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**REDOX CYCLING BY CATECHOL METABOLITES
OF ENDOGENOUS ESTROGENS AS POTENTIAL CONTRIBUTORS
TO THE INITIATION OF BREAST CANCER**

by

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A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Joint Graduate Program in Toxicology

written under the direction of

Professor Jeffrey D. Laskin, Ph.D.

and approved by

New Brunswick, New Jersey

May 2011

ABSTRACT OF THE DISSERTATION

Redox Cycling By Catechol Metabolites Of Endogenous Estrogens As Potential
Contributors To The Initiation Of Breast Cancer

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Jeffrey D. Laskin, Ph.D.

Human idiopathic post-menopausal mammary adenocarcinoma is a tumor of breast epithelial tissue with a high incidence in women. The etiology of this disease stems from both genetic and environmental factors. A major environmental component thought to be important in the development of breast cancer is estrogen exposure; indeed at least one environmental estrogen is known to have tumorigenic properties. Several studies have attempted to characterize the role of exogenous estrogens in initiating breast cancer; however little work has been done using endogenous human estrogens. The present studies investigated a mechanism by which endogenous estrogens may contribute to idiopathic breast tumor formation. In this mechanism, endogenous estrogens are first metabolized by cytochrome P450s to catechol metabolites. Catechols are known to be reactive and can generate highly toxic and mutagenic reactive oxygen species (ROS) by redox cycling. We have shown that the flavoenzymes NADH-cytochrome b₅ reductase and NADPH-cytochrome P450 reductase can mediate redox cycling of catechol estrogen metabolites and generate hydrogen peroxide, superoxide anion, and, under conditions

favorable for Fenton chemistry, hydroxyl radicals. Moreover, we show that this process can damage DNA causing strand breakage and nucleotide base oxidation. We also demonstrated that redox cycling by catechol metabolites of endogenous estrogens can occur in breast epithelial cells. Cell lysates from three human mammary epithelial cell lines, MCF-7 (estrogen receptor alpha positive, tumorigenic), MDA-MB-231 (estrogen receptor alpha negative, tumorigenic), and MCF-10A (estrogen receptor alpha negative, non-tumorigenic), were found to redox cycle catechol estrogens and generate ROS. Additionally, these metabolites were found to stimulate hydrogen peroxide release by intact cells as measured using an extracellular electrochemical microsensor. In all cases, the three cell lines were found to be equally active in mediating redox cycling and generating ROS. These data indicate that redox cycling can occur in breast epithelial cells; however, ROS production appears to be independent of either estrogen receptor status or tumorigenic stage. Therefore, catechol estrogen metabolite redox cycling must be a constitutive property of the breast epithelial cells and is not acquired during breast tumor development. Because we can measure release of hydrogen peroxide by intact breast epithelial cells by catechol estrogens, redox cycling must be significant enough to overwhelm protective cellular antioxidant defense systems. Taken together, these data indicate that endogenous estrogen metabolism to catechols and subsequent flavoenzyme-mediated redox cycling and generation of ROS may contribute to breast tumor development.

DEDICATION

To my parents Michael and Mary, who bequeathed to me their curiosity and stubbornness, and have encouraged and aided me immeasurably in life...

...to Maria and Kiersten whose intelligence and wit encouraged me to ask the stupid questions...

...to Josie and Cristina because your friendship and ideas were invaluable, and because tea-time was definitely cheaper than therapy...

...and to Steven, Dr. Peggy, Sammy, and Ava who cheerfully donated their washer, dryer, and the contents of their fridge to this project; and without whom this dissertation would not be possible.

ACKNOWLEDGEMENTS

I would like to acknowledge the important contribution of my mentor and advisor, Dr. Jeffrey Laskin to the success of this project. It would not have been possible without his expert and excellent guidance. I would also like to thank Dr. Michael Gallo for his invaluable input and assistance. I value his friendship enormously and am grateful for his interest and enthusiasm for this project even though I was not technically his student. I would also like to acknowledge the important contributions of the other members of my thesis committee, Drs. Andrew Gow and Donald Gerecke, for all their help, encouragement, and advice throughout graduate school.

Thanks are also due to my partners in crime in the Laskin laboratories: Adrienne, Josh, Vladimir, Wendy, Shao, and Yi-Hua, as well as Angie, Carol, and Agnes. I hope you learned half as much from me as I learned from you. I want to acknowledge the important contribution of the EOHSI support staff, especially Toni, Sandi, Liz, and Bernadine for helping me negotiate not one, but two, bureaucracies. To this end, Dr. Kenneth Reuhl was invaluable for both his support and his enormous influence with the university authorities.

Finally, I have to acknowledge the Rutgers Association of Toxicology Students for their enormous and continued support. I could not have wished for a better class of colleagues and I will miss them terribly.

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ABBREVIATIONS

1A1	Cytochrome P450 1A1
1B1	Cytochrome P450 1B1
2-OH TPT	2-Hydroxyterephthalate
2MeOE2	2-Methoxyestradiol
2OHE2	2-Hydroxyestradiol
2OHE3	2-Hydroxyestriol
4OHE1	4-Hydroxyestradiol
4OHE2	4-Hydroxyestradiol
8-oxo G	8-oxy-2-deoxyguanosine
AFU	Arbitrary fluorescence units
b5R	NADH-cytochrome b ₅ reductase
DMSO	Dimethyl sulfoxide
DPI	Diphenyleneiodinium
ds	Double-strand
E1	Estrone
E2	Estradiol
E3	Estriol
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FeCl ₃	Iron (III) chloride
FMN	Flavin mononucleotide
H ₂ O ₂	Hydrogen peroxide

HMEC	Human mammary epithelial cells
K_M	Michaelis-Menten kinetic constant
mg	Milligram
min	Minutes
mM	Millimolar
NADH	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
nM	Nanomolar
nmol	Nanomoles
pA	Picoamperes
pg	Picograms
pmol	Picomoles
POR	NADPH-cytochrome P450 reductase
ROS	Reactive oxygen species
s	Seconds
SEM	Standard Error of the Mean
ss	Single-strand
V_{max}	Maximal reaction rate
α POR	Antibody to NADPH-cytochrome P450 reductase
μ M	Micromolar

INTRODUCTION

Idiopathic post menopausal breast cancer is a neoplastic disease of the mammary ductal tissue diagnosed in more than one in every thousand US women each year (Horner *et al.*, 2009). Extensive research into the etiology of the disease has revealed both genetic (e.g. BRCA genes) and environmental (e.g. xenoestrogen) pathophysiology (Hall *et al.*, 1990b; Mitlak and Cohen, 1997). However not all estrogens are exogenous. Research into the mechanism of action of both xeno- and endogenous estrogens has largely centered on hormonal control of tumor progression and promotion. As a result, the role of estrogens, especially endogenous estrogens, in breast cancer initiation is poorly understood.

A. The Breast

1. Structure

The breasts are symmetrical bilateral organs consisting of three tissue types: adipose, fibrous, and epithelial. The entire organ is a modified apocrine sweat gland and develops similarly in both sexes until the onset of puberty (McKiernan *et al.*, 1988). Under the influence of ovarian and pituitary hormones, the female breasts proliferate and differentiate into the final mammary gland (Howard and Gusterson, 2000). The majority of the developed breast is composed of adipose tissue which is divided and structurally supported by fibrous septae. Embedded in the adipose and fibrous tissues are around 20 separate glandular lobes and the connecting lactiferous duct that drains the lobe towards the areola on the surface of the nipple. Within each lobe this duct branches into a series of smaller ducts each leading to a lobule made of acini (Young and Heath, 2000).

Surrounding and supporting each lobule is dense fibrous tissue composed primarily of collagen (Howard and Gusterson, 2000).

This basic structure is dynamic, however. From puberty to menopause, the ductal epithelia enlarge and shrink mildly with the ovarian cycle (Engel, 1947). During pregnancy, the lobules proliferate greatly increasing the numbers of secretory acini and produce colostrum in the latter stages of pregnancy (Howard and Gusterson, 2000). Upon parturition, milk secretion begins and continues until weaning. After weaning, the lactating breast regresses with the resumption of the menstrual cycle (Engel, 1947). During menopause, the hormonal control of the breast ceases and the breasts progressively atrophy and regress (Howard and Gusterson, 2000).

As involution occurs, both the ducts and lobules disappear and are replaced by adipose. This regression results in the loss of the extensively branching duct system or arbor present in the premenopausal breast. As the fibrous tissue recedes, the few acini and ducts that remain are in closer proximity to adipose tissue (Howard and Gusterson, 2000). Under the microscope, the terminal duct lobular units appear to retain both layers of cells: an epithelial layer (luminal) and myoepithelial layer (basal) which surrounds the luminal layer. This discontinuous myoepithelial layer is made up of stellate-shaped cells that stain positive for actin and contract in response to oxytocin propelling lactation (Young and Heath, 2000). Both myoepithelial and luminal cells are capable of undergoing adenosis, however luminal hyperplasia represents the first step in the bulk of idiopathic breast cancers (Burkitt *et al.*, 1996).

2. Non-neoplastic and neoplastic breast disease

A number of non-neoplastic breast syndromes result in palpable tumor formation or dense spots in a mammograph. In all cases, however, these conditions involve stromal, glandular, and/or epithelial changes. For instance, fibrocystic change involves ductal dilation, apocrine metaplasia, and stromal fibrosis. Fibrocystic change is so common that is considered physiologically normal. It may however create firm palpable masses and cysts mistaken for carcinoma and therefore requiring biopsy (Howard and Gusterson, 2000).

An epithelial hyperplasia is a more serious lesion. Unlike adenosis, a benign glandular proliferation, epithelial hyperplasia causes an increase in the numbers of cell layers in the ductal epithelium. The loss of the normal two-layer structure produces a detectable mass, but does not increase risk for malignancy. If however, the epithelial hyperplasia is atypical, it significantly increases the risk of progressing to ductal carcinoma *in situ* (DCIS) (Mallon *et al.*, 2000).

The fibroadenoma is the most common benign neoplasia of the breast. These are isolated, localized proliferations of the ducts and stroma most common in women in their late twenties and early thirties. It is usually considered a tumor but may be a nodular form of benign mammary hyperplasia. Like mammary hyperplasia, the lesion is composed of epithelial and fibrous components; however, the mass is encapsulated in connective tissue. The epithelia are glandular in structure and resemble ductal epithelium. Hormonal changes may induce proliferation of these cells (Howard and Gusterson, 2000).

Malignant tumors of the breast are among the most common cancers affecting women (Horner *et al.*, 2009). Most are adenocarcinomas developing from the epithelial

cells of the lobules (lobular carcinoma) or the ducts (ductal carcinoma). In both cases, the development of invasive cancer is usually preceded by a carcinoma *in situ* in which the proliferation has yet to breach the basement membrane and invade the stroma (an invasive carcinoma). However, an *in situ* malignancy can still proliferate down the mammary ducts onto the surface of the nipple, resulting in Paget's disease (Mallon *et al.*, 2000).

Advances in screening technologies have improved early detection, such that more breast malignancies are caught in the *in situ* stage in the parts of the world where mammography is routine. As a result, the incidence of adenocarcinoma *in situ* is increasing despite an overall decrease in breast cancer incidence in the United States (Altekruse *et al.*, 2010). DCIS is graded according to the degree of differentiation within the tumor. Highly differentiated lesions (low grade) confer only a moderate risk, while poorly differentiated lesions (high grade) are likely to become invasive ductal carcinomas. However, low grade DCIS shares many histological features with atypical ductal hyperplasia, and can be misdiagnosed (Mallon *et al.*, 2000).

The most important factors influencing the prognosis of breast carcinoma patients are the histological type and grade of the tumor, the tumor size, and the presence of metastases (Mallon *et al.*, 2000). However, breast malignancies are often hormonally driven; therefore, tumors which express estrogen, progesterone, and HER-2/neu receptors increase the number of therapeutic options and have improved prognoses (Zhu *et al.*, 1997; Chen and Colditz, 2007).

B. Neoplastic transformation

1. Introduction

Neoplasia differs from hyperplasia in that neoplasia describes autonomous cell division. Like hyperplastic tissues, abnormal cellular proliferation occurs, however in a neoplasm growth does not cease upon removal of the proliferative stimulus. A neoplasm is deemed benign, if the abnormal cell growth is localized and has well defined margins, or malignant, if the neoplastic margins extend into the surrounding tissue. Benign neoplasms are generally less serious than malignant ones, however they can be life-threatening if the location of the tumor itself exerts harm (e.g. a meningioma), a condition known as positional malignancy. Malignant neoplasms can grow quite extensively into the surrounding tissue, destroying it in a process known as invasion. Some malignancies, including breast tumors, can spread locally into surrounding vascular, lymphatic, and organ tissues. Additionally, individual cells or groups of cells may break away from the primary tumor to colonize other organs and tissues in a process known as metastasis (Burkitt *et al.*, 1996). Thus, malignancies are defined by two heritable properties: they reproduce in defiance of the normal social constraints of the surrounding tissue and they invade and colonize tissues normally reserved for other cell types (Alberts, 1998).

In addition to abnormal cell proliferation, neoplasia is characterized by abnormal cell maturation. During normal growth, cells may acquire specialized structures adapted for specific tissue functions as cellular maturation occurs. This is known as differentiation. The conclusion of this process produces particular cell lines which are considered highly differentiated from their undifferentiated stem cell precursors.

Neoplastic cells on the other hand exist in multiple states of differentiation. Benign neoplasms are considered well differentiated, while malignant ones display variable degrees of differentiation. In general, poorly differentiated malignancies tend to be more aggressive and invasive than their well-differentiated counterparts are (Burkitt *et al.*, 1996).

2. Initiation of idiopathic carcinogenesis

Idiopathic cancers are a complicated constellation of diseases whose origins are genetic. Such malignancies differ from other genetic diseases (e.g. inborn errors of metabolism) because the pathological changes in the genetic information underlying cancer are primarily somatic and not germ-line mutations (Burkitt *et al.*, 1996).

Carcinogenic mutations free cells from normal controls on cell proliferation and survival. These mutations are heritable by any subsequent daughter cell and take two forms. Gain-of-function mutations in a pro-proliferation gene (proto-oncogene) causing the overexpression or hyperactivity of the resulting protein increase the probability of excessive cellular replication. Conversely, loss-of-function mutations of an antiproliferation (tumor suppressor) gene release the cell from normal proliferation restraints with much the same effect. In either case, natural selection of these mutations give the mutant cells a competitive advantage over their surrounding normal neighbors (Russo *et al.*, 2002).

Many carcinogenic mutations occur spontaneously. Natural mutations regularly occur as a result of fundamental limitations in the accuracy of the repair and replication of DNA. Such spontaneous mutations occur at an estimated rate of one mutation per gene per 10^6 cell divisions. If approximately 10^{16} such divisions occur over the human

lifetime, every gene in a single human genome has undergone mutation on about 10^{10} separate occasions (Alberts, 1998). However, many of these mutations are either silent or lethal. Of the remainder, few of these mutations offer the cells the competitive advantage required for natural selection. Immune surveillance identifies and destroys many of the rest. The remaining cells, however, have enhanced DNA replication and cellular division capabilities which further increase the likelihood of mutations because less time is spent on DNA repair.

Other carcinogenic mutations form as the result of mutagens. These genotoxic compounds directly or indirectly alter the DNA sequence of expressed tumor suppressor and oncogenes (Russo *et al.*, 2002). This results in either modified protein products or changes in the epigenetic regulation of the expression of pertinent genes. In either case, the mutations formed must offer an evolutionary advantage to the cellular clones that carry it in order to be carcinogenic.

Dozens of physical and chemical environmental toxicants are suspected to be human carcinogens, however there are only about a hundred known human carcinogens which have been well characterized (National Toxicology Program, 2009). For example, N-methyl-N-nitrosourea (MNU) and ionizing radiation have been causatively linked to breast carcinogenesis. Mutations initiated by MNU include activation of Ras, an oncogene. Ras is a membrane-bound G-protein that functions as a molecular switch mediating growth factor response. Mutations in Ras reduce its GTPase activity, locking Ras in the GTP-bound (active) state and leading to uncontrolled proliferation (Alberts, 1998). In contrast, previous chest exposure to ionizing radiation (often used to treat lymphomas and other forms of cancer) produces missense mutations in the coding

sequence for p53, a tumor suppressor gene. These mutations allow the faulty p53 protein to dimerize with a wild-type p53 protein inactivating it. Fully 50% of all human malignant tumors have p53 mutations (Chang *et al.*, 1993).

Not all mutagens are exogenous environmental compounds. Some natural mutations are the result of endogenous carcinogens. For example, fatty acid chains in lipids can form radicals that if not rapidly quenched, can form malondialdehyde. This reactive aldehyde forms adducts with deoxyguanosine and deoxyadenosine; therefore malondialdehyde is considered a genotoxicant and, if not repaired by base excision, a mutagen (National Toxicology Program, 2009).

Many independent mutations are required to initiate idiopathic carcinogenesis. These mutations do not occur at once. Decades of exposure to mutagens or errors in DNA replication and repair are required; therefore, idiopathic malignancies are typically diseases of mid-life to old age (Alberts, 1998). Germ-line tumor suppressor and oncogene mutations do exist and are heritable (e.g. those of the BRCA family); however, the resulting cancers occur with high frequency and at an earlier age (Hall *et al.*, 1990a). Such malignancies are not considered idiopathic.

3. Cancer cell proliferation and clonal progression

It is important to note that mutagenesis of tumor suppressor and oncogenes is only the initial step in the tumorigenic pathway. The production of idiopathic invasive metastatic malignancies necessarily requires progression and expansion of the new clone. Prolonged exposure to nongenotoxic or epigenetic carcinogens promotes clonal expansion by enhancing proliferative signaling. Nonmutagenic carcinogens fall into four

classes: mitogens and chemicals that promote mitogenic signaling, proinflammatory cytokines and toxicants which promote sustained cellular injury, endogenous or exogenous chemicals that interact with transcription factors to alter mRNA synthesis, and chemicals which alter the methylation and/or acetylation status of critical gene promoter regions and the neighboring histone proteins. Together these mechanisms enable the cell to progress from a single mutated cell to a clonal focus.

Mitogens are compounds which accelerate the transition through the interphase of the cell cycle (G_1 -, S-, and G_2 -phases) and into mitosis (M). Mitogens and mitogenic signaling shorten interphase by overriding some of the cell cycle checkpoints necessary to ensure genomic fidelity. This increases the spontaneous mutation rate in affected cells and promotes the proliferation of all cells responsive to the mitogen, mutant or normal. (All growth factors are by definition mitogenic to their target cell types.) However, as the mutant clones have an additional proliferative phenotype, these cells will out proliferate their non-mutant neighbors increasing the size of the burgeoning neoplasm. Sustained chronic inflammation and cellular injury are indirectly mitogens because a major aspect of the immune response is the release of mitogenic cytokines and growth factors from responding immune and endothelial cells. Normally mitogen exposure ceases with the resolution of the inflammation; however chronically injured tissues are exposed continuously, which may transform the affected cells.

Some mitogens and a large number of other non-genotoxic compounds (e.g. TCDD) are capable of altering gene expression in conjunction with a wide range of transcription factors. These carcinogens progress tumorigenesis by altering the transcriptional activity of the promoter regions of tumor suppressor and proto-oncogenes.

Induction of proto-oncogene protein produces final results similar to the gain-of-function mutation to an oncogene discussed above. Similarly, decreases in the cellular concentration of tumor suppressor proteins advance carcinogenesis with results similar to loss-of-function mutations in tumor suppressor genes. It is important to note that progression of carcinogenesis via a transcriptional mechanism is independent of any carcinogenic mechanism involving mutagenesis. If both mechanisms were to occur concurrently, the effect would be synergistic.

Alterations of transcriptional activity can also occur because of changes in the DNA methylation/histone acetylation status of both tumor suppressor and proto-oncogenes. Methylation of CpG dinucleotides in the promoter region increases histone acetylation and methylation, effectively silencing the downstream gene. Under demethylated conditions, however the gene promoter region is exposed. If the appropriate factors are available for that particular gene, transcription will occur. Any toxicant is carcinogenic which causes an imbalance in the activities of the enzymes that alter the methylation and/or acetylation status of DNA and histone proteins in a direction favoring transcription. So too is any cause of DNA hypomethylation (e.g. S-adenosyl-methionine depletion).

4. Tumor promotion and unchecked growth

As the progressive phase of carcinogenesis occurs, the clonal cells become substantially altered. Under normal conditions, the cell would detect these alterations before exiting the G₁-phase in the cell cycle and either initiate repair before continuing or slate the cell for destruction. Cell death can occur in two ways: necrosis (cellular

explosion) or apoptosis (programmed cellular implosion); though, both types of cell death are controlled by the mitochondria and have several features in common.

During necrosis, the mitochondria increase calcium uptake and decrease inner membrane potential. Loss of membrane potential uncouples the electron transport chain producing reactive oxygen and nitrogen species and the rapid depletion of cellular ATP. This in turn causes a rapid increase in the permeability of the mitochondrial inner membrane. The mitochondrial permeability transition (MPT) produces a rapid influx of water, causing the mitochondria to swell and burst. If enough of the mitochondria undergo MPT, cellular homeostasis is interrupted. Under the resulting osmotic pressure, the cell swells and bursts releasing its contents into the surrounding environment.

While the necrotic cell swells and lyses, the apoptotic cell shrinks, breaks into small apoptotic bodies, and is phagocytosed. Cellular death via apoptosis is a highly regulated, cascade-like process which disassembles the cell. Apoptosis is controlled by two pathways: the extrinsic pathway that is triggered by stimulation of death receptors on the surface of the cell membrane, and/or the intrinsic apoptotic pathway governed by the mitochondria themselves. Like necrosis, the intrinsic apoptotic pathway begins with calcium accumulation, dissipation of the membrane potential, production of ROS/RNS, and the MPT. However, during apoptosis mitochondrial cytochrome c is released into the cytoplasm. Cytochrome c release activates a signaling cascade that ends with activation of a series of caspases that cleave specific cellular proteins. The cell begins to shrink, its contents condense, and it forms membrane bound apoptotic bodies which are the remnants of the cell. These bodies are phagocytosed and their contents recycled by surrounding cells. Regardless of whether the signal is communicated via the extrinsic or

intrinsic pathways, fundamentally apoptosis is controlled by a balance of pro- and anti-apoptotic factors. Many of these factors are nuclear and maintain the balance between ensuring DNA replication and its coding fidelity. For the overall good of the organism, the cell is programmed to eliminate itself in the event of DNA damage.

Unsurprisingly then, initiated pre-neoplastic cell clones have a higher rate of apoptosis than their normal counterparts do. Many tumor suppressor proteins, such as p53, are also pro-apoptotic factors, preventing the proliferating clone from being promoted into a full-fledged malignancy. For promotion to occur, the apoptotic machinery must be suppressed. Non-genomic carcinogens which activate anti-apoptotic pathways and repress pro-apoptotic signaling (e.g. peroxisome proliferators) accelerate the growth of pre-neoplastic foci into tumors.

In addition to the suppression of apoptosis, the clonal foci are expanding because they fail to terminate cell proliferation. This occurs for several reasons. As mentioned earlier, the increased mitotic activity promotes further mutagenesis because less time is available for DNA repair during a shorted G_1 -phase. Increased mitotic activity also reduces maintenance methylation of the DNA daughter strands in the shortened G_2 -phase, thereby altering tumor suppressor and oncogene expression. As proliferation increases the clone expands to form larger foci and tumors. Finally, cell-to-cell communication is disrupted. Decreases in intercellular communication both isolate the clonal cells from the stop signals emitted from the surrounding normal tissue and decrease the reliance of the clone on outside information. Disruption in intercellular communications through gap-junctions and endocrine hormones is a major factor contributing to the invasiveness of

the resulting tumor. At this point, the clone has expanded to the point where it is considered a malignancy.

5. Summary

These tumorigenic mechanisms are cooperative and can occur concurrently. Many compounds exert their carcinogenicity via multiple mechanisms and can have modes of action in all three phases of tumorigenesis: initiation, progression, and promotion. Such toxicants are usually referred to as complete carcinogens. For example, DNA viruses are complete carcinogens because insertion of the viral genome often causes mutations in that of the host (initiation), because the inflammatory response of the host is mitogenic (progression), and because the virus itself inhibits apoptosis/necrosis by the host cell to ensure its own survival (proliferation). Thus, viruses are both genotoxic and non-genotoxic carcinogens. It is important to note that all three phases of carcinogenesis must occur for a neoplasm to become an invasive malignant tumor.

C. Oxidative Stress

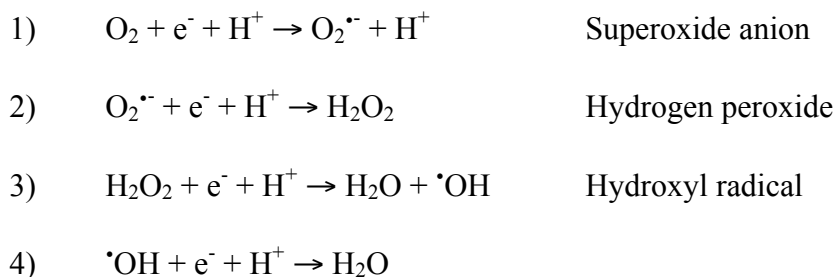
1. Introduction

A major consequence of mitochondrial oxidative phosphorylation is the production of reactive oxygen species (ROS) by reduction of molecular oxygen. The majority of this ROS is generated when electrons from cytochrome c oxidase in complex IV directly reduce oxygen, a process termed electron transport chain leakage. Under homeostatic conditions, ROS generation is closely balanced by endogenous antioxidant mechanisms. However, any alteration in ROS generation can impact the cellular

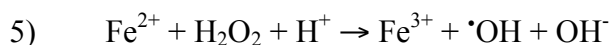
oxidative balance. If ROS concentrations increase in excess of the detoxification capability of the antioxidants, the resulting imbalance is termed oxidative stress. This imbalance prevents antioxidant regeneration, initiates signal transduction pathways, damages DNA and other cellular macromolecules, and may ultimately result in either apoptosis or mutations and initiation of carcinogenesis.

2. Formation of reactive oxygen species

ROS are formed by the partial reduction of molecular oxygen. During this incomplete reduction, each atom in molecular oxygen undergoes a two-electron reduction to form a molecule of water (Scheme 1). In the process, superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet OH$) are formed, as shown in the following reactions:



Hydroxyl radicals can also be generated via the interaction of hydrogen peroxide and, to a lesser extent, superoxide with transition metal ions. Transition metals such as copper and iron exist as cations with multiple oxidation states. In their more reduced states, they are stable yet willing electron donors. In its reduced form (Fe^{2+}), iron, in particular, is known to generate hydroxyl radicals from hydrogen peroxide in a process known as the Fenton reaction:



For this reason, the state of iron(II) is tightly regulated. The concentration of intracellular iron is minimized by sequestration in ferritin as Fe^{3+} ; however, free Fe^{2+} may be released by either the reduction of Fe^{3+} by superoxide anion or the degradation of iron-containing enzymes and ferritin.

The reactivity of each ROS species is determined by its oxidative potential. By far, the most reactive species are the hydroxyl radicals, which have a half-life on the order of a billionth of a second. Hydroxyl radicals, therefore, are only able to diffuse very short distances (a few molecular diameters) from their origin before reacting nonspecifically with any neighboring cellular macromolecule. Superoxide anion radicals are much less reactive with a half-life on the order of a millionth of a second. Although less reactive than the other ROS species, superoxide production is important nonetheless because it leads to the formation of other ROS, including hydroxyl radicals and hydrogen peroxide.

