NADPH Oxidase Activity in Retinal Pigmented Epithelium as a Target for Prevention of Age-related Macular Degeneration

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The author is co-inventor and co-owner of a U.S. patent covering nutraceutical uses of phycocyanobilin-enriched spirulina extracts

Abstract
Considerable evidence points to macular oxidative stress as a key driver of age-related macular degeneration (AMD). In particular, retinal pigmented epithelial (RPE) cells subjected to oxidative stress are prone to apoptosis or senescence; lose their tight junctions; decrease their production of factors which provide trophic support for retinal photoreceptors, suppress choroidal neovascularization, and control complement activation; and boost their production of vascular endothelial growth factor (VEGF), a major stimulant to neovascularization – all effects which are consistent with the pathogenesis of AMD. There is good reason to suspect that NADPH oxidase activity in RPE cells is the key source of the oxidative stress that compromises RPE function in AMD. Studies with other cell lines indicate that a number of risk factors for AMD – including cigarette smoke, homocysteine, hyperglycemia, cadmium, and up-regulated activation of the alternative complement cascade – have the ability to stimulate NADPH oxidase activity, as does all-trans-retinal, a suspected pathogenic factor in AMD. Hence, measures which can down-regulate NADPH oxidase activity may have potential for preventing or controlling AMD. These may include high-dose statins – found to reverse features of early AMD in about half of patients in a recent pilot study – and spirulina, a rich source of the chromophore phycocyanobilin (PhyCB). PhyCB is a biliverdin derivative that can mimic the intracellular physiological role of bilirubin as an NADPH oxidase inhibitor. Patients with Gilbert syndrome or relatively high plasma unconjugated bilirubin may be at lower risk for AMD owing to bilirubin’s inhibitory impact on NADPH oxidase; this prediction could be readily tested in epidemiological studies.
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Pathophysiology of AMD

Age-related macular degeneration (AMD) is the leading cause of vision loss and partial blindness among elderly Americans 60 and older. The National Eye Institute estimates that there are presently about 1.75 million people in the U.S. with advanced AMD and associated vision loss. The number is expected to grow to approximately 3 million by 2020. Men and women are about equally affected, and the incidence of AMD in blacks is lower than it is in whites. AMD is the most common cause of visual impairment in the developed world.

AMD is a degenerative disease of the macula, the part of the retina responsible for the sharp central vision needed to read or drive; central vision loss is likely to occur. AMD may be manifested as a sudden worsening and distortion of central vision and may progress rapidly with a course of only weeks or months. Typically, AMD has a preclinical, asymptomatic phase, in which extracellular waste material known as "drusen" accumulates in the space between the basement membrane—Bruch’s membrane—and the pigmented epithelial layer (comprised of retinal pigmented epithelial cells—RPE).

RPE cells are remarkably versatile in their contribution to healthful retinal function, and it is generally believed that dysfunction or death of these cells is crucial to AMD pathogenesis. The retinal pigmented epithelium serves as the blood-retinal barrier, and regulates the transport of oxygen, substrates and waste products between the choroidal vasculature and the retina. Loss of RPE tight junctions—which evidently compromises this barrier function—is a hallmark of AMD. RPE cells also generate growth factors that aid the survival of retinal photoreceptors and other cellular constituents of the inner retina, while suppressing pathological choroidal neovascularization.

The most curious function of RPE cells is to phagocytize photoreceptor outer segments (POS). While doing this, they isomerize all-trans retinyl esters derived from POS back to 11-cis-retinal, which is then returned to the photoreceptors for use in rhodopsin synthesis; this is a crucial function, as photoreceptors are incapable of performing this isomerization themselves. The phagocytic function of RPE cells entails continual lysosomal activity; when this activity is sub-optimally efficient, as it appears to be in AMD, lipofuscin deposits accumulate in these cells. Lipofuscin has photosensitizing activity, and components of lipofuscin—notably A2-E, a retinoid metabolite—can impair lysosomal function, in part by blocking the proton pump that acidifies lysosomes. (This raises the specter of a vicious cycle in which lipofuscin accumulation encourages further accumulation of lipofuscin). Proteomic studies indicate that the drusen that characterizes AMD is derived largely from inadequately degraded POS components as well as lysosomal proteins—suggesting that stressed RPE cells are disgorging half-digested lysosomal contents into the sub-epithelial space. Drusen is also rich in complement metabolites; this accords well with the discovery that AMD is more common in individuals who carry reduced-function alleles of key proteins that regulate the complement cascades.

Degeneration of the RPE is considered the most important hallmark of AMD. This is characterized by pigment mottling, accumulation of intercellular lysosomal lipofuscin and extracellular drusen, loss of tight junctions, and apoptotic cell death. As discussed below, chronic oxidative stress is strongly linked to RPE senescence and the pathogenesis of AMD.

