Forum Review Article

Oxidative Stress, Lens Gap Junctions, and Cataracts

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Abstract

The eye lens is constantly subjected to oxidative stress from radiation and other sources. The lens has several mechanisms to protect its components from oxidative stress and to maintain its redox state, including enzymatic pathways and high concentrations of ascorbate and reduced glutathione. With aging, accumulation of oxidized lens components and decreased efficiency of repair mechanisms can contribute to the development of lens opacities or cataracts. Maintenance of transparency and homeostasis of the avascular lens depend on an extensive network of gap junctions. Communication through gap junction channels allows intercellular passage of molecules (up to 1 kDa) including antioxidants. Lens gap junctions and their constituent proteins, connexins (Cx43, Cx46, and Cx50), are also subject to the effects of oxidative stress. These observations suggest that oxidative stress-induced damage to connexins (and consequent altered intercellular communication) may contribute to cataract formation. *Antioxid. Redox Signal.* 11, 339–353.

The Eye Lens

THE LENS IS A TRANSPARENT AND AVASCULAR ORGAN whose main function is to transmit light and focus it on the retina. The lens is comprised of an anterior epithelial cell layer and of fiber cells that form the bulk of the organ. At the lens equator, epithelial cells differentiate into fiber cells, a process that involves cell elongation and loss of organelles. This differentiation process occurs throughout the lifespan of the organism. Most of the metabolic, synthetic, and active transport machinery in the lens is localized to surface (nucleated) cells (92). Fiber cell survival and the maintenance of transparency depend on the function of epithelial cells and on intercellular communication.

Gap Junctions and Intercellular Communication in the Lens

Lens intercellular communication is facilitated by an extensive network of gap junctions. Gap junctions are membrane specializations that contain clusters of intercellular channels that are permeable to ions and molecules up to 1 kDa. Lens fiber cells share ions and small metabolites through gap junction channels, and therefore behave as a functional syncytium (45, 91). Epithelial and fiber cells contain morphologically and physiologically distinct gap junctions (95, 123).

The lens appears to have an internal "circulation" in which flow of water and ions drives the movement of solutes throughout the organ (90). A model of this circulation has been developed based on surface currents recorded from lenses (90, 115, 133). In this model, current (*i.e.*, flow of ions and associated water) enters the lens along the extracellular spaces between cells at the anterior and posterior poles, it crosses fiber cell membranes in the lens interior, and it flows back to the surface at the equator (through a cell-to-cell pathway). This model is consistent with the distribution of ion channels and pumps in the lens (29). The lens circulatory system provides a pathway for internal fiber cells to obtain essential nutrients, remove potentially toxic metabolites, and maintain resting potentials (43, 118). Therefore, it has been proposed that maintenance of lens intercellular communication is crucial for the support of fiber cells (44).

Gap junction channels are oligomeric assemblies of members of a family of related proteins called connexins (Cx) (14). Connexins contain four transmembrane domains with the amino and carboxyl termini on the cytoplasmic side. Channels formed by diverse connexins differ in physiological properties, including unitary conductance, permeability, gating, and regulation by different protein kinase-dependent pathways (reviewed in 51, 52, 138).

Three connexins have been identified in the lens with somewhat overlapping expression patterns (Fig. 1). Cx43 is expressed in lens epithelial cells (99), but its expression is
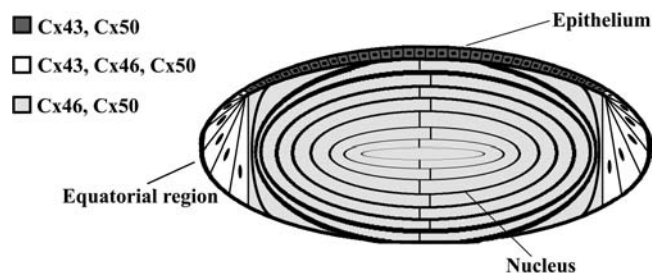
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FIG. 1. Diagram of the lens showing the distribution of connexin isoforms. Cells from the anterior epithelial layer express Cx43 and Cx50, cells from the equatorial region express Cx43, Cx46, and Cx50, and fiber cells (including those of the nucleus) contain Cx46 and Cx50.

turned off as epithelial cells in the equatorial region differentiate into fiber cells. Cx50 is also expressed in epithelial cells (24, 135, 158). Cx46 and Cx50 become abundantly expressed in the differentiating cells and are the two most abundant connexins in lens fiber cells (116, 170). Cx46 and Cx50 co-localize at gap junctional plaques and form mixed hexamers (60, 116). Recently, transcripts for another connexin, Cx23, have been detected in the zebrafish embryo lens (56) and in the mouse lens (121). Based on its pattern of expression and the lack of proper elongation of fiber cells in mice expressing a missense mutation of Cx23, Puk *et al.* (121) have proposed that Cx23 is involved in fiber cell differentiation. Lens cells from other mammalian species may also express Cx23, since it has been identified as an expressed sequence in mRNA from whole eyes or lenses (accession numbers for *Oryctogalus cuniculus* EST from eye: EB384661.1, EB384662.1; *Bos taurus* EST from lens: EG705869.1; *Macaca mulatta* EST from lens: EG703836). However, detection of Cx23 protein in lens cells, its abundance, and its contribution to lens gap junctional intercellular communication relative to the other lens connexins have not been reported. Therefore, Cx23 is not included in Fig. 1, nor further considered in this review.

The internal circulation between lens cells may change with aging, at least in humans. Sweeney and Truscott (157) and Moffat *et al.* (97) have shown restricted diffusion of GSH and water beyond the cortex/nucleus boundary in lenses from human subjects starting at middle age; while the restricted diffusion of GSH is observed in most lenses from subjects over the age of 30, the restriction to diffusion of water is observed much later (>65 years). These authors speculate that this barrier contributes to the development of age-related nuclear cataracts. The molecular basis for this restricted diffusion is unknown, but it is possible that connexin modifications in the aging lens lead to a decrease or loss of intercellular communication between cortical and nuclear fiber cells (preventing delivery of small molecules into the lens nucleus).

Cataracts

Clinical manifestation, classification, and etiology

Cataracts are a common pathological abnormality of the lens characterized by loss of lens transparency. They are the

leading cause of blindness worldwide (129). Because of its high prevalence, this disease has substantial public health implications. In many countries, cataractous lenses that impair vision are surgically removed and replaced with prosthetic intraocular lenses. With increasing life expectancy, the corresponding increase in age-related cataracts will produce an even greater worldwide financial burden. Therefore, many efforts have been devoted to determine the factors that lead to cataract formation and to develop treatments to prevent their formation. Although the specific associated biochemical and histological changes may be diverse, a common biochemical change is the presence of high molecular weight insoluble protein aggregates.