Additionally, several radicals are produced by ROS in subsequent reactions. These radicals are formed primarily during lipid peroxidation and radical propagation reactions and include alkoxyl radicals (RO^\bullet), peroxy radicals (ROO^\bullet), and organic hydroperoxides (ROOH). Singlet molecular oxygen ($^1\text{O}_2$) is also produced as a result of photochemical reactions and is a highly reactive radical that oxidizes cellular macromolecules. Together with the ROS species mentioned previously, these molecules are important in generating conditions of oxidative stress.

Despite the fact that it is not a radical, hydrogen peroxide is a more potent oxidant than superoxide, though less potent than hydroxyl radicals. Hydrogen peroxide is both neutral and symmetric; therefore, it is non-polar and can readily cross cell membranes. It

is highly stable, except in the presence of oxidizing and reducing agents, and will traverse great distances from its origination point. These qualities and its role in hydroxyl radical production form the basis for its toxicity as a species of ROS.

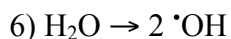
3. Sources of reactive oxygen species

As mentioned previously, electron leakage from the mitochondrial electron transport chain is responsible for the majority of endogenously generated ROS. However, leakage from a second electron transport chain located in the endoplasmic reticulum also contributes. NADPH-cytochrome P450 reductase (POR) is an enzyme which strips electrons from the electron donor NADPH and passes them one at a time down an electron transport chain to a network of heme-containing mixed-function oxidases. Like the cytochrome c oxidase to which it is related, POR is also capable of directly reducing molecular oxygen and constitutes a substantial percentage of ROS production in the cell types in which it is expressed.

Myriad other enzymes are also involved in the generation of ROS. Xanthine oxidase is an enzyme that produces superoxide anion as a byproduct of *de novo* nucleotide synthesis. The neutrophil membrane protein NADPH-oxidase produces large amounts of ROS as part of the respiratory burst response of the immune system. Many other enzymes which catalyze redox reactions are also capable of producing ROS, either as a direct reaction product, or because of electron leakage. Many of these enzymes are also mediators of environmental sources of ROS.

The environment in which and organism lives and breathes significantly impacts its cellular redox balance. Exogenous sources of ROS include both physical and chemical

toxigants. Ionizing radiation such as gamma-rays, X-rays, alpha- and beta-particles induces radiolytic fission, generating hydroxyl radicals as shown below:



A common mechanism by which metals and chemicals produce ROS is that of redox cycling. This occurs when the parent redox cycling agent (e.g. the herbicide diquat) is either oxidized or reduced by one electron. This reaction usually requires both an electron acceptor or donor and an enzymatic mediator. The resulting toxicant radical is unstable and immediately transfers the electron to molecular oxygen, producing superoxide anion (which then generate other ROS as described before) and regenerating the parent toxicant. Given an unlimited supply of molecular oxygen and reducing equivalents, very few molecules of toxicant are required to generate large quantities of ROS. Not all redox cycling agents are exogenous. For example, the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) is a redox-active molecule which produces ROS in the substantia nigra region of the brain. These ROS disturb dopamine storage and may participate in the neuronal degeneration typical of Parkinsonism.

Many classes of compounds have redox cycling properties. Transition metals form cations which are capable of carrying more than one oxidation state. For instance, copper cations can carry both +1 and +2 charges. Such metals are so ideally suited to redox chemistry that as free cations they will readily react with cellular macromolecules. This toxicity means that in biological systems, transition metal cations must be sequestered into the active sites of catalytic enzymes.

Most redox cycling agents, however, are organic. In order to be capable of redox cycling, these compounds must have two features: a nucleophilic or electrophilic atom

which can readily change oxidation state (e.g. nitrogen or oxygen), and a high degree of molecular conjugation (π -bonding) in a position to stabilize the resulting cation or anion radical. The amount of conjugation needed typically implies, but not requires, an aromatic ring structure. Several classes of molecules meet these requirements. For instance pyridinium cations (e.g. diquat) are positively charged, aromatic, nucleophilic, tertiary amine, Lewis bases capable of distributing the cation radical charge among the conjugated π -bonds of the aromatic ring. On the other hand, quinones are electrophilic Lewis acids which contain two carbonyl groups placed *ortho*- or *para*- to each other around a 6-carbon conjugated aliphatic ring (e.g. 1,2- or 1,4-benzoquinones). *Meta*-carbonyl arrangements cannot redox cycle as at least one of the carbonyl groups is necessarily out of conjugation by this arrangement (e.g. dicoumarol). Thus *ortho*- and *para*-quinones have the redox potential to generate ROS, while *meta*-arranged hexene rings do not and are not considered true quinones.

4. Oxidative damage

At normal physiologic levels, ROS act as important molecular signals required to maintain homeostasis and mediate pathways that control cellular proliferation, differentiation, and apoptosis. At raised concentrations, ROS induce conditions of oxidative stress. This results in damage to DNA, proteins, and lipids.

a. DNA damage

Acute and chronic oxidation of DNA damages it in a mutagenic manner and may contribute to the initiation phase of carcinogenesis. Five types of DNA damage are among the most common: base oxidation, strand breakage, intercalation, adduct

formation, and base depurination. If hydroxyl radicals react with DNA bases, hydroxylation occurs of which, 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxo-dG) formation is the most common. 8-oxo-dG generation increases C-G \rightarrow A-T transversions, causing mispairing during replication and, provided these mutations are not silent, single amino acid substitutions. Additional base oxidation reactions can form pyrimidine glycols and hydroperoxides. On the other hand, if the hydroxyl radicals react with the DNA deoxyribose backbone, single strand breaks may result. Both base oxidation and single strand breakage are mended by base excision repair in which the unaffected strand serves as the template. As the frequency of single strand breakage increases, the probability of two single strand breaks occurring on opposing strands in close proximity to each other also increases. This effectively produces double strand breaks that are particularly hazardous because neither strand is available for use as a sequence template. Genome rearrangement often results.

The final three forms of DNA damage: intercalation, adduct formation, and base depurination are often functions of the same genotoxicant. For example, the chemotherapeutic drug cisplatin is both an intercalating and a potent DNA crosslinking agent. This is due in part to its small flat molecular structure which allows cisplatin to fit between the base pairs of the macromolecular DNA ladder (intercalate). The reactive platinum(II) center can react with nucleotide bases on both strands (bifunctional adduct formation), effectively linking the two halves of the DNA zipper together and triggering apoptosis. Some DNA adducts, once formed, undergo additional reactions that result in the loss of the DNA base either spontaneously, or via base excision repair. If this gap in the ladder remains, or DNA repair is performed incorrectly, DNA mutations will occur.

b. Protein damage

Protein oxidation usually results in altered or lost function. These effects could be due to direct binding of ROS at the active site of an enzyme, or result from conformational changes brought by ROS covalent binding to the side chains of other tyrosine, methionine, serine, and especially cysteine amino acid residues. If the sulfhydryl groups of several cysteine residues are oxidized, they may form disulfide bonds, rearranging the tertiary structure of the protein. Through a series of reactions, protein oxidation results in the generation of an excess of carbonyl groups which has served as a useful metric of the level of oxidative damage to proteins.

c. Lipid damage

Lipid peroxidation is perhaps the most widely recognized feature of oxidative macromolecular damage. In this type of damage, the unsaturated fatty acids in the lipid tails react with ROS to form lipid hydroperoxides. These hydroperoxides are themselves radicals and will react with neighboring lipids, propagating the reaction. Lipid hydroperoxides are also vulnerable to reaction with transition metals forming alkoxyl and peroxy radicals. Lipid peroxidation alters cellular signaling both directly by the production of mediators (e.g. 4-hydroxynonenol) and indirectly via increases in the intracellular calcium concentration. Both lipid peroxidation and modification of key residues on redox-sensitive proteins have been shown to activate numerous signaling cascades. These signal transduction pathways are complex and the interaction between ROS and the kinase cascades is not well understood at this time; however, depending on the duration and amount of ROS exposure, these non-genotoxic alterations in cell

signaling can be mitogenic or apoptotic. Thus, ROS can be classified as complete carcinogens.

5. Cellular defense mechanisms

To prevent oxidative damage, a number of cellular detoxification mechanisms have evolved. As removal of ROS is critical for cell survival, several antioxidant enzymes exist to convert ROS into less harmful compounds. The major enzymes: superoxide dismutase, catalase, and the peroxidases provide the primary antioxidant defense system. These enzymes operate by completely oxidizing or reducing the ROS to form either molecular oxygen or water. Additional ROS removal is provided by glutathione, an antioxidant tripeptide, either via glutathione peroxidase or secondarily by removal of the macromolecular oxidation products via glutathione-S-transferases.

a. Superoxide dismutase

Superoxide dismutase (SOD) is an enzyme which catalyzes the one-electron oxidation of superoxide anion to molecular oxygen by harnessing the one-electron reduction of another superoxide anion molecule to hydrogen peroxide.

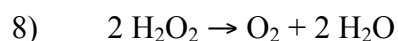


Three isoforms of this enzyme exist and are generally identified by the metal(s) in their active sites. Eukaryotic cytosolic SOD (CuZnSOD or SOD1) contains both copper and zinc cations, mitochondrial SOD (MnSOD or SOD2) contains a single manganese cation, and prokaryotic SOD (FeSOD) contains iron. While SOD is generally thought to provide the primary defense against ROS by oxidizing superoxide anion to molecular oxygen,

overexpression of CuZnSOD has been shown to increase cellular oxidative stress due to a corresponding elevation in hydrogen peroxide formation.

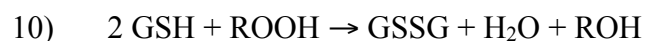
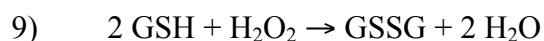
b. Catalase and peroxidases

Both catalase (also known as hydrogen peroxide dismutase) and all peroxidases catalyze the reduction of hydrogen peroxide to water; however, the reactions differ in the reducing agent required for catalysis. Like SOD, catalase requires two molecules of ROS for activity. One molecule of hydrogen peroxide undergoes a two-electron oxidation to molecular oxygen, while the second undergoes a two-electron reduction to water.



This reaction occurs very rapidly, as catalase has one of the highest measured k_{cat} constants of any known enzyme allowing it to rapidly decompose high hydrogen peroxide concentrations without the use of cellular reducing equivalents. However, its low binding affinity (K_M) makes it ineffective for the detoxification of lower concentrations of hydrogen peroxide that would otherwise be toxic to the cell.

To eliminate reduced hydrogen peroxide concentrations, eukaryotic cells have evolved a number of peroxidase enzymes which exploit cellular reducing equivalents to oxidize any number of peroxides, including hydrogen peroxide. The most ubiquitous of these enzymes is glutathione peroxidase, whose enzymatic reactions occur as follows:



c. Glutathione conjugation

As hydroxyl radicals have such a short half-life (10^{-9} seconds), no antioxidant enzyme is capable of detoxifying them. Instead, the cellular strategy is to remove the

macromolecular oxidation products by conjugation to reduced glutathione. This allows cells to eliminate damaged molecules. Glutathione conjugation can occur spontaneously, however a large superfamily of glutathione-S-transferase (GST) enzymes has evolved to accelerate and regulate this reaction. These enzymes also prevent the further oxidative stress by directly eliminating ROS and lipid peroxidation species.

D. Estrogens

1. Introduction

Estrogens are among the major steroid hormones and act as the primary female sex hormone with many functions in women including regulation of the menstrual cycle. Three endogenous estrogens are known to exist in humans: estrone (E1), estradiol (E2), and estriol (E3), including human males at reduced doses. The ovarian estrogens E1 and E2 are produced from the androgens androstenedione and testosterone respectively by aromatase. Additionally, E1 and E2 are also interconverted by 17 β -hydroxysteroid dehydrogenase. In sexually mature, non-pregnant women, E2 is the predominant estrogen; however, after menopause the ovaries atrophy and the non-ovarian estrogen E3 dominates. E3 is also the primary estrogen of pregnancy.

As widespread hormones, estrogens control a wide variety of functions. In its capacity as a proliferative agent, it initiates female pubertal growth and regulates the formation of secondary sex characteristics, including the breasts. During the menstrual cycle, estrogen stimulates endometrial growth. A drop in circulating estrogen is responsible for the sudden atrophy of uterine lining which begins menstruation. Estrogens are key to the maintenance of blood vessel and skin elasticity, increase synthesis of bone,

and reduce bone resorption. Estrogen mediated changes in cellular signaling increase the serum concentration of many prothrombotics, but decrease cholesterol and triglyceride levels. Many, if not most, sexually dimorphic characteristics are linked to estrogen function.

2. Modes of action

Regardless of structure, all estrogens have identical modes of action. As steroid hormones, estrogens readily diffuse across membranes and through tissues. Inside cells, estrogens bind cytoplasmic estrogen receptors that dimerize and translocate to the nucleus. These activated estrogen receptor dimers are potent transcription factors which regulate the expression of genes with upstream estrogen response elements (EREs). Two nuclear estrogen receptors have been described in humans: alpha ($ER\alpha$) and beta ($ER\beta$). $ER\alpha$ is widely expressed in endometrium, breast, ovarian stroma, and the hypothalamus, while expression of $ER\beta$ is located in endothelial, brain, bone, kidney, heart, lungs, intestinal mucosa and prostate cells. Due to alternative splicing, at least three $ER\alpha$ and five $ER\beta$ isoforms are known to exist. Estrogen receptors can form both homodimers ($\alpha\alpha$ or $\beta\beta$) and heterodimers ($\alpha\beta$); however, since not all cell types express both receptors, the number of combinations is limited in these cells.

Evidence also suggests that E2 can associate with the endoplasmic reticulum membrane and bind G-protein coupled receptor 30 (GPR30) (Revankar *et al.*, 2005; Kleuser *et al.*, 2008; Madak-Erdogan *et al.*, 2008). Activated GPR30 mobilizes calcium and nuclear phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) synthesis. Both calcium mobilization and PIP_3 synthesis activate downstream kinases, stimulating both growth

and cell survival pathways. Thus, E2 (and probably E1 and E3) are both genomic and non-genomic proliferation agents.

a. Endocrine disruption

Endogenous estrogens are not the only chemicals capable of activating estrogen receptors. Many compounds, including drugs and environmental pollutants are ligands for ER α and ER β . In some cases, the xenoestrogens activate ER-mediated transcription (agonism); while in others, transcription is inhibited (antagonism). These ER ligands often selectively agonize or antagonize one receptor type over another and are termed selective estrogen receptor modulators (SERMs). For example, the phytoestrogen genistein binds to ER β with greater affinity than ER α . A number of SERMs have been identified (e.g. ethinyl estradiol, equilin, methoxychlor, nonyl-phenol, bisphenol A, and some PCBs); however, with the exception of the pharmaceuticals, ethinyl estradiol and equilin, these agonists have relatively low binding affinities to the ERs. Some controversy remains over whether environmental exposures are sufficient to cause toxicity to humans.

Pharmaceutical SERMs, on the other hand, have been designed for their potency and selectivity. Depending on their treatment use, these SERMs can be ER agonists, antagonists, or partial agonists/antagonists (agonist in one or more tissues and antagonist in another). For example, tamoxifen is a drug which was designed to be a selective antagonist for ER α in the breast, but is actually classified as a partial agonist because it acts as an ER α agonist in endometrial uterine tissue. Whether a SERM is an agonist or an antagonist is primarily controlled by the position of helix 12 of the ER upon drug binding. Helix 12 belongs to the AF-2 domain of the receptor and controls binding to

coactivator and corepressor proteins. If estrogen binding causes conformational changes in the receptor that opens up helix 12 to coactivator binding, then the estrogen will act as an agonist. If helix 12 remains in a position favorable for corepressor binding, the estrogen is an antagonist. Since cell types express a different constellation of coactivator and corepressor proteins and the receptors themselves may be composed of different isoforms, estrogen response will vary with tissue type.

b. Role of estrogen in breast carcinogenesis

Exposure to both endogenous and exogenous estrogens has been well characterized as a major risk factor for breast-cancer development. Metaanalysis of 9 studies demonstrates a dose-response relationship between estrogen exposure and increased breast cancer risk. (Key *et al.*, 2002) Epidemiology has linked early menarche and late menopause with an increased prevalence of breast tumors in post-menopausal women (Kato *et al.*, 1988).

In 2002, a randomized control trial of Premarin was halted early due in part to a 24% increase in breast cancer incidence between the experimental and control arms of the study. (Rossouw *et al.*, 2002) One of the most commonly prescribed hormone replacement therapies (HRTs) since 1942, Premarin is a HRT used to treat the symptoms of menopause. The increase in breast cancer incidence was given significant media attention and sales of HRT dropped by 2003. As a result, the age-adjusted incidence of breast cancer in the US also fell 6.7% that year. (Ravdin *et al.*, 2007) Furthermore, non-neoplastic breast tissue of women receiving Premarin had a significant increase in DNA damage compared with tissues from women who were not. (Malins *et al.*, 1996; Malins *et al.*, 2006; Karihtala and Soini, 2007) This implies that in addition to their known role as

promoters of random carcinogenic mutations, equine estrogens (the major components of Premarin) can also act as tumor initiators.

3. Estrogen metabolism

A traditional mode of action whereby the SERMs stimulate cellular proliferation by binding nuclear estrogen receptors, altering transcription, cannot explain this. Neither can activation of non-genomic proliferation pathways involving membrane-bound estrogen receptors. (Revankar *et al.*, 2005; Song *et al.*, 2006) An alternate mode of action involving bioactivation of the equine estrogens to catechol metabolites in the endoplasmic reticulum of breast epithelial cells (Scheme 2), and then subsequent oxidation to redox-active ortho-quinones, has been suggested. This hypothesis is supported by the increase in DNA-damage in breast tissue of women on HRT as well as the increase in lipid-peroxides in neoplastic compared to normal breast tissue. (Boyd and McGuire, 1991) Additionally, genetic polymorphisms of CYP450 which increase the activity of isoforms 1A1 and 1B1 have been linked to increased risk for breast cancer. (Kisselev *et al.*, 2005; Wen *et al.*, 2007) Expression of 1A1 and 1B1 is also found to be higher in breast tissue of cancer patients than those without breast cancer. (Singh *et al.*, 2005) It is likely that estrogen-induced initiation of breast cancer involves semiquinone radical production and subsequent alkylation and oxidative stress.

Cytochromes P450 1A1 and 1B1 are isozymes known for Phase I metabolism of estrogens. (Aoyama *et al.*, 1990; Kerlan *et al.*, 1992; Savas *et al.*, 1994; Sutter *et al.*, 1994) CYP1A1 catalyzes hydroxylation at the 2- position of the endogenous estrogens estradiol (E2) and estrone (E1), however the enzyme is only 80-85% selective for the two

position. (Weisz *et al.*, 1992; Spink *et al.*, 1997; Spink *et al.*, 1998; Badawi *et al.*, 2000; Badawi *et al.*, 2001; Cribb *et al.*, 2006) The remaining 15-20% of 1A1 and all 1B1 estrogen hydroxylase activity occurs at the 4-position (Scheme 3). (Weisz *et al.*, 1992; Savas *et al.*, 1994; Sutter *et al.*, 1994; Sissung *et al.*, 2006) *O*-quinones formed from the oxidation of 2-hydroxy estradiol (2-OHE2) and 2-hydroxy estrone (2-OHE1) by NADPH-cytochrome P450 reductase are thought to be less toxic than their 4-hydroxy counterparts, despite being the major catechol estrogen metabolite in most mammals. (Liehr *et al.*, 1986b; Li and Li, 1987; Martucci and Fishman, 1993; Newbold and Liehr, 2000) This observation is only partly explained by the induction of 1B1 in neoplastic breast tissue making 4-OHE2 the major estrogen metabolite in the breast. (Rajapakse *et al.*, 2005) The difference in half-lives of the resulting 2- and 4-OHE1-*o*-quinones (47 seconds and 12 minutes, respectively) also partly contributes. (Iverson *et al.*, 1996)

a. Adduct formation

Activation of endogenous 2- and 4-hydroxyestrogens to semiquinone radicals increases the magnitude of the redox potential, and therefore reactivity, of these estrogen metabolites. These radicals can directly react with DNA nucleotide bases, forming adducts. The mutagenic potential of these adducts varies between 2- and 4-hydroxyestrogen species. 2-hydroxyestradiol and 2-hydroxyestrone semiquinones reacted with guanine at N3 forming stable adducts that are usually identified and repaired before replication. In contrast, 4-hydroxyestradiol and 4-hydroxyestrone semiquinones preferred adduct formation with guanine at N7. These N7 adducts further react by cleaving the glycosidic bond releasing the now aminated estrogen and depurinating the guanine residue. Apurinic sites are poorly recognized by DNA repair machinery and are easily

misread during transcription. As a result, depurinating DNA adducts are significantly more mutagenic than non-depurinating ones. This is the major reason why 2-hydroxy catechol estrogen metabolites are thought to be less toxic than their 4-hydroxylated counterparts.

b. Catechol estrogen induced oxidative stress

In addition to their ability to directly modify DNA nucleotide bases, catechol metabolites of exogenous estrogens redox cycle and cause oxidative stress. Activation of equilin to catechol metabolites, semiquinones, and quinones depletes reducing agents and generates reactive oxygen species causing oxidative stress. (Liehr *et al.*, 1986c; Bolton *et al.*, 2000) *O*-quinones are known to be redox active. After reduction by redox active ions or enzymes, the spontaneous oxidation of the semiquinone radical generates superoxide. Superoxide radicals are dismutated by Superoxide dismutase (SOD) to hydrogen peroxide and molecular oxygen. Catalase then dismutates the hydrogen peroxide to molecular oxygen and water. However, this process also restores the quinone, which can then be reoxidized and cycle again. The result of this redox cycling is the production of substantial amounts of reactive oxygen species (ROS). (Bolton *et al.*, 2000) As the antioxidant systems become overwhelmed, more ROS are produced including the highly reactive hydroxyl radical in the presence of transition metals like iron. Hydroxyl radicals are known to alkylate DNA, increase lipid peroxidation, and oxidize proteins and are considered highly mutagenic.

Unsurprisingly then, catechol estrogen metabolites have been demonstrated to increase lipid peroxidation, (Roy and Liehr, 1992; Wang and Liehr, 1995) oxidize proteins, (Winter and Liehr, 1991) and alkylate DNA. (Liehr *et al.*, 1986c; Roy and

Liehr, 1988) Lipid peroxidation is a result of hydroxyl radical reactions with membrane lipids. Released lipid peroxide radicals induce DNA alkylation both directly and secondarily through reactive aldehyde byproducts of lipid peroxidation. (Liehr *et al.*, 1986a) Many protein and lipid peroxide byproducts also double as cell signaling molecules.

Catechol estrogen metabolites also oxidize proteins, particularly redox-active proteins like non-specific-quinone oxidoreductase 1 (NQO-1) (Bianco *et al.*, 2003; Cassidy *et al.*, 2006) and glutathione-S-transferase P1-1. (Chang *et al.*, 2001; Abel *et al.*, 2004) Both of these enzymes are potential catechol estrogen detoxification pathways. Another protein target for estrogen quinones, thioredoxin-1 regulates both constitutive and inducible gene expression of the CYPs that metabolize E1 and E2 into catechol estrogens, 1B1 and 1A1. (Husbeck and Powis, 2002) At high concentration, catechol estrogen metabolites have also been shown to be irreversible inactivators of redox-sensitive enzymes. (Yao *et al.*, 2002) This occurs through both oxidation and alkylation mechanisms and is suppressed by α -naphthoflavone, a CYP1A and 1B inhibitor. (Liehr *et al.*, 1991) In addition to its primary function, each of these proteins acts as a sensor of the cellular “redox state”. These sensors maintain the redox balance of cells and tissues and advances cellular proliferation or apoptosis through the stress response cascade if necessary.

E. Summary and rationale for the dissertation

Clearly, the relationship between long term estrogen exposure and breast tumorigenesis is very complex. In at least two modes of action, changes in gene

expression or as direct modifiers of cell signaling pathways, estrogens act as proliferators of spontaneous mutations. However, estrogens are also mutagenic and therefore complete carcinogens. In a third mode of action, estrogens are metabolized to catechols and semiquinone radicals which directly form DNA adducts. DNA adduct formation may account for estrogen's role as a mutagen; however, a fourth possibility is also suggested by the literature. It is likely that the catechol metabolites of estrogens are redox cycling agents, producing ROS that are capable of damaging DNA, oxidizing proteins, and forming lipid peroxides (Scheme 4). The resulting oxidative stress is not only mutagenic, but also carcinogenic via non-genomic mechanisms.

Premarin-induced carcinogenesis has been widely attributed to the production of redox-active catechol metabolites of equilin, its active ingredient, by cytochrome P450 isozymes. These catechols readily undergo one-electron oxidation by NADPH-cytochrome P450 oxidoreductase to semiquinone intermediates in the presence of electron donors NADH and NADPH. The equilin-semiquinones react with nearby DNA, forming adducts (Bolton, 2002). Alternatively, equilin-semiquinones are further oxidized by cytochrome P450 reductase to ortho-quinones, or redirect their unpaired electron to elemental oxygen forming superoxide, a reactive oxygen species (ROS), and restoring the catechol to react again in a process called redox cycling. Cellular superoxide dismutases (SODs) convert the superoxide to hydrogen peroxide, which endogenous transition metals can convert to highly mutagenic hydroxyl radicals (Liehr and Jones, 2001). These hydroxyl radicals cause lipid peroxidation, protein oxidation, and oxidative DNA damage. Thus metabolism of equilin to a catechol is shown to have widespread effects on

cellular homeostasis, including both mutagenic and carcinogenic effects in tissues with a high-degree of cytochrome P450 reductase activity, especially human mammary tissue.

While several studies have attempted to characterize the role of exogenous estrogens in the initiation phase of breast cancer, little work has been done examining the mechanisms by which endogenous estrogens contribute to the initiation phase of idiopathic breast carcinogenesis. To date, the capability of endogenous catechol estrogen metabolites to form ROS remains poorly characterized. Furthermore, whether endogenous catechol estrogen redox cycling occurs in breast epithelial cells has not been studied. This fourth mode of estrogen carcinogenicity is likely a contributing factor in the development of idiopathic breast cancer although the interaction between the mechanisms involved is not completely understood.

METHODOLOGY

A. Rationale

If equilin can be metabolized to catechols which redox cycle and produce mutagenic ROS, then perhaps endogenous estrogens can also become mutagenic via a similar mechanism. Three predominant human estrogens are known to exist. The most prevalent endogenous estrogen in adult pre-menopausal women is estradiol (E2), which can be biologically interconverted to estrone (E1). However, after menopause, production of gonadal estrogens slows and estriol (E3) from the adrenal gland and adipocytes becomes the major estrogen. Each of the three endogenous estrogens is a substrate for cytochromes P450 1A1 and 1B1 (Aoyama *et al.*, 1990; Kerlan *et al.*, 1992; Savas *et al.*, 1994; Sutter *et al.*, 1994). These enzymes are adjacent to two redox-active oxidoreductases in the microsomal electron-transport chain (Scheme 2) which are well-known mediators of redox cycling.

Because of this reductase activity and the proximity to the cytochromes in which the parent estrogens are metabolized to catechols, cytochrome b₅ and cytochrome P450 oxidoreductases seemed likely mediators of catechol estrogen redox cycling. Using this information, we investigated whether the catechol metabolites of all three human estrogens were also capable of redox cycling and producing ROS in a mechanism analogous to that of equilin. Using four catechol metabolites of the three endogenous human estrogens, we explored whether redox cycling could be mediated by recombinant cytochrome b₅ and P450 reductases as well as human mammary epithelial cells.

