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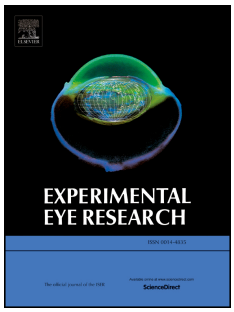

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Small molecules, both dietary and endogenous, influence the onset of lens cataracts

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ABSTRACT

How the lens ages successfully is a lesson in biological adaption and the emergent properties of its complement of cells and proteins. This living tissue contains some of the oldest proteins in our bodies and yet they remain functional for decades, despite exposure to UV light, to reactive oxygen species and all the other hazards to protein function. This remarkable feat is achieved by a shrewd investment in very stable proteins as lens crystallins, by providing a reservoir of ATP-independent protein chaperones unequalled by any other tissue and by an oxidation-resistant environment. In addition, glutathione, a free radical scavenger, is present in mM concentrations and the plasma membranes contain oxidation-resistant sphingolipids, so what compromises lens function as it ages? In this review, we examine the role of small molecules in the prevention or causation of cataracts, including those associated with diet, metabolic pathways and drug therapy (steroids).

INTRODUCTION

A central question of great current interest in cataract research is whether there is a role for small molecules coming either from the diet or as part of cellular metabolism in the maintenance the eye lens in a healthy, optically clear and functional state. A review on the nutritional modulation of cataracts appeared in 2014 (Weikel et al., 2014), which focused on familiar dietary components. This current review examines recent exciting developments in sterol-based small molecule biochemistry in the lens and what led to these findings in order to focus attention on the opportunity to deliver small molecules to prevent and treat lens cataract.

PHYSIOLOGY AND BIOCHEMISTRY OF THE LENS

First and foremost, the fundamental feature of the lens is that it is one of the most optically transparent tissues in the body. This is achieved by a series of distinctive cell biological events to deliver this essential property for its function. These include the orchestrated spatial organisation of the differentiating lens fibre cells to minimize light scatter, the expression of stable proteins so that concentrations of up to 800 mg/ml protein have been reported in some vertebrate lenses (Mirarefi et al., 2010) to refract light onto the retina, the development of a graded refractive index system to minimize spherical aberration (Land, 2012) and lastly the removal of all organelles that would otherwise scatter light, e.g., nuclei, mitochondria and endoplasmic reticulum (Bassnett et al., 2011). As only the first few outermost layers of cells in the lens retain their organelles, all the other differentiated fiber cells that comprise the bulk of the lens are unable to synthesize new proteins (Lieska et al., 1992) despite the presence of mRNAs (Jaworski and Wistow, 1996). Nevertheless, as the lens ages, lens proteins are subjected to continuous and also significant post-translational modifications (Truscott and Friedrich, 2015) that accumulate as the lens cells age leading to the loss of protein function (Zhu et al., 2010b).

This presents a biological paradox for the lens. It must age successfully and avoid pathological consequences, i.e., cataracts, but without replenishing its original protein complement (Lynnerup et al., 2008; Zhu et al., 2010b). A possible replenishment by a soluble protein exchange process (Shestopalov and Bassnett, 2003) has yet to be fully assessed (Stewart et al., 2013). The lens fiber cells have biochemical mechanisms to ensure that proteins synthesized early in life resist becoming misfolded with time and the eventual aggregation and cataract formation (Truscott and Friedrich, 2016). Also, transporters ensure that avaricious proteases lying in wait in the fiber cells are denied the calcium ions needed for their activity (Duncan and Jacob, 1984). Other membrane proteins such as the aquaporins regulate water flow in and out of the lens cells, as occurs during accommodation (Gerometta et al., 2007) and in a pH dependent manner (Nemeth-Cahalan et al., 2004). This activity of the aquaporins relies on the lipid environment that they occupy (Laganowsky et al., 2014; Tong et al., 2013).

