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We have previously reported that 17β-Estradiol (17β-E_2) can protect human lens epithelial cells against oxidative stress by preserving mitochondrial function, acting as a positive regulator of the MAPK signal transduction pathway. While pERK plays a significant role in stabilizing the inner mitochondrial membrane to maintain the mitochondrial membrane potential during oxidative stress, the protective mechanisms activated by 17β-E_2 are probably multifactorial acting via both genomic and non genomic pathways. This study examined the effects of 17β-E_2 on the expression and activity of MnSOD, which is present exclusively in the mitochondria, as a possible mechanism by which it affords protection against oxidative stress. Our results demonstrate that 17β-E_2 rapidly increases the activity of MnSOD in a time dependent manner. This augmentation of activity of MnSOD by17β-E_2 is seen in the absence of a corresponding increase in the mRNA and protein expression, thereby indicating that it is a non genomic response. A better understanding of the mechanisms by which estrogens protect the cells against oxidative stress will help us in developing estrogens to be useful therapies for the prevention of cataract in postmenopausal women and non feminizing estrogens may provide similar protection in men.

MODULATION OF MANGANESE SUPEROXIDE DISMUTASE BY

17-BETA ESTRADIOL

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Modulation of manganese superoxide dismutase activity by 17-β estradiol

THESIS

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CHAPTER 1

Background

Cataract, a leading cause of blindness worldwide, is a multifactorial eye disease [1]. Cataract formation represents a serious problem in the elderly, with approximately 25% of the population aged >65 years and about 50% aged 80 years experiencing a serious loss of vision as a result of this condition [2]. In the United States, approximately 1.35 million cataract surgeries are performed annually at a cost of more than $3 billion [3] and this accounts for 12% of all medicare expenses in U.S [4] thus representing a large financial burden on the health care systems. Surgical removal of cataract may not represent the optimal solution. Although generally recognized as one of the safest operations, there is a significant complication rate associated with this surgical procedure. Some complications include opacification of the posterior lens capsule (30-50%), retinal detachments (0.8%), corneal problems (1%) and endopthalmitis (0.1%). Although the risks are very small, the large number of procedures performed means that approximately 30,000 individuals develop serious complications as a result of cataract surgery in US alone. Thus, there is a need for pharmaceutical agents which prevent or delay the onset of cataract [2].

A. Oxidative Stress and Cataract

Oxidative stress, which refers to cellular damage caused by reactive oxygen species

(ROS), has been implicated in many disease processes, especially age related disorders including cardiovascular diseases, arthritis, brain dysfunction, emphysema and cataract [5]. ROS are constantly produced intracellularly as a result of normal metabolic activity. Cells of the body may also be exposed to exogenous pollutants, chemicals and environmental radiations, which generate high levels of intracellular ROS and produce a state of intracellular oxidative stress [6]. ROS are oxygen containing molecules that have higher chemical reactivity than ground state oxygen. Though the primary ROS made by mitochondria is superoxide radical [O^{2-} ; 7] other ROS include hydroxyl radical ($HO^{\cdot}$), and molecules such as singlet oxygen and hydrogen peroxide.

Lens cells are particularly subject to oxidative stress due to photo-oxidative processes and are consequently prone to cellular oxidative damage [8]. Furthermore the fiber cells of the lens are not renewed and therefore have to last a lifetime. A number of publications suggest that oxidative stress leads to modification of a number of cellular constituents such as proteins, cytoskeletal elements, glutathione levels and cause alterations in transport systems in the epithelium resulting in lens opacification and cataracts [9]. Different agents are thought to cause oxidative damage in the lens, leading to cataractogenesis. The oxidative agent hydrogen peroxide (H_2O_2) is present at concentrations of 20-30μM in normal aqueous humor and is reported to be raised (up to 660μM) in patients with cataract [10]. In vitro lens culture experiments have demonstrated that H_2O_2 at these high concentrations caused lens opacification and produced a pattern of oxidative damage similar to that found in human cataract. Lipid peroxidation (LPO) due to oxidative stress has been implicated in human cataract and

lens opacity because the toxic peroxidation products induce fragmentation of soluble lens proteins and damage vital membrane structures leading to changes in the refractive properties of the lens. [11,12]. It is known that lipid peroxides undergo degradation to form toxic reactive aldehydes, such as 4-Hydroxynonenal (HNE). It has been reported that HNE can mediate oxidative stress-induced cell death in many cell types including lens epithelial cells [13]. Accumulation of sugar metabolites within the lens and glycation of proteins can also lead to the development of cataracts. Autoxidation of sugars is regarded as a source of ROS, which along with glycation products cause an alteration in the refractive properties of the lens ultimately leading to cataract formation [14,15].

B. Oxidative stress and Mitochondria

Mitochondrial membrane potential is the central parameter that controls respiratory rate, ATP synthesis and the generation of reactive oxygen species. The fundamental functions of ATP generation, Ca^{2+} uptake and storage, and the generation and detoxification of ROS, are driven by the mitochondrial membrane potential, $\Delta\psi_m$. Many apoptotic signals initiate apoptosis by disturbing mitochondrial function. Mitochondria are a major source of ROS which formed as a toxic by product of ATP synthesis. Oxygen radicals, in turn, damage the mitochondria and the surrounding cell. When sufficient oxidative damage accumulates in the mitochondria and the cell, the cell dies. Hence, the chronic level of mitochondrial oxidative stress is believed to help determine an individuals aging rate and susceptibility to a variety of age related diseases such as cardiovascular disease, stroke, diabetes, memory loss, forms of deafness and cataract formation. Mitochondria have been found to be especially susceptible to oxidative damage. Oxidant damage to the

mitochondria can cause release of calcium, protein oxidation, depletion of ATP, lipid peroxidation,DNA damage and cytochrome c release leading to apoptosis. A vicious circle of oxidative stress and damage to cellular structures can lead to either cell death by apoptosis or to a cellular energetic decline and ageing [16,17].

C. ROS and Antioxidative Enzymes

The primary antioxidant enzymes that protect cells from oxidative damage include the Superoxide dismutase (SOD) family, catalase (CAT) and Glutathione (GSH) family which includes Glutathione peroxidase (GPX) and Glutathione reductase (GR). Among these groups of antioxidant enzymes that provide a defense system to protect cells against oxidative stress, SOD is one of the key enzymes that detoxifies the O^{2-} radical and generates H_2O_2 which is further broken down into water by Glutathione peroxidase (GPX)

and catalase (9, Figure 1-1).

Superoxide Dismutases:

At least three isoforms of SOD have been found in eukaryotes. Mitochondrial manganese SOD (MnSOD) is a tetrameric protein that is localized primarily in the mitochondrial matrix [18] and its purpose in this location is to remove O^{2-}, which is generated by leakage of single electrons from the electron transport chain. It is encoded by nuclear a gene and is made as a precursor protein which is transported to the mitochondria after post translational modification. This protein is further clipped to a 24 Kd protein in the mitochondria.

Copper/Zinc SOD (Cu/ZnSOD) is a dimeric protein that is localized primarily in the

cytoplasm (19) and nucleus and is thought to remove O^{2-} generated by ER and cytosolic as well as membrane oxidases. Recent literature suggeststhat it is also present in the mitochondrial intermembrane space and is thought to remove O^{2-} which is generated by complex III of the mitochondrial electron transport chain.

The third isoform of SOD, Extracellular SOD (ecSOD) is a tetrameric protein that is found in the extracellular space and may be important in removing O^{2-} generated by membrane related oxidases [20].

Reaction:

$$O^{2-} + O^{2-} + 2\,H^{+} \xrightarrow{SOD} H_2O_2 + O_2$$

Glutathione Peroxidase and Glutathione Reductase:

Glutathione peroxidase catalyzes the reduction of intracellular hydroperoxides including hydrogen peroxide and protects the cell from oxidative damage. Glutathione peroxidase reduces H_2O_2 to H_2O by oxidizing glutathione (GSH) (Equation A). Reduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase (Equation B).

Reaction:

A) $$R\text{-}O\text{-}O\text{-}H + 2GSH \xrightarrow{GPX1} R\text{-}O\text{-}H + GSSG + H_2O$$

B) $$GSSG + NADPH + H^{+} \xrightarrow{GR} 2GSH + NADP^{+}$$

GPX 1(Cellular GPX)

As the first discovered GPX family, the classical cytosolic/mitochondrial GPX1 (cGPX) is a selenium-dependent enzyme. With the main role of regulating intracellular ROS, GPX 1 is known to prevent sclerosis of the arteries.

GPX 2 (gastrointestinal GPX)

Known as gastrointestinal GPX (GI-GPX), GPX 2 is an intracellular enzyme expressed only at the epithelium of the gastrointestinal tract

GPX 3 (extracellular GPX)

Extracellular plasma GPX (pGPX or GPX3) is mainly expressed by the kidney from where it is released into the blood circulation

GPX 4 (Phospholipid hydroperoxide)

Expressed in most tissues, GPX 4 can reduce many hydroperoxides including hydroperoxides integrated in membranes and hydroperoxy lipids in low density lipoprotein or thymine. Different from other GPX isoforms, which have tetrameric structures, GPX 4 is monomer.

Catalase:

An enzyme that brings about (catalyzes) the reaction by which hydrogen peroxide is decomposed to water and oxygen. Catalase prevents the accumulation of and protects the body tissues from damage by peroxide. In most mammalian cell types, catalase is exclusively found in the perixisomes. It is not clear to what extent catalase is involved in detoxifying hydrogen peroxide in the cytosoplasm [21]

Reaction:

$2H_2O_2 \rightarrow 2H_2O + O_2$

D. Estrogen and Lens

Recent epidemiological studies have shown that there is a higher incidence of cataract formation in postmenopausal women as compared to men of the same age, suggesting that the absence of estrogens may contribute to the increased risk [22]. The Beaver dam eye study [23] and Salisbury eye study [24] have both indicated a protective association between the use of estrogen and the risk of cataract development. The results of these epidemiologic studies indicate that estrogen replacement therapy in postmenopausal women is associated with a reduced risk of cataract formation. These epidemiologic findings are further supported by various studies using tissue culture and rodent models [25,26]. It has been reported that there is an increased formation of cataract in transgenic mice expressing a dominant negative form of the estrogen receptor (ER) supporting the notion that inhibition of estrogen action promotes cataractogenesis. Using methylnitrosourea (MNU) treated ovariectomized rats, as a model for cataractogenesis in postmenopausal women, it has been demonstrated that estrogen treatment diminished the incidence of cataract formation. In cultured rat lenses, estrogen protected lenses against cataracts induced by transforming growth factor-β [TGFβ, 25].