B. Chemicals and reagents.

Amplex Red (10-acetyl-3, 7-dihydroxyphenoxazine), ROX (a mixture of 5- and 6-carboxy-X-rhodamines), and Quant-iT™ PicoGreen® dsDNA reagent were from obtained from Invitrogen (Eugene, OR). Recombinant human cytochrome P450 oxidoreductase + cytochrome b₅ control Supersomes™ were purchased from BD Biosciences (San Jose, CA). Supersomes are recombinant cDNA-expressed enzymes prepared from a baculovirus-infected insect cell system. A plasmid for the expression of human recombinant soluble cytochrome b₅ reductase was a gracious gift of Dr. Lauren A. Trepanier (University of Wisconsin-Madison). The expression of these plasmids in *E.coli* and subsequent purification of cytochrome b₅ reductase was performed as previously described (Trepanier *et al.*, 2005). The estrogens, NADPH, dihydroethidium, terephthalate, calf-thymus DNA, and all other chemicals were from Sigma-Aldrich (St. Louis, MO). All graphical, statistical, and kinetic analyses were performed using the data analysis software GraphPad Prism® for Windows® v. 5.03 (La Jolla, CA).

C. Attempt to measure NAD(P)H depletion

The process of redox cycling is comprised of two discrete steps. In the first, an oxidoreductase mediating enzyme transfers an electron from a reducing equivalent to the redox cycling agent, forming a radical. In the second step, the radical spontaneously passes the electron to molecular oxygen, forming superoxide anion and restoring the redox cycling agent (Scheme 5). The resulting superoxide can be dismutated into hydrogen peroxide, which in turn can form hydroxyl radicals in the presence of transition metals (Fenton chemistry). Since the restored redox cycling agent can re-react with the

mediating enzyme, the cycle continues, generating toxic concentrations of reactive oxygen species, depleting reserves of reducing equivalents, and producing hypoxic conditions in a closed system. Therefore, in order to characterize catechol estrogens as redox cycling agents, they must both decrease key substrates (NAD(P)H and molecular oxygen) and produce reactive oxygen species when catalyzed by a mediating enzyme.

We began our characterization by attempting to measure a decrease in reducing equivalents. Due to their adenine base structure the oxidized equivalents NAD^+ and NADP^+ both absorb ultraviolet light with a peak absorption at about 260 nm. Similarly, the reduced equivalents NADH and NADPH also share a peak absorption at around 340 nm. No matter which electron donor is used, it should theoretically be possible to monitor the rate of NAD(P)H oxidation using the ratio of absorbances at 340 and 260 nm. Unfortunately, the estrogen species also strongly absorbed light at these wavelengths, confounding any attempt to quantify reducing equivalent depletion.

C. Enzyme assays for reactive oxygen species generation

1. Hydrogen peroxide generation

Next we determined whether recombinant cytochrome b_5 and P450 reductases mediate reactive oxygen species generation by 2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2), 4-hydroxyestrone (4OHE1), and 2-hydroxyestriol (2OHE3). Since peroxides are among the most stable of the reactive oxygen species, we first examined the role of these estrogens in hydrogen peroxide production, measured using the Amplex Red™/horseradish peroxidase method as previously described (Gray *et al.*, 2007; Mishin *et al.*, 2010). This assay consists of a non-fluorescent substrate, Amplex-

Red, which is converted by horseradish peroxidase into a highly fluorescent product resorufin in the presence of hydrogen peroxide. The Amplex-Red/horseradish peroxidase assay has a lower limit of detection of 5.0 pmol H_2O_2 /well and a lower limit of quantitation of 30 pmol H_2O_2 /well, making it 20 times more sensitive than the previous ferrous thiocyanate method. This linear dynamic range extended to 400 pmol/assay ($r^2=0.995$) (Mishin *et al.*, 2010). However, this conversion of substrate to product is enzymatic; at high H_2O_2 concentration the enzyme can become saturated and becomes the rate limiter in the conversion of Amplex Red to resorufin. Thus any kinetic analysis represents a composite of two enzymatic reactions, the initial generator of hydrogen peroxide and the secondary production of the fluorescent indicator. Under ideal conditions, the assay uses concentrations of horseradish peroxidase that far exceed that required to process any generated hydrogen peroxide; however, at extreme rates of hydrogen peroxide generation, this may not be kinetically feasible.

Assays for hydrogen peroxide production were analyzed at 37°C and contained 70 mM NaCl in potassium phosphate buffer (30 mM, pH 7.8) supplemented with 100 μM NADPH, 100 μM Amplex Red, 1.1 unit/ml horseradish peroxidase, 5 $\mu\text{g/ml}$ recombinant protein or 50 $\mu\text{g/ml}$ cell lysate protein, and appropriate concentrations of estrogens or estrogen metabolites. The fluorescent product, resorufin (excitation 540 nm/emission 595 nm), was recorded using a SpectraMax M5 fluorescent microplate reader (Molecular Devices, Sunnyvale, CA).

2. Superoxide anion generation

It is generally thought that hydrogen peroxide is generated from the dismutation of superoxide anions, either spontaneously or as catalyzed by the family of superoxide

dismutase enzymes. Therefore, we next determined whether recombinant cytochrome b₅ and P450 reductase mediated redox cycling by catechol estrogen metabolites generates superoxide anions. Several methods for the quantification of superoxide exist; the most popular methodology measures spectrophotometrically the spontaneous reduction of acetylated cytochrome c by superoxide. However, using this technique we found that acetyl-cytochrome c reduction was dependent on the NADPH and enzyme concentrations, but not the estrogen substrate concentrations. Since both oxidoreductases have cytochrome c binding domains and reduction of cytochrome c is one of the functions of both proteins, it seems likely that the recombinant cytochrome b₅ and P450 reductases are able to directly reduce acetylated cytochrome c.

As a result, we explored other dyes for detecting superoxide generation, including adrenochrome (poor sensitivity) and DCFH (poor selectivity), before selecting the dihydroethidium fluorescence method. This method had greater sensitivity than adrenochrome reduction, though overall it was still poor. The basis of this assay is a small shift in the fluorescence emission spectrum resulting in a partially hydroxylated ethidium molecule (Zhao *et al.*, 2003; Robinson *et al.*, 2006). To maximize the sensitivity of this reaction, we measured fluorescence excitation and emission spectra of a reaction mix lacking enzyme before and after the addition of solid potassium superoxide. We used the wavelengths (excitation 510 nm/emission 595 nm) corresponding to the maximal difference in further experiments. Final reaction mixes in PBS contained 100 μ M NADPH, 5 μ g/ml recombinant protein, 80 μ M dihydroethidium (hydroethidine) and appropriate concentrations of either estrogens or estrogen metabolites in a total reaction

volume of 100 μ l. Fluorescence was measured using a SpectraMax M5 fluorescent microplate reader (Molecular Devices, Sunnyvale, CA).

1. Hydroxyl radical generation

The hydrogen peroxide generated by redox cycling is considered more toxic because it can form hydroxyl radicals with reduced transition metal ions. In particular, ferrous iron is known to generate hydroxyl radicals from hydrogen peroxide in a process known as the Fenton reaction. These hydroxyl radicals are widely known to be mutagenic; various labs have demonstrated DNA strand breakage and base oxidation resulting from hydroxyl radical generation (Lemon *et al.*, 2008).

Drs. Mishin and Thomas had previously developed an HPLC method for the detection of hydroxyl radicals (Mishin and Thomas, 2004). In this assay, any hydroxyl radicals produced by Fenton chemistry are scavenged by the non-fluorescent substrate terephthalate. Because of the molecular symmetry of terephthalate, the resulting hydroxylation reaction produces a single fluorescent product, 2-hydroxyterephthalate (2-OH-TPT, excitation 315 nm/emission 425 nm). While this assay is sensitive (lower limit of quantitation = 690 pmol/assay) and specific, it is also low-throughput, as each sample requires at least 20 minutes for analysis.

However, the product of the hydroxyl radical scavenging reaction, 2-OH-TPT, is highly fluorescent, while the substrate, terephthalate is not. This makes the assay ideal for adaptation to a somewhat less sensitive but more high-throughput microtiter platereader assay. Additionally, the platereader assays have the advantage of being ‘live’; therefore kinetic information can be obtained from repeated readings of the same sample, whereas the HPLC assay must be stopped at a single timepoint for analysis. We began by

measuring the fluorescence of 0-10 μM synthetic 2-hydroxyterephthalate in assay reaction mixes ($r^2 = 0.99$). We found a lower limit of detection (3 SD above background) of 3.5 pmol 2-OH-TPT/assay and a lower limit of quantitation (10 SD above background) of 10 pmol 2-OH-TPT/assay, making it 69 times more sensitive than the previous HPLC method.

To test whether we could then measure Fenton chemistry, we used this standard curve to determine the 2-OH-TPT generated by 0-100 μM hydrogen peroxide in the presence of 100 μM FeCl_3 and 110 μM EDTA. The resulting 2-OH-TPT concentrations linearly correlated with those of hydrogen peroxide ($r^2 = 0.87$) over a range of 0-10 μM . We measured a lower limit of quantitation of 40 pmol/assay for hydrogen peroxide, making terephthalate a somewhat less sensitive assay than Amplex Red. However, the slope measured (0.0039 μM 2-OH-TPT for every μM hydrogen peroxide used) indicates that this correlation is not 1:1. If every molecule of hydrogen peroxide is consumed in the reaction, as hydrogen peroxide is not the limiting reagent, then only 1 hydroxyl radical is detected of every 250 radicals produced.

This low efficiency is reasonable, as hydroxyl radicals are known to react rapidly and nonspecifically with whatever happens to be within a few molecular diameters. If this molecule happens to be water, then the hydroxyl radical will take a hydrogen atom, forming water and another hydroxyl radical in a propagation reaction. If the hydroxyl radical meets a larger molecule, such as terephthalate or a macromolecule, the radical will spontaneously bind to it in a scavenging reaction, forming an additional hydroxyl bond. Hydroxyl radicals can also be self-quenching; that is, two radicals react with each other forming hydrogen peroxide. Thus, most of the hydroxyl radicals formed under

conditions of Fenton chemistry react with cellular macromolecules and each other, instead of the terephthalate.

Previously, Mishin and Thomas rejected the cuvette terephthalate method because they found a fluorescence emission intensity of 1.2 times initial background after a 15 minute 37°C incubations required microsomal concentrations that exceeded that which produced a linear reaction rate with time. We made similar observations using our platereader method, however we were able to reduce the enzyme concentration by at least one log by extending the duration of the assay to one to four hours. Since we were able to analyze 96 samples in this timeframe, this still represents an 8- to 16-fold increase in time-savings when compared to the original HPLC assay. The use of more high-throughput microtiter formats (i.e., 384-well) could further decrease the time required per sample.

Because many organic solvents are also hydroxyl radical scavengers and the estrogens are sparingly soluble in buffer systems, stock solutions of estrogens (100x) dissolved in isopropanol were added to the microtiter plates and evaporated overnight before the addition of other reagents. Final reaction mixes in PBS contained 100 μ M NADPH, 5 μ g/ml recombinant protein or 50 μ g/ml cell lysate protein, 100 μ M FeCl₃, 110 μ M EDTA, 1 mM terephthalate and appropriate concentrations of either estrogens or estrogen metabolites in a total reaction volume of 100 μ l. Fluorescence was measured using a SpectraMax M5 fluorescent microplate reader (Molecular Devices, Sunnyvale, CA).

C. Enzyme assays for DNA damage

General estrogen stimulated genotoxicity was measured using DNA melting curves. Calf-thymus DNA (2000 ng/ml) in Tris-EDTA pH 7.4 buffer was exposed to estrogen stimulated ROS generation (10 µg/ml recombinant cytochrome P450 reductase, 200 µM NADPH, 200 µM FeCl₃, and increasing estrogen concentrations) at 37°C. After one hour, an equal volume of 2X PicoGreen and ROX solution was added for a final reaction volume of 100 µl. The 96-well polypropylene PCR plate was sealed and centrifuged for 5 minutes at 1000 x g to eliminate bubbles before it was placed in a Applied Biosystems 7300 real-time thermocycler (Life Technologies, Carlsbad, CA) for melting curve analysis.

DNA nucleotide base oxidation was quantified using a commercially available 8-hydroxy-2-deoxyguanosine EIA Kit (Cayman Chemical, Ann Arbor, MI). Reaction mixes in Tris-EDTA pH 7.4, containing 100 µM NADPH, 5 µg/ml recombinant cytochrome P450 reductase protein, 100 µM FeCl₃, 50 µg/ml calf-thymus DNA and 100 µM of either estrogens or estrogen metabolites in a total reaction volume of 100 µl, were allowed to produce ROS at 37°C. After one hour, 1 µl of 1 mg/ml nuclease P1 stock was added and allowed to digest at 37°C for 30 minutes. The pH of the solution was adjusted to 8.0 with 10 µl of 1M Tris buffer, followed by addition of 2 units of alkaline phosphatase and re-incubation at 37°C for another hour to free the nucleotide bases. Any further enzyme activity was eliminated by boiling the sample at 99°C for ten minute and then cooling on ice. These aliquots were then diluted 1:1 in EIA buffer from the kit to a final volume of 200 µl each. Each sample was plated into the kit in triplicate (50 µl/well) and processed according to the manufacturers instructions. Briefly, the samples were

incubated overnight at 4°C with acetylcholine esterase tracer and antibody to 8-hydroxy-2-deoxyguanosine. In the morning, all wells were triple-rinsed and developed using Ellman's reagent. The sample absorbances at 410 were quantified after 90 minutes using a SpectraMax M5 fluorescent microplate reader (Molecular Devices, Sunnyvale, CA) and compared to standard curve wells for analysis.

D. Cell culture

Several model systems are available to investigate the actions of mammary carcinogens on the breast. The effects on the entire breast may be determined *in vivo* using whole animals or explants from either humans or animals. While these studies can provide information about the global effects of a particular carcinogen on the breast, they cannot provide detailed mechanistic information for a particular cell type such as the mammary epithelium. By culturing these cells, examination of the molecular and cellular processes which result in breast tumor initiation is possible. Primary human mammary epithelial cells are commercially available from several vendors; however, each lot of these cells are specific to a single donor, making lot to lot genetic variability an added variable. Furthermore, these cells are expensive and difficult to culture. For these reasons, we instead used three commercially available immortalized cell lines to model breast epithelial cells at varying stages of the tumorigenic process. All three cell lines were grown on the plastic of tissue culture dishes in 5% CO₂ at 37°C and subcultured biweekly.

The first mammary epithelial cell line, MCF-10A, was isolated from a 36 year-old Caucasian female with fibrocystic disease. This cell line is a non-tumorigenic epithelial

line derived from adherent cells in a population cultured long term in serum free medium with a low calcium concentration. Although MCF-10A cells are non-tumorigenic, they are already transformed and can be used over more than several passages. However, because these cells are non-cancerous, they do require insulin, glucocorticoid, cholera enterotoxin, and epidermal growth factor signals for colony growth. Furthermore, growth of these cells can be stopped by contact inhibition and cessation of growth biochemical signals. MCF-10A cells are positive for epithelial sialomucins, normal mammary cytokeratins, and milkfat globule antigen. They exhibit three-dimensional growth in collagen, and display characteristics of luminal ductal cells. MCF-10A cells were cultured in mammary epithelial basal medium with the addition of insulin, hydrocortisone, 100 ng/ml cholera enterotoxin, recombinant epidermal growth factor, bovine pituitary extract, gentamycin, and amphotercin B (ATCC, 2010). The MCF-10A cell line will be used to model normal, non-cancerous breast epithelia.

The second mammary epithelial cell line, MCF-7, was isolated from the pleural effusion from the adenocarcinoma of a 69 year-old Caucasian female. This cell line is a tumorigenic epithelial line that has retained several characteristics of differentiated mammary epithelium including cytoplasmic estrogen receptors. These cells are negative for HER2/neu, and positive for estrogen receptor alpha (ER α). Growth can be modulated using anti-estrogen treatment. MCF-7 cells were cultured in modified Eagle's growth medium with the addition of 10% fetal bovine serum, penicillin, and streptomycin (ATCC, 2010). The MCF-7 cell line will be used to model low-grade, highly differentiated breast epithelial adenocarcinoma cells.

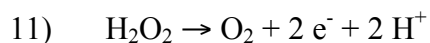
The final mammary epithelial cell line, MDA-MB-231, was isolated from the pleural effusion of the adenocarcinoma of a 51 year-old Caucasian female. This cell line is a tumorigenic breast epithelial line that is highly undifferentiated. These cells are positive for the HER2/neu receptor, but negative for estrogen receptor alpha (ER α). Growth can be modulated using trastuzumab. MDA-MB-231 cells were cultured in modified Eagle's growth medium with the addition of 10% fetal bovine serum, penicillin, and streptomycin (ATCC, 2010). The MDA-MB-231 cell line will be used to model high-grade, poorly differentiated breast epithelial adenocarcinoma cells.

MCF-7, MDA-MB-231, and MCF-10A cell lines were obtained from the American Type Culture Collection. MCF-7 and MDA-MB-231 cells were maintained in Dubecco's Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Mediatech, Manassas, VA), 100 units/ml penicillin, 100 μ g/ml streptomycin (both from Gibco, Grand Island, NY). MCF-10A cells were maintained in mammary epithelial basal media supplemented with insulin (CC-4021G 0.5 ml), hydrocortisone (CC-4031G 0.5 ml), recombinant human epithelial growth factor (CC-4017G 0.5 ml), bovine pituitary extract (CC-4009G 2 ml), gentamicin (CC-4081G 0.5 ml), and amphotericin (MEGM BulletKit, Lonza, Walkersville, MD). All cell lines were incubated at 37°C in 5% CO₂ in a humidified incubator. To prepare lysates, cells were resuspended in phosphate-buffered saline (10⁶ cells/ml) and disrupted on ice using a sonic membrane dismembrator (Artek Systems Inc., Farmingdale, NY). Protein concentrations were quantified using the DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

E. Electrochemical determination of reactive oxygen species generation by intact cells

As hydrogen peroxide is a non-polar small molecule, it can be released by redox cycling cells. We quantified this release from cultured mammary epithelial cells using platinum amperometric microsensors as previously described with minor alterations (Twig *et al.*, 2001). When placed near cells, these microsensors can electrochemically measure the concentration at a point in space of cell permeable molecules moving along a gradient. Our collaborators at the Marine Biological Laboratory in Woods Hole, Massachusetts are experts in the development and manufacture of these microsensors. Indeed, their laboratory regularly uses these electrodes to measure such disparate physiological phenomena as proton or cation gradients across membranes and oxygen or glucose consumption by cells. In all cases, these electrodes harness the chemical oxidation and reduction reactions of these compounds to detect the concentration of specific molecules of interest at the electrode tip.

The measurement of hydrogen peroxide generation is the reverse of that of oxygen consumption. As hydrogen peroxide is oxidized, it forms molecular oxygen in the following half-reaction which occurs at a potential of 0.6 V using a AgCl standard electrode.



Thus when a potential of 0.6 V is applied to a platinum electrode, any molecule of hydrogen peroxide at the electrode tip will be oxidized to molecular oxygen, releasing two electrons which can be detected as increase in current. This potential is chosen because it is specific for this particular half-reaction, thereby making the electrode highly selective for hydrogen peroxide.

These microsensors are able to determine the concentration of hydrogen peroxide with exquisite spatial and temporal sensitivity (submicromolar, it varies somewhat with each electrode). By keeping the position of the electrode in the dish constant, they can be used to determine small changes in the gradient of hydrogen peroxide release by cells in response to various treatments added to the culture buffer.

However, this strength also provides the limitations of this assay. These electrodes are highly sensitive to variables that cannot be controlled except within a single assay. Changes in the distance between the microsensor and the cells, as well as the number of responding cells in local area of the electrode make an enormous difference in the detected response. These variables are difficult to control because the microsensors themselves are very fragile and cannot be allowed to touch anything solid. Each is hand-manufactured and displays unique noise characteristics which are evident in the tracings. Selection of a new electrode can take days. Furthermore, the electrochemical properties of the microsensors themselves alter slowly as the electrode ages. This is primarily due to electrode ‘fouling’ with protein, dust, and cellular debris, but also due to minute changes in the surface area of the electrode as pits and imperfections in the platinum wire increase in size. We did our best to insure consistent results, but measurements of this kind are as much of an art as a science.

Just before data collection, the culture medium from 60-80% confluent cell layers was replaced with Krebs-Ringer bicarbonate buffer (4 mM glucose, 140 mM NaCl, 30 mM HEPES, 4.6 mM KCl, 1 mM MgSO₄, 0.15 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 5 mM NaHCO₃, 2 mM CaCl₂ and 0.05% BSA, pH 7.4, osmolarity 284 mosM) (Heart and Smith, 2007). After measurement of basal hydrogen peroxide release by the cells,

increasing volumes of an estrogen or estrogen metabolite (10 mM in isopropanol) were added to the reaction buffer. All experiments were performed at room temperature.

RESULTS

A. Role of cytochrome b₅ reductase in mediating reactive oxygen species production by catechol metabolites of endogenous estrogens

In initial studies, we characterized redox cycling of catechol metabolites of estrogens by recombinant cytochrome b₅ reductase. We found that this enzyme was able to generate hydrogen peroxide with four estrogen metabolites (2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2), 4-hydroxyestrone (4OHE1), and 2-hydroxyestriol (2OHE3). Hydrogen peroxide production was concentration- and time-dependent and required NADPH (Fig. 1 and data not shown). Using Michaelis-Menten analysis, the apparent K_M's for the reaction ranged from 1.6 and 4.6 μM and the V_{max}'s were generally similar (5.5 and 6.7 pmol hydrogen peroxide/min/U cytochrome b₅ reductase (Fig. 2 and Table 1). To test whether recombinant cytochrome b₅ reductase is actually a required mediator of ROS production, hydrogen peroxide generation was measured in the presence and absence of catalase and a polyclonal antibody which our lab has previously shown to inhibit cytochrome P450 reductase activity. Although the antigen used to raise the antibody was from the active site of cytochrome P450 reductase, similarities in the active sites of both enzymes, specifically the presence of a flavin cofactor, led us to believe that this polyclonal antibody may react with and inhibit the activity of cytochrome b₅ reductase as well. Both catalase and the polyclonal antibody prevented hydrogen peroxide formation (Fig. 3).

It is generally thought that ROS generation during redox cycling is a result of the univalent reduction of oxygen producing superoxide anion. In this reaction, one molecule of oxygen is reduced by two molecules of NADPH generating two molecules of

superoxide anion. We found that redox cycling of the catechol estrogens readily produced superoxide anion (Figs. 4 and 5). In these experiments 2OHE2 produced more superoxide anion than any other catechol estrogen (rank order: 2OHE2 >> 4OHE2 = 2OHE3 > 4OHE1). Superoxide anion production by the catechol estrogen metabolites was inhibited by superoxide dismutase (Fig.6).

In the presence of transition metals, especially iron, hydrogen peroxide can undergo Fenton chemistry producing highly mutagenic hydroxyl radicals. We next determined if catechol estrogen redox cycling can lead to the production of hydroxyl radicals. Each of the catechol estrogens was found to generate hydroxyl radicals (Figs. 7 and 8). As with the superoxide anion production, 2OHE2 was the most efficient generator of hydroxyl radicals followed by 4OHE2, 4OHE1, and 2OHE3. Hydroxyl radical production by all four catechol estrogens was NADPH-dependent and prevented by diphenyleneiodonium (DPI), an inhibitor of flavoproteins, DMSO, a hydroxyl radical scavenger, and catalase (Fig 9).

B. Role of cytochrome P450 reductase in mediating reactive oxygen species generation by catechol metabolites of endogenous estrogens

We next determined if the flavoenzyme cytochrome P450 reductase can also mediate catechol estrogen redox cycling. We found that 2OHE2 and 4OHE2, but not the parent estradiol (E2) or the methoxy-metabolite 2-methoxyestradiol (2MeOE2), stimulated hydrogen peroxide production (Fig. 10). Both E2 and 2MeOE2 have the chemical resonance which stabilizes the semiquinone radical produced by a one electron reduction, but lack the ortho-positioned hydroxyl groups required for semiquinone redox

cycling. The catechol estrogens tested including 2OHE2, 4OHE2, 4-hydroxyestrone (4OHE1), and 2OHE3 generated similar quantities of hydrogen peroxide. The apparent K_M 's for hydrogen peroxide production by cytochrome P450 reductase ranged from 43 and 77 μM and V_{max} 's from 3.8 and 4.3 pmol hydrogen peroxide/min/U P450 reductase activity (Fig. 11 and Table 2). Similar experiments with E2, estrone (E1), estriol (E3), and 2MeOE2 failed to stimulate hydrogen peroxide generation (data not shown). Both catalase and a polyclonal antibody to cytochrome P450 reductase prevented catechol estrogen redox cycling (Fig. 12).

Under conditions favorable to Fenton chemistry, recombinant cytochrome P450 reductase also mediates formation of hydroxyl radicals during redox cycling. This occurred in the presence of iron (Fig 13). As with hydrogen peroxide, the catechol estradiol metabolites 2OHE2 and 4OHE2, but not E2 or 2MeOE2 stimulated hydroxyl radical production. The apparent K_M 's for hydroxyl radical production by redox cycling of 2OHE2, 4OHE2, 4OHE1 and 2OHE3 ranged from 25 and 177 μM and the V_{max} 's ranged from 29 to 189 pmol 2-hydroxyterephthalate/min/U POR activity (Fig. 14). E1, E2, E3, and 2MeOE2 did not stimulate hydroxyl radical production (data not shown). As with the cytochrome b_5 reductase, 2OHE2 \ll 4OHE2 = 2OHE3 < 4OHE1 at generating hydroxyl radicals during redox cycling. Hydroxyl radical formation was dependent on iron (not shown) and inhibited by DPI, DMSO, and catalase (Fig. 15).

All four catechol estrogens also generated superoxide anion. The K_M 's for redox cycling reaction to generate hydroxyl radicals for the four catechol estrogens ranged from 14 and 1500 μM , while maximal rates varied between 0.077 and 3.2 AFU/min/U POR

activity (Fig. 16). Superoxide anion production was inhibited by superoxide dismutase (Fig. 17).

C. Redox cycling by catechol metabolites of endogenous estrogens damages DNA

It is well established that ROS can damage DNA, including nucleotide base oxidation, single- and double-strand DNA breaks, adduct formation, and thymidine bridge formation. To date however, these structural changes must be identified individually; technique to identify broad-spectrum genotoxicity are not readily available.

We developed an *in vitro* technique for quantifying different forms of DNA damage based on analysis of the melting curve. In this assay, calf-thymus DNA is incubated with a DNA damaging agent on a 96-well PCR plate. After the addition of a double-stranded DNA specific dye, the plate is sealed and placed in a RT-PCR thermocycler. Depending on the type of genotoxicant, the melting curve will be altered. This can include changes in initial fluorescence, initial melting temperature, and melting rate (Fig. 18).

We next applied this technique to characterize the effects of catechol estrogen-stimulated redox cycling and ROS formation on DNA damage. In a redox cycling system containing NADPH and recombinant cytochrome P450 reductase, increasing concentrations of the catechol estrogen metabolites 2OHE2, 4OHE2, 4OHE1, and 2OHE3 readily damaged DNA causing single- and double-strand breaks (Fig. 19). Genotoxicity was not observed with the parent estrogens (E1, E2, or E3), or 2MeOE2, none of which redox cycle.

We next determined if catechol estrogens induced ROS generation can oxidize DNA. For these studies we measured the formation of 8-hydroxy-2-deoxyguanosine, a biomarker of DNA damage. Under conditions favorable to both ROS generation and Fenton chemistry (i.e., hydroxyl radical formation) we quantified 8-hydroxy-2-deoxyguanosine by enzyme immunoassay (Fig. 20). Treatment with all four catechol estrogens increased formation of 8-hydroxy-2-deoxyguanosine; increases in DNA base oxidation by redox cycling with 2OHE2, 4OHE2, and 4OHE1 were statistically significant. As with the melting curve assay, neither the parent estrogens, nor the methoxy metabolite generated significant quantities of 8-hydroxy-2-deoxyguanosine. Catechol estrogen metabolite-stimulated 8-hydroxy-2-deoxyguanosine formation was cytochrome P450 reductase-dependent, indicating that nucleotide oxidation is the result of redox cycling and ROS production.

