THE ROLE OF ESTROGEN AND SUPEROXIDE DISMUTASE IN CATARACTOGENESIS

Thesis for the degree of Doctor of Medicine

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ABSTRACT

Cataract is an opacification of the eye lens, constituting the major cause of blindness globally. Oxidative stress is a key factor in the formation of cataract and female gender is a known risk factor for age-related cataract. The aim of this thesis was to investigate the role of estrogen and the antioxidant enzyme superoxide dismutase (SOD) in cataractogenesis.

Human lens epithelial cells (HLECs) obtained during cataract surgery at the Department of Ophthalmology at Sahlgrenska university hospital in Mölndal (SU/M) were used to study the effects of the major estrogen, 17β-estradiol (E2), on proliferation, cell viability, intracellular redox status, SOD and estrogen receptors (ERs). H_2O_2-induced oxidative stress was used to study the antioxidative properties of E2 in HLECs. Two genetic association studies were performed to investigate if genetic variations in estrogen-related and in SOD genes were associated with increased risk of cataract in an Estonian population, consisting of 492 patients with age-related cataract and 185 controls. Patients and controls were also recruited from the Eye Clinic at SU/M for a study on possible correlations between E2 levels and SOD activity.

The effects of E2 at pharmacological concentrations in HLECs were; increased apoptosis and cell death, reduced cell viability and proliferation as well as increased intracellular levels of reactive oxygen species (ROS). At lower (physiologic) concentrations, increased proliferation, reduced cell death, stabilization of mitochondrial membrane potential and protection against oxidative stress by reduction of ROS were observed. At these concentrations total SOD activity was increased and protein expression levels of ERs were altered. However, no change in neither gene nor protein expression levels of SODs was seen. A linear correlation between increasing age and declining E2 serum levels was evident in cataract patients and controls. Men exhibited higher E2 levels compared to postmenopausal women. However, no correlation between serum levels of E2 and SOD activity was found in our study subjects. The genetic association studies showed that genetic variations in SOD and estrogen-related genes were not associated with increased risk of cataract.

In conclusion, no correlation between SOD activity and E2 serum levels in cataract patients and controls was found and genetic variations in SOD or estrogen-related genes showed no association with increased risk of cataract in our subjects. The observed increase in SOD activity after exposure to E2 and reduction of ROS after preincubation with E2 in oxidatively stressed cells, support a role for E2 in the protection against oxidative stress in HLECs. The antioxidative effect of E2 in lens epithelial cells appears to be induced by non-genomic mechanisms.

Keywords: antioxidant enzyme, cataract, estrogen, gender, lens epithelial cells, oxidative stress, polymorphism, superoxide dismutase

LIST OF PAPERS

This thesis is based on the following research papers, referred to in the text by their Roman numerals:

I. **Dragana Čelojević***, Anne Petersen, Jan-Olof Karlsson, Anders Behndig, Madeleine Zetterberg. Effects of 17β-estradiol on proliferation, cell viability and intracellular redox status in native human lens epithelial cells. *Molecular Vision. 2011; 17:1987-1996.*

II. **Dragana Čelojević***, Staffan Nilsson, Anders Behndig, Gunnar Tasa, Erkki Juronen, Jan-Olof Karlsson, Henrik Zetterberg, Anne Petersen, Madeleine Zetterberg. Superoxide dismutase gene polymorphisms in patients with age-related cataract. *Ophthalmic Genetics. 2013; 34(3):140-145.*

III. **Dragana Škiljić**, Staffan Nilsson, Mona Seibt Palmér, Gunnar Tasa, Erkki Juronen, Anders Behndig, Jan-Olof Karlsson, Anne Petersen, Henrik Zetterberg, Madeleine Zetterberg. Estrogen–related polymorphisms in Estonian patients with age-related cataract. *Submitted manuscript, 2014.*

IV. **Dragana Škiljić**, Anne Petersen, Jan-Olof Karlsson, Anders Behndig, Staffan Nilsson, Madeleine Zetterberg. Effects of 17β-estradiol on activity, gene and protein expression of superoxide dismutases in human lens epithelial cells. *Manuscript.*

V. **Dragana Škiljić**, Staffan Nilsson, Anne Petersen, Jan-Olof Karlsson, Anders Behndig, Lada Kalaboukhova, Madeleine Zetterberg. Estradiol levels and superoxide dismutase activity in patients with age-related cataract. *Manuscript.*

* Paper published under former name Čelojević.

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ABBREVIATIONS

AP-1	activator protein 1
ATP	adenosinetriphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CO_2	carbon dioxide
COMT	catechol-O-methyltransferase
Ct	cycle threshold
CYP	cytochrome P450
DCFH-DA	2',7'-dichlorofluorescein diacetate
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E1	estrone
E2	estradiol (17β-estradiol)
E3	estriol
EPHA2	ephrin receptor A2
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HET	hydroethidine, dihydroethidium
HLE-B3	human lens epithelial B3 (transformed) cells
HLEC	human lens epithelial cells
HRP	horseradish peroxidase
HRT	hormone replacement therapy
HSP	heat shock protein
HWE	Hardy-Weinberg equilibrium
JC-1	5,5′,6,6'-tetrachloro-1,1',3,3′- tetraethylbenzimidazolylcarbocyanine iodide
LD	linkage disequilibrium

LDS	lithium dodecyl sulfate
MAPK	mitogen-activated protein kinase
MCB	monochlorobimane
MEM	minimum essential medium
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
$NO^{\bullet}$	nitric oxide
NO^{-}	nitric oxide anion
NOS	nitric oxide synthase
O_2	oxygen
$O_2^{\bullet -}$	superoxide
$^1O_2$	singlet oxygen
$ONOO^{-}$	peroxynitrite
$^{\bullet}OH$	hydroxyl radical
OH^{-}	hydroxide anion
OR	odds ratio
PBS	phosphate buffered saline
PCO	posterior capsular opacification
PCR	polymerase chain reaction
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
Prx	peroxiredoxin
PSC	posterior subcapsular cataract
qPCR	quantitative polymerase chain reaction
RFU	relative fluorescence unit
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SHBG	sex hormone-binding globulin
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
TGFβ	transforming growth factor β
V_{max}	maximum velocity

INTRODUCTION

The human eye lens

The lens is an important component of the optical system of the eye, together with the cornea responsible for refraction of light. For light to be transmitted and focused on the retina, the most important feature of the lens is transparency. Transparency is due to a highly organized system of lens cells, so called lens fibers. The shape, arrangement, internal structure and biochemistry of these cells make the lens unique, resulting in a transparent and avascular tissue, which receives its nourishment from the aqueous and vitreous humors. The lens is positioned in front of the vitreous body, behind the iris and is enclosed in a capsule (Figure 1).[1, 2]

The lens capsule is an elastic thick basement membrane, produced by the lens epithelium and it is attached to the zonular fibers at the lens equator. The zonules mediate movements from the ciliary muscle to the lens through the capsule. This is called accommodation and when the shape of the lens changes, it results in altered refractive power. Oxygen, glucose, amino acids, fatty acids and other nutrients pass through the capsule to the lens and waste products as lactate and CO_2 are transported out.[1, 3]

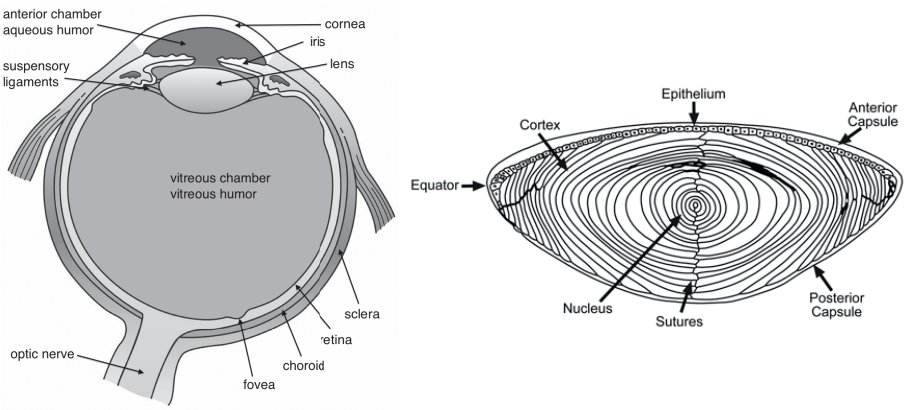

Figure 1. The human eye (left) and schematic drawing of the human lens (right).

The lens epithelium is a single layer of epithelial cells on the anterior surface of the lens, inside the capsule. As the cells divide they migrate to the lens equator, where differentiation takes place. The cells are then elongated and packed towards the center, the nucleus, of the lens (Figure 1). When the cells differentiate into thin fiber cells, they are tightly packed with minimal

extracellular space and lose all organelles as well as nuclei, processes that contribute to the lens transparency.[3, 4]

The lens fibers mostly contain water-soluble proteins, α-, β- and γ-crystallins, residues from the epithelial cells where they are produced. The most metabolically active part of the lens is the lens epithelium located on the anterior and pre-equatorial region of the lens. As the cells are packed towards the nucleus they lose their organelles, however the cells between the nucleus and equator, in the superficial lens cortex, can still be nucleated and metabolically active.[2, 3] Cortical lens fibers, as well as fibers in the nucleus, have been reported to exhibit some enzyme activity.[5, 6] The lens epithelium is a major site of detoxification and defense against oxidative stress and enzyme activity of several antioxidant defense systems have been detected.[7]

Aging of the lens

The primary lens fibers are formed before birth, during lens formation and are part of the lens throughout life in the innermost part of the lens nucleus. The lens epithelial cells continue to divide throughout life and more lens fibers are produced that are then tightly packed towards the nucleus, consequently leading to thickening of the lens during aging. Also, the curvature of the anterior surface of the lens changes so that the lens becomes more convex and the insertion point of the zonules is altered due to the age-related changes to the lens and capsule. Together with other age-related changes such as reduced elastic properties of the lens and a weakening of the ciliary muscle, a gradual decrease in accommodation is experienced from the age of forty, resulting in diminished ability to focus on near objects, a condition called presbyopia[8, 9]

Age-related changes of the lens proteins include post-translational modifications, conformational changes and loss of chaperone function. The ubiquitin-proteasome pathway is involved in degradation and removal of oxidized proteins and the activity of the ubiquitin conjugation activity decreases in the aging lens – contributing to accumulation of damaged and aggregated proteins resulting in loss of transparency, increased coloration and light scattering with age.[9]

During life we are constantly exposed to external physical and chemical agents and this is also the case for the lens where the lens epithelium is a major site of detoxification. However, the enzyme activity levels of several of the defense systems are reduced in the lens epithelium during aging. The nucleus, where the oldest lens fibers are found, has been demonstrated to have least protection and is particularly at risk of damage.[9, 10]

Cataract

Cataract is defined as opacification (clouding) of the normally transparent lens, which causes impaired visual function due to light-scatter. Cataractogenesis is the process of cataract formation and it is considered the leading cause of visual impairment and the most common cause of blindness in the world. The most recent report on visual impairment globally, describes cataract as being responsible for 51% of all blind patients and 33% of all patients with visual impairment, the second major cause after uncorrected refractive errors.[11]

As mentioned previously, increased aggregation and insolubility of proteins, light scattering and loss of transparency are features of the aging human lens and can be seen as precursors of cataract.[9] Age-related cataract (also called senile cataract) is a multifactorial disease and besides the major risk factor – aging – there are several other factors that contribute to development of lens opacities such as; gender, smoking, genetic predisposition, diabetes, ultraviolet (UVB) and ionizing radiation. Drugs or mechanical trauma can also induce cataract. Ethnicity, obesity, hypertension, socioeconomic status, estrogen exposure, antioxidants and alcohol are also factors that have been linked to the disease.[12-17]

