus
H_2O_2	Hydrogen peroxide
HUVEC	Human umblical vein and endothelial cells
IGF-IR	Insulin-like growth factor I receptor
IP3	Inositol trisphosphate
$LOO^{\cdot}$	Peroxyl radicals
MAPK	Mitogen activating protein kinase
MKP	Mitogen activated protein kinase phosphatase
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NRF-1	Nuclear respiratory factor 1
$O_2^{\cdot -}$	Superoxide
$^1O_2$	Singlet oxygen
OH^-	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PDGF	Platelet derived growth factor
Prx	Peroxiredoxin
PTEN	Phosphatase and tensin homolog
PTK	Protein tyrosine kinase

PTP	Protein tyrosine phosphatase
PTP1B	Protein-tyrosine phosphatase 1B
PI3K	Phosphatidylinositol 3-kinases
LMW-PTP	Low molecular weight PTP
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RS^-	Thiolate
RSH	Thiol
RSO^-	Sulfenate
RTK	Receptor Tyrosine Kinase
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
$-SO_2H$	Sulfinate
$-SO_3H$	Sulfonate
SAPK	Stress activated protein kinase
TNFα	Tumor necrosis factor alpha
Mn(SOD)	Superoxide dismutase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRF-1LKO	NRF-1 liver knock-out
NO_2	Nitrogen dioxide
$NO^·$	Nitric oxide
TAM	Tamoxifen

TGFα	Transforming growth factor beta 1
TGFβ1	Transforming growth factor beta 1
Trx	Thioredoxin
4-OHT	4-hydroxy-tamoxifen

I. INTRODUCTION

The fact that a majority of breast cancer express estrogen receptor alpha (ERα), has informed the use of antiestrogens as one of the drugs of choice for ER+ breast cancers. Anti-estrogens which act by inhibiting the function of ERs include tamoxifen, raloxifen and fulvestrant[1]. Tamoxifen is the most commonly used treatment for patients with ER+ breast cancer and its use as a breast cancer preventative agent and an adjuvant therapy in early cancer has been shown to improve the overall survival[2, 3]. Furthermore, the extensive use of tamoxifen has made a significant contribution to the reduction in breast cancer mortality seen over the last two decades.

However, in spite of the obvious benefits of tamoxifen in breast cancer treatment, almost all patients with metastatic breast disease and as many as 40% of patients receiving adjuvant tamoxifen eventually develop resistance and die from their disease[1]. Hence the issue of tamoxifen resistance has been a major setback in an otherwise successful treatment of ER+ breast cancer.

Though a number of pathways have been proposed and explored, to date there has been no clear mechanism implicated in the development of anti-estrogen resistance. A hitherto unexplored mechanism involves the role of reactive oxygen species (ROS) in the mechanism of tamoxifen resistance. (ROS) comprise of either partially reduced metabolites of oxygen such as superoxide anions ($O_2^{-\bullet}$); peroxyl radicals ($LOO^{\cdot}$) and hydroxyl radicals ($OH^{\cdot}$) or non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$); which have a higher reactivity than molecular oxygen[4]. They are

products of cellular metabolism which have been shown to induce oxidative stress. An excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer[5, 6]. Furthermore, ROS induced oxidative stress has been shown to initiate a number of different responses including cell proliferation and transformation; alteration of intracellular redox state and the oxidative modification of certain signaling proteins resulting in the post translational modification[7] of their downstream targets. Interestingly, like estrogen, tamoxifen has been shown to induce ROS formation, making it probable that tamoxifen induced ROS generated as a result of prolong tamoxifen treatment of breast cancer patients may play a vital role in the development of tamoxifen resistance.

As protection against oxidative stress, cells have several enzymic and nonenzymic antioxidants or reductants that maintain the intracellular redox environment in a principally reduced state. Typically the enzyme superoxide dismutase (SOD) catalyses the conversion of O_2^- to H_2O_2, and then the H_2O_2 generated is degraded to H_2O by several cellular enzymes, usually Catalase or glutathione peroxidase coupled with glutathione reductase[8, 9]. It is however interesting to note that in most cancer tissue the levels/ activity of manganese superoxide dismutase and Catalase have been found to be almost always low. Making a case for the existance of a pro-oxidative environment in cancer cells that may result in an oxidative stress surge and redox signaling culminating in uninhibited cell proliferation.

Hypothesis and Specific Aims

We hypothesize that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment, as a result of chronic oxidative stress. We further postulate that an increase in reactive oxygen species (ROS) levels promotes either the loss of p27 inhibitory function or NRF-1 activation through the inactivation of protein tyrosine phosphates (PTPs) with consequent changes in p27 and/or NRF-1 phosphorylation.

To test this hypothesis, we explored the following Specific Aims:

Aim 1. To investigate whether anti-estrogens prevent estrogen-mediated progression of cell cycle by counteracting estrogen-induced ROS signaling. To further investigate if reducing the oxidative environment of anti-estrogen-resistant breast cancer cells restores the anti-proliferative action of tamoxifen.

Aim 2. To determine mechanisms by which a pro-oxidative state may promote p27 inactivation, and thus cell cycle progression.

Aim 3**.** To determine mechanisms whereby a pro-oxidative state may promote oxidation of protein tyrosine phosphatase PTEN, NRF-1 activation and cell cycle progression.

Findings of this study will elucidate the roles of the cellular redox state, redox signaling pathways, PTPs and p27 in anti-estrogen resistance, and may lead to new therapeutic strategies to delay or even prevent this important clinical problem. This is a new line of

research that may lay the groundwork for clinical trials of anti-estrogens plus antioxidant-based drugs for the prevention and treatment of estrogen-dependent breast cancer.

II. LITERATURE REVIEW:

THE ROLE OF REDOX SIGNALING IN THE MOLECULAR MECHANISM OF TAMOXIFEN REISTANCE IN BREAST CANCER TREATMENT

Abstract

Anti-estrogens are one of the classes of drugs used to treat ER+ breast cancer. Since their discovery they have been the drugs of choice in breast cancer treatment; prevention and adjuvant therapy for early breast cancer cases. Although anti-estrogens have been beneficial to a large number of patients, unfortunately about 40% of breast cancer patients and a majority of patients with metastatic breast disease who were initially responsive to the drugs will relapse and die of the disease. In spite of the current break-through in our knowledge of the molecular mechanisms that contribute to resistance, anti-estrogen resistance remains a major setback in the treatment of ER+ breast cancer. In this review we examine the previously unexplored role of reactive oxygen species (ROS) and redox signaling in the molecular mechanism that contribute to breast cancer resistance to anti-estrogens.

Introduction

In the majority of invasive breast tumors estrogen receptor alpha (ERα) is often up-regulated while ERβ is down-regulated. This finding is the basis for the therapeutic use of

antiestrogens, which are drugs that disrupt the actions of estrogen and ERα[1]. The types of antiestrogens used include those that inhibit the function of estrogen receptors such as tamoxifen, raloxifene, and ICI 182,780 (fulvestrant), or the aromatase inhibitors such as letrozole and exemestane which block the conversion of androgens into biologically active estrogens.Over the years the successful use of these groups of drugs has been plagued by the emergence of drug resistance, which to date remains a major problem in the treatment of breast cancer.

Herein, we reviewed the role of estrogen and its receptors in the development of breast cancer and the function of tamoxifen as an antiestrogen. We also examined the role of reactive oxygen species (ROS) as signaling molecules and how they may induce antiestrogen resistance by altering the post-translational status of certain signaling proteins which are key players in the cell cycle.

An overview of Estrogen and ER action

The human breast like that of other mammalian species contains both epithelial and mesenchymal components and a vast majority of malignant tumors are epithelial in origin[1].

In normal breast cells, estrogen and estrogen receptors are critical regulators which enable the mammary epithelial cells to undergo proliferation, differentiation, apoptosis and remodeling in the course of the mammalian reproductive lifespan[1, 10, 11].

Studies have shown that estrogens initiate different growth responses in tissues depending on the cell type, the dose and the timing of exposure [12]. Moreover, it is now generally accepted that, in addition to its role in the promotion of breast tumors, estrogen also supports the growth of pre-neoplastic and malignant cells through ER-mediated signaling pathways[13].

The conventional model of estrogen action is based on its binding to ERα/β receptors, which initiates transcription either directly by binding to estrogen response elements (EREs) in the promoter region of target genes or indirectly through the phosphorylation of signaling proteins involved in cell cycle progression [14, 15]. Overall, estrogen regulates the expression of many genes important for normal cell physiology and growth of some breast tumors [16].

Both experimental and epidemiologic data suggest that estrogen plays a significant role in carcinogenesis[17 , 15]. In malignant breast tissue, the action of estrogen and its steroid receptors is dysregulated, resulting in a shift to proliferation without differentiation and apoptosis, and an increase in the percentage of epithelial cells expressing ERα. Additionally, cell proliferation makes a change from paracrine to autocrine growth [18] . It is noteworthy that the risk of breast cancer is, in part, due to the duration of proliferation without differentiation.

Estrogen Receptors (ER)

Normal human breast cells contain two types of E2 receptors, namely ERα and ERβ, which are the products of separate genes. ERα is expressed in 15 – 30% of the luminal epithelial cells (so called estrogen receptor positive cells) while ERβ is more widely expressed in the mammary tissue[1;19, 20]. ERβ is co-expressed with ERα in the luminal epithelial cells, but can also be found in the myoepithelial cells as well as in the stromal cells. In the normal luminal epithelial breast cells, estrogen-dependent proliferation occurs in a paracrine manner with ERα containing cells producing growth factors that induce proliferation in adjacent estrogen-negative cells.

Deletion of both ERα and ERβ genes has revealed the significance of E2 for normal female sex organ development and function [20;21]. Both *in vivo* and *in vitro* studies in mammals have postulated that E2 plays a considerable role in the maintenance of bone[22], vascular integrity, and brain function. Estrogen has also been shown to be a significant growth and survival factor for human breast cancer cells [23, 24]. ERs act principally as nuclear transcription factors, an event that is enhanced by ligand binding. Though a separate pool of receptors for E2 in the cytoplasm and plasma membrane have been identified, the mechanisms of action and cellular functions of these proteins are just beginning to be unraveled.

Estrogen Receptors: Nuclear/genomic pathway

The 'genomic action' of E2 occurs after a time-lag of at least 2 h following E2 stimulation. This action accounts for some hormone functions in physiological and

pathological situations [25, 26].The nuclear ER contains domains lettered A through F, with activation function (AF)-1 (ligand independent) and AF-2 (ligand dependent) facilitating the transactivation of target genes[27].

In the so called ligand dependent pathway, ER functions in the nucleus as a transcriptional regulator of specific genes. The receptor protein has a ligand-binding domain (AF-2), several transcription activation domains, and a DNA-binding domain that interacts with specific regions in the promoter of target genes, including sites known as estrogen-responsive elements (ERE) [28-30]. The binding of estrogen to ER induces phosphorylation of the receptor, alters its conformation, and triggers receptor dimerization.

The ER protein in the nucleus can also modify transcription of genes through protein-protein interactions. In this way, ER can function much like a coactivator protein itself by binding to other transcription factors and recruiting acetyltransferases to complexes bound to activator protein or SP-1 sites on DNA[31]. This is how estrogen helps to regulate genes encoding proteins such as cyclin D1, insulin-like growth factor I receptor (IGF-IR), and collagenase. ER has also been to be able to decrease the expression of many genes[32].

In the ligand-independent pathway, the estrogen receptor translocates to the nuclear membrane from the cytosol and can activate transcription in a ligand-independent fashion[33]. These transcriptional activities of ER have been called its classical or genomic

activity. From a functional perspective, a more appropriate term is nuclear-initiated steroid signaling.

Membrane-initiated Estrogen signaling

In contrast to the nuclear effects, ER functions that can occur very rapidly in the cell before new gene transcription takes place have been identified. This membrane-initiated steroid signaling could occur outside the nucleus or even in the cell membrane [34]. A number of studies based on endothelial and breast cancer cells have been able to show that a small group of ER is positioned outside the nucleus in the cytoplasm or bound to the plasma membrane[35, 36 37, 38]. It is thought that the presence of a membrane-bound ER may explain the previously identified short-term effects of estrogen (occurring within minutes) in cultured cells[39, 40]. Further investigations have indicated that E2 rapidly activates signaling, such as calcium flux,[41] phospholipase C activation [42] and inositol trisphosphate (IP3) generation. Most studies suggest that these actions necessitates E2 binding to ERs. In neural cells, ERs can activate protein kinase C and protein kinase A. They can also uncouple opioidergic and gabanergic receptors from their effector signaling molecules[40]. These signaling events are likely to be as a result of E2 activation of G proteins. Therefore, ERs appear to be part of the large family of G-protein-coupled receptors (GPCR). After several G proteins are activated, E2-ER can then trigger signaling cascades that terminate in a cell biological function.

Antiestrogen (Tamoxifen) Action

Tamoxifen belongs to a class of therapeutic agents called the selective estrogen receptor modulators (SERMs). These drug function as estrogen receptor (ER) antagonists in breast tissue and as ER agonists in the endometrium, heart and bones[43]. 4-hydroxy-tamoxifen is an active metabolite of tamoxifen, which has been shown to compete for binding with natural estrogen to the ERα with high affinity[44, 45].

Subsequent to the antagonist binding to ERα, a different 3-dimensional structure is elicited in the receptor, which renders it unable to enhance specific gene expression. Therefore antiestrogens interrupt the estrogen-induced signals, which can result in the inhibition of cell proliferation, tumor growth arrest, and induction of apoptosis[46; 47, 48].

In vitro tamoxifen has been shown to induce G1 phase cell cycle arrest in ER-positive breast cancer cells exposed to estrogens. [1; 49, 50;51] creating an avenue by which genes that control cell cycle progression could significantly impact drug sensitivity and resistance. Furthermore, tamoxifen has the capacity to indirectly decrease levels of cyclin D1[52] .

In vivo, tamoxifen inhibits estrogen-stimulated growth of MCF7 xenografts in mice and prevents development of breast cancer in the NMU and DMBA rodent models[53, 54]. Similar to estrogens, tamoxifen increases the expression of IGF-I and vascular endothelial cell growth factor in the uterus.[55, 56] In clinical specimens acquired sequentially from patients with breast cancer, administration of tamoxifen has been

shown to reduce proliferation, upregulate ER expression, downregulate TGFα and induce stromal TGFβ1[57-60].

Other classes of therapeutic agents are the selective estrogen receptor down regulators (SERDs) and aromatase inhibitors (AI). SERDs include ICI182780 (Fulvestrant), which is a pure estrogen antagonist with a 100-fold greater affinity for ER than tamoxifen. By binding ER it inhibits receptor dimerization and abrogates estrogen signaling[17].
Aromatase inhibitors are a second line therapy for post–menopausal patients who have progressed after tamoxifen treatment. They act by inhibiting the action of aromatase which is an enzyme necessary for the conversion of androgens to estrogens.
It is interesting to note that the majority of tumors that develop resistance over time continue to express ERα[10;11] . Furthermore, many of the tamoxifen-resistant tumors still respond to fulvestrant and AIs, indicating that estrogen remains an important regulator of tumor growth under these circumstances[10]. These data provide support for the idea that endocrine targeted therapies can lead to the activation of novel signaling pathways that evade the effects of antiestrogens.

Redox signaling

Redox Signaling is process whereby activated or free radicals and reactive oxygen species act as messengers in the biological system. It involves a vital and continuous process by which human cells communicate with each other and carry out essential functions in the body. Redox signaling often entails oxidation-reduction specific reactions.

Unlike signal transduction which usually involves both reversible and irreversible modifications of second messengers and proteins, redox signaling entails at least one reversible reaction involving the oxidation of a signaling molecule by a reactive species. It may therefore be inferred that the reaction of reactive oxygen or nitrogen species with their target is akin to the on-off signaling associated with phosphorylation than it is to the nonenzymatic lipid peroxidation.

According to Forman et al., [61] redox signaling occurs when at least one step in a signaling pathway involves one of its components being specifically modified by a reactive species through a reaction that is chemically reversible under physiological conditionsand/or enzymatically catalyzed.

Signaling molecules: Reactive Oxygen or Nitrogen species

Both reactive oxygen and nitrogen species are known redox signaling molecules. Reactive nitrogen species (RNS) are molecules having nitrogen atoms, which because of their chemistry, are regarded as being highly reactive towards other cellular components. These molecules may or may not have unpaired electrons. Examples of RNS include radicals like Nitric Oxide ($NO^{\cdot}$), Nitrogen dioxide ($NO_2^{\cdot}$) and non rasicals like Peroxynitrite ($ONOO^{-}$). On the other hand, Reactive oxygen species (ROS) constitutes either partially reduced metabolites of oxygen such as superoxide anions ($O_2^{-\bullet}$); peroxyl radicals ($LOO^{\cdot}$) and hydroxyl radicals ($OH\cdot$) or non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$); which have a higher reactivity than molecular

oxygen. ROS are by-products of normal cellular aerobic metabolism and the exposure of cells to certain forms of stress results in an increase in the level of ROS generated. It is note worthy that high level of reactive oxygen species are injurious to cells because of their tendency to react with a number of intracellular targets, such as proteins, lipids, and DNA. Conversely, quite a number of studies have shown that reactive oxygen species posses a variety of physiologic functions at low concentrations. These functions include but are not limited to: the regulation of gene transcription [62], signal transduction pathways [63-66] mitosis [67], apoptosis [67, 68], and senescence [68, 69]. Additionally, it has been shown that the stimulated production of oxidants plays a vital role in the mitogenic response to many growth factors [63, 66, 70] For example, ROS have been shown to play a vital role in cell growth mediated by 17β-estradiol (E2),[71] peptide growth factors and cytokines including PDGF [66], vascular endothelial growth factor, insulin, and tumor necrosis factor[72].

Regarding their role as signaling molecules, some reactive oxygen species such as H_2O_2 , are considered key signaling molecules, while others appear to be particularly harmful to living systems[73]. In higher organisms, low to moderate concentrations of NO and ROS are utilized as signaling molecules for other normal cellular functions like: their physiological roles in the cellular responses to oxygenation, as in the defense against infectious agents; the initiation of a number of cellular signaling systems and the induction of a mitogenic response [72, 74, 75]. Furthermore, exposure to a variety of non-physiologically significant concentrations of ROS or RNS that induce oxidative stress but do not kill cells can stimulate responses such as repair, adaptation, or transformation[76].

On the other hand, excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer [5, 5, 6]. as well as other metabolic diseases like diabetes mellitus[72]. Tumor cells have been shown to have a high and persistent oxidative stress [77]. Free radicals are involved in initiation [78] as well as promotion/ progression stages of tumorigenesis [79, 80] while inhibitors and scavengers of ROS inhibit these stages of tumor development [81, 82].

Based on work done on the release of ROS by non phagocytic cells, and on cells where ROS have no apparent role, ROS has been shown to be key signaling molecules, even though their exact mode of action was relatively unknown. Currently several studies have shown the ability of ROS to induce necrosis at high concentrations, as well as its role in the induction/inhibition of cell proliferation, and in the activation/inhibition of apoptosis[83, 84].

Sources of reactive oxygen species

ROS (usually $O_2^{-\bullet}$) can be generated intracellularly by a number of enzymes including NADPH oxidase which upon stimulation undergo a respiratory burst, with the release of superoxide into the phagosome[85, 86] the endoplasmic reticulum which is another site of electron transport, where $O_2^{-\bullet}$ is generated by the leakage of electrons from NADPH cytochrome P450 reductase[87] cytosolic oxidases; xanthine oxidase and lipoxygenase, and the mitochondrion. In the mitochondria, reactive oxygen species like $O_2^{-\bullet}$ and H_2O_2 are often generated as by-products of electron transfer reactions that occur during the operation of the mitochondrial electron transport chain. ROS is generated in the

mitochondrial electron transport chain as a result of "leakage"[88] of electrons from electron carriers that are passed directly to oxygen, reducing it to $O_2^{\bullet -}$. An evidence of this mode of ROS generation is substantiated by the fact that the mitochondria contain their own superoxide dismutase, an inducible Mn^{2+}-dependent enzyme [89], for rapid elimination of such reactive species and from a study by Felty et al.[90] which identified the mitochondrion as a major source of estrogen-induced ROS in breast cancer cells.

ROS is also generated by the metabolism of arachidonic acid by the enzymes cyclooxygenases and lipoxygenases. Prostaglandin H synthase is a major enzyme in the biosynthesis of prostaglandins, prostacyclins, and thromboxanes. Prostaglandin H synthase possesses both cyclooxygenase and hydroperoxidase activity[91], hence it has the capacity to generate ROS as oxidizing equivalents via side-chain reactions that are dependent upon the presence of a suitable reducing substrate such as NADH or NADPH .

Nitric oxide ($NO^{\bullet}$) is generated from the conversion of the amino acid l-arginine to l-citrulline by NOS. $NO^{\bullet}$ is an important reactive species containing both nitrogen and oxygen. Production of $NO^{\bullet}$ by neutrophils is well documented, but there are important interspecies differences in the amount of $NO^{\bullet}$ produced: rodent neutrophils produce substantially more ROS than human cells [92;93].

17β-estradiol (Estrogen) induces ROS formation

Presently there are several postulates that E2-induced mitochondrial ROS is involved in the growth and proliferation of estrogen-dependent cells. Felty et al.[94] reported that

physiological concentrations of E2 stimulate a rapid generation of intracellular ROS and intracellular ROS have been implicated in the promotion of rapid cell cycle activity in neoplastic cells.

One attribute of rapidly dividing cancer cells is their ability to generate significant quantities of intracellular ROS. Studies have shown that estrogen-induced stimulation of both MCF-7 and macrophage cells is partly due to the effect of ROS[95, 96]. In an unpublished data by Singh et al., it was observed that scavengers of ROS such as *N*-acetylcysteine, ebselen, and catalase inhibited the estrogen-induced growth of MCF-7 cells, lending credence to the proposed role of ROS as an autocrine growth signal in estrogen-induced cell proliferation[97]. Based on a study by Taylor et al.[98], which established that oxidative stress modifies mitochondrial matrix protein thiols, Felty and Roy [97] extrapolated that an estrogen-mediated cell growth through mitochondrial-generated ROS signaling molecules may exist. This was based on the reasoning that since exposure to estrogen can generate mitochondrial ROS, the oxidation of thiols in response to estrogen converts the oxidative stress to a change in protein function that is involved in cell growth.

In a study of E2-induced ROS generation in MCF-7 and other cells, the measurable events occurred earlier than a regular ER-mediated genomic action. This lead to the belief that E2-stimulated ROS production does not depend solely on the presence of the ER in breast cancer cells. Further more, because the ER-negative cell line MDA-MB 468 upon E2 stimulation was able to produce ROS equal in amount to that of ER-positive cell lines

such as MCF7, T47D, and ZR75, it was inferred that, ROS formation upon E2 exposure might explain oxidative damage to hormone-dependent tumors and ensuing genetic alterations [99, 100]. A few studies have also revealed that E2-induced production of ROS also provides systematic support for the generation of mutations by physiological concentrations of estrogens[101, 102].

In 2006, Felty[103] showed that estrogen exposure of human umblical vein and endothelial cells (HUVECs) stimulated a rapid production of intracellular ROS that is involved in signaling endothelial cell growth. He also showed that the early E2 signaling did not require ER-mediated genomic signaling, because E2-induced growth could be inhibited by antioxidants. The functional outcomes of E2-induced ROS formation include an increase in phosphorylation of the signaling proteins C-jun and CREB, as well as the activation of the binding of three oxidant-sensitive transcription factors AP-1, CREB, and possibly nuclear respiratory factor 1(NRF-1)[90].