D. Redox cycling of catechol metabolites of endogenous estrogens in breast epithelial cell lines

We also characterized redox cycling by estradiol and several of its metabolites using lysates from MCF-7, MDA-MB-231, and MCF-10A breast epithelial cells by quantifying hydrogen peroxide production in enzyme assays. Neither the parent estrogen, estradiol (E2), nor the methoxy-estrogen metabolite, 2MeOE2 was able to stimulate hydrogen peroxide production; however the catechol estrogens 2OHE2 and 4OHE2 readily generated hydrogen peroxide. (Fig. 21). The rates of hydrogen peroxide formation were determined for E2, 2OHE2, 4OHE2, and 2MeOE2. For comparison, we also analyzed E1, 4OHE1, E3, and 2OHE3. Neither the parent estrogens E1, E2, and E3,

nor the methoxy-metabolite 2MeOE2 produced hydrogen peroxide; however the four catechol metabolites 2OHE2, 4OHE2, 4OHE1 and 2OHE3 generated hydrogen peroxide in a time- and concentration-dependent manner in all three breast epithelial cell lines with K_M 's ranging from 3 to 10 μ M (Fig. 22 and Table 3). Redox cycling in cell lysates by the catechol estrogen metabolites was inhibited by catalase as well as DPI. These latter findings indicate that redox cycling in the breast epithelial cells was mediated by a flavoenzyme (Fig. 23).

We next determined if hydroxyl radicals could be formed by redox cycling of the catechol estrogens in the breast epithelial cell lysates. As observed with hydrogen peroxide production, the catechol estradiols stimulated hydroxyl radical formation that was both time- and concentration-dependent (Fig. 24). The K_M 's for hydroxyl radical formation ranged from 10 to 100 μ M (Fig. 25). As observed with hydrogen peroxide generation, neither the parent estrogens (E1, E2, and E3), nor the methoxy-estradiol metabolite (2MeOE2) generated hydroxyl radicals (Fig. 24 and not shown). Accumulation of hydroxyl radicals during catechol estrogen redox cycling was inhibited by DPI, catalase, and DMSO (Fig. 26).

Hydrogen peroxide is membrane permeable. If it is formed inside cells during redox cycling, it can be released by the breast epithelial cells, damaging surrounding cells. We next used a hydrogen peroxide microsensor to quantify hydrogen peroxide release from intact breast epithelial cells. These microsensors, which are sensitive to hydrogen peroxide in the range of 1-30 μ M (Fig. 27), were readily able to detect the release of this ROS by breast epithelial cells. We found that the four redox active catechol estrogens (2OHE2, 4OHE2, 4OHE1, and 2OHE3) stimulated hydrogen peroxide

release by intact MCF-10A cells. This response was concentration-dependent. In contrast, neither the parent estrogens (E1, E2, or E3) nor the methoxy-metabolite (2MeOE2), generated hydrogen peroxide at concentrations up to 30 μ M (Figs. 28 and 29). Similar results were found using MCF-7 and MDA-MB-231 cells (Fig. 29 and not shown).

To compare hydrogen peroxide release by each catechol estrogen metabolite, the measured electric potentials were used to define a Michaelis-Menten relationship and the data were reported as a percentage of V_{max} (Fig. 30 and not shown). Using this analysis, we determined that hydrogen peroxide release by cells was indistinguishable with respect to both the identity of the catechol estrogen and the cell line. To characterize the relationship between the enzyme mediating redox cycling in the cultured cells and the catechol estrogen metabolites, MDA-MB-231 cells were treated with DPI. Pretreatment of the cells with DPI inhibited 2OHE3-stimulated hydrogen peroxide production. DPI also reduced 2OHE3-stimulated hydrogen peroxide generation. Addition of extracellular catalase further reduced 2OHE3-stimulated release of hydrogen peroxide (Fig. 31). Taken together these data indicate that flavoenzymes play an important role in the redox cycling of catechol estrogen metabolites.

DISCUSSION

Previous work has demonstrated that endogenous estrogens are metabolized into 2- and 4-hydroxyestrogen catechols by cytochromes P450 1A1 and 1B1, respectively. The catechols then undergo univalent oxidation by redox active enzymes to form *o*-semiquinones which are capable of reacting with nucleotide bases, forming DNA adducts. The adducts formed from 2-hydroxyestrogens are stable; while adducts formed by 4-hydroxyestrogens are unstable and react further. DNA adducts formed from these catechol estrogens can result in depurination, potentially resulting in mutagenesis. Based on these data, it has become generally accepted that 4-hydroxyestrogens are toxic, while the 2-hydroxyestrogen metabolites are comparatively benign (Cavalieri *et al.*, 2000; Li *et al.*, 2004; Cavalieri *et al.*, 2006). However, if both 2- and 4-hydroxyestrogen semiquinones are capable of redox cycling, resulting in the generation of mutagenic ROS, then this assumption may not be true. ROS, particularly hydroxyl radicals, are known to be potent carcinogens, acting via both genotoxic (mutagenic) and non-genotoxic modes of action (Totter, 1980; Ames, 1989).

A. Role of cytochrome b₅ reductase in mediating reactive oxygen species production by catechol metabolites of endogenous estrogens

NADH-cytochrome b₅ reductase is a single-subunit, FAD containing enzyme that forms an integral part of the microsomal electron-transport chain. This oxidoreductase catalyzes the two-electron oxidation of NAD(P)H, passing the electrons to neighboring heme-containing cytochrome proteins in two subsequent univalent reduction reactions (Strittmatter, 1965). The most common, but by no means only, cytochrome b₅ reductase

electron acceptor is cytochrome b₅. During this reaction, an intermediate flavin semiquinone anion-NAD cation is formed in the active site of the enzyme (Iyanagi, 1977). This semi-oxidized flavin intermediate is highly reactive with one-electron oxidation and reduction potentials of 88 and -147 mV, respectively. In an aerobic environment, the FAD-semiquinone readily reduces molecular oxygen to superoxide anion with a turnover number of 2.1 s⁻¹. This ‘electron leakage’ restores the FAD cofactor in the enzyme to its original oxidized state (Iyanagi *et al.*, 1984). However, ‘electron leakage’ is not the only mechanism of ROS generation by cytochrome b₅ reductase. The addition of a redox cycling substrate (e.g., mitomycin C) substantially increases molecular oxygen reduction by this enzyme (Maeda *et al.*, 1999).

Due to its proximity to the estrogen metabolizing enzymes cytochromes P450 1A1 and 1B1, its activity as a univalent oxidoreductase, and its demonstrated capability to mediate redox cycling by heterocyclic compounds, cytochrome b₅ reductase seemed a probable mediator of catechol estrogen redox cycling. Accordingly, we measured redox cycling of four catechol estrogen metabolites by cytochrome b₅ reductase. As expected, these catechol estrogens were capable of redox cycling, generating hydrogen peroxide. The rates of hydrogen peroxide production were similar for all four endogenous catechol estrogens (2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2), 4-hydroxyestrone (4OHE1), and 2-hydroxyestriol (2OHE3) and were dependent on NAD(P)H reducing equivalents. This activity was suppressed by DPI, an inhibitor of flavoproteins. This suggests that catechol estrogen share a mechanism of ROS production. Generation of hydrogen peroxide was found to be linear with respect to time and protein concentration. While NADH is the preferred substrate, cytochrome b₅ reductase can also utilize

NADPH. Therefore, it is not surprising that at saturating concentrations of NADH or NADPH, no differences in the rate of ROS production were apparent.

The rate of hydrogen peroxide generation is closely linked to the concentration of catechol estrogen metabolites. Since the spontaneous reduction of molecular oxygen by the semiquinone estrogen metabolite occurs rapidly, the enzymatic univalent oxidation of the catechol (or reduction of the quinone) is the rate-limiting step during redox cycling. By varying the concentration of each catechol estrogen metabolite, we were able to determine several kinetic parameters for univalent catechol estrogen oxidation (or quinone reduction) by cytochrome b₅ reductase. Generation of hydrogen peroxide by catechol estrogen metabolites was saturable and reversible, with apparent K_M 's between 1.6 and 4.6 μM and V_{max} 's between 5.5 and 6.7 pmol $\text{H}_2\text{O}_2/\text{min}/\text{U}$ cytochrome b₅ reductase. As the concentration of catalytic sites was constant, the measured V_{max} is proportionate to the turnover number for catechol estrogen oxidation. These values are small, indicating poor catalytic efficiency at high substrate concentrations. Thus, this activity of cytochrome b₅ reductase is not catalytically efficient and therefore highly dependent on the catechol estrogen metabolite concentration. The observed K_M values represent the concentrations of substrate required to achieve a half-maximal reaction rate. These values are also small, indicating that cytochrome b₅ reductase has a high binding affinity for these catechol estrogen metabolites. Taken together, these data indicate that cytochrome b₅ reductase is ideally suited to mediate redox cycling by low doses of catechol estrogen metabolites.

Since this reaction meets all the assumptions necessary for Michaelis-Menten analysis, together, K_M and V_{max} can be used to determine the concentration of catechol

estrogens required to generate statistically significant amounts of hydrogen peroxide (v_α). Since V_{\max} is by definition, the reaction rate quantified at saturating substrate concentrations, then:

$$11) \quad v_\alpha = \alpha \cdot V_{\max}$$

$$12) \quad v_\alpha = V_{\max} \cdot [S_\alpha] / (K_M + [S_\alpha]) \quad \text{Michaelis-Menten equation}$$

$$13) \quad [S_\alpha] = v_\alpha \cdot K_M / (V_{\max} - v_\alpha)$$

where α is an acceptable type-1 error. Assuming a value for α of 0.05 (a single-tailed 95% confidence interval), as little as 84-240 nM of these endogenous catechol estrogen metabolites are required for cytochrome b_5 reductase to produce statistically significant amounts of hydrogen peroxide.

Catechol estrogen metabolite-stimulated hydrogen peroxide generation was ameliorated in a two stage process by the antioxidant enzyme, catalase. Catalase is a potent divalent dismutase which harnesses the oxidation of one molecule of hydrogen peroxide to molecular oxygen to reduce another hydrogen peroxide molecule to water. To prove that the hydrogen peroxide generation was mediated by cytochrome b_5 reductase, we used polyclonal antibodies to cytochrome P450 reductase. Since both oxidoreductases require flavin-cofactors for mediation of redox cycling, it is not surprising that antibodies to this site would inhibit both enzymes. The addition of catalase and anti-cytochrome P450 reductase to the redox cycling reaction effectively prevented accumulation of hydrogen peroxide.

It is widely accepted that hydrogen peroxide is produced from the enzymatic and non-enzymatic dismutation of superoxide radical anion, producing one molecule of hydrogen peroxide and one molecule of molecular oxygen for every two molecules of

superoxide. Accordingly, we quantified the ability of cytochrome b₅ reductase mediated redox cycling of the catechol estrogen metabolites to generate superoxide anion radicals. All four catechol estrogen metabolites readily produced superoxide anion, however 2OHE2 generated substantially more of this radical than the other three catechols. This would imply that 2OHE2 is a better redox cycling agent, and therefore more toxic by this mechanism, than the other catechol metabolites. Regardless, in all cases the production of superoxide anion was found to be linear with respect to time and protein concentration, dependent on NADPH reducing equivalents, and suppressed by DPI. Accumulation of superoxide anion radical was effectively prevented by the addition of the antioxidant enzyme superoxide dismutase (SOD).

While superoxide anion dismutation occurs spontaneously, it is relatively slow and therefore, biological systems have evolved to produce the protein SOD to enhance the rate of this reaction. Under redox cycling conditions, any molecular oxygen formed by superoxide anion dismutation is then re-reduced to form another molecule of superoxide anion. As long as the concentration of reducing equivalents remains saturated (as with an NAD(P)H regenerating system), all the superoxide anion generated by redox cycling will eventually form hydrogen peroxide.

The rate of dismutation of superoxide anion is diffusion limited; thus, hydrogen peroxide formation is only limited by the frequency of the collision between molecules of SOD and superoxide radical anion. SOD also has a low binding efficiency (large K_M) (Forman and Fridovich, 1973). As interaction between superoxide and organic macromolecules are 'spin forbidden' and dismutation by SOD is unlikely, at low (physiological) concentrations any superoxide anion formed react with other radical

species like nitric oxide, forming peroxynitrite (Koppenol *et al.*, 1992). Peroxynitrite can react with DNA and other cellular macromolecules (Beckman *et al.*, 1990; Koppenol *et al.*, 1992), thereby increasing superoxide anion toxicity. However, at high concentrations of superoxide anion, such as those produced during redox cycling, SOD dismutation is so rapid that virtually all superoxide is converted to hydrogen peroxide. Reactions between hydrogen peroxide and cellular macromolecules are not 'spin-forbidden' and known to be carcinogenic.

Additionally, hydrogen peroxide can spontaneously form highly mutagenic hydroxyl radicals in the presence of transition metals. When iron was added to the redox cycling assay mixture, all four catechol estrogens (2OHE2, 4OHE2, 4OHE1, and 2OHE3) were capable of producing hydroxyl radicals. As with superoxide anion generation, hydroxyl radical production was NADPH dependent and inhibited by DPI. Hydroxyl radical generation was ameliorated by addition of catalase and the hydroxyl radical scavenger, DMSO.

At high concentrations, 2OHE2 generates more hydroxyl radicals than the other three catechol estrogen metabolites, producing 8-14 times more ROS at the predicted V_{max} . Since the kinetic rate of hydroxyl radical synthesis at enzyme saturation (V_{max}) is a direct function of catalytic turnover of substrate to product in the active site of the enzyme, the increased hydroxyl radical formation can only be accounted for by the increased turnover efficiency of 2OHE2 in the active site. Therefore, the chemical nature of the hydroxyl groups at carbons 2 and 17 must interact with the active site of cytochrome b_5 reductase to destabilize the 2OHE2 catechol. Regardless, as the most efficient generator of mutagenic hydroxyl radicals with cytochrome b_5 reductase, by this

mechanism of carcinogenesis 2OHE2 is a more potent mutagen than the other catechol estrogens tested.

B. Role of cytochrome P450 reductase in mediating reactive oxygen species generation by catechol metabolites of endogenous estrogens

NADPH-cytochrome P450 reductase is a single-subunit, FAD and FMN containing enzyme which is also an integral part of the microsomal electron-transport chain. This oxidoreductase catalyzes the two-electron oxidation of NADPH, passing the electrons to neighboring cytochrome P450 proteins either directly or via cytochrome b_5 (Lu *et al.*, 1969). Under anaerobic conditions, the active site of this reductase, the FAD cofactor, oxidizes NAD(P)H and passes the electrons one at a time to the FMN cofactor which then reduces the substrate protein in the electron transport chain (Guengerich, 2005). Under aerobic conditions, however, the electron can ‘leak’ to reduce molecular oxygen instead, forming superoxide anion. This ‘electron leakage’ is analogous to that of cytochrome b_5 reductase previously discussed. Cytochrome P450 reductase has also been well characterized as a mediator of redox cycling. The addition of a redox cycling substrates (e.g., diquat) substantially increases molecular oxygen reduction and NADPH consumption by this enzyme (Gage, 1968), much like the increases we observed in cytochrome b_5 reductase mediated catechol estrogen redox cycling.

As with cytochrome b_5 reductase, the proximity of cytochrome P450 reductase to the estrogen metabolizing enzymes cytochromes P450 1A1 and 1B1, its activity as a univalent oxidoreductase, and its demonstrated capability to mediate redox cycling by heterocyclic compounds, make it a likely mediator of catechol estrogen redox cycling. To

investigate this possibility, we quantified cytochrome P450 reductase mediated redox cycling by four catechol estrogen metabolites (2OHE2, 4OHE2, 4OHE1, and 2OHE3), three parent estrogens (E1, E2, and E3), and one methoxy-metabolite (2MeOE2). As expected, these catechol estrogens readily redox cycle, while similar concentrations of the parent estrogens (E1, E2, or E3) or the methoxy-metabolite (2MeOE2) failed to produce ROS. For each endogenous catechol estrogen, the cytochrome P450 reductase mediated hydrogen peroxide accumulation was reduced by the addition of catalase or polyclonal anti-cytochrome P450 reductase antibody to the assay mixture.

Neither the parent estrogens nor the methoxy-metabolite is capable of producing ROS, because they lack the basic catechol structure required. While all eight compounds tested had the high degree of conjugation needed to stabilize the semiquinone radical intermediate, only the catechols had adjacent hydroxyl groups. In addition to its role as another potential conjugated pi-bond, the extraneous phenol bond probably undergoes acid-base chemistry within the active site of cytochrome P450 reductase, briefly forming an electrophilic carbonyl group. This stabilizes the deprotonation of the hydroxyl group, forming the semiquinone radical.

Kinetic analysis of catechol estrogen redox cycling mediated by cytochrome P450 reductase revealed that while the maximal rate of hydrogen peroxide production was similar to that mediated by cytochrome b₅ reductase, 15- to 40-fold more catechol estrogen was required to achieve a half-maximal rate of reaction. All other things being equal, this means cytochrome P450 reductase is less likely than cytochrome b₅ reductase to mediate redox cycling by catechol estrogen metabolites at physiological concentrations.

Conditional to this argument, however, is the assumption that both enzymes and the catechol estrogen substrates freely diffuse throughout the lipid bilayer of the endoplasmic reticulum. As these proteins are part of the same microsomal electron transport chain, this is not necessarily the case. Both cytochrome P450 and cytochrome b₅ reductases interact catalytically with a number of heme-containing proteins, including cytochromes b₅, P450, and most significantly, the estrogen synthesis enzyme aromatase. The strength of these non-covalent interactions will necessarily alter the diffusion patterns of these proteins (and the estrogens which interact with these enzymes) from simple Brownian motion, to a model which more resembles discrete protein ‘islands’ in a sea of lipids. Such ‘islands’ would necessarily be dynamic, as individual proteins are free to join or leave the loose association at any time. The ‘island’ composition may vary, and the enzymes participating in estrogen synthesis (aromatase), metabolism to catechols (cytochromes P450 1A1 and 1B1), and redox cycling mediation (cytochrome b₅ and cytochrome P450 reductases) may be in close proximity to each other. Therefore, it is impossible to determine from these data whether either, or both, of these oxidoreductases are the more likely mediators of redox cycling by physiologically relevant catechol estrogen metabolite concentrations.

Irrespective of which enzyme mediates redox cycling by the endogenous catechol estrogen metabolites, in the presence of iron, the hydrogen peroxide produced readily forms mutagenic hydroxyl radicals. Accordingly, we tested whether hydroxyl radicals could be generated by cytochrome P450 reductase mediated redox cycling by the eight endogenous estrogens (E2, 2OHE2, 4OHE2, 2MeOE2, E1, 4OHE1, E3, and 2OHE3). As with hydrogen peroxide production, neither the parent estrogens (E1, E2, and E3) nor the

methoxy-metabolite (2MeOE2) produced hydroxyl radicals. This is consistent with the lack of redox cycling capability by these compounds. The endogenous catechol estrogen metabolites, on the other hand, readily generated hydroxyl radicals under conditions favorable to Fenton chemistry. As with cytochrome b₅ reductase, hydroxyl radical generation was NADPH dependent, inhibited by DPI, and ameliorated by addition of catalase or the hydroxyl radical scavenger, DMSO.

While all four catechol estrogens produce hydroxyl radicals, at saturating concentrations 2OHE2 produced more of this ROS than the other metabolites. However, this three- to seven-fold difference was half the size of that resulting from cytochrome b₅ reductase mediated redox cycling. As with cytochrome b₅ reductase, the increased hydroxyl radical formation by the higher 2OHE2 concentrations must be due to its increased turnover efficiency in the active site. (Measurements of superoxide anion radical generation support this.) Therefore, the hydroxyl groups at carbons 2 and 17 must also destabilize the 2OHE2 catechol in the active site of cytochrome P450 reductase and assist in the univalent transfer of the electron from the flavin-semiquinone to 2OHE2 in the enzyme's active site. Again assuming a ROS mediated mechanism of mutagenesis, 2OHE2 is potentially a more potent mutagen than the other catechol estrogens tested, as its cytochrome P450 reductase mediated redox cycling can generate more hydroxyl radicals.

C. Redox cycling by endogenous catechol estrogen metabolites damages DNA

Hydroxyl radical generation has been previously characterized as highly mutagenic (Totter, 1980). These radicals can oxidize DNA, which if not repaired, results

in C-G → A-T transversions, strand breakage, mispairing during replication, and single amino acid substitutions in the gene products. If these gene mutations confer an evolutionary advantage to the mutant cell over its neighbors, they contribute to the proliferation and survival of the clone. Several mutations of this sort together make up the initiation phase of carcinogenesis.

To investigate whether redox cycling by endogenous estrogens could cause genotoxicity, we examined the melting curves resulting from exposure of DNA to these reactions under conditions favorable for Fenton chemistry. The DNA melting is directly proportional to the amount of hydrogen bonding between the base pairs. As DNA is damaged, the strength of the bonding changes, altering DNA melting. We quantified cytochrome P450 reductase mediated genotoxicity resulting from redox cycling by the four catechol estrogen metabolites (2OHE2, 4OHE2, 4OHE1, and 2OHE3), the three parent estrogens (E1, E2, and E3), and the methoxy-metabolite (2MeOE2). Alterations in the melting curves were observed when DNA was co incubated with the redox cycling catechol estrogens; however, neither the parent estrogens (E1, E2, or E3) nor the methoxy-metabolite (2MeOE2) altered DNA melting. This is as expected, since the catechol metabolites are the only estrogens tested that are capable of producing ROS. The degree of melting curve perturbation observed was directly proportional to the concentration of catechol estrogen metabolite, indicating that genotoxicity is a function of the amount of ROS produced.

In addition to quantifying the amount of genotoxicity, the DNA melting curves also identify the type of damage incurred. This assay can differentiate between three of the most common types of DNA damage: strand breakage, intercalation, and adduct

formation. If the major source of DNA damage resulting from the synthesis of endogenous estrogen semiquinone radicals is from direct adduct formation, as has been previously reported (Cavalieri *et al.*, 2000), then the DNA melting curves produced by treatment with catechol estrogens should reflect increases in the initial melting rate and a decreased temperature of initial melting.

On the other hand, if hydroxyl radical generation through Fenton chemistry is the ultimate genotoxicant, then strand breakage from radical hydroxylation of the deoxyribose backbone is likely to be the primary mode of DNA damage. With increasing frequency, these single-strand breaks begin to occur in close proximity to each other also on opposing DNA strands, forming double-strand breaks.

We found that the DNA melting curves resulting from redox cycling by the four catechol estrogen metabolites (2OHE2, 4OHE2, 4OHE1, and 2OHE3) displayed clear evidence of strand breakage, including an increased rate of initial melting and a decrease in initial melting temperature. The dye used to monitor DNA melting is a dsDNA specific intercalating agent whose fluorescence is enhanced by non-covalent interaction between the dye and the terminal ends of dsDNA. Therefore, single-strand breaks that perturb the dsDNA structure, and therefore dye intercalation, resulting in an initial loss of fluorescence. However, if the single-strand breaks formed are in close proximity to more breaks on the other DNA strand, a double-stranded break is formed. This produces two new dsDNA termini, enhancing initial fluorescence, but also increasing the initial melting rate and decreasing the initial melting temperature, when compared to controls. The DNA melting curves of cytochrome P450 reductase mediated redox cycling by 2OHE2, 4OHE2, and 2OHE3 indicated formation of double-strand breaks. This was especially

apparent in DNA treated with 2OHE2; however, double-strand breakage was concentration dependent in all three cases. Double-strand breaks are considered particularly mutagenic because neither strand can be used as a sequence template during DNA repair. Therefore, by an ROS-mediated mechanism, 2OHE2 is probably the most mutagenic endogenous catechol estrogen metabolite.

Hydroxyl radicals can also directly react with DNA nucleotide bases. Base oxidation was quantified using 8-hydroxy-7,8-dihydro-2-deoxyguanosine (8OHdG), a rearrangement product of 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxo-dG), as a biomarker. Guanosine oxidation increases C-G \rightarrow A-T transversions, causing replication mispairing and gene mutations (Greenberg, 2004). We quantified cytochrome P450 reductase mediated base oxidation resulting from redox cycling by the four catechol estrogen metabolites (2OHE2, 4OHE2, 4OHE1, and 2OHE3), the three parent estrogens (E1, E2, and E3), and the methoxy-metabolite (2MeOE2) in the presence of calf-thymus DNA. Under conditions favorable for Fenton chemistry, all eight estrogens increased 8OHdG formation, no significant DNA base oxidation was detected with either the parent estrogens, or the methoxy-metabolite. In contrast, cytochrome P450 reductase mediated redox cycling and ROS production by the four catechol estrogen metabolites oxidized DNA nucleotide bases, producing 8OHdG. This oxidation was statistically significant in the case of three of the endogenous catechol metabolites: 2OHE2, 4OHE2, and 4OHE1; however, the substantial ROS produced by 2OHE3 failed to achieve statistical significance. As before, 2OHE2 produced the greatest amount of DNA damage, but the relative difference between 2OHE2, 4OHE2, 4OHE1, and 2OHE3 treatments was much smaller. The quantities of 8OHdG produced by catechol estrogen redox cycling were not

substantially different from that generated by direct treatment with 100 μ M hydrogen peroxide. As expected, the base oxidation by endogenous catechol estrogens was both NADPH and cytochrome P450 reductase dependent, indicating that generation of ROS is required for this form of genotoxicity.

D. Role of human breast epithelial cells in the production of reactive oxygen species by catechol metabolites of endogenous estrogens

To better understand how the biochemical mechanism of ROS generation interacts with other estrogen modes of action, we characterized endogenous human estrogens stimulated ROS generation by breast epithelial cells. Three different cell lines were compared: MCF-7, MDA-MB-231, and MCF-10A cells. These cell lines represent human mammary epithelial cells at various stages of breast cancer development. Both MCF-7 and MDA-MB-231 cells are cancerous, originating in the plural effusion from two human breast adenocarcinomas. However, these cell lines differ in their tumor characteristics.

MCF-7 cells have retained several characteristics of differentiated mammary epithelium including cytoplasmic estrogen receptors. These cells are negative for HER2/neu, and positive for estrogen receptor alpha (ER α); therefore, growth can be modulated using anti-estrogen treatment, but not trastuzumab (ATCC, 2010). These cells are highly differentiated and are commonly used to model low-grade breast epithelial adenocarcinoma cells. In contrast, MDA-MB-231 cells are poorly differentiated, positive for the HER2/neu receptor, and negative for estrogen receptor alpha (ER α). Growth can be modulated using trastuzumab, but anti-estrogen treatments are ineffective (ATCC,

2010). We use MDA-MB-231 cells to model high-grade, poorly differentiated breast epithelial adenocarcinoma cells.

In contrast, MCF-10A cells are transformed, but not tumorigenic. These cells originate from the fibrocystic lesion of a premenopausal woman and are positive for many mammary epithelial markers, including: epithelial sialomucins, normal mammary cytokeratins, and milkfat globule antigen. They exhibit three-dimensional growth in collagen, display characteristics of luminal ductal cells, and are responsive to insulin, glucocorticoids, cholera enterotoxin, and epidermal growth factor (ATCC, 2010). The MCF-10A cell line will be used to model normal, non-cancerous breast epithelial cells. Each of the three cell lines has a unique combination of characteristics (i.e. tumorigenicity and ER expression). Therefore, we can compare similar experiments using each cell line to draw additional conclusions about the role of catechol estrogen stimulated ROS generation at varying stages of the carcinogenic process.