Advanced forms of AMD include both dry (geographic atrophy) and wet (neovascular) AMD. The dry form of AMD is far more common, but the wet form occurs together with the dry form in about 15% of cases. Dry AMD is characterized by progressive apoptosis within the light-sensitive retinal macula, required for
fine vision; apoptotic loss of photoreceptors, RPE cells, and the underlying cells in the choroidal capillary layer is noted. Wet AMD is characterized by choroidal neovascularization, with vascular leakage into subretinal spaces, and can lead to sudden, catastrophic, and permanent visual loss.

Subjects with a family history of AMD, smokers, and people who are obese or sedentary are at increased risk for this syndrome. People who are genetically prone to increased activation of the alternative complement pathway – such as those carrying certain alleles of the gene for complement factor H – are at greatly increased risk, perhaps reflecting a role for complement in drusen formation.

Oxidative Stress Plays a Key Role in the Pathogenesis of AMD

Although the pathogenesis of AMD remains murky, there is a growing consensus that oxidative stress plays a key role in driving this syndrome. Chemical markers of oxidative stress have consistently been found to be elevated in the maculas of patients with AMD. In cell culture studies, exposure of RPE cells to oxidative stress has been shown to impair the function and survival of these cells – inducing a loss of tight junctions, impairing production of pigmented epithelium-derived factor (PEDF – a growth factor that provides trophic support for photoreceptors while inhibiting choroidal neovascularization) and of complement factor H (impaired function of which has been linked to increased AMD risk), boosting expression of vascular endothelial growth factor (VEGF – a key driver of angiogenesis), and promoting apoptosis and/or senescence– effects that are all consistent with the pathology of AMD. The oxidant-induced shift in the balance of PEDF and VEGF production in RPE cells seems likely to be a key mediator of choroidal neovascularization. Moreover, oxidative stress can also induce apoptosis in photoreceptors, an effect which seems likely to contribute to the photoreceptor loss characteristic of dry AMD. And the most well characterized risk factor for AMD is cigarette smoking, which is well known to promote oxidative stress in tissues; in mice exposed chronically to cigarette smoke, increased oxidative stress and apoptosis is noted in RPE cells, accompanied by thickening of Bruch’s membrane.

A further line of evidence implicating oxidative stress in AMD is the fact that transgenic mice lacking superoxide dismutase activity have been shown to develop a syndrome very analogous to AMD as they age; drusen, thickened Bruch’s membrane, and choroidal neovascularization are all observed, and the junctions linking retinal pigment endothelium are disrupted – features characteristic of human AMD. Increased light exposure accelerates accumulation of drusen in these mice, presumably because light promotes retinal oxidative stress.

Clearly, some of the oxidative stress that contributes to AMD pathogenesis results from light exposure. The macula is exposed to blue light that generates singlet oxygen and other reactive oxygen species when it interacts with photosensitizing chemicals; the lipofuscin that accumulates in stressed RPE cells is rich in such photosensitizers, some of which, such as A2-E, are derived from all-trans-retinal.

Photoreceptors are exceptionally susceptible to oxidative damage, as their membranes are extremely rich in the long-chain omega-3 polyunsaturate docosahexaenoic acid (DHA); DHA can constitute up to half of the fatty acids in photoreceptor outer segments (POS). This presumably explains why POS membranes are also unusually rich in xanthophyll carotenoids capable of quenching the singlet oxygen that otherwise might peroxidize DHA. The xanthophylls also are found in photoreceptor axons, where their presumed key function is to absorb blue light before it can...
penetrate to deeper levels of the macula and generate oxidants. The fact that the xanthophyll content of the macula is subnormal in patients with AMD might in some measure be a consequence of the disease, but it is also reasonable to suspect that this deficit contributes to disease progression by lessening the macula’s antioxidant protection.29-31

NADPH Oxidase – A Key Source of Pathogenic Oxidative Stress in AMD

There is good reason to suspect that NADPH oxidase may be a key source of oxidant stress in RPE cells. RPE cells act as phagocytes, engulfing outer segments of retinal rod photoreceptors; like other phagocytes, RPE possess NADPH activity that increases during phagocytosis.32, 33 More recent work establishes that RPE expresses p22phox, a key membrane component of NADPH oxidase complexes.34 Intriguingly, viral delivery of small interfering RNA for p22phox to the subretinal space prevents choroidal neovascularization in a mouse model of AMD (involving laser disruption of Bruch’s membrane).35 The authors conclude that “NADPH oxidase-mediated ROS production in RPE cells may play an important role in the genesis of neovascular AMD, and this pathway may represent a new target for therapeutic intervention in AMD.” In addition, NAPDH oxidase activity is expressed by retinal neurons; this activity increases when growth factor support is withdrawn (as might be expected if death or dysfunction of retinal pigment epithelium impairs its trophic function), and there is suggestive evidence that NADPH oxidase activation may drive the apoptotic death of cone cells in retinitis pigmentosa.36, 37

At least 5 risk factors for AMD – cigarette smoke, homocysteine, hyperglycemia, cadmium, and up-regulated activation of the alternative complement cascade – have the potential to activate NADPH oxidase in RPE cells, as documented below. Hence, a case can be made that up-regulated activity of NADPH oxidase in RPE cells plays a key role in driving the AMD syndrome.