SUSTAINING THE SOLUBILITY OF LENS PROTEINS

Lens cells have a large reservoir of particular protein chaperones called α-crystallins (Horwitz, 1992). These are members of the small heat shock protein family (Garrido et al., 2012). The α-crystallins can suppress the aggregation of off-pathway intermediates in refolding human γC-, γD- and γS-crystallins (Acosta-Sampson and King, 2010) and so are thought to be a major factor in securing lens function. The paradox, however, is that in the centre of the lens, there is very little full length αA- or αB-crystallin (Grey and Schey, 2009; Truscott and Friedrich, 2015). The truncated products in combination with other age-related post-translational modifications such as deamidation can preserve some chaperone activity (Asomugha et al., 2011), but some peptides derived from these lenticular chaperones have compromised activity (Kannan et al., 2013; Santhoshkumar et al., 2011). So, while the chaperone reservoir is one of the biochemical mechanisms maintaining lens transparency over the lifetime of the tissue, it is not without ageing effects.

Another mechanism is the innate stability of the proteins responsible for the refractive properties of the lens (Slingsby et al., 2013). There are often structurally related to enzymes

(Bloemendal et al., 2004; Piatigorsky et al., 1994; Wistow, 2012) and were also called crystallins (Morner, 1894), but they are quite distinct from the α-crystallin protein chaperones. In mammalian lenses these are the β- and γ-crystallin protein families (Slingsby et al., 2013). The combination, therefore, of the most abundant lens proteins being very stable proteins and the presence of the largest reservoir of protein chaperones in the vertebrate body being located in the lens loads the die in favour of sustained protein function throughout the lifetime of the lens (Slingsby et al., 2013), although not all the mechanistic details are currently known.

As with other protein chaperones, α-crystallins also assist in the assembly/disassembly of protein complexes and also influence phospholipid head group organization (Zhu et al., 2010a), They are also nucleotide-independent protein chaperones, a distinct advantage in the low oxygen tension environment of the lens (Barbazetto et al., 2004; Beebe et al., 2014) where ATP is replenished by glycolysis (Cheng et al., 1991). The lens α-crystallins comprise two proteins, αA- and αB-crystallin. They form large, dynamic protein complexes (Hochberg and Benesch, 2014). The applications of advanced physical techniques such as ion mobility mass spectrometry (Hilton et al., 2013; Hochberg and Benesch, 2014; Hochberg et al., 2014) and nuclear magnetic spectroscopy (Roos et al., 2015) have produced important new insights into oligomer dynamics, subunit organization and therefore the structure-function relationship.

ANTIOXIDANTS AND OTHER OXIDATION LIMITING MECHANISMS IN THE LENS

Besides the switching off of DNA transcription and protein synthesis (Jaworski and Wistow, 1996; Lieska et al., 1992; Lynnerup et al., 2008; Stewart et al., 2013) and a reduction in metabolism to a maintenance level (Dovrat et al., 1986; Dovrat et al., 1984; Scharf et al., 1987; Zhu et al., 2010b), a third biochemical process occurs to assist the lens in preventing premature protein denaturation, namely the presence of antioxidants in or near the lens. The anterior side of the lens is washed with the aqueous humor, a selected ultrafiltrate of the blood. In animals such as humans that operate in the light rather than the dark, the aqueous humor and the lens epithelial cells (but not the fiber cells) contain millimolar concentrations of ascorbate due to an ascorbate transporter in the ciliary epithelium (Helbig et al., 1989). In the lens cells there are

millimolar concentrations of the tripeptide glutathione (GSH). High-resolution magic angle spinning proton NMR spectroscopy of ocular tissues revealed that GSH is present at much higher concentrations in the lens (Kryczka et al., 2014). GSH can react with free radicals and other oxidants generated by the formation of singlet oxygen ($^1O_2$) from the interaction between ultraviolet photons and tryptophan residues on the proteins (Ortwerth et al., 2009) and can also react with the oxidized form of ascorbate, dehydroascorbate, to regenerate ascorbate (Sasaki et al., 1995). The resulting oxidized glutathione (GSSG) is reduced back to GSH by NADPH, produced by residual metabolic activity (the pentose phosphate shunt) in the lens (Ganea and Harding, 2006). An important question is where GSH comes from (Srinivas, 2014). There is evidence for transporters for glutamate and cysteine in the differentiated fiber cells in the lens (Lim et al., 2013), thereby offering a continuing source of GSH. Whether this extends to the nuclear region of the lens remains to be seen. It is possible that like other biochemical entities in the lens, GSH in the nuclear region is generated during the undifferentiated, epithelial stage of lens cells and is therefore "old", particularly as GSH exchange between nuclear and cortical regions is compromised by the barrier that develops in older human lenses (Sweeney and Truscott, 1998). This would imply that there would be a loss of GSH with aging in this critical region of the lens (Srinivas, 2014; Truscott, 2005).