To investigate this, we quantified breast epithelial cell lysate mediated redox cycling by four catechol estrogen metabolites (2OHE2, 4OHE2, 4OHE1, and 2OHE3), three parent estrogens (E1, E2, and E3), and one methoxy-metabolite (2MeOE2). As with cytochrome b5 and cytochrome P450 reductases, the endogenous catechol estrogens readily redox cycled, but similar concentrations of the parent estrogens (E1, E2, or E3) or the methoxy-metabolite (2MeOE2) failed to produce ROS. The ROS generated by the breast epithelial cell lysates was produced in a time- and estrogen concentration-dependent manner. For each endogenous catechol estrogen metabolite, the breast epithelial cell lysate mediated ROS accumulation was reduced by the addition of DPI, catalase, or DMSO (hydroxyl radicals only) to the assay mixture.

In all cases of ROS generation, a similarity between the three cell lines was observed. Neither the parent estrogens (E1, E2, and E3) nor the methoxy-metabolite (2MeOE2) redox cycled; however, lysates from all three mammary epithelial cell lines mediated ROS generation by endogenous catechol estrogen metabolites. This ROS production was virtually identical between these cell lines in terms of the amount, rate, reversibility, and saturation point. As the three cell lines differ in both their ER α expression and their tumorigenic status, ROS generation by mammary epithelial cells must be independent of estrogen receptor or tumorigenicity. Therefore, endogenous estrogen stimulated ROS generation cannot be explained by its canonical mode of action, nuclear receptor modification of gene expression. Furthermore, this ROS production is a fundamental activity of human mammary epithelial cells, and may occur before, during, and after any phase of the carcinogenic process.

Kinetic analysis of hydrogen peroxide generation by these cells suggest that estrogen metabolite semiquinone synthesis had apparent K_M 's between 2.3 and 19 μ M and V_{max} 's between 2.7 and 6.4 pmol H₂O₂/min/mg total protein. As the concentration of cell lysate protein, and therefore number of catalytic sites, was held constant for each cell line, the measured V_{max} is proportionate to the turnover number for catechol estrogen oxidation. As observed with reductase-mediated redox cycling, these values are small, indicating poor catalytic efficiency at high substrate concentrations. Thus, semiquinone production is not catalytically efficient and highly dependent on the catechol estrogen metabolite concentration. The observed K_M values (the concentrations of substrate needed to achieve a half-maximal rate) lie between those predicted using recombinant cytochrome b₅ and cytochrome P450 reductases, suggesting that both

cytochrome b5 and cytochrome P450 reductases could mediate redox cycling. However, the same observed K_M values more closely resemble those produced using cytochrome b₅ reductase, indicating that this enzyme may be the greater contributor to hydrogen peroxide production by endogenous catechol estrogen metabolites. Taken together, these data indicate that breast epithelial cell lysates readily mediate redox cycling by low doses of catechol estrogen metabolites.

Since the above reaction meets all the assumptions necessary for Michaelis-Menten analysis, K_M and V_{max} can be used to determine the concentration of catechol estrogens required to generate statistically significant amounts of hydrogen peroxide (v_a).

Assuming a value for α of 0.05 (a single-tailed 95% confidence interval), less than one μM of these endogenous catechol estrogen metabolites are required for breast epithelial cell lysates to produce statistically significant amounts of hydrogen peroxide.

To model ROS generation by endogenous catechol estrogen metabolites in breast epithelial cells more realistically, we modified Clark-type microelectrodes to measure the hydrogen peroxide rather than oxygen concentration in the vicinity of intact breast epithelial cells. Because both hydrogen peroxide and estrogens are cell membrane permeable, it is possible to treat an adherent cell population with an estrogen and monitor any resulting generation of hydrogen peroxide.

Treatment with increasing concentrations of endogenous catechol estrogen metabolites (2OHE2, 4OHE2, 4OHE1, and 2OHE3) increased hydrogen peroxide production from MCF-7, MDA-MB-231, and MCF-10A cells. In contrast, neither the parent estrogens (E1, E2, and E3) nor the methoxy-metabolite (2MeOE2) raised the extracellular hydrogen peroxide concentration. The increased generation of hydrogen

peroxide by the cells was prevented by pretreatment with DPI and ameliorated by both DPI and catalase. These data indicate that the hydrogen peroxide generation is mediated by one or more flavoproteins, which is consistent with redox cycling by both cytochrome P450 and cytochrome b₅ reductases. Kinetic analysis of these data demonstrates similarities in endogenous catechol estrogen metabolite stimulated response. Each catechol estrogen metabolite generated similar concentrations of hydrogen peroxide, as a percentage of V_{max}, indicating a lack of specificity between the endogenous catechol and its enzymatic mediator of redox cycling. Thus, catechol estrogen stimulated ROS production appears to occur via a common mode of action at the cellular level.

Because our measurements were extracellular, this hydrogen peroxide must be beyond that which is cleared by intracellular antioxidant mechanisms. These changes in hydrogen peroxide concentrations should be thought of as changes at a fixed point in space along a gradient. Closer to the point of generation, and even within the cells, the concentration is higher. Thus, these data underestimate the hydrogen peroxide concentration inside the cells, and therefore underestimate the amount of mutagenic ROS at the site of nuclear DNA. Given enough endogenous catechol estrogen production, ROS mediated DNA mutagenesis seems probable.

Since normal circulating serum estrogen levels in premenopausal females range from 75-2000 pg/ml (depending on the estrogen, the individual, and the phase of the menstrual cycle) (Caron *et al.*, 2009), it seems unlikely at first glance that enough catechol estrogen metabolites would form to generate ROS. However, the localized estrogen metabolite concentration in the endoplasmic reticulum of human breast epithelial cells is likely to be much higher than the serum concentration. This cell type

expresses the nuclear estrogen receptors (ERs). These high-affinity binding proteins are capable of pulling a concentration gradient into the cell, geometrically increasing the intracellular estrogen concentration (Jensen and Jordan, 2003). Additionally, the enzymes governing estrogen synthesis (aromatase), estrogen metabolism to catechols (cytochromes P450 1A1 and 1B1), and estrogen redox cycling (cytochrome b₅ and P450 oxidoreductases) are adjacent to one another on the microsomal electron transport chain (Lu *et al.*, 1969). As a result, the localized endoplasmic reticulum membrane concentration of endogenous catechol estrogen metabolites is likely to be significantly greater than levels of estrogen in the serum and therefore, well within a plausible range for ROS generation.

While this ROS production could be significant enough to saturate the antioxidant defense mechanisms and diffuse out of the cells, it is more probable that *in vivo*, the ROS concentration due to endogenous catechol estrogen redox cycling is smaller and more localized. Often these ROS will be scavenged by antioxidant pathways (Klaunig *et al.*, 2010); however some of the ROS can damage intracellular macromolecules, including DNA, proteins, and lipids (Klaunig *et al.*, 2010). Over decades of exposure, this small amount of added oxidative stress may increase the mutation rate of human breast epithelial cells (Klaunig *et al.*, 2010) and contribute to overall estrogen carcinogenicity and the initiation phase of breast cancer.

CONCLUDING REMARKS

Estrogens are known human carcinogens which are well-known for their role in the progression and promotion of hormonally sensitive cancers. We have characterized a novel and previously unrecognized mode of action of endogenous estrogens by which they could act as mutagens during the initiation phase of breast cancer. Endogenous estrogens are metabolized to catechols by cytochromes P450 1A1 and 1B1. These catechol metabolites of endogenous estrogens are capable of redox cycling, readily generating mutagenic reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and especially hydroxyl radicals.

Using recombinant sources of human cytochrome b₅ and cytochrome P450 reductases, we determined that these enzymes do indeed mediate redox cycling by catechol metabolites of endogenous estrogens. At low catechol estrogen concentrations, cytochrome b₅ reductase produces more ROS, indicating that it is more likely than cytochrome P450 reductase to mediate redox cycling by catechol estrogen metabolites. A variety of antioxidants and inhibitors of these enzymes prevent accumulation of ROS. ROS generation was sufficient to oxidize nucleotide bases and break the DNA backbone.

Using three breast epithelial cell lines: MCF-10A, MCF-7, and MDA-MB-231, we determined that breast epithelial cells do mediate redox cycling by catechol metabolites of endogenous estrogens. ROS generation by these catechols was substantial enough to overwhelm the antioxidant detoxification pathways, and diffuse out of the cells. This signifies firstly, that ROS generation is sufficient to distribute into the nuclear compartment of the cell in DNA damaging quantities, and secondly, that ROS generated by one cell could diffuse into and alter macromolecular structures in neighboring cells,

including epithelia, adipocytes, or fibroblasts. The production of ROS is inhibited by addition of antioxidant enzymes or diphenyleneiodinium, suggesting that the enzyme(s) mediating catechol estrogen redox cycling is/are flavoprotein(s), consistent with a reaction mediated by cytochrome b₅ and cytochrome P450 reductases. ROS production was equivalent between the cell lines, indicating that redox cycling capability was not related to the carcinogenic stage or the estrogen receptor status of the breast epithelial cells. Therefore, breast epithelial cell-mediated ROS generation by catechol metabolites of endogenous estrogens could arise both before and throughout breast tumor formation and must occur via a mechanism unrelated to the canonical mode of estrogen action.

Taken together, these results suggest a new mode of endogenous estrogen breast carcinogenesis, whereby metabolism of these estrogens to catechols and subsequent redox cycling, produces ROS capable of mutating DNA. Thus, by varying modes of action these estrogens probably are factors in the initiation, progression, and promotion phases of tumorigenesis, making endogenous human estrogens complete carcinogens.

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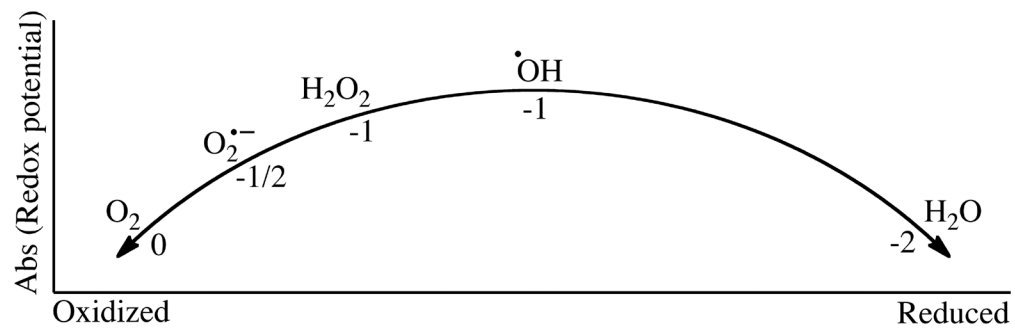
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FIGURES AND TABLES

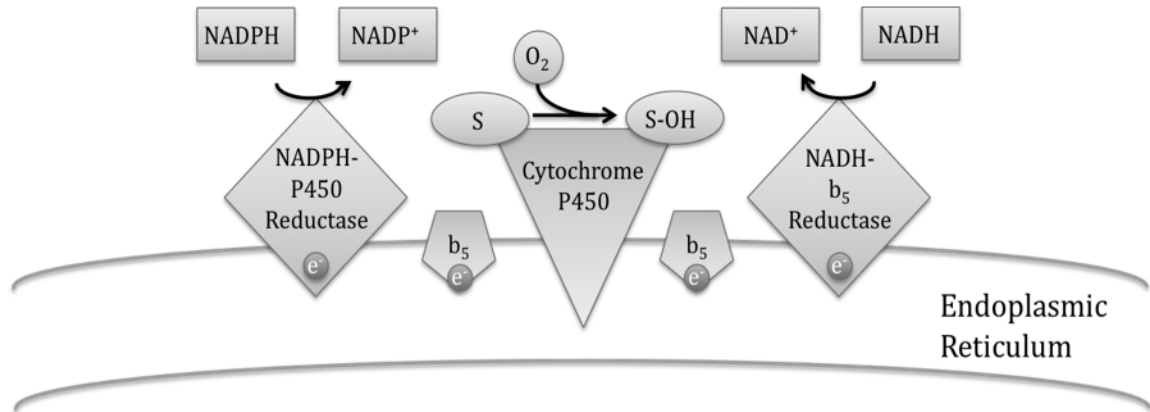
Scheme 1. Reactive oxygen intermediates are partially reduced oxygen species. Oxygen can exist in one of several oxidation states from zero (O_2) to negative two (H_2O). These oxygen species are stable; however, the intermediate oxidation states are occupied by oxygen species that have an increased oxidation potential. As a result, these partially reduced or oxidized oxygen species are more reactive and can damage cellular macromolecules, including DNA, proteins, and lipids.

Scheme 1.



Scheme 2. The mixed-function oxidase cytochrome P450 is part of the microsomal electron transport chain in the endoplasmic reticulum of cells. Electrons required for P450 substrate metabolism are provided from the oxidation of the reducing equivalents NADH and NADPH by the flavoproteins NADH-cytochrome b₅ reductase (b₅R) and NADPH-cytochrome P450 reductase (POR). These electrons are then passed along the microsomal electron transport chain, either directly to the heme-cofactor in the active site of cytochrome P450 or indirectly via cytochrome b₅.

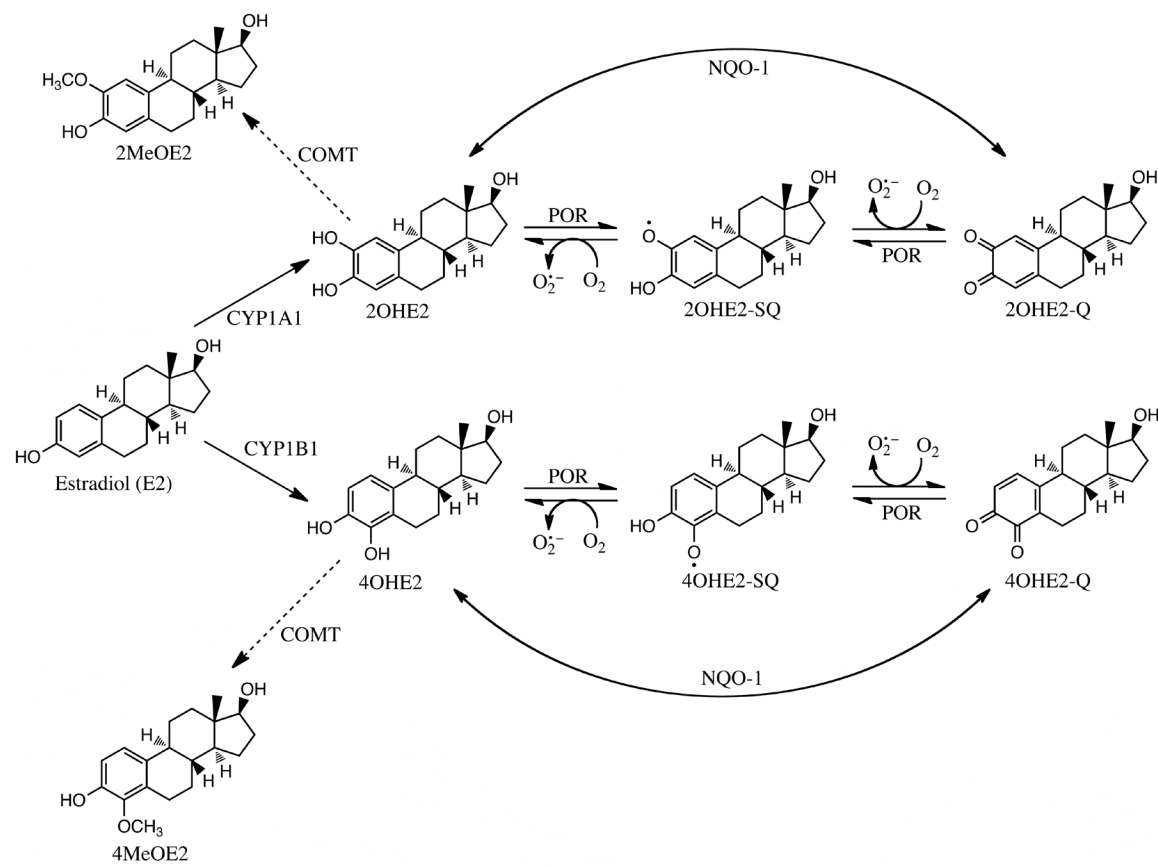
Scheme 2.



Scheme 3. Estradiol is metabolized to catechols by cytochrome P450s 1A1 and 1B1.

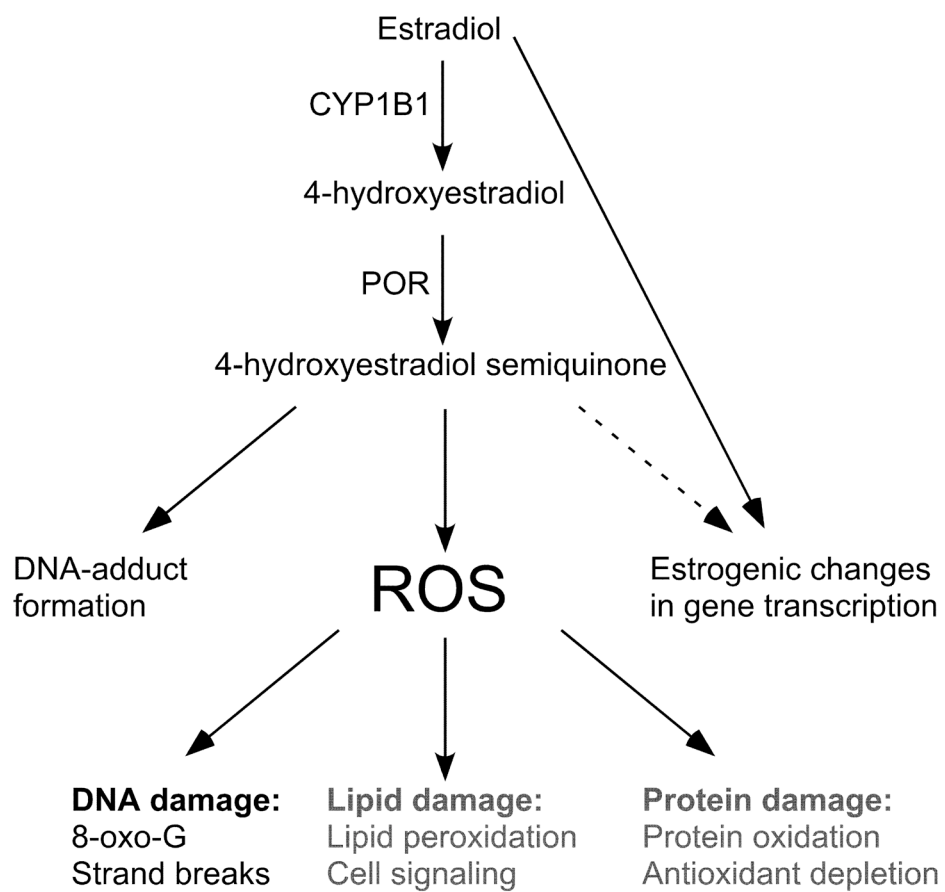
Oxidation of these metabolites to semiquinones and quinones by NADPH-cytochrome P450 reductase (POR) and subsequent redox cycling results in the production of ROS.

Scheme 3.



Scheme 4. Metabolism of endogenous estrogens to catechols and subsequent redox cycling generates reactive oxygen species. The reactive oxygen species produced are capable of oxidative damage to cellular macromolecules including DNA, proteins, and lipids.

Scheme 4.



Scheme 5. Redox cycling by endogenous catechol estrogens generates superoxide. The superoxide produce can be dismutated to hydrogen peroxide which can further react to form highly mutagenic hydroxyl radicals under conditions favorable for Fenton chemistry.

Scheme 5.

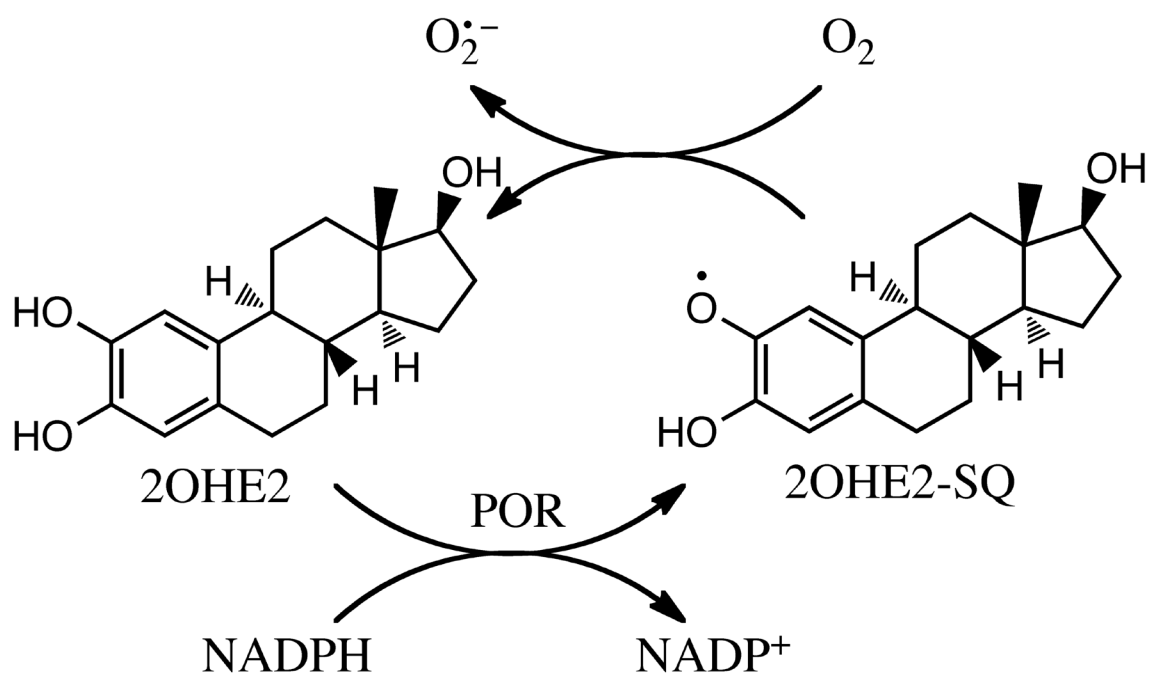


Figure 1. Redox cycling of catechol estrogens by recombinant cytochrome b₅ reductase generates H₂O₂. Assay mixtures contained 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) cytochrome b₅ reductase, and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 1.

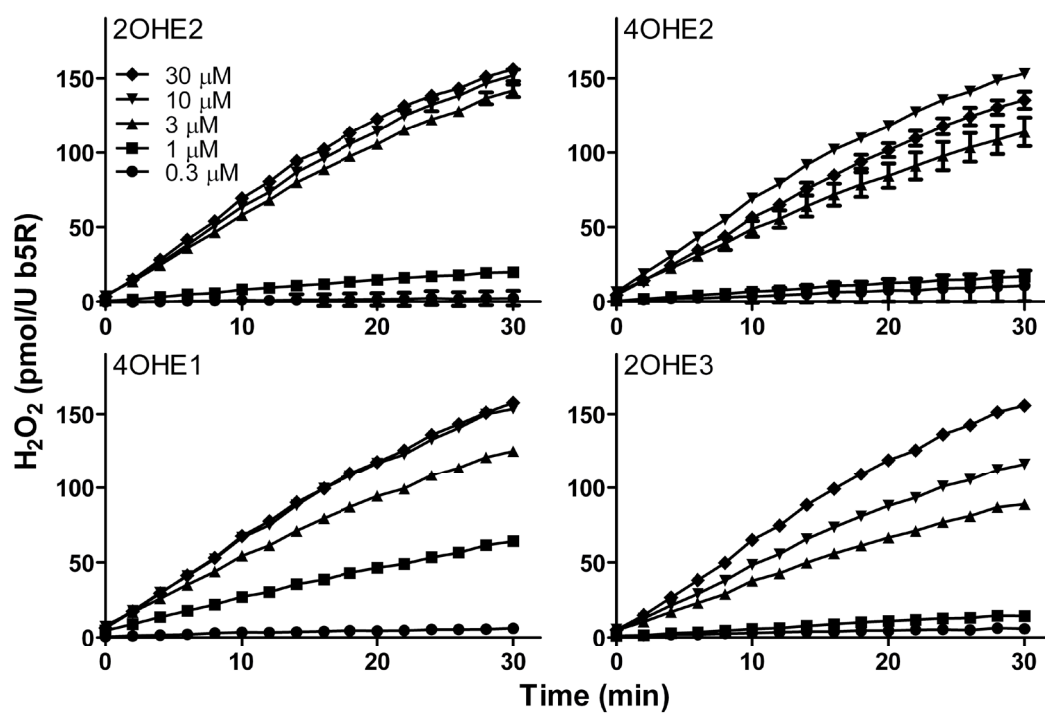


Figure 2. Rates of catechol estrogens redox cycling by cytochrome b₅. Assay mixtures contained 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) cytochrome b₅ reductase, and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 2.

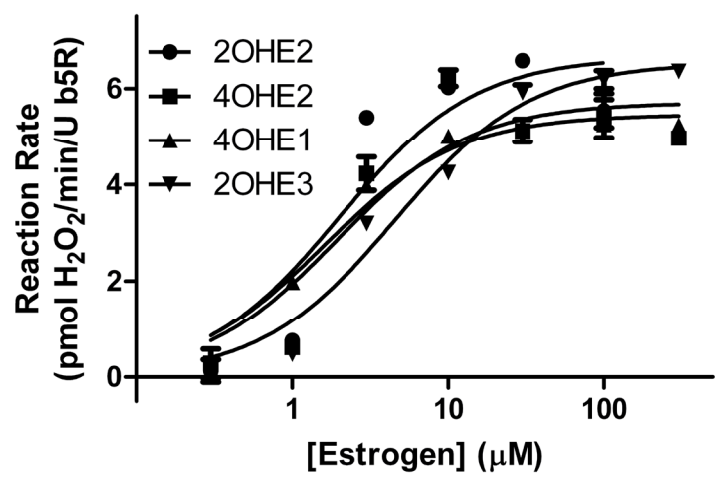


Table 1. Effects of catechol estrogen treatment on the rate of H_2O_2 generation by cytochrome b₅ reductase

	H_2O_2	
	V _{max} (pmol/min/U b ₅ R)	K _M (μM)
2OHE2	6.7 ± 0.82	2.0 ± 1.1
4OHE2	5.7 ± 0.56	1.9 ± 1.0
4OHE1	5.5 ± 0.21	1.6 ± 0.34
2OHE3	6.5 ± 0.29	4.6 ± 0.97

Data are reported as mean ± SEM (n = 3)

Figure 3. Effects of inhibitors on catechol estrogen redox cycling. Assay mixtures contained 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) cytochrome b₅ reductase, and 10 μ M 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Where indicated, reactions were supplemented with a 1:100 dilution of polyclonal antibodies to P450 reductase (α POR, 10 μ g/ml) or 1000 U/ml catalase. Data are expressed as mean \pm SEM (n = 3).

Figure 3.

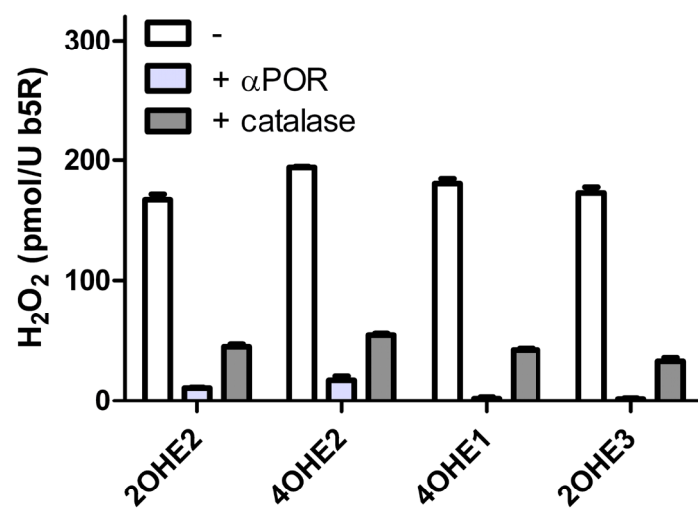


Figure 4. Catechol estrogen redox cycling generates superoxide anion. Assay mixtures contained 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) cytochrome b₅ reductase, and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 4.