Additional findings by Felty et al. suggest that E2-induced mitochondrial ROS modulate G1 to S transition and some of the early G1 genes through a nongenomic, ER-independent signaling pathway resulting in the conclusion that estrogen-induced mitochondrial oxidants control the early stage of cell cycle progression, which could provides the basis for the discovery of novel antioxidant-based drugs or antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer [104].

Tamoxifen induces ROS formation

In addition to its role in inhibiting the growth of ER-positive breast cancer cells through its antiestrogenic properties [105], tamoxifen also appears to have effects on many ER-negative cancer cells[106-109]. Though the mechanism of this action is not known, as in the case of E2, studies have found the effect of tamoxifen on ER-negative cells to be related to its non-genomic actions.

It has been reported that tamoxifen-induced apoptosis in ER-negative HepG2 human hepatoma cells is mediated by increased intracellular Ca2+ [110] and a consequent generation of ROS [111]. In the study by Lee et al.[111], ROS appeared to be a downstream signal of elevated intracellular Ca2+ , given that their generation was temporally preceded by elevation of intracellular Ca2+ within a time frame, and completely inhibited by intracellular and extracellular Ca2+ chelating agents. Another study by Kallio et al [112] showed that at high doses tamoxifen was able to induce apoptosis through the production of ROS in MCF 7 cells.

Tamoxifen-induced ROS generation has been shown to be produced by membrane bound NADPH oxidase[113] and not to the activation of enzymes like microsomal enzyme, cytochrome P-450, and numerous catalytic cytosolic enzymes, including cyclooxygenase. The is because ROS production was not altered by treatment with specific inhibitors of these enzymes[111]. NADPH oxidase has also been functionally active in nonphagocytic cells, including endothelial cells[114], vascular smooth muscle cells,[115] neuroepithelial bodies of the lung [116] and type I cells of the carotid body[117]. The components of the

NADPH oxidase have been expressed in HepG2 cells[118, 119] and the enzyme appears to be a major source of ROS produced by hypoxia[118]. Lee et al [111]. also reported that the NADPH oxidase acts as a major site of the tamoxifen-induced ROS production.

Tamoxifen as a scavenger of ROS

Several studies including a study by Kuo et al [120] have shown that 4-OHT (an active metabolite of Tamoxifen) possesses a potent superoxide anion radical-scavenging tendency. The presence of phenolic groups in tamoxifen, (much like raloxifen and E2) confers its antioxidant action chiefly because phenolic structures have the capacity to bind iron, in addition to reducing peroxyl or alkoxyl radicals [121]. In addition, several studies [239;122, 123;124;125] including one by Arteaga et al.[126, 127] have shown that tamoxifen is a scavenger of ROS.

The role of ROS in oxidative stress and redox signaling

The harmful effect of free reactive oxygen and/or nitrogen species resulting in possible biological damage is called oxidative stress and nitrosative stress respectively[128-130]. Oxidative and nitrosative stress occur in biological systems following an over production of ROS/RNS as well as an accompanying insufficiency of enzymatic and non-enzymatic antioxidants. Simply put, oxidative stress refers to a disturbance in the balance between pro-oxidant/antioxidant reactions in living organisms. As mentioned previously in this review, excess ROS can damage cellular lipids, proteins, or DNA by hindering their natural function. Hence it comes as no surprise that oxidative stress has been implicated in a number of human diseases as well as in the ageing process[131].

Redox regulation, on the other hand, is a very important system responsible for the fairly fragile balance between the beneficial and the harmful effects of ROS/RNS. According to Droge [72], the redox regulation process protects living organisms from a variety of oxidative stresses and maintains the so-called "redox homeostasis" by controlling the redox status in the living system[132].

Redox balance, which is the ratio between oxidizing and reducing species within the cell, plays a significant role in the regulation of signaling pathways, including kinase and phosphatase activity as well as gene expression through regulation of the function of transcription factors [7, 133, 134, 134]

As protection against oxidative radical stress, cells have several enzymic and nonenzymic anti-oxidants or reductants that maintain the intracellular redox environment in an especially reduced state. Typically the enzyme superoxide dismutase (SOD) catalyses the conversion of O_2^- to H_2O_2, and then the H_2O_2 generated is degraded to H_2O by several cellular enzymes, usually catalase or glutathione peroxidase coupled with glutathione reductase [135, 136] (see eqn 1).

$$O_2 + e^- \rightarrow O_2^{-\bullet} \xrightarrow{\text{SOD}} H_2O_2 \xrightarrow[\text{GSH peroxidase}]{\text{Catalase}} H_2O \quad \text{(eqn1)}$$

Other relevant scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which uses GSH as a substrate. GSH plays a central role in maintaining redox homeostasis.The GSH to oxidized glutathione ratio (2GSH/GSSG) provides an estimate of cellular redox buffering ability[137]. Forman et al.[138] have argued that in

contrast to oxidative stress which is induced as a response to damaged molecules, redox signaling always involves responses that are specific to oxidation reduction reactions.

Oxidative stress often initiates a number of varied responses ranging from cell proliferation and transformation to apoptosis and senescence. The type of effect induced depends on the types and amounts of ROS and reactive nitrogen species (RNS) that are generated, the duration of the oxidative burst, the cellular antioxidant defense systems, and the cellular context in which oxidative stress occurs. For example, low concentration exposure of cells to hydrogen peroxide (H_2O_2) have been shown to mediate platelet-derived growth factor (PDGF)-induced vascular smooth muscle proliferation [139], whereas moderate concentrations of H_2O_2 induce growth arrest while high concentrations induce apoptosis and/or necrosis[140]. Enzymes that are involved in oxidant generation or oxidant scavenging are also critically involved in the regulation of cell growth[141-144]. It has also been documented in several biochemical studies that, in most cancer tissue the levels/ activity of manganese superoxide dismutase and catalase are almost always low while that of copper, zinc superoxide dismutase is usually low. Conversely, the activity of glutathione peroxidase in tumor tissue is variable if measured with biochemical methods using tissue homogenates[145-148].These findings show an association between low antioxidant levels vis a viz oxidative stress and cancer.

Chemistry of ROS

Presently, there are two main models of ROS signaling namely: (i) alteration of intracellular redox state and (ii) oxidative modification of proteins[7]. In the alteration of

intracellular redox state, the cytosol is usually maintained in strong reducing conditions often achieved by the redox balancing action of intracellular thiols like glutathione (GSH) and thioredoxin (TRX).

Studies have shown that depending on their local environment, thiolates (S^-) react with H_2O_2 a lot faster than thiols (SH)[149]. It has also been established that in the active sites of proteins like TRX and Prx one cysteine exists in the thiolate form making it more amenable to reacting with H_2O_2. In the following redox reactions involving H_2O_2 and a thiolate (RS^-)[61] (see fig. 1A), the sulfenate (RSO^-) that is formed then reacts with a thiol to form a disulphide bond (see fig.1B). Then the original thiolate is restored by exchange with another thiolate (see fig 1C).

Oxidative modification of proteins

ROS can change the structure and function of proteins by modifying certain critical amino acid residues, thereby inducing protein dimerization, and interaction with Fe-S moieties or other metal complexes[7]. The most recognized oxidative modifications of critical amino acids within the functional domain of proteins involve cysteine residues. The sulfhydryl group (-SH) of a single cysteine residue may be oxidized to form sulfenic (-SOH), sulfinic ($-SO_2H$), sulfonic ($-SO_3H$), or S-glutathionylated (-SSG) derivatives[7, 150]. When the critical cysteine is located within its catalytic domain, an alteration of this nature could vary the enzyme activity[151]. On the other hand, when the critical protein is within the DNA-binding motif of a transcription factor, the ability of the transcription factor to bind DNA is affected[152]. For example, the active site of all but one of the protein

peroxiredoxin (Prx) contains cysteines out of which one is a thiolate (S^-). Prx will react with ROS to form sulfenic acid (see fig. 2A). A second thiol then reacts with the sulfenate to form an intramolecular disulphide (see fig 2B). In the third and final step, all but one Prx use TRX to restore the original thiolate (see fig 2C).

Redox signaling and its effect on signaling pathways

Oxidative and reductive stress can induce redox surges with a resultant change in the thiol status of the proteins. Post-translational modifications of proteins or changes in the thiol status of proteins due to changes in the redox environment of the cell are two of the major pathways for signaling in cells. Characteristically, changes in the cellular redox environment could result in modification of signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, as well as regulation of the cell cycle[153-155;156;157;137, 158, 159].

A number of studies have shown that ROS and RNS have the ability to change or modify several signaling pathways[72, 160-165, 165]. Among these pathways are the mitogen-activated protein kinases (MAPK), which has been shown to be activated by both exogenous, and receptor stimulated H_2O_2[166]; Protein tyrosine phosphatases (PTPs) and thioredoxin (TRX). Modification of signaling pathways is possible because certain key proteins such as PTPs and TRX contain essential cysteines which serve as targets for ROS and RNS[167, 168]. Transcription factors like AP-1 and NF-kB, and Caspases are considered redox sensitive because it has been biochemically demonstrated that their critical cysteines are

in the thiolate form accounting for the ease with which they are oxidized and reduced by oxidants and enzymes[169-171].

The first evidence of reversibility of thiols oxidized by ROS and RNS was demonstrated with PTPs at a time when PTPs were first recognized as important players in signaling. Over the years, redox-mediated regulation of PTPs has been supported by several in vivo studies. The following PTPs are known targets for ROS and RNS, PTP1B, SHP-2, LMW-PTP, PTEN as well as CDC25[172].

ROS induces the oxidative modification of Protein tyrosine phosphatases (PTPs)

Protein tyrosine phosphatases (PTPs) are important enzymes in the control of cell cycles and signal transduction. They act in conjunction with protein tyrosine kinases to regulate the phosphorylation of protein tyrosine in response to cellular signals[173, 174]. Many studies have revealed that PTKs may be directly activated by the inhibition of PTPs by ROS [167, 175, 176]. Reversible inactivation of PTPs by ROS (especially H_2O_2) plays an important role in redox control and cell signaling[132]. Based on the work done by Rhee's laboratory, we now know that stimulation of A431 cells with epidermal growth factor (EGF) leads to the generation of H_2O_2 and a consequent inhibition of PTP1B[70]. Other biochemical studies also showed that an increase in the production of intracellular oxidants could contribute to enhanced tyrosine phosphorylation-dependent signaling, such as signaling in response to growth factors[70;66], by transiently stemming the enzymatic activity of members of the PTP family, and promoting a burst of PTK activity[177, 178]. However, it is unclear if this phenomenon is the same for every member of the PTP family.

ROS and the cellular redox state are able to regulate the activity of PTPs. PTPs have a functional motif that contains a constant cysteine residue, which acts as an electron donor in catalysis[179, 180]. As a result of its unsually low pKa, the functional cysteine residue is prone to oxidation and thus inactivation. Only oxidations that do not advance beyond sulfenic acid (S–OH) are reversible[181, 182]. A vital requirement for regulating the activity of PTPs (or any regulating molecule) is that the modification be specific and reversible. However, the cysteine sulfenic acid formed at the active site of phosphatases can be oxidized further to non-reversible cysteine-sulfinic acid and cysteine-sulfonic acid, within the cell [183]. This effect is believed to be countered by glutathionylation[184].

Glutathionylation is a reaction of either cysteine or cysteine sulfenate with GSH within the cell to form a mixed disulfide bond. The formation of a mixed disulfide between PTP1B and GSH (or GSSG) would prevent irreversible oxidation of the active site cysteine and provide for the reversible reduction either chemically or enzymatically. Barrett et al. [185] have reported that PTP1B is inactivated by the formation of a mixed disulfide with glutathione and that this inactivation is reversed not only by DTT but also more importantly by thioltransferase, a thiol-disulfide oxidoreductase that is specific for glutathionyl mixed disulfide substrates[186, 187] and specifically utilizes GSH as co-substrate[188]. The glutathionylation occurs on Cys215, the active site cysteine. This mechanism suggests an alternative modification to the redox regulation of cysteine in PTP1B and suggests a possible *in vivo* mechanism in the regulation of phosphatase

activity. Also, reversible S-glutathionylation also appears to form the basis for redox regulation of c-Jun DNA binding[169].

PTP-1B is directly inactivated by ROS-induced reversible oxidation of its catalytic site, Cys215, and this has been proposed as a mechanism for EGF-mediated mitogenic signaling[151; 189]. Studies on B lymphocytes reveal that, following B cell receptor activation, the ROS produced by DUOX1 enhance prompt generation of a signal by reversible inhibition of SHP1, which then results in enhanced tyrosine phosphorylation and activation of Lyn kinase [190]. Superoxide radical has been shown to regulate the activity of PTPs (especially PTP1B) very efficiently, again through cysteine residues [132].

Phosphatase and Tensin Homologue (PTEN) and Reactive Oxygen Species

The tumor suppressor PTEN (phosphatase and tensed homologue) regulates cell growth, survival and migration by the removal of the 3'-phosphate of phosphoinositides. It has been reported that the exposure of cells or purified PTEN to H_2O_2 resulted in inactivation of PTEN in a time- and H_2O_2 concentration-dependent manner [167, 189, 191]. As with other phosphatases, H_2O_2 induces the reversible inactivation of PTEN through oxidation of the essential Cys124 [167; 192]. This forms cysteine-sulfenic acid, which reacts with the so-called backdoor cysteine (Cys71 substrate) to form a disulfide. The disulfide formed is then further reduced to its active form by thioredoxin (TRX)[193].

Though the stimulation of various receptors induces H_2O_2 production, Lee et al.[194] proposed that the receptor-mediated activation of phosphoinositide 3-kinase (PI3K) alone

does not does not account for the accumulation of 3'-phosphorylated phosphoinositides, and that the simultaneous inactivation of PTEN by H_2O_2 produced in response to receptor stimulation might also be essential for this event[195]. A high frequency of PTEN mutations and subsequent loss of function has been reported in several tumor types like endometrial carcinoma, brain, and breast cancer[196]. It is documented the loss of PTEN function results in an increased Akt activity and, subsequently, cell survival[197]. Evidence exists that inactivation of PTEN by oxidation might be a physiological mechanism for regulation of this enzyme, not only by oxidative stress, but by ROS produced in other circumstances such as the stimulation of cells by growth factors [198, 199].

The cyclin-dependent kinase inhibitor $p27^{Kip1}$, a target of Akt, has been proposed as a downstream mediator through which PTEN may positively regulate cell cycle progression[200]. In prostate cancer, 16–68% of the cases show an association between the low grade expression or loss of p27 protein with either adverse or impaired prognosis[201-204;205].

CDC25 Phosphatases and Reactive Oxygen Species

Cdc25 phosphatases also known as the dual-specificity phosphatases, are key activators of the CDK/cyclin complex, hence they serve as regulators of normal cell division. There are three isoforms of Cdc25, namely Cdc25A, Cdc25B, and Cdc25C. These three phosphatases function to dephosphorylate the CDK/cyclin complex on pThr14 and/or pTyr15 residues, counteracting the phosphorylating effect of the Weel and Myt1 kinase. This dephosphorylation prompts the ultimate activation of CDK/cyclin activity during

normal cell cycle progression[206, 207]. Cdc25A controls both the G1-to-S and G2-to-M transitions, whereas Cdc25B and Cdc25C are regulators of the G2-to-M transition.

The Cdc25 phosphatases also play an important role in the checkpoint response that prevents CDK/cyclin activation following DNA damage[208, 209]. Confirming an important role for the Cdc25 phosphatases in cancer, Cdc25A and Cdc25B, but not Cdc25C, are over expressed in many different primary human cancers[210].

Like all other PTPs Cdc25 phosphatases have two cysteine molecules in their functional site. One of the cysteines exists in the very reactive thiolate (S^-) form while the other one exists as a less reactive thiol. The thiolate in Cdc25's functional motif has been shown to be oxidized by low concentrations of ROS resulting in the formation of a reversible cyseine-sulfenic acid (see eqn 13). The resultant sulfenic acid then forms a mixed disulfide bond with the second thiol to prevent further oxidation by ROS to an irreversible sulfinic acid. The reduction of the disulphide bond to the original thiolate is carried out by either TRX or GSH. H_2O_2 causes oxidation of the active site cysteine residue in Cdc25- PTP, with a subsequent binding to 14-3-3 proteins for nuclear export and subsequent degradation[211; 212, 213]. Oxidative stress has also been shown to induce the nuclear export of Cdc25B through protein kinase B/Akt-dependent phosphorylation on Ser353 [214].

An association between mitogenic signaling and the cell cycle mechanism has been suggested by studies which showed that the tyrosine phosphatase Cdc25A, among others,

could have an effect on Raf-1, a key component of the MAPK pathway[215, 216]. In 1995, Conklin et al. showed that Raf-1, which is known to bind 14-3-3 proteins, appeared to associate with Cdc25A and stimulate its phosphatase activity[217]. Galaktionov et al. [218] also showed an association between the Cdc25 phosphatase and Raf-l in somatic mammalian cells and in meiotic frog oocytes. These authors also suggested that activation of the cell cycle by the Ras/Raf-l pathways might partially be mediated by Cdc25. Another study also showed that dephosphorylation of Raf-1on tyrosine by Cdc25A resulted in a significant decrease in the kinase activity of Raf-1[219], and consequently of ERK. On the flip side, this implies that inactivation of CDC25A could lead to an increase in the kinase activity of Raf-1 and thus the phosphorylation and activation of ERK.

Recently, a study by Vogt et al.[220] showed that overexpression of Cdc25A in whole cells resulted in the dephosphorylation of ERK, which was reversible by an inhibitor of Cdc25A (2-mercaptoethanol)-3-methyl- 1,4-naphthoquinone (compound 5). The high point in this study is that a Cdc25 inhibitor increased phosphorylation and nuclear accumulation of ERK thereby underscoring the proposition that Cdc25A regulates the phosphorylation state of ERK. More recently, in addition to showing a physical interaction between Raf-1 and Cdc25A, Nemoto et al. [221] were also able to show that the phosphatase Cdc25A regulates Raf-1/MEK/ERK kinase activation in human prostate cancer cells. Additional work done by Wang et al.[222] suggested that phospho-ERK is possibly another substrate of Cdc25A. Based on the above studies, one can infer that the oxidation (downregulation) of CDC25A by ROS (which renders Cdc25A inactive) may

also result in the phosphorylation of ERK that could ultimately phosphorylate a number of transcription factors, as well as the CDK inhibitor p27.

Mitogen Activated Protein Kinase- Phosphatase (MKPs) and Reactive Oxygen Species

These are dual specificity phosphatases which have the ability to inhibit the mitogen activated protein kinases (MAPKs) in several cell types[223-229]. Like other dual specificity phoshatases, mitogen activated protein kinase phosphatases (MKPs) have recently been added to the redox sensitive phosphatases list. Seth and Rudolf [230] showed that, like other PTPs, MKPs are able to form cysteine-sulfenic acid upon oxidation by ROS. Unlike other PTPs, however, the sulfenic acid formed does not form a disulfide with proximate cysteine or sulfenyl amide bond species with neighboring residue, but rather utilizes various cysteines distributed in both the N-terminal substrate-binding domain (Cys147 in particular) and the C-terminal catalytic domain (Cys218) to protect its active site from further oxidation.

MAPKs play a central role in mediating intracellular signaling events triggered by mitogens, growth factors, and stress[231, 232]. The MAPKs are activated by specific upstream dual specificity kinases (MAPK kinases, MKKs) through phosphorylation on both threonine and tyrosine residues in the TXY motif. Extracellular stimuli activate MAPKs leading to the phosphorylation of an array of cellular substrates and nuclear transcription factors.

In mammals, three distinct MAPK families have been studied extensively. The extracellular signal-regulated kinase (ERK) family is activated by growth and differentiation factors and by phorbol esters [233]. The c-Jun NH2-terminal kinase (JNK) family (or stress-activated protein kinase (SAPK) and p38 MAPK families are activated by pro-inflammatory cytokines and environmental stress. It is noteworthy that the three aforementioned subgroups of MAPKs have been implicated in both cell growth and cell death, hence the need to tightly regulate these pathways is principal in determining cell fate[234]. Therefore the injurious effect of non-stop activation of MAPK pathways may include excessive production of MAPK-regulated genes, uncontrolled proliferation, and unscheduled cell death [235].

A number of cellular stimuli that induce ROS production are also capable of activating MAPK pathways concurrently, in multiple cell types. This was established by studies which showed that prevention of ROS accumulation by antioxidants blocks MAPK activation after cell stimulation with cellular stimuli[235-237]. Additionally, it has been documented that direct exposure of cells to exogenous H_2O_2, to imitate oxidative stress, resulted in the activation of MAPK pathways[238, 239]. Though the mechanism(s) by which ROS can activate the MAPK pathways, is still not well defined and because ROS can change protein structure and function by modifying critical amino acid residues of proteins[7, 238, 239], we could therefore infer that the oxidative modification of signaling proteins by ROS may a probable mechanism for the activation and or inactivation of MAPK pathways.

The Effect of ROS on Transcription Factors

AP-1 and NFκB

The tumor progression stage of carcinogenesis has been shown to be modulated by transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappaB (NFκB). A causal relation has been well documented between neoplastic transformation and transcription factor AP-1 transactivation [240-243]. Infact, a constitutive increase of AP-1 activity has been associated with the malignant conversion of papillomas to carcinomas [244]. There is an increase in NFκB transactivation during tumor progression [245].This NFκB transactivation is also increased by agents that elevate AP-1 activity during tumor progression while its inhibitors also attenuate AP-1 activity [246, 247] suggestive of common upstream signaling cascades that mediate elevated AP-1 in addition to NFκB transactivation during tumor progression.

ROS have been shown to act as activators of transcription factors which modulate their activity either directly or indirectly by activating other signaling cascades. NFκB transactivation is considered redox sensitive because (i) The phosphorylation of its inhibitor I-κB is mediated by oxidation; and (ii) the Ref/thioredoxin-dependent binding of the p50 subunit to the DNA is controlled by the reduction of an essential cysteine group [248] (cite article). NFκB activation can be be induced by several activators/oxidants in the absence of any physiological stimulus and is inhibited by a wide range of antioxidants [249-251]. On the other hand AP-1 DNA binding activity has also been shown to be modulated by Ref/thioredoxin-dependent reduction of cysteines in c-Jun and c-Fos [252].

Persistently high ROS levels activate redox-sensitive transcription factors, such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), which may then act as molecular switches that convert normal cells into premalignant cells, with ensuing clonal expansion to form solid tumor[253]. Hence, abnormal activation of NF-κB and AP-1, which results in the transcriptional activation of genes involved in inflammation, cellular proliferation, and growth, has been implicated in pathophysiology of various malignancies [253, 254].

Mitogen-activated protein kinases (MAPKs) have been shown to modulate transcription factor activities in addition to being part of kinase cascades that serves as transmitters connecting extracellular stimuli to specific transcription factors thus allowing these signals to regulate specific gene expression. Studies have shown that JNKs and p38 MAPK are a part of the stress response pathways activated by cellular stress induced by agents like heat, UV and ionizing radiation and inflammatory cytokines mediating inhibition of cell proliferation or cell death [255, 256]. AP-1 activity has been shown to be modulated by MAPKs in response to various stimuli[257]. NFκB activation has been reported to be modulated by MEKK1, a kinase upstream of JNKs [258] as well as p38 MAPK [259]. There is evidence that antioxidants can attenuate MAPK activation [6, 255, 260], thereby suggesting that MAPK signaling cascades are additional targets affected by ROS levels in cells.