Cataracts can be subdivided according to their anatomical location within the lens (*e.g.*, cortical, nuclear, subcapsular), their appearance (*e.g.*, total, pulverulent), and most commonly by a combination of these two parameters (*e.g.*, nuclear pulverulent). They can also be subdivided according to their etiology (*e.g.*, congenital, disease-related, or age-related). Many disease- and age-related cataracts may result from oxidative stress.

In this review, we will present an overview of the relationship between oxidative stress and cataract formation, the pathways involved in generating reactive oxygen species (ROS) in the lens, and pathways that ameliorate (protect and repair) the effects of oxidative stress upon lens components. Then, we will examine the effects of oxidative stress on lens gap junctions and how they may contribute to cataract formation. Finally, we will highlight the challenges for future research in this area.

Contribution of oxidative stress to cataract formation

The participation of oxidative stress in cataract formation was inferred after comparing the lipid composition, protein modifications, and concentrations of antioxidants between cataractous and clear lenses. Oxidative stress and hyperbaric oxygen therapy both cause similar biochemical changes and cataracts, further supporting the hypothesis (88, 113). Substantial evidence has accumulated to support the conclusion that ROS and resulting oxidative damage are factors contributing to the development of various types of cataracts (148, 155).

Oxidation of proteins, lipids, and DNA has been observed in cataractous lenses (6, 28, 64, 152, 180). Proteins from cataractous lenses lose sulfhydryl groups, contain oxidized methionines, become thiolated or crosslinked by nondisulfide bonds, form high molecular weight aggregates, and become insoluble (reviewed in 127, 161). All of these modifications may result from changes in the redox state of the lens. In fact, the normally high concentrations of reduced glutathione (GSH) are decreased in cataractous lenses (147), a decrease that may result from inactivation of glutathione reductase by disulfide bond formation and protein unfolding (174). Depletion of GSH in early postnatal mice causes cell damage to epithelial and fiber cells (70, 89), an effect that can be prevented by pretreatment with glutathione ester (89). Mixed protein–thiol and protein–protein disulfide bonds have been reported in lens proteins (23, 82, 83, 181). Because the formation of disulfide bonds precedes the characteristic morphological changes of cataracts, it has been proposed
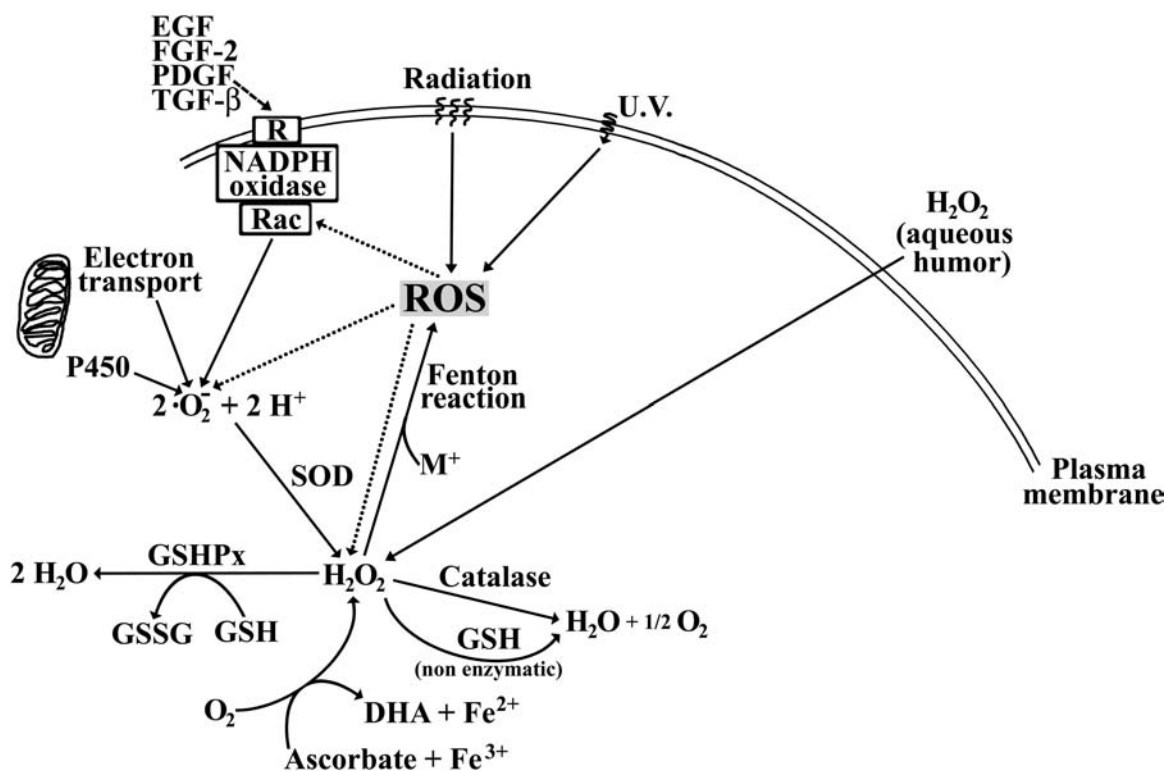
FIG. 2. Pathways for generation of ROS in the lens. Normal electron transport and activity of cytochrome P450 (P450) can generate $\cdot O_2^-$. The NADPH oxidase complex, which assembles with participation of Rac GTPases and is associated with activation of plasma membrane receptors (R) by extracellular signals (*e.g.*, growth factors), can also generate $\cdot O_2^-$. Growth factor receptors may also be activated by UV radiation-generated reactive oxygen species (ROS). H_2O_2 within the cell can be produced by the activity of superoxide dismutase (SOD) on $\cdot O_2^-$ or it can originate by diffusion through the plasma membrane from the aqueous humor. H_2O_2 can also be generated by ascorbate and molecular oxygen in the presence of ferric ions. Reaction of H_2O_2 with metal ions (M^+) can generate other ROS including $\cdot OH$ or $\cdot O_2^-$ through the Fenton reaction. H_2O_2 can be decomposed enzymatically by GSH-dependent peroxidases (GSHPx) or catalase, and nonenzymatically by reduced glutathione (GSH).

that this modification contributes to cataract formation following oxidative stress (82).

Oxidative stress may also have a role in selenite-induced and diabetic cataracts. In the selenite-induced cataract, several of the selenite-induced effects can be ameliorated by diethyldithiocarbamate which has antioxidant properties (57). In diabetic cataracts, hyperglycemia leads to an increase in superoxide in mitochondria (108, 134), and increased superoxide dismutase (SOD) activity has been observed by some investigators (140), but not others (110).