The lens plasma membranes are a further barrier to oxidation. Those membranes in the centre (nucleus) of the lens are less permeable to oxygen than those in the lens cortex and ageing decreases further that permeability to oxygen (Raguz et al., 2015; Subczynski et al., 2012). The nuclear membranes of the human lens are rich in sphingomyelin and dihydro-sphingomyelin that can help protect cholesterol against free-radical mediated oxidation (Sargis and Subbaiah, 2006). Nonetheless, despite these protective mechanisms, 7-ketocholesterol accumulates in ageing human lenses (Rodriguez et al., 2014) and 7β-hydroxycholesterol, 7-ketocholesterol, 5α, 6α-epoxycholestanol, 20α-hydroxycholesterol and 25-hydroxycholesterol are all found in human cataract (Girao et al., 1998). So, these mechanisms minimize, but don't prevent lipid oxidation events in the lens. Oxidation of the gap junction proteins may alter the passage of

small molecules (<1,000 Da) between the cells of the lens (Berthoud and Beyer, 2009), although this may be relatively, non-selective.

LENS PROTEIN MODIFICATIONS

Despite large concentrations of antioxidants in the lens system, extensive posttranslational modifications (PTMs) of lens proteins occur with aging (Truscott and Friedrich, 2015). The most common PTM, however, does not come from interaction with oxidants. Instead, it is a purely chemical process whereby asparagine and glutamine residues undergo deamidation to form aspartate, isoaspartate and glutamate residues (Robinson et al., 2005). The other PTMs represent many types of oxidation reactions. Some of these are due to UV light ($^1O_2$) and result in oxidation of tryptophan, tyrosine, cysteine, methionine and histidine residues (Andley et al., 1985; Goosey et al., 1980; Ortwerth et al., 1998) and can induce disulphide bond cleavage (Wang and Wen, 2010), loss of chaperone activity (Mafia et al., 2008) to promote protein aggregation. Others involve dehydroascorbate-induced modifications (Dickerson et al., 1995; Linetsky et al., 2008; Nemet and Monnier, 2011) and include α-crystallin protein chaperones (Dickerson et al., 1995). Proteins on long storage (not just in the lens) undergo the Maillard reaction between their free lysine and arginine amino groups and aldehyde and ketone groups coming from monosaccharides and oxidized lipids. These rearrange to form Amadori products that can form crosslinks between proteins and are prevalent in patients with diabetes (Smuda et al., 2015).

Another process that alters the lens proteins is proteolysis. Using a variety of methods, including imaging mass spectrometry, extensive C-truncation has been shown to occur of the α-crystallins (Grey and Schey, 2009). For αA-crystallin in rats, this process has already begun by the stage of weaning (Stella et al., 2010). It continues with aging. αA-crystallin truncated forms from residues 1-100 are restricted to the nuclear zone of the lens. At weaning, the full length αA-crystallin is found throughout the cortical region and in the undifferentiated outermost epithelial cell layer, but not in the nuclear region (Stella et al., 2010). As rats age, full length αA-crystallin is only found in the newest epithelial cell layer (Anderson et al., 2015). The reason for

C-truncation is multi-factorial. The other crystallins as well as cytoskeletal and integral membrane proteins are all proteolysed during the ageing process (Korlimbinis et al., 2009; Lampi et al., 2014; Sandilands et al., 1995; Truscott and Friedrich, 2016). It may be due to the activity of calcium-dependent proteases such as calpain, alternative enzymatic capabilities of other lens proteins, or by purely chemical processes that occur over the long period of life.