NRF-1

Nuclear respiratory factor 1(NRF-1) is a protein that encodes genes that code for a phosphorylated nuclear protein with a bZIP domain. The encoded protein forms a homo-

or heterodimer, which functions as a transcription factor that activates the expression of some key metabolic genes. These key metabolic genes regulate cellular growth and nuclear genes required for respiration, heme biosynthesis, mitochondrial DNA transcription and replication[261, 262] Initially, NRF-1 was described as an activator of cytochrome c expression and afterwards was found to act on many nuclear genes essential for mitochondrial respiratory function[263, 264] such as genes encoding respiratory subunits [265], the rate-limiting heme biosynthetic enzyme[266] as well as mtDNA transcription and replication factors[267, 268].

NRF-1 binds to the antioxidant response element (ARE) in the promoters of genes which are involved in response to cellular stress[269] to induce the expression of phase II detoxifying enzymes and oxidative stress-inducible proteins, like NADPH, quinone oxidoreductase, and glutathione S-transferaseA2 [270, 271]. Studies have shown that NRF-1's ability to regulate the ARE of stress-related genes and TNFα is based on its interaction with transcription factors such as AP1 proteins (c-Jun, JunB, and JunD)[272, 273]. In addition, NRF-1 has been shown to play an important role in cellular growth and differentiation[274, 275]. A biochemical study by Chang et al[276]. implicated NRF-1 protein with the regulation of neurite outgrowth.

Studies have shown that NRF-1 is phosphorylated both *in vivo* and *in vitro* on serine residues within a concise amino-terminal domain[277]. The phosphorylation of these sites *in vitro* augments NRF-1 DNA binding action, surmising the capacity of such modifications to regulate NRF-1 function. Herzig et al.[278] showed that the activity of NRF-1 is

enhanced by phosphorylation, which occurs in cells that have been stimulated with serum. It is assumed that a phosphorylation such as this allows the nuclear transcriptional apparatus to respond both to extracellular signals and intracellular ATP concentrations in controlling the expression of the respiratory chain[278]. The post-transcriptional modification of the N-terminus of NRF-1 has been shown to play an important role in controlling DNA-binding activity[277]. It has been established that P/CAF, which is a p300/CBP (cAMP response-element-binding protein (CREB)-binding protein)-associated factor, can interact directly with the N-terminal domain of NRF-1 and modify it by acetylation. The ensuing modification significantly stimulates the DNA-binding activity of NRF-1[279]. A study by Cho et al.[280] showed that the methylation on the NRF-1 sites may be a means of silencing the Tfam promoter with an ensuing decrease of mitochondrial biogenesis[281]. According to Narayanan et al.[282] NRF-1 often exists as a complex with Keap1 (inhibitor) but as a result of stress, NRF-1 separates from Keap1 and becomes active.

NRF-1 and 17 β-Estradiol (E2)

Estrogen increases the transcription of NRF-1[283, 284]. Results of work done by Rodríguez-Cuenca et al.[285] show that E2 appears to affect the mitochondrial biogenic program in two ways. The first is inhibiting it through the downregulation of NRF-1, gabpa, and tfam mRNA[286]; or second, downregulating PTEN mRNA levels in cell-cultured brown adipocytes, implying an activation of the PI3K-Akt pathway and therefore a mitochondrial recruitment-stimulatory signal. E2 has been shown to inactivate PTEN activity by phosphorylation and, hence, influence PTEN activity, suggesting both a

chronic and an acute control mechanism of PTEN by estrogens [287]. Felty et al. [288] showed that E2-induced oxidants increase the transcriptional activity or binding of AP-1, CREB, and NRF-1 to their individual DNA response elements. The study actually showed that at physiological concentrations E2 stimulates a rapid production of intracellular ROS resulting in the phosphorylation of c-jun and CREB as well as increased activity of redox-sensitive transcription factors NRF-1, c-jun, and CREB, which are known to be involved in the regulation of cell cycle genes[289].

NRF-1 and Cancer

Xu et al. [290] have been able to demonstrate a connection between cancer and NRF-1, based on the result of a study which observed changes in NRF-1LKO (NRF-1 liver knock-out) livers. The livers were characterized initially by cell death, proliferation, and inflammation, followed by the appearance of dysplastic cells and eventually cancer. The X gene of hepatitis B virus (HBV) which is one of the chief factors in HBV-induced hepatocarcinogenesis is necessary to achieve a productive HBV replication in vivo. Recently, Tokosumi and others[291] showed that NRF-1 exclusively binds the 21-bp minimal promoter and contributes to transcription of the X gene. They also suggested that concurrent activation of the X gene and mitochondrial genes by NRF-1 may allow the X protein to target mitochondria most efficiently[292]. A study by Auf dem Keller et al. [293] showed that NRF-mediated gene expression in keratinocytes is dispensable for wound healing but crucial for skin tumor prevention.

NRF-1 and cell cycle

The transition of cells from quiescence (G_0) to proliferation (G1) increases the cellular energy (ATP) demand. The cell responds to this increase in demand for ATP by modulating the activity of the respiratory chain components, and it is the duty of NRF-1 to regulate the expression of a number of genes required for mitochondrial respiratory function [264].

A recent study demonstrated that NRF-1 activity is enhanced by phosphorylation upon serum-induced proliferation, leading to transcriptional induction of cytochrome *c*, a major component of the respiratory apparatus[294]. The induction of cytochrome *c* was associated [295] with enhanced energy production by the mitochondria in preparation for entry to the cell cycle.

Results from work done by Herzig et al. [296] show that NRF-1 functions to induce gene expression and maintain the level of cytochrome *c* in actively dividing cells. Additionally, the results also suggested the possibility that activated NRF-1 may contribute to the synthesis of other respiratory chain constituents later in the cell cycle. It can therefore be inferred that the phosphorylation of NRF-1 may be a significant regulatory event in the transition from quiescence to active cell division.

Virbasius et al. [297] showed that NRF-1 sites are found in genes that may be directly involved in cell cycle regulation (like cdc2, RCC1) or are regulated by cell growth (like omithine decarboxylase, DNA polymerase-a, and GADD153). Their study concluded that

even though the most current and best defined biological role for NRF-1 is in the nuclear control of mitochondrial function, the NRF-1 protein or related proteins having the NRF-1 DNA-binding domain may possess the ability to integrate various functions required for cell maintenance, growth, and proliferation.[298]

NRF-1 has been shown to mediate the apoptotic function of *c-myc* in a study which also shows that cytochrome *c* and other nuclear-encoded mitochondrial genes are also regulated by NRF-1 and provides evidence of a link between the induction of NRF-1 target genes and sensitization to apoptosis on serum depletion[299]. The finding by Felty et al. [300] that E2-induced mitochondrial ROS control the expression of early G1 phase genes (Felty and Roy, unpublished results), imply that E2-induced ROS increase the transcriptional activity or binding of AP-1, CREB, and NRF-1 to their respective DNA response elements responsible for the expression of early cell cycle genes. Based on previous studies (already mentioned in this review) which showed that redox-sensitive transcription factors c-jun, CREB, and NRF-1 regulate cell cycle genes[262, 301, 302], Felty et al.'s finding could be a pointer that E2-induced ROS may play a role in cell cycle progression of estrogen-dependent cells [303].

NRFI and ROS

A study by Suliman et al.[262] showed that ROS signaling is necessary for hepatic mitochondrial biogenesis accompanying cell proliferation, which is linked to activation of pro-survival PI3K-Akt pathways. The PI3K-Akt pathway in turn leads to NRF-1 activation and subsequent DNA binding. Furthermore, ROS originating in mitochondria,

cytoplasm, or outside the cell could activate Akt and promote NRF-1 translocation. Hence, PI3K-Akt activation may regulate both mitochondrial biogenesis and cell proliferation after oxidative stress.

Already mentioned in this review is the fact that cysteine-dependent phosphatases, like PTEN, which are reciprocal regulators of PI3K, are sensitive to inactivation by oxidants. This is substantiated in a recent study by Piantadosi and Suliman [304], which provided evidence that the stimulation of PI3K by extracellular oxidants activate Akt and promote NRF-1 phosphorylation as well as nuclear translocation. NRF-1 phosphorylation increases its ability to stimulate transcription of Tfam, a downstream nuclear-encoded gene for a mitochondrial protein required for mtDNA transcription and replication.

Based on work done on HeLa cells, Miranda et al.[305] have been able to show that cells depleted of mitochondrial DNA (ρ^0) have an increased amount of intracellular ROS, and that under these conditions, NRF-1 and Tfam are up regulated, further supporting the postulated role of ROS in mitochondrial signaling to the nucleus. NRF-1 has also been found to play a role in the regulation of genes involved in glutathione synthesis, therefore signifying a basis for an equally low GSH concentration and reduced stress response in a study which also found that interference with the NRF-1 gene results in hypersensitivity of fibroblasts to the noxious effects of various oxidizing agents[306]. Work done by Chen et al. [307] showed that NRF-1 may be required to protect fetal liver cells from endogenous TNF-induced apoptosis. The study concluded that NRF-1 plays an important role in maintaining redox balance in the fetal liver cells signifying a cell-specific and

developmental stage-specific function of NRF-1 in protecting liver cells from apoptosis during development.

Cell Cycle

Cell cycle control

Simply put, a cell cycle refers to a programmed life cycle of a dividing cell. The process of cell-cycle transition can be divided into four phases: the G1 phase, where mRNA and proteins are synthesized in preparation for DNA synthesis; the S phase where DNA synthesis occurs; the G2 phase, where mRNA is synthesized in preparation for the fourth phase, mitosis (M phase). Quiescent cells are found in the G_0 phase and exhibit minimal mRNA and protein synthesis. Stimulation of quiescent cells (G0) results in their entry into the G1 phase which in turn induces a number of proteins that regulate and control cell growth and proliferation [229;308].

In addition to the four cell cycle phases, there are two main checkpoints that regulate cell-cycle progression. The G1/S checkpoint prevents the replication of damaged DNA. This checkpoint is regulated by a balance between growth-stimulating and growth-inhibitory responses. The second checkpoint, which is G1/S to G2/M checkpoints control the sequence and timing of cell-cycle transitions, enabling the assimilation of cell division with environmental stimuli as well as the monitoring of DNA damage to maintain genomic integrity. An additional spindle assembly checkpoint functions to delay mitosis until the mitotic spindle is correctly formed [309].

The cell cycle process is controlled by the following proteins: The cyclins, namely cyclins A, D, and E; the cyclin dependent kinases CDK2, 4, and 6; the cyclin kinase inhibitors (CKIs), namely $p16^{INK4a}$ (p16), $p21^{CIP1/WAF1}$(p21), and p27; and other negative regulators such as the retinoblastoma protein (Rb) and p53, which regulate the effects of both CKI p21 and cyclin A protein[310]. During the G1phase, cyclins D1 and E are rapidly synthesized and bind to CDK4 and CDK2, respectively. The resulting cyclinD-CDK4/6 complex then phoshorylates the retinoblastoma protein (Rb). Phosphorylation of Rb releases E2F which mediates gene expression and the induction and the formation of cyclin E- CDK2 complex.

The cyclin E- CDK2 complex phosphorylates a broad variety of proteins, including Rb with a consequent release of E2F, which promotes cell-cycle progression to late G1, leading to the induction and formation of the cyclin A/CDK2 complex.

The cyclin A/CDK2 complex functions to advance cell-cycle progression through the G1/S phase, into S, as well as late S and S/G2 interphase (cyclin A/CDK1). Cyclin A/CDK1 collaborates with Cyclin B/CDK1 to regulate G2 to M phase transition. In its phosphorylated state, cyclin B complex is inactive and requires dephosphorylation on Tyr 15 by CDC25 to activate it. The active cyclin B complex promotes cell division (M phase)[229].

The active cyclin D complex is inhibited by CDK inhibitor p16 to induce early G1 arrest and prevent the phosphorylation of Rb. It has also been shown that •NO has the potential

to induce p21, leading to the inhibition of the action of cyclin E complex. Proliferating cell nuclear antigen (PCNA), a cofactor for DNA polymerase is negatively regulated by p21 [311]. Inhibition of CDK2 activity by p27 can prevent the phosphorylation of transcription factors critical for entry into the S phase [312]. In G2, p53 can induce late G2 arrest (inhibition of active cyclin B action) or mediate early G2 arrest via p21 inhibition of Cdc25.

The role of p27 in cell cycle control

The CDK inhibitor p27 has a negative effect on the activity of CDK2-cyclin complexes during G1 phase and CDK1 (Cdc2) complexes during G2 phase. In breast cancer, cyclins D1 and E, in addition to the cyclin-dependent kinase inhibitors p21 and p27, play very important roles in cell-cycle control and as potential oncogenes or tumor suppressor genes. Reduced expression of p27 has been associated with the poor survival of cancer patients[313].

In breast cancer cells, CDK inhibitors are regulated following mitogenic stimuli such as activation of receptor tyrosine kinases and steroid hormone receptors. Their deregulation frequently impacts on breast cancer outcome, including response to therapy[314]. The expression of p27 is regulated through transcriptional and translational control mechanisms, by modulation of both protein stability and changes in subcellular localization.

According to Medema et al. [315], even though the transcription of the p27 gene is regulated by factors like AFX-like Forkhead transcription factors, the abundance of p27 during the

cell cycle is mostly mediated post-transcriptionally. As cells exit quiescence and progress into S phase, the levels of p27 protein decline as a result of decreased translation of p27 mRNA and targeted proteolysis[316].

In the G1 and S phase, degradation of p27 is regulated by two RING-finger E3 ubiquitin ligase-containing complexes with contrasting subcellular locations. These complexes respectively ubiquitylate p27 and target it for proteosomal degradation. In addition to promoting p27 degradation, phosphorylation also regulates p27 subcellular localization. Following mitogen stimulation, this p27 is likely to be phosphorylated on serine 10 by human kinase-interacting stathmin (hKis), which promotes nuclear export[317]. According to work done by Liang and Slingerland, [318] p27 is also phosphorylated on two threonine residues, Thr157 and Thr198, by Akt, which results in the cytoplasmic retention of p27 . The resultant cytoplasmic localization following the phosphorylation of p27 at Thr157 is due to inhibition of its nuclear import. Overall, this suggests a model in which mitogenic signaling pathways promote cytoplasmic localization of p27 and its subsequent degradation by the Kip1 ubiquitination-promoting complex (KPC). This will increase the activity of nuclear cyclin E-CDK2 resulting in p27 Thr187 phosphorylation and degradation of p27, leading to a positive feedback loop for enhancement of cyclin E-CDK2 activity.

Sakakibara et al. showed that the ERK subfamily of MAPK is both essential and adequate in the regulation of p27 and a number of other studies have also established that ERK activity also contributes to the down-regulation of p27 [319; 320-324].

The effects of ROS on regulators cell cycle

There is increasing evidence suggesting that ROS and RNS play a part in the regulation of cell proliferation and cell-cycle control. Studies have shown that there is a significant similarity between the pathway that regulates cell cycle progression and the one that regulates the induction of apoptosis. These similar pathways are intimately interconnected by critical regulatory molecules and signal transduction cascades[325].

Reactive oxygen and nitrogen species have a wide range of effects on cell-cycle advancement. The growth-inducing actions of ROS/RNS may be exerted via the activation of kinase signaling cascades, redox regulation of tyrosine phosphatases, and direct activation of transcription factors[144].

Low concentrations of H_2O_2 increase cell proliferation through increased cyclin D expression and G1/S transition in fibroblasts[326] and by means of decreased expression of p27 in prostate tumor spheroids[327]. Arnold et al. [328] have also shown that H_2O_2 mediates cell growth and cell-cycle regulation caused by targeted overexpression of the Nox1 subunit of the NADPH oxidase[328]. The ability of ROS to promote the G1/S transition is further substantiated by the finding that treatment with antioxidants will repeatedly block proliferation and cell-cycle progression. This can be corroborated by the work of Menon et al. [329], which showed that the antioxidant *N*-acetyl-L-cysteine induces G1 arrest by decreasing cyclin D1, increasing p27 protein levels, and leading to hypophosphorylation of Rb[329]. It is also noteworthy that low to moderate ROS can induce MAPK pathways

that lead to cell growth and proliferation, whereas high ROS induce DNA damage and/or MAPK pathways that activate p53, cell arrest, and apoptosis[326].

Growth arrest associated with higher, sustained levels of ROS and RNS appears to involve the induction of inhibitory cell-cycle control proteins, especially p21, and the repression of cyclins[330]. More recently, the redox-sensitive family of Forkhead box class O (FOXO) transcription factors has been shown to promote cell-cycle arrest in G1 by transcriptional up-regulation of the CDK inhibitor p27 and down-regulation of cyclin D1[331].

Very little is known about the redox control of the spindle assembly checkpoint. It has recently been suggested that mitotic spindle pole formation is an important component of H_2O_2 -induced mitosis in type II pneumocytes [332]. Cdc2 and other mitotic protein kinases have also been shown to play a role in spindle regulation,[333] suggesting a possible role of redox control of Cdc2 activation by Cdc25C in the spindle checkpoint. The MAPK pathways, especially p38 and ERK1/2 ,[334] which are regulated by ROS appear to play a key role in mitotic spindle assembly.

The role of Redox signaling in Anti estrogen Resistance

Based on the above review, we may therefore infer that redox signaling could contribute to anti-estrogen resistance by the following biological processes: induction of ROS by anti-estrogens and a subsequent inactivation of PTPs or MKPs resulting in the activation and hyper-stimulation of either the MAPK pathway or the PI3K/Akt pathway, which could either affect the cell cycle through the activation of transcription factors or the

CDKI leading to a change from the tamoxifen sensitive phonotype to a tamoxifen resistant one.

Cancer cells are characterized by uncontrolled proliferation and altered energy metabolism. In response to the activation of many oncogenes, continuous mitogenic signaling augments the production of ROS. Additionally, mitogens like estrogen and anti-estrogens such as tamoxifen have been shown lead to ROS production when they are exposed to cancer cells both in vitro and in vivo. The ROS and redox signaling that ensue result in the induction of replication stress and DNA damage response, which in conjunction with the functional checkpoint, induces senescenes, often considered a barrier to tumorigenesis [335, 336]. Interestingly, once cancer cells have escaped the mechanisms that counteract mitogenic signaling, they advance to a pro-oxidative state typified by unbridled proliferation and altered energy metabolism, including defects in mitochondrial respiration.

Defects in mitochondrial respiration cause inactivation of PTEN in a redox-dependent manner and activation of the pro-survival factor Akt [337]. Akt is one of the kinases that phosphorylate p27 on the threonine 157 position resulting in cytoplamic sequestration of p27 with an ensuing loss in its cyclin kinase inhibitory function; a consequent unrestrained cell proliferation and a change from a tamoxifen sensitive to a tamoxifen resistant phenotype. Futhermore, activated Akt has been shown to phosphorylate the redox sensitive transcription factor NFR-1 thereby activating it and leading to its binding to the promoter region of cell cycle genes[338].

It is also probable that NRF-1 could be part of a pathway that connects the mitogen-activated signaling cascade (either the MAP kinase or the PI3K Akt pathway) to cell cycle progression. Following ROS production and the induction of a pro-oxidative state in cancer cells, PTPs are oxidized in a redox dependent manner, rendering them unable to carry out their usual function. In the case of Cdc25A this function is to inactivate the MAPKKK Raf-1, with which it has been shown to have an on/off switch like relationship[339]. The inability of oxidized Cdc25A to inactivate Raf-1 results in a continuous activation of the MAP kinase pathway, with a consequent upregulation and nuclear translocation of ERK. The nuclear translocation of ERK enables it to phosphorylate NRF-1 protein causing it to bind to the promoter of certain cell cycle genes (unpublished work done in Dr Roy's lab) inducing their transcription and promoting cell proliferation.

Tumor suppressor genes also play an important role in linking metabolism to cell growth. A central regulator of metabolism is the PI3K/AKT pathway, which controls metabolism through several mechanisms, including enhancement of protein translation through mTOR[340]. The c-Myc oncoprotein also plays a vital role in regulating metabolic changes required for cell division[341]. Furthermore, the pro-oxidative state of cancer cells is typified by adjustive responses that include changes in the expression of antioxidant enzymes and increased production of GSH. Together the interconnected disturbance in metabolism and oxidant production common to most tumor cells provide therapeutic

opportunities that do not depend on targeting a single oncogene or tumor suppressor gene.

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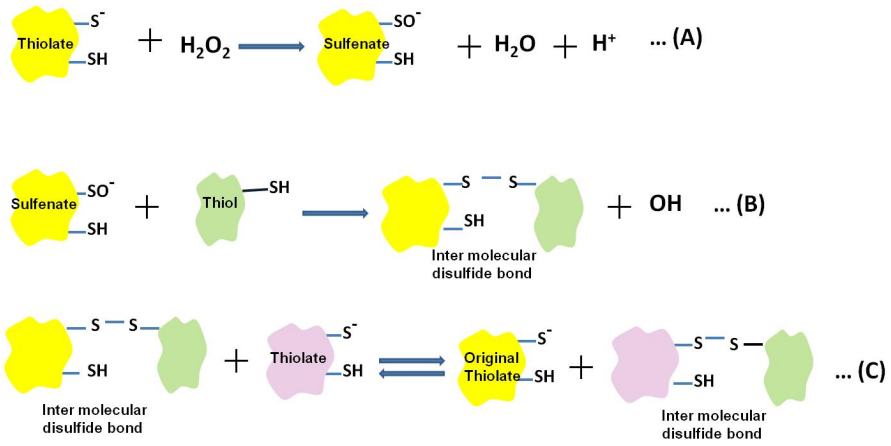
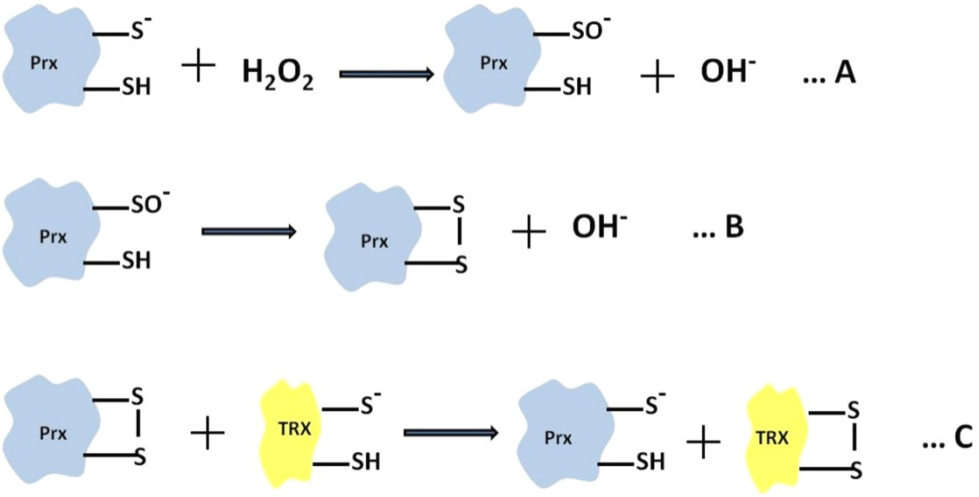
Figures and Legend

Figure1-R



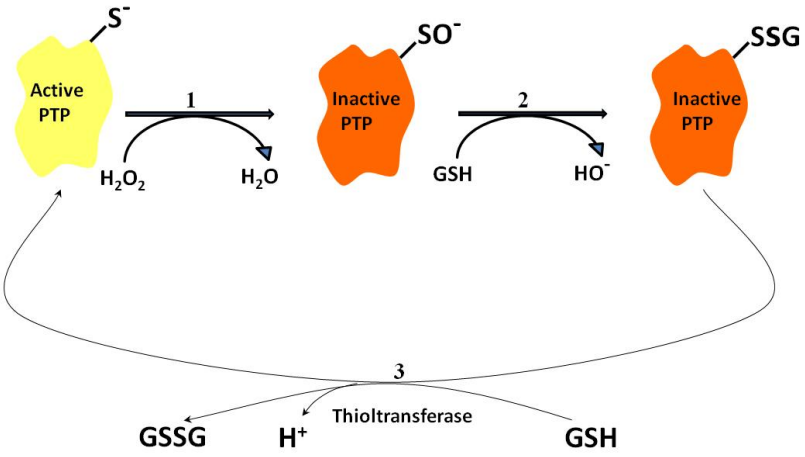
Redox reactions involving hydrogen peroxide (H_2O_2) and a thiolate (RS^-). Thiolate reacts easily with H_2O_2 forming a sulfenate (RSO^-) (see 1A), the sulfenate that is formed then reacts with a thiol to form a disulphide bond (see 1B). Then the original thiolate is restored by exchange with another thiolate (see 1C).