Photooxidation may also contribute to age-related cataracts. Ultraviolet (UV) rays present in sunlight induce formation of ROS. In humans, the free UV filters (tryptophan derivatives) decrease with aging (16) whereas the levels of protein-bound UV filters which generate peroxides (mainly H_2O_2) upon UVA illumination (*i.e.*, behaving as photosensitizers; 114) increase with age (96). Thus, the decrease in free UV filters and their binding to lens proteins cause the lens to be progressively more predisposed to UV damage and oxidation (16, 96, 114).

In summary, in response to oxidative stresses, lens proteins (including enzymes, crystallins, and other chaperones) may become modified, denatured, and aggregated, contributing to cataract formation.

Redox Systems in the Lens

ROS generation systems

Free radicals or ROS including the superoxide anion ($\cdot O_2^-$), hydroxyl free radicals ($\cdot OH$), and H_2O_2 may contribute to cataract formation. Among these, $\cdot OH$ is the most reactive oxygen species. Because of its small size and neutral charge, H_2O_2 can cross the plasma membrane; therefore, its generation outside the lens may lead to oxidative stress in the lens and cataract formation. Whereas the normal concentration of H_2O_2 in the aqueous humor and lens is 25–30 μM, a three-fold increase has been found in lenses from several cataract patients (36, 149).

ROS can originate endogenously in different cellular compartments (*e.g.*, mitochondria, peroxisomes, cytoplasm) as a result of normal metabolism through the enzymatic activities of lipoxygenases, NADPH oxidase and cytochrome P450, and mitochondrial electron transport, as a response to paracrine signals (*e.g.*, inflammatory cytokines), or through the effects of exogenous factors (*e.g.*, UV and ionizing radiation). Moreover, ROS may be generated by the interaction of metals with H_2O_2 through the Fenton reaction, especially in cataractous lenses that have high concentrations of redox-active iron (34). A summary of these pathways is presented in Fig. 2.
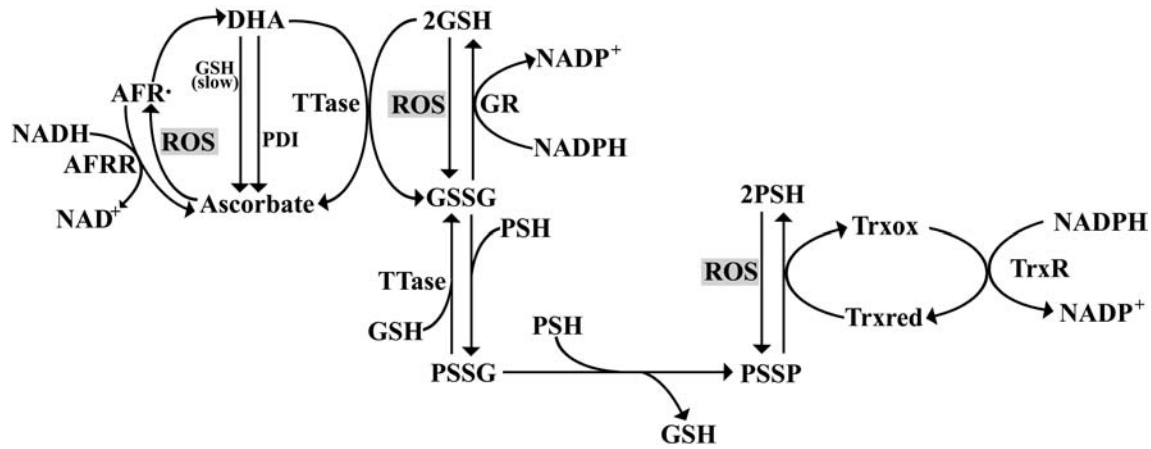
FIG. 3. Redox pathways involved in oxidative stress in the eye lens. AFR·, ascorbate free radical; AFRR, NADH-dependent ascorbate free radical reductase; DHA, dehydroascorbate; GR, GSSG reductase; GSSG, oxidized glutathione; GSH, reduced glutathione; PDI, protein disulfide isomerase; PSH, sulfhydryl-containing protein; PSSG, glutathionated protein; PSSP, oxidized disulfide bond-containing protein; ROS, reactive oxygen species; TrxR, thioredoxin reductase; Trxox, oxidized thioredoxin; Trxred, reduced thioredoxin; TTase, thioltransferase.

The NADPH oxidase system may participate in generating ROS in the lens. Immunoblotting and/or RT-PCR results have shown that different components of the NADPH oxidase complex (including p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox}, gp91^{phox}, Rac1, and Rac2) are expressed in the lens; the specific components expressed vary between human and mouse lenses (126). This complex has been most extensively characterized in phagocytes (reviewed in 169). An active phagocyte oxidase complex is formed upon stimulation by translocation of p40^{phox}, p47^{phox}, p67^{phox}, and the GTPase Rac from the cytosol to the plasma membrane where they interact with the membrane-associated heterodimer of p22^{phox} and gp91^{phox} (the NADPH oxidase catalytic core). The active oxidase transfers electrons from NADPH to reduce oxygen and form superoxide. The p47^{phox} subunit drives assembly, triggering the translocation of and interaction between the different subunits of the complex. Phosphorylation of the C-terminus of p47^{phox} by protein kinase C (PKC), Akt, and/or mitogen-activated protein kinases disinhibits the autoinhibition of p47^{phox} and drives assembly and activation.

ROS degradation/inactivation systems

Due to its external location and because its function is to transmit light to the retina, the lens is constantly exposed to light and radiation; therefore, it is one of the organs most prone to suffer oxidative stress produced in response to exogenous sources. However, the lens contains several nonenzymatic and enzymatic mechanisms to protect itself from oxidative stress and to repair oxidatively damaged cell components. The nonenzymatic mechanisms include the presence of high concentrations of ascorbate, reduced glutathione (GSH), crystallins (which act as protein chaperones), and free UV filters (*e.g.*, tryptophan derivatives in the human lens and NAD(P)H in rabbit, guinea pig and frog lenses (2, 16, 125, 127).