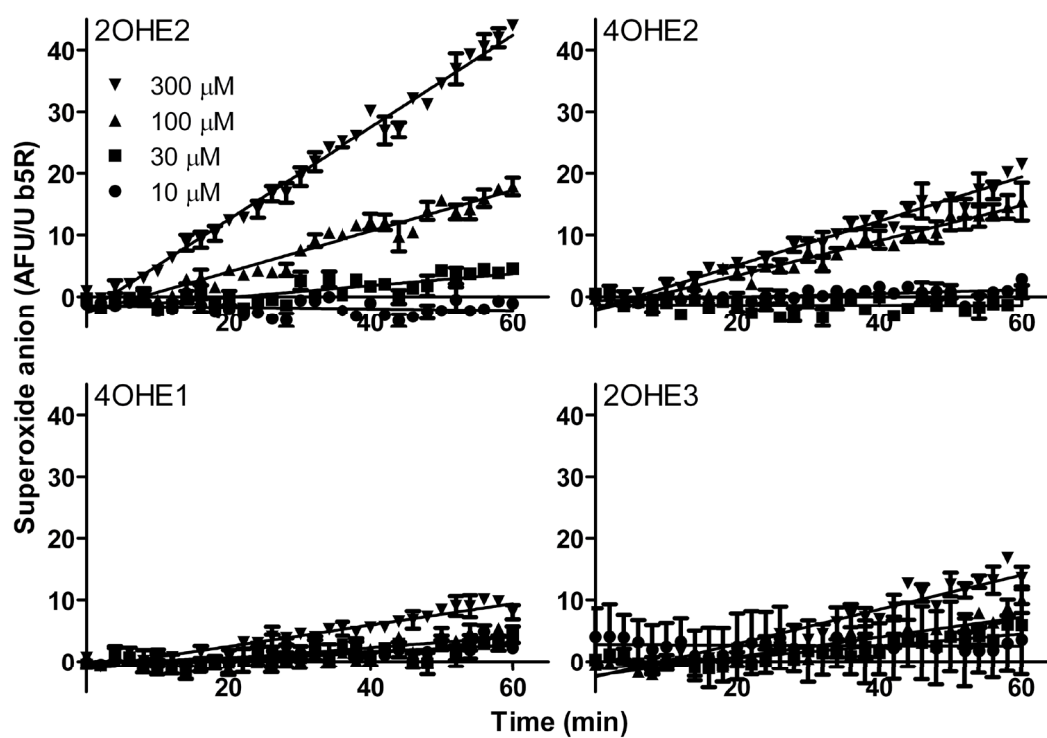


Figure 5. Rates of superoxide anion formation during catechol estrogen redox cycling.

Assay mixtures contained 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) cytochrome b₅ reductase, and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 5.

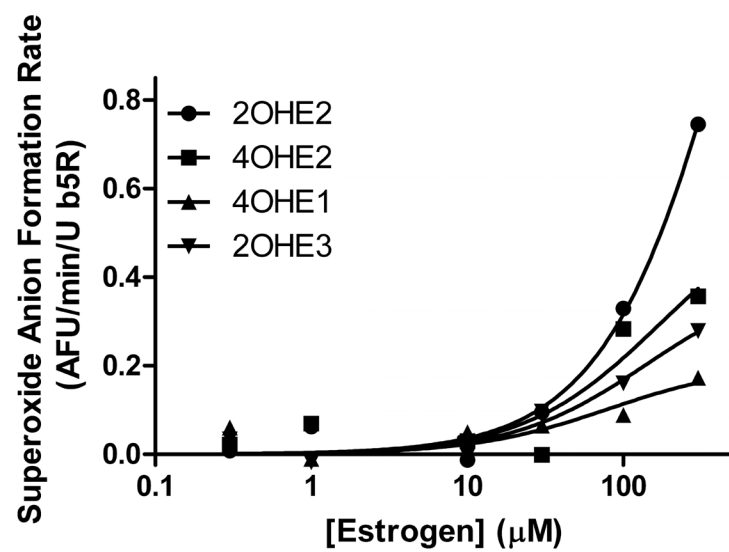


Figure 6. Inhibition of catechol estrogen redox cycling by superoxide dismutase. Assays were run in the absence and presence of 1 U superoxide dismutase (SOD) and 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) cytochrome b₅ reductase, and 100 μ M 4OHE2. Data are expressed as mean \pm SEM (n = 3).

Figure 6.

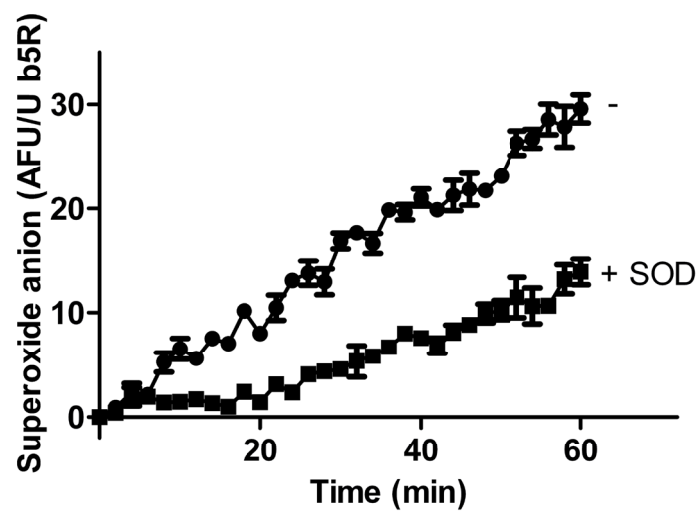


Figure 7. Redox cycling of catechol estrogens generate hydroxyl radicals. Assay mixtures contained 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) of purified cytochrome b₅ reductase, and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 7.

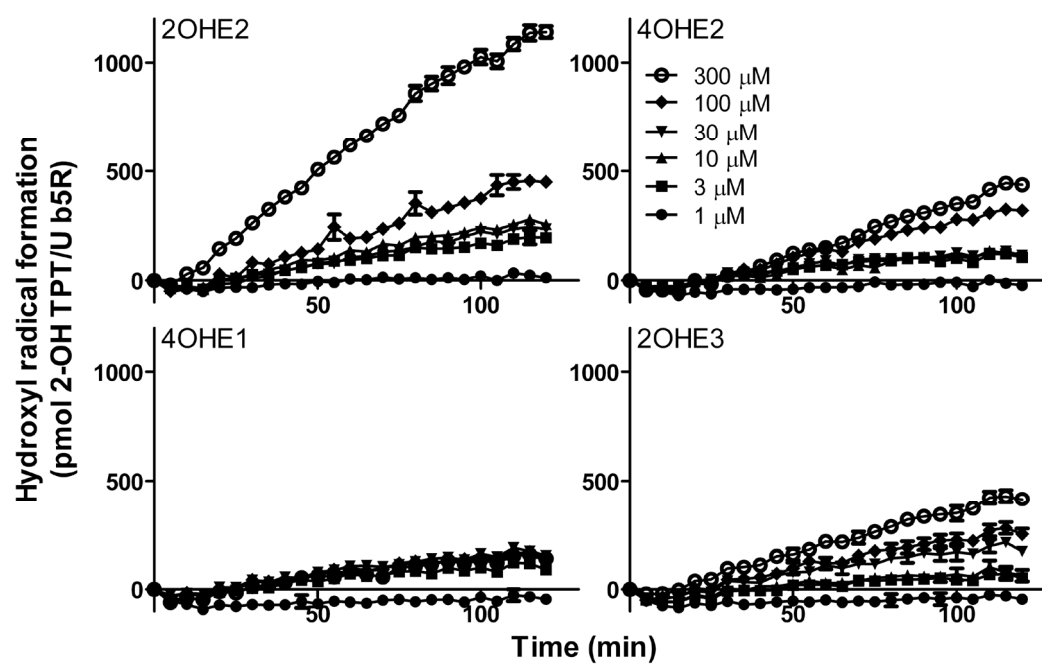


Figure 8. Rates of hydroxyl radical production by redox cycling of catechol estrogens.

Assay mixtures contained 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) cytochrome b₅ reductase, and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 8.

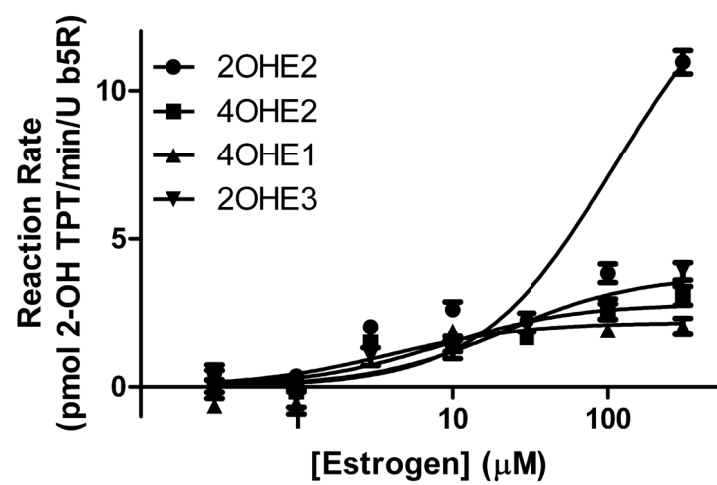


Figure 9. Effects of inhibitors on hydroxyl radical production during redox cycling.

Assay mixtures contained 100 μ M NADPH, 2.92 U/ml (5 μ g/ml) cytochrome b₅ reductase, and 30 μ M 2OHE2, 4OHE2, 4OHE1, or 2OHE3, supplemented with either 10 μ M DPI, 1% DMSO, or 1000 U/ml catalase. Data are expressed as mean \pm SEM (n = 3).

Figure 9.

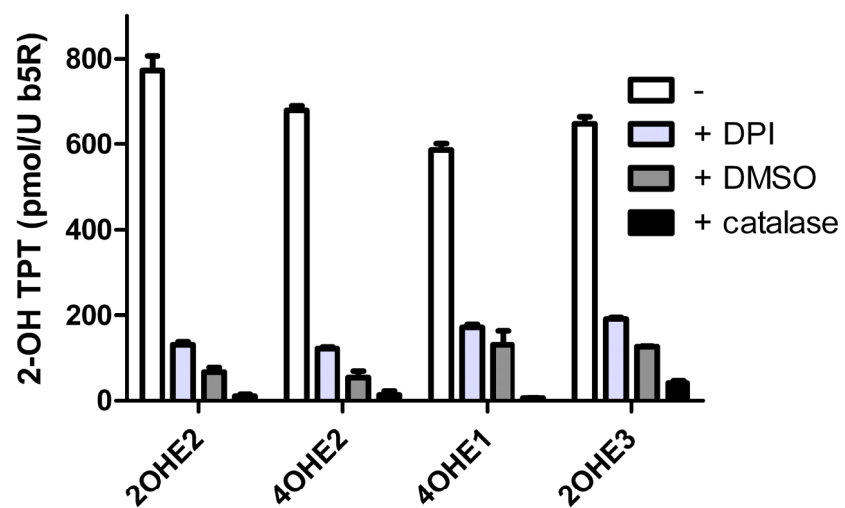


Figure 10. Recombinant cytochrome P450 reductase redox cycles catechol metabolites of estradiol and generates H_2O_2 . Assay mixtures contained 100 μ M NADPH, Supersomes containing 3.95 U/ml (5 μ g/ml) cytochrome P450 reductase (POR), and increasing concentrations of E2, 2OHE2, 4OHE2, or 2MeOE2. Data are expressed as mean \pm SEM (n = 3).

Figure 10.

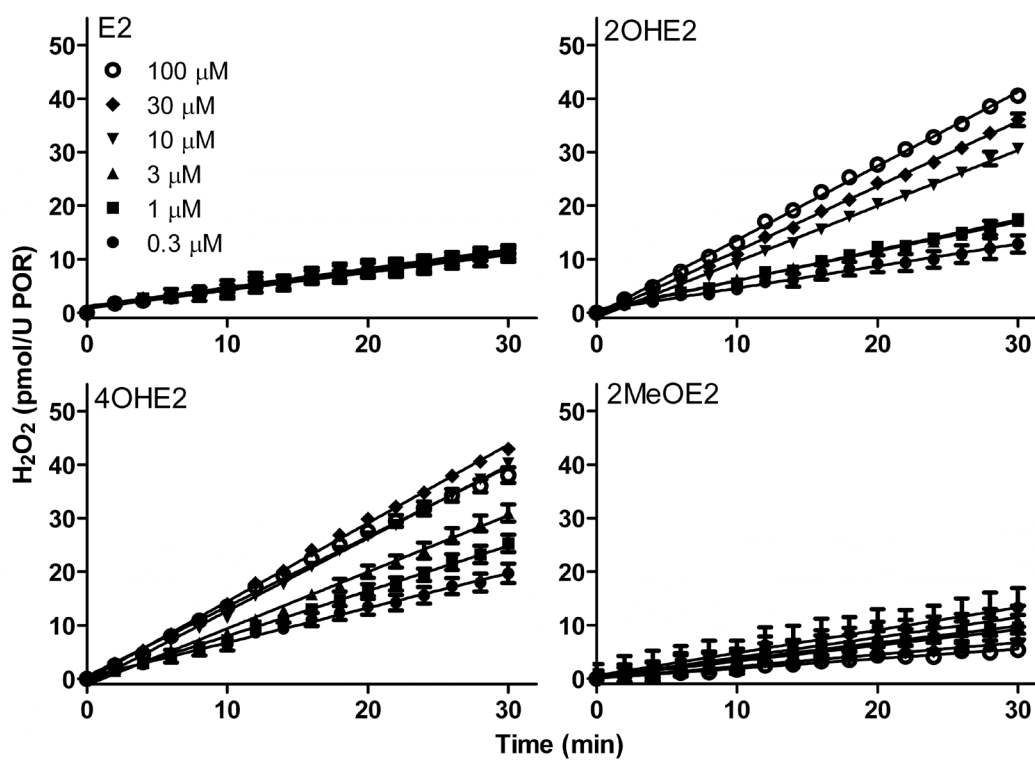


Figure 11. Cytochrome P450 reductase mediated H_2O_2 production by redox cycling of estrogen metabolites. Assay mixtures contained 100 μ M NADPH, Supersomes containing 3.95 U/ml (5 μ g/ml) cytochrome P450 reductase (POR), and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 11.

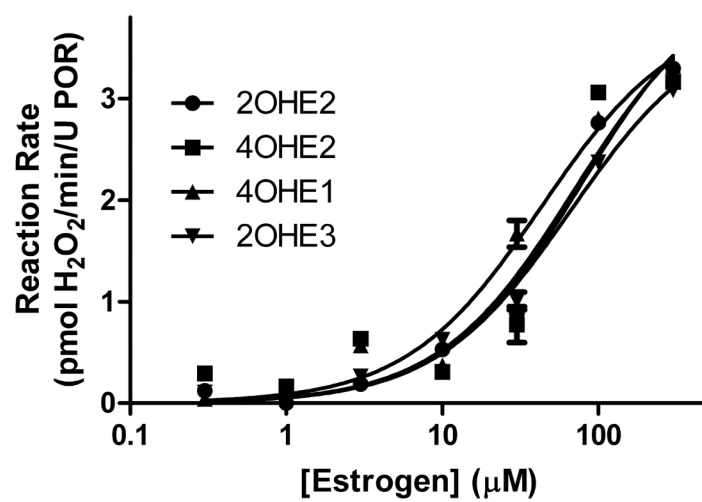


Table 2. Effects of catechol estrogen treatment on the rate of H_2O_2 generation by cytochrome P450 reductase

	H_2O_2	
	Vmax (pmol/min/U POR)	K_M (μM)
2OHE2	4.3 ± 0.43	77 ± 21
4OHE2	4.2 ± 0.80	70 ± 37
4OHE1	3.9 ± 0.30	43 ± 11
2OHE3	3.8 ± 0.21	67 ± 11

Data are reported as mean \pm SEM (n = 3)

Figure 12. Redox cycling by catechol estrogens is inhibited by catalase and antibodies to cytochrome P450 reductase. Assay mixtures contained 100 μ M NADPH, Supersomes containing 3.95 U/ml (5 μ g/ml) cytochrome P450 reductase (POR), and 10 μ M 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Where indicated, reactions were supplemented with a 1:100 dilution of antibodies to cytochrome P450 reductase (α POR) or 1000 U/ml catalase. Data are expressed as mean \pm SEM (n = 3).

Figure 12.

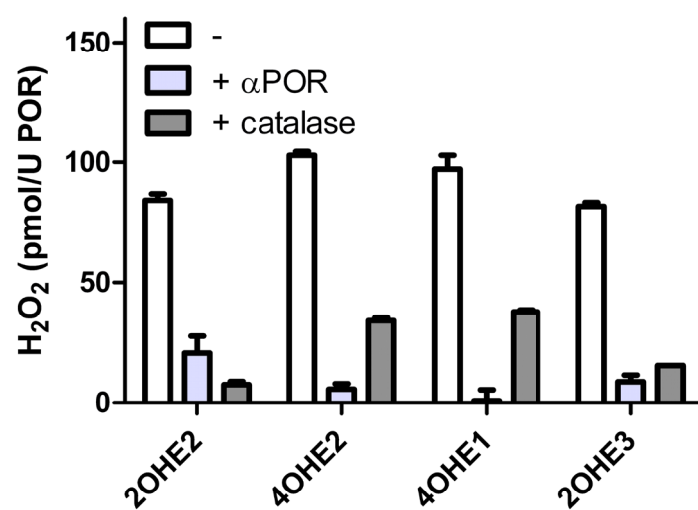


Figure 13. Cytochrome P450 reductase redox cycles catechol metabolites and generates hydroxyl radicals. Assay mixtures contained 100 μ M NADPH, 3.95 U/ml (5 μ g/ml) Supersomes containing cytochrome P450 reductase (POR) and increasing concentrations of E2, 2OHE2, 4OHE2, or 2MeOE2. Data are expressed as mean \pm SEM (n = 3).

Figure 13.

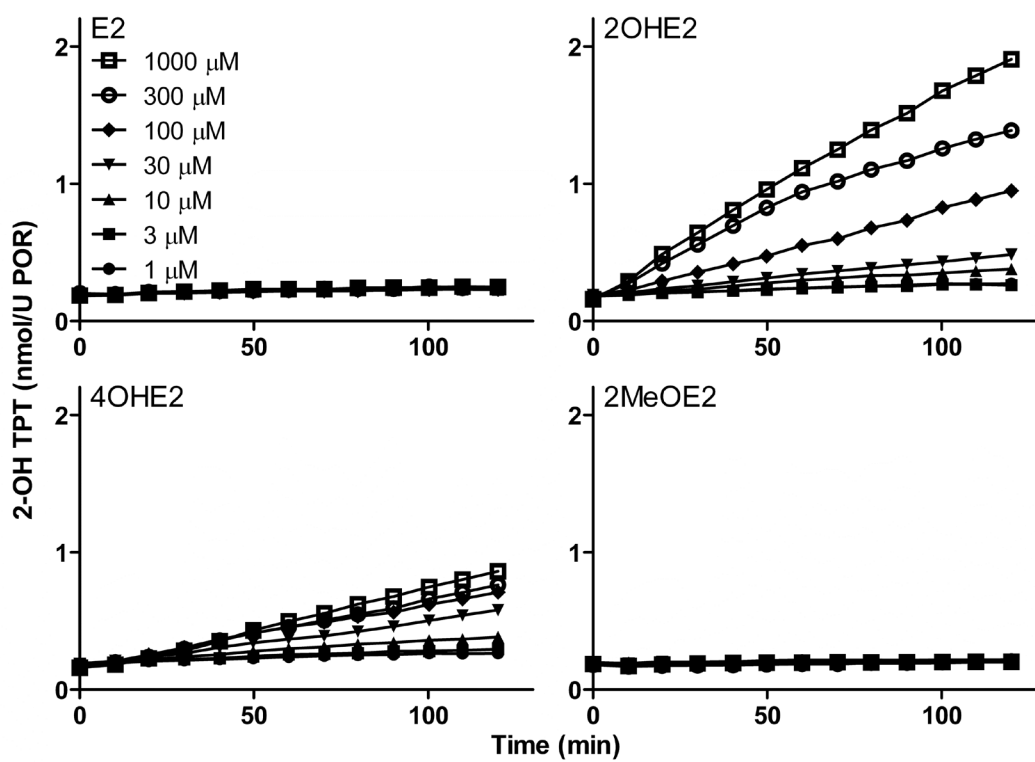


Figure 14. Rates of hydroxyl radical production by redox cycling of estrogen

metabolites. Assay mixtures contained 100 μ M NADPH, 100 μ M FeCl₃, 110 μ M EDTA, 3.95 U/ml (5 μ g/ml) Supersomes containing cytochrome P450 reductase (POR), and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 14.

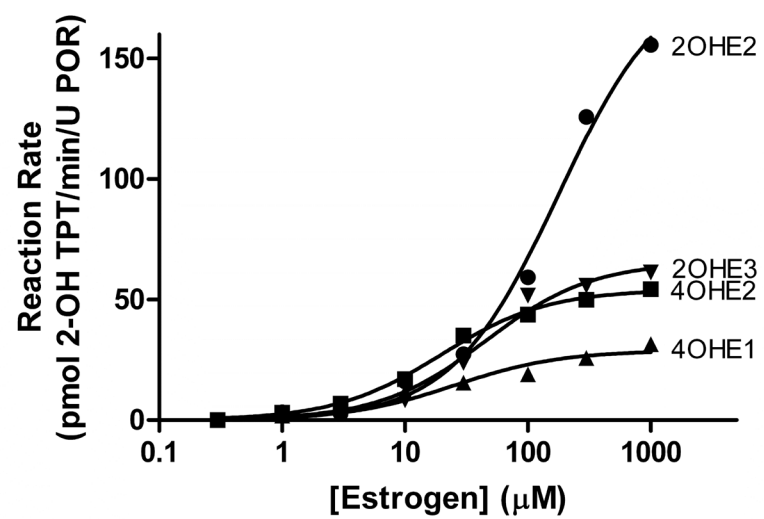


Figure 15. Hydroxyl radical production during redox cycling is inhibited by diphenyleneiodium, catalase, and DMSO. Assay mixtures contained 100 μ M NADPH, 100 μ M FeCl₃, 110 μ M EDTA, 3.95 U/ml (5 μ g/ml) of Supersomes containing cytochrome P450 reductase (POR), and 30 μ M 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Where indicated, assays also contained 1% DMSO, 10 μ M DPI or 1000 U/ml catalase. Data are expressed as mean \pm SEM (n = 3).

Figure 15.

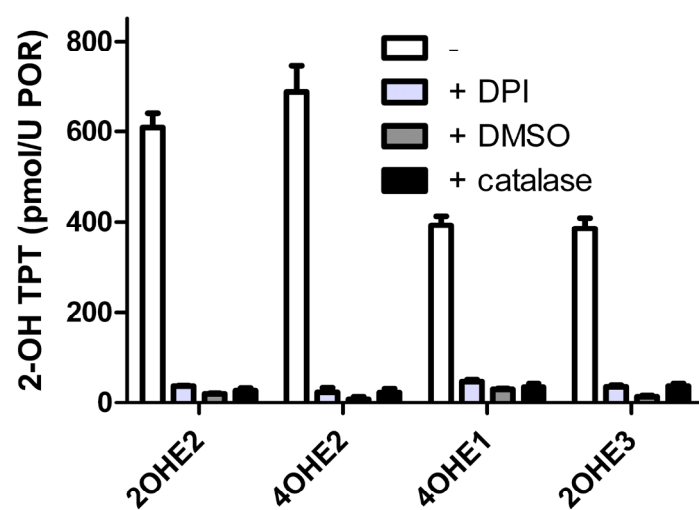


Figure 16. Rates of superoxide anion production by redox cycling of estrogen metabolites. Assay mixtures contained 100 μ M NADPH, 3.95 U/ml (5 μ g/ml) Supersomes containing cytochrome P450 reductase (POR), and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data were obtained as arbitrary fluorescence units (AFU) and expressed as mean \pm SEM (n = 3).

Figure 16.

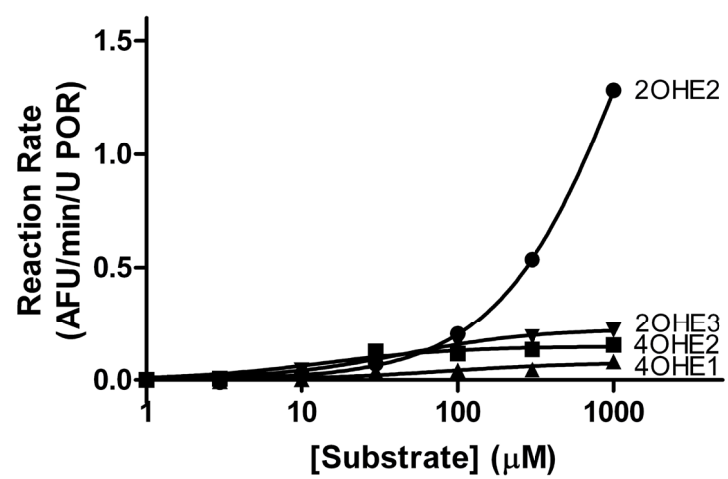


Figure 17. Effects of superoxide dismutase on superoxide anion production during catechol estrogen redox cycling. Assay mixtures with or without 1U superoxide dismutase contained 100 μ M NADPH, 3.95 U/ml (5 μ g/ml) Supersomes containing cytochrome P450 reductase, and 100 μ M 4OHE2. Data are expressed as mean \pm SEM (n = 3).

Figure 17.

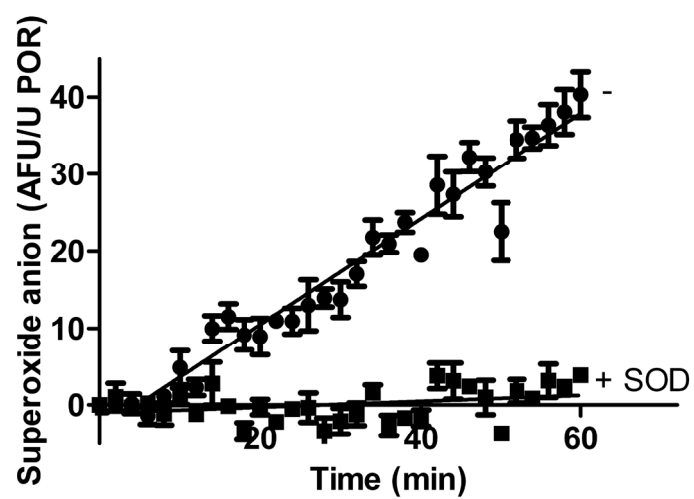


Figure 18. Genotoxins alter the melting curve of DNA. Treatment of DNA with a damaging agent modifies the melting curves in a manner characteristic of the type of DNA damage incurred. Thus this technique can be used both as an assay to broadly screen for genotoxicity, and a method to measure both the type and degree of genotoxicity sustained.

Figure 18.