Less is known about other compounds arising from the diet itself, intermediates in normal cellular metabolism or from modifications of endogenous or exogenous substances by the gut microbiota, that may have an impact on the lens cell system. While it has been suggested that fatty acids in the diet do not influence the lipid composition of the lens (Nealon et al., 2008), both sterols and flavonoids in the diet can prevent or even reverse cataract in animal and *ex vivo* human models (Weikel et al., 2014). One of the impacts of modern –omics is that investigators are returning to a close examination of the metabolome, the summation of all the known physiological pathways as well as pathways of the organism's associated microbiota. The analytical approaches have revealed previously unidentified small molecules that were not detected in the heyday of pathway research.

DIETARY FACTORS ASSOCIATED WITH ALTERED LENS CATARACT RISKS

A. Compounds with antioxidant properties

The diet provides many sources of the antioxidant ascorbic acid, particularly in fruits and vegetables. However, it alone does not necessarily prevent lens cataracts. Introduction of a human ascorbate transporter to increase ascorbate concentrations in the aqueous humor of mice, led to a more rapid onset of lens cataracts (Fan et al., 2006), likely by elevated glycation as a result of ascorbic acid oxidation products (Linetsky et al., 2014; Linetsky et al., 2008). It is possible that the increased ascorbate in aqueous humor also increased dehydroascorbate and outstripped the capacity of the GSSG-NADH regenerative cycle. Other plant phytochemicals that are part of the diet have shown preventive activities in models of oxidative stress. Ellagic acid inhibited the formation of cataracts induced by selenite in Wistar rats (Sakthivel et al.,

2008) and prevented alterations in lens proteins (Sakthivel et al., 2011). The polyphenols in *Moringa oleifera* also prevented cataract formation in selenite-treated rat pups (Sasikala et al., 2010). Diabetic cataracts in streptozotocin-induced diabetic rats were prevented by soy isoflavones (Lu et al., 2008a; Lu et al., 2008b) although it was suspected that isoflavones were increasing insulin secretion/susceptibility rather than having antioxidant activity. It's worth noting that Chacko et al. have shown that isoflavones are PPAR-γ agonists (Chacko et al., 2007; Chacko et al., 2005). Soy isoflavones also inhibited cataract in galactose-induced cataracts (Huang et al., 2007). However, in a model of age-related senile cataracts, the Ihara Cataractous Rat strain f (ICR/f), where insulin or its action where not deficient, soy isoflavones were not preventive and even accelerated the early onset of cataracts (Floyd et al., 2011). This result appears to confirm an earlier report using the Royal College of Surgeons Pink Eyed Rat, that less than 1% of animals on a soy-free AIN76A diet developed lens cataracts with aging, as opposed to 29% of those on a lab chow diet containing copious amounts of soy (Hess et al., 1985). Grape seed proanthocyanidins were shown to delay the late stages of development of cataracts in ICR/f rats (Yamakoshi et al., 2002) and the selenite model (Zhang and Hu, 2012).

Another dietary antioxidant, vitamin E, prevents lens cataract in selenite-induced cataracts (Mathew et al., 2003). Other studies suggest a role for vitamin E in prevention of cataracts in other rodent models (Haque and Gilani, 2005; Kojima et al., 2002; MacDonald-Wicks and Garg, 2003) but not in humans (Olmedilla et al., 2003). A meta-analysis of epidemiological data revealed that dietary, but not supplemental vitamin E reduced the risk of age-related cataracts (Zhang and Hu, 2012).

In contrast, some dietary supplements and drugs increase the risk of lens cataracts by absorbing incident light at non-UV wavelengths and generating $^1O_2$ and other oxidation products through their photophore properties. Examples are intermediates in porphyrin synthesis and metabolism (Roberts and Dillon, 1987), compounds in dietary supplements such as hypericin in St John's wort (Schey et al., 2000) and drugs such as ciprofloxacin (Zhao et al., 2010).