ROS are degraded through the enzymatic activities of superoxide dismutase, catalase, and peroxidases (glutathione peroxidase, peroxiredoxins, and microperoxidases) (127, 132, 151). Superoxide dismutase catalyzes the conversion of $\cdot O_2^-$

to H_2O_2. H_2O_2 can be degraded enzymatically by catalase, an enzyme that specifically uses H_2O_2 as a substrate, or by glutathione peroxidase (GSHPx-1), a selenoenzyme that reduces peroxides and requires GSH. Microperoxidases (which are heme peptide peroxidases derived from enzymatic cleavage of cytochrome c) also hydrolyze peroxides (151). H_2O_2 can be degraded nonenzymatically by reduced glutathione. Synthesis of the cofactor, GSH, involves the enzymes γ-glutamyl cysteine synthetase and glutathione synthase. In the biochemical reactions that require GSH, GSH is converted to its oxidized form, GSSG; GSSG is then converted back to its reduced form by GSSG reductase, an enzyme that requires NADPH (largely produced through the pentose phosphate pathway) as a cofactor (127). There likely is redundancy in the systems for degradation of peroxides since the lenses from animals lacking catalase or GSHPx-1 appear normal (53, 150). However, while lenses from catalase knockout mice do not exhibit increased sensitivity to oxidative stress (53), those from GSHPx-1-null mice are somewhat more sensitive to oxidative stress (150). Moreover, lenses from SOD-1 (CuZn-SOD)-null mice (containing twice the concentration of $\cdot O_2^-$ of wild-type mice) appear normal, but they are more prone to develop photochemical cataracts (8). Lenses from peroxiredoxin 6-null mice have some histological abnormalities (*i.e.*, abnormal cell localization in the bow region, some lens epithelial cells migrating to the region posterior to the equator, impaired loss of nuclei, and thickening of the capsule) and are more sensitive to oxidative stress (31).

Systems that protect/repair proteins from oxidative stress

Several protein modifications may result from oxidative stress including oxidation of methionines, oxidation of cysteines (with formation of protein–protein disulfide bonds and/or protein–thiol mixed disulfide bonds), and cross-linking of proteins through amino acids other than cysteine. These modifications can lead to formation of the high molecular weight insoluble aggregates that are common in cataractous lenses (17, 161, 162).

Repair of lens proteins after oxidative damage involves

the participation of GSH-dependent thioltransferase, the NADPH-dependent thioredoxin/thioredoxin reductase system, and methionine sulfoxide reductases (as illustrated in Fig. 3). GSH-dependent thioltransferase catalyzes the dethiolation of protein–thiol mixed disulfides (124). Thioredoxin reduces protein–protein disulfide bonds in a reaction that leads to oxidation of thioredoxin. Oxidized thioredoxin is reduced by thioredoxin reductase, a homodimeric selenoenzyme that uses NADPH as a cofactor (177). Methionine sulfoxide reductases convert oxidized methionine (methionine sulfoxide) back to methionine in a reaction that involves thioredoxin reductase, the thioredoxin system, and NADPH (153). The expression of cystathionine-β-synthase in the lens has been reported (117). This enzyme is rate-limiting in a transsulfuration pathway that may contribute to the production of cysteine and glutathione (117). These enzymes work together to protect the lens from oxidative stress.

In the human lens, levels of GSH decrease from the cortical region towards the nucleus, whereas cysteine levels increase from the cortical region towards the nucleus (27). Since the nucleus and organelles are lost during fiber cell differentiation, it is not surprising that epithelial and differentiating fiber cells contain most of the redox systems of the lens. Indeed, many components of the redox system (including SOD, catalase, GSSG reductase, NADPH oxidase, peroxiredoxin 6, thioltransferase, thioredoxin reductase, ascorbate free radical reductase, methionine sulfoxide reductase) have much higher activities/levels in the epithelial/surface cells than in the rest of the lens (Fig. 4) (7, 15, 63, 65, 124, 126, 143, 153, 154, 177). Two enzymes that do not follow this pattern are GSHPx-1 (whose activity is comparable in the epithelium and in the rest of the mouse lens; 154) and peroxiredoxin 3 (a mitochondrial peroxidase with higher abundance in fiber cells than in epithelial cells; 72).

Physiological Roles of ROS

ROS effects on enzymes

Oxidative stress can alter enzymatic activities directly by protein modification. For example, thiolation of an SH group at the active site of glycolytic enzymes (*e.g.*, hexokinase or pyruvate kinase) leads to loss of activity, whereas thiolation of fructose 1,6-bisphosphatase, a gluconeogenic enzyme, leads to its activation (122). Oxidative stress may also affect enzymes indirectly (through regulation of gene expression). For example, exposure of porcine lenses to H_2O_2 results in induction of thioltransferase and thioredoxin/thioredoxin reductase (98). Similarly, exposure of cultured lens epithelial cells to low concentrations of H_2O_2 results in induction of peroxiredoxin 3 (72) and peroxiredoxin 6 (112). These compensatory changes may help the lens respond to oxidative stress and regain its normal redox state.

ROS effects on cell proliferation

ROS generation is a component of several signaling cascades involved in several processes including cell proliferation. In lens epithelial cells, several growth factors [epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2), platelet-derived growth factor (PDGF), and transforming growth factor β (TGF-β)] increase ROS production; this effect can be blocked by NADPH oxidase inhibitors
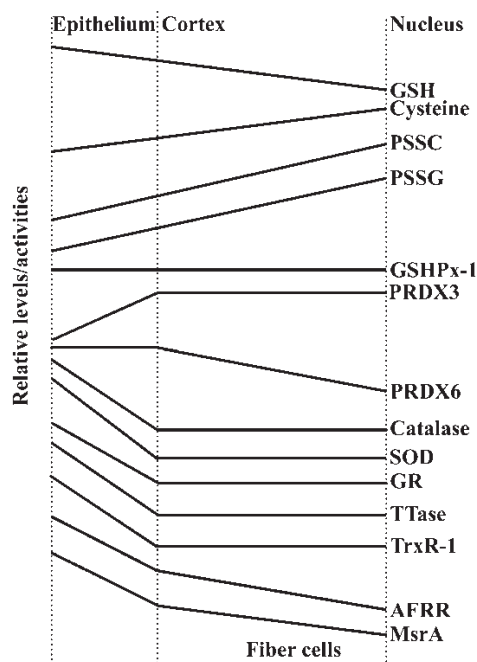

FIG. 4.   Regional distribution of components of the redox system within the lens. This graphical representation summarizes data from many studies regarding the relative levels/activities (shown in arbitrary units) of the different molecules/enzymes in the different regions of the lens. The levels/activities of most components of the redox system are higher in the epithelium. AFRR, NADH-dependent ascorbate free radical reductase; GR, GSSG reductase; GSH, reduced glutathione; GSHPx-1, glutathione-dependent peroxidase-1; MsrA, methionine sulfoxide reductase A; PRDX3, peroxiredoxin 3; PRDX6, peroxiredoxin 6; PSSC, protein-bound cysteine; PSSG, glutathionated protein; SOD, superoxide dismutase; TrxR-1, thioredoxin reductase 1; TTase, thioltransferase.