Estrogen exerts its effects via both genomic and non genomic actions. These so-called "non-genomic" effects are independent of gene transcription or protein synthesis and involve steroid-induced modulation of cytoplasmic or of cell membrane-bound regulatory proteins. Estrogen receptors act via the regulation of transcriptional processes, involving

nuclear translocation and binding to specific response elements, thus leading to regulation of target gene expression, called the genomic actions of estrogen.

Previous data from our laboratory indicate that cell death induced by H_2O_2 in cultured human lens epithelial cells is associated with accumulation of intracellular ROS, collapse of mitochondrial membrane potential ($\Delta\psi_m$), and depletion of ATP, and both 17α- and 17ß-E_2 can preserve mitochondrial function, cell viability, and ATP levels in human lens cells during oxidative stress [27]. More recent studies from our lab demonstrate that 17β-E_2 stabilizes $\Delta\psi_m$ in cultured human and bovine lens epithelial cells, acting as a positive regulator of the MAPK signal transduction pathway. A positive correlation was observed between the relative degree of ERK phosphorylation and attenuation of mitochondrial membrane depolarization, regardless of acute oxidative stress [28]. Further Studies in our lab demonstrate that when ERK2 is silenced using small interfering RNA (siRNA), there is a significant increase in mitochondrial depolarization in HLE-B3 cells and this depolarization was prevented by pretreatment with 17β-E_2 [29]. These studies indicate that, though ERK2 plays a regulatory role on mitochondrial membrane potential, estrogen blocks the mitochondrial membrane depolarization via an ERK independent mechanism. While data from our lab indicates a significant role of pERK in stabilizing the inner mitochondrial membrane to maintain $\Delta\psi_m$ during oxidative stress, the protective mechanisms activated by 17β-E_2 are likely to be multifactorial and function by both genomic and non-genomic pathways. These different mechanisms then integrate at the mitochondria and regulate the mitochondrial defense state in order to advance anti-oxidative protection.

E. MnSOD and Estrogens

The mitochondrial enzyme MnSOD may be involved in protecting the lens against free-radical–induced injury. It has been reported that interleukin (IL)-1, tumor necrosis factor (TNF)-α, radiation, which are mediators of oxidative stress can induce MnSOD. It has been demonstrated that MnSOD exerts a protective effect against H_2O_2 induced oxidative stress in cultured lens epithelial cell line SRA 01/04. This effect of MnSOD was studied by up and down regulating the enzyme levels. Cells with elevated enzyme levels were more resistant to the cytotoxic effect of H_2O_2 and demonstrated greater cell viability. On the other hand MnSOD-deficient cells showed dramatic mitochondrial damage when exposed to 50 μM H_2O_2 for 1 hour. When these cells were oxidatively challenged by H_2O_2, photochemically generated reactive oxygen species, or UVB irradiation there were a greater number of DNA strand breaks in MnSOD-deficient cells than in those in which the enzyme was upregulated [9]. These findings demonstrate the protective effect of MnSOD in antioxidant defense of cultured lens epithelial cells. MnSOD –deficient cells showed dramatic mitochondrial damage, cytochrome C leakage, caspase 3 activation and increased apoptotic cell death when they were challenged with O^{2-}. A number of studies demonstrate the effect of estrogens on the RNA, protein and the activity levels of MnSOD [30]. There have been some studies on the effect of 17β-E_2 on the expression and activity of MnSOD [7,31,32,33]. These studies indicate a positive effect of estrogens on the expression and activity of MnSOD. In this study we have tried to determine whether 17β-E_2 has a similar effect on the modulation of MnSOD in HLE-B3 cells.

F. Significance

Although the effect of estrogens on antioxidant genes have been elucidated in other cell lines, similar studies have yet to be performed in human lens cells to demonstrate the relation between estrogens and antioxidant genes. Data from our lab demonstrates that 17β-E_2 protects against oxidative stress by providing mitochondrial stabilization. One of the aspects our lab had investigated was the effect of estrogens on the MAPK/ERK pathway. It was demonstrated that estrogen upregulates the MAPK pathway and stabilizes $\Delta\psi_m$ in cultured human and bovine lens epithelial cells. We have recently demonstrated that estrogen can stabilize the mitochondrial membrane potential independent of ERK stimulation. These results indicate that the protective mechanisms activated by 17β-E_2 are probably multifactorial, acting via both genomic and non genomic pathways to stabilize the inner mitochondrial membrane and maintaining $\Delta\psi_m$ during oxidative stress. The overall goal of this study is to have a better understanding of the mechanism by which 17β-E_2 exerts its effects. In the current study we seek to examine one of the possible ways by which estrogens may be stabilizing the mitochondria, thereby protecting the cells against oxidative stress. This will help us in developing estrogens to be useful therapies for the prevention of cataract in postmenopausal women and non feminizing estrogens may provide similar protection in men.

REFERENCES:

1. Harding JJ. Pharmacological treatment strategies in age related cataracts. Drugs aging 1992; 4: 287-300.

2. Babizhayev MA, Deyev AI, Yermakova VN, Brikman IV, BoursJ. Lipid peroxidation and cataracts: N-acetylcarnosine as a therapeutic tool to manage age-related cataracts in human and in canine eyes Drugs R D 2004; 3:125-39.

3. Steinberg EP, Javitt JC, Sharkey PD, et al. The content and cost of cataract surgery. Arch opthalmol 1993; 111:1041-1049.

4. John RH, James FH, Quingling H. Identification and functional clustering of global gene expression differences between human age-related cataract and clear lenses. Molecular vision 2003; 9:515-37.

5. Ames BN, Shigenaga MK, Hagen TM, Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Natl Acad. Sci. USA 1993; 90: 7915–7922.

6. Wetterhahn, KE, Dudek, EJ. Environmental metal carcinogens: genotoxicity by direct metal- mediated and indirect oxidative pathways. New J. Chem. 1996; 20:199-203

7. Strehlow K, Rotter S, Wassmann S, Adam O, Grohé C, Laufs K, Böhm M, Nickenig G. Modulation of antioxidant enzyme expression and function by estrogen. Circ Res, 2003; 93:170-7

8. Edward JD, Fu S, and Allen T. H_2O_2 mediated oxidative stress activates NF-kB in lens epithelial cells. Free Radical Biology & Medicine 2001; 31: 651-658.

9. Matsui M, Lin LR, Ho YS, Reddy VN. The effect of up- and downregulation of MnSOD enzyme on oxidative stress in human lens epithelial cells. Invest Ophthalmol Vis. Sci 2003; 44:3467–3475.

10. Spector A and Garner WH, Hydrogen peroxide and human cataract. Exp. Eye. Res 1981; 3: 673-681.

11. Babizhayev MA, Deyev AI., Linberg LF, Lipid peroxidation as a possible cause of cataract. Mech. Aging Dev 1988; 44: 69–89.

12. Awasthi S, Srivastava SK, Piper JT, Singhal SS, Chaubey M, Awasthi YC. Curcumin protects against 4-hydroxy-2-nonenalinduced cataract formation in rat lenses. Am. J. Clin. Nutr 1996;64: 761–766.

13. Choudhary S, Zhang W, Zhou F, Campbell GA, Chan LL,Thompson EB, Ansari NH. Cellular lipid peroxidation endproducts induce apoptosis in human lens epithelial cells. Free Radic.Biol. Med 2002;32: 360–369.

14. Monnier V. Non-enzymatic glycosilation, the Maillard reaction and the aging process. J. Geront 1990;45, B105–B111.

15. Thornalley P, Wolff S, Crabbe J, Stern A. The autoxidation of glyceraldehyde and other simple monosaccharides under physiological conditions catalysed by buffer ions. Biochim. Biophys. Acta 1984;797: 276–287

16. Nicholls DG. Mitochondrial membrane potential and aging. Aging Cell 2004;3:35-4017.

17. Crawford DR, Wang Y, Schools GP, Kochheiser J, Davies KJ. Down-regulation of mammalian mitochondrial RNAs during oxidative stress. Free Radic Biol Med 1997; 22:551-9.

18. Beckman JS, Carson M, Smith CD and Koppenol WH. ALS, SOD and peroxynitrite. Nature (Lond), 1993; 364: 584.

19. Chang LY, Slot JW, Geuze HJ, and Crapo JD. Molecular immunocytochemistry of the CuZn superoxide dismutase in rat hepatocytes. J. Cell Biol, 1998;107: 2169-2179

20. Zhang Y, Zhao W, Zhang HJ, Domann FE, and Oberley LW. Overexpression of copper zinc superoxide dismutase suppresses human glioma cell growth. J Cancer Research 2002; 62: 1205-1212

21. J.G. Scandalios. Oxidative stress: Molecular perception and transduction of signals triggering antioxidant gene defenses. Brazilian Journal of Medical and Biological Research 2005; 38: 995-1014

22. Klein BE, Klein R, Linton KL. Prevalance of age-related lens opacities in a population. The Beaver Dam Eye Study. Opthalmology. 1992;99: 546-552.

23. Klein BE, Klein R, Lee KE. Incidence of age related cataract: the beaver dam eye study. Arch opthalmol. 1998;116:219-225

24. Freeman EE, Munoz B, Schein OD, West SK. Hormone replacement therapy and lens opacities: the Salisbury eye evaluation project. Arch Opthalmol. 2001; 119: 1687-1692.

25. Hales AM, Chamberlain CG, Murphy CR, McAvoy JW. Estrogen protects lenses against cataract induced by transforming growth factor-beta (TGF-beta). J Exp Med 1997; 185: 273-280.