Figure 2-R



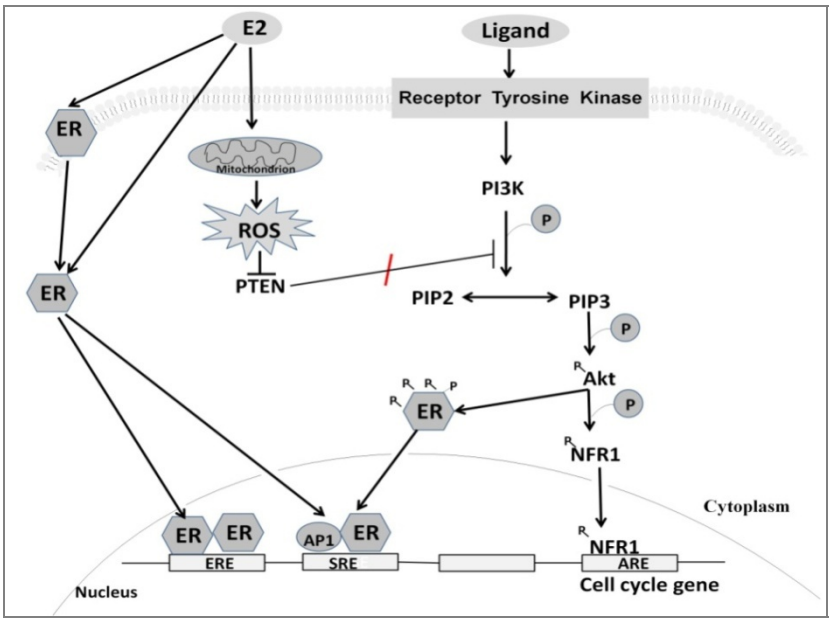
The oxidation of Prx by H_2O_2. Prx reacts with H_2O_2 to form a sulfenate (fig.2A). A second thiol then reacts with the sulfenate to form an intra-molecular disulphide (fig.2 B). In the third and final step, TRX used to restore the original thiolate (fig. 2C).

Figure 3-R



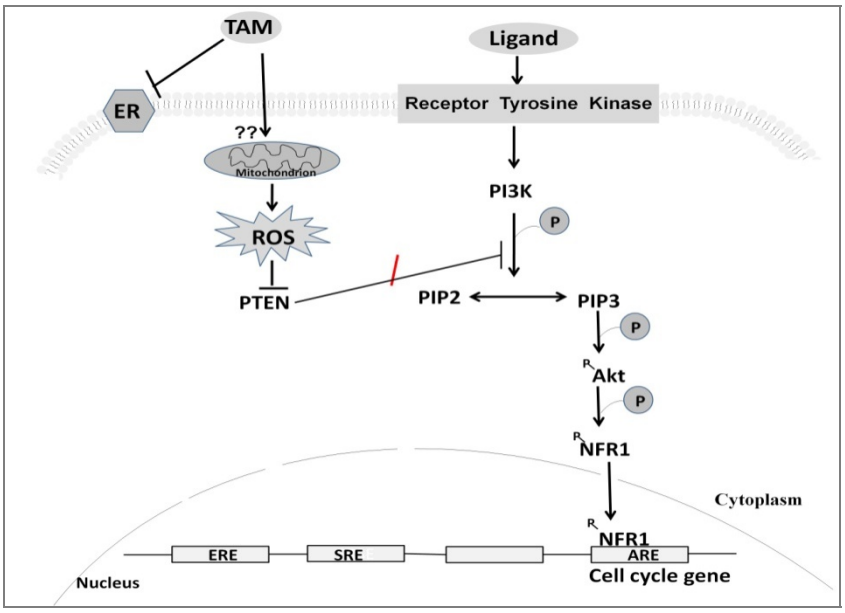
Reversible PTP inactivation by H_2O_2. In step 1, an active PTP containing a thiolate reacts with H_2O_2 to form an inactive but reversible PTP-sulfenate (SO^-). In step 2, the PTP-sulfenate reacts with GSH within the cell to form a mixed disulfide bond which would prevent irreversible oxidation of the active site cysteine and allow for the reversible reduction. In step 3, the mixed disulfide bond on the inactive PTP is reversed back to a thiolate and PTP becomes active again.

Figure 4-R



The effect of E2-induced ROS on regular mitogenic signaling. E2 crosses the cell membrane and goes to the mitochondrium where it induces ROS formation. The oxidants formed inhibit PTEN rendering it unable to inhibit the PI3K/Akt pathway resulting in the hyper-stimulation of the pathway and a continuous phosphorylation of Akt. Phosphorylated Akt then activates NRF-1 by phosphorylation enabling the now active protein to binds to the promoter region of certain cell cycle genes.

Figure 5-R



The effect of tamoxifen-induced ROS on regular mitogenic signaling. Tamoxifen-induced oxidants inhibit PTEN rendering it unable to inhibit the PI3K/Akt pathway resulting in the hyper-stimulation of the pathway and a continuous phosphorylation of Akt. Phosphorylated Akt then activates NRF-1 by phosphorylation enabling the now active protein to binds to the promoter region of certain cell cycle genes.

III REDOX SIGNALING CONRIBUTES TO THE DEVELOPMENT OF TAMOXIFEN RESISTANCE IN BREAST CANCER TREATMENT

Abstract

Tamoxifen is a selective estrogen receptor modulator (SERM) which is used to treat estrogen receptor positive (ER+) breast cancer. It is also used in breast cancer prevention

and as an adjuvant in early breast cancer cases. Only about 70% of breast cancer patients will initially respond to tamoxifen treatment. About 40% of patients who were initially responsive to the drug and majority of the patients with metastatic breast disease will develop resistance over time. Therefore the problem of tamoxifen resistance has been a major setback in the otherwise successful treatment of ER+ breast cancer patients. Although there has been an improvement in our current understanding of the molecular mechanism(s) that result in the development of tamoxifen resistance, there is still a lot of ground to cover on the subject. In this study we explored the role of Reactive oxygen species (ROS) in the evolution of tamoxifen sensitive breast cancer to tamoxifen resistant breast cancer. ROS are products of cellular metabolism which have been shown to induce oxidative stress. An excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer. Furthermore, ROS induced oxidative stress has been shown to initiate various cellular responses including cell proliferation and transformation; alteration of intracellular redox state and the oxidative modification of certain signaling proteins resulting in the post translational modification of their downstream targets. Interestingly, like estrogen, tamoxifen has been shown to induce ROS formation, making it probable that tamoxifen induced ROS generated as a result of prolong tamoxifen treatment of breast cancer patients may play a vital role in the development of its resistance. Using tamoxifen resistant LCC2 cell and standard laboratory techniques, we examined the role of ROS in the evolution of breast cancer from a tamoxifen sensitive phenotype to a resistant one. We were interested in finding out how and if ROS induces the post translational modification of p27 resulting in the loss of its inhibitory function and if this process can be reversed by pre-exposing the cells

to biological or chemical antioxidants. We discovered that, as a result of tamoxifen exposure, an increasingly pro-oxidant environment induced cell proliferation by diminishing p27 expression and increasing its phosphorylation on Threonine157 and Threonine187. This is accompanied by an ensuing reduction of p27 binding to Cyclin E/Cdk2 complex and loss of p27 stability culminating in the loss of sensitivity to tamoxifen. We also found that Pre-exposing cells to either biological or chemical anti-oxidants, restored tamoxifen sensitivity by reversing the previous expression and phosphorylation of p27.

Introduction

Tamoxifen resistance has remained a major setback in an otherwise successful treatment of estrogen receptor positive (ER+) breast cancer. Most breast cancers are estrogen-receptor positive (ER+). Approximately 70% of patients with ER+ cancer will respond to anti-estrogen therapy, such as tamoxifen[1]. In addition, a significant number of tumors that initially respond to tamoxifen treatment often progress into a resistant phenotype over time, in spite of sustained expression of ERα [2, 3].

Over the years, several pathways and postulates have been proposed and examined as possible mechanisms of breast cancer resistance to tamoxifen. While some of these have translated into beneficial therapies to a large number of patients, tamoxifen resistance still remains a significant problem. In addition, though studies have examined the roles of ERα and β, and their co-regulatory proteins; growth factor receptors and the activity of their downstream kinases; Cas/c-Src/BCAR3 and cell cycle regulators like Cyclins D/E

and p27, currently there is no study investigating the effect of Reactive oxygen species (ROS) on p27 in tamoxifen resistance.

Cancers have been shown to be under persistent oxidative stress [4]. Oxidative stress, which is triggered by an imbalance between the production and detoxification of ROS, [5] has been implicated in the therapeutic resistance to tamoxifen [6]. Tamoxifen, a selective estrogen receptor modulator (SERM), has been reported to possess both pro- and anti-oxidant properties. Among its antioxidant actions are: its cardio-protective effect in the prevention of atherosclerosis [7, 8]; its inhibition of lipid peroxidation [9] and its ability to protect low density lipoproteins (LDL) against copper ion-mediated oxidative damage [10] in humans. Conversely, its pro-oxidant effects include its ability to induce oxidative liver damage [11] and its explication as a hepato-carcinogen in rodents due to ROS over-production that occurs during its metabolism [12, 13]. Additionally, it has been suggested that oxidative stress might trigger the pathogenesis of tamoxifen-induced toxicity [14]. Though it has not been ascertained whether the elevated oxidative state of tamoxifen resistant breast cancer is due to prolong tamoxifen exposure, the possibility that the pro-oxidant property of prolonged tamoxifen exposure may play a role in breast cancer resistance cannot be ignored.

Reactive oxygen species consist of a number of partially reduced metabolites of oxygen such as superoxide anions ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$), characterized by higher reactivity than molecular oxygen. These reactive species are known to play a vital role in cell growth mediated by 17β estradiol (E2) [15]. Oxidative

stress has been shown to instigate a number of varied responses including cell proliferation and transformation. Furthermore, an excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of cancer [16]. Redox balance is achieved by various enzymatic and non enzymatic antioxidant systems that counteract the harmful effect of ROS. For example, superoxide dismutase (SOD) catalyses the dismutation of the superoxide anion into H_2O_2, while Catalase and glutathione peroxidase (GPx) metabolize H_2O_2 into water and molecular oxygen [17, 18]. It is interesting to note that physiological ROS play a significant role in the regulation of signaling pathways such as kinase and phosphatase activation/inactivation.

Oxidative stress-induced redox surges could bring about changes in the thiol status of signaling proteins with alterations in their Phosphorylation/dephosphorylations functions. These modifications could lead to changes in cellular signaling transduction pathways, DNA and RNA syntheses, protein syntheses, enzyme activation and cell cycle regulations [19-27]. Signaling proteins such as mitogen-activated protein kinases (MAPK) [27] and protein tyrosine phosphatases (PTPs) like PTEN (Phosphatase and tensin homolog deleted on chromosome ten) and CDC25 [28] have been shown to be susceptible to oxidation because they contain essential cysteines which serve as possible targets for ROS in different pathways [30, 31].

Protein tyrosine phosphatases (PTPs) are important enzymes in cell cycle control and signal transduction. In conjunction with protein tyrosine kinases (PTKs), PTPs regulate levels of protein tyrosine phosphorylation in response to cellular signals [32, 33]. Many

studies have shown that PTKs may be directly activated through the inhibition of PTPs by ROS [34, 35]. For example, PTEN is a redox sensitive phosphatase which has been shown to be inactivated by ROS. The inactivation of PTEN leads to the sustained phosporylation and activation of AKT. AKT has been shown to phosphorylate p27 on threonine 157 with a resultant cytoplasmic sequestration and a consequent inhibition of its nuclear function.

p27 is a Cyclin-kinase inhibitor (CKI) which was originally discovered as a protein whose expression was induced by different growth inhibitory conditions/agents such as serum starvation, contact inhibition, transforming growth factor-α or by lovastatin [36]. Its expression is reduced by mitogens like epidermal growth factors and estrogens. Mice with p27 knockout develop multiorgan hyperplasia and pituitary tumors, thus underscoring a role for p27 in both proliferation and differentiation [37]. p27 is also well-known for its ability to inhibit G1 Cyclin/CDK complexes. It is noteworthy that its activity is regulated by its post-translational modification [38, 39]. Hence one may infer that because of their capacity to oxidize and inactivate PTEN, ROS may be able to regulate p27 function by modulating its post-translational modification.

We therefore hypothesized that (i) in addition to its known action at the ER, tamoxifen also prevents estrogen-mediated progression of cell cycle by counteracting estrogen-induced reactive oxygen species signaling, and (ii) as a result of chronic oxidative stress, and the conversion of estrogen-sensitive breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment. Therefore, an increase in ROS levels promotes the loss of p27 inhibitory function through the

inactivation of protein tyrosine phosphates (PTPs) and a consequent change in p27 phosphorylation.

To test these hypotheses, we conducted experiments to i) determine the relationship between tamoxifen and oxidative stress, ii) determine the effect of ROS on p27 expression and its phoshorylation in tamoxifen resistant LCC2 cells, iii) determine if pre-treatment of LCC2 cells with biological or chemical antioxidants can restore tamoxifen sensitivity by re-establishing redox balance within the cells, and iv) determine the effect of antioxidants on p27 stability and its inhibitory function.

Materials and methods

Materials

Antibodies to p27, Cyclin E, CDK2 and Phosphorylated p27 on Threonine187 were purchased from Santa Cruz Biotechnology. T157 Phosphorylated p27 antibody was purchased from Abcam. Beta Actin, 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA), DMSO, Ebselen and cyclohexamide were purchased from Sigma Aldrich. Exactacruz immune- precipitation kit was purchased from Santa Cruz Biotechnology. BrdU kit was purchased from ROCHE. Protein A Agarose was purchased from Invitrogen. The adenoviruses AdEmpty (EV), AdMnSOD (SOD), and AdCatalase (CAT) were purchased from ViraQuest, Inc. (North Liberty, IA, USA).

Cell culture

LCC2 cells were provided by Dr Slingerland (Braman Family Breast Cancer Institute, Miami)). Cells were cultured in DMEM (Gibco) containing 5% Fetal bovine serum (FBS), 25,000 units of penicillin (base) and 25,000 µg of Streptomycin at 37°C in a fully humidified atmosphere of 5% CO_2 in air. For experiments, 40% Confluent cells were further cultured in DMEM containing 5% Charcoal Stripped FBS for 24hours to minimize the effect of serum and synchronize the cells in G1. Synchronized cells were always treated with 0.01% DMSO, 1uM Tamoxifen (TAM), 100pg E2 or 1uM fulvestrant.

Adenoviral Transduction

The adenoviruses AdEmpty (EV), AdMnSOD (SOD), and AdCatalase (CAT) were manufactured at ViraQuest, Inc. (North Liberty, IA, USA) by inserting either Ad5 E1/partial E3-deleted replication-deficient adenoviral vector only (EV) or the vector containing MnSOD (SOD) or Catalase (CAT) gene in the E1 region of the vector.

LCC2 cells were seeded in 100-mm dishes at a density of 1×10^6 cells/dish in the medium containing charcoal stripped FBS for 24hrs. The medium was then aspirated and replaced with 5 ml of serum-free medium containing 50pfu of SOD, CAT or EV adenovirus. After 2 hours of incubation, 10% of charcoal stripped serum was added to the medium making it 5% charcoal stripped medium. Medium was changed after 24 hours of adenoviral exposure and treatment(s) were added to pre-designated plates and incubated for another 48 hours.

Ebselen Pre-treatment

$1x10^6$ LCC2 cells were seeded per 100mm dish in medium with 5% Charcoal stripped serum. 21 hours post seeding 20μM of Ebselen was added to the culture and incubated for 3hours. Desired treatments were then added to the culture and incubated for 48 hours.

BrdU Incorporation Assay

For BrdU incorporation, LCC2 cells were seeded in 96-well plates at a density of 2500/well and incubated in 5% CO2 incubator at 37°C. 24 hours after seeding, cells were either infected with EV, CAT or SOD at a multiplicity of infection (m.o.i) of 50pfu/cell as described above or pre-treated with 20 μM of Ebselen. After exposure to antioxidants cells were then stimulated with either 0.01% DMSO, E2 (100pg), TAM (1uM) or E2 + TAM for 48hours. Following the above treatment, BrdU incorporation assay was carried out using Cell Proliferation ELISA, BrdU labeling Kit (Roche Molecular Biochemical) in accordance with manufacturer's recommendation. Colorimetric changes were acquired at 370 nm with a Tecan Genios microplate reader.

2′, 7′-dichlorofluorescin diacetate (DCFH-DA)

To carry out the DCFH-DA Assay, 10,000/well of LCC2 cells were seeded in a 96-well plate and cultured in 10% growth medium for 24 hours. After 24 hours growth medium was aspirated and replaced with serum free medium for another 24 hours after which the starvation media was aspirated and replaced with 100uL/well of HBSS containing 10 μM DCFDA pre-diluted with Pluronic F-127 and incubated at 37°C for 20 mins. At the end of the incubation, DCFH-DA solution was gently aspirated and pre-designated wells were

then stimulated with 100ul of HBSS containing either 0.01% DMSO (vehicle control), E2 (100pg), TAM (1uM) or E2 + TAM. Cells were further incubated for 5mins after which fluorescence readings were taken using 485 nm and 535 nm as excitation and emission filters, at intervals of 5 mins. Optical density readings were obtained using a Tecan Genios microplate reader.

Western blot

1 x 10^6 LCC2 cells per 100mm dish were initially exposed to antioxidants as described above and then stimulated with either 0.01% DMSO, 1μM TAM, or 1μM fulvestrant for 48hours. Post treatment, cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer [25 mM Tris HCl (pH 7.4), 25 mM NaCl, 1 mM sodium orthovanadate,10 mM sodium pyrophosphate, 10 mM NaF, 0.5 mM EGTA, 1.0% Triton X-100, 1 mM PMSF, and 10 mM okadaic acid] for 15 min on ice. Samples were then briefly sonicated and centrifuged at 10,000 rpm for 10 min at 4°C. The total protein concentration of the resulting supernatant was determined by Pierce BCA quantification kit. Equal amount of protein extracts (50 μg) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PVDF), blocked in 1% BSA and fat free milk solution containing 50 mM NAF for 1hour and blotted with antibodies. Labeled proteins are visualized with an ECL system (Amersham Biosciences). Band intensity was determined using densitometric analysis (NIH ImageJ software). The level of p27 was expressed as its ratio to β-actin.

For chronic exposure, after exposing cells to antioxidants cells were stimulated with either DMSO, TAM, or fulvestrant for 21 days after which the cells were harvested for lysate preparation and Immunoblotting as described above.

Co-immunoprecipitation

For co-immunopreipitation to determine Cyclin E complexes, the Exactacruz protocol was followed according to the manufacturer's recommendation. Briefly, after stimulation, lysate was prepared using ice-cold Nonidet P-40 lysis buffer (0.1% Nonidet P-40, 50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 0.02 mg each of aprotinin, leupeptin, and pepstatin per ml). The lysate was then sonicated briefly and centrifuged at 10,000 rpm for 10 mins. Supernatant was saved and then pre-cleared using 30 µl of Exactacruz pre-clearing matrix for 30minutes at 4^{o}C while rotating. Matrix was pelleted by microcentrifugation at maximum speed for 30 seconds at 4°C and the supernatant (cell lysate) was transferred to a new micro centrifuge tube and BCA assay performed.

IP antibody-IP matrix complex was formed by adding 2 µg of Cyclin E antibody to be immunoprecipitated to 40 µl of suspended IP matrix and 500 µl PBS and incubating at 4° C on a rotator overnight. After overnight incubation, matrix was pelleted, supernatant discarded and pelleted matrix was washed two times with 500 µl of PBS, each time while repeating the above centrifugation and aspiration steps. After the second wash and micro-centrifugation, 300 µg of pre-cleared lysate was added to the IP antibody-IP matrix complex, and incubated overnight at 4° C after which samples were microcentrifuged at

maximum speed for 30 seconds at 4° C to pellet IP matrix and the pelleted matrix washed 2-4 times with NP40 lysis buffer and pelleted. After the final wash, 40 µl of 2X reducing electrophoresis buffer was added to the pellet, boiled for 3 mins, quick-spinned and equal volumes loaded onto gel, resolved, transferred, and blotted with Cyclin E, Cdk2, and p27 antibodies. Antibody alone, and whole cell lysate controls were run alongside immunoprecipitates. The same was done for CDK2 and p27. Western blot anti-body was probed using the appropriate HRP conjugated ExactaCruz reagent.

Soft Agar Colony Formation Assay.

To assess the effect of antioxidants on anchorage-independent cell growth, a soft agar colony formation assay using either plain or antioxidant pretreated (Ebselen, Catalase or MnSOD) LCC2 cells was performed. LCC2 cells (1000/well) were suspended in 0.2ml of charcoal stripped culture medium containing 0.25% agar and appropriate treatment (0.01% DMSO or 1µM Tamoxifen or fulvestrant) and poured over a pre-hardened feeder layer of agarose comprising 0.2 ml of the charcoal stripped medium, containing 0.5% agar, in a 48 well plate. Cells were fed every 4 days and allowed to incubate for 30 days. After 14 days of incubation at 37°C in a humidified CO2 incubator, colonies were counted excluding any colonies with a diameter ≤ 60 µ meter.

P27 protein stability

To determine the effect of antioxidants on p27 stability, 1 x 10^6 LCC2 cells were seeded in 100 mm plates in medium with charcoal stripped FBS for 24hours and then some cells were exposed to either Ebselen or 50 pfu of CAT, SOD or EV. After 24 hours media was

replaced with fresh medium with charcoal stripped FBS alone for 40 hours. After which 50 μM of cycloheximide was added to media for the indicated period spanning the next 8 hours. Cells were lysed in RIPA buffer for 60 min at 4°C. Samples were then centrifuged at 10,000g for 10 min at 4°C. The total protein concentration of the resulting supernatant was determined by BCA quantification. Equal amount of protein extracts (50 μg) were separated by SDS-PAGE, transferred to a PVDF membrane, blocked in 1% BSA and fat free milk solution containing 50 mM NAF for 1 hour and blotted with antibodies. Labeled proteins are visualized with an ECL system (Amersham Biosciences). Band intensity was determined using densitometric analysis (NIH ImageJ software). The level of p27kip was expressed as its ratio to β-actin.