The main types of age-related cataract are divided into nuclear, cortical and posterior subcapsular cataract (PSC) or a mixture of these types, depending on where the lens opacities are located. Nuclear cataracts occur in the lens nucleus, the central part of the lens, composed of fiber cells that are present at birth. Cortical cataract occurs in the fibers in the outer part of the lens, the cortex, most often starting in the equatorial parts of the lens, while posterior subcapsular opacities are located in the central part of the posterior superficial lens fibers. PSC is considered as being formed from fiber cells that fail to differentiate properly, resulting in migration and accumulation at the posterior pole of the lens. However, this type of cataract is the least common. The prevalence of the different types of cataract differs between ethnicity and different regions of the world, but nuclear cataract is reported as being the most common type overall.[18]

Nuclear cataract

Nuclear cataract is characterized by oxidation, loss of reduced glutathione (GSH), increased coloration and modifications of nuclear proteins such as insolubilization and cross-linking. The lens fibers are relatively intact and loss of transparency is instead caused by protein aggregates that cause light scatter and the coloration i.e. yellow/brown pigmentation which absorbs light. The reduced

transparency results in more impaired distant vision than near vision. A typical finding in nuclear cataract is also the loss of GSH in the nuclear region, which is due to oxidation – a key feature in the formation of nuclear cataract.[19, 20]

Smoking is a consistent risk factor for nuclear cataracts and the risk for developing nuclear opacities increases with the amount and duration of smoking.[14, 21, 22] Nuclear opacities have also been associated with white race, lower education and family history of cataract.[15]

Cortical cataract

As opposed to nuclear cataract, cortical cataract shows several major histological changes of the lens fiber arrangement. The changes involve disruption of fiber cell membranes resulting in swelling due to leakage of cytoplasmic content of damaged fiber cells. The effect on vision varies and depends on the location of the opacities.[2]

Most epidemiologic studies indicate that female gender is most strongly associated with cortical cataract and to some, but lesser extent, with nuclear cataract.[13, 16, 23] Also, non-white race, diabetes, heredity and UVB exposure have been associated with increased risk of cortical cataract.[17, 24-26] The Salisbury Eye Evaluation project demonstrated that exposure to UVB radiation from sunlight increases the risk of developing cortical lens opacities by 10%.[27] Several experimental studies have confirmed the increased risk of UVB exposure with cataract formation.[17, 28]

Posterior subcapsular cataract

Posterior subcapsular opacities are formed from fiber cells that fail to differentiate properly. Instead of cells elongating, they migrate and accumulate at the posterior surface of the lens. As in cortical cataract, disruption and swelling of lens fibers occur. Near vision is often more reduced than distant vision.[2]

Long-term use of steroids such as glucocorticoids is a major risk factor of developing PSC and this has been confirmed in both epidemiologic and experimental studies.[29, 30] Diabetes has also been associated with increased risk for PSC, as well as male gender and ionizing radiation.[31, 32]

Oxidative stress

Aerobic metabolism generates reactive oxygen species (ROS), a term that is often used to describe molecules or free radicals that can cause oxidative stress. Disturbance of the balance between ROS generated by normal metabolism or exogenous sources, and the antioxidant defense leads to oxidative stress, which can cause oxidative damage to biomolecules such as protein, lipids, carbohydrates and DNA.[33, 34] However, oxidative stress does not always lead to oxidative damage depending on severity of the oxidative stress and cell type. Cells can respond to mild oxidative stress by proliferation or adapting and upregulating defense systems, which results in cells becoming more resistant to higher levels of oxidative stress. But if defense systems cannot handle the increased production of ROS, then oxidative stress can lead to oxidative damage to biomolecules, senescence and even cell death.[33]

Oxidative stress plays a key role in cataractogenesis, which has been shown in experimental as well as in epidemiological studies. Elevated hydrogen peroxide levels have been demonstrated in lenses and aqueous humor from cataract patients.[35] With increasing age, the lens as well as other tissues is more susceptible to oxidative stress and less able to repair oxidative damage. Increased oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) in the nuclear region of the lens as well as oxidation resulting in extensive modification of nuclear proteins have been demonstrated in the lens.[19, 20, 36] Several of the risk factors for cataract identified in epidemiological studies - smoking, radiation and diabetes - results in oxidative stress. Smoking is a well-known risk factor and has been associated with nuclear cataract in several studies.[14, 21, 22] Other source of oxidative damage is radiation and especially UV. As we age the eyes are more susceptible to UV damage due to decrease of UV filters in our lenses.[37] The incidence of cataract is higher in diabetics as compared to non-diabetics and a combination of glycemic and oxidative stress is implied in the pathogenesis even though the exact mechanisms are not fully elucidated.[38]

As oxidative mechanisms are of importance in cataract formation, several large clinical trials have investigated if dietary antioxidants could have beneficial effects on cataract prevention or not, but the results have been inconsistent. A randomized clinical trial that included 4757 participants, The Age-Related Eye Disease Study (AREDS), found no effect on cataract incidence after supplementation with vitamin C, E and β-carotene.[39] Neither in the Australian, The Vitamin E, Cataract and Age-related macular degeneration Trial (VECAT), no effects of high dose vitamin E on cataract incidence or

progression were found.[40] However, small protective effects of antioxidant mixture of vitamin C, E and β-carotene were found in a smaller trial, The Roche European-American Anticataract Trial (REACT).[41]

Reactive oxygen species & Aging

In 1956 Denham Harman introduced "the free radical theory of aging" which suggested that free radicals cause accumulative and irreversible damage to macromolecules, loss of cellular function resulting in cell death – impacting both health and lifespan.[42] The importance of ROS in the aging process has been supported by a number of studies. Maximal lifespan of species is inversely correlated with the ROS-generating potential in many tissues but positively associated with the antioxidant capacity and it can also be prolonged by overexpression of antioxidant enzymes.[43, 44] Mitochondria are a major source of ROS production and have been implied as a target of oxidative damage during aging.[45] Additionally, more ROS and less ATP is produced in mitochondria during aging in mammalian tissue.[46] Interestingly, the age-related dysfunction of mitochondria exhibits gender-related differences, something that has been suggested to contribute to the difference in lifespan between male and females. Borras *et al.* have demonstrated that mitochondria from female rats generate half the amount of peroxides as compared to those from male rats, and this was not evident in ovariectomized rats. They also demonstrated that mitochondrial DNA from males exhibits 4 times higher levels of oxidative damage and that mitochondria from female rats have higher expression of the antioxidant enzymes.[47]

Reactive oxygen species in the lens

The lens metabolism is predominantly anaerobic and aerobic metabolism takes place solely in the lens epithelium. Oxygen can enter the lens via diffusion from the surrounding aqueous and vitreous humor. In the lens epithelium and superficial cortical lens fibers, mitochondrial respiration accounts for approximately 90% of the oxygen consumed by the lens, however other non-mitochondrial consumers of oxygen have also been demonstrated.[48] During mitochondrial respiration, ROS are produced via the electron transport chain where inefficient electron coupling leads to the formation of superoxide. ROS are also produced during cellular response to inflammation and viral infections and besides these endogenous sources of ROS, the lens is also exposed to exogenous sources of oxidative stress such as UVB exposure, ionizing radiation, cigarette smoke and drugs.[12, 14, 17, 32] ROS generated from the iris, ciliary body or

corneal endothelial cells can be accumulated in the anterior chamber, as hydrogen peroxide for example, and may then diffuse into the lens. In addition, ROS generated from the retina can be transported through the vitreous body and diffuse posteriorly into the lens. Hence, the anatomical location of the lens makes it relatively accessible and susceptible to ROS.[49]

ROS is a collective term that includes not only free radicals (with unpaired electrons) but also some non-radical derivates from oxygen. They are by-products or produced in different redox reactions i.e. when atoms undergo reduction (gain of electrons) or oxidation (loss of electrons).[33] Here are some of the most important ROS described in the lens:

Singlet oxygen ($^1O_2$) is more oxidizing than ground-state oxygen but still not a free radical since it does not have unpaired electrons. It is generated through absorption of photochemical energy in photosensitized reactions, such as radiation.[33]

Superoxide ($O_2^{\bullet-}$) is a radical formed when oxygen is reduced, i.e. one electron is added to the ground-state oxygen, and it can be generated by NADPH oxidases, xanthine oxidase or through autoxidation of molecules such as GSH. The major source of $O_2^{\bullet-}$ is the mitochondria, during energy production. $O_2^{\bullet-}$ is also a by-product of inflammatory response and generated in photosensitized reactions. Even though it is a radical it is not highly reactive but at high levels it can still cause extensive damage to proteins (containing iron-sulfur clusters) and most importantly it forms reactive intermediates such as hydroxyl radicals and peroxynitrite. The radical will not readily cross cell membranes and in aqueous solution one $O_2^{\bullet-}$ is oxidized to O_2 and another is reduced to hydrogen peroxide in a dismutation reaction (Figure 2).[33]

Hydrogen peroxide (H_2O_2) moves through cell membranes and is generated by several enzymes. It is poorly reactive and a weak oxidizing or reducing agent. However, it can still be cytotoxic, capable of inactivating enzymes, increasing $O_2^{\bullet-}$ production by activating NADPH oxidases, as well as forming damaging species such as hydroxyl radicals (Figure 2). It can be generated through the dismutation of $O_2^{\bullet-}$, by xanthine oxidase and several other oxidases.[33] H_2O_2 is often used to induce oxidative stress in lens epithelial cells in an experimental setting.

Hydroxyl radical (•OH) is a highly reactive free radical that can be generated by reaction with metal ions with H_2O_2, from ozone or peroxynitrite or when hypochlorous acid reacts with $O_2^{\bullet-}$. It can also be generated by other sources such as ultrasound, UV and ionizing radiation. •OH has high positive redox potential and can react with many different molecules, causing damage to amino acids, carbohydrates, phospholipids and DNA bases. H_2O_2 can accelerate •OH production with transitional metals such as iron (Fe^{2+}) in the Haber-Weiss and Fenton reactions (Figure 2).[33]

Nitric oxide (NO•), also called nitrogen monoxide, is a gaseous free radical that can react with $O_2^{\bullet-}$ to form peroxynitrite, a reaction that is competitive with the dismutation reaction. NO• is synthesized by the nitric oxide synthase (NOS) enzymes (Figure 2).[33]

Peroxynitrite ($ONOO^-$) is a non-radical formed from NO^- and O_2 or by the more common combination of NO• and $O_2^{\bullet-}$ (Figure 2). It is fairly unreactive although it can oxidize thiols and methionine among other molecules. It can also cause damage through oxidation of lipids, nitration of amino acids and DNA bases, resulting in DNA strand breaks and inactivation of enzymes.[33]

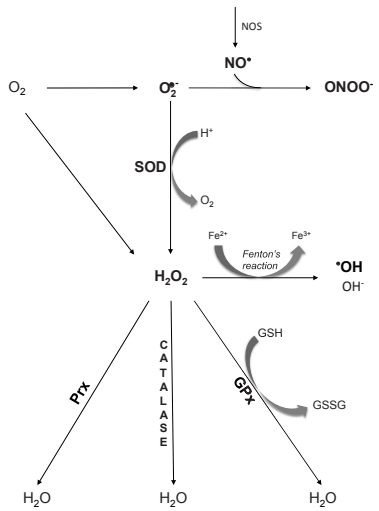

Figure 2. Major reactive oxygen species (ROS) and antioxidant enzymes in the lens.

O_2: oxygen; $O_2^{\bullet-}$: superoxide; NO•: nitric oxide; NOS: nitric oxide synthase; $ONOO^-$: peroxynitrite; SOD: superoxide dismutase; H^+: hydrogen; H_2O_2: hydrogen peroxide; Fe^{2+}/Fe^{3+} iron; •OH hydroxyl radical; OH^-hydroxide anion; Prxs: peroxiredoxins; GPx: glutathione peroxidase; GSH: reduced glutathione; GSSG: oxidized glutathione.