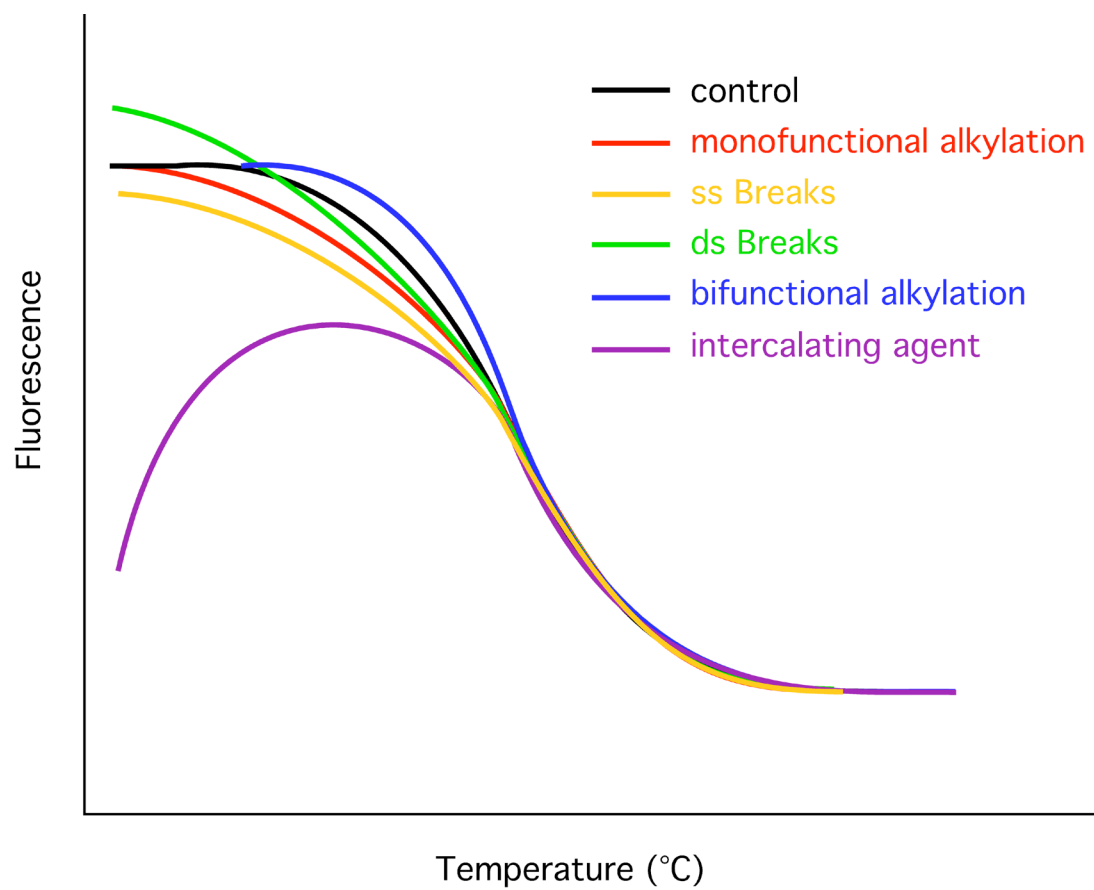


Figure 19. Catechol estrogen stimulated redox cycling damages DNA. Assay mixtures contained 100 μ M NADPH, 3.95 U/ml (5 μ g/ml) Supersomes containing cytochrome P450 reductase, and increasing concentrations of E2, 2OHE2, 4OHE2, 2MeOE2, E1, 4OHE1, E3, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 19.

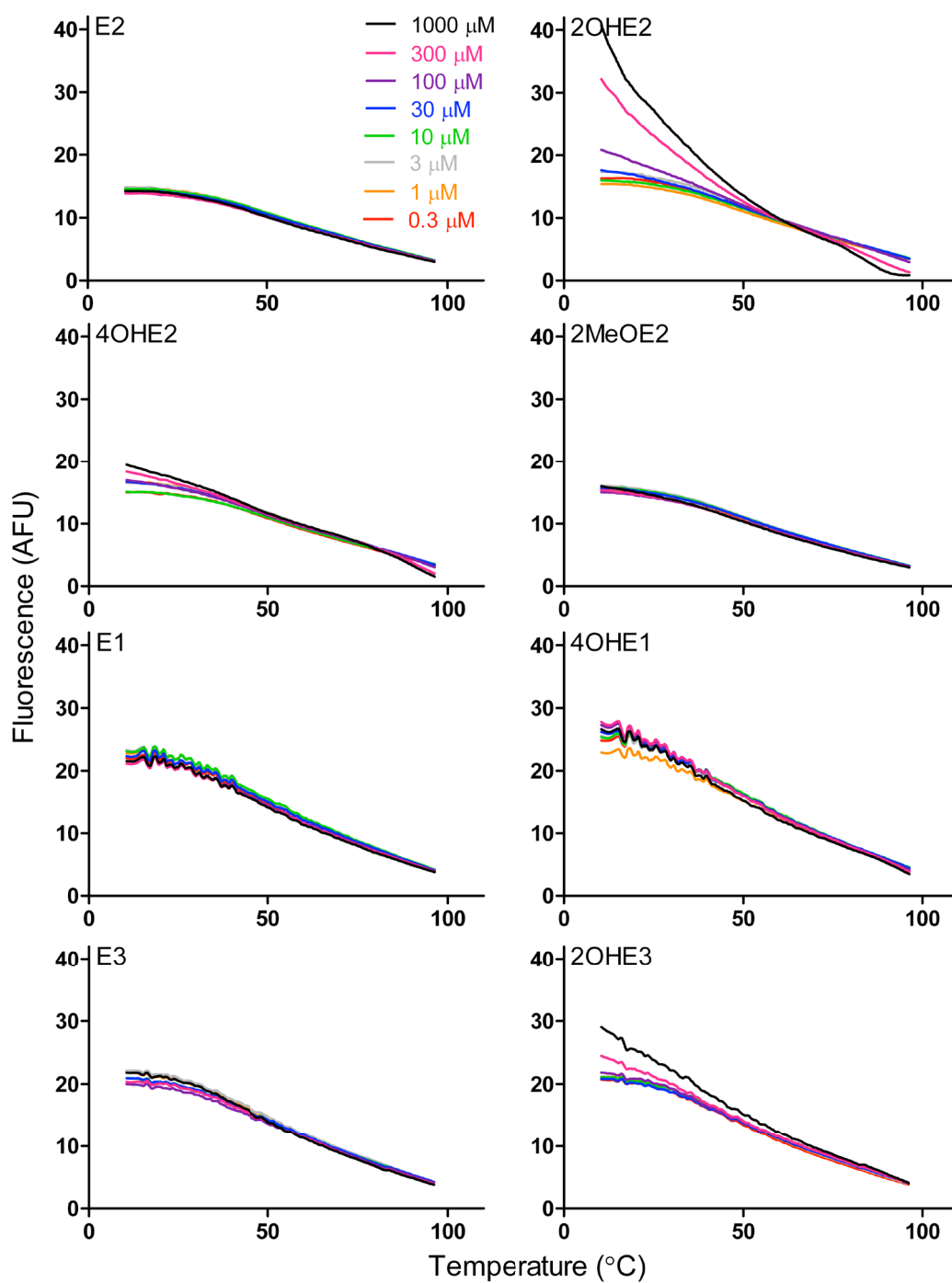


Figure 20. Redox cycling of catechol estrogens stimulates DNA base oxidation. DNA base oxidation was measured by the formation of 8-hydroxy-2-deoxyguanosine. Assays were run with 1 µg/ml calf-thymus DNA, 100 µM NADPH, 100 µM FeCl₃, 110 µM EDTA, and 3.95 U/ml (5 µg/ml) Supersomes containing cytochrome P450 reductase.

Left Panel: Redox cycling by catechol estrogen metabolites increased DNA base oxidation. The concentration of 8-hydroxy-2-deoxyguanosine was measured in the absence and presence of 100 µM E2, 2OHE2, 4OHE2, 2MeOE2, E1, 4OHE1, E3, or 2OHE3. H₂O₂ (100 µM) was used as a positive control. All samples were compared with the negative control to determine significance. *Right Panel:* Catechol estrogen stimulated base oxidation is POR dependent. Samples were compared with the 2OHE2 sample to determine significance. Data are expressed as mean ± SEM (n = 3). Significance was determined by one-way ANOVA with an ad hoc Bonferroni corrected t-test as follows: * $0.01 \leq P \leq 0.05$, ** $0.001 \leq P \leq 0.01$, *** $P < 0.001$.

Figure 20.

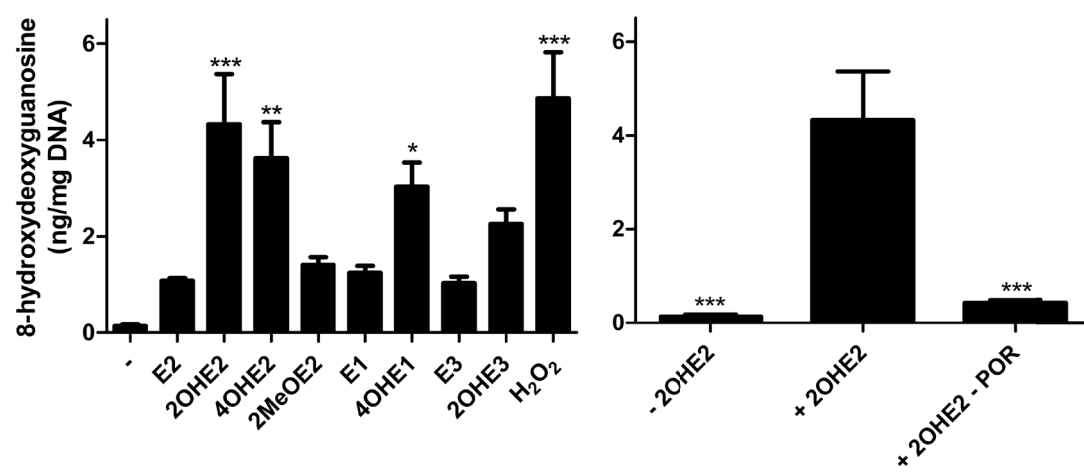


Figure 21. Cell lysates from breast epithelial cell lines redox cycle catechol metabolites of estradiol and generate H_2O_2 . Assay mixtures contained 100 μ M NADPH, 50 μ g/ml lysate protein from MCF-7, MDA-MB-231, or MCF-10A cells and increasing concentrations of either E2, 2OHE2, 4OHE2, or 2MeOE2. Data are expressed as mean \pm SEM (n = 3).

Figure 21.

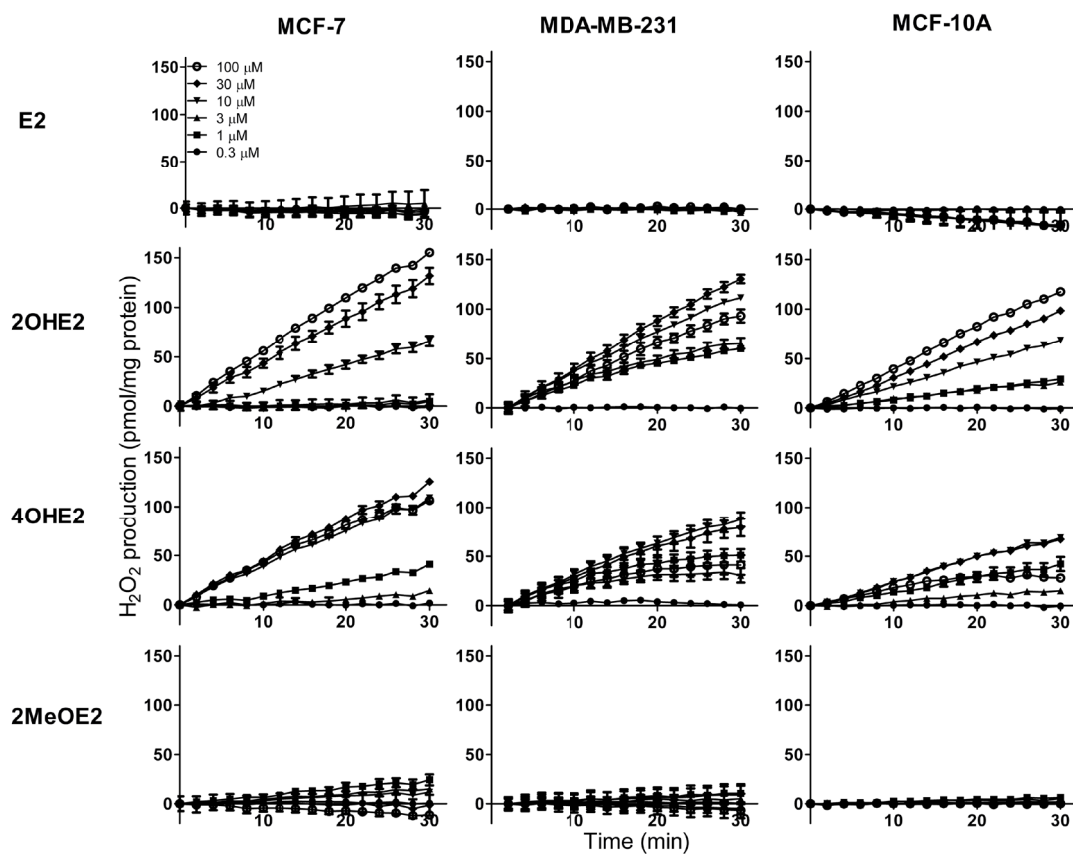


Figure 22. H₂O₂ production by redox cycling of estrogen metabolites. Assay mixtures contained 100 μ M NADPH, 50 μ g/ml cell lysate protein from MCF-7 (upper panel), MDA-MB-231 (center panel), or MCF-10A cells (lower panel), and increasing concentrations of either 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 22.

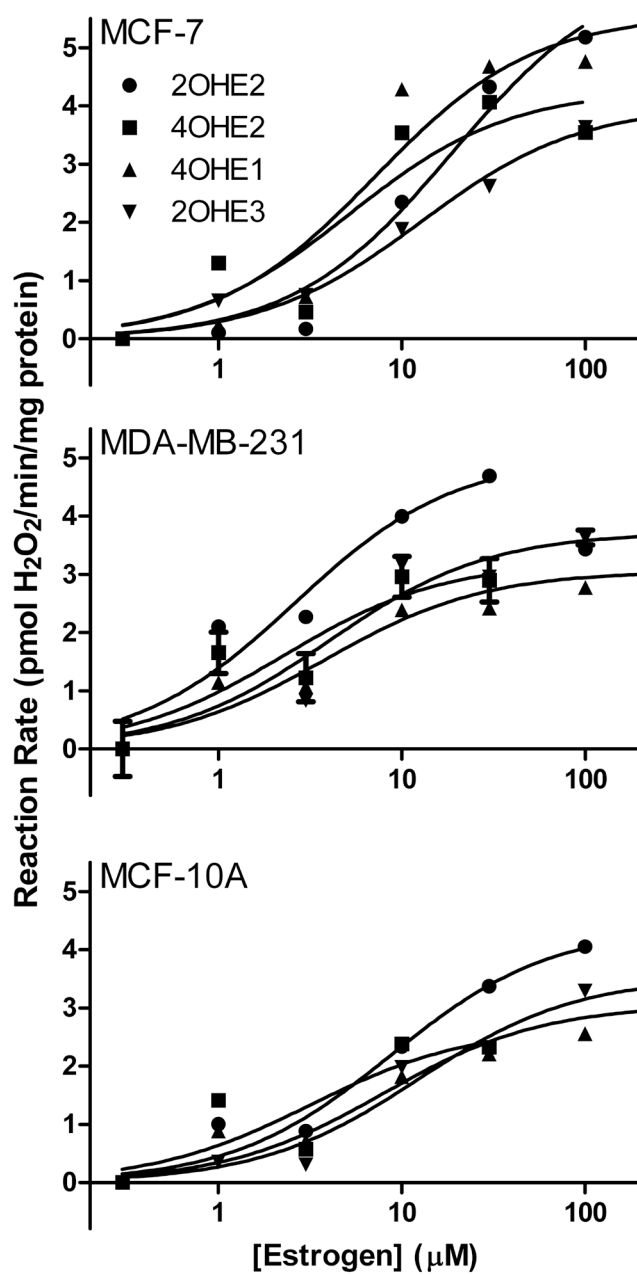


Table 3. Effects of catechol estrogen treatment on the rate of H_2O_2 generation by MCF-7, MDA-MB-231, and MCF-10A breast epithelial cells.

	MCF-7		MDA-MB-231		MCF-10A	
	Vmax (pmol/min/ mg protein)	K _M (μ M)	Vmax (pmol/min/ mg protein)	K _M (μ M)	Vmax (pmol/min/ mg protein)	K _M (μ M)
2OHE2	6.4 \pm 0.76	19 \pm 6.6	5.0 \pm 0.65	2.6 \pm 1.2	4.4 \pm 0.36	8.7 \pm 2.6
4OHE2	4.3 \pm 0.79	5.2 \pm 3.7	3.2 \pm 0.66	2.3 \pm 1.7	2.7 \pm 0.83	3.2 \pm 3.4
4OHE1	5.6 \pm 0.39	7.1 \pm 2.6	3.1 \pm 0.18	3.8 \pm 1.2	3.1 \pm 0.20	7.9 \pm 2.7
2OHE3	4.0 \pm 0.22	13 \pm 3.4	3.7 \pm 0.31	4.0 \pm 1.9	3.5 \pm 0.22	12 \pm 3.6

Data are expressed as mean \pm SEM (n = 3)

Figure 23. Effects of inhibitors on catechol estrogen redox cycling. The generation of H_2O_2 was determined in assay mixtures contained 100 μM NADPH, 10 μM of either 2OHE2, 4OHE2, 4OHE1, or 2OHE3, and 50 $\mu\text{g/ml}$ cell lysate protein from MCF-10A cells in the absence or presence of 10 μM diphenyleneiodonium (DPI) or 1000 U/ml catalase. Data are expressed as mean \pm SEM ($n = 3$).

Figure 23.

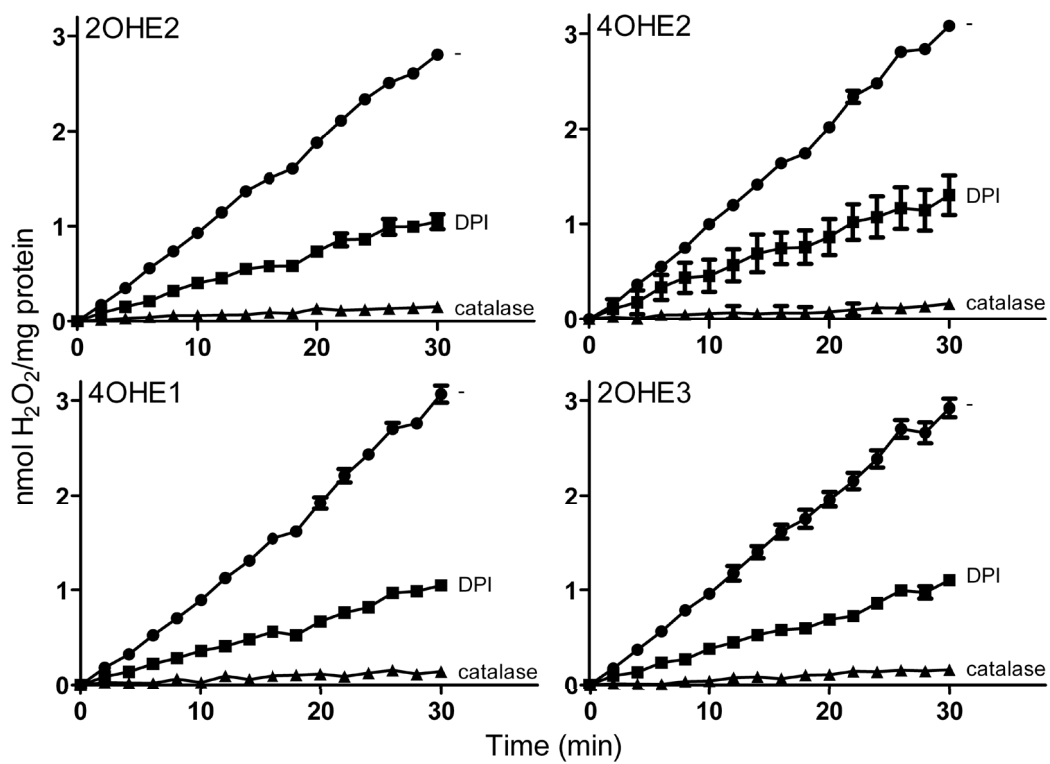


Figure 24. Hydroxyl radical production by redox cycling of estrogen metabolites. Assay mixtures contained 100 μ M NADPH, 100 μ M FeCl₃, 110 μ M EDTA, 50 μ g/ml cell lysate protein from MCF-7, MDA-MB-231 or MCF-10A cells and increasing concentrations of either E2, 2OHE2, 4OHE2, or 2MeOE2. Data are expressed as mean \pm SEM (n = 3).

Figure 24.

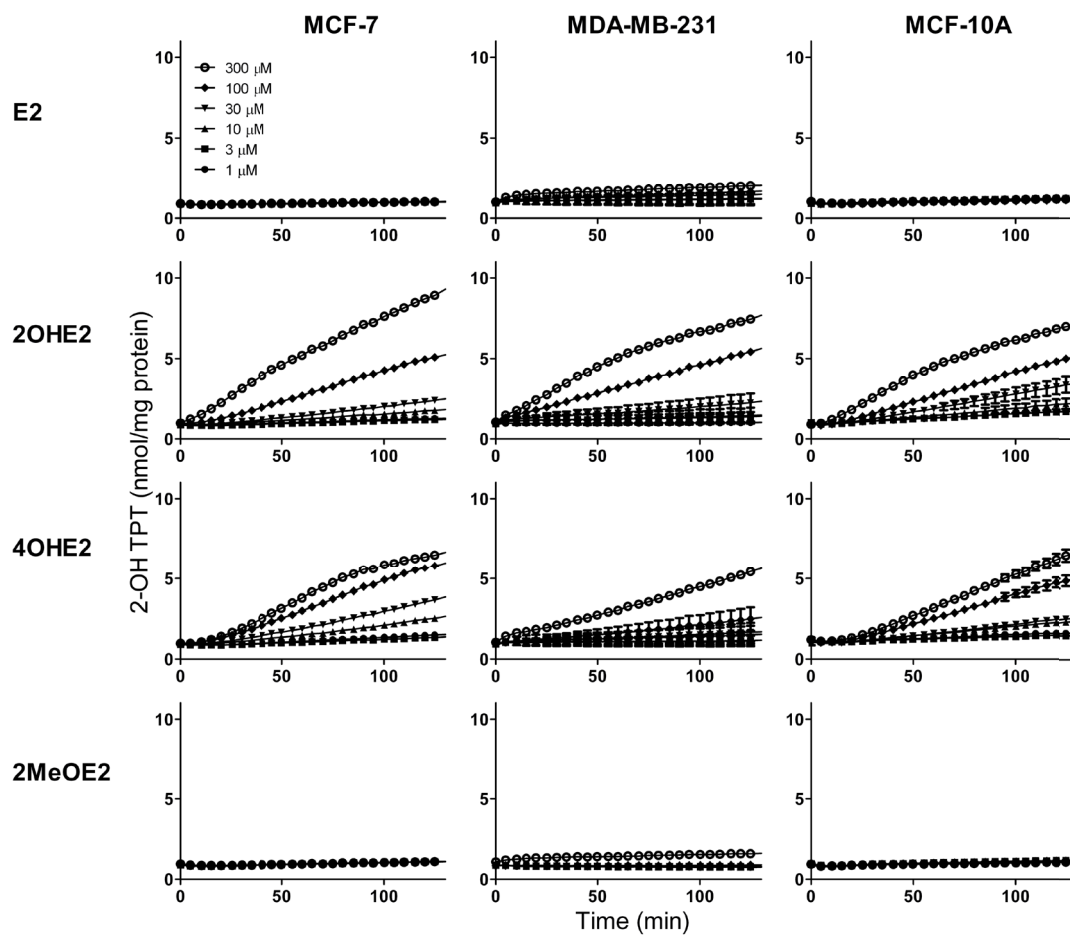


Figure 25. Rates of hydroxyl radical production by redox cycling of estrogen metabolites.

Assay mixtures contained 100 μ M NADPH, 100 μ M FeCl₃, 110 μ M EDTA, 50 μ g/ml cell lysate protein from MCF-7 (upper panel), MDA-MB-231 (center panel), or MCF-10A cells (lower panel), and increasing concentrations of 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Data are expressed as mean \pm SEM (n = 3).

Figure 25.

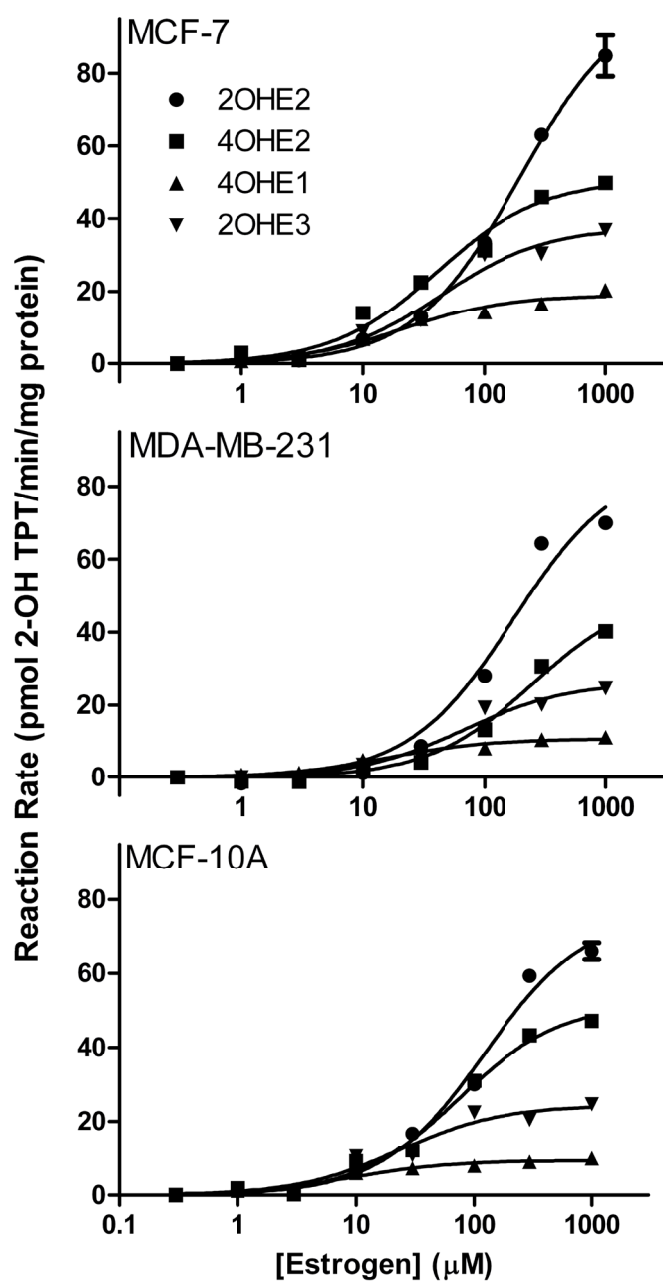


Figure 26. Effects of inhibitors on hydroxyl radical production during catechol estrogen redox cycling. Assay mixtures contained 100 μ M NADPH, 100 μ M FeCl₃, 110 μ M EDTA, 50 μ g/ml MCF-10A cell lysate protein, and 10 μ M 2OHE2, 4OHE2, 4OHE1, or 2OHE3. Where indicated, assays also contained 10 μ M DPI, 1000 U/ml catalase or 1% DMSO. Data are expressed as mean \pm SEM (n = 3).

Figure 26.