Results

Antioxidants mitigate ROS formation in TAM resistant LCC2 cells

To determine the effect of antioxidants on TAM-induced ROS in LCC2 cells, a DCFH-DA assay was performed using cells with or without antioxidants. Data reveals that LCC2 cells generate ROS when exposed to TAM and E2 respectively and the ROS generated was significantly inhibited by the co-treatment of TAM with E2 (Figs. 1A)**.** This observation implies that while individual treatment of LCC2 cells with E2 and TAM are pro-oxidant, TAM possesses an anti-oxidant effect in the presence of E2. Co-treatments of cells with either TAM or E2 and ROS modulators (biological or chemical) also inhibited TAM-induced ROS (Figs. 1B, C)**.**

Antioxidants inhibits BrdU incorporation in TAM resistant cells

A BrdU incorporation assay was carried out to determine the effect of antioxidants on ROS induced DNA synthesis in LCC2 cells. Experimental outcomes show that E2 and TAM-induced cell proliferation in LCC2 cells is significantly inhibited by the co-treatment of TAM with E2 (Fig. 2A) and by co-treatment of TAM or E2 with Biological (CAT or SOD) or chemical (Ebselen) ROS modulators (Figs. 2B, C)**.** The ability of ROS modulators to inhibit TAM or E2 induced DNA synthesis points to the pro-oxidant effect of TAM or E2 on LCC2 cells and the inherent capacity of this pro-oxidant environment to promote cell proliferation.

Antioxidants mitigate anchorage independent growth in TAM resistant LCC2 cells.

To determine the effect of antioxidants on anchorage-independent growth in TAM resistant cells, a colony assay was performed using LCC2 cells**.** Anchorage independent growth was significantly inhibited by fulvestrant but not by TAM or DMSO (Fig.3A). fulvestrant was included in this experiment as a positive control because LCC2 cells are sensitive to it. Ebselen pretreatment or the over expression of CAT or SOD in TAM resistant LCC2 cells significantly inhibited anchorage independent growth compared to EV infected or uninfected cells (Figs. 3B, C). This result further substantiates the pro-oxidant, growth inducing effect of TAM**.**

Antioxidants increased p27 expression in TAM resistant cells

Western blotting was carried out to determine the effect of antioxidants on TAM resistant cells. Fig. 4A reveals that the treatment of LCC2 cells with fulvestrant significantly

increased p27 expression compared to TAM. Ebselen pretreatment or the over expression of CAT or SOD in LCC2 cells also resulted in a significant increase in p27 expression when treated with TAM compared to EV infected cells and uninfected cells (Fig.4B, C). This is similar to the effect observed following exposure to fulvestrant in Fig.4A**.** It may thus be inferred that the TAM induced pro-oxidant environment may promote LCC2 cell proliferation by decreasing p27 expression.

Antioxidants decreased p27 phosphorylation in TAM resistant cells

Western blotting was also carried out to determine the effect of antioxidants on p27 phosphorylation. Panel I. Results obtained from this experiment show that treatment of uninfected LCC2 cells with fulvestrant resulted in significantly reduced phosphorylation of p27 on Threonine 157(T157) relative to TAM treated cells which showed a phosphorylation level that was ≥ T157 phosphorylation in cells exposed to DMSO control (Fig.5A). Over expression of CAT or SOD or Ebselen pretreatment in LCC2 cells leads to a significant decrease in T157 phosphorylation when treated with TAM compared to either EV infected control or uninfected cells. (Figs.5B, C). Once again the cells exposed to ROS modulators acted like those exposed to fulvestrant by showing a decrease in p27 phosphorylation on T157 unlike the unexposed cells which showed an increase in T157 phosphorylation. It is noteworthy that phosphorylation of p27 on T157 results in increased cytoplasmic sequestration of p27 and vice versa.

Panel II. In Fig.5A Experimental outcome reveals a significant reduction of p27 phosphorylation on Threonine 187 (T187) in LCC2 cells exposed to fulvestrant compared

to TAM. (B) LCC2 cells over expressing CAT or SOD or pretreated with Ebselen also shows a significant reduction in the level of p27 phosphorylation on T187 when treated with TAM compared to either EV control or uninfected cells.

A result similar to the one obtained for T157.

Prolonged exposure of LCC2 cells to antioxidants and TAM treatment increased p27 expression

Immunoblotting was carried out to determine the effect of chronic antioxidant and TAM exposure on p27 expression. Results obtained show that prolonged (21 days) exposure of LCC2 cells to fulvestrant significantly increased p27 expression compared to TAM (Fig. 6A). Over expression of CAT or SOD in LCC2 cells also resulted in a significant increase in p27 expression when treated with TAM compared to EV infected cells and uninfected cells (Fig.6B). This shows that chronic exposure to antioxidants and TAM have the same outcome as short term (48hrs) exposure.

Prolonged exposure of LCC2 cells to antioxidants and TAM treatment reduced p27 phosphorylation

The following are results from Immunoblotting to determine the effect of antioxidants on p27 phosphorylation. Panel I. Chronic treatment of uninfected LCC2 cells with fulvestrant resulted in a significant reduction in the phosphorylation of p27 on Threonine 157(T157) compared to TAM treated cells which showed an increase in the level of p27 phosphorylation on T157(Fig.7A). Treatment of LCC2 cells over expressing CAT or

SOD also resulted in a reduction in phosphorylation when compared to either EV control or uninfected cells exposed TAM (Fig.7B).

Panel II. Fig.8A shows that chronic exposure of LCC2 cells to fulvestrant resulted in a significant reduction in the level of p27 phosphorylation on Threonine 187 (T187) compared to TAM. LCC2 cells over expressing CAT or SOD upon treatment with TAM showed a significant reduction in phosphorylation when compared to either EV control or uninfected cells exposed to TAM.

Antioxidants increased p27 binding to CylinE and CDK2 in TAM resistant cells

To determine the effect of Antioxidants on p27 binding to CDK2 and or CyclinE a co-immunoprecipitation was performed. Treatment of LCC2 cells with fulvestrant resulted in an increase in p27 binding to CDK2 and CyclinE compared to TAM treatment (Fig. 8A). Ebselen pretreatment or the Over expression of CAT or SOD in LCC2 also resulted in increased binding of p27 to CDK2 and CyclinE when treated with TAM compared to either EV infected controls or uninfected cells exposed to TAM (Fig.8B, C).

Antioxidants increased CDK2 binding to P27 and CyclinE in TAM resistant cells To determine the effect of Antioxidants on p27 binding to CDK2 and or CyclinE a co-immunoprecipitation was performed. Treatment of LCC2 cells with fulvestrant resulted in an increase in CDK2 binding to p27 and CyclinE compared to TAM treatment (Fig. 9A). Ebselen pretreatment or the Over expression of CAT or SOD in LCC2 also resulted

in increased binding of CDK2 to p27 and CyclinE when treated with TAM compared to either EV infected controls or uninfected cells exposed to TAM (Fig.9B, C).

Antioxidant Increased CylinE binding to CDK2 and p27 in TAM resistant cells

To determine the effect of antioxidants on CyclinE binding to CDK2 and or p27 a co-immunoprecipitation was performed. In (Fig. 10A)**,** treatment of LCC2 cells with fulvestrant resulted in an increase in CyclinE binding to CDK2 and p27 compared to TAM while data from (Fig.10B, C) also showed increased CyclinE binding to CDK2 and p27 in LCC2 cells over expressing CAT or SOD compared to either EV infected cells or uninfected cells exposed to TAM.

P27 expression is less in LCC2 cells compared to MCF7 when synchronized

Pursuant to the finding in Figures 4 and 5 Immunoblotting was performed to compare the levels of p27 in TAM resistant LCC2 cells to its parent MCF7 cells. Results obtained showed the level of p27 expression in LCC2 to be about 40% less than that in MCF7 cells of equal protein concentration, exposed to the same experimental conditions**.**

Antioxidants Increased p27 stability in TAM resistant cells

To determine p27 stability a stability assay was performed using cycloheximide. LCC2 cells not pre-exposed to antioxidants had a shorter half life (2.42hours) compared to CAT or SOD over expressing cells.

Discussion

In this study, we hypothesized that, (i) In addition to its known action at the ER, TAM also prevents estrogen-mediated progression of cell cycle by counteracting estrogen-induced ROS signaling, and (ii) As a result of chronic oxidative stress, the conversion of estrogen-sensitive breast tumors to a Tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment. We therefore propose that an increase in ROS levels promotes the loss of p27 inhibitory function through the inactivation of protein tyrosine phosphates (PTPs) and a consequent change in p27 phosphorylation.

TAM, a known selective estrogen receptor modulator (SERM), has been widely shown to possess either pro- or antioxidant properties [7-13]. Our results show that TAM possesses the capacity to act like both an oxidant and an antioxidant. Exposure of LCC2 cells to TAM or E2 induced the formation of reactive oxidants (Fig.1A). These reactive oxidants were then inhibited by the co-treatment of E2 with TAM, much like the ROS inhibition observed in cells pre-treated with antioxidants (Figs.1B & C). We further demonstrated that E2 and TAM induced ROS in LCC2 cells were capable of inducing cell proliferation which was then inhibited by pre-exposure of the cells to biological or chemical antioxidants or the co-treatment of TAM with E2 (see Figs.2 & 3). These findings make a case for the dual role of TAM as both a pro- and an antioxidant. Based on these results, it appears that the antioxidant effect of TAM is observed in the presence of E2.

Exposure of TAM resistant LCC2 cells to TAM resulted in a decrease in p27 expression in contrast to the increase in p27 expression observed when the same cells were treated

with fulvestrant (see Fig.4A). Fulvestrant is an anti-estrogen to which LCC2 cells are known to be sensitive. We also observed that the exposure of these cells to TAM increased p27 phosphorylation on T157 and T187 while exposure to fulvestrant appeared to have the opposite effect (Fig5. Panels 1& IIA).

Conversely, following the antioxidant pre-treatment/over expression, a fulvestrant-like effect i.e., an increase in p27 expression (see Figs. 4B &C) and a decrease in p27 phosphorylation on T157 and T187 was observed in TAM treated cells compared to cells which were not pre-exposed to antioxidants (Figs. 5 Panels I & II B & C). It can thus be inferred that the over expression of antioxidant in LCC2 cells leads to an increase in p27 expression and a decrease in its phosphorylation on T157 and T187. This is in line with other previous studies [40 - 42], which showed that treatment with anti-estrogen drugs like TAM or fulvestrant caused cell cycle arrest, with up-regulation of p21 and p27 levels, an increase in their binding to Cyclin E–Cdk2, and kinase inhibition [40]. Additionally, results from our comparison of p27 expression between Tamoxifen sensitive parental MCF7 and Tamoxifen resistant LCC2 cells showed a 40% decrease in p27 expression in LCC2 cells compared to MCF7 (Fig.11). This implies that TAM resistance could be associated with a decrease in p27 expression rather than the loss p27 function in which case, an increase in p27 expression following antioxidant pre-treatment is a significant finding.

Though the observed experimental outcomes imitate the effect of fulvestrant on LCC2 cells, we cannot yet interpret this to either denote an increase in TAM sensitivity or the growth inhibitory function of p27. This is because previous studies have indicated that

p27 potentially has dual role(s) in tumor – suppression and promotion [43-46] depending on its subcellular redistribution and its binding to the Cyclin-Cdk complex. The binding of p27 to the Cyclin E/Cdk2 complex in the nucleus promotes its function as a CKI while cytoplasmic sequestration takes it away from its nuclear Cyclin/Cdk target to the growth promoting interaction with Cyclin D/Cdk4/6 complex [47].

In line with previous studies [40], we also found an increase in the binding of Cyclin E–Cdk2 and p27 in TAM treated cells over expressing antioxidant (CAT and SOD) compared to control cells not exposed to antioxidants (see Figs. 8, 9 & 10). In the context of our initial finding of increased p27 expression and decreased phosphorylation, these results of increased binding of p27 to Cyclin E–Cdk2 and increased p27 stability in cells over expressing antioxidants (Fig. 1**2**), are highly suggestive of an increase in the TAM sensitivity, most likely due to p27 inhibitory function in antioxidant over expressing LCC2 cells.

In summary, we demonstrate that TAM has the capacity to induce ROS formation and act as an antioxidant in the presence of E2. Following prolonged exposure to TAM and an increasingly pro-oxidant environment, the oxidants formed are able to promote cell proliferation. This could be by decreasing p27 expression and regulation of its activity by post-translational modification involving an increase in p27 phosphorylation on T157 and T187. This is accompanied by a resultant cytoplasmic sequestration, decreased binding to Cyclin E/Cdk2 complex and loss of p27 stability resulting in the loss of sensitivity to TAM. Clearly, these events are oxidant driven, therefore they can be reversed by

antioxidant over expression or pre-treatment, with a resultant growth inhibitory effect and increased TAM sensitivity.

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(44) Kaldis, P. Another piece of the p27Kip1 puzzle. *Cell* 2007; 128, 241–4.

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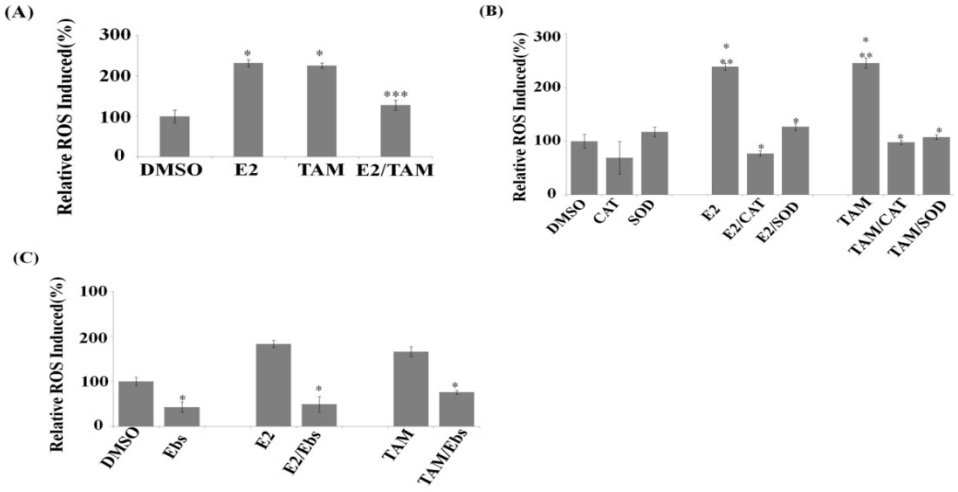
Figures and Legend

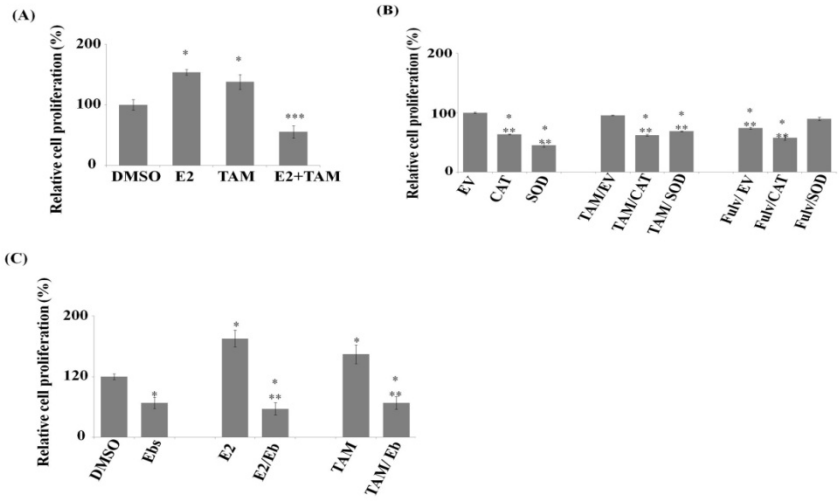
Fig 1-LCC2.



Antioxidants mitigate ROS formation in TAM resistant LCC2 cells. To determine the effect of antioxidants on TAM induced ROS in LCC2 cells, 1 x10^4cells/well were seeded in a 96-well plate overnight and serum starved for 24 hrs. Post-starvation 100 µL/well of HBSS containing 10 µM DCFH-DA pre-diluted with Pluronic F-127 was added to each well and incubated at 37^{o}C for 20 mins. DCFH-DA solution was then aspirated and replaced with 100 µL of HBSS containing the desired treatments. Cells were further incubated for 5 mins and fluorescence readings were taken using 485 nm and 535 nm as excitation and emission filters, at intervals of 5mins. A) LCC2 cells treated with TAM, E2 or TAM/E2; B) TAM resistant cells over expressing Catalase (CAT) and MnSOD (SOD) were treated with TAM, E2 or TAM/E2; C) LCC2 cells co-treated with Ebselen and TAM, E2 or TAM/E2. Assay was performed 3x and data is expressed as mean percentage change from control +/- SE, (p 0.05). (*) denotes significance when treatment is compared to DMSO control, (**) indicates significance when compared to EV/DMSO

control while (***) indicates significance when treatment is compared to DMSO and each individual treatment.

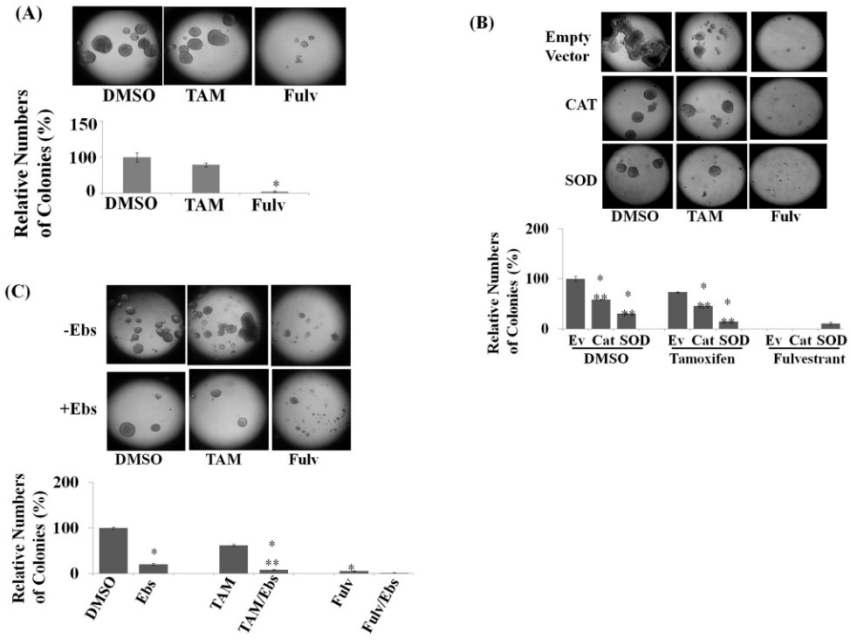
Fig 2-LCC2



Antioxidants inhibit BrdU incorporation in TAM resistant cells. To determine the effect of antioxidants on ROS induced DNA synthesis in LCC2 cells, 2.5 x 10^3 cells/well were seeded in a 96-well plate overnight and exposed to chemical or biological antioxidants as described in methods. After exposure to antioxidants, cells were then stimulated with E2, TAM or fulvestrant for 46 hrs and pulsed labeled with BrdU for 2 hrs. BrdU assay was then carried out as recommended by manufacturer. Colorimetric changes were acquired at 370 nm with a Tecan Genios microplate reader at 5 mins interval. A) LCC2 cells treated with TAM, E2 or TAM/E2; B) TAM resistant cells over expressing CAT and SOD and treated with TAM, E2 or TAM/E2; C) LCC2 cells co-treated with Ebselen and TAM, E2 or TAM/E2. Assay was performed 3x and data is expressed as mean percentage change from control +/- SE, (p 0.05). (*) denotes significance when treatment is compared to DMSO control, (**) indicates significance

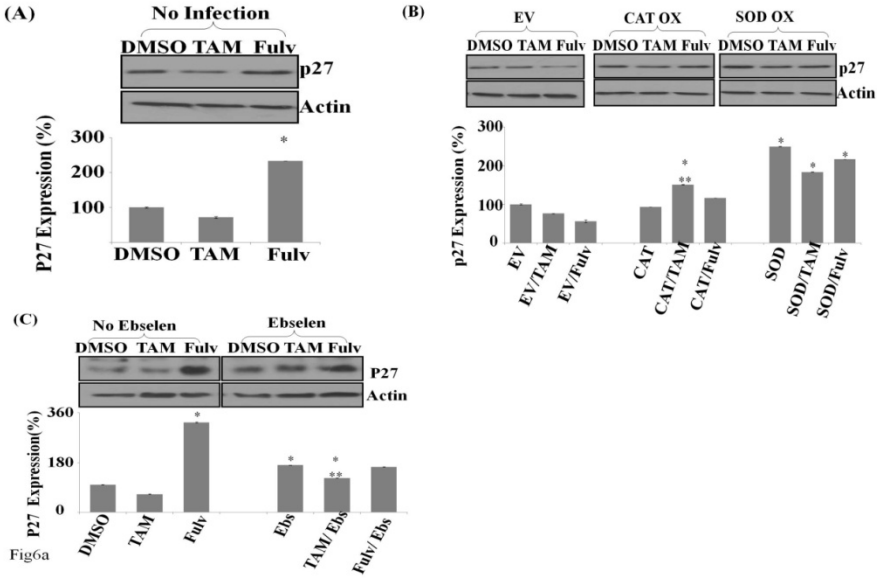
when compared to EV/DMSO control while (***) indicates significance when treatment is compared to DMSO and each individual treatment.

Fig 3-LCC2



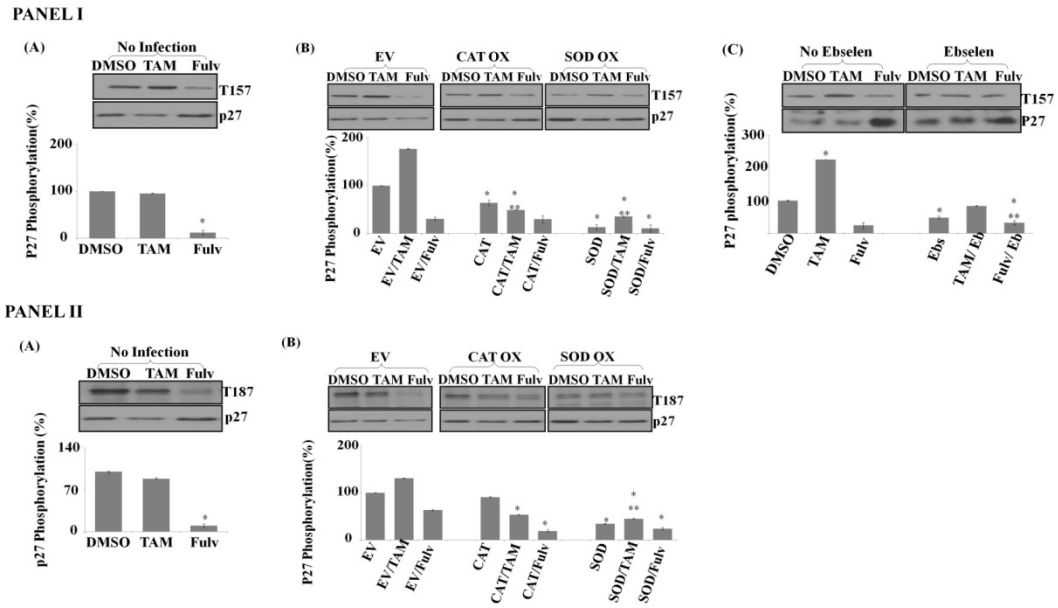
Antioxidants mitigate anchorage independent growth in TAM resistant LCC2 cells. To determine the effect of antioxidants on anchorage-independent growth in TAM resistant cells, LCC2 cells were incubated on soft agar with or without antioxidants exposure for 21 days and colonies ≥60 microns in diameter were enumerated. A) Anchorage independent growth of TAM resistant cells; B) LCC2 cells over expressing Catalase (CAT) or MnSOD (SOD); C) LCC2 cells pretreated with chemical antioxidant. Assay performed 3x and data is expressed as mean percentage change from control +/- SE, (p 0.05). (*) denote significance when treatment is compared to DMSO control while (**) indicates significance when compared to EV/DMSO control.