(126). Chen *et al.* (20) have suggested that G-protein coupled receptors and EGF receptors can modulate the mitogenic action of PDGF. This cellular signaling may also be affected by external influences, since UV radiation leads to ROS production and transient activation of the EGF receptor which induces translocation of Rac2 and NADPH oxidase activity (62).

Gap Junctions and Cataracts

As stated above, intercellular communication mediated by gap junction channels has been proposed to have a major role in the maintenance of lens transparency. This hypothesis has been substantiated by a number of genetic studies in mice. Targeted deletion of Cx46 or Cx50 results in the development of cataracts in homozygous mice (41, 171). The severities of the cataracts in both Cx46- and Cx50-null mice depend on the genetic background (35, 40). Furthermore, the cataract trait in several mutant mouse strains has been mapped to the lens connexin loci. Steele *et al.* (156) demonstrated that the *No2* mouse, which develops congenital cataracts (especially in homozygotes), carries a missense mutation within the coding region of Cx50, resulting in a change of amino acid residue 47 from aspartate to alanine

Table 1.   Summary of Human Cx46 and Cx50 Mutants Associated with Cataract Formation

| Mutant connexin | Disease phenotype | | Reference |
	Cataract appearance	Other features	
Cx46			
D3Y	Zonular pulverulent		1
L11S	"Ant-egg"		49
V28M	Variable		25
F32L	Nuclear pulverulent		59
R33L	Finely granular embryonal		47
W45S	Nuclear		86
P59L	Nuclear punctuate		9
N63S	Zonular pulverulent		87
R76G	Total		25
R76H	Lamellar nuclear opacity surrounding pulverulent nuclear opacities.	Dominant inheritance with incomplete penetration	18
T87M	"Pearl box"		48
P187L	Homogeneous zonular pulverulent		128
N188T	Nuclear pulverulent		74
fs380	Zonular pulverulent		87
Cx50			
R23T	Nuclear		172
V44E	Total	Microcornea and variably associated with myopia	26
W45E	Jellyfish-like	Associated with microcornea	165
D47N	Nuclear pulverulent		4
E48K	"Zonular nuclear" pulverulent		10
V64G	Nuclear		86
V79L	"Full moon" with Y-sutural opacities		163
P88Q	Lamellar pulverulent		5
	Balloon-like with Y-sutural opacities		164
P88S	Zonular pulverulent		142
P189L	Star-shaped nuclear opacity with a whitish central core	Associated with microcornea	50
R198Q	Not available	Microcornea and variably associated with myopia	26
fs203	Total	Recessive inheritance; associated with nystagmus and amblyopia.	120
I247M	Zonular pulverulent		119
S276F	Nuclear pulverulent		175

(Cx50D47A). Similarly, mice carrying a Cx50 mutation at amino acid residue 64 (changing from valine to alanine, Cx50V64A) exhibit dominantly-inherited cataracts (46). Another mouse with cataracts, *Lop10*, carries a missense mutation at amino acid residue 22 of Cx50 (Cx50G22R) (19).

Most importantly, mutations in lens connexins have been associated with human disease. Missense and frame-shift mutations of the Cx46 and Cx50 genes have been identified in members of families with inherited cataracts (usually dominant) of various different phenotypes. These mutants and their associated cataract phenotypes are summarized in Table 1.

Gap Junctions and ROS

Because gap junction channels facilitate transfer of ions and molecules up to 1 kDa among coupled cells, they can allow sharing of molecules involved in maintaining a reduced environment in the lens including cysteine, ascorbate, free UV filters (*e.g.*, tryptophan derivatives), and GSH. In fact, the permeability of Cx43 gap junction channels to GSH has been

directly demonstrated (39). Thus, it is expected that gap junction function plays a pivotal role in maintaining the redox state of the lens. However, this function may be impaired by oxidative stress as demonstrated by the cellular uncoupling observed after treatment of cells with toxic compounds (*e.g.*, CCl_4 and paraquat) that lead to generation of free radicals (136, 137). Paradoxically, treatment of primary cultures of lens epithelial cells with 7-ketocholesterol, a cholesterol oxide that is increased in human cataracts, induces an increase in intercellular dye transfer; this effect is associated with an increase in the phosphorylated forms of Cx43, insolubility in 1% Triton X-100 and Cx43 at the plasma membrane (37).

Post-Translational Modification of Connexins

Like other proteins, connexins are subject to post-translational modification. Many of the connexins are phosphoproteins. A number of phosphorylated residues and the responsible kinases have been identified, and these phosphorylation events have been associated with various steps in the connexin life cycle (reviewed in 68; for Cx43, see

146). Connexins are also post-translationally modified by ubiquitinylation (66), S-nitrosylation (130), fatty acylation (80), hydroxylation (80), and γ-carboxyglutamylation (80). The functional role of many of these modifications remains unknown.

Cx43, Cx46, and Cx50 are phosphoproteins (61, 99, 158). Shearer *et al*. (141) have demonstrated acetylation of the second amino acid residue (glycine) and oxidation of methionines in the bovine orthologs of Cx46 and Cx50 (Cx44 and Cx49, respectively), and deamidation of asparagine121 in Cx49; however, the authors caution that both methionine oxidation and deamidation may occur during sample preparation (141).

Cx43 phosphorylation has been extensively studied in several different cell types. (The results may be extrapolated to Cx43 in lens epithelial cells.) Modification of Cx43 by phosphorylation decreases the electrophoretic mobility of the protein, resulting in an immunoblot pattern that contains three major immunoreactive Cx43 bands (designated NP, P1, and P2) (100). Depending on the cell type, the Cx43 band initially designated NP (for nonphosphorylated) may contain Cx43 phosphorylated at a site that does not alter its electrophoretic mobility (144). The P2 phosphoform has been correlated with localization of the protein to gap junctional plaques and insolubility in Triton X-100 (101). A P3 form with an electrophoretic mobility slower than P2 is observed in some cell lines during mitosis (67).

Several bands corresponding to different phosphorylated forms of Cx46 and Cx50 are detected in lens homogenates by immunoblotting. Phosphorylation of the lens fiber cell connexins has been implicated in regulation of intercellular communication, protein stability, and proteolytic cleavage (11–13, 21, 178). Ovine Cx49, the ortholog of Cx50, is phosphorylated by casein kinase I, and inhibition of casein kinase I increases intercellular communication between cultured lens cells (21). Phosphorylation may regulate stability of Cx56, the chicken ortholog of Cx46, since the phosphorylated forms of Cx56 with lower electrophoretic mobility show longer half-lives (11). Casein kinase II-mediated phosphorylation of Ser363 in Cx45.6, the chicken ortholog of Cx50, regulates its cleavage by a caspase-3-like protease (178).