Defense systems in the lens

Imbalance between produced ROS and the antioxidant defense in cells leads to oxidative stress. This can result from reduced antioxidant defense and/or increased production of ROS.[33] Here are some of the antioxidant enzymes described in the lens:

Superoxide dismutase is involved in the dismutation of $O_2^{\bullet -}$ into O_2 and H_2O_2, described in detail later.

Catalase is normally found in the peroxisomes where it detoxifies H_2O_2 into water. Even though it has been demonstrated that increasing catalase expression in human lens epithelial cells (HLECs) protects against H_2O_2-induced oxidative stress,[50] lenses from mice lacking catalase did not show increased susceptibility to oxidative stress.[51] Hence, suggesting that catalase is not the most important enzyme involved in H_2O_2 scavenging.

Peroxiredoxins (Prxs), there are six different Prxs found in different organelles of mammalian cells; peroxisomes, mitochondria and endoplasmic reticulum, as well as in the cytosol. They are peroxide scavengers that have redox active cysteines and use the thioredoxin system as electron donor to scavenge H_2O_2 and $ONOO^-$ among other hydroperoxides.[52] In HLECs and fiber cells, significant mRNA and protein levels of one of the Prxs found in mitochondria (Prx 3) have been detected and this Prx was also induced by H_2O_2 in HLECs, suggesting that Prx 3 has an important role in detoxifying H_2O_2 in the lens.[53]

Glutathione peroxidase (GPx or GSHPx) reduces H_2O_2 into water through oxidation of GSH, which donates hydrogen and forms GSSG.[54] In a study, lenses from transgenic mice with elevated GPx activity was compared with lenses from GPx knockout mice. The lenses were exposed to H_2O_2 and the lenses from mice with elevated GPx levels showed significantly less cytotoxic effects and DNA strand breaks, when evaluating morphological changes in the epithelium and superficial cortex, compared to knockout lenses, thus suggesting that increased GPx activity protects the lens against H_2O_2-induced damage.[55] Furthermore, it has also been demonstrated that GPx activity is reduced in cataractous lenses, suggesting that increased oxidative stress is involved in cataract formation.[56]

Glutathione

There are several other defense systems involved in protecting the lens from oxidative damage besides antioxidant enzymes, such as ROS scavengers. Those are non-enzymatic molecules that bind and detoxify ROS. One of the major ROS scavengers in the lens is reduced glutathione (GSH), unusually abundant in the lens compared to other tissues. GSH is predominately found in its reduced form in the lens epithelium during normal metabolism and also found in lens fibers in high levels. The lens epithelium contains an active glutathione redox cycle, which reduces the oxidized form of glutathione, GSSG, back to GSH in a reaction catalyzed by glutathione reductase (GR) using NADPH as reducing agent.[20, 57] As mentioned previously, decreased levels of reduced GSH is seen in aging and cataractous lenses, as well as decreased GR activity levels. As reduced GSH is decreased, the oxidized form GSSG increases.[36, 58] The function of GSH in the lens is to preserve protein thiol groups in their reduced form which maintains normal protein function. When the levels of GSH are decreased and GSSG is formed in the lens, this is believed to increase the rate of posttranslational modifications of crystallins and damage key proteins containing –SH groups and proteins associated with membrane permeability.[59, 60] Ultimatley, these oxidatively induced protein modifications lead to increased cross-linking and formation of light scattering protein aggregates.

Other powerful ROS scavengers are vitamin C (ascorbate), vitamin E and carotenoids such as lutein and zeaxanthin. There are also other defense systems in the lens; free metal detoxifiers, protein repair systems, reducing systems and chaperone proteins.[61]

Superoxide dismutase

Superoxide dismutase (SOD) is involved in the detoxification of superoxide ($O_2^{\bullet-}$) by dismutation and is one of the major antioxidant enzymes. Dismutation is a reaction in which the same species is both oxidized and reduced, in this case superoxide, when one $O_2^{\bullet-}$ is oxidized to O_2 and another is reduced to H_2O_2.[33] Since superoxide not readily crosses cell membranes there are three SOD isoenzymes in mammalians; SOD-1, SOD-2 and SOD-3. They are encoded by three separate genes and confined to separate compartments of the cell. These metalloenzymes use copper-zinc or manganese to scavenge superoxide.[62]

SOD-1

The first SOD isoenzyme to be discovered was SOD-1, also called CuZn-SOD or erythrocuprein. This dimeric protein binds one copper and one zinc atom to each subunit. The copper atom is important for the catalytic activity and although zinc is not involved in the enzymatic activity it is important for maintaining the structure. The primary location of SOD-1 is in the cytosol but it is also found in the nucleus.[63] This enzyme is encoded by *SOD1* located on chromosome 21. Several mutations in this gene have been described in individuals with familial amyotrophic lateral sclerosis.[64, 65] In Down syndrome, triplication of chromosome 21 include the *SOD1* gene, resulting in elevated levels of SOD-1 activity. Paradoxically, this SOD-1 overexpression does not increase the antioxidant capacity of the cells, but instead appears to generate more oxidative stress.[66, 67]

SOD-2

The second isoenzyme discovered was the manganese-containing enzyme, SOD-2, also called Mn-SOD. This tetrameric protein contains Mn in the active site and it is primarily located in the mitochondrial matrix. It is highly important in detoxifying $O_2^{\bullet-}$ produced during mitochondrial respiration.[68] Mice deficient in SOD-2 exhibit high neonatal lethality, whereas mice lacking SOD-1 or SOD-3 have less pronounced effects on survival.[69-71] SOD-2 is encoded by *SOD2* on chromosome 6.[72, 73] Genetic polymorphisms in the gene have been associated with increased risk of diseases such as Alzheimer's disease, Parkinson's disease, prostate and breast cancer,[74-79] as well as aging and longevity.[80]

SOD-3

The last discovered isoenzyme was SOD-3, also called CuZn-SOD, due to the same metals in the active site as SOD-1. Unlike SOD-1 it is a tetrameric protein with higher molecular weight. It has a heparin-binding domain and is primarily found in the extracellular space (therefore also called EC-SOD), but it can also bind to cell surfaces through heparin sulfate proteoglycans.[81] In contrast to SOD-1 and SOD-2, the expression of SOD-3 appears restricted to only a few cell types in several tissues. SOD-3 can be proteolytically modified resulting in different forms of SOD-3 with altered affinity for heparin.[82] SOD-3 is encoded by *SOD3* located on chromosome 4.[83] A mutation in this gene have been shown to reduce heparin affinity resulting in a 10-fold increase of SOD-3 in human plasma.[84]

Superoxide dismutase in cataractogenesis

Given the composition of the eye lens, which is largely built up of tightly stacked lens fibers containing cytoplasm devoid of organelles, it is not surprising that SOD-1 is the predominant isoenzyme. The amount of SOD-2, which is likely confined to the lens epithelium and the superficial lens fibers, is relatively low in the human eye lens and the content of SOD-3 is negligible as the lens comprise very little extracellular space. However, SOD-3 is produced and secreted by lens epithelial cells and can be detected in the cell culture medium when the cells are cultured.[85] Although SOD activity is relatively low in the human lens as compared to other tissues, the role of SOD in the lens and in the pathogenesis of cataract may still be of importance.

Protective effects of SOD-1 against H_2O_2-induced oxidative damage have been demonstrated in whole rat lenses when SOD-1 protein and activity levels were overexpressed.[86] Protective effects were also seen when SOD-2 levels were upregulated in a transformed human lens epithelial cell line, as cells were more resistant to oxidative damage and showed greater cell viability.[87] Also, lenses from *Sod1* knockout mice developed age-related lens opacities earlier than wild-type mice, suggesting that SOD participates in the protection against age-related cataract.[88] Studies have also demonstrated reduced SOD activity in cataractous lenses as compared to clear lenses from humans.[56, 89] In addition, this decline in SOD activity has been demonstrated in aging lenses.[90] Rajkumar *et al.* have also demonstrated that SOD activity declines gradually with age; the highest levels of SOD were found in samples from patients 50 years of age or younger. They also showed varying levels of total SOD activity in patients with cataract depending on cataract subtype. SOD activity was highest in lens capsules samples from cortical cataract patients.[91] There have been reports of increased SOD activity in erythrocytes in cataract patients compared to controls and the POLA study group also showed an increased incidence of cortical cataract in patients with high SOD activity in erythrocytes.[92-94] However, conflicting data on SOD activity levels also exist and there are studies showing decreased SOD activity levels in erythrocytes, sera and lenses from cataract patients compared to controls.[95-97]

Studies with synthetic SOD mimics, Tempol and the reduced form Tempol-H, have showed protective effects against lens opacifications in organ culture and animal models. The synthetic compounds inhibited opacification of H_2O_2-induced damage in lenses in culture as well as protected against x-ray induced lens damage in rabbit.[98, 99]

Cataract & Gender

Female gender is consistent as a risk factor for cataract in several epidemiologic studies.[13, 16, 100-102] As mentioned, most epidemiologic studies indicate that female gender is most strongly associated with cortical cataract and to some, but lesser extent, with nuclear cataract.[13, 16, 23] Several lifestyle-related factors generally associated with cataract, such as UVB exposure and smoking habits, cannot explain the gender difference, since UVB exposure is higher and smoking more prevalent in men. It has also been suggested that there are gender-related differences in self-assessment of visual function and/or different demands for good visual acuity between men and women depending on their respective everyday activities or differences in longevity, which could contribute to this difference.[100, 103] However, the higher frequency of cataract surgery in women corresponds well with the higher prevalence of lens opacities in women, thus indicating that female gender truly increases the risk of cataract.[100, 101]

The higher prevalence of cataract in women has led to extensive investigations about the effect of endogenous as well as exogenous estrogen in cataract formation. Data are conflicting, but the majority of studies in this area suggest a protective role for estrogen. Studies have shown that earlier menarche and/or later menopause, causing extended period of reproductive years, are associated with decreased risk of cataract, thus indicating that estrogens may have protective effects on the lens.[104-107] Also, data from the Salisbury Eye Evaluation Project and the Beaver Dam Eye Study showed an association of increasing number of live births in younger women and protection against lens opacities.[108, 109] However, other studies did not find support for such associations.[110, 111]

Regarding exogenous estrogen, conflicting data exist both for the use of oral contraceptive pills and postmenopausal hormone replacement therapy (HRT). The Blue Mountains Eye Study showed a weak protective effect of oral contraceptive pills against the development of cortical cataract,[110] but no such association could be found in other studies.[104, 108] As for HRT, several studies indicate a protective effect of postmenopausal estrogen use against cataract.[106-108, 111] Other studies found no difference in overall cataract prevalence between HRT users and HRT non-users,[110] and a few studies even demonstrate an increased incidence of cataract extraction among long-term users of HRT.[112, 113]

Epidemiologic studies have shown that the gender difference in cataract prevalence occur in higher age-groups, after menopause, and that men, premenopausal women and women just entering menopause have the same prevalence of lens opacities and cataract extraction.[16, 100] It has therefore been

hypothesized that the increased risk of cataract in women is due to the dramatic decrease in estrogen levels at menopause, i.e. a withdrawal effect of the potentially protective estrogen, in contrast to the more steady estrogen concentration in men. In addition, androgen deprivation in the treatment of prostate cancer has been linked to increased risk of cataract in a large epidemiological study, showing that hormonal status may be important in cataractogenesis in both genders.[114] Also interesting, when comparing the serum concentration of 17β-estradiol (E2), the major estrogen in both genders before menopause, men have E2 levels in the same range as postmenopausal women (Table 1). There are even studies that have reported higher E2 levels in men compared to postmenopausal women.[115, 116]

TABLE 1. Reference range for 17β-estradiol in men and women

Women (menstrual cycle)	**17β-estradiol** (pmol/L)
Follicular phase	77-921
Periovulatory	139-2382
Luteal phase	77-1145
Postmenopausal	<36-103
Men	<40-162

The serum concentration of 17β-estradiol (E2) is shown for pre- and postmenopausal women and for men. Reference range from Sahlgrenska University Hospital, Gothenburg, Sweden, updated values from 2012. Concentrations account for total E2, both free and bound, in serum.