Fig 4-LCC2



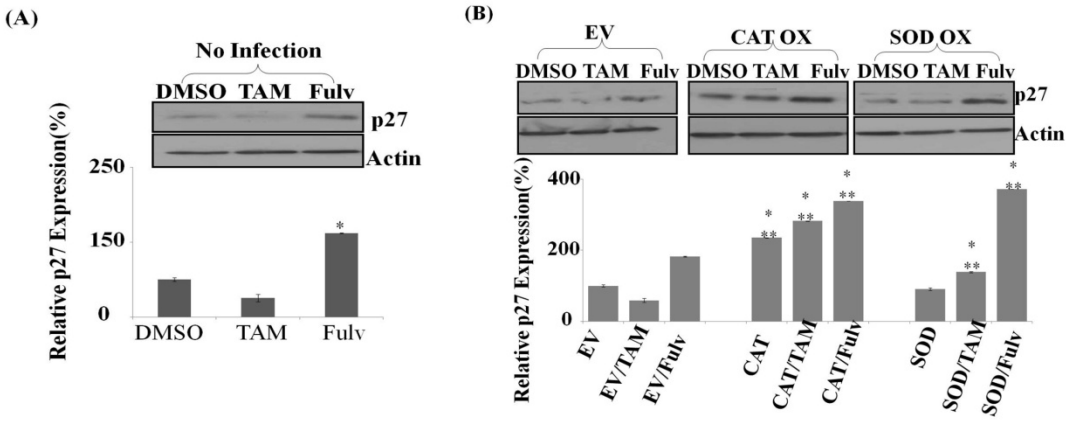
Antioxidants Increased p27 expression in TAM Resistant cells. LCC2 cells were exposed to antioxidants as described in methods and treated with TAM and fulvestrant respectively. After 48 hrs incubations, cells were harvested with lysis buffer and 50 ug whole cell lysate (WCL) were fractionated on 12% SDS-PAGE gel. Immunoblots were probed with p27 or β actin antibodies respectively. A) LCC2 cells treated with TAM or fulvestrant, B) LCC2 cells over expressing CAT or SOD, then treated with TAM and Fulv, C) LCC2 cells co-treated with chemical antioxidant and TAM or Fulv. Assay was performed 3x. P27 protein level was determined by densitometric analysis and expressed as the percentage mean of its ratio with β actin relative to control +/- SE, (p 0.05). (*) denotes significance when treatment is compared to DMSO control while (**) indicates significance when compared to EV/DMSO control.

Fig 5-LCC2



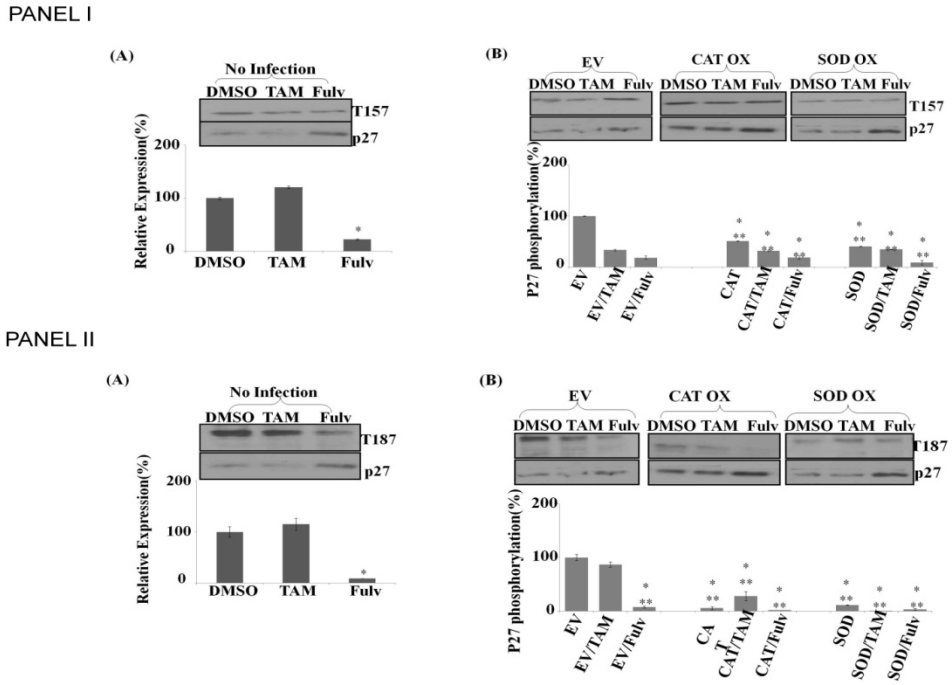
Antioxidants decreased p27 phosphorylation in TAM Resistant cells. (Panel I) A) LCC2 cells treated with TAM or Fulv and probed with p27 or p27 phosphorylated on Threonine 157 (T157); B) LCC2 cells over expressing CAT or SOD were treated with TAM and Fulv, then probed with p27 or T157; C) LCC2 cells co-treated with chemical antioxidant and TAM or Fulv, then probed with p27 or T157. (Panel 2) A) LCC2 cells treated with TAM or Fulv and probed with p27 or p27 phosphorylated on Threonine 187 (T187); B) LCC2 cells over expressing CAT or SOD were treated with TAM and Fulv, then probed with p27 or T187; C) LCC2 cells co-treated with chemical antioxidant and TAM or Fulv, then probed with p27 or T187. Assay was performed 3x. Phosphorylated p27 levels were determined by densitometric analysis and expressed as the percentage mean of their ratios to the ratio of p27 with β actin relative to control +/- SE, (p 0.05). (*) is denotes significance when compared to DMSO control while (**) indicates significance when compared to EV/DMSO control.

Fig. 6-LCC2



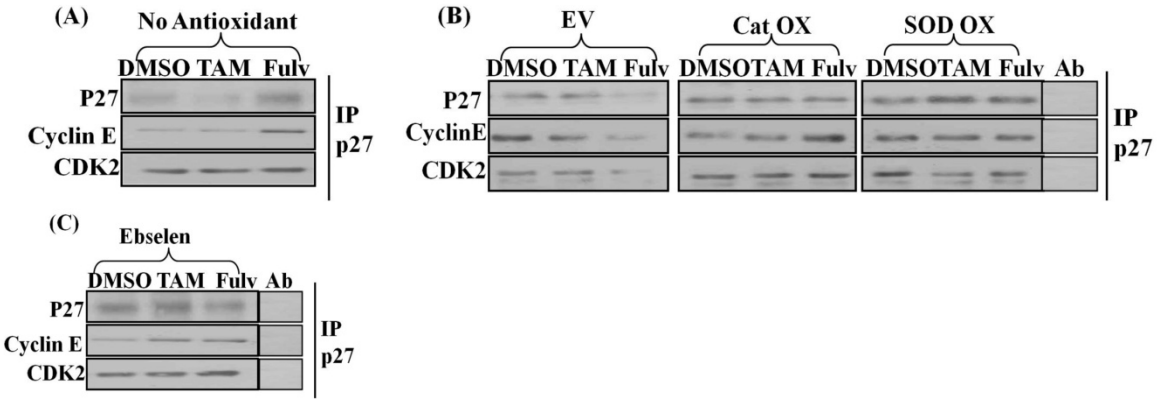
Prolonged antioxidant treatments increased p27 expression in LCC2 cells. LCC2 cells over expressing CAT or SOD were exposed to either TAM or Fulv for 21 days. A) Uninfected cells were treated with TAM or Fulv and probed for p27 and actin; B) CAT or SOD infected cells treated with TAM or Fulv and probed for p27 and β actin. Assay was performed 3x. P27 protein level was determined by densitometric analysis and expressed as the percentage mean of its ratio with β actin relative to control +/- SE, (*p* 0.05). (*) denotes significance when treatment is compared to DMSO control while (**) indicates significance when compared to EV/DMSO control.

Fig 7-LCC2



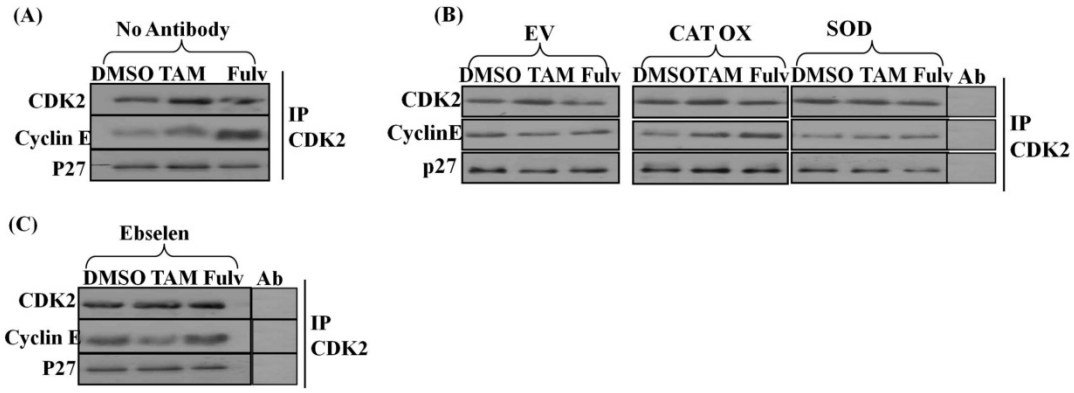
Prolonged exposure of LCC2 cells to Antioxidants decreased p27 phosphorylation in LCC2. (Panel I) A) LCC2 cells treated with TAM or Fulv for 21 days and probed with p27 or T157; B) LCC2 cells over expressing CAT or SOD were treated with TAM and Fulv for 21 days, and then probed with p27 or T157. (Panel II) A) LCC2 cells treated with TAM or Fulv for 21 days and probed with p27 or T187; B) LCC2 cells over expressing CAT or SOD were treated with TAM and Fulv for 21 days, and then probed with p27 or T187. Assay was performed 3x. Phosphorylated p27 levels were determined by densitometric analysis and expressed as the percentage mean of their ratios to the ratio of p27 with β actin relative to control +/- SE, (p 0.05). (*) denotes significance when compared to DMSO control while (**) indicates significance when compared to EV/DMSO control.

Fig 8-LCC2.



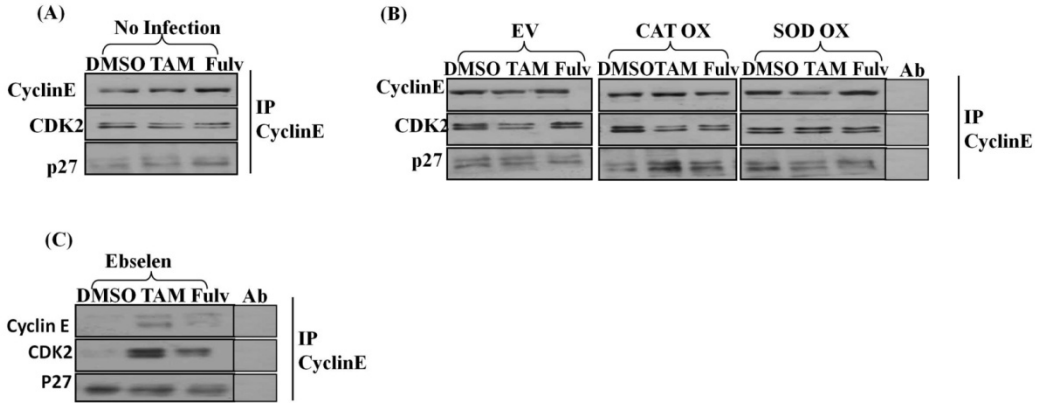
Antioxidant Increased p27 binding to CylinE and CDK2 in TAM resistant cells. LCC2 were exposed to antioxidants as described in methods and treated with TAM and fulvestrant respectively. After 48 hrs incubations, cells were harvested with lysis buffer and 350 ug WCL was immunoprecipitated (IP) with p27 antibody using Exactacruz kit as recommended. Eluent was fractionated on 12% SDS-PAGE gel. Immunoblots were probed with p27, CDK2 or CyclinE antibodies respectively. A) LCC2 cells treated with TAM or Fulv, B) LCC2 cells over expressing CAT or SOD, then treated with TAM and Fulv, C) LCC2 cells co-treated with chemical antioxidant and TAM or Fulv.

Fig 9- LCC2



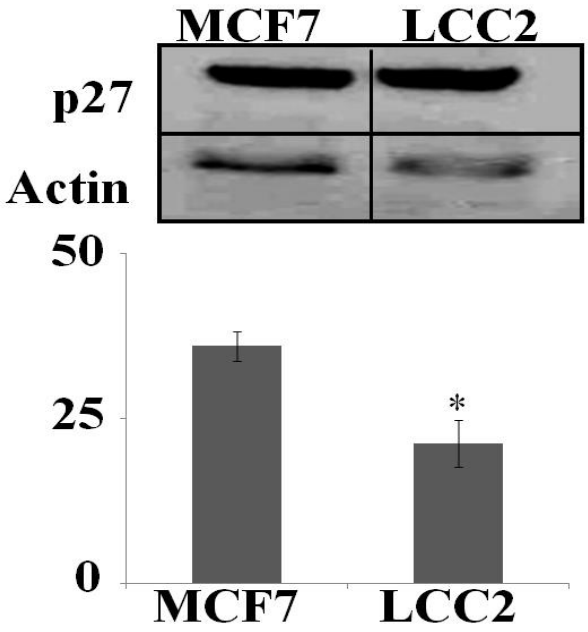
Antioxidants Increased CDK2 binding to CylinE and p27 in TAM resistant cells. LCC2 were exposed to antioxidants as described in methods and treated with TAM and fulvestrant respectively. After 48 hrs incubations, cells were harvested with lysis buffer and 350 ug WCL was immunoprecipitated (IP) with CDK2 antibody using Exactacruz kit as recommended. Eluent was fractionated on 12% SDS-PAGE gel. Immunoblot were probed with, CDK2, p27 or CyclinE antibodies. A) LCC2 cells treated with TAM or Fulv, B) LCC2 cells over expressing CAT or SOD, then treated with TAM and Fulv, C) LCC2 cells co-treated with chemical antioxidant and TAM or Fulv.

Fig10-LCC2



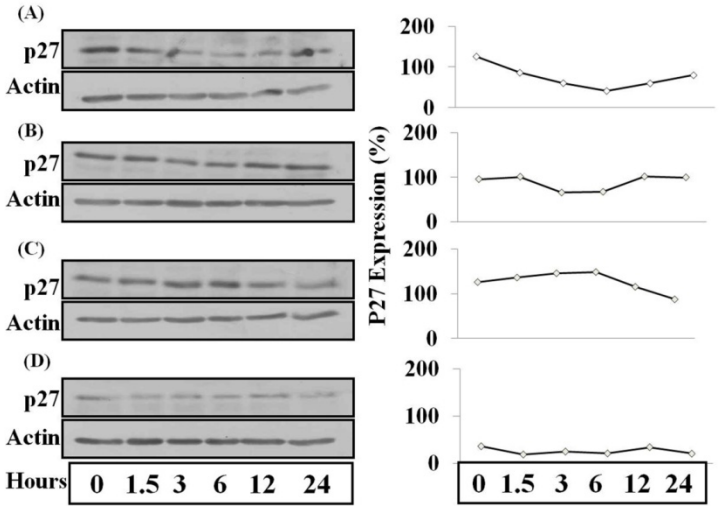
Antioxidant Increased CylinE binding to CDK2 and p27 in TAM resistant cells. LCC2 were exposed to antioxidants as described in methods and treated with TAM and fulvestrant respectively. After 48 hrs incubations, cells were harvested with lysis buffer and 350 ug WCL was immunoprecipitated (IP) with CDK2 antibody using Exactacruz kit as recommended. Eluent was fractionated on 12% SDS-PAGE gel. Immunoblots were probed with, CyclinE, p27 or CDK2, antibodies respectively. A) LCC2 cells treated with TAM or Fulv, B) LCC2 cells over expressing CAT or SOD, then treated with TAM and Fulv, C) LCC2 cells co-treated with chemical antioxidant and TAM or Fulv.

Fig11-LCC2



P27 expression is less in LCC2 cells compared to MCF7. 1million LCC2 and MCF7 cells were seed and serum starved for 48 hours after which cells were harvested with lysis buffer and 50 ug whole cell lysate (WCL) were fractionated on 12% SDS-PAGE gel. Immunoblot were probed with p27 or beta actin antibodies respectively. Figure shows p27 and Actin expression in LCC2 and MCF7 cells. Assay was performed 3x. P27 protein level was determined by densitometric analysis and expressed as the percentage mean of its ratio with β actin relative to control +/- SE, (p 0.05 (*) is indicates significance when p27 expression in LCC2 cells is compared to that of MCF7 cells.

Fig12-LCC2



Antioxidants increased p27 stability. Uninfected LCC2 cells (A) and LCC2 cells over expressing CAT(B), SOD(C) or EV(D) were treated with 50uM cycloheximide (CHX) for the time periods indicated, and p27 protein levels were determined by immunoblotting and densitometry with β actin as internal control.

IV ESTROGEN INDUCED ROS MEDIATES IN VITRO CELL PROLIFERATION AND GROWTH THROUGH PTEN OXIDATION AND AKT-NRF-1 PHOSPHORYLATION

Abstract

Tamoxifen resistance is still a significant problem in the treatment of estrogen receptor positive breast cancer. Due to *de novo* resistance, only about 70% of breast cancer patients will initially respond to tamoxifen treatment. About 40% of patients who were initially responsive to the drug and majority of the patients with metastatic breast disease

will develop an *acquired* resistance to the drug over time. Although there has been an improvement in our understanding of the subject, the molecular mechanism(s) that result in the evolution of tamoxifen resistant breast cancer is still unclear. In this study we explored the role of reactive oxygen species (ROS) in the advancement of breast cancer from a tamoxifen-sensitive to a tamoxifen-resistant phenotype. ROS are products of cellular metabolism and an excessive and/or sustained increase in their production has been implicated in the pathogenesis of cancer. Furthermore, ROS induces oxidative stress which has been shown to initiate various cellular responses including alteration of intracellular redox state and the oxidative modification of certain signaling proteins resulting in the post translational modification of their downstream targets. Interestingly, tamoxifen like estrogen, has been shown to induce ROS formation, making it probable that tamoxifen induced ROS generated as a result of prolong tamoxifen treatment of breast cancer patients may play a critical role in the development of its resistance. Using MCF7 breast cancer cells and standard laboratory techniques, we explored the role of ROS and redox signaling in development of tamoxifen resistance in breast cancer cells. We were interested in finding out how and if ROS, through the oxidation of Phosphatase and tensin homolog (PTEN) induces the phosphorylation and activation of nuclear respiratory factor 1(NRF-1) resulting in its binding to the promoter region of certain cell cycle genes, thereby promoting cell proliferation and a transformation of the cells to a resistant phenotype. We also wanted to find out if this process can be reversed by pre-exposing the cells to biological or chemical antioxidants. Our finding was that, tamoxifen-induced ROS oxidizes PTEN resulting to the hyper-stimulation of the Akt/PI3K pathway and a consequent NRF-1phophorylation/activation. Activated NRF-1

then binds to the promoter region of some cell cycle genes inducing cell proliferation. We also found that pre-treating the cell with anti-oxidants restored sensitivity to tamoxifen.

Introduction

PTEN (phosphatase and tensin homologue deleted from chromosome 10) is a redox sensitive dual specificity phosphatase [1]. It is a known tumor suppressor because of its ability to regulate the cell cycle either by preventing cells from dividing too fast or dividing in an uncontrolled manner [2] such as that seen in cancer cells. The protein which is a negative-regulator of the phosphatidylinositol 3-Kinase (PI3K)/Akt pathway acts by dephosphorylating phosphatidyl-inositol3,4,5-trisphosphate (PI(3,4,5)P3) at the 3' position of the inositol ring, thereby counteracting the effect of PI3K and inactivating this key player in the survival pathway [3].

The tumor suppressor aspect of PTEN function is pertinent in the context of human disease since a number of studies have found PTEN deficiency, which could arise either by polymorphic mutation or gene deletion, to be a cause of cancer [4, 5]. Indeed one of the reasons for the activation of Akt signaling in cancers is the mutation or inactivation of PTEN [6]. However, PTEN inactivation and the resulting PI3K/Akt pathway hyper-activation could occur through mechanisms other than those that target the integrity of the gene [7].

Alternate mechanisms of the down regulation of PTEN activity by posttranslational modifications such as phosphorylation and oxidation, though not directly implicated in

cancer, have been documented [8-11]. Furthermore, reactive oxygen species (ROS) have been shown to oxidize PTEN at its active site ensuing in the formation of a disulphide bond and a subsequent PTEN inactivation. Several studies have demonstrated the ability of reactive oxygen species to reversibly oxidize PTEN and other dual specificity phosphatases, leading to their temporary inactivation [12 -13]. Indeed a number of studies have looked into the temporary inactivation of PTEN by ROS and its effect on downstream molecules like Akt and its substrates. For example Connor et al and others have shown that PTEN oxidation enables PtdIns(3,4,5)P3 to directly activate Akt, which in turn activates p70 S6 kinase and inhibits the Akt substrate, glycogen synthase kinase-3 thereby regulating cellular metabolism and the cell cycle [13,14].

A less well known Akt substrate is NFF1 (nuclear respiratory factor-1/α-palindrome-binding protein) [15] which is a redox sensitive transcription factor [16] that has been shown to regulate metabolism and cell proliferation [17-20]. A study by Piatandosi and Suliman (2006), has demonstrated how stimulation of PI3K by exogenous oxidants activate Akt and promotes NRF1 phosphorylation and nuclear translocation [15]. Additionally, it has been shown that exposure of breast cancer cells to estrogen (E2) induces cell growth [21] and an increase in NRF1 expression [22]. Since estrogen has been shown to promote ROS formation [23], we considered it pertinent to examine a hitherto unexplored possibility that E2-induced ROS could result in PTEN inactivation, a consequent increase in NRF1 expression and phosphorylation and an effect on the cell cycle. In the event of this concept being cogent, we also wanted to determine the effect of antioxidants on our proposed pathway.

In this study, we show how estrogen induced PTEN oxidation in MCF7 results in the hyper-activation of the PI3K/Akt signaling pathway with a consequent increase in the expression and phosphorylation of NRF1 and a resultant binding of NRF1 to the promoter region of cell cycle genes. We also show how pre-exposing MCF7 cells to antioxidants is able to reverse the PTEN oxidation and its downstream effects.

Materials and methods

Cell culture and Materials

MCF7 cells were obtained from Gainsville. Cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum, 25,000 units of penicillin (base) and 25,000 µg of Streptomycin at 37°C in a fully humidified atmosphere of 5% CO2 in air. For experiments, 70% Confluent cells were further cultured in serum free DMEM to minimize the effect of serum and synchronize the cells in G0. Synchronized cells were always stimulated with 0.01% DMSO or 100pg E2.

Antibodies to NRF1 was obtained from Rockland, PTEN was obtained from cell signaling while p27, AKT and PAKT ser 473 were purchased from Santa Cruz biotechnology. T157 Phosphorylated p27 antibody was purchased from Abcam. Beta Actin, 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA), DMSO and Ebselen were purchased from Sigma Aldrich. BrdU kit was purchased from ROCHE. Protein A Agarose was purchased from Invitrogen. The adenoviruses AdEmpty (EV), AdMnSOD (SOD), and AdCatalase (CAT) were manufactured at ViraQuest, Inc. (North Liberty, IA, USA). Primers for CyclinB1, CDC2, CDC25C, PRC1and PCNA where purchased from

Applied Biosystems. The Upstate kit for ChIP assay was purchased from upstate biotechnology.