26. Bigbsy RM, Cardenas H, Caperell-Grant A, Grubbs CJ. Protective effects of estrogen in a rat model of age related cataracts. Proc Natl Acad Sci USA. 1999; 96: 9328-9332.

27. Wang J, Simpkins JW, Dykens JA, Cammarata PR. Oxidative damage to human lens epithelial cells in culture: estrogen protection of mitochondrial potential, ATP, and cell viability. Invest Ophthalmol Vis Sci 2003; 44:2067–2075.

28. Moor AN, Flynn JM, Gottipati S, Giblin FJ, Cammarata PR. 17beta-estradiol stimulates MAPK signaling pathway in human lens epithelial cell cultures preventing collapse of mitochondrial membrane potential during acute oxidative stress. Mitochondrion 2005; 5:235-47.

29. Flynn J, Lannigan D, Clark D, Garner M, Cammarata P. RNA suppression of ERK2 leads to collapse of mitochondrial membrane potential with acute oxidative stress in human lens epithelial cells. Am J Physiol Endocrinol Metab 2008: [Epub ahead of print]

30. V.N. Reddy, E. Kasahara, M. Hiraoka, L.R. Lin and Y.S. Ho, Effects of variation in superoxide dismutases (SOD) on oxidative stress and apoptosis in lens epithelium. Exp. Eye Res 2004; 79: 859–868.

31. Borras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann G E, Vina J. 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade. Aging Cell 2005; 4:113–118.

32. Razmaraa A, Ducklesa SP, Krausea DN, Procaccioa V. Estrogen suppresses brain mitochondrial oxidative stress in female and male rats. Brain Research 2007; 1176:71-81

33.Pedram A, Razandi M,. Wallace DC, Levin ER. Functional Estrogen Receptors in the Mitochondria of Breast Cancer Cells. Mol Biol Cell 2006; 17:2125-37

34. Zubkova EV, Robaire B. Effect of glutathione depletion on antioxidant enzymes in the epididymis, seminal vesicles, and liver and on spermatozoa motility in the aging brown norway rat. Biology of Reproduction 2004;71:1002-1008.

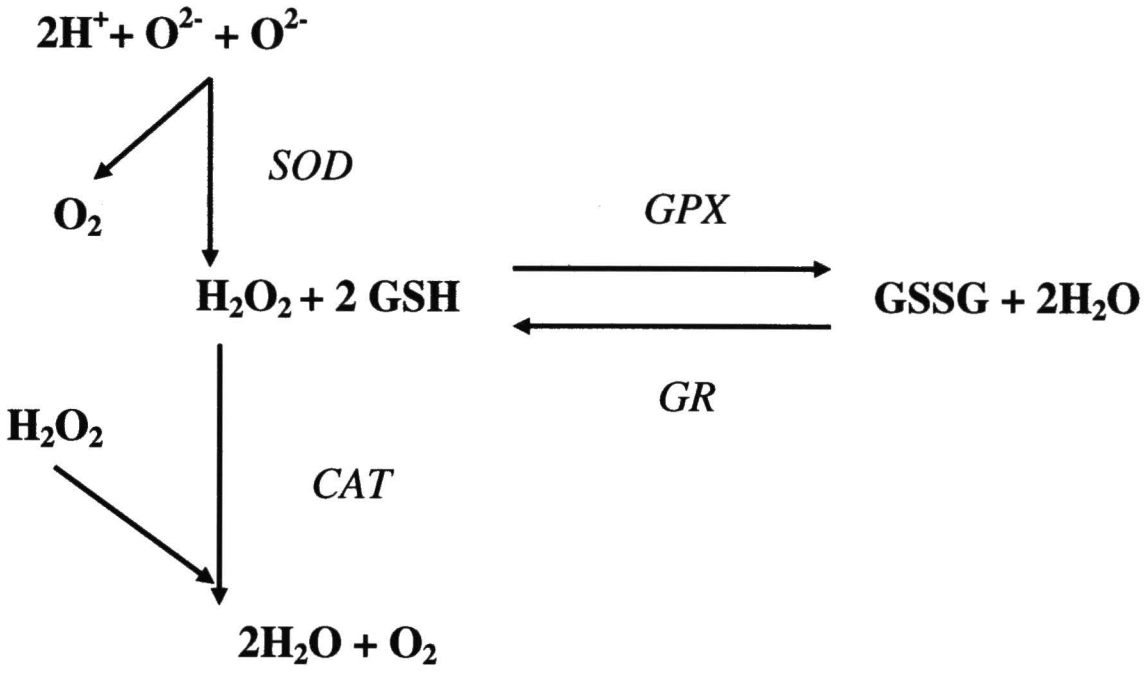

Figure 1. Diagrammatic representation of the relationship between the various antioxidant enzymes, GSH and GSSG. SOD, Superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSH, oxidized glutathione; CAT, catalase, O^{2-}, superoxide anion .

(Modified from Zubkova et al, 34)

Chapter II: RESULTS

Mitochondrial superoxide dismutase activation with 17-β estradiol-treated human lens epithelial cells.

Research Article (Submitted to Molecular Vision, Mar 2008)

Chapter II

Manuscript:

Mitochondrial superoxide dismutase activation with 17- β estradiol-treated human lens epithelial cells.

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Abstract

Purpose: 17 beta estradiol (17β-E_2) protects human lens epithelial cells against oxidative stress by preserving mitochondrial function, in part, via the non-genomic rapid activation of prosurvival signal transduction pathways. The study described herein examined whether 17β-E_2 also elicits genomic protection by influencing the expression (and activity) of mitochondrial-associated manganese superoxide dismutase (MnSOD) as a possible parallel mechanism by which 17β-E_2 protects against oxidative stress. **Methods:** Virally-transformed human lens epithelial cells (HLE-B3) were preincubated with 17β-E_2 and mRNA or protein lysates collected over a time course ranging from 90 min to 24 h. Positive expression of lens epithelial cell MnSOD mRNA was determined by semiquantitative RT-PCR and its levels monitored by real-time PCR up to 24 h post 17β-E_2 administration. Western blot analysis was used to examine the pattern of protein expression as influenced by 17β-E_2 treatment. MnSOD activity, as influenced by 17β-E_2, was determined by measuring enzymatic activity. **Results:** A significant rapid increase in activity of MnSOD was observed with HLE-B3 cells by 90 min post-bolus addition 17β-E_2 which returned to control level by 240 min. Neither an increase in MnSOD mRNA nor protein expression was detected up through 24 h. **Conclusions:** These data demonstrate that 17β-E_2 rapidly and transiently increases the activity of MnSOD but influences neither mRNA expression nor protein expression. These results suggest that MnSOD may be important in protecting lens epithelial cell mitochondria.

Introduction

Epidemiological studies have indicated a higher incidence of cataract formation in postmenopausal women as compared to men of the same age, suggesting that the absence of estrogens may contribute to their increased risk [1]. The Beaver dam eye study [2] and Salisbury eye study [3] both found a protective association between the use of estrogen and the risk of cataract development. These findings have been further substantiated in studies using rodent models and cell cultures. Using a transgenic mouse model expressing a dominant-negative form of estrogen receptor α, which inhibits estrogen receptor α function, it was demonstrated that female mice spontaneously formed cortical cataracts after puberty which progressed with age, thereby suggesting that repression of (nuclear) estrogen action induces cortical cataract [4]. Estrogen treatment diminished the incidence of cortical cataracts in ovariectomized rats treated with methylnitrosourea (MNU) [5]. It has also been reported that estrogen protected lenses against cataracts induced by transforming growth factor-β (TGFβ) in cultured rat lenses [6]. Numerous studies have established that the cytoprotective benefits of estrogens are achieved by its ability to act via both, genomic and non-genomic pathways [7].

Cataract is a worldwide leading cause of blindness and is a multifactorial eye disease. While surgical procedures can correct vision loss, this presents a large financial burden on national health care systems mandating the search for pharmaceutical agents which prevent or delay the onset of cataract [8, 9]. Oxidative damage resulting from free radicals and/or H_2O_2 is considered to be a major risk factor in the pathogenesis of both age related and diabetic cataract [10-13]. Elevated levels of H_2O_2 have been reported in

the aqueous humor of cataract patients and free radicals and H_2O_2 have been implicated in cataract formation [14, 15]. Mitochondria are especially sensitive to oxidative stress. H_2O_2 can cause the collapse of mitochondrial membrane potential ($\Delta\psi_m$) in many cell types including lens epithelial cells, exacerbating free radical production [16, 17]. It has been reported that 17β-E_2 can protect human lens epithelial cells against oxidative stress by preserving mitochondrial function [17]. 17β-E_2 stabilizes $\Delta\psi_m$ in cultured human and bovine lens epithelial cells, acting as a positive regulator of the MAPK signal transduction pathway [18]. These effects did not require prolonged exposure to estrogens, suggesting that estrogens are acting, at least in part, via rapid non-genomic pathways. Studies from our laboratory recently demonstrated that silencing ERK2 dramatically increased membrane depolarization compared to non-specific control siRNA. That is, ERK2 regulates mitochondrial membrane permeability transition in humans lens epithelial cells, supporting the notion that estrogen-induced activation of ERK2 acts to protect cells from acute oxidative stress. Furthermore, despite the fact that ERK2 plays a regulatory role on mitochondrial membrane potential, it was reported that estrogen blocked mitochondrial membrane depolarization via an ERK-independent mechanism [19]. Future studies aimed at discovering the means by which phosphorylated ERK prevents mitochondrial membrane permeability transition, as well as the means by which estrogen might directly associate with elements of the mitochondrial transition pore or indirectly activate/promote phosphorylation of components opposing the cell death machinery will undeniably be of great importance to understanding the non-genomic estrogen-mediated prevention of mitochondrial membrane permeability transition.

The study reported herein, illustrates the effects of 17β-E_2 on the expression and activity of MnSOD. A decrease in SOD has been reported in diabetic patients [20]. It has also been reported that MnSOD exerts a protective effect against H_2O_2 induced oxidative stress in cultured lens epithelial cell line SRA 01/04 [21]. Cell cultures made deficient in MnSOD by downregulating the enzyme have been shown to display a pattern of mitochondrial damage, cytochrome C leakage, caspase 3 activation and increased apoptotic cell death when challenged with superoxide anion [22].