Estrogen

The main source of circulating estrogen in premenopausal non-pregnant women is the ovaries. After menopause estrogen is formed in peripheral tissues and the mesenchymal cells of the adipose tissue overtake the role as the main source of estrogen. Cells in the testes produce estrogen but both intratesticular and extragonadal production, like adipose tissue, are of physiologic importance as sources of estrogen in men.[117] Estrogens are steroid hormones that exist in three major naturally occurring forms. Estradiol (E2) is the predominant estrogen during the reproductive years in women compared to estrone (E1), which is the major estrogen after menopause and estriol (E3), which dominates during pregnancy when it is synthesized by the placenta. E2 possess 12 times higher estrogenic potency than E1 and 80 times higher than E3.[118, 119] Their structures differ in the number of hydroxyl (OH) groups attached, as implied by their names (Figure 3).

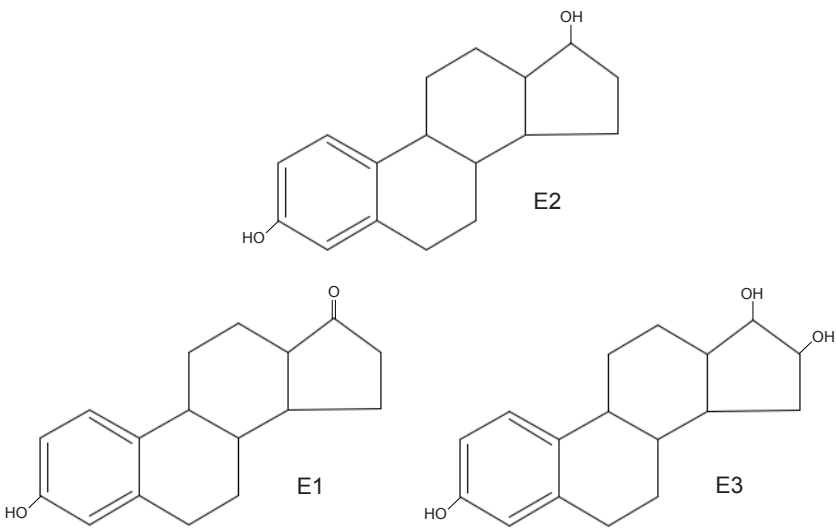

Figure 3. Chemical structures of estrogens; estrone (E1), estradiol (E2) and estriol (E3).

In the ovaries and testes estrogens are biosynthesized from cholesterol and testosterone respectively whereas in extragonadal sites estrogens are derived from C_{19} androgenic precursors.[120] Cholesterol is converted into pregnenolone and then into progesterone and eventually into C_{19} androgenic precursors; dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), androstenediol and androstenedione, and the latter two can be converted into testosterone. The final step in the biosynthesis of E2 is through aromatization of testosterone or from E1 (Figure 4). E2 is bound to sex hormone-binding globulin (SHBG) or albumin and only a small fraction of E2 circulates unbound and free in the blood. E3 is formed both from E1 and E2 by conversion in the liver.[118, 119] Estrogen metabolism include both phase I enzymes and phase II enzymes. Cytochrome P450 (CYP) are phase I enzymes and CYP17A1 (17α-hydroxylase or 17,20 lyase) and CYP19A1 (aromatase) are some of the major isoforms of these enzymes, involved in the biosynthesis of estrogens.[121] CYP1A1 is involved in the hydroxylation of E1 and E2 into their respective catechol estrogens, which results in lowered estrogenicity.[122] The estrogen inactivation is then catalyzed by phase II enzymes, one of which is catechol-O-methyltransferase (COMT) that converts catechol estrogens through O-methylation into methoxy metabolites with low or no affinity for estrogen receptors (ERs).[123, 124]

The biological effects of estrogens are many; both clinical and experimental studies show estrogens to be key regulators in tissue homeostasis by sensitizing cells to both mitogenic and apoptotic signals and by inducing expression of growth factors and cytokines.[125] Estrogen is generally associated with a proliferative response and is mainly considered anti-apoptotic. However, under certain circumstances, estrogen may also initiate apoptosis, hence the expression "the estrogen paradox".[126] Estrogens have been ascribed both antioxidative and pro-oxidative actions.[127] Estrogens have been reported to induce mitochondria-dependent ROS generation, whereas other studies show an estrogen-dependent inhibition of ROS production.[128, 129] The role of estrogens as ROS scavengers has therefore been argued. All estrogens are antioxidants since they contain a phenolic OH group at the C_3 position in the A ring (Figure 3). The free radical scavenging effect of this phenol group is thought to mediate an ER-independent protection against neurodegeneration, and blocking of this ring leads to elimination of neuroprotection.[130-132] However, the phenolic OH group may act both as a proton donor and as an electron acceptor, thus exhibiting both pro- and anti-oxidative properties.

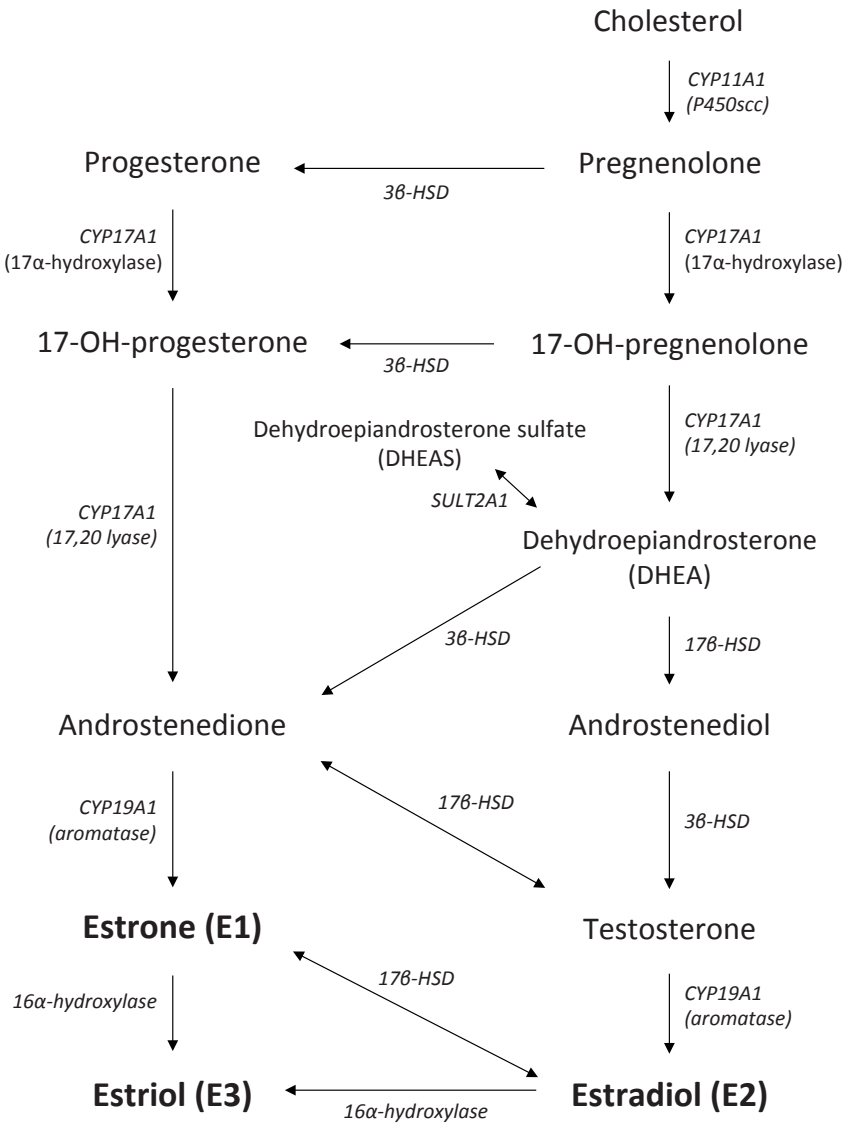

Figure 4. Biosynthesis of estrogens; estrone (E1), estradiol (E2) and estriol (E3).
CYP11A1: cytochrome P450 (CYP) 11A1 or P450scc (side-chain cleavage enzyme); CYP 17A1: cytochrome P450 (CYP) 17A1 or 17α-hydroxylase/17,20 lyase; 3β-HSD: 3β-hydroxysteroid dehydrogenase; SULT2A: sulfotransferase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; CYP19A1: cytochrome P450 (CYP) 19A1 or aromatase.

Estrogens can exert their actions by binding to their receptors ERα and ERβ. Several isoforms have been found of both the classical ERα and the more recently discovered ERβ. As for their affinity for different ligands, E2 binds equivalently to ERα and ERβ, whereas E1 prefers ERα and E3 binds preferentially to ERβ.[133, 134] ERs located in the cytoplasm are kept in an inactive state by heat shock proteins (HSPs). Like other steroid hormones, estrogens can exert their actions through the classic genomic pathway by binding to ERs, which releases HSPs and exposes nucleus localization sequences, leading to translocation into the cell nucleus and binding to specific sites in DNA at the promoter site of target genes, called estrogen response element (ERE). This is followed by recruitment of co-regulators to the transcription start site leading to induced gene expression.[135, 136] Apart from this classical signaling, another type of genomic mechanism of ER-signaling exists; transcription factor cross-talk, when ligand-activated ERs interact with with co-regulators and transcription factors, such as activator protein 1 (AP-1) and transcription factor Sp1, leading to induced gene expression.[137, 138]

Non-genomic effects of steroids do not depend on gene transcription or protein synthesis and involve cytoplasmic or membrane-bound regulatory proteins or membrane-localized ERs. There are also ligand-independent pathways where ER activity can be regulated through activation of several different signal transduction pathways such as extracellular signal-regulated kinases (ERKs) included in the mitogen-activated protein kinase (MAPK) pathway.[139] In addition, E2 have also been show to activate GPR30, belonging to the family of G-protein-coupled receptors, which is believed to be responsible for many of the rapid actions of estrogen.[140]

Estrogen in cataractogenesis

Both ERα and ERβ have been demonstrated in lenses from animals and humans.[141-144]As stated above, after menopause estrogens are no longer produced by the ovaries but are instead formed in peripheral tissues. In the rat retina, synthesis of E2 from pregnenolone and testosterone has been demonstrated, suggesting that some of the estrogen affecting the lens may come from intraocularly produced estrogen.[145] Estradiol has been identified in the aqueous humor of humans, but the investigators did not find any differences between cataract patients and controls nor between men and women, when measuring E2 levels in aqueous humor and serum.[146]

Since epidemiologic data shows increased risk of cataract in postmenopausal women, several groups have set out to investigate the effects of estrogen on the

lens in experimental models. Bigsby *et al.* showed in a rat model of age-related cataract that ovariectomized rats developed significantly less opacification of the lens if they received hormone replacement with E2 or E1.[141] This was also supported by another study where lenses from ovariectomized rats were treated with E2 were protected from opacification by transforming growth factor β (TGFβ), indicating that E2 confers protection against induced cataract in female rats, since female rats were more resistant to TGFβ-induced cataract compared to males.[147] Bigsby *et al.* also demonstrated that lens cells express both ERα and ERβ, thus implying that the protection by estrogen against cataract may be receptor-mediated.[141] In addition, transgenic mice expressing a dominant negative form of ERα, inhibiting the receptor, spontaneously developed cortical cataract.[148]

Given the antioxidative properties of estrogen described above, several investigators have examined the role of estrogens in human lens epithelial cells (HLECs) under conditions of oxidative stress. Estrogen-mediated protection from H_2O_2-induced oxidative stress in HLECs has been demonstrated in several studies.[149-151] Gajjar *et al.* demonstrated that the protective effect of E2 was rapid and seen already within 5 minutes of pretreatment, indicating a non-genomic mode of action.[150] Evidence for non-receptor mediated antioxidative protection also comes from Wang *et al.*, who demonstrated a dose-dependent increase in cell viability and intracellular ATP levels by E2, effects that were not abolished by an estrogen receptor antagonist. Gottipati *et al.* have demonstrated a significant increase in SOD-2 activity levels in transformed lens epithelial cells (HLE-B3) after incubation with E2 without any changes in either mRNA or protein expression levels, suggesting non-genomic mechanisms of E2. [152] Flynn *et al.* demonstrated that E2 protected against H_2O_2-induced oxidative stress in HLECs from both male and female donors. However, silencing of ERβ resulted in collapse of mitochondrial membrane potential, indicating that although the estrogen-mediated protection against oxidative stress was gender-independent, it was ERβ-dependent.[153] The exact mechanism, or relative contribution of genomic and non-genomic pathways in estrogen-mediated antioxidative protection remains to be elucidated.