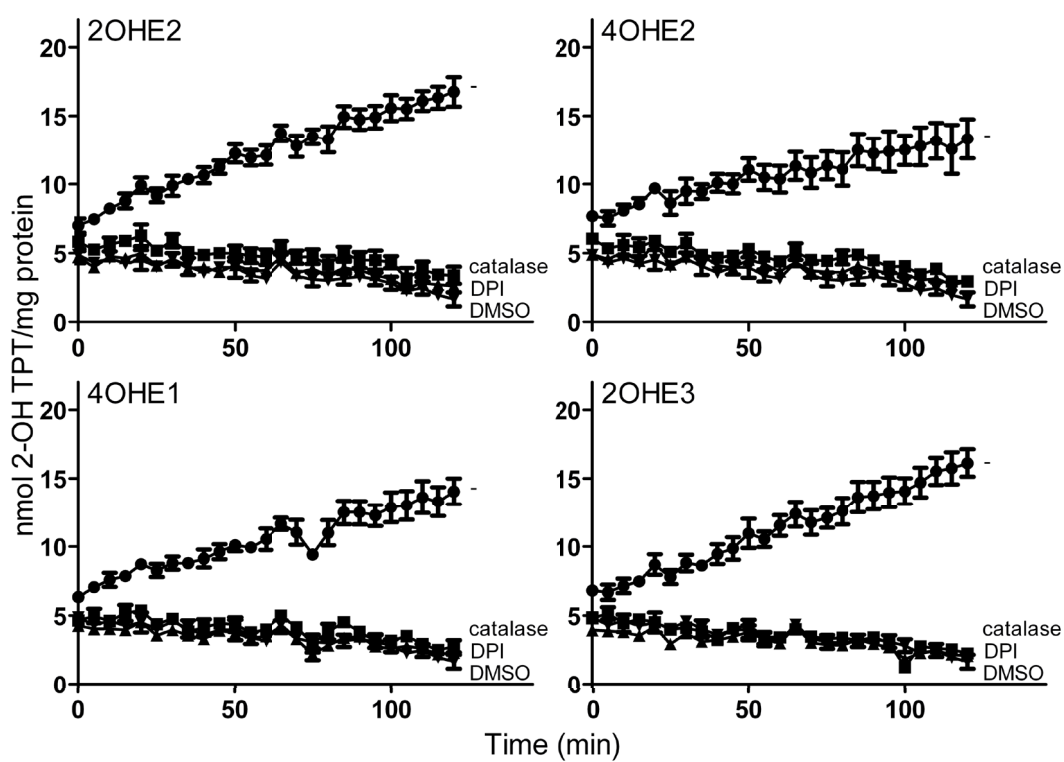


Figure 27. Measurement of H_2O_2 release by cells using an electrochemical microsensor.

Panel A, microsensor placed near the cells. *Panel B*, calibration of microsensor following the addition of H_2O_2 to the buffer. Removal of the H_2O_2 by the addition of catalase reduced the signal to background. Each microsensor is custom fabricated, resulting in unique signal-to-noise characteristics. *Panel C*, calibration curve of H_2O_2 in buffer. Note that the response is linear up to at least 30 μM .

Figure 27.

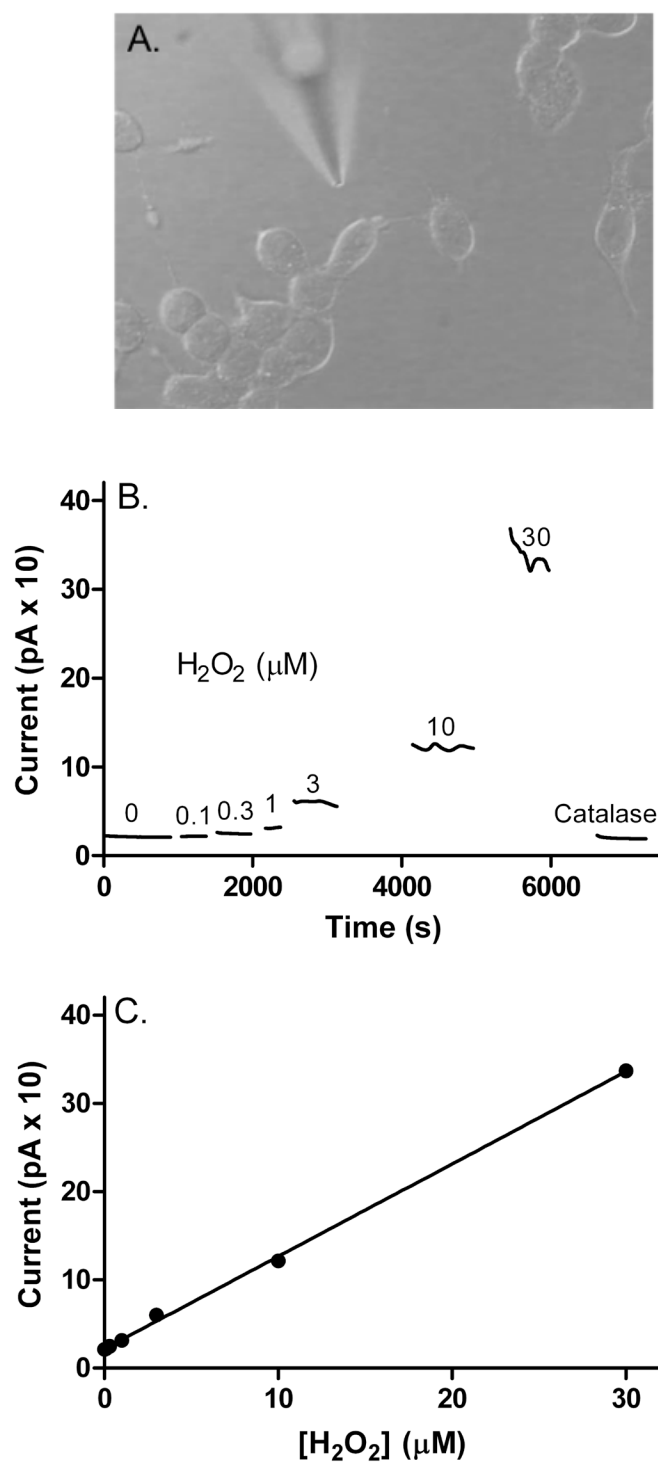


Figure 28. Catechol estrogen metabolites stimulate H_2O_2 release by MCF-10A cells.

Intact cells were treated with the parent- (E2, E1, or E3), methoxy- (2MeOE2), or catechol estrogens (2OHE2, 4OHE2, 4OHE1, 2OHE3) and then analyzed for extracellular H_2O_2 release using a H_2O_2 microsensor. Note that H_2O_2 was only detected near cells treated with the dose response of catechol estrogens.

Figure 28.

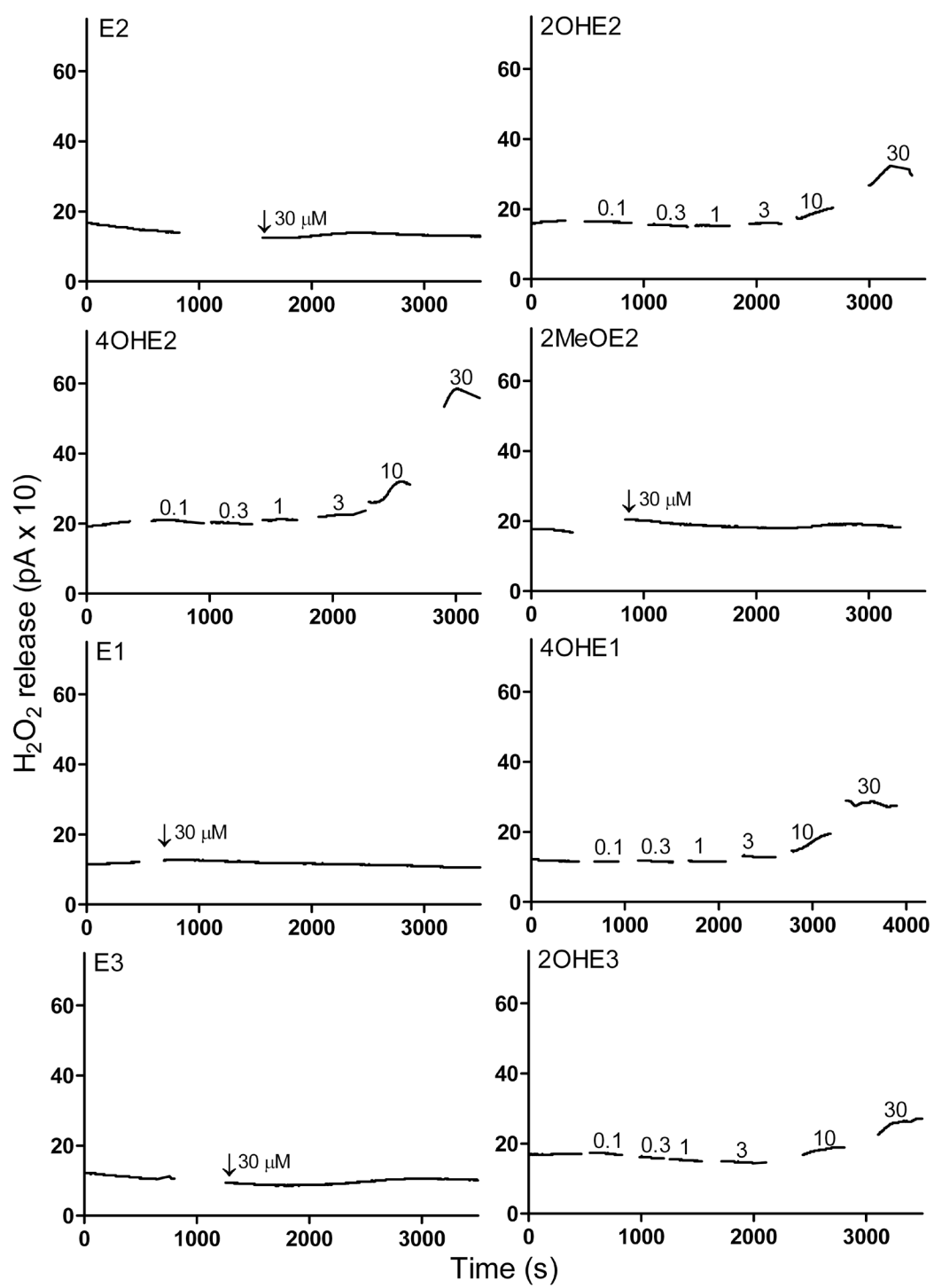


Figure 29. Comparison of H_2O_2 release by breast tumor cell lines. H_2O_2 released by intact MCF-7, MDA-MB-231, or MCF-10A cells treated with catechol estrogens was assayed using a microsensor.

Figure 29.

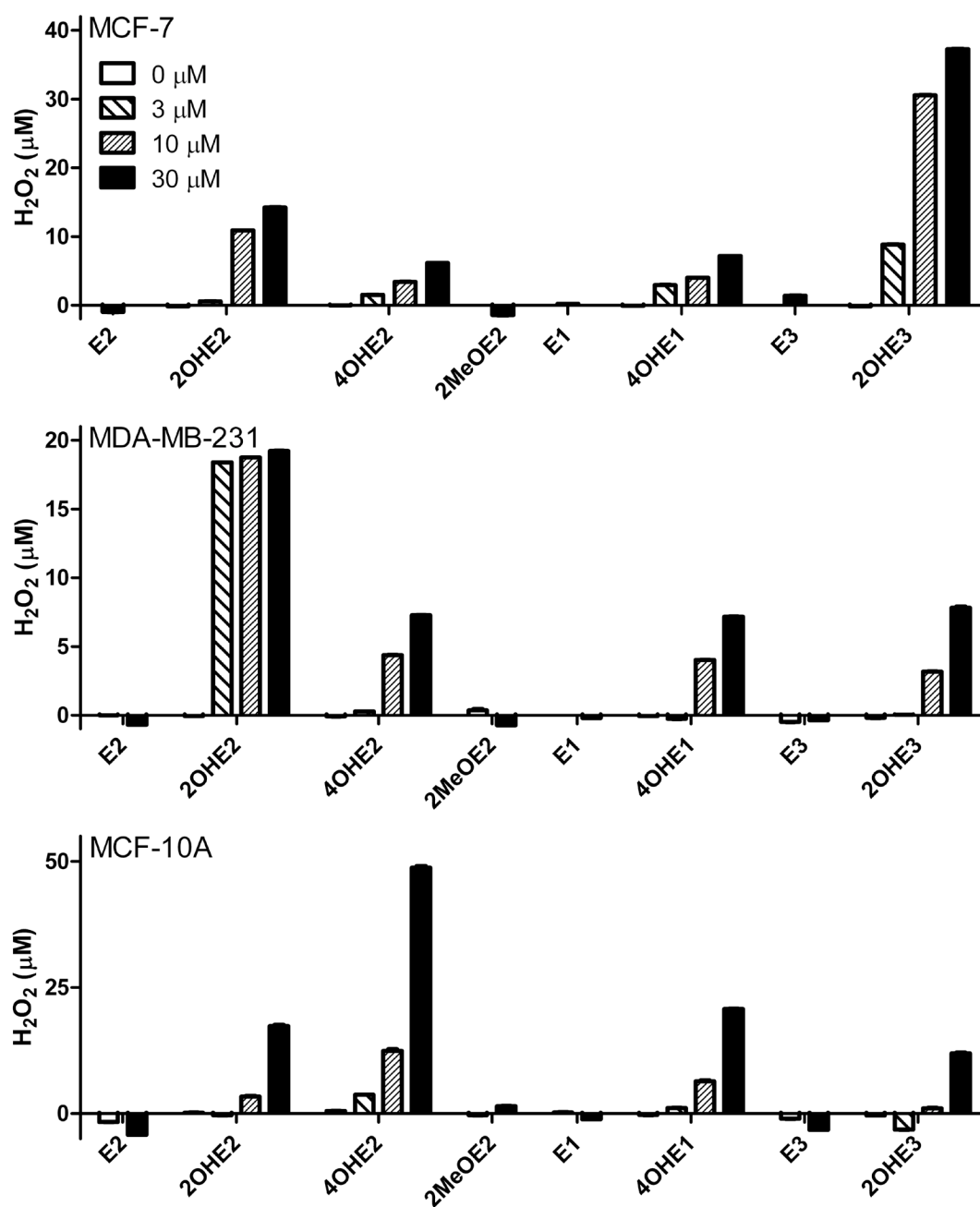


Figure 30. Catechol estrogen metabolites stimulate H_2O_2 release by MCF-7 cells. Cells were treated with increasing concentrations of estrogens to stimulate H_2O_2 production. Data shown represent temporal averages of representative experiments (mean \pm SEM (n \geq 182))

Figure 30.

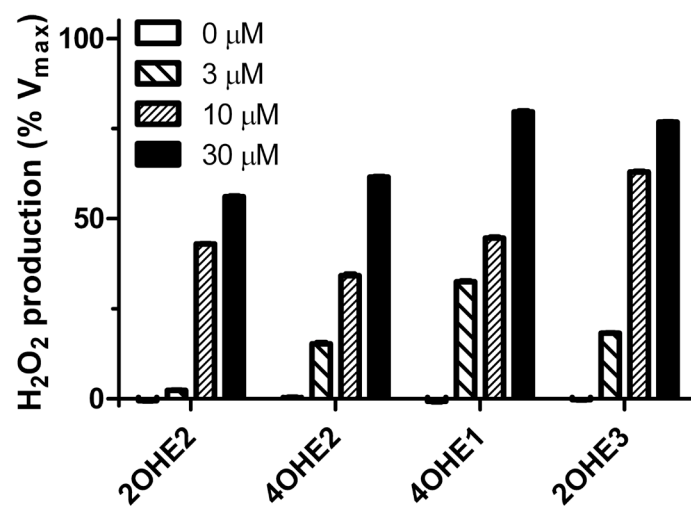
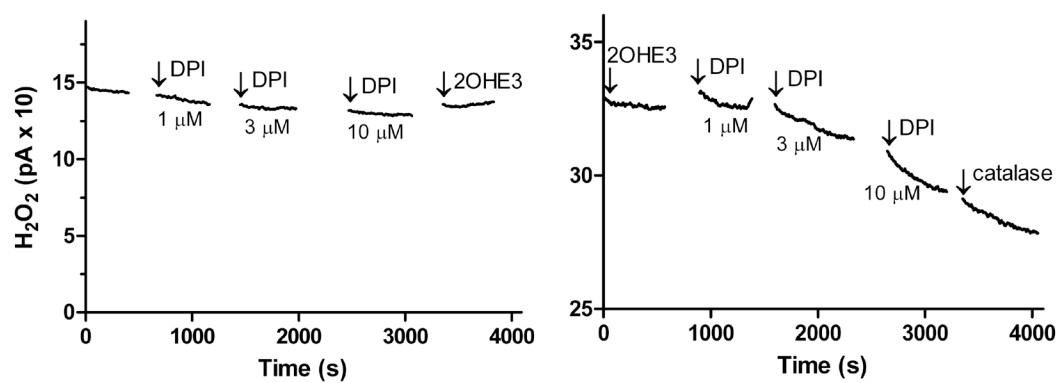


Figure 31. Effects of inhibitors on H₂O₂ generation by MDA-MB-231 cells. Intact cells were treated with increasing concentrations of the flavoenzyme inhibitor DPI before (left panel) or after (right panel) the addition of 10 μ M 2OHE3. Note that in the left panel DPI prevented 2OHE3-stimulated H₂O₂ production, while in the right panel DPI inhibits 2OHE3-stimulated H₂O₂ generation. Sample data shown.

Figure 31.



CURRICULUM VITAE

Karma Fussell

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Skills and Techniques

All the standard molecular biology and biochemistry techniques, including HPLC, tandem mass spectrometry, enzymology and enzyme kinetics, agarose gels, pull-down assays, 1D and 2D electrophoresis, immunoblotting, ELISA, EIA, PCR and QPCR, cell culture, tissue histology, immunostaining, electrochemical microsensors, fluorescence microwell assays, and more. I speak fluent English and conversational German.

Professional Experience

Graduate Student, Rutgers University, New Brunswick/Piscataway, NJ, August 2006-current

- Researched the mechanisms of toxicity of reactive oxygen and nitrogen species (Laboratories of Jeffrey Laskin and Michael Gallo). Investigated the biochemical modes of toxicity of oxidative stressors, including chemicals that redox cycle and non-ionizing radiation.

Visiting Scientist, Marine Biological Laboratory, Woods Hole, MA, summers of 2007-2009

- Studied the dynamic change in oxygen consumption of living cells in culture in response to xenobiotic stressors using a self-referencing microelectrode assembly (Laboratories of Jeffrey Laskin and Diane Heck).

Teaching Assistant, Rutgers University, New Brunswick/Piscataway, NJ August 2006-May 2009

- Assisted faculty with classroom setup, exam preparation, grading, and technical support for smart classrooms. Aided students with questions.

Associate Scientist, RapidFire™, Biotrove Inc., Woburn, MA February 2004-August 2006

- Developed 5 cytochrome P450 drug-drug interaction assays and contributed significantly to the development of another 13 custom assay protocols for clients. Used three proprietary RapidFire ultra high throughput robotic HPLCs connected to three MDS-SciEx API 4000 mass spectrometers to screen over 7 million drug library compounds for drug efficacy.

Undergraduate Fellow, Pharmaceutical Science Department, University of Montana, Missoula, MT, May 2001-May 2003

- Developed an enzymatic assay to measure inhibition of thermolysin by α -aminophosphothioic acid derivatives.

Teaching Assistant, Department of Chemistry, Gonzaga University, Spokane, WA, 2000-2003

- Assisted professor with laboratory preparations, grading papers, and aiding students.

Education

Bachelor of Science, Chemistry and Biochemistry, Gonzaga University, Spokane, WA, May 2003

Doctor of Philosophy, Toxicology, Rutgers University/ University of Medicine and Dentistry of New Jersey, Piscataway, NJ, defended, September 2010

Research Interests

Currently I am working in the laboratories of Drs. Jeffrey Laskin and Michael Gallo studying the toxic effects of reactive oxygen and nitrogen species on living cells in culture. Our laboratory investigates the biochemical modes of toxicity of oxidative stressors, including both endogenous and exogenous chemicals which redox cycle and non-ionizing radiation.

Professional Affiliations

New York Academy of Science, Student Member, September 2009- current

Society of Toxicology, Student Member, January 2007-current

Molecular Biology Specialty Section of the Society of Toxicology, Student Member, January 2008-current

Mid-Atlantic Society of Toxicology, Student Member, January 2007-current

American Chemical Society, Member 2002-2003

Gonzaga University Chapter - American Chemical Society, Founding Member 2002-2003

Professional Development

Mid-Atlantic Society of Toxicology Regional Meeting "Epigenetics: Toxic Responses and Disease", Bordentown, NJ, November 2010

Mid-Atlantic Society of Toxicology Regional Meeting "Toxicants in the News", Iselin, NJ, May 2010

Society of Toxicology 49th Annual Meeting, Salt Lake City, UT March 2010

Mid-Atlantic Society of Toxicology Regional Meeting "Metabolic Syndrome and Related Conditions: Pathogenesis to Therapy", Bordentown, NJ, October 2009

Gordon Research Conference "Hormone Action in Development and Cancer", Holderness, NH, July 2009

Mid-Atlantic Society of Toxicology Regional Meeting "Toxicants in the News", Iselin, NJ, May 2009

Society of Toxicology 48th Annual Meeting, Baltimore, MD March, 2009

Mid-Atlantic Society of Toxicology Regional Meeting "Toxicology in the Mid-Atlantic Region: Hot Topics, New Developments & Emerging Issues", Iselin, NJ, October 2008

Mid-Atlantic Society of Toxicology Regional Meeting "Sex-Differences and/or Polymorphisms, Toxic Insult and Susceptibility", Bordentown, NJ, May 2008

Society of Toxicology 47th Annual Meeting, Seattle, WA March 2008

Mid-Atlantic Society of Toxicology Regional Meeting "Toxics to Therapeutics", Piscataway, NJ, May 2007

225th American Chemical Society National Meeting, New Orleans, LA, March 2003

Publications and Presentations

Patents:

Fussell, K. C., J. D. Laskin. A Sensitive High-throughput Method for DNA Damage and Repair. *Patent pending*

Publications:

Fussell, K. C. Redox Cycling by Catechol Metabolites of Endogenous Estrogens as Potential Contributors to the Initiation of Breast Cancer. *Ph.D. Dissertation defended September 2010.*

Fussell, K. C., J. P. Gray, P. J. S. Smith, D. E. Heck, J. D. Laskin. Redox Cycling and Increased Oxygen Utilization Contributes to Oxidative Stress and Cytotoxicity in Chinese Hamster Ovary cells overexpressing NADPH-cytochrome P450 Reductase. *Free Radical Biology and Medicine* **50** (7) 874 - 882 (2011)

Fussell, K. C., R. G. Udasin, P. J. S. Smith, M. A. Gallo, J. D. Laskin. Characterization of Redox Cycling and Generation of Reactive Oxygen Species by Catechol Estrogen Metabolites in Breast Epithelial Cells. *Manuscript under review*

Fussell, K. C., R. G. Udasin, Y. Wang, M. A. Gallo, J. D. Laskin. Role of cytochrome P450 reductase and cytochrome b₅ reductase in catechol estrogen redox cycling. *Manuscript in process*

Fussell, K. C., J. D. Laskin. A novel sensitive high-throughput assay to identify DNA and repair. *Manuscript in process*

Abstracts:

Udasin, R. G., K. C. Fussell, Y. Wang, V. Mishin, D. E. Heck, P. J. S. Smith, D. L. Laskin, J. D. Laskin. Hydroxyl Radical Formation by Redox Cycling Of Quinones in Diesel Exhaust Particles. *Presented at the 2011 annual meeting of the Society of Toxicology in Washington, DC.*

Fussell, K. C., R. G. Udasin, P. J. S. Smith, M. A. Gallo, J. D. Laskin. Redox cycling by endogenous 2- and 4-hydroxyestrogen catechol metabolites is associated with oxidative stress in human breast epithelial cell lines. *Presented at the 2010 annual meeting of the Society of Toxicology in Salt Lake City, UT and the Spring 2010 meeting of the Mid-Atlantic Society of Toxicology, Iselin, NJ.*

Udasin, R. G., K. C. Fussell, Y. Wang, D. E. Heck, V. Mishin, P. J. S. Smith, D. L. Laskin, J. D. Laskin. Role of cytochrome P450 reductase in mediating redox cycling of 9,10-phenanthrenequinone. *Presented at the 2010 annual meeting of the Society of Toxicology in Salt Lake City, UT.*

Fussell, K. C., M. A. Gallo, J. D. Laskin. Role of catechol estrogen redox cycling in mediating oxidative stress in breast epithelial cells. *Presented at the Fourth International Conference on Oxidative/Nitrosative Stress and Disease in New York, NY, October 28 2009.*

Udasin, R. G., K. C. Fussell, V. Mishin, J. D. Laskin. A high-throughput method to specifically measure hydroxyl radical formation by the Fenton reaction. *Presented at the Fourth International Conference on Oxidative/Nitrosative Stress and Disease in New York, NY, October 28 2009.*

Fussell, K. C., M. A. Gallo, J. D. Laskin. Redox cycling and production of reactive oxygen intermediates by 2- and 4-hydroxyestrogen catechol metabolites is associated with oxidative damage in breast epithelial cells. *Presented at the 2009 Gordon Conference on Hormone Action in Development and Carcinogenesis in Holderness, NH.*

Fussell, K. C., M. A. Gallo, J. D. Laskin. NADPH-cytochrome P450 oxidoreductase mediates redox cycling by 2- and 4-hydroxyestrogen catechol metabolites. *Presented at the 2009 annual NJCCR-New Jersey Cancer Retreat in Piscataway, NJ.; and at the Spring 2009 meeting of the Mid-Atlantic Society of Toxicology in Iselin, NJ.*

Fussell, K. C., M. A. Gallo, J. D. Laskin. NADPH-cytochrome P450 oxidoreductase mediates redox cycling by 2- and 4-hydroxyestradiol catechol metabolites. *Presented at the 2009 annual meeting of the Society of Toxicology in Baltimore, MD.*

Fussell, K. C., J. P. Gray, P. J. S. Smith, D. E. Heck, J. D. Laskin. Increased oxygen utilization and oxidative stress in CHO cells during diquat redox cycling. *Presented at the 2008 annual meeting of the Society of Toxicology in Seattle, WA.; and at the Spring 2008 meeting of the Mid-Atlantic Society of Toxicology in Bordentown, NJ.*

Gray, J. P., E. Heart, K. Fussell, S. Jamil, P. J. S. Smith, and J. D. Laskin. Intracellular acidification promotes alloxan-induced toxicity in pancreatic islets in vitro. *Presented at the 2008 annual meeting of the Society of Toxicology in Seattle, WA.*

Gray, J. P., K. Fussell, S. Jamil, P. J. S. Smith, E. Heart, and J. D. Laskin. Intracellular acidification promotes alloxan-induced toxicity in pancreatic islets in vitro. *Presented at the Fall 2007 meeting of the Boston-Ithaca Islet Club, Burlington, VT.*

Özbal, C., W. LaMarr and K. Fussell. "Label-Free Screening by High-Throughput Mass Spectrometry," *Presented at the Tenth Annual Society for Biomolecular Screening Conference, Orlando, FL, 2005*

Thompson, C.M., K.C. Fussell, T.M. Voelker, P. Tongcharoensirikul, A.I. Suarez. "Development of a rapid high-performance liquid chromatography-mass spectrometry assay for the inhibition of thermolysin by enantio-enriched APTA analogs," *Presented at the 225th American Chemical Society National Meeting, New Orleans, LA, 2003*

Awards and Service

Honorable Mention Student Poster Competition, Molecular Biology Specialty Section, Society of Toxicology Annual Meeting 2010, Salt Lake City, UT

Second Place Student Poster Competition, Mid-Atlantic Society of Toxicology Spring Meeting 2009

Student Representative 2009-2011 Molecular Biology Specialty Section of the Society of Toxicology