B. Lipids and fatty acids

A feature of the maturing lens is gradual association of the α-crystallins with membrane components (Borchman and Tang, 1996; Cobb and Petrash, 2002; Grami et al., 2005). It therefore is important to understand how the lens lipid composition changes with age. In humans, the relative amounts of sphingolipids increase with aging (Huang et al., 2007). However, this may be more a reflection of the disappearance of the other major membrane lipids, the phosphatidylcholines and phosphatidylethanolamines (Li et al., 1987; Zigman et al., 1984). Although not proven, long chain unsaturated fatty acids released from PCs and PEs may be the source of energy needed to maintain lens function over a lifetime, although the low levels of butyrate measured in the lens (Kryczka et al., 2014) and the spatial restriction for fatty acid oxidation to the lens epithelium and outer cortex because of the lack of mitochondria (Bassnett et al., 2011) challenges this concept. Based on the $^{14}C/^{12}C$ isotopic ratio of the lens lipid matching that of the ratio of their year of birth, lipid replacement in lens membranes is minimal (Hughes et al., 2015). Nonetheless, the changing lipid composition, paralleled by the establishment of membrane domains in the aging lens nucleus (Raguz et al., 2015), may alter the distribution of the crystallins (and other proteins) within the differentiated, old fiber cell. With the increase in sphingolipids in the nuclear lens membranes, α-crystallins then bind more strongly to these membranes (Grami et al., 2005) and in doing so may recruit other lens proteins and thereby increase total light scattering, again an ageing signature for the lens (Michael and Bron, 2011).

C. Sterols and other small molecules

Two recent studies have revealed the role of compounds in the cholesterol biosynthesis and metabolism pathways. In the first (Zhao et al., 2015), clever analysis of genetic mutations of two family members with frank lens cataracts revealed a mutation in the active site of lanosterol synthase (LSS), the enzyme responsible for the conversion of a linear unsaturated hydrocarbon, 2,3-oxidosqualene, into the steroid ring conformation of mammalian sterols, steroids and bile acids. Examination of a further 150 subjects with lens cataracts identified a

second mutation in the active site of LSS (Zhao et al., 2015). Expression of the two mutant LSS demonstrated that both mutations led to loss of LSS activity. However, this was not the first finding concerning the association of loss of LSS activity and cataracts. It had been previously shown that in the Shumiya rat that cataracts were associated with deletion of exon 4 of LSS (Mori et al., 2006). In addition, drug candidates for inhibiting cholesterol synthesis, each inhibitors of LSS, caused the formation of lens cataracts (Fouchet et al., 2008; Funk and Landes, 2005; Pyrah et al., 2001). A mechanism of action was proposed by (Cenedella et al., 2004) where inhibition of lanosterol synthesis (and therefore products downstream of it) resulted in a "stiffer" membrane, thereby altering the way in which proteins associate with the membranes. The α-crystallins become attached to the lipid membrane (Borchman and Tang, 1996; Grami et al., 2005; Maddala and Rao, 2005) and their association with lens membranes coincides with decreased lens compliance (Heys et al., 2007). The α-crystallins influence the head group organization of the phospholipids (Zhu et al., 2010a). Other lens proteins also become associated as the membranes age (Truscott et al., 2011) and it is thought that this will also contribute to the complexity of the protein complement on the membranes in aged lenses. Zhao et al. (2015) found that applying lanosterol, but not cholesterol, to the eyes of dogs with age related (non-diabetic) cataract, cells transfected with mutant αA-crystallin and to *ex vivo* cataractous rabbit lens resulted in solubilization of the cataracts and solubilisation of the protein aggregates. In the latter case, lanosterol-induced solubilization obeyed first order kinetics with a $T_{1/2}$ of 4 h. Excitingly, a nanoparticle preparation of lanosterol when applied as eye drops to one eye of a dog with bilateral cataracts for 6 weeks led to a marked dissolution of that cataract. This remarkable study suggests that dietary-derived small molecules may not only reduce the risk of lens cataracts, but may even be used to effect a therapeutic reversal of cataracts.

A second study, recently published in *Science* (Makley et al., 2015), took a completely different approach but arrived nevertheless at cholesterol-related small molecules to help reverse cataract caused by α-crystallin mutations. From a relative small screen (~2,500) of bioactives, two oxysterols were eventually identified (5α-cholestan-3β-ol-6-one and 5-cholesten-3β,25-

diol) that reversed cataract caused by R120G αB-crystallin and R49C αA-crystallin. Molecular modeling suggested that these molecules bind at the dimer interface of the crystallin domain for αB-crystallin.