Cataract & Heredity

Genetic variation constitutes the basis for making individuals unique, but it also influence the susceptibility to genetic diseases. The most common form of genetic variation is called single nucleotide polymorphism (SNP), which is a change in a single base in our DNA. The variants of a SNP are called alleles and the two alleles carried by an individual together make up the genotype of that person. An individual with the same alleles on both chromosomes is homozygous and if the alleles are different, the individual is heterozygous for that specific SNP. Genetic association studies investigate if allele and genotype frequencies of SNPs occur in different frequencies in individuals with a certain disease compared to individuals who do not have the disease i.e. control subjects.[154, 155]

Complex diseases or multifactorial diseases results from a combination of lifestyle related factors and a large number of common genetic variations, each contributing with only a minor increase in risk of disease. Unlike congenital cataract, which is largely inherited in a classical Mendelian manner involving single genes inherited in specific patterns, age-related cataract is a complex disease. Sibling correlations analyses from the Beaver Dam Eye Study suggested that a major gene could account for 58% and 35% of the genetic risk of developing cortical and nuclear cataract, respectively.[26, 156] However, the relative contribution of genetic versus environmental effects were not measured in these studies. Twin studies performed by Hammond *et al.* have showed that environmental effects are in fact less important than previously believed and that genetic factors have the highest impact on risk of cataract, even more than aging itself. Heredity accounted for 58%, environment for 26% and age for 16% of the clinical variance for cortical cataract.[157] For nuclear cataract the variance explained by genetic factors was 48%, environmental effects accounted for 14% and age for 38% of the variance.[158]

A couple of candidate genes have been reported to be associated with age-related cataract such as *GALK1*, *GSTM1* and *EPHA2*.[159] The gene encoding ephrin receptor A2 (EPHA2) on chromosome 1 has been associated with both congenital and age-related cataract in several studies.[160-163]

AIMS

The overall aim of this thesis was to study the role of estrogen and superoxide dismutase in cataractogenesis, including both cell culture and clinical studies.

The specific aims of each paper were:

Paper I

- to study the effects of estrogen on proliferation, cell viability and intracellular redox status, as well as possible antioxidative properties of estrogens in human lens epithelial cells.

Paper II

- to investigate genetic variations of the three different superoxide dismutase genes in an association study including patients with cataract and controls.

Paper III

- to study genetic variations of several estrogen-related genes in an association study including patients with cataract and controls.

Paper IV

- to investigate the effect of estrogen on superoxide dismutase activity and expression as well as the effect on expression and subcellular distribution of estrogen receptors in human lens epithelial cells.

Paper V

- to measure serum levels of estradiol and superoxide dismutase activity in patients with cataract and in controls and to investigate a possible correlation between estradiol and superoxide dismutase.

MATERIAL & METHODS

Cell culture

Human lens epithelial cells

Human lens capsule epithelium specimens were obtained during cataract surgery (phacoemulsification) at the Eye Clinic, Sahlgrenska University Hospital, in Mölndal, Sweden. The capsulorhexis specimens, usually 5 mm in diameter, were immediately after surgery put in tubes with cell culture medium. The lens epithelium specimens were then transferred from the tubes to 24-well culture dishes in a humidified 5% CO_2 incubator at 37°C, after which the human lens epithelial cells (HLECs) started to proliferate. When confluent, the HLECs were subcultured by trypsin-EDTA treatment, followed by resuspension in cell culture media; Eagle's minimum essential medium (MEM) or RPMI-1640 with phenol red supplemented with penicillin, streptomycin, L-glutamine, amphotericin B and 10% fetal bovine serum (FBS).

Each cell line from one individual was subcultured and passages between IV and XV were used for experiments. Despite the HLECs being derived from cataractous lenses, morphology of the cells exhibited a normal epithelial pattern, with no gross deviations from normal lens epithelial cell appearance.

Exposure to 17β-estradiol and oxidative stress

Stock solution of E2; 17β-estradiol (10 mM) was prepared in 99.5% ethanol. In paper I, HLECs were incubated in triplicates with E2 (0.0001, 0.001, 0.01, 0.1, 1 and 10 μM) in serum free cell culture media, RPMI-1640 without phenol red for 24 h at 37°C in a humidified 5% CO_2 incubator. For experiments where the antioxidative effect of E2 was studied, HLECs were preincubated with E2 for 4h prior to addition of 25 μM H_2O_2 for 24h. In paper IV, prior to each experiment, cells were washed with Dulbecco's phosphate buffered saline (PBS), after which the medium was changed to Eagle's MEM without phenol red and 5% FBS for 22-24 h before exposure to E2 in serum free medium. HLECs were incubated in triplicates with E2 (0.1 μM and 1 μM) for 1.5 h or 24 h. Control cells were incubated simultaneously, in an ethanol concentration equivalent to the highest E2 concentration, for 1.5 h or 24 h.

Cell viability & Cell death

MTT assay

MTT (3-[4, 5- dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide) is cleaved by mitochondrial dehydrogenases to insoluble formazan crystals in metabolically active cells and this method was used to detect viable cells and to measure cell viability in paper I. MTT was added to HLECs cultured in a 96-well plate and the cells were then incubated for 4 h, after which formazan crystals were solubilized in DMSO. Absorption was measured and the difference between the sample wavelength (570 nm) and the reference wavelength (650 nm) was calculated.

Hoechst

Hoechst 33342 is a specific stain for AT-rich regions of double-stranded DNA and used for the visualization of chromatin distribution in living cells. In paper I Hoechst was used to study the morphology of cell nuclei in HLECs in order to detect mitosis and apoptosis. Hoechst was also used to visualize nuclei for immunocytochemistry in paper IV. Cultured HLECs were fixed in 4% paraformaldehyde in PBS for 30 min, after which cells were stained with Hoechst for 15 min at 37°C. The coverslips were then mounted on chamber slides with Dako's fluorescent mounting media followed by counting of stained cells in a fluorescence microscope.

Caspase-3

Caspases are proteases involved in apoptosis and caspase-3 is one of the executive caspases. In paper I, caspase-3 activity was measured to detect and quantify apoptosis. After exposure, HLECs were centrifuged at 350 g for 5 min. The medium was removed, and the well plates were immediately frozen at −152 °C for at least 30 min. The frozen HLECs were thawed incubated in CHAPS-containing buffer including protease inhibitors; trypsin inhibitor, pepstatin, leupeptin and PMSF for 30 min at room temperature. The synthetic fluorogenic substrate, Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-AMC) and the reducing agent dithiothreitol (DTT) were added to the cell lysates. The fluorescent cleavage product of the substrate was measured during 2 h (Ex 380 nm, Em 460 nm) and V_{max} was determined in the linear interval. Proteolytic activity was expressed as the increase in relative fluorescence units per second and gram of protein (RFU $s^{-1}g^{-1}$).

Propidium iodide

Propidium iodide (PI) is a stain that binds to double-stranded DNA and can enter and stain non-viable cells, but it cannot cross the membranes of viable cells. In paper I, PI was used to determine the percentage of non-viable cells. PI was used in cell culture media without phenol red, and added to the cells for 3 min, after which fluorescence background was measured (Ex 540 nm, Em 620 nm). In order to determine the total number of cells, CHAPS-containing buffer was added and the cells were frozen at −80 °C. After lysis of cells, HLECs were thawed and total cell lysate/density was measured. This method was also used as reference for relative cell density in other methods in paper I, i.e. peroxides, superoxides and glutathione levels.

Intracellular redox status

Peroxides

The change in peroxide levels in paper I was measured using the non-fluorescent, 2',7'-dichlorofluorescein diacetate (DCFH-DA). In the cell, DCFH-DA is cleaved by esterases yielding polarized DCFH trapped within the cells, which is then oxidized to fluorescent DCF by several ROS including different peroxides (but not superoxide and hydrogen peroxide). The amount of formed DCF may therefore be considered a measurement of peroxide levels in the cell. The cells were incubated with DCFH-DA for 30 min at 37 °C and peroxide levels were measured (Ex 490 nm, Em 535 nm). The method is often referred to as a general method of monitoring ROS generated in cells.

Superoxides

In paper I, superoxide levels were measured in HLECs using dihydroethidium (also called hydroethidine; HET), which is oxidized by superoxides to the fluorescent substance ethidium. Changes in ethidium concentration can hence be used as a measure of superoxide levels. The cells were preloaded with HET for 10 min at 37 °C, after which the cells were rinsed in PBS and cell culture media without phenol red was added. Changes in superoxide levels were measured after incubation in 37 °C for 30 min (Ex 510 nm, Em 600 nm).

Glutathione

The level of reduced glutathione (GSH) levels in HLECs was determined using monochlorobimane (MCB), in paper I. MCB forms a fluorescent conjugate together with GSH in a reaction catalyzed by glutathione S-transferase (GST).

The final measurements were performed after 2 h (Ex 380 nm, Em 460 nm). Lower GSH levels indicate oxidation and the formation of oxidized glutathione (GSSG).

Mitochondrial membrane potential – JC-1

JC-1 (5,5′,6,6’-tetrachloro-1,1’,3,3′- tetraethylbenzimidazolylcarbocyanine iodide) was used in paper I to determine changes in mitochondrial membrane potential ($\Delta\psi$). HLECs were incubated with JC-1, after which the dye was removed, the cells were rinsed in PBS and serum free cell culture media was added. The green (Ex 485 nm, Em 535 nm) and red (Ex 540 nm, 590 nm) JC-1 signals were then measured. Oxidative stress usually causes a disruption of the mitochondrial membrane potential and the JC-1 dye indicates mitochondria depolarization by a decrease in the red to green fluorescence intensity ratio. In normal cells, due to the electrochemical potential gradient, the JC-1 dye is concentrated in the mitochondrial matrix, where it forms red fluorescent aggregates. A disruption of the mitochondrial membrane potential prevents the accumulation of the JC-1 dye in the mitochondria and thus, the dye is dispersed throughout the entire cell leading to a shift from red (JC-1 aggregates) to green fluorescence (JC-1 monomers).

Protein analyses

Protein expression

In paper IV, protein expression analyses were performed using western blot technique. HLECs were rinsed in ice cold PBS after exposure, followed by lysis in modified NuPage 0.5% lithium dodecyl sulfate (LDS) sample buffer. The cell lysate was heated at 70°C for 10 minutes and sonicated for 20 seconds at 50% amplitude. Immediately before gel loading, the reducing agent (DTT) was added. Triplets of the samples were loaded on NuPage 4-12% Bis-Tris gradient minigels using NuPage MES or MOPS SDS running buffer and sharp pre-stained protein standard. After electrophoresis, the proteins were transferred to nitrocellulose membranes followed by a blocking in 5% non fat milk powder in PBS over night in +4 °C. Primary antibodies used for Western blotting included; polyclonal rabbit anti- ERα, ERβ and SOD-1 as well as monoclonal mouse anti- SOD-2 and β-actin. Primary antibody binding was detected with the corresponding secondary antibodies conjugated to horseradish peroxidase. Protein expression bands were visualized with Luminata Forte Western HRP Substrate in the ImageQuant LAS 500, followed by densitometric analysis using ImageJ software version 1.37.