Protein Kinase C

Protein kinase C (PKC) comprises a family of serine/threonine kinases that have central roles in cell signaling. The PKC isoenzymes have been grouped into three classes depending on the cofactors required for enzyme activation. Conventional PKC isoenzymes (α, β, and γ) require diacylglycerol, Ca^{2+}, and phosphatidylserine for activity; the novel PKC isoenzymes (δ, ε, θ, and η/L) do not require Ca^{2+} for activation; and the atypical PKC isoenzymes (ζ, ι/λ) require neither Ca^{2+} nor diacylglycerol (reviewed in 105). The PKC activators, diacylglycerol and phorbol esters (*e.g.*, 12-O-tetradecanoyl phorbol ester, TPA), bind to cysteine-rich domains (C1) in conventional and novel PKCs. Because PKC activation may result from oxidative stress or lead to ROS generation, we will give special attention to the PKC-dependent effects on connexins and gap junction function.

Expression of protein kinase C in lens cells

The chicken lens expresses PKCα, γ, ε, and ι (13). Most of the PKC enzyme immunoreactivity detected with isoenzyme-specific antibodies localizes to the epithelium and annular pad. PKCγ and ε are present at lower levels in fiber cells where at least some PKCγ is localized at the plasma membrane (13). The specificity of expression of PKC isoenzymes is maintained in primary cultures of lens cells (13), where cells differentiate to form birefringent structures called lentoids that share similarities to differentiating fiber cells including expression of fiber cell connexins (11, 61, 93). Cultured ovine lens epithelial and lentoid cells have been reported to express PKCα; however, treatment with TPA does not induce translocation of PKCα to the plasma membrane of epithelial cells (159). The rabbit lens epithelial cell line, N/N1003A, expresses PKCα and γ (167). Saleh *et al*. (139) analyzed rat lenses and found expression of PKCγ in epithelium, the equatorial (bow) region, and the cortex; PKCα was not found in the cortex of these lenses, the only region analyzed for this isoenzyme. In cultured bovine lens epithelial cells, PKCα and γ were detected by immunoblotting (42).

Effects of PKC activation

In many cell types that express Cx43, treatment with TPA decreases intercellular communication. This change in gap junction function is associated with a relative increase in the P1 and P2 phosphoforms and a decrease in NP, a change associated with differential phosphorylation of Cx43 (reviewed in 138, 145). Serines at positions 368, 279/282, and 328 in Cx43 are phosphorylated after treatment with PKC activators (146). PKC-dependent phosphorylation of Ser368 in Cx43 leads to decreased intercellular communication due to a change in single channel behavior (69) and altered permeability (30); phosphorylation of Ser368 has also been implicated in trafficking/assembly of gap junctions (144).

As in these other systems, treatment of epithelial cells with phorbol esters (such as TPA) leads to a decrease in intercellular communication (81, 131, 159) associated with increased Cx43 phosphorylation (detectable by ^{32}P incorporation) (131). Several mechanisms have been proposed for the TPA-induced decrease in intercellular communication. In cultured bovine epithelial cells, it has been suggested that the TPA-induced phosphorylation of Cx43 targets the protein (or converts the protein into a better substrate) for proteasomal degradation, because it is associated with an MG132-sensitive decrease in Cx43 levels (38). In cultures of ovine lens epithelial cells, TPA induced a gradual disappearance of Cx43 from gap junction plaques and a transient decrease in Cx43 levels which the authors interpreted as a transitory modification that affected its antigenicity (159). In these cultures, TPA also induces a decrease in levels of Cx49 mRNA and the disappearance of this connexin from gap junction plaques over a few hours (159). In contrast, activation of PKCα and γ in N/N1003A cells by treatment with TPA leads to a decrease in the number of gap junctional plaques without changes in Cx43 levels, suggesting redistribution of Cx43 (167). Similar results are obtained after treatment of N/N1003A cells with diacylglycerol (DAG), insulin-like growth factor 1 (IGF-1), or lens epithelium-derived growth factor (LEDGF), suggesting that IGF-1 and LEDGF may correspond to the endogenous agonists that trigger activation of PKC (78, 107). PKC involvement may also explain the decreased intercellular communication of lens epithelial cells elicited by extracellular ATP (84); this effect is sensitive to PKC and phospholipase C inhibitors and requires a serine
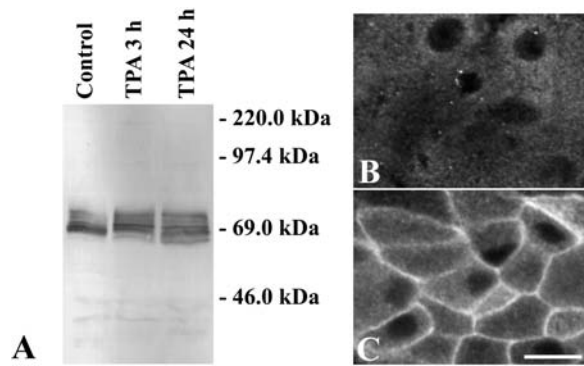
FIG. 5. Effects of TPA on Cx56 and PKCγ in cultured chicken embryonic lens cells. (**A**) Immunoblot of Cx56 from homogenates of lentoid-containing cultures that were left untreated (Control) or that were treated with 200 nM TPA for 3 or 24 h. The migration positions of the molecular mass standards are indicated. (**B, C**). Photomicrographs show the distribution of anti-PKCγ immunoreactivity in lentoid cells left untreated (**B**) or treated with 200 nM TPA for 30 min (**C**). TPA treatment activated PKCγ (as demonstrated by its translocation to the plasma membrane) and produced a change in the immunoblot pattern of phosphorylated Cx56 forms. *Bar*, 12 μm in **B** and 15 μm in **C**.

at position 368 even though there is no requirement for a change in the phosphorylation state of this residue (85).