Materials and Methods

HLE-B3 cells, a human lens epithelial cell line immortalized by SV-40 viral transformation [23] were obtained from Usha Andley (Washington University School of Medicine, Department of Ophthalmology, St Louis, MO). Cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, nonessential amino acids and 0.02 g/L gentamycin solution (Sigma Chemical Co., St Louis, MO) at 37 °C and 5%CO_2/95%O_2. All experiments were performed with monolayers of HLE-B3 cells between passages 15-22. In order to deplete the cell cultures of estrogens, cells were maintained in 20% FBS MEM for 24–48 h then switched to 2% charcoal dextran-stripped FBS (Gemini Bio-Products, Woodland, CA) (CSFBS) MEM for up to 18 h with a final medium change to 0.5% (CSFBS) MEM for 24 h.

1,3,5(10) estratrien-3, 17β-DIOL (17β-E_2) was purchased from Steraloids, Inc. (Newport, RI). The hormone was dissolved in 100% ethanol and stock solutions of hormone were prepared fresh for each experiment and diluted in culture medium to a working concentration of 1 μM. Control cells received an equivalent aliquot of ethanol. Rabbit polyclonal antibody against human SOD 2 (MnSOD) was purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibody against actin and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Reverse transcriptase/Polymerase Chain Reaction

HLE-B3 cells maintained in 75 cm^2 culture flasks were harvested by scraping and rinsed once with 1× PBS (pH 7·4) and pelleted by centrifugation at 3000g for 5 min. Total RNA was extracted using either a TRIzol® Reagent (Invitrogen Corp. Carlsbad, CA, USA) or a Trizol kit (Tel-Test, Friendwood, TX, USA) according to the supplier's protocol. RNA pellets were air dried for 10 min and subsequently treated with Turbo DNA-free (Ambion, Austin, TX, USA) and dissolved in deionized water as per manufacturer's protocol. The concentration and purity of the RNA preparation were determined by measuring the absorbance of RNA at wavelengths 260 and 280 nm (Hitachi Instruments Inc., Tokyo, Japan). RNA was stored at –80°C for subsequent experiments.

cDNA was prepared with AMV reverse transcriptase (Promega, Madison, WI, USA) using random hexamer primers (Promega, Madison, WI, USA). RNA was initially denatured at 85°C for 3 min then placed on ice for 3–5 min. The reaction was performed in a total volume of 20 µl containing: 1.0 µg of total RNA, 10 U of AMV reverse transcriptase, 25 ng μl^{-1} random hexamer, 4 µl of 5× AMV reverse transcriptase buffer, 4 µl of 5 mM $MgCl_2$, 1 mM dNTPs (Promega, Madison, WI, USA) and 2U/µl RNasin (Promega, Madison, WI, USA). The reaction was incubated at 42°C for 45 min. PCR primers were specifically designed for MnSOD using Primer 3 (MIT, Cambridge, MA) and synthesized by Sigma Genosys (Spring, TX, USA).

For PCR reactions, 2·5 µl of cDNA from the reverse transcriptase reactions was amplified in a total volume of 50 µl containing 0.2 µM of target gene primers (sense and

antisense), 0.75 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP and dTTP, 1 U of Taq polymerase (Promega, Madison, WI, USA) and 5 µl of 10× PCR buffer (Promega, Madison, WI, USA). Samples were overlaid with 200 µl mineral oil. Amplification was performed on a Perkin Elmer DNA Thermal Cycler 480 (Perkin Elmer, Boston, MA) for 35 cycles. The authenticity of PCR products were confirmed by DNA sequencing (Seqwright, Houston, TX, USA) and a BLAST search of the sequence through the National Center for Biotechnology Information (NCBI) database (data not shown).

Real-Time PCR

Real-time PCR was performed (Mx3000P Real-Time System; Stratagene La Jolla, CA) with PCR master mix (SYBR Green; Stratagene). Each reaction contained 12.5µl of 2X master mix, 500nM MnSOD forward and reverse primers and 2.5ng cDNA from HLE-B3 cells treated with estrogen or ethanol (controls) at various time points of 0, 1.5, 3, 6, 12 and 24 hours.

Cycle threshold (Ct) values were normalized to the housekeeper TATA binding protein (TBP) and comparative quantification was performed based on 2-deltadeltaCt calculation method. The specific primers used for MnSOD were 5': CTGATTTGGACAAGCAGCAA and MnSOD 3': CTGGACAAACCTCAGCCCTA, product size: 199 bp; and for TBP 5': GAAACGCCGAATATAATCCCA and TBP 3': GCTGGAAAACCCAACTTCTG, product size 181 bp.

Cell lysis, electrophoresis and Western blot

Total cell lysates were collected from HLE-B3 cultures after treatments by rinsing adherent cells with ice-cold 1× phosphate buffered saline (PBS) pH 7.4 immediately

followed by addition of lysis buffer [25 mM HEPES, pH 7.4, 0.25 NaCl, 0.5% IGEPAL (NP-40), 0.2% Triton X-100, 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 10 mM NaF, 0.1 mM Na_3VO_4 and a cocktail of protease inhibitors (Sigma-Aldrich, St Louis, MO)] to the monolayers for 30 min at 4°C. Lysates were collected, sonicated for 5 s and sampled for protein concentration using the Bio-Rad protein assay buffer (Bio-Rad Laboratories, Hercules, CA). 3×SDS (Laemmli) buffer was added to the lysates which were subsequently boiled for 3 min and the proteins resolved by electrophoresis on 10% SDS-polyacrylamide gels (20 μg protein per lane). Proteins were transferred to nitrocellulose (Scheicher and Schuell, Keene, NH) and the membranes were blocked with 1% bovine serum albumen (BSA) and 0.02% NaN_3 in Tween-Tris-buffered saline (TTBS) for 15 min. Membranes were probed for 3h at room temperature and overnight at 4°C with primary antibodies (see Materials and Methods), rinsed in TTBS (4×5 min washes) and incubated in goat anti-rabbit horseradish peroxidase conjugate for 1h at room temperature. Required concentrations of antibodies were determined according to the manufacturer's protocols. Membranes were again rinsed in TTBS (4×5 min washes) and proteins were detected using a SuperSignal west pico chemiluminescent kit from Pierce (Rockford, IL). Probed membranes were exposed to Kodak BioMax Light Film (Kodak Scientific Imaging, Rochester, NY).

SOD enzyme activity assay

For MnSOD activity evaluation, cells were lysed, collected with a rubber policeman and sonicated in cold 20mM HEPES buffer, pH 7.2 (EGTA 1mM, 210 mM mannitol, and 70 mM sucrose) followed by centrifugation at 1,500x g, 4 °C for 5 min. The supernatant

was subsequently centrifuged at 10,000 x g, at 4°C for 15 min. The supernatant was evaluated for MnSOD activity evaluation using a Superoxide Dismutase Assay kit (SOD) (#706002, Cayman Chemicals Inc., Ann Arbor, Michigan) according to the manufacturer's protocol. The assay kit is designed to measure total SOD activity (cytosolic and mitochondrial). Separation of the two enzyme activities was achieved as directed by the manufacturer's protocol. Briefly, the 1,500 x g supernatant of the cell lysate is re-centrifuged at 10,000 x g for 15 min at 4^0C. The resulting supernatant contains the cytosolic enzyme (Cu/Zn-SOD) and the pellet the mitochondrial enzyme (MnSOD). The pellet is homogenized as per the manufacturer's protocol. The addition of 1-3 mM potassium cyanide to the assay inhibits both Cu/Zn-SOD and extracellular SOD, resulting largely in the detection of MnSOD alone.

Statistical analysis

For the MnSOD enzyme assay significant differences between groups were determined by an independent sample Student's t-test (2-tailed) using SPSS version 12.0 for Windows. For QPCR, in all cases normalized expression data were compared by two-way analysis of variance (ANOVA). For all experiments, data are reported as mean ± S.E. or S.D. as indicated and P values <0.05 were considered significant.

Results

RT-PCR detection of MnSOD mRNA

Total RNA was extracted from cultured HLE-B3 cells and subjected to RT-PCR for the detection of mitochondrial-associated MnSOD. The resulting cDNA product yielded one band at the correct predicted molecular weight (Figure 1).

Effect of 17β-E_2 on MnSOD mRNA expression

HLE-B3 cells were maintained in 75 cm^2 culture flasks in 20% FBS MEM for 24–48 h then switched to 2% charcoal dextran-stripped FBS (CSFBS) MEM for up to 18 h with a final medium change to 0.5% (CSFBS) MEM for 24 h. Cells were incubated with either 1 μM 17β-E_2 or ethanol-substituted control for 1.5, 3, 6, 12 and 24 h. Each collected time point was compared to its parallel control population of cells which had not been treated with 17β-E_2. MnSOD mRNA was analyzed by real-time PCR (Figure 2). The level of expressed MnSOD with 17β-E_2 treatment was comparable to its non-treated counterpart. The addition of 17β-E_2 did not elicit any statistically significant change in the levels of expressed MnSOD over the duration of the time course.

Effect of 17β-E_2 on MnSOD protein expression

HLE-B3 cells were maintained in 0.5% CSFBS (see Materials and Methods). Cells were incubated with 1 μM 17β-E_2 for 1.5, 3, 6, 12 and 24 h. The 0 h and one of the 24 h time points (indicated as *24 in Figure 3), served as controls and received an equivalent ethanol concentration without estrogen. MnSOD protein expression was monitored by Western blot analyses. No demonstrable change in the level protein expression of MnSOD was detected over the entire time course of collected samples.