Protein determination

Protein concentration of cell lysates was determined using the BCA protein assay reagent with BSA as the standard and absorption was measured at 570 nm. This method was used in paper I, for caspase-3 assay and in paper IV for the SOD activity assay.

Immunocytochemistry

Immunocytochemistry was used in paper IV to visualize immunolabeling of ERα, ERβ, SOD-1 and SOD-2. After E2 exposure, the cells were rinsed in PBS and fixed in 4% paraformaldehyde (pH 7.4). The cells were rinsed again and permeabilized by 0.25% triton-X in PBS for 10 min at room temperature. Following standard protocols for immunocytochemistry, the cells were labeled with antibodies against ERα, ERβ, SOD-1 and SOD-2 and visualized by Alexa Fluor 488 Goat Anti-Rabbit or Anti-Mouse IgG (H+L) antibodies. Nuclear morphology was viewed using Hoechst 33342. Prior to fixation, cells were incubated with MitoTracker Deep Red FM, which was used for mitochondrial localization. The cells were viewed using a fluorescence microscope.

Superoxide dismutase activity

In paper IV, HLECs exposed to E2 were rinsed with PBS and the cell pellets were sonicated after which SOD activity was measured using the Superoxide Dismutase Assay kit, according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). Absorbance was measured at 440 nm on the microplate reader Infinite M200 PRO (Tecan group Ltd., Männedorf, Switzerland). The SOD assay uses tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit (U) of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measured total SOD activity (U/ml). In paper V, erythrocytes were collected from whole blood samples after centrifugation at 800 x g for 10 min at 4°C. The erythrocytes were then lyzed in four times its volume of ice-cold distilled water after which the samples were centrifuged at 10.000 x g for 15 min at 4°C. The erythrocyte lysate (supernatant) was then collected and aliquots were stored at -80°C, after which SOD activity was measured.

Estradiol levels

In paper V, venous blood samples were drawn from patients and controls and collected using tubes with clot activator and gel separator for serum samples. The serum sample tubes were centrifuged at 1800 x g for 10 minutes after coagulation, within 3 hours after sampling. Sera was then collected and aliquots were stored at -80°C. Serum E2 (17β-estradiol) analyses were performed with radioimmunoassay (Spectria Estradiol RIA, Orion Diagnostica, Espoo, Finland) with an extraction step prior to quantification, yielding a sensitivity of 4 pmol/L. The assay measures total estradiol serum concentrations *i.e.* both free E2 and previously bound to sex hormone-binding globulin (SHBG) or albumin.

Molecular genetics

SNPs & Haplotypes

A combination of alleles from adjacent SNPs located on the same parental chromosome is called haplotype. Genetic recombination is a natural event during meiosis caused by pairing of parental chromosomes, which results in gametes of alternated segments of different parental origin. Adjacent SNPs are less likely to be recombined and will therefore be inherited together and will be correlated, which is referred to as SNPs being in linkage disequilibrium (LD). Groups of adjacent SNPs form LD blocks, also called haplotype blocks. Due to specific patterns in these blocks, specific SNPs can predict the other SNPs within the same block. These representative SNPs, called tag SNPs, can cover the entire variation of a gene or a region. Tag SNPs were used for haplotype analyses of *SOD2* in paper II.

All SNPs in paper II and III were checked for Hardy-Weinberg equilibrium (HWE), which is a measure of the correspondence between allele and genotype frequencies in a population where random mating without selection pressure occurs. Genotyping or data handling errors can be detected if analyses show deviation from HWE, i.e. significant difference between expected and observed allele frequencies (using χ^2-test) in the disease and control group. Part of the reason for checking deviations from HWE is to test if a population is of homologous ethnic origin to avoid spurious association due to population stratification, but often lack of HWE is an indication of genotyping errors.

Genotyping

All the SNPs in paper II and III were genotyped using genomic DNA extracted from whole blood samples with TaqMan SNP Genotyping Assays. TaqMan Drug Metabolism Genotyping Assays were used for some of the SNPs in paper III. The TaqMan assays were used according to the TaqMan Allelic Discrimination technology. The technology uses fluorescent probes in polymerase chain reaction (PCR). Two probes are used, each sequence-specific for each allele and each are labeled with one fluorescent reporter dye each (VIC and FAM), together with a quencher that absorbs fluorescence at the other end of the probe. During the PCR when DNA polymerase extends the newly synthesized DNA strand, the probe is cleaved if it has bound (matched) the sequence specific for the allele and then the quencher will no longer be attached to the probe, resulting in increased fluorescence intensity from the specific

probe. If the probe does not bind (mismatch) the sequence in the allele, it will not be cleaved by the polymerase and there will not be an increase in fluorescence intensity. After the PCR amplification, the fluorescent signals are measured and plotted in an allelic discrimination plot, making it possible to discriminate between the genotypes. The signal from only one probe means that the individual is homozygous for the allele represented for that probe. If the individual is heterozygous, signals from both alleles will be detected (Figure 5).

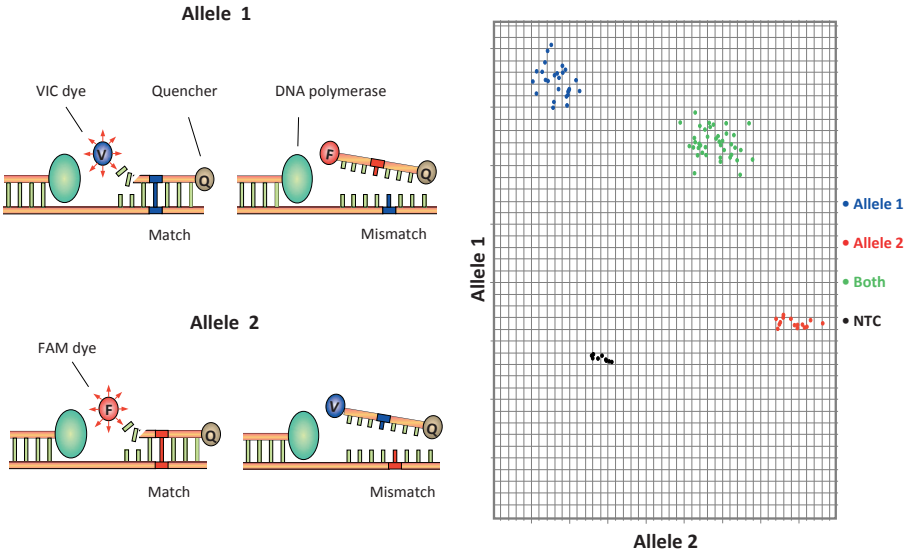

Figure 5. TaqMan Allelic Discrimination technology according to Livak *et al.* Fluorescent reporter dye (VIC and FAM) together with a quencher (Q), cleaved by DNA polymerase if the probe is a match (yielding fluorescence) and if mismatched, the probe will be displaced (no fluorescene). The allelic discrimination plot displays genotypes for each individual; homozygous for allele 1 (blue), heterozygous (green) and homozygous for allele 2 (red). Non-template control (NTC) are also used to exclude any contaminations.

Gene expression

In paper V TaqMan Gene Expression Assays were used specific for the studied (target) genes; *SOD1*, *SOD2* and *SOD3*, and reference genes; *RPLP0* and *PPIA*. Quantitative PCR (qPCR) was used to measure gene expression by quantifying the amount of mRNA, which first was converted to the more stable complementary DNA (cDNA) by reverse transcription PCR (RT-PCR), before it was used in the qPCR. The fluorescence from the sequence-specific TaqMan probes used in the qPCR, was measured in real-time. The fluorescence intensity for each sample is plotted against the amplification cycle and when calculating the relative quantification the cycle at threshold (Ct) is used. A threshold is set in the exponential phase of the amplification curves and cycle at threshold is read for each sample (Figure 6). The Ct is inversely related to the amount of mRNA in the initial sample, i.e. the higher Ct value, the less amount of mRNA and lower gene expression. Data (Ct values) is then normalized to one or several reference genes to account for differences in starting material. Calculations of the relative gene expression are performed according to Livak and Schmittgen described here:

$Ct_{\text{target gene}} - Ct_{\text{reference gene}} = \Delta Ct$

$\Delta Ct_{\text{exposed group}} - \Delta Ct_{\text{unexposed group}} = \Delta\Delta Ct$

$2^{-\Delta\Delta Ct}$ = fold change of relative expression

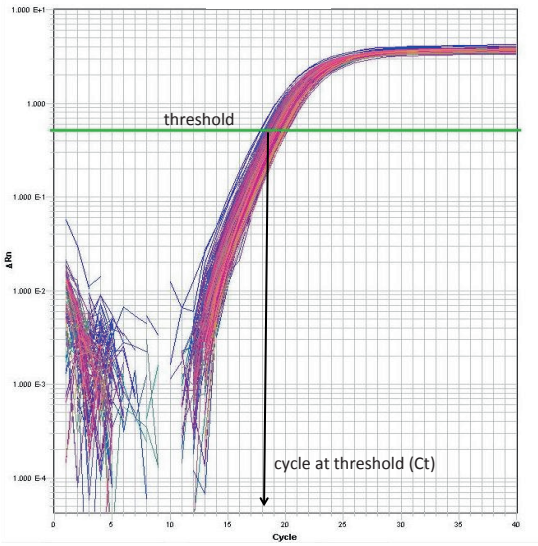

Figure 6. Example of amplification plot where fluorescence intensity is plotted against amplification cycle. Threshold (green line) is set in the exponential phase of the amplification curves and cycle at threshold (black line) is read for each sample.

Patients

In paper II and III, Estonian patients with age-related cataract and controls were genotyped. The studied subjects consisted of 492 patients with age-related cataract and 185 controls, recruited from two ophthalmic clinics in Tartu and the South Estonian area. The cataracts were determined and classified into the following subtypes; cortical cataract (n=151), posterior subcapsular cataract (n=119), nuclear cataract (n=75) and mixed cataract (n=147). Classification was done prior to surgery, using biomicroscopy and ophthalmoscopy, and patients with secondary cataracts were excluded. Subjects without cataract, uveitis, and glaucoma were included as controls. Data on smoking (current and former smoking habits) was obtained for all individuals and age was reported at the time of surgery or when included in the study as controls.

In paper V, patients were recruited at the Eye Clinic, Sahlgrenska University Hospital, in Mölndal, Sweden. The study subjects consisted of 103 patients with age-related cataract and 22 controls. Cataracts were classified as nuclear, cortical, or posterior subcapsular using biomicroscopy and patients with secondary cataracts were excluded. Controls were either spouses or relatives to glaucoma patients, had glaucoma themselves or were recruited from the emergency ophthalmic clinic, with diagnoses such as vitreous detachment or blepharitis. Exclusion criteria for controls were previous cataract surgery or lens opacities and age <60 years. Data on smoking (current/former smoking habits), hormones (current/former use of contraceptives and/or hormone replacement therapy), diabetes and age at menarche/menopause was obtained for all individuals and age was reported at the time of sample collection.

Ethics

In all the studies included in this thesis, written informed consent was obtained and the studies were conducted following the tenets of the Declaration of Helsinki. The Regional Research Ethics Committee in Gothenburg approved the studies in paper I, IV and V. The Ethical Commission at the University of Tartu in Estonia approved the studies in paper II and III.

Statistics

In the cell culture studies, paper I and IV, one-way ANOVA with Dunnett's post hoc test were used to compare exposed groups with unexposed control group. The gene expression data, in paper IV, was normalized to the reference genes, *RPLP0* and *PPIA*, and compared to the expression in control cells according to the $2^{-\Delta\Delta Ct}$ method, using a linear mixed model.