Steroids are associated with cataracts – use of prednisone to treat lupus, glucocorticoid injections for Addison's disease and other conditions – are they displacing "beneficial" oxysterols? Of course, the Zhao paper (Zhao et al., 2015) identified mutations in lanosterol synthase as the genetic basis of inherited cataract in humans, an observation made also for the Shumiya rat cataract model (Mori et al., 2006). This quite clearly establishes lanosterol and cholesterol metabolism as key factors in preventing cataract, complementing the observation that patients with Smith-Lemli-Opitz syndrome (Cotlier and Rice, 1971; Fitzky et al., 1998), mevalonic aciduria (Hoffmann et al., 1986; Schafer et al., 1992) and cerebrotendidinous xanthomatosis (Chen et al., 1996; Yoshinaga et al., 2014) and those affected by Coats' Disease with ocular deposits of cholesterol (Beby et al., 2005; Chang et al., 1984) also present with cataract. The debate over the increased risk or otherwise of cataract associated with statin use continues (Desai et al., 2014; Kostis and Dobrzynski, 2015). There are many documented changes in lipid composition that accompany cataract in human lenses (Borchman and Yappert, 2010). Dietary linoleic acid levels are correlated with increased nuclear cataract (Lu et al., 2007), although dietary fatty acids do not influence the lipid composition of the rat lens (Nealon et al., 2008), the inability to synthesise ether lipids in the plasmalogen biosynthesis pathway is still linked to cataract in mice (Gorgas et al., 2006; Liegel et al., 2011; Park et al., 2014) and humans (Buchert et al., 2014; Liegel et al., 2013). Nevertheless, lipids and sterols are clearly linked to cataractogenesis and its prevention.

Comparing the three sterols (Figs. 2A-C) that have been shown to improve mutant αA-crystallin solubility or clarify pre-existing lens cataracts with three well known examples of xenobiotics (therapeutic agents and a botanical) (Figs. 2D-F) whose use leads to cataract formation, a common structural feature in the former but not the latter is the presence of a hydrophobic side chain. Thus, prednisone, while having a steroid ring nucleus, lacks the side chain of the

sterols. While this would appear to advocate for a beneficial effect of a more familiar sterol, cholesterol, a previous study showed cholesterol led to a more tightly packed bilayer than lanosterol (Cournia et al., 2007). Accordingly, lanosterol–enriched membranes had a lower melting temperature (increased fluidity). In contrast, hypericin increased membrane stiffness (Chaloupka et al., 1999).

FUTURE DIRECTIONS

Lipids and their exchange through the lens over short (week) timescales compared to protein exchange and turnover in the membrane and soluble fractions are clearly areas of interest. This is because of the possibility of pharmacological alternatives to surgery for the treatment and prevention of cataract in humans and in animals. The lens also offers insight into the ageing process itself and the biochemical and cell biological processes that accompany the ageing of the lens and the consequences for its proteins and lipids. In the lens, time is measured in decades and therefore that parameter is an area of the biochemical and cell biological time scale that is rarely considered let alone studied, but is key to understanding how to age successfully. The recent reports that certain sterols prevent and even reverse lens cataract will encourage a more thorough understanding of the role of the changing lipid environment with aging on the primary function of the lens, to remain clear and pass observed light to the rest of the visual apparatus. Finally, delivery of these sterols or lipid domain modifiers to the lens environment, particularly orally via the diet, will remain a challenge.

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FIGURE LEGENDS

Fig. 1. A summary of the major ageing effects on lens proteins and plasma membranes. The recent discovery of the effects of selected sterols (Makley et al., 2015; Zhao et al., 2015) on lens transparency and the data from ^{14}C distribution in the soluble protein fraction of human lenses (Stewart et al., 2013) suggest the protein and sterol content of the lens nucleus exchanges with the cortex.