Effect of 17β-E_2 on MnSOD activity

Two experiments, using separate cell populations, were performed with the cells maintained in 0.5% serum as described above (Table 1; upper and lower panel). Cell cultures were incubated with 17β-E_2 for 30 min, 90 minutes and 6 h after which MnSOD activity was immediately determined. The 90 minute incubation with 1 μM 17β-E_2 significantly increased MnSOD activity as compared to the untreated controls. No statistically significant difference was observed in the samples treated with and without 17β-E_2 for 30 minutes nor was there any observed increase in MnSOD activity with estrogen treatment (upper panel; Table 1). A second set of cells were likewise maintained in 0.5% serum but this time incubated with 17β-E_2 for 90 minutes and 6 h. As with the first set of treated cells (upper panel) 90 min of incubation with 1μM 17β-E_2 increased MnSOD activity as compared to its untreated counterpart. No statistically significant difference was observed in the samples treated with 17β-E_2 at 6 hours as compared to its untreated counterpart and, more importantly, MnSOD activity had returned to a level equivalent to that observed with 30 min of treatment (lower panel; Table 1).

Discussion

Cellular damage caused by accumulation reactive oxygen species (ROS), has been implicated in many disease processes, especially age related disorders. Lens cells are particularly subject to oxidative stress due to photo-oxidative processes and are consequently prone to cellular oxidative damage [24]. ROS can cause oxidative modification of a number of cellular constituents, such as proteins, cytoskeletal elements, membrane sulfhydryls, glutathione levels, and alterations in transport systems in the epithelium. These changes in turn are thought to result in lens opacification and nuclear cataracts [21, 25, 26]. Mitochondria have been found to be especially susceptible to oxidative damage. Oxidant damage to the mitochondria can cause release of calcium, protein oxidation, depletion of ATP, lipid peroxidation and DNA damage [27]. The lens is equipped with antioxidant enzymes which can prevent the toxic effects of free radicals. Superoxide anion is dismutated to yield H_2O_2 by the enzyme superoxide dismutase (SOD), which is then eliminated by glutathione peroxidase and catalase [28]. There are three isoforms of this enzyme that include the cytosolic-CuZnSOD (SOD1), mitochondrial superoxide dismutase MnSOD (SOD2) and the extracellular SOD (ecSOD).

Various studies using tissue culture and animal models have demonstrated the beneficial effects of estrogen in lens to prevent or delay the onset of cataract formation (4-6). The classic model that describes the action of 17β-E_2 depicts its binding to a receptor and transfer of the complex to specific promoter-regulatory DNA elements prompting nuclear gene transcription activation or repression and subsequent protein

synthesis [7, 29]. However, non-genomic actions of steroid hormones have been described. Recent work from this laboratory has focused on investigating such alternative non-genomic pathways in order to explain the rapid cytoprotective effects of estrogens (17-19).

With respect to genomic response, there are several recent studies, with opposing results, relating to the effect of estrogen on the expression and activity of MnSOD. Using cultured vascular smooth muscle endothelial cells, it has been reported that the expression and the activity of MnSOD is enhanced by estrogens. Preincubation with 1 μM 17β-E_2 elevated the expression of MnSOD mRNA after 12 hours. The protein level of MnSOD was enhanced after 14 hours followed by an increase in MnSOD activity 24 hour post-incubation. Furthermore, these effects on MnSOD expression and activity were reported to be mediated by the activation of estrogen receptors, as the increased expression of MnSOD mRNA was blocked by incubation with estrogen receptor antagonist ICI 182,780 [30]. In another study, it was reported that MCF-7 cells incubated with physiological concentrations of estrogen (0.02nM) for a period of 48 hours demonstrated an increase in the expression of MnSOD mRNA and the upregulation of MnSOD was linked to MAPK and NFkB signaling pathways [31]. It was recently reported that 17β-estradiol significantly reduced the rate of superoxide production in a receptor dependent manner using rat pheochromocytoma cells (PC-12 cells). Gonadectomized animals were treated with testosterone, dihydrotestosterone and estrogen to assess the in vivo effects of gonadal hormones on brain mitochondrial oxidative stress in male and female rats. Only estrogen decreased brain mitochondrial

ROS production in vivo. However, in apparent opposition to the two studies reported above [30, 31], estrogen was reported to increase MnSOD activity levels *without* affecting the protein levels of MnSOD in mitochondria [32]. This observation has also been independently substantiated in that estrogen upregulates the activity of MnSOD in a rapid manner (10-20 min) but does not alter protein expression of MnSOD in the mitochondria [33]. Thus, 17β-E_2 seems to enhance mRNA transcription, protein levels and activity of MnSOD in some cell systems, while only increasing MnSOD activity in others. Furthermore, it is noteworthy to point out that there is considerable variability as to the timeframe required (i.e., minutes vs. hours) for the estrogen-mediated response to increase MnSOD activity.

The study described herein also examined the effect of estrogens on the expression and activity of MnSOD, as part of our preliminary investigation into possible cytoprotective genomic responses by estrogen in cultured human lens epithelial cells. With the cultured lens epithelium system, no change in the expression of MnSOD mRNA was observed over 24 h post-incubation with estradiol as determined by real-time PCR (Figure 2). HLE-B3 cells treated with 17β-E_2 over a similar time course also showed no alteration in protein expression for MnSOD as determined by Western blot analyses (Figure 3). However, a rapid and transient increase in the activity of MnSOD was observed such that activity peaked by 90 min and returned to control levels by 6 h (Table 1). This rapid and transient increase in MnSOD activity unaccompanied by a coupled change in its mRNA and protein expression argues for non-genomic action of estradiol with cultured human lens epithelial cells.

What then is the potential mechanism and beneficial effects of a mitochondrial MnSOD response mechanism that senses a rapid (and as a result, transient) "spike" in reactive oxygen species (ROS)? The accumulation of ROS, produced by a wide variety of exogenous chemical and metabolic processes, can play a dual role in biological systems dependent on the relative level of ROS intracellular concentration, in that, ROS can either be beneficial or harmful [34]. At low concentration, ROS can act as second messengers in signaling cascades leading to induction of mitogenic responses, whereas at higher concentrations ROS cause damage to various cellular structures, including lipids, proteins and nucleic acids [35]. Mitochondria are considered to be a major source of ROS, which include superoxide anion ($O_2^{\bullet -}$), H_2O_2, and the hydroxyl free radical. These compounds may play a key role as signaling molecules regulating mitochondrial dysfunction and subsequent apoptotic events. It has been reported that exposure to estrogen stimulates the rapid production of intracellular ROS in human umbilical vein endothelial cells [36]. Estrogen-induced mitochondrial reactive oxygen species act as signal-transducing messengers [37]. Studies have shown that estrogen causes an increase in mitochondrial calcium [38]. Ca^{2+} can further enhance the dislocation of cytochrome c from the inner mitochondrial membrane, either by competing with cardiolipin binding sites thereby resulting in the blocking of complex III, which would have the effect of enhancing ROS generation [39]. An increased mitochondrial formation of ROS triggers the intrinsic pathway of apoptosis by increasing the permeability of the outer mitochondrial membrane through the opening of the mitochondrial permeability transition pore [40]. MnSOD preserves mitochondrial function by regulating the sensitivity of the

permeability transition pore to ROS [41]. In our hands, the endogenous accumulation of reactive oxygen species (ROS) was assessed in HLE-B3 cells treated with estrogen by loading cells with H_2DCF-DA, which upon oxidation in the presence of ROS transitions to the fluorescent compound, DCF. A statistically significant increase in ROS was observed, only after three days, in estrogen-treated cells relative to parallel control cell cultures, consistent with the likelihood that mitochondrial reactive oxygen species were moderated by activated MnSOD until such time as the oxidative insult overwhelmed the cellular system (data not shown).

Data presented herein supports the notion that the bolus addition of 17β-E_2 causes a transient increase in the production of ROS in HLE-B3 cells but at the same time constitutively induces MnSOD activity thereby quenching the ROS formed in the mitochondria thus preserving mitochondrial function. Chen et al. (42) have demonstrated that, "a low level of reactive oxygen species plays an important role in host defense and mediating mitogen-stimulated cell signaling." This group has shown that the endogenous generation of ROS initiates redox signaling, resulting in, among other things, activation of mitogen-activated protein kinases (MAPKs). To that end, it is noteworthy to mention that we have previously reported that cell death induced by H_2O_2 in cultured human lens epithelial cells is associated with accumulation of intracellular ROS, collapse of mitochondrial membrane potential ($\Delta\psi_m$), and depletion of ATP and that estrogens mitigated mitochondrial collapse of $\Delta\psi_m$, ATP depletion and cell death by increasing the amount of Ca^{2+} or H_2O_2 required to collapse mitochondrial membrane potential [17]. It

has also been shown that 17β-E_2 acts as a positive regulator of the survival signal transduction pathway, MAPK which, in turn, acts to stabilize $\Delta\psi_m$ [18,19].

Our conceptual framework regarding the protective mechanism(s) activated by 17β-E_2 is based on the premise that cytoprotection will prove to be complex and multi-faceted, including both non-genomic and genomic aspects. That is, we support the notion that the estrogen-driven responses driving mitochondrial protection against membrane potential loss are likely to prove to be dynamic in that the protective stabilization of mitochondrial membrane potential by estrogens may be attributed to consolidation of several mechanisms of action working in concert. To that end, we can now report that there is, at least, a coupled duality to the mechanism of estradiol's cytoprotective action against acute oxidative stress. Estradiol administration to HLE-B3 cells initiates a rapid increase in intracellular (mitochondrial) ROS, transiently increasing MnSOD activity, which immediately acts to lower the ROS concentration before mitochondrial damage might ensue and, at the same time, estradiol prompts a signaling cascade culminating in the activation of the ERK/MAPK pathway, which exerts a positive effect by attenuating the extent of depolarization of mitochondrial membrane potential in the face of acute oxidative stress, thereby preventing entry into the cell death pathway.

Other mitochondrial protective mechanisms against oxidative stress are likely. The restraint of $\Delta\psi_m$ collapse might be explained by a repression of Ca^{2+} uptake into the mitochondria, increased tolerance to mitochondrial calcium sequestration, increased Ca^{2+} efflux from the mitochondria, increased resorption of Ca^{2+} into endoplasmic reticulum and/or increased efflux of Ca^{2+} via the plasma membrane. An estrogen redox-cycle has

been proposed (43) which may control glutathione and NAD(P)H flux and in conjunction with "classic" antioxidant responses (44) may equally act as defense mechanisms against reactive oxygen species.