In paper II, III and V, demographic differences between cataract patients and control subjects were analyzed using Student's t-test for continuous parameters and Pearson's chi-square test (or Fisher's exact test when appropriate) for categorical parameters. Also one-way ANOVA was used when comparing age between the different subtypes of cataract. In paper II and III, single marker association tests were performed using logistic regression in an additive model (homozygote for major allele=0, heterozygote=1 and homozygote for minor allele=2), including relevant risk factors for age-related cataract; age, sex and smoking, as covariates in the analyses. Binary logistic regression was also used when performing single marker associations between the different subtypes. Differences between allele frequencies were analyzed with chi-square test. All SNPs were analyzed for deviation from Hardy-Weinberg equilibrium and haplotype analysis (paper II) was performed using Haploview 4.2. Bonferroni correction was used to adjust for multiple testing in paper III. In paper V, multiple linear regressions were used to analyze potential predictors of the dependent variables; E2 levels and SOD activity.

Statistical analyses were performed using IBM SPSS Statistics versions 18-21 (IBM Corp., Armonk, NY, USA) and p-values ≤ 0.05 were considered statistically significant.

RESULTS & DISCUSSION

Effects of estrogen in HLECs (paper I and IV)

Since estrogens have been ascribed both antioxidative and pro-oxidative actions and the effects of different doses differ between cell lines, dose-response experiments were performed initially. The effects of 17β-estradiol (E2) were then studied at concentrations between 0.1 nM and 10 μM (paper I), which are relevant physiologic and pharmacological concentrations. Two concentrations, 0.1 μM and 1 μM, were chosen for further studies (paper IV).

Oxidative effects of estrogen

In paper I, HLECs exposed to 10 μM E2 resulted in a significantly lower number of viable cells, decreased number of mitotic nuclei, an increase in the relative number of non-viable cells i.e. increased cell death, as well as increase in the number of apoptotic nuclei and increased caspase-3 activity. Both an increase in peroxide and superoxide levels was observed. There was also a decrease in GSH levels at the same concentration of estrogen. Results from paper I therefore suggest pro-oxidative effects of the pharmacological (10 μM) E2 concentration (Table 2). However, mitochondrial membrane potential was not affected, indicating that this increased ROS production did not interfere with mitochondrial function.

Table 2. Effects of E2 at the pharmacological concentration, 10 μM

Cell viability (MTT)	↓
Mitosis (Hoechst)	↓
Cell death (PI)	↑
Apoptosis (Caspase-3)	↑
Apoptosis (Hoechst)	↑
ROS (Peroxides)	↑
ROS (Superoxides)	↑
Reduced glutathione (GSH)	↓

The effects of 17β-estradiol (E2) were compared to control cells (0 μM E2).

The exact mechanism for estrogen-mediated apoptosis is not clear; both extrinsic and intrinsic apoptotic pathways exist, the latter being triggered by collapse of mitochondrial membrane potential. Our data demonstrate caspase-dependent apoptosis at pharmacological concentration of E2, without mitochondrial depolarization.

Proliferative effects of estrogen

In paper I, lower concentrations of E2 (1 nM), showed an increase in the number of mitotic nuclei and decrease in cell death, which was observed with Hoechst and PI, respectively. Results from paper I suggest proliferative effects of E2 at lower concentration, 1nM and the stimulatory effect of physiologic concentrations of estrogen on proliferation of cultured HLECs in this study is especially interesting from an ophthalmologic view since female gender has been suggested as a risk factor for developing posterior capsular opacification (PCO).[164] Data from our group also show a slightly higher rate of cell growth in capsule-epithelium specimens derived from female cataract patients compared to men.[165]

Estrogen protection against oxidative stress

To examine the effect of E2 in oxidatively stressed HLECs in paper I, the cells were preincubated with E2 at different concentrations for 4 h and then simultaneously exposed to 25 μM H_2O_2 and E2 for 24 h. At lower concentrations, 0.01 to 1 μM, E2 protected against oxidative stress, as evident by decreased peroxide and superoxide levels as compared to control cells exposed to H_2O_2 alone (Figure 7). The peroxide levels in HLECs incubated with E2 were reduced to the same level as in control cells not exposed to H_2O_2. Superoxide levels were also decreased but not to the same extent as in cells without H_2O_2 exposure. An increase in the mitochondrial membrane potential (JC-1) was seen in the lower range of E2 concentrations used, 0.1–10 nM. However, E2 did not reverse the effects of 25 μM H_2O_2 on mitochondrial membrane potential or GSH levels. Other studies have also demonstrated estrogen-mediated protection against H_2O_2-induced oxidative stress in a transformed human lens epithelial cell line (HLE-B3). Pretreatment with E2 caused a dose-dependent preservation of mitochondrial potential, intracellular ATP-levels and increased cell viability. However, no effects on ROS levels were seen in the same study at concentrations ranging from 1 nM to 10 μM E2, when using 100 μM H_2O_2 to oxidatively stress lens epithelial cells.[166] This is thus in contrast to our data from paper I where E2 reversed the increase in peroxide

and superoxide levels seen in HLECs exposed to 25 μM H_2O_2. The H_2O_2-concentration, 25 μM, used in experiments in paper I was well within the reported range of H_2O_2 found in aqueous humor of cataract patients,[35] and was chosen since this level of H_2O_2 induced ROS-production but did not cause apoptosis. Even though we did not detect estrogen-mediated protection of mitochondrial membrane potential against H_2O_2-induced oxidative stress, the results from paper I still indicate protection against oxidative stress, suggesting antioxidative effects of E2 at lower (0.01 to 1 μM), more physiologic concentrations.

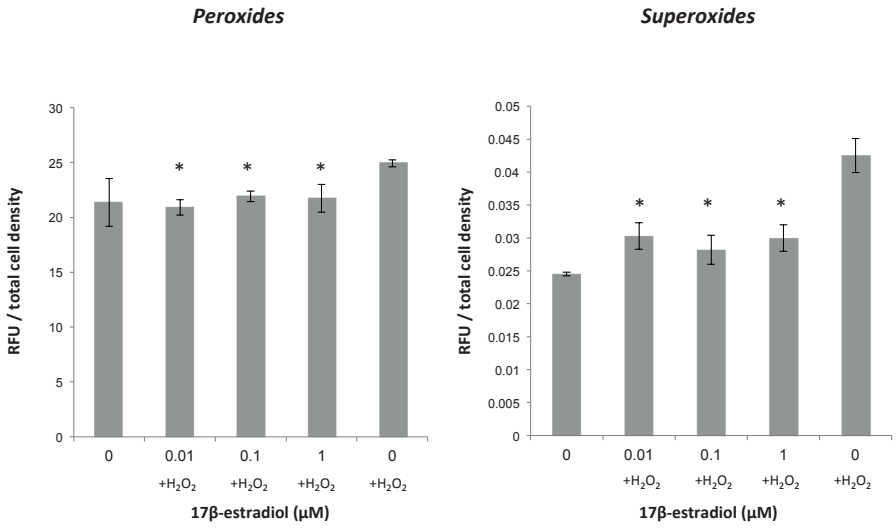

Figure 7. Antioxidative effects of 17β-estradiol (E2) against oxidative stress. Human lens epithelial cells preincubated with E2 for 4 hours after which cells were simultaneously exposed to 25μM hydrogen peroxide (H_2O_2) for 24 h. A significant decrease in peroxide (left) and superoxide (right) levels compared to control cells exposed to H_2O_2 but not E2. Peroxide levels reduced to same levels as unexposed cells. Asterisks indicate statistical significance $p<0.05$.

Effects of estrogen on SOD expression and activity

In paper IV, strong immunolabeling with SOD-1 was seen in the cytosol and nucleus in contrast to SOD-2 where mitochondrial localization dominated for SOD-2. No subcellular redistribution of SOD-1 or SOD-2 was seen with E2 exposure. SOD-1 is the predominant isoenzyme in the lens and the amount of

SOD-2, which is likely confined to the lens epithelium, is relatively low in the human eye lens and the content of SOD-3 is negligible.[85] Therefore it is not surprising that we observed lower gene expression levels of *SOD3* in HLECs as compared to *SOD1* and *SOD2* levels. However, we found no significant changes in *SOD1*, *SOD2* or *SOD3* gene expression neither after 1.5 h nor after 24 h exposure to 0.1 μM and 1 μM E2, when compared to the expression in control cells and after normalization to reference genes. A slight increase in both SOD-1 and SOD-2 protein expression was seen at 0.1 μM after 1.5 h and elevated SOD-2 levels were also seen at 1 μM E2 after 24 h. However, these results were not statistically significant compared to control cells. A significant increase in total SOD activity was seen in whole cell lysate from HLECs after exposure to 0.1 μM and 1 μM E2 for 1.5 h. By 24 hours however, SOD activity was back to baseline values. Thus, the effects of E2 on SOD activity did not correlate with protein or gene expression of the different SODs. The results from paper IV are in accordance with Gottipati *et al.* who showed a significant increase in SOD-2 activity levels in HLE-B3 cells after exposure to E2 without any changes in either mRNA or protein expression levels.[152] A few other studies have also reported increased SOD-2 activity levels without alteration of SOD-2 protein levels in mitochondria after E2 exposure.[167, 168] However, yet other studies report both upregulated gene and protein expression of SOD-2 and SOD-3 in an E2 concentration- and time-dependent manner mediated by ERs, in vascular smooth muscle endothelial cells.[169] Also, E2 showed antioxidative effects by upregulation of GPx and SOD-2 expression via activation of the MAPK pathway through ERK phosphorylation.[170] The discrepancies regarding E2 effects on SOD in both protein and gene expression may be attributed to differences between cell lines. The exact mechanism, or relative contribution of genomic and non-genomic pathways in estrogen-mediated antioxidative protection remains to be elucidated. Results from paper IV demonstrate that E2 have effects on SOD activity but not on gene or protein expression of SODs, thus implying non-genomic mechanisms of E2.

Protein expression and immunolocalization of estrogen receptors

In paper IV, we showed that both ERα and ERβ were present in the nucleus and mitochondrial localization of ERβ was evident by colocalization with MitoTracker. Our results also showed reduced ERα expression levels as well as the reversed effect, elevated ERβ expression levels and increased immunolabeling, with increased E2 concentration. This may be explained by a study showing that ERβ exhibits an inhibitory effect on ERα-mediated gene

expression, when ERs are coexpressed.[134] The mitochondrial localization and elevated expression levels of ERβ due to E2 exposure indicate mitochondrial involvement, and this supports that the suggested E2-induced mitochondrial cytoprotective effects are mediated through ER-dependent mechanisms in HLECs.

Native human lens epithelial cells vs. transformed lens epithelial cell line

Results are preferably compared to experiments using the same cells as in your own experiments, but this may be difficult since few studies use the exact same cells. In paper I and IV, native HLECs were used in contrast to most studies performed so far on estrogen and ERs in lens epithelial cells, in which the transformed human lens epithelial cell line, HLE-B3, was used. The relative abundance and preferential subcellular distribution of the two ERs and their respective isoforms exhibit substantial variations between species as well as between freshly isolated capsulorhexis specimens and cultured HLECs and also in native versus transformed cells.[171, 172] The intracellular localization and ratio between ERα and ERβ have been suggested to account for much of the differences seen in estrogen response in various cell types and may also explain discrepancies between studies performed on native HLECs and HLE-B3. Flynn *et al.* have demonstrated a difference in ERβ variants distribution and RNA expression as well as responsiveness to oxidative stress between HLECs and HLE-B3.[173] However the subcellular localization of wild-type ERβ were the same for the different cell lines and in accordance with our results.