The effects of phorbol esters and PKC activation on fiber cell connexins have been studied in lentoid-containing cultures and lens organ cultures. TPA treatment of cultures from chicken lenses decreases dye coupling between lentoid cells (13). The TPA-induced decrease in dye coupling is associated with activation of PKCγ and a change in the immunoblot pattern of Cx56 (Fig. 5). The altered immunoblot pattern is associated with an increase in phosphorylation at Ser118, suggesting that phosphorylation leads to channel closure (12). Rat Cx46 hemichannels are also modulated by PKC phosphorylation; OAG reduces the amplitude of hemichannel currents and leads to their inactivation after prolonged incubation, an effect that can be inhibited by PKC inhibitors (106). PKCγ and Cx46 have been reported to co-localize in the equatorial and cortical regions of rat lenses, and 10% of the total PKCγ in lens cortex was co-precipitated with anti-Cx46 antisera (139).

Although we have shown that phorbol esters lead to uncoupling of lentoid cells derived from chicken lenses (13), the effects of these PKC activators on fiber cell communication have varied in different systems (and may result, at least in part, from species differences). For example, TenBroek *et al.* (159) found no effect of phorbol esters on dye coupling in lentoids from ovine lenses. Cx49, the ovine Cx50 ortholog, appears not to be a substrate for PKC (3), while Cx45.6, the chicken ortholog, is a good PKC substrate (61). Treatment of whole rat lenses with TPA leads to an increase in PKCγ activity associated with a decrease in anti-Cx50 immunoreactivity in gap junctions, an increase in anti-Cx50 immunoreactivity in the nonjunctional plasma membrane (hemichannels), and a decrease in Lucifer yellow transfer in the lens cortex measured in whole lenses. Co-precipitation of PKCγ with anti-caveolin-1 antibodies was also detected

after TPA treatment, suggesting that PKCγ translocates to caveolin-1-containing membrane fractions, which also contain Cx46 and Cx50. These results suggest that TPA-induced activation of PKC leads to undocking and dispersion of Cx50 hemichannels (179).

Effects of Oxidative Stress on Lens Connexins

Oxidative stress may be linked to changes in connexins and gap junctions. We have observed that treatment of chicken lentoid-containing cultures with H_2O_2 leads to dose- and time-dependent changes in the immunoblot pattern of Cx56 (Fig. 6). These results suggest that H_2O_2 leads to differential phosphorylation of Cx56. The appearance of a cleaved form when cultures are treated with 500 μM H_2O_2 is associated with cell death, suggesting that strong oxidizing treatments lead to deleterious effects that surpass the restoring capacity of the cells. Treatment of N/N1003A cells with H_2O_2 leads to an increase in PKCγ activity, an effect that appears to result from direct oxidation of the enzyme with formation of disulfide bonds. Activation of PKCγ resulted in increased phosphorylation of Cx43 in Ser368 as detected by immunoblotting, a decrease in the number of gap junction plaques, and a decrease in dye coupling (77). Similar effects on lens fiber connexins were observed when rat lenses were treated with H_2O_2 (*i.e.*, H_2O_2 induced activation of PKCγ and increased phosphorylation of Cx46 and Cx50) (75). In both N/N1003A cells and lenses, PKCγ co-precipitated with connexins using anti-caveolin-1 antibodies, leading the authors to conclude that activated PKCγ translocated to and phosphorylated connexins in lipid rafts. In the selenite-induced cataract, a decrease in the relative levels of the Cx46 phosphorylated form with the slowest electrophoretic mobility and an increase in the levels of the cleaved form of Cx50 in the lens cortex have been reported (33).
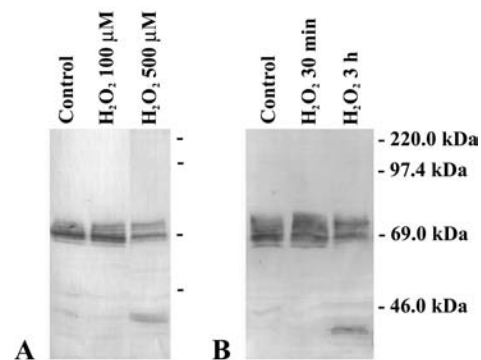

FIG. 6. Effects of oxidative stress on Cx56 in cultured embryonic chicken lens cells. (**A**) Immunoblot of Cx56 from homogenates of lentoid-containing cultures that were left untreated (Control) or that were treated with 100 or 500 μM H_2O_2 for 3 h. (**B**) Immunoblot of Cx56 from homogenates of lentoid-containing cultures that were left untreated (Control) or that were treated with 500 μM H_2O_2 for 30 min or 3 h. The migration positions of the molecular mass standards are indicated. Only subtle changes in the immunoblot pattern of Cx56 bands were detected after treatment with 100 μM H_2O_2 for 3 h. A prominent degradation product of Cx56 was generated after treatment with 500 μM H_2O_2 for 3 h.

ROS and Apoptosis

Oxidative stress corresponds to an imbalance between the rate of ROS production and their rate of degradation, leading to accumulation of oxidized cell components. When this exceeds the cell's ability to repair these components, or the oxidizing stress is too high, apoptosis occurs. Apoptosis may play important roles in lens development and pathology (reviewed in 173, 176). Cellular stresses like H_2O_2 (76) or activators of the intrinsic pathway of apoptosis (160) can cause cell death of lens epithelial cells. Gap junction channels may propagate apoptosis signals through a bystander-like mechanism as proposed previously for other cell types (22, 32, 79, 94). However, other studies suggest a role for PKC-dependent closure of gap junction channels as a protective/survival mechanism, because the induced channel closure might prevent the intercellular transmission of apoptotic signals (77). Thus, published data suggest that PKC activation by H_2O_2 can either generate apoptotic signals (that may be propagated through gap junction channels) or lead to closure of those channels (blocking that propagation).

Involvement of Gap Junctions in Growth Factor Signaling

ROS may influence connexins and gap junction-mediated intercellular communication through their involvement in cellular signaling under normal physiological conditions. As noted above, stimulation of several growth factor receptors leads to ROS generation. Activation of various growth factor receptors leads to Cx43 phosphorylation and decreased intercellular communication in various non-lens cells (168). In chicken embryo lens cells, FGF treatment or ERK activation increases intercellular coupling (71). This difference may be due to the expression of additional connexins in lens cells (71).

Nitrogen Oxides in the Lens in Relation to Connexins and Cataract Formation

This review would not be complete without considering the possible role of nitrogen oxides in cataract formation. Nitric oxide (NO) is generated by nitric oxide synthases (NOS). NO can generate several different nitrogen oxide species including peroxynitrite which is formed after reaction with $\cdot O_2^-$. Peroxynitrite is a highly reactive species that can oxidize proteins and nonprotein sulfhydryl groups. Nitrogen oxides can modify proteins by nitration and nitrosation of their aromatic amino acid residues. For example, incubation of α-crystallin with the nitrite ion results in oxidative damage of the protein similar to the modifications found in cataracts (111).