References

1. Klein BE, Klein R, Linton KL. Prevalance of age-related lens opacities in a population. The Beaver Dam Eye Study. Opthalmology 1992; 99:546-552.

2. Klein B, Klein R, Ritter L. Is there evidence of an estrogen effect on age-related lens opacities? The Beaver Dam Eye Study. Arch Ophthalmol 1994; 112:85-91

3. Freeman EE, Munoz B, Schein OD, West SK. Hormone replacement therapy and lens opacities: the Salisbury eye evaluation project. Arch Opthalmol 2001; 119:1687-1692.

4. Davis VL, Chan CC, Schoen TJ, Couse JF, Chader GJ, Korach KS. An estrogen receptor repressor induces cataract formation in transgenic mice. Proc Natl Acad Sci U S A 2002; 99:9427-32.

5. Bigsby RM, Cardenas H, Caperell-Grant A, Grubbs CJ. Protective effects of estrogen in a rat model of age-related cataracts. Proc Natl Acad Sci U S A 1999; 96:9328-32.

6. Hales AM, Chamberlain CG, Murphy CR, McAvoy JW. Estrogen protects lenses against cataract induced by transforming growth factor-beta (TGFbeta). J Exp Med 1997; 185:273-80.

7. Levin ER. Integration of the extranuclear and nuclear actions of estrogen. Mol Endocrinol 2005;19:1951-9.

8. Harding JJ. Pharmacological treatment strategies in age related cataracts. Drugs aging 1992;24:287-300.

9. Javitt JC, Wang F, West SK. Blindness due to cataract: epidemiology and prevention. Annu Rev Public Health 1996;17: 159-77.

10. Ozmen B, Ozmen D, Erkin E, Guner I, Habif S, Bayindir O. Lens superoxide

dismutase and catalase activities in diabetic cataract. Clin Biochem 2002;35: 69-72.

11. Giblin, FJ, McCready, JP, Reddy, VN. The role of glutathione metabolism in the detoxification of H_2O_2 in rabbit lens Invest Ophthalmol Vis Sci 1982; 22:330-335.

12. Giblin, FJ, McCready, JP. The effect of inhibition of glutathione reductase on the detoxification of H_2O_2 by rabbit lens Invest Ophthalmol Vis Sci 1983; 24:113-118.

13. Spector, A. The search for a solution to senile cataracts. Proctor Lecture Invest Ophthalmol Vis Sci 1984; 25:130-146.

14. Spector A, Garner WH. Hydrogen peroxide and human cataract. Exp Eye Res 1981; 33:673-81.

15. Xing KY, Lou MF. Effect of H_2O_2 on human lens epithelial cells and the possible mechanism for oxidative damage repair by thioltransferase. Exp Eye Res 2002; 74:113-22.

16. Dykens JA. Isolated cerebellar and cerebral mitochondria produce free radicals when exposed to elevated Ca^{+2} and Na^{+}: implications for neurodegeneration. J Neurochem 1994; 63:584–591.

17. Wang J, Simpkins JW, Dykens JA, Cammarata PR. Oxidative damage to human lens epithelial cells in culture: estrogen protection of mitochondrial potential, ATP, and cell viability. Invest Ophthalmol Vis Sci 2003; 44:2067–2075.

18. Moor AN, Flynn JM, Gottipati S, Giblin FJ, Cammarata PR. 17beta-estradiol stimulates MAPK signaling pathway in human lens epithelial cell cultures preventing collapse of mitochondrial membrane potential during acute oxidative stress. Mitochondrion 2005; 5:235-47.

19. Flynn J, Lannigan D, Clark D, Garner M, Cammarata P. RNA Suppression of ERK2 Leads to Collapse of Mitochondrial Membrane Potential with Acute Oxidative Stress in Human Lens Epithelial Cells. Am J Physiol Endocrinol Metab 2008; March 294 (3):589-599.

20. Oda A, Bannai C, Yamaoka T, Katori T, Matsushima T, Yamashita K. Inactivation of Cu,Zn-superoxide dismutase by in vitro glycosylation and in erythrocytes of diabetic patients. Horm Metab Res 1994; 26:1-4

21. Matsui M, Lin L.-R., Ho Y.-S, Reddy V.N. The effect of up- and downregulation of MnSOD enzyme on oxidative stress in human lens epithelial cells. Invest Ophthalmol Vis. Sci 2003; 44:3467–3475.

22. V.N. Reddy, E. Kasahara, M. Hiraoka, L.R. Lin and Y.S. Ho, Effects of variation in superoxide dismutases (SOD) on oxidative stress and apoptosis in lens epithelium. Exp. Eye Res 2004; 79: 859–868.

23. Andley UP, Rhim JS, Chylack LT Jr, Fleming TP. Propagation and immortalization of human lens epithelial cells in culture. Invest Ophthalmol Vis Sci 1994; 35:3094-102.

24.Edward JD, Fu S, and Allen T. H_2O_2 mediated oxidative stress activates NF-kB in lens epithelial cells. Free Radical Biology & Medicine 2001; 31:651-658.

25. Garadi, R, Giblin, FJ, Reddy, VN. Disulfide-linked membrane protein in X-ray-induced cataracts Ophthalmic Res 1987; 19:141-149

26. Padgaonkar, V, Giblin, FJ, Reddy, VN. Disulfide cross-linking of urea-insoluble protein in rabbit lenses treated with hyperbaric oxygen Exp Eye Res 1989;49:887-889

27. Crawford DR, Wang Y, Schools GP, Kochheiser J, Davies KJ. Down-regulation of

mammalian mitochondrial RNAs during oxidative stress. Free Radic Biol Med 1997; 22:551-9.

28. Bhuyan KC, Bhuyan DK. Superoxide dismutase of the eye: relative functions of superoxide dismutase and catalase in protecting the ocular lens from oxidative damage. Biochim. Biophys. Acta 1978; 542:28–38.

29. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem 1994; 63:451–486.

30.Strehlow K, Rotter S, Wassmann S, Adam O, Grohé C, Laufs K, Böhm M, Nickenig G. Modulation of Antioxidant Enzyme Expression and Function by Estrogen.Circ. Res 2003; 93:170-177.

31. Borras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann G E, Vina J. 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade. Aging Cell 2005; 4:113–118.

32. Razmaraa A, Ducklesa SP, Krausea DN, Procaccioa V. Estrogen suppresses brain mitochondrial oxidative stress in female and male rats. Brain Research 2007; 1176:71-81

33.Pedram A, Razandi M,. Wallace DC, Levin ER. Functional Estrogen Receptors in the Mitochondria of Breast Cancer Cells. Mol Biol Cell 2006; 17:2125-37

34. Valko M, Izakovic M, Mazur M, Rhodes CJ , Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol. Cell. Biochem 2004; 266: 37–56.

35. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interactions 2006; 160: 1-40.

36. Felty Q. Estrogen-induced DNA synthesis in vascular endothelial cells is mediated by ROS signaling. BMC Cardiovasc Disord. 2006; 11: 6-16.

37. Felty Q, Xiong WC, Sun D, Sarkar S, Singh KP, Parkash J, Roy D. Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. Biochem 2005; 44: 6900-6909.

38. Nilsen J, Brinton RD. Mechanism of estrogen-mediated neuroprotection: Regulation of mitochondrial calcium and Bcl-2 expression. Proc Natl Acad Sci U S A. 2003; 4: 2842–2847

39. Grijabla MT, Vercesi AE, and Schreier S. Ca2+ induced increased lipid packing and domain formation in submitochondrial particles. A possible early step in the mechanism of Ca2+ stimulated generation of reactive oxygen species by the respiratory chain. Biochemistry 1999; 38: 13279-13287.

40. Julio F. Turrens. Mitochondrial formation of reactive oxygen species. J physiol 2003; 552: 335-344.

41. Van Remmen HV, Williams MD, Guo Z, Estlack L, Yang H, Carlson EJ, Epstein CJ, Huang TT, Richardson A. Knockout mice heterozygous for Sod2 show alterations in cardiac mitochondrial function and apoptosis. Am J Physiol Heart Circ Physiol 2001; 281: H1422-H1432.

42. Chen KC, Zhou Y, Xing K, Krysan K, Lou MF. Platelet derived growth factor (PDGF)-induced reactive oxygen species in the lens epithelial cells: the redox signaling. Exp Eye Res. 2004; 78:1057-1067.

43. Prokai L, Prokai-Tatrai K, Perjesi P, Zharikova AD, Simpkins JW. Quinol-based metabolic

cycle for estrogens in rat liver microsomes. Drug Metab Dispos 2003; 31:701–704.

44. Lee W, Wells T, Kantorow M. Localization and H_2O_2-specific induction of PRDX3 in the eye lens. Mol Vis. 2007; 13:1469-1474.

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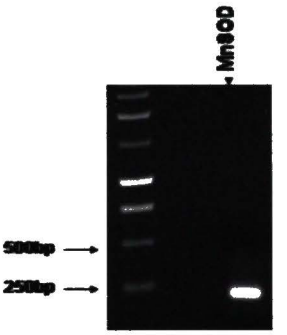
Figure Legends:

Figure 1: RT-PCR expression of MnSOD in HLE-B3 cells. Total RNA was extracted from confluent HLE-B3 cells and subjected to RT-PCR for MnSOD. The cDNA product yielded one band at the correct predicted molecular size(199 bp). The authenticity of PCR products was verified by DNA sequencing and a BLAST search of the sequence

Figure 2: Real-Time PCR expression of MnSOD in HLE-B3 cells. HLE-B3 cells were incubated with 1 μM17β-E_2 for 0, 1.5, 3, 6, 12 and 24 hours. Expression of MnSOD mRNA was analyzed by quantitative real-time PCR. There was no statistically significant change, as determined by Two Way ANOVA, in MnSOD mRNA at any time point after estrogen treatment as compared to control (untreated). Data are expressed as mean $\pm$ SD values. Significance was taken at $P<0.05$ (n =3).