Fig. 2. Chemical structures of small molecules that influence the solubilities of lens proteins. The sterols, lanosterol (A), 5α-cholestan-3β-ol-6-one (B) and 5-cholesten-3β,25-diol (C), have recently been shown (Makley et al., 2015; Zhao et al., 2015) to solubilize mutant αA-crystallin and proteins in lens cataracts. In contrast, the therapeutic agents, prednisone (D) and ciprofloxacin (E) and the botanical, hypericin (F), are associated clinically with the formation of lens cataract.

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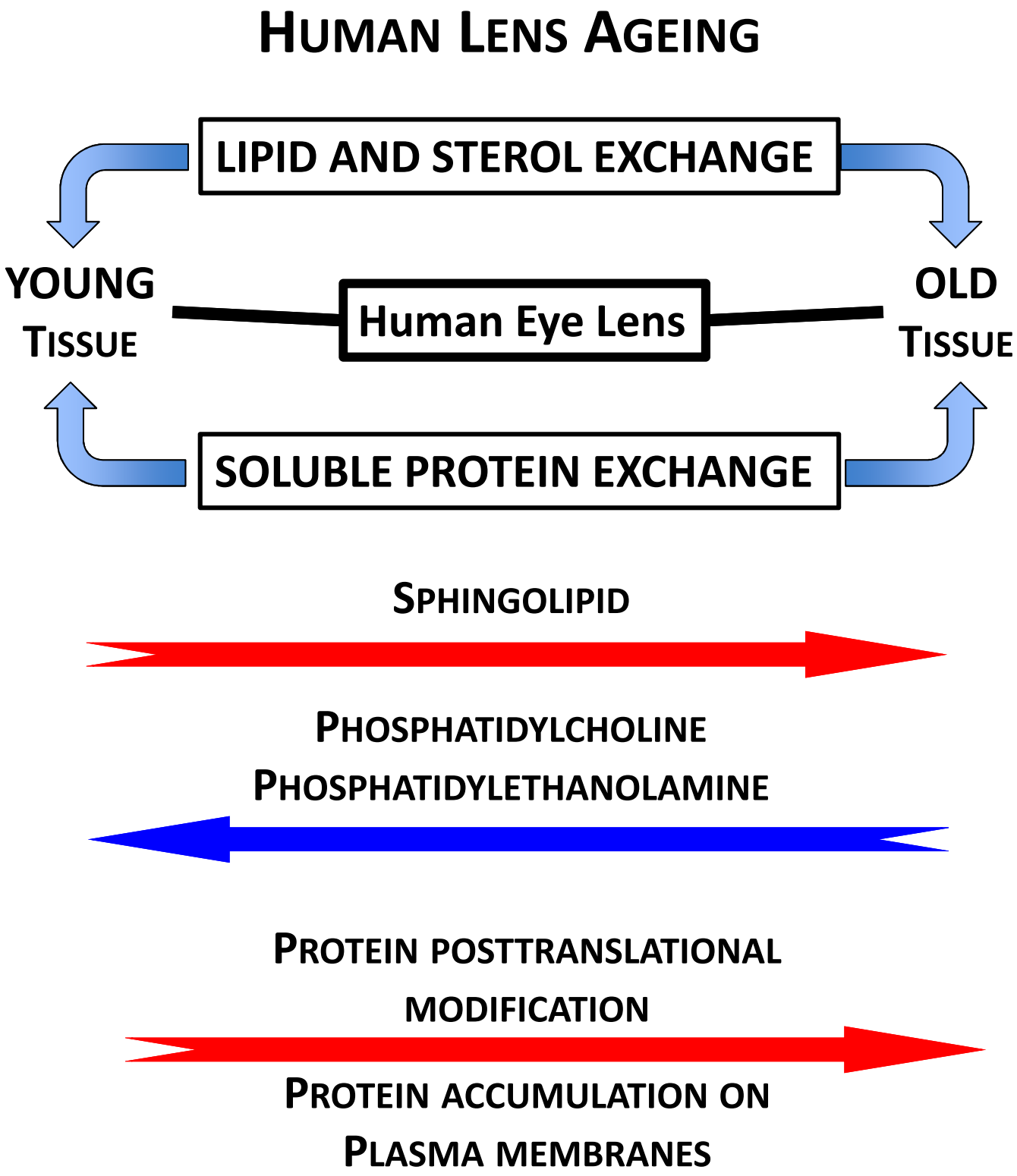
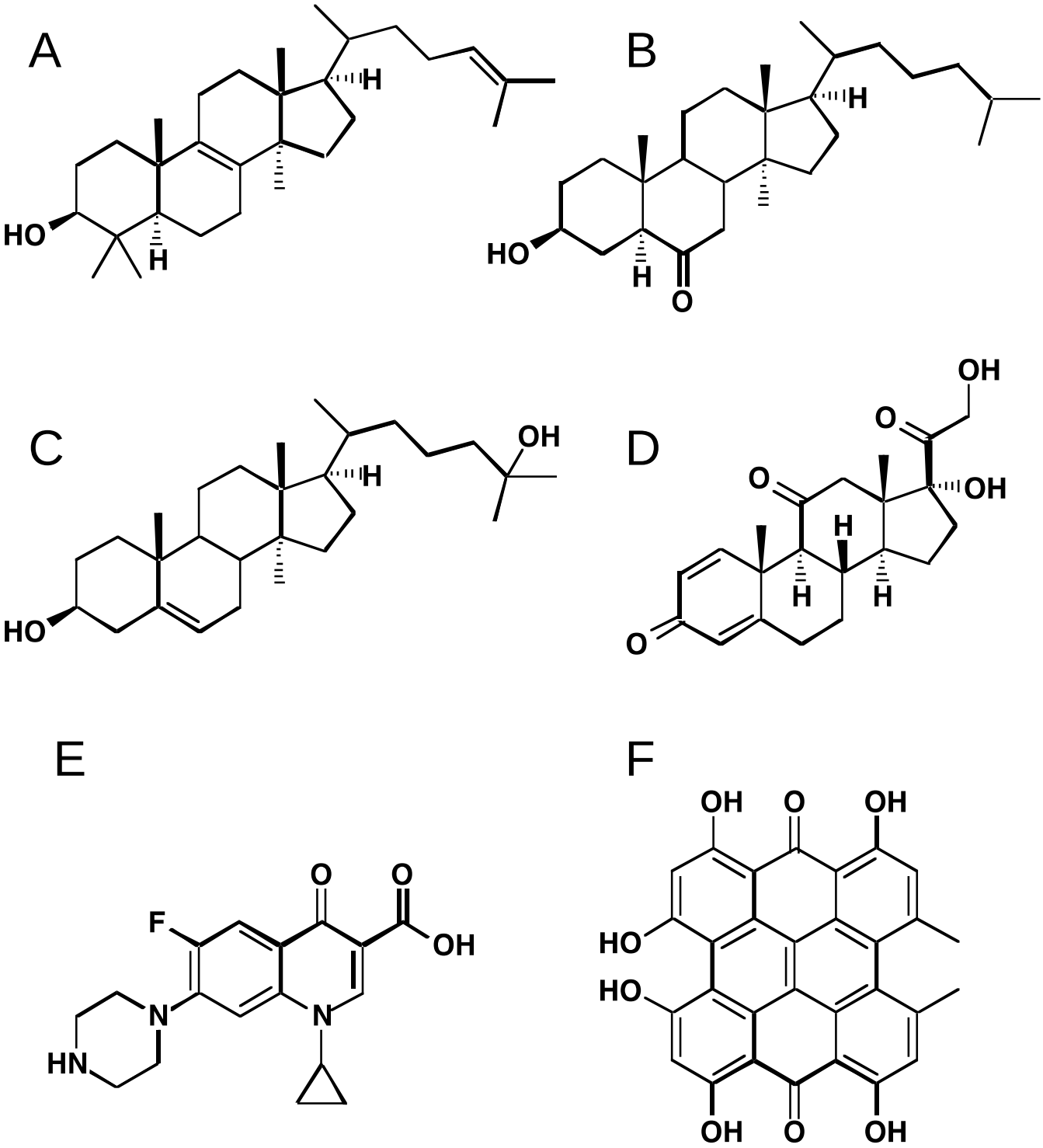


Fig. 1 Barnes and Quinlan



Barnes and Quinlan Fig. 2