Adenoviral Transduction

The adenoviruses AdEmpty (EV), AdMnSOD (SOD), and AdCatalase (CAT) were manufactured at ViraQuest, Inc. (North Liberty, IA, USA) by inserting either nothing (EV) or the MnSOD or Catalase gene into the E1 region of an Ad5 E1/partial E3-deleted replication-deficient adenoviral vector.

MCF7 cells were plated in 100-mm dishes at a density of 1×10^6 cells/dish in charcoal stripped medium. The following day, the medium was aspirated and replaced with 5 ml of serum-free medium containing 200pfu of SOD, CAT or EV adenovirus for 24hours after which cells were stimulated with required treatments for the desired amount of time.

Ebselen Pre-treatment

1million MCF7 cells were plated per 100mm dish in 10% serum medium. 21hours post seeding 20uM of Ebselen was added to the medium for 3hours after which cells were stimulated with the desired treatments for 24 hours.

BrdU Incorporation Assay

For BrdU incorporation, MCF7 cells were seeded in 96-well plates at a density of 2500/well and incubated in 5% CO2 incubator at 37^{o}C. 24 hours after seeding, cells were either infected with AdCatalase, AdMnSOD or AdEV at a multiplicity of infection (m.o.i) of 290pfu/cell as described above or pre-treated with 40uM of Ebselen. After exposure to antioxidants cells were then stimulated with either 0.01% DMSO or E2 (100pg) for 24hours. After stimulation, BrdU incorporation was carried out using Roche Bioscience (Cell Proliferation ELISA, BrdU Kit; Roche Molecular Biochemical, Indianapolis, IN) labeling kit in accordance with manufacturer's recommendation. Colorimetric changes acquired at 370 nm with a Tecan Genios microplate reader.

2′, 7′-dichlorofluorescin diacetate (DCFH-DA)

To carry out the DCFH-DA Assay, 10,000/well of MCF7 cells were seeded in a 96-well plate and cultured in 10% growth medium for 24hours. After 24hours growth medium was aspirated and replaced with starvation medium for another 24hours after which the starvation media was aspirated and replaced with 100uL/well of HBSS containing 10uM DCFDA pre-diluted with Pluronic F-127 and incubated at 37oC for 20mins. At the end of the incubation, DCFH-DA solution was gently aspirated and pre-designated wells were then stimulated with 100ul of HBSS containing either 0.01% DMSO (vehicle control) or E2 (100pg). Cells were further incubated for 5mins after which fluorescence readings were taken using 485 nm and 535 nm as excitation and emission filters, at intervals of 5mins. Optical density readings were obtained using a Tecan Genios microplate reader.

Westernblot

1 million MCF7 cells per 100mm dish were initially exposed to antioxidants as described above and then stimulated with either 0.01% DMSO, or E2 30mins. Post treatment, cells were harvested and lysed in radioimmunoprecipitation assay buffer [25 mM Tris HCl (pH 7.4), 25 mM NaCl, 1 mM sodium orthovanadate,10 mM sodium pyrophosphate, 10 mM NaF, 0.5 mM EGTA, 1.0% Triton X-100, 1 mM PMSF, and 10 mM okadaic acid] for 15 min on ice. Samples were then briefly sonicated and centrifuged at 10,000rpm for 10 min at 4°C. The total protein concentration of the resulting supernatant was determined by BCA quantification. Equal amount of protein extracts (50ug) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, blocked in 1% BSA and fat free milk solution containing 50mM NAF for 1hour and blotted with antibodies. Labeled proteins are visualized with an ECL system (Amersham Biosciences). Band intensity was determined using densitometric analysis (NIH ImageJ software). The level of protein level was expressed as its ratio to βactin.

Soft Agar Colony Formation Assay.

To assess the effect of antioxidants on anchorage-independent cell growth, a soft agar colony formation assay using either plain or Ebselen pretreated MCF7 cells was performed. MCF7 cells (1000/well) were suspended in 0.2ml of charcoal stripped culture medium containing 0.25% agar and appropriate treatment (0.01%DMSO, 100pg E2, 1uM Tamoxifen, or co-treatment of TAM with E2) and poured over a pre-hardened feeder layer of agarose comprising 0.2 ml of the charcoal stripped medium, containing 0.5% agar, in a 48 well plate. Cells were fed every 4 days and allowed to incubate for

30days. After 14 days of incubation at 37°C in a humidified CO2 incubator, colonies counted excluding any colonies with a diameter ≤ 60.

Identification of Reduced and Oxidized Forms of PTEN by Immunoblot Analysis.

After stimulation, I million/100mm dish of MCF7 cells in 1 ml of HBSS were scraped into 0.2 ml of ice-cold 50% trichloroacetic acid and transferred to microfuge tubes. The cell suspensions were sonicated briefly and then centrifuged at 2000*g* for 5 min. The supernatants were removed, and the pellets were washed with acetone and then solubilized in 0.2 ml of 100 mM Tris-HCl (pH 6.8) buffer containing 2% SDS and 40 mM NEM. 5ul of the solubilized pellets were subjected to SDS-PAGE under nonreducing conditions, and the separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane.

The membrane was then subjected to immunoblot analysis with either rabbit antibodies to PTEN or monoclonal antibody to Beta Actin. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Biosciences). The intensity of PTEN bands was quantitated with NIH ImageJ.

Chromatin Immunoprecipitation (ChIP) Assay

This assay was carried out using the upstate protocol that came with the kit. Briefly, after stimulation, protein complex was cross-linked to DNA by adding formaldehyde directly to culture medium to a final concentration of 1% and incubate for 10 minutes at 37C. Cells were washed twice with ice cold PBS containing protease inhibitors scraped into

conical tube and Pelleted for 4 minutes at 2000 rpm at 4ºC. Cell pellet was then lysed in SDS Lysis buffer for 10 minutes on ice. Lysate was sonicated (5 x 15pulses) and centrifuged for 10 minutes at 13,000 rpm at 4°C. 150 ul of the sonicated cell pellet suspension was transferred to a new 1.5 ml eppendorf tube and diluted 10times in ChIP Dilution buffer containing protease inhibitors. 1% (~15 ul) was kept aside as input/starting material. 1.5 ml diluted cell pellet suspension was pre-cleared with 60 ul of Salmon Sperm DNA/Protein A Agarose-50% slurry for 2 hours at 4ºC with agitation. Agarose was pelleted by brief centrfugation (1min @ 1000rpm). The supernatant fraction was collected and the immunoprecipitating antibody added to the 1.5 ml supernatant fraction and incubate overnight at 4ºC with rotation. A non-specific antibody for immunoprecipitation as negative control. 40 ul of Salmon Sperm DNA/Protein A Agarose Slurry was added for another two hour at 4ºC with rotation to collect the antibody/protein complex. Agarose was then pelleted, supernatant was discarded and washed with different wash buffers for 3-5minutes per wash. The histone complex was then eluted from the antibody and all samples including the input were reverse cross-linked by heating at 65ºC for 6 hours. DNA was then recovered by ethanol precipitation and PCR analysis performed.

Immunofluorescence Labeling

Cells were seeded ($1.0x10^4$ cells/chamber) and stimulated in chamber slides as indicated in the legends of the figures. After treatment, cells were fixed with ice cold methanol for 15mins, and permeabilized with 0.5% Triton X-100 for 30min. Then the cells were blocked with 1% normal goat sera for 1 hr after which they were probed simultaneously

with antibodies diluted 1: 500 for NRF1; or 1:1500 for phosphor serine. Alexa Fluor labeled secondary antibodies directed against primary antibodies was diluted 1:1000. The confocal fluorescence images were scanned on a Nikon TE2000U inverted fluorescence microscope equipped with a Nikon D-Eclipse C1 laser scanning confocal microscope system (Nikon Corp., USA). The z-series scanning were done at every 1 μm up to a z-depth of 10 μm by using a Nikon 40 x 1.30 NA DIC H/N2 Plan Fluor oil immersion objective. The built-in Nikon EZ-C1 software was used for confocal image acquisition and analyses.

Results

Estrogen induces ROS in MCF-7. To determine whether E2 induces ROS in breast cancer cells, MCF-7 cells were treated with either E2 or E2 with ROS modulators as described in methods. Results showed that cells exposed to E2 induced ROS formations (Fig.1A). However, co-treatment of cells with E2 and ROS modulators (CAT or Eb) inhibited estrogen's ability to induce ROS formations (Fig.1B).

Estrogen induced ROS mediates in vitro proliferation and growth of MCF-7 cells. To ascertain whether E2 induced ROS facilitates in vitro proliferation and growth of breast cancer cells, MCF-7 cells were seeded for BrdU and soft agar assay as described in methods. Cells were subsequently treated with E2 or E2 and ROS modulators. BrdU incorporation, which is a marker of cells proliferation and soft agar colony formation, which is a hallmark of anchorage independent cancer growth, were analyzed and enumerated as described in methods and legends. Data indicate that E2 induced

proliferation of MCF-7 cells were abrogated by ROS modulators (Fig 2). Similarly, E2 induced anchorage independent growth of breast cancer cells were abolished by ROS modulators (Fig 3). These findings indicate that estrogen induced proliferation and growth of breast cancer cells are redox dependent.

Estrogen induced ROS oxidizes PTEN in MCF-7 cells. Oxidation of PTEN by ROS has been demonstrated to promote carcinogenesis and growth of cancer cells (PMID: 15534200). We investigated whether E2 induced ROS also oxidizes PTEN thereby promoting E2 induced proliferation and growth of breast cancer cells. Western blot analysis indicates that while H2O2 are potent oxidizers of PTEN (Fig. 4), E2 induced ROS can similarly oxidize PTEN albeit to a lesser extent compared to H2O2 (Fig. 5A). However, when cells were co-treated with either Catalase or Ebselen, E2 induced PTEN oxidation were significantly reversed (Figs. 5B & C). These findings indicates that E2 induced ROS can lead to PTEN oxidation which could alter downstream signaling process that favors breast cancer proliferation and growth in response to estrogens.

Estrogen induced PTEN oxidation activates Akt phosphorylation in MCF7 cells. Western blot analysis were carried out to determine whether E2 induced PTEN oxidation would lead to increased Akt activation and whether ROS modulators would attenuate Akt activation in E2 treated breast cancer cell. Our study showed that E2 induced significant Akt phosphorylation compared to vehicle treated cells (Fig. 6A). When cells were co-treated with E2 and ROS modulators, Akt activations were significantly reduced (Figs.6B&C). This data indicates that E2 induced PTEN inactivation and activation of

Akt signaling cascade in breast cancer cells are redox dependent. Activated Akt can in turn phosphorylate downstream substrates such as transcription factors that favor survival and growth of breast cancer cells.

Estrogen induces NRF1 expression and phosphorylation in MCF7 cells. To determine the effect of Estrogen induced PTEN oxidation on downstream Akt signaling substrates, Immunohistochemistry assays were carried out to determine how E2 induced ROS affect NRF1 expression and phosphorylation. Results show an increase in NRF1 expression and phosphorylation in E2 treated cells compared to DMSO control (Fig.7).

Estrogen induced ROS mediates NFR-1 binding to promoter regions of cell cycle genes in MCF7 cells. A chromatin immunoprecipitation assay was carried out to determine the effect of E2 on NRF1 binding as described in methods. Result obtained shows an E2 induced increase in NRF1 binding to the promoter region of the following cell cycle genes: CyclinB1, CDC2, PCNA, CDC25C and PRC1 (Fig. 8, Panels I &IIA). Conversely, Antioxidant pre-treatment significantly inhibited the E2 induced NRF1 binding to the promoter region of the same genes (Fig. 8, Panels I &IIB).

Estrogen induced ROS mediates transcription of Cell cycle genes. RT-PCR was carried out to determine the effect of E2 induced ROS on the transcription of cell cycle genes as shown in methods. Result obtained show an up-regulation in the transcription of CyclinB1, PCNA, CDC25C and PRC1 (Fig.9, Panels I&IIA). However, when cells were

co-treated with either CAT or Eb, there was a significant inhibition of E2 induced transcription upregulation of the same genes (Fig.9, Panels I&IIB).

Discussion

The results of our study show that estrogen-induced reactive oxidants have the capacity to stimulate cell proliferation in MCF7 breast cancer cells through the oxidation of PTEN, and the post translational modification of its downstream target.

In line with previous work done [23], we are able to show here that estrogen-induced reactive oxygen species (ROS) is capable of promoting DNA synthesis and cell proliferation, which can be inhibited by exposure of the same cells to either biological or chemical antioxidants, Catalase or Ebselen (See figs. 1, 2 and 3).

In this study, we have been able to show for the first time in MCF7 cells that estrogen-induced ROS oxidizes PTEN within 30 minutes of exposure. In addition, we reveal here-in the ability of either Catalase or Ebselen to reverse the oxidizing effect of estrogen-induced ROS. See figs.4 and 5. We also show here that estrogen-induced PTEN inactivation in MCF7 cells results in an increase in the phosphorylation of one of its downstream target kinases, Akt. This is in line with previous studies which showed an increase in Akt phosphorylation following PTEN inactivation by agents other than estrogen [13, 24, 25]. Moreover, we have also been able show that the pre-exposure of MCF7 cells to either Catalase or Ebselen significantly reverses the E2-induced effect. See fig.6. Also demonstrated in this study is a corresponding increase in both the expression and

phosphorylation of NRF1, which is a known Akt substrate that has been implicated in cell growth and proliferation [18-20]. (See figure 7).

Based on the work done by Cam et al. [18], which showed NRF1 could collaborate with E2F family members to regulate the expression of genes that are involved in cellular proliferation**,** we explored the possibility of phosphorylated (active) NRF1 binding to the promoter region of cell cycle genes. Evident in our study for the first time in MCF7 cells, is an increase in the binding of Akt phosphorylated NRF1 to the promoter region of the following cell cycle genes: CyclinB1; CDC25A; CDC2; PCNA and PRC1. It is also worthy of mention that this promoter binding was significantly inhibited by the pre-exposure of MCF7 cells to either Catalase or manganese superoxide dismutase (MnSOD). See fig. 8. We were further able to reveal a consequent increase, in the transcription of these gene, which was also inhibited by pre-exposure to Catalase and MnSOD (See fig. 9).

In summary we have been able to show for the first time in MCF7 cells, that estrogen-induced ROS inactivates PTEN with an ensuing increase in Akt phosphorylation. This Increase in Akt phosphorylation results in an increase in NRF1 expression and phosphorylation leading to the activation and binding of NRF1 to the promoter region of some cell cycle genes, namely CyclinB1, CDC25A, CDC2, PCNA and PRC1 leading to cell proliferation. We also show a corresponding increase in the transcription of these genes. Additionally, we show for the first time that that pre-exposure of MCF7 cells to

either biological or chemical antioxidants significantly inhibited the effect of estrogen-induced ROS on PTEN and its downstream substrates (See fig. 10).

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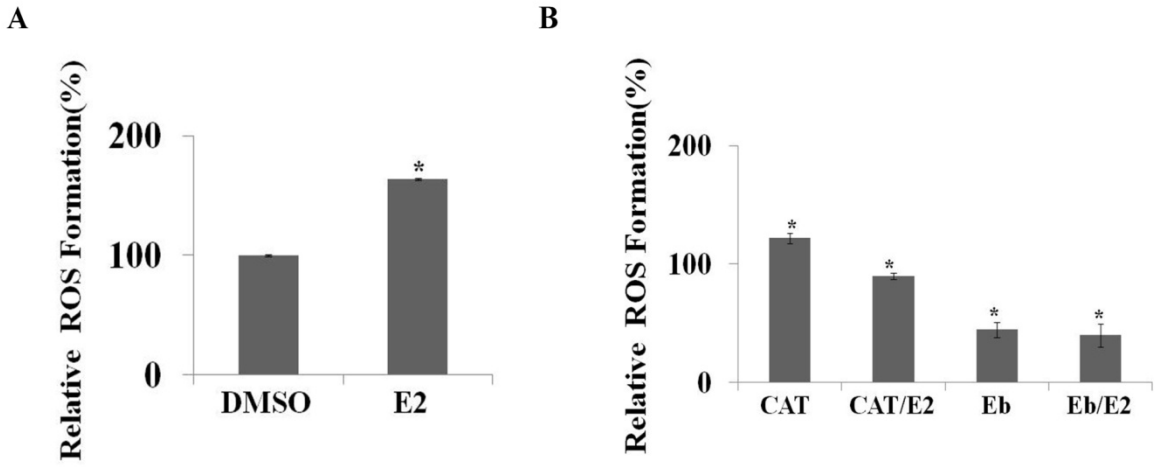
Figures and Legend

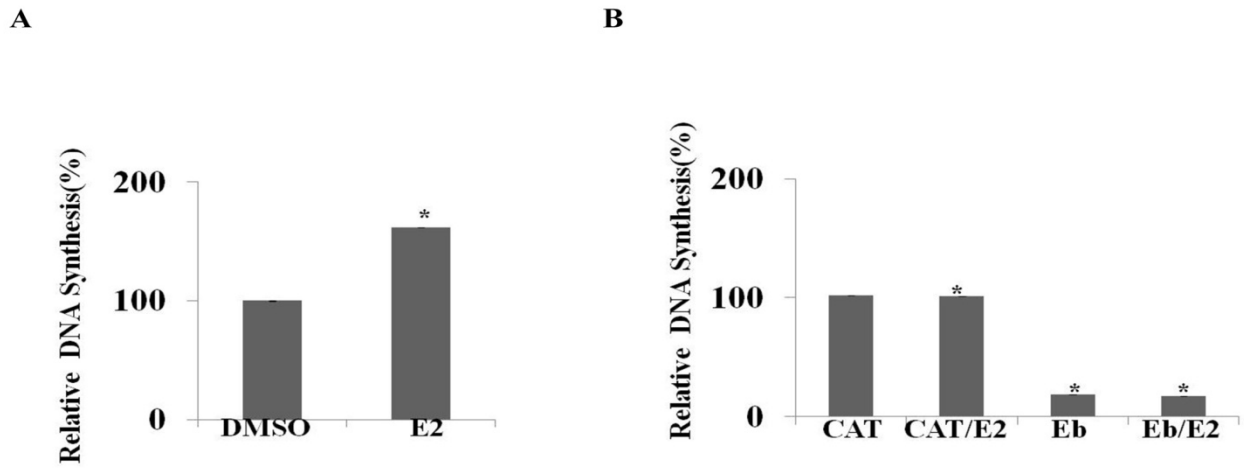
Fig 1-MCF7



Antioxidants inhibit E2 induced ROS in MCF7 cells. 1 x10^4cells/well were seeded in a 96-well plate overnight and serum starved for 24 hrs. Post-starvation 100uL/well of HBSS containing 10uM DCFH-DA pre-diluted with Pluronic F-127 was added to each well and incubated at 37^{o}C for 20mins. DCFH-DA solution was then aspirated and replaced with 100ul of HBSS containing the desired treatments. Cells were further incubated for 5mins and fluorescence readings were taken using 485 nm and 535 nm as excitation and emission filters, at intervals of 5mins. A) MCF7 cells treated with E2. B) MCF7 cells over expressing Catalase (CAT) or pre-treated with Ebselen were exposed to either E2 or DMSO. Assay was performed 3x and data is expressed as mean percentage

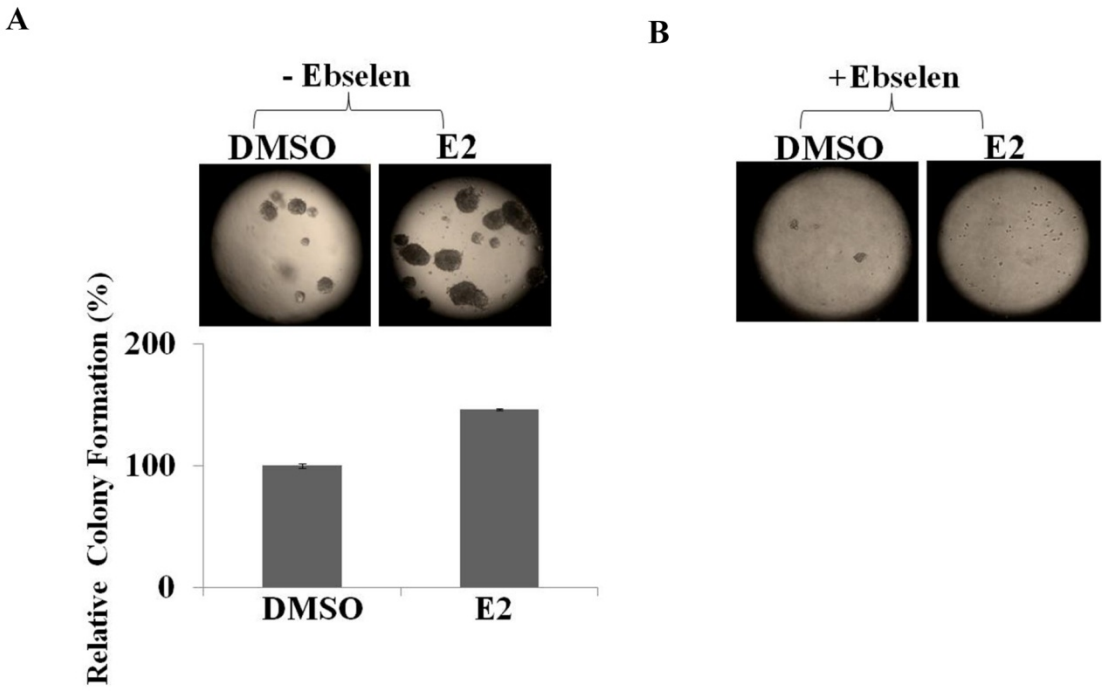
change from control +/- SE, (p 0.05). (*) denotes significance when treatment is compared to DMSO control.

Fig 2-MCF7



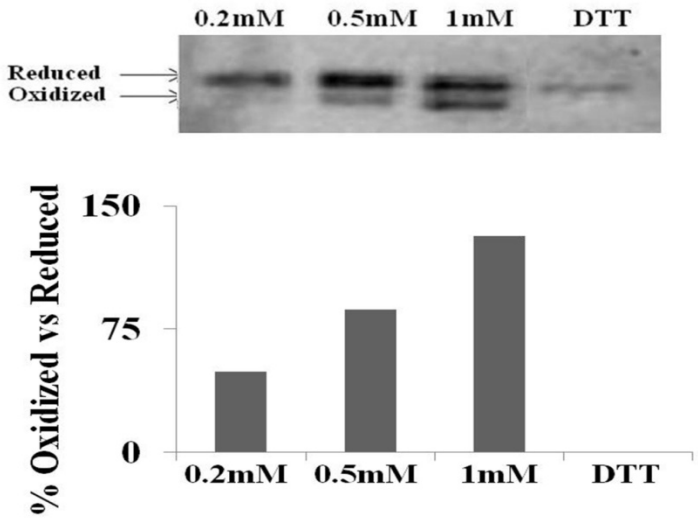
Antioxidants inhibit DNA synthesis in MCF7 cells. 2.5 x 10^3 cells/well were seeded in a 96-well plate overnight and exposed to chemical or biological antioxidants as described in methods. After exposure to antioxidants, cells were then stimulated with E2 for 22 hrs and pulsed labeled with BrdU for 2 hrs. BrdU assay was then carried out as recommended by manufacturer. Colorimetric changes were acquired at 370 nm with a Tecan Genios microplate reader at 5 mins interval. A) MCF7 cells treated with either E2 or DMSO B) MCF7 cells over expressing Catalase (CAT) or pre-treated with Ebselen were treated with either E2 or DMSO. Assay was performed 3x and data is expressed as mean percentage change from control +/- SE, (p 0.05). (*) denotes significance when treatment is compared to DMSO control.