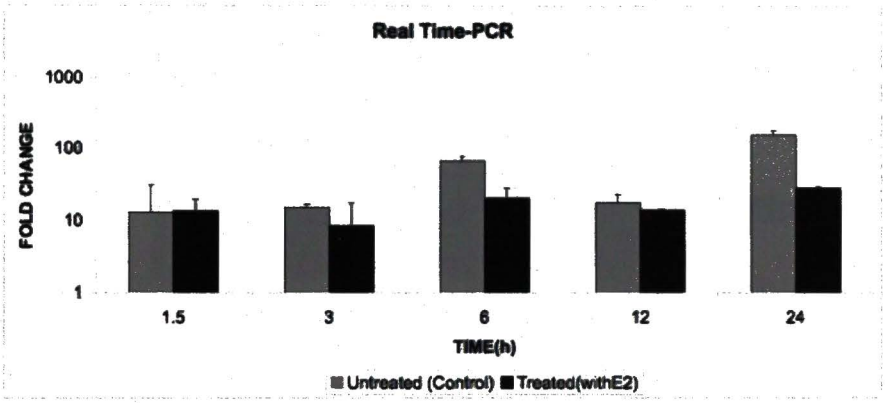
Figure 3: Western blot analyses of MnSOD expression in HLEB3 cells. Total cell lysates were collected from HLE-B3 cells grown in 0.5 % serum and stimulated by exposure to 1 μM 17β-E_2 for 0, 1.5, 3, 6, 12 and 24 hours. There was no change in the expression of MnSOD at any of the time points. The time points at 0 and 24* hours did not receive estrogen.

Table 1: 17β-E_2 upregulates MnSOD activity. HLEB3 cells maintained in 0.5 % serum were exposed to 1 μM 17β-E_2 for 30 and 90 min (upper panel, n=16, of two separate cell populations) and 90 min and 6 hours (lower panel, n=8 from a single cell population) and MnSOD activity measured (refer to Materials and Methods). A significant increase in activity was detected with the 90 min incubation. The results are reported as Units/mL and Units/mg protein.



PCR product run on 1% agarose gel

Figure II-1


Real Time-PCR
1000
100
10
1
FOLD CHANGE
1.5
3
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TIME(h)
Untreated (Control)
Treated(withE2)

Figure II- 2

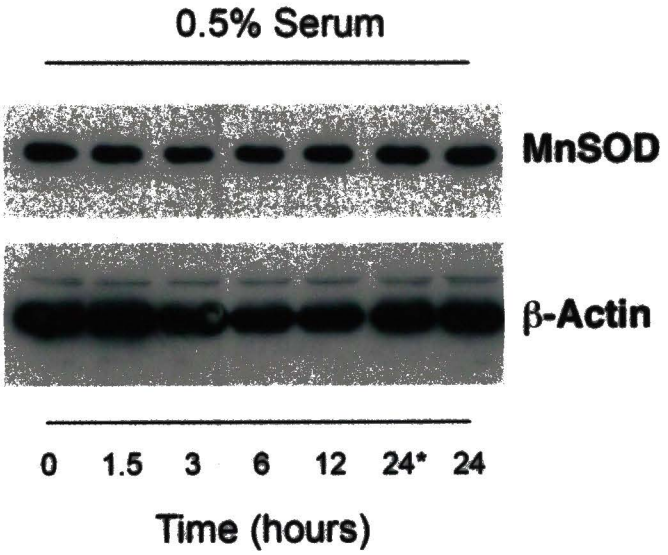

Figure II- 3

U/ml ± SEM			U/mg protein ± SEM		
	Control	(+) 17β E2		Control	(+) 17 β E2
30 min	54.9 ± 6.1	67.7 ± 6.5	30 min	24.5 ± 5.3	29.4 ± 5.1
90 min	66.4 ± 9.1	10 4± 12.1*	90 min	27.4 ± 3.8	49.6 ± 7.4*
90 min	20.4 ± 4.4	91 ± 10.8*	90 min	11.6 ± 3	62 ± 8*
6 hrs	45 ± 12	49 ± 6	6 hrs	30 ± 8	33 ± 5

Table II-1.

CHAPTER III: DISCUSSION

Modulation of manganese superoxide dismutase activity by 17-β estradiol

CHAPTER III

In this study we examined the effect of estrogens on the expression and activity of MnSOD, as part of our investigation into possible cytoprotective genomic responses by estrogen in cultured human lens epithelial cells. The present study demonstrates a rapid and transient increase in the activity of MnSOD which is unaccompanied by changes in its mRNA and protein expression which argues for non-genomic action of estradiol with cultured human lens epithelial cells.

A. 17β-E_2 and its effect on MnSOD mRNA expression and protein: There have been several recent papers [1,2,3,4] which have examined the effect of estrogens on the expression and activity of MnSOD. 17β-E_2 seems to enhance mRNA transcription, protein levels and activity of MnSOD in some cell systems, while only increasing MnSOD activity in others. Furthermore, there is considerable variability as to the timeframe required (i.e., minutes vs. hours) for the estrogen-mediated response to increase MnSOD activity. The results of these papers are summarized briefly in the table below.

Table III-1. Representation of the results from 5 groups in a tabular form.

Groups	Group 1	Group 2	Group 3	Group 4	Group 5
Cells and Models used	i) Rat VSMC's ii) C57 BL6 mice iii)Mono-cytes from patients receiving GnRH	MCF-7 Cells	MCF-7 Cells	i) PC -12 Cells ii) Male fisher 344 rats	HLE-B3 Cells
ROS and 17β-E_2	17β-E_2 prevented angiotensin II induced ROS production in rat VSMC's	17β-E_2 prevented UV mediated ROS production in MCF- 7 Cells	17β-E_2 decreased H_2O_2 levels in MCF-7 Cells	i)17β-E_2 decreased mito-chondrial O^{2-} production in PC-12 Cells ii) 17β-E_2	17β-E_2 causes a slow increase in ROS in HLE-B3 cells over a period of

				decreased mito-chondrial ROS in gonadec-tomized male and female rats	3 periods
MnSOD mRNA expression	Increased expression after 12 hours exposure to 17β-E_2	Not determined	Increased expression after 48 hours exposure to 17β-E_2	Not determined	No change in the expression of MnSOD mRNA over a period of 90 minutes to 24 hours

MnSOD Protein	Increased expression in 14 hours	No change in expression (at 4 hours)	Not determined	No change on 3-4 weeks of exposure to17β-E_2 in male and female rats	No Change in protein amount over a period of 0-24 hours
MnSOD Activity	Increased activity observed in 24 hours	Increased activity observed in 10-20 minutes	Not determined	Increased activity on 3-4 weeks of exposure to17β-E_2 in male and female rats	Increased activity observed in 90 minutes
Receptor mediated	Yes	Yes	Yes	Yes	Not determined

Concentration of 17β-E_2 used	1μm/L (1nm/ml)	10nm/ml	0.02nm/ml	Physiological levels	1μm/ml
Other significant results	17β-E_2 replacement decreases the ROS production and prevented the down-regulation of MnSOD in ovarecto-mized mice. Increased MnSOD levels were found in monocytes		17β-E_2 activates MAPK pathway and promotes NFκB transloctionto the nucleus and increases MnSOD and GPx expression by binding to the promoter region	17β-E_2 was shown to decrease oxidative stress in brain mitochondria. In vivo treatment of gonadec-tomized animals with 17β-E_2 increases MnSOD activity in both male and female	

	of females, receiving GnRH and FSH, undergoing in vitro fertilization (this treatment causes increase in the circulating estrogen levels)			rat while testosterone and DHT had no effect on male rats	

Group 1: Strehlow *etal* [1]

Group 2: .Pedram *etal* [2]

Group 3: Borras *etal* [3]

Group 4: Razmaraa *etal* [4]

Group 5: Gottipati .S, Cammarata PR [submitted for publication to Molecular Vision]

VSMC; Vascular Smooth Muscle Cells, MCF-7 –Breast adenocarcinoma cell line, UV; Ultraviolet, DHT; Dihydrotestesterone, FSH; Follicle Stimulating Hormone, GnRH; Gonadotropin Releasing Hormone.

Different authors have found varying results.

MnSOD mRNA Expression:

Two groups have reported an increase in MnSOD mRNA in cell cultures. Group 1 reported an increase in mRNA expression in 12 hours, group 3 in 48 hours, whereas we did not observe any change in mRNA expression when HLE-B3 cells were stimulated with 17β-E_2 over a period of time (90 minutes to 24 hours) as determined by Real Time PCR. A possible explanation might be the concentration of estrogen used in the experiments. Group 1 used 1μm/L (1nm/ml) and group 2 used 0.02nm/ml, whereas we used a concentration of 1μm/ml of 17β-E_2. It is possible that the increased expression of MnSOD mRNA is seen at lower concentrations of 17β-E_2.

However group 1 also conducted studies in human subjects who were recruited for in vitro fertilization. The women were treated with a GnRH analogue which led to pituitary desensitization and a suppression of estrogen release. Subsequent to GnRH treatment, women received FSH. Under this treatment, estrogen levels increased "substantially". The increasing estrogen levels correlated with MnSOD expression on circulating monocytes. Thus 17β-E_2 induces MnSOD expression in monocytes in an in vivo model, possibly at higher (supraphysiological) levels.

MnSOD protein expression:

Only group 1 reported an increase in protein expression at 14 hours. Group 2 measured

the protein at 4 hours post exposure to and did not observe any change in its expression. We stimulated HLE-B3 cells with 17β-E_2 and measured the protein expression of MnSOD over a timeframe of 0-24 hours. No change of MnSOD protein was observed in our cells.

Possibly the use of lower concentration of 17β-E_2causes an increase in the mRNA expression of MnSOD, which then leads to an increase in the protein amount.

MnSOD Activity:

Irrespective of the cell type or the concentration of 17β-E_2 used all groups which measured MnSOD activity report an increase in its activity, though the time at which the activity goes up is different in each study.

Why does the activity of MnSOD increase on treatment with 17β-E_2 even in the absence of corresponding increase/change in mRNA and protein expression?

B. Enzyme Kinetics, MnSOD and 17β-E_2

Enzymes are protein catalysts that, like all catalysts, speed up the rate of a chemical reaction without being used up in the process. They achieve their effect by temporarily binding to the substrate and, in doing so, lowering the activation energy needed to convert it to a product.