Whenever possible, using native cells - as in paper I and IV - is preferable. The use of immortalized and transformed cell lines such as HLE-B3 has been questioned since the cells are adapted and likely to lose characteristics that are critical to their function in vivo.[174]

Genetic variations in superoxide dismutase and estrogen-related genes in cataract (paper II and III)

Superoxide dismutase genes

In paper II, polymorphisms in the three SOD genes (*SOD1*, *SOD2* and *SOD3*) were studied in Estonian patients with age-related cataract and controls. The eight studied SNPs were either functional, disease-associated or tag SNPs. However, none of the studied SNPs showed significant associations with risk of cataract, neither in univariate analyses nor when having the known risk factors; age, sex and smoking as covariates in multivariate analyses. These results were consistent also after stratifying for type of cataract. Paper II, thus does not support a major role for SOD gene variations in cataractogenesis.

Estrogen-related genes

In paper III, eight either functional or disease-associated SNPs in estrogen-related genes were studied in the same Estonian population as in paper II. The estrogen-related genes encoded ERs (*ESR1, ESR2*) and enzymes involved in the biosynthesis (*CYP17A1, CYP19A1*), bioavailability (*CYP1A1*) and inactivation (*COMT*) of estrogens. None of the SNPs showed significant associations with risk of cataract when comparing all cases and controls, neither in the univariate analyses nor when adding the known risk factors (age, sex and smoking) as covariates in the analyses. After stratification by cataract subtype, the nuclear cataract group had a significantly lower minor (C) allele frequency of rs10046 (*CYP19A1*) compared to the control group ($p=0.026$). After including covariates in the analysis the adjusted odds ratio (OR) was 0.56 with a 95% confidence interval of 0.35 to 0.90, $p=0.016$. However, after correcting with Bonferroni for multiple testing of the eight SNPs in the four different cataract subtypes, this finding was no longer significant ($p=0.51$). Thus, paper III does not support a major role for genetic variations in estrogen-related genes to be involved in cataractogenesis.

Estonian patients and controls

There was no difference in gender distribution between patients and controls in the Estonian study population. The higher frequency of nuclear opacities among smokers in the Estonian cohort is also in accordance with epidemiologic data reporting smoking as a risk factor for nuclear cataract.[14, 21, 22]

A limitation of both paper II and III was the number of study participants, especially controls, included in the Estonian study population which may have contributed to the inconclusive results. Other limitations were that the mean age was significantly lower in the control group, resulting in a number of "false negative" control subjects who may develop cataract in the next few years. In addition, there was no information about the composition of mixed cataracts, which - if available - would have enabled sorting of mixed cataracts into defined subtypes.

Estrogen levels and superoxide dismutase activity in cataract patients and controls (paper V)

To date, no other study has investigated both serum estradiol levels and erythrocyte superoxide dismutase activity in patients with cataract. This makes the investigation in paper V, unique. Comparisons between the cataract group and control group have been performed in this study even though a weakness was the number of controls, only 22 subjects were included compared to 103 cataract patients. As in the Estonian study population in paper II and III, there was no difference in gender distribution between patients and controls. In this study we could however divide the patient by type of lens opacities so that a patient with mixed cataract could be included in several subtypes, and this is considered an advantage. However, the diabetic incidence was higher among the patients while none of the controls had diabetes something that may be explained by the increased risk of developing lens opacities when having diabetes.[25]

Estradiol levels in serum

In our studied subjects, in paper V, men had significantly higher E2 levels compared to postmenopausal women ($p<0.001$). It has previously been reported that serum E2 levels are higher in older men than in older women due to higher levels of testosterone and DHEA in men, resulting in higher E2 through peripheral aromatization, thus yielding higher E2 levels in men compared to women after menopause.[115, 116] This means that the potentially protective effects of E2 that are dramatically reduced at menopause are even below men's levels, therefore possibly contributing to increased risk of cataract. This would also contribute to the reduced risk of cataract in women using HRT, as implied by several studies.[106, 107, 111]As expected a linear relation between increasing age and declining E2 levels i.e. a negative correlation was observed.

Multivariate analysis showed significant impact of both age (p=0.021) and sex (p=<0.001) on E2 levels, but cataract diagnosis on the other hand did not.

Superoxide dismutase activity in erythrocytes

No correlation was seen with SOD activity and age, gender or cataract diagnosis in paper V. There have been reports of increased SOD activity in erythrocytes in cataract patients compared to controls as well as an increased incidence of cortical cataract in patients with high SOD activity in erythrocytes.[92-94] Rajkumar *et al.* demonstrated increased SOD activity in lens capsules samples from cortical cataract patients and the activity declined gradually with age in all samples; the highest levels of SOD were found in samples from patients 50 years of age or younger.[91] However, conflicting data on SOD activity levels also exist and there are studies showing decreased SOD activity levels in erythrocytes, sera and lenses from cataract patients compared to controls.[95-97] Our data could not confirm any correlations with SOD activity in erythrocytes.

Estradiol vs. Superoxide dismutase

No correlation between SOD activity and E2 levels was found, not even when including age, gender and diagnosis in the model. Even though in vitro studies have demonstrated that E2 can influence SOD expression and/or activity, we could not find any correlation between SOD activity and E2 concentration in our studied subjects.

CONCLUSIONS

Effects of estrogen in human lens epithelial cells were

- increased proliferation, reduced cell death, stabilization of mitochondrial membrane potential and protection against oxidative stress at lower (physiologic) concentrations.

- increased caspase-3 activity, apoptosis, cell death and intracellular reactive oxygen species as well as reduced GSH levels, cell viability and proliferation at higher (pharmacological) concentrations.

Genetic variation in superoxide dismutase and estrogen-related genes was

- not associated with increased risk of cataract in an Estonian population.

Effects of estrogen on superoxide dismutase and estrogen receptors in human lens epithelial cells were

- increased total superoxide dismutase activity.

- unchanged protein and gene expression of superoxide dismutases.

- altered protein expression levels of estrogen receptors.

Estrogen levels and superoxide dismutase activity in cataract patients and controls showed

- a linear correlation between increasing age and declining estradiol levels.

- higher estradiol levels in men compared to postmenopausal women.

- no correlation between superoxide dismutase activity and estradiol levels in our studied subjects.

Concluding remarks

Cataract is the most common cause of blindness in the world and the only treatment for cataract today is to surgically remove the lens, most often replacing it with a plastic lens prosthesis. In the western world, cataract extraction is the most frequent surgical procedure and as lifespan is getting longer, an increased number of people requiring cataract surgery can be anticipated. Cataract surgery is not without risks and postoperative complications, together with high healthcare costs for society as well as visual impairment and disability affecting people's everyday life, are reasons to continue investigating the underlying mechanisms and to find preventive actions against cataract formation. The idea has been launched of a chemical, rather than the present surgical solution to the cataract disease.[175] Even if a non-surgical treatment of cataract cannot be invented, the possibility of delaying the onset of the disease would also have a great impact on the prevalence of the disease. Studies have shown that if one succeeds to find ways to delay cataract formation with 10 years, the number of required cataract surgeries would be reduced by 45%.[176]

Many lifestyle-related factors are exogenous sources of oxidative stress associated with cataractogenesis. Therefore many suggested preventative actions involve these factors, such as UVB exposure and smoking. Wearing a hat and UVB-protecting sunglasses and avoiding direct sunlight at peak hours of UVB radiation have been suggested as powerful preventative measures. Studies have shown dose-response associations between smoking and risk of cataract among current and former smokers and it has been demonstrated that the risk of developing cataract decreases with time in former smokers, thus promoting smoking cessation. The association between cataract and smoking was also confirmed in the present thesis. Dietary antioxidants have been suggested to reduce the risk of cataract but the results have been inconsistent. It has also been suggested that the possible protective effect of antioxidant supplements in the middle age is not sufficient to reduce oxidative damage that has already been caused and therefore lifelong vitamin supplementation would be required.[177] Whether or not the lifestyle changes described above are feasible in order to avoid cataract surgery, must be decided by each individual.

The most common postoperative complication after cataract surgery, posterior capsular opacification (PCO), is caused by proliferation of residual lens epithelial cells on the lens capsule. Our results indicate proliferative effects by low levels of E2 exposure, which would have negative effects in this context. The biological effects of estrogens are many and are even associated with

increased risk of developing certain hormone-sensitive cancers. Even if our results showed estrogen-mediated protection against oxidative stress, they do not encourage intake of estrogens. As mentioned, estrogen exerts different effects in different cell lines and estrogen-mediated protection is both concentration- and time-dependent. The mechanism for possible estrogen-mediated protection against cataract formation is still not clear. The main finding of this thesis is that estrogen exerts both pro- and anti-oxidative effects on the lens. Estrogen-mediated protection has been implied in several other age-related diseases as well. Therefore, studies on lens epithelial are important since the lens may serve as a useful model for studying estrogen-mediated protective and/or harmful effects in other age-related diseases and in biological aging in general.

SUMMARY IN SWEDISH / SVENSK SAMMANFATTNING

Katarakt (grå starr) är en synstörande grumling av ögats lins och den största riskfaktorn för katarakt är åldrande. En av de viktigaste bidragande faktorerna till sjukdomen är oxidativ stress och epidemiologiska studier har visat att kvinnor har högre risk än män att få katarakt. Den här könsskillnaden i kataraktrisk uppstår först i högre åldrar, d.v.s. efter klimakteriet hos kvinnor och i motsvarande ålder hos män. Man tror att östrogen kan ha en skyddande effekt mot katarakt och att de minskade östrogen-nivåerna efter klimakteriet kan vara orsaken till denna könsskillnad. Orsaken till att östrogen tros skydda mot katarakt är inte känd men experimentella studier har visat att östrogen kan verka antioxidativt, dvs. skydda mot fria radikaler (oxidativ stress). Syftet med den här avhandlingen var att undersöka mekanismen bakom ett eventuellt östrogen-medierat skydd mot katarakt samt att studera hur östrogen påverkar ett av de viktigaste antioxidativa systemen i cellen, superoxiddismutas.

Humana linsepitelceller, vilka erhållits vid kataraktkirurgi odlades och exponerades sedan för olika koncentrationer av 17β-östradiol (den vanligaste formen av östrogen) varefter effekten av detta hormon studerades. Cellerna utsattes också för oxidativ stress genom exponering för väteperoxid, med och utan östrogen, för att studera detta hormons eventuella antioxidativa effekter. I kliniska studier undersöktes serum-koncentrationerna av östrogen och aktiviteten hos superoxiddismutas hos kataraktpatienter respektive hos kontrollpersoner av båda könen. Dessutom studerades genetiska variationer (polymorfismer) i östrogen-relaterade gener samt i generna för superoxiddismutas, hos kataraktpatienter och kontrollpersoner.

Sammanfattningsvis visade våra studier att östrogen ökade aktiviteten av superoxiddismutas och minskade de fria radikalerna i humana linsepitelceller. Detta visar att östrogen kan verka antioxidativt och kan skydda mot oxidativ stress i humana linsepitelceller. De kliniska studierna gav dock inget stöd för att genetiska variationer i östrogen-relaterade eller i superoxiddismutas-gener skulle öka risken att få katarakt. Det fanns inte heller något samband mellan serum-koncentrationerna av östrogen och aktiviteten hos superoxiddismutas hos kataraktpatienter och kontrollpersonerna. Katarakt är idag den vanligaste orsaken till blindhet globalt sett och kataraktextraktion är det vanligaste kirurgiska ingreppet i västvärlden, inklusive Sverige. Detta innebär stora samhällsekonomiska kostnader och orsakar funktionshinder hos patienter med nedsatt syn tiden före operation. Det är därför viktigt att fortsätta sökandet efter de underliggande orsakerna till katarakt och försöka hitta förebyggande åtgärder mot utvecklingen av katarakt. Katarakt uppvisar också likheter med andra vanliga åldersrelaterade sjukdomar och kan därför användas som en modell i studier av biologiskt åldrande.

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