Fig 3-MCF7



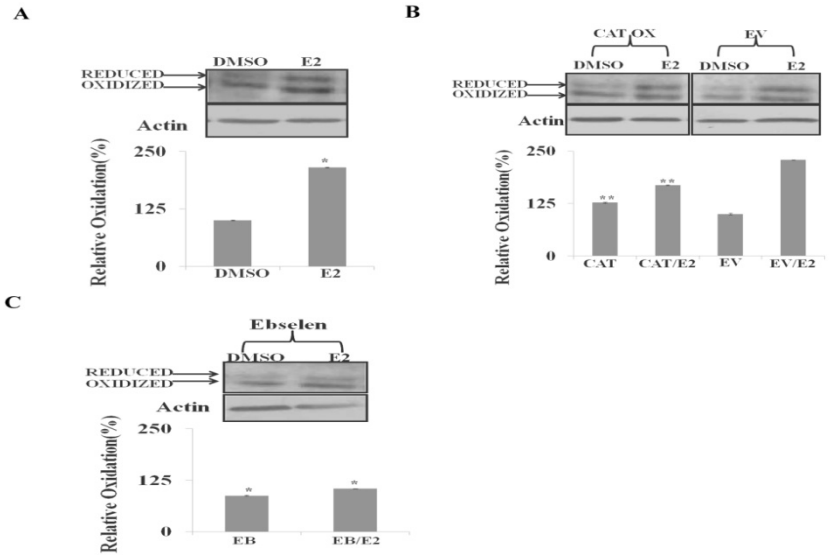
Antioxidants inhibit E2-induced anchorage independent growth in MCF7 cells. Cells were incubated on soft agar with or without antioxidants exposure for 21 days and colonies ≥60 microns in diameter were enumerated. **A)** Anchorage independent growth of MCF7 cells exposed to either DMSO or E2 **B)** MCF7 cells pretreated with chemical antioxidant. Assay performed 3x and data is expressed as mean percentage change from control.

Fig 4-MCF7



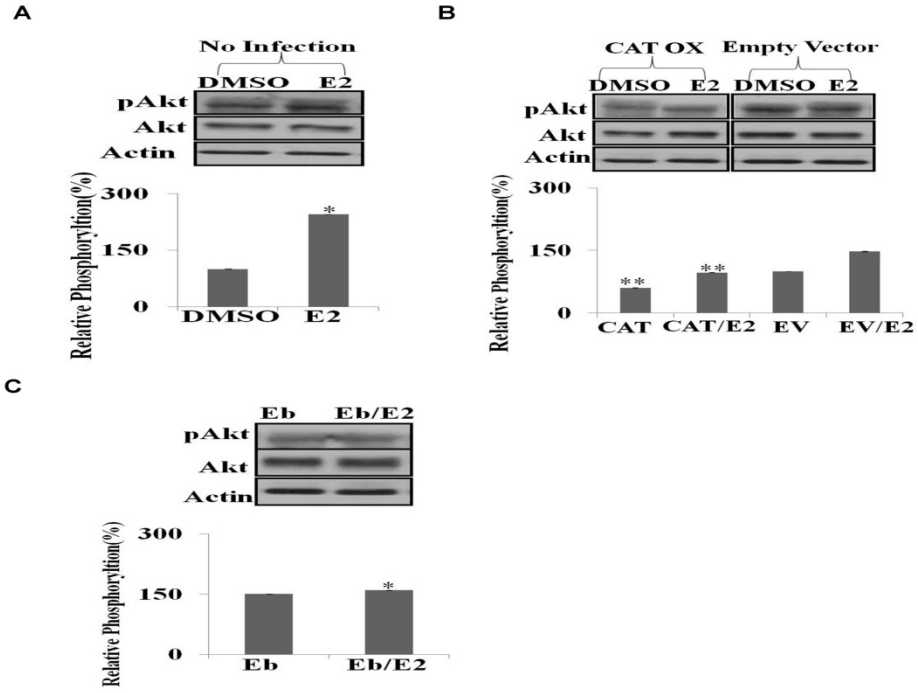
Hydrogen Peroxide (H_2O_2) oxidizes PTEN in a dose dependent manner in MCF7 cells. MCF7 cells were seeded and stimulated with different doses of H_2O_2 for 30min and then harvested. Cellular protein extracts were then alkylated with NEM and subjected to non-reducing SDS-PAGE followed by immunoblot analysis with antibodies to PTEN. Assay was performed 3x and data is expressed as mean percentage change from control.

Fig 5-MCF7



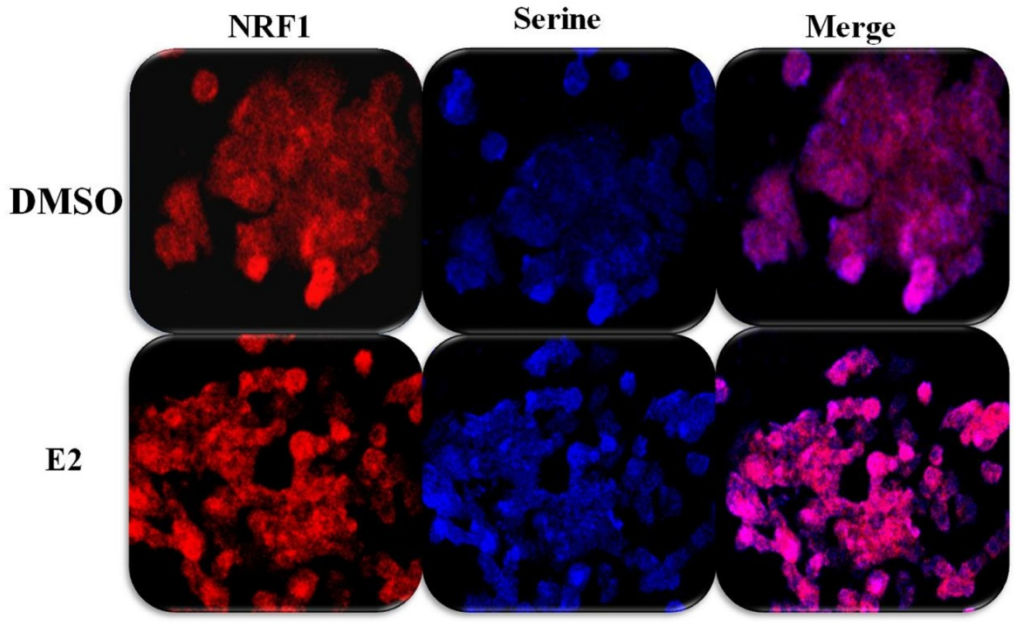
Antioxidants inhibit the E2-induced PTEN oxidation in MCF7 cells. Cells were pre-treated with Ebselen for 3hrs and stimulated with either DMSO or E2 for 30mins. Cellular protein extracts were then alkylated with NEM and subjected to nonreducing SDS-PAGE followed by immunoblot analysis with antibodies to PTEN. A) MCF7 cells treated with either E2 or DMSO. B) MCF7 cells over-expressing Catalase (CAT) or Empty Vector (EV) treated with DMSO or E2. C) MCF7 cells pre-treated with Ebselen were treated with either DMSO or E2. Assay was performed 3x and data is expressed as mean percentage change from control +/- SE, (p 0.05). (*) denotes significance when treatment is compared to DMSO control while (**) denotes significance when treatment is compared EV control.

Fig 6-MCF7



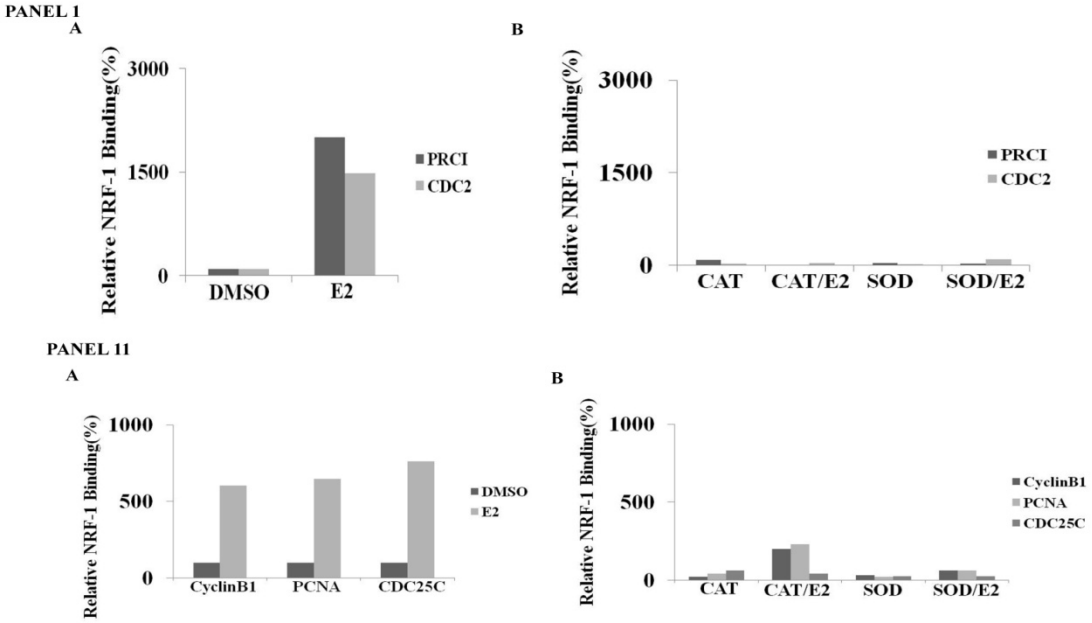
E2-induced PTEN oxidation activates Akt phosphorylation in MCF7 cells. Akt and phospho-Akt levels were determined post PTEN oxidation by immunoblotting using MCF7 cells pre-exposed with either biological (CAT) or chemical (Ebselen) antioxidants and then treated with E2 for 30min to induce PTEN oxidation. **A)** MCF7 cells treated with either E2 or DMSO **B)** MCF7 cells over-expressing Catalase (CAT) or Empty Vector (EV) treated with DMSO or E2. **C)** MCF7 cells pre-treated with Ebselen were treated with either DMSO or E2. Assay was performed 3x and data is expressed as mean percentage change from control +/- SE, (p 0.05). (*) denotes significance when treatment is compared to DMSO control while (**) denotes significance when treatment is compared EV control.

Fig7-MCF7.



E2 induces NRF1 expression and phosphorylation in MCF7 cells. 1.0 x10^4 cells/chamber were treated for 45mins with E2. Cells were then fixed with ice cold methanol for 15mins, and permeabilized with 0.5% Triton X-100 for 30mins after which they were blocked with 1% normal goat serum for 1hr and then probed simultaneously with antibodies for NRF1 (red) phosphoserine (blue).The merged phosphorylated NRF1 is in pink.

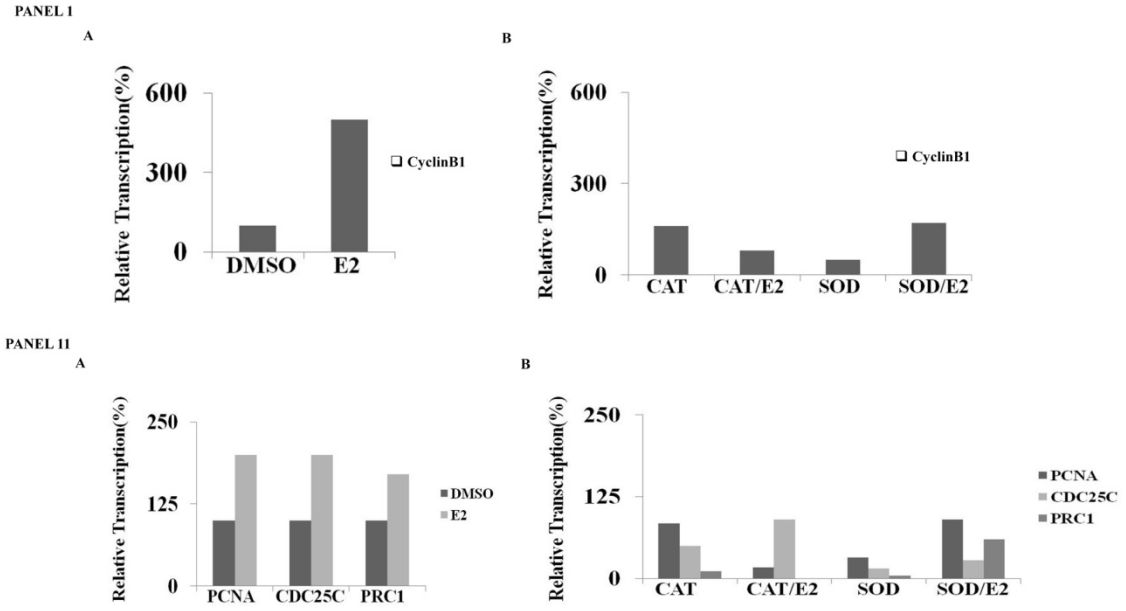
Fig 8-MCF7



E2-induced ROS mediates NFR-1 binding to the promoter region of cell cycle genes. MCF7 cells were seeded, serum starved for 24 hrs and either infected with Adenovirus over- expressing MnSOD (SOD) or Catalase (CAT) at m.o.i of 200pfu as shown in the methods section. Cells were then exposed to either DMSO or E2 for 16hrs and harvested for ChIP assay as described in methods. PCR were run on ABI Biosystem 7300 thermocycler with the following cycle conditions: 95 ºC, 10 min**;** 40 cycles of (95 ºC, 15 sec; 60 ºC, 60 sec). PANEL I: PCR were run with primers for PRC1 and CDC2. A) MCF7 cells were treated with either E2 or DMSO B) MCF7cells over expressing CAT or SOD were treated with either DMSO or E2. Assay was performed 3x and data is expressed as mean percentage change from control. PANELII: PCR were run with primers for PCNA, CyclinB1 and CDC25C. A) MCF7 cells were treated with either E2 or DMSO B) MCF7 cells over expressing CAT or SOD were treated with either DMSO

or E2. Assays was performed 3x and data is expressed as mean percentage change from control.

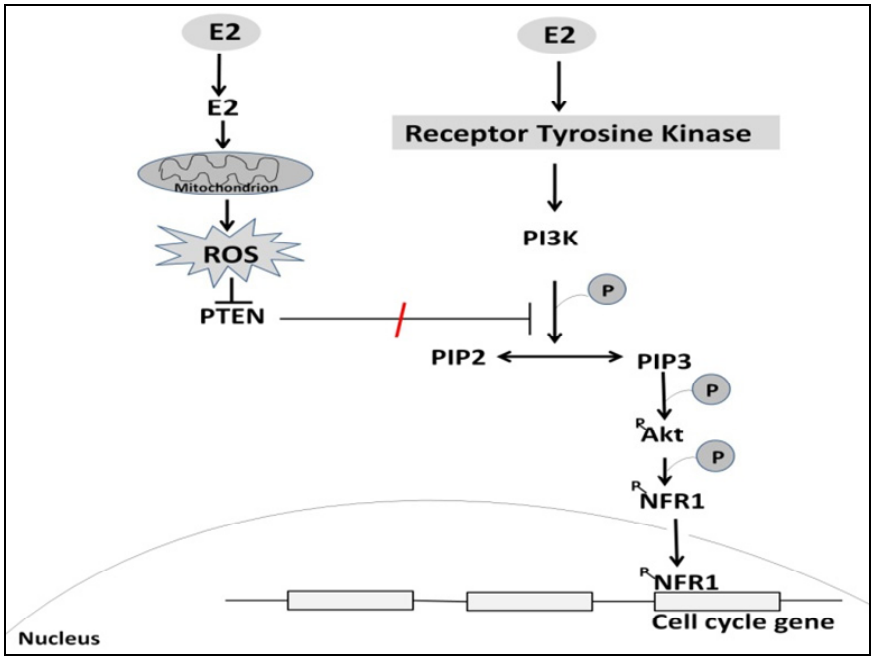
Fig 9-MCF7



E2-induced ROS mediates transcription of cell cycle genes. MCF7 cells were seeded, serum starved for 24 hrs and either infected with Adenovirus over- expressing MnSOD (SOD) or Catalase (CAT) at m.o.i of 200pfu as shown in the methods section. Cells were then exposed to either DMSO or E2 for 24hrs and harvested for RT-PCR as described in methods. RT- PCR were run on ABI Biosystem 7300 thermocycler with the following cycle conditions: initial initiation Step set at 48^{o}C for 30mins; and 95 ^{o}C for 10min (1 cycle) and the PCR step at 95^{o}C for 15sec and 60sec for 1min (40 cycle). PANEL I: RT-PCR were run with primers for CyclinB1. A) MCF7 cells were treated with either E2 or DMSO B) MCF7cells over expressing CAT or SOD were treated with either DMSO or E2. Assay was performed 3x and data is expressed as mean percentage change from

control. PANELII: RT-PCR was run with primers for PCNA, PRC1 and CDC25C. A) MCF7 cells were treated with either E2 or DMSO B) MCF7cells over expressing CAT or SOD were treated with either DMSO or E2. Assays was performed 3x and data is expressed as mean percentage change from control.

Fig 10-MCF7



A schematic showing our hypothesized pathway. Estrogen-induced ROS inactivates PTEN with an ensuing increase in Akt phosphorylation. This Increase in Akt phosphorylation results in an increase in NRF1 expression and phosphorylation leading to the activation and binding of NRF1 to the promoter region of some cell cycle genes.

V CONCLUSION

The goal of this dissertation was to investigate and hopefully reveal the role of ROS in the conversion of tamoxifen sensitive breast tumors to a tamoxifen-resistant phenotype.

Our research was based on the working hypothesis that excess ROS oxidizes protein tyrosine phosphatases (PTPs), thereby changing the phosphorylation state and altering the functions of certain key signaling proteins such as p27 (a Cyclin dependent kinase inhibitor) and nuclear respiratory factor 1(NRF-1) (a transcription factor).We further postulated that if ROS/redox signaling has a role in the development of tamoxifen resistance, then we may be able to restore sensitivity to tamoxifen by pre-exposing resistant breast cancer cells to antioxidants.

In the course of our research, using tamoxifen resistant LCC2 breast cancer cells, we found tamoxifen to be highly pro-oxidant, based on its ability to induce ROS formation when it is used to stimulate LCC2 cells. We further discovered that tamoxifen acts as an ROS scavenger (antioxidant) in the presence of estrogen. Upon further investigation we found that stimulating LCC2 cells with tamoxifen resulted in a reduction in the expression of p27 protein and an increase in its phosphorylation on threoning 157 and 187 respectively which correlated with an increase in cell proliferation. Interestingly, we were able to reverse these findings using biological (MnSOD and Catalase) and chemical (Ebselen) antioxidants. Meaning that LCC2cells which were pre-exposed to antioxidants showed a significant increase in p27 expression and a reduction in the phosphorylation of p27 implying a possible growth inhibitory effect.

Our findings then raised a question about what aspect of p27 was really affected in the development of tamoxifen resistance. Was it a decrease in p27 expression or just the inability of the protein to carry out its growth inhibitory function? So we compared the

level of p27 in tamoxifen sensitive MCF7 breast cancer cells to that in tamoxifen resistant breast cancer cells and found that the level of p27 in LCC2 cells was significantly less than in MCF7 demonstrating that if tamoxifen resistance is as a result of a reduction in p27 expression, then our finding that pre-exposure to anti-oxidants t increases p27 expression was a significant one. We then conducted a p27 stability assay comparing cells pre- exposed to anti-oxidant to those which were not. Again we found that those cells pre-exposed to anti-oxidants showed more p27 stability compared to those which were not. We also conducted a functional assay to determine the effect of our findings on the function of p27, again we found that cells which were pre-exposed to anti-oxidants were more functional because they exhibited more binding capacity of p27 to CyclinE and Cdk2 compared to cells which were not exposed to anti-oxidants. As mentioned previously in this dissertation, the growth inhibitory effect of p27 is based on its ability to bind to the CylinE-Cdk2 complex.

Putting all our findings together we may therefore infer that, the development of tamoxifen resistance is due to the ability of tamoxifen to produce a sustained increase in ROS which results in a pro-oxidative environment. In a pro-oxidative environment the expression and stability of p27 is diminished resulting in loss of its inhibitory function because there is not enough p27 to bind and inhibit the growth promoting CyclinE-CDK2 complex. Therefore our findings that anti-oxidants increased p27 expression, stability and binding are very significant.

To determine the mechanism of tamoxifen resistance, we used Tamoxifen sensitive MCF7 cells to determine the effect of ROS on the loss of tamoxifen sensitivity and demonstrated that in a pro-oxidant environment, the sustained increase in ROS level leads to the oxidation and inhibition of the phosphatase PTEN with a consequent increase in the phosphorylation of Akt. An increase in Akt phosphorylation then results in the phosphorylation and activation of NRF-1. We then demonstrated that activated NRF-1 then binds to the promoter region of the following cell cycle genes: PCNA, cyclinB1, CDC25A and PRC1 leading to their transcription and culminating in a sustained cell proliferation. This finding could also be used to explain what happens in the case of p27 because Akt has been shown to phosphorylate p27 on T157 resulting in the cytoplasmic sequestration of p27 and loss of its inhibitory function.

In a nutshell, we have been able to experimentally demonstrate that ROS/redox signaling contributes to the evolution of breast cancer from a tamoxifen sensitive to a tamoxifen resistant phenotype. The Findings of this study will elucidate the roles of the cellular redox state, redox signaling pathways, PTPs and p27 in anti-estrogen resistance, and may lead to new therapeutic strategies to delay or even prevent this important clinical problem. This is a new line of research that may lay the groundwork for clinical trials of anti-estrogens plus antioxidant-based drugs for the prevention and treatment of estrogen-dependent breast

Directions for future research

As mentioned in the conclusion part of this dissertation, using MCF 7 cells we could also demonstrate the effect of Akt phosphorylation on T157 and subsequently p27 function. We have conducted preliminary studies in our lab and the results are encouraging.

Since our current research was done *in vitro*, another area for further research would be a test of our hypothesis in vivo. Xenografted tumors in nude mice will be used to determine if ROS inhibitors or glutathione or thioredoxin modifiers can cooperate with anti-estrogens to inhibit the proliferation of anti-estrogen-resistant breast cancer cells. Findings of this study will reveal the roles of the cellular redox state, redox signaling pathways and p27/NRF-1 in anti-estrogen resistance, and may lead to new therapeutic strategies to delay or even to prevent this important clinical problem. This is a new line of research that may lay the groundwork for clinical trials of anti-estrogens plus antioxidant-based drugs for the prevention and treatment of estrogen-dependent breast cancer.

VITA

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Publications and Abstracts

Garba NA., Roy, D. 2011. Estrogen Induced ROS mediates in vitro cell proliferation and growth through PTEN oxidation and AKT-NRF1 phosphorylation (Prepared for submission).

Garba, NA., Joyce Slingerland., Roy, D., Felty, Quentin. 2011. The Role of Redox Signaling in the development of Tamoxifen Resistance in Breast Cancer (Prepared for submission)

Garba, NA., Joyce Slingerland., Roy, D. 2011. Redox Signaling Contributes to development of Tamoxifen Resistance in Breast Cancer (Prepared for submission).

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