Participation of inducible nitric oxide synthase (iNOS) and nitrite ion in cataractogenesis has been proposed in several models. In selenite-induced cataracts, an increase in nitrite and iNOS is observed within one day of treatment; the development of opacification can be inhibited by inhibitors of NOS and accelerated by the NOS substrate, arginine (57, 58). In the Shumiya cataract strain, appearance of opacities is preceded by increases in iNOS, Ca^{2+}, and proteolysis; treatment with aminoguanidine, an inhibitor of iNOS, leads to a decrease in iNOS protein and Ca^{2+}. The authors have suggested that the increase in Ca^{2+} leads to calpain-mediated proteolysis, decreased protein solubility, and opacification (54, 55). In the UPL rat, another hereditary cataractous strain, the appearance of opacities is associated with a decrease in ATP and GSH levels, a decrease in cytochrome c oxidase activity, and increased NO and Ca^{2+} levels, Na^+/K^+ ratio, and water-insoluble/water-soluble protein ratio. Treatment with NOS inhibitors delays the development (or decreases the severity) of cataracts (102, 103). The temporal course of these changes has led to the proposal that the increase in iNOS leads to excess production of NO which in turn leads to mitochondrial genome damage and cytochrome c oxidase dysfunction with a consequent decrease in ATP production, a decrease in Ca^{2+}-ATPase function and an increase in lens Ca^{2+}, which leads to opacification (103). In the SOD-1 (CuZn-SOD)-null mice, development of high glucose-induced cataracts can be delayed by NOS inhibitors and increased by NO donors (109). Nitric oxide donors also lead to decreases in ATP and GSH levels in the lens in organ culture (166). Treatment of the human lens epithelial cell line, SRA 01/04, with interferon-γ and LPS leads to iNOS expression, a decrease in ATP levels, and an increase in Ca^{2+}-ATPase activity, effects that are attenuated by NOS inhibitors (73, 104).

Direct modification of connexins by nitrogen oxides has been reported (130). *S*-nitrosylation of Cx43 has been associated with opening of connexin hemichannels (measured by dye uptake) and an increase in the number of channels at the plasma membrane in cultured astrocytes (130); the dye uptake can be reduced by high intracellular concentrations of glutathione (130). A similar protective mechanism could operate in the Cx43-expressing nucleated cells of the lens as long as their normal high levels of glutathione are maintained.

Concluding Remarks

Because epithelial cells differentiate into fiber cells throughout the life span of the individual, somatic DNA mutations in epithelial cells acquired through oxidative stress may have detrimental consequences for lens homeostasis and transparency. Similarly, during aging, cells in the lens interior may accumulate modifications in enzymes and other components that are important for maintenance of a reduced environment and become more dependent on repair mechanisms (provided by the more externally located cells via intercellular communication). It is thus possible that protein or gene modifications in nucleated lens cells that result in decreased antioxidant activity would lead to cataract formation. Similarly, mutations that reduce intercellular communication would also lead to cataracts, because antioxidant molecules would not reach the interior of the lens. The effects of oxidative stress on lens proteins, lipids, and DNA may result in changes in intercellular communication because of changes in the lipid environment, activation of signaling pathways/enzymes that affect post-translational modification of connexins, direct oxidation of connexin molecules, or somatic mutations in genes, including those of connexins.

To elucidate the roles of altered gap junction function in oxidative stress-induced cataracts, it still remains to determine the temporal correlation between changes in connexin-mediated function and levels of antioxidants, ATP, and Ca^{2+}, and the activities of enzymes involved in maintaining the re-

dox state in an oxidative stress model of cataract. Biochemical determination of connexin protein modification can help elucidate whether direct oxidation of lens connexins occurs during cataract formation. Such analysis could clarify the relation between connexin modifications and changes in intercellular communication, and whether they precede the development of lens opacities. Care should be taken when performing these experiments, because proteins may be oxidized or some modifications may be lost (or acquired) during tissue dissection and sample preparation.

The real challenge to study the relationship between oxidative stress, connexin function, and cataracts lies in designing a good model to study this interrelationship. When considering this model, one has to take into account that the anterior of the lens is bathed by the aqueous humor, while the posterior of the lens is bathed by the vitreous. (Both humors contain signaling molecules that may affect how lens cells respond to a given stimulus.) To mimic the situation *in vivo*, an ideal *in vitro* model would consist of lenses correctly oriented in organ cultures in a container with two-chambers (one containing media mimicking the vitreous and the other the aqueous); this model would permit application of oxidative stresses to the lens from either chamber. It would even be better to develop techniques that would allow assessment of connexin function *in vivo* to study this interrelationship.

Taken together, there is strong evidence linking oxidative stress to cataract formation, oxidative stress to altered gap junction-mediated intercellular communication, and altered intercellular communication to cataracts. These results suggest that oxidative stress may lead to decreased connexin function and cataract formation, but the direct relationship between all three: oxidative stress, connexin function, and cataracts remains to be demonstrated.

Abbreviations

AFR·, ascorbate free radical; AFRR, NADH-dependent ascorbate free radical reductase; C1 domain, cysteine-rich region of protein kinase C isoenzymes; Cx, connexin; DAG, diacylglycerol; DHA, dehydroascorbate; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; EST, expressed sequence tag; FGF-2, basic fibroblast growth factor; GR, oxidized glutathione reductase (GSSG reductase); GSSG, oxidized glutathione; GSH, reduced glutathione; GSHPx, glutathione-dependent peroxidase; IGF-I, insulin-like growth factor I; iNOS, inducible nitric oxide synthase; LEDGF, lens epithelium-derived growth factor; LPS, lipopolysaccharide; MsrA, methionine sulfoxide reductase A; NO, nitric oxide; $\cdot O_2^-$, superoxide anion; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; $\cdot OH$, hydroxyl free radical; P1, P2 and P3, different phosphorylated forms of Cx43; PDI, protein disulfide isomerase; PDGF, platelet-derived growth factor; PKC, protein kinase C; PRDX3, peroxiredoxin 3; PRDX6, peroxiredoxin 6; PSH, sulfhydryl-containing protein; PSSC, protein-bound cysteine; PSSG, gluthationated protein; PSSP, oxidized disulfide bond-containing protein; ROS, reactive oxygen species; SH, sulfhydryl; SOD, superoxide dismutase; TGF-β, transforming growth factor β; TPA, 12-O-tetradecanoyl phorbol ester; TrxR, thioredoxin reductase; Trxox, oxidized thioredoxin; Trxred, reduced thioredoxin; TTase, thioltransferase.

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