The rate at which an enzyme works is influenced by several factors, e.g.,

1. Concentration of enzyme and substrate molecules
2. Temperature (as the temperature rises, molecular motion and hence collisions between enzyme and substrate speed up)

3. pH: the conformation of a protein is influenced by pH and as enzyme activity is crucially dependent on its conformation, its activity is likewise affected

4. Presence of inhibitors

5. Activators/Modifiers

6. Cofactors can also modulate the enzyme activity [5,6].

Manganese superoxide dismutase (Mn-SOD) is encoded by nuclear chromatin, synthesized in the cytosol, and imported posttranslationally into the mitochondrial matrix. The mature protein is assembled into a tetramer. Human MnSOD is a tetrameric enzyme with four identical subunits each harboring a Mn+3 atom [7,8]. Superoxide dismutases (SODs) are antioxidant metalloenzymes catalysing the dismutation of superoxide radical, $O_2\cdot^-$

$2O_2\cdot^- + 2H^+ \rightarrow O_2 + H_2O_2$

An increase in MnSOD activity could be due to any of the reasons outlined above.

Enzyme Concentration: Some authors do see a change in mRNA expression and protein expression of MnSOD, this may lead to a change in the enzyme concentration. However most of the groups including our lab do not observe any change in expression at mRNA or protein level. Some mechanism other than just an increase in the enzyme concentration is responsible for the increased enzymatic activity of MnSOD.

Cofactor (Manganese): Metal ions can modify the activity of MnSOD. It has been proposed that Mn(III) can increase the cell content of content MnSOD by speeding the conversion of the apoenzyme to the holoenzyme [12]. Pugh and Fridovich have suggested that the regulation of MnSOD involves autogenous repression by apo-SOD

and posttranslational control based on competition between manganese and iron ions for the metal-binding site of apo-MnSOD and only the former conferring catalytic activity [13]. One recent study demonstrated that in a mouse model of hemochromatosis (*Hfe–/–*), iron accumulation in the liver was confined to the cytosol where it was associated with decreased mitochondrial accumulation of other metals including manganese, copper,and zinc. Mitochondria from *Hfe–/–* mice were dysfunctional and manifested increased oxidant damage and decreased function of MnSOD, but correcting the mitochondrial manganese deficit largely reversed this damage [14]. In a recent study it was found that, in the absence of a parallel increase of the manganese ion, MnSOD mRNA induction was not accompanied by a higher enzymatic activity, furthermore increase in the manganese content of the cells was associated with increased MnSOD activity [15]. Estrogens have been shown to increase the manganese content in an animal model. In ovarectomized rats the level of manganese in teeth as well as in mandible and administration of 17β-E_2 caused increase of manganese content [16]. Estrogens can mediate increased absorbtion of manganese [17], however it is not known whether estrogens can cause an increase in the cellular content of manganese and the time required for estrogens to increase the manganese content of the cells. It is a possibility that estrogens are increasing the MnSOD activity by making available more manganese in the cells and thus activating the inactive enzyme already present in the cells.

Post-translational modification: Previous reports found that posttranslational modification is the main mechanism for the activation of SOD isoenzymes, particularly Cu/Zn-SOD [18]. MnSOD appears to be regulated at multiple levels, both, at

transcriptional and the posttranscriptional levels [19]. The levels of MnSOD mRNA expression do not always correlate with its increased or decreased activity. In the failing myocardium a decrease in MnSOD activity was observed in the setting of increased mRNA expression [20]. Using Listeria Monocytogens it has been demonstrated that the cytosolic MnSOD is less active when it is phosphorylated at both the serine and threonine residues and the nonphosphorylated MnSOD is active [21]. Though no studies have been done using human cells, it is possible that a similar mechanism is increasing the MnSOD activity even in the absence of a corresponding increase in mRNA or protein expression.

C. Conclusions and Future Directions:

In the present study we examined the effect of estrogens on the expression and activity of MnSOD, as part of our investigation into possible cytoprotective genomic responses by estrogen in cultured human lens epithelial cells. With the cultured lens epithelium system, no change in the expression of MnSOD mRNA was observed over 24 hour post-incubation with estradiol as determined by real-time PCR. HLE-B3 cells treated with 17β-E_2 over a similar time course also showed no alteration in protein level of MnSOD as determined by Western blot analyses. However, a rapid and transient increase in the activity of MnSOD was observed such that activity peaked by 90 min and returned to control levels by 6 h. This rapid and transient increase in MnSOD activity unaccompanied by a change in its mRNA and protein expression argues for non-genomic action of estradiol with cultured human lens epithelial cells.

Different labs have found varying results on the effect of estrogens on the expression and activity of MnSOD. As was discussed previously, one of the possible reasons might be

the low (physiological) concentrations of 17β-E_2 used in the experiments where the authors found an increase in the expression of mRNA and protein of MnSOD. Similar experiment can be performed using HLE-B3 cells, where HLE-B3 cells will be stimulated with a low (1nm/ml) concentration of 17β-E_2 and followed over a time course of 0 to 24 hours to determine any change in mRNA expression by QPCR and a change in protein expression can be determined by Western blot analyses.

While there is a difference in the observed results for the mRNA and protein expression, all the groups which have examined the effect of 17β-E_2 on MnSOD have reported an increase in the activity of the enzyme. One of the possible explanations might be that the enzyme is undergoing a posttranslational modification, i.e. dephoshorylation, which then converts the inactive enzyme already present in the mitochondria to a more active form. Such a mechanism of posttranscriptional modification of the enzyme, MnSOD has been demonstrated in Listeria Monocytogens, where phosphorylation on the serine and threonine residues makes the enzyme inactive and dephosphorylation makes the enzyme active again. No evidence exists that a similar mechanism of modifying SOD activity is present in humans. We would like to examine in detail whether phosphorylation and dephosphorylation events play a role in the modulation of MnSOD activity in HLE-B3 cells.

A better understanding of the mechanism by which 17β-E_2 exerts its effects will help us in developing estrogens to be useful therapies for the prevention of cataract in postmenopausal women and non feminizing estrogens may provide similar protection in men.

REFERENCES:

1. Strehlow K, Rotter S, Wassmann S, Adam O, Grohé C, Laufs K, Böhm M, Nickenig G. Modulation of Antioxidant Enzyme Expression and Function by Estrogen.Circ. Res 2003; 93:170-177.

2.Pedram A, Razandi M,. Wallace DC, Levin ER. Functional Estrogen Receptors in the Mitochondria of Breast Cancer Cells. Mol Biol Cell 2006; 17:2125-37

3. Borras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann G E, Vina J. 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade. Aging Cell 2005; 4:113–118.

4. Razmaraa A, Ducklesa SP, Krausea DN, Procaccioa V. Estrogen suppresses brain mitochondrial oxidative stress in female and male rats. Brain Research 2007; 1176:71-81

5. http://www.chemsoc.org/networks/learnnet/cfb/enzymes.htm

6. intranet.bell.ac.uk/.../biology/Lecture%20Notes/Cell%20Metabolism%20-%20Lecture%204%20-%20Enzyme%203a.ppt

7. 18. Beckman JS, Carson M, Smith CD and Koppenol WH. ALS, SOD and peroxynitrite. Nature (Lond), 1993; 364: 584.

8. Borgstahl GEO, Parge HE, Hickey MJ, Beyer WF Jr, Hallewell RA, Tainer JA. The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. Cell 1992;71: 107-118.

9. Gregory EM, Fridovich I. Induction of superoxide dismutase by molecular oxygen. J. Bacteriol 1973; 114:543-548.

10. Sarsour EH, Venkataraman S, Kalen AL, Oberley LW, Goswami PC. Manganese

superoxide dismutase activity regulates transitions between quiescent and proliferative growth. Aging Cell. 2008 Mar 10 [Epub ahead of print]

11. Allen RG, Balin AK. Effects of oxygen on the antioxidant responses of normal and transformed cells. Exp Cell Res 2003;289:307-16.

12. Pugh, SYR, DiGuiseppi JL, Fridovich I. Induction of superoxide dismutase in Escherichia coli by manganese and iron. J. Bacteriol 1984; 160:137-142.

13. Pugh, SYR, Fridovich I. Induction of superoxide dismutase in Escherichia coli B by metal chelators. J. Bacteriol 1985;162:196-202.

14. Jouihan HA, Cobine PA, Cooksey RC, Hoagland EA, Boudina S, Abel ED, Winge DR, McClain DA. Iron-mediated inhibition of mitochondrial manganese uptake mediates mitochondrial dysfunction in a mouse model of hemochromatosis. Mol Med. 2008; 14: 98–108

15. Koch O, Farré S, De Leo ME, Palozza P, Palazzotti B, Borrelo S, Palombini G, Cravero A, Galeotti T. Regulation of manganese superoxide dismutase (MnSOD) in chronic experimental alcoholism: effects of vitamin E-supplemented and –deficient diets. Alcohol and Alcoholism 2000;35: 159-163

16. Rahnama M, Błoniarz J, Zareba S, Swiatkowski W. Study of estrogen deficiency impact on manganese levels in teeth and mandible of rats after ovariectomy. Rocz Panstw Zakl Hig. 2003;54:33-8.

17. http://www.nzhealth.net.nz/dis_ease/cjd.shtml

18. Jensen LT, Culotta VC. Activation of CuZn superoxide dismutase from Caenorhabditis elegans does not require the copper chaperone CCS. J Biol Chem 2005;280: 41373–9.

19. Touati D. Transcriptional and posttranscriptional regulation of manganese superoxide dismutase biosynthesis in Escherichia coli, studied with operon and protein fusions. J Bacteriol 1988 ;170:2511-20

20. Sam F, Kerstetter DL, Pimental DR, Mulukutla S, Tabaee A, Bristow MR, Colucci WS, Sawyer DB. Increased reactive oxygen species production and functional alterations in antioxidant enzymes in human failing myocardium. J Card Fail. 2005 ;11:473-80.

21. Archambaud C, Nahori MA, Pizarro-Cerda J, Cossart P, Dussurget O. Control of Listeria superoxide dismutase by phosphorylation. : J Biol Chem. 2006; 281:31812-22. Epub 2006 Aug