Cigarette Smoke - Cigarette smoking is one of the best established risk factors for AMD, and the most important of the modifiable risk factors.38 The highly active oxidants in inhaled smoke that are traumatic to the lungs are unlikely to mediate this risk, as their half-lives are very short. However, cigarette smoke extract (CSE) also contains a range of more stable compounds capable of exerting systemic effects, including agents such as acrolein that can react with sulfhydryl groups in proteins via Michael additions. In cell culture studies, CSE has been shown to activate NADPH oxidase in a range of cell types; this activation, in turn, stems from activation of classical isoforms of protein kinase C (PKC).39-43 Acrolein and methyl vinyl ketone, prominent components of CSE, have been found to be particularly active in this regard.43 While exposure to RPE cells to CSE has been shown to evoke oxidative stress, studies examining the role of NADPH oxidase in this regard have not been published; nonetheless, results in other cell lines are consistent with the possibility that NADPH oxidase is a key source of oxidative stress in CSE-exposed RPE cells.

Homocysteine - Considerable epidemiology, both prospective and case-control, has associated modest elevations of plasma homocysteine with increased risk for AMD – as confirmed in a recent meta-analysis.44 Moreover, there is reason to suspect that this association is causal. The Women’s Antioxidant and Folic Acid Cardiovascular Study (WAFACS) randomized 5,442 women at high cardiovascular risk (with pre-existing cardiovascular disease or with 3 or more cardiovascular risk factors) to receive a supplement providing 2.5 mg folic acid, 50 mg pyridoxine, and 1 mg cyanocobalamin, or matching placebo.45 5,205 of these women did not have a diagnosis of AMD at baseline. Although the primary intent
of this study was to examine the impact of homocysteine-lowering supplementation on cardiovascular risk, the authors prospectively decided to include assessment of new AMD incidence in their protocol. During an average follow-up of 7.3 years, the participants reported 55 new cases of AMD in the supplemented group and 82 in the placebo group (RR 0.66; CI: 0.47-0.93; P=0.02); visually significant new AMD was reported by 26 subjects in the treated group, vs. 44 in the placebo group (RR 0.59; CI: 0.36-.095; P=0.3). A sub-group analysis indicated that homocysteine levels fell by an average of 18% in the treated group, and the authors suggested that this may have contributed to the observed benefit.

The vascular toxicity of hyperhomocysteinemia is suspected to reflect induction of oxidative stress in the vascular system. Homocysteine has been shown to activate NADPH oxidase in vascular endothelium, and this effect likewise appears to reflect activation of PKC. Homocysteine thiolactone, a cyclic derivative of homocysteine produced by enzymatic activity within cells, reacts readily with protein lysine groups, and seems likely to be the true mediator of this effect. It is curious that, whereas folate supplementation has not been found to confer protection from myocardial infarction in subjects with moderately elevated homocysteine levels, it has been found to decrease risk for ischemic stroke; this possibly reflects the fact that expression of NADPH oxidase is far higher in the cerebral vasculature than in other vascular beds.

**Hyperglycemia** - Both diabetes and frequent consumption of high-glycemic-index foods have emerged as risk factors for AMD is some though not all pertinent studies. Hyperglycemia induces oxidative stress in RPE cells and in the retinas of diabetic mice. Hyperglycemia has been shown to induce NADPH oxidase activation in a number of cell types, and, once again, PKC activation contributes to this effect. Indeed, there is reason to suspect that activation of NAPDH oxidase is a key mediator of the complications of diabetes. Presumably, episodic elevations of plasma glucose associated with high-glycemic-index meals likewise could modestly boost NADPH oxidase activity.

**Cadmium** - Non-occupational cadmium exposure has been linked to increased risk for vascular diseases, various cancers, osteoporosis, nephropathy, and other disorders. The broad risks associated with high-normal body cadmium levels may reflect the ability of this toxic metal to promote oxidative stress. In particular, a number of cell culture studies point to NADPH oxidase as the source of this oxidative stress. In light of these considerations, it should not come as a surprise that recent epidemiological studies have linked high-normal levels of cadmium in urine, blood, or vitreous fluid to increased risk for AMD. In light of the fact that high-dose supplemental zinc can function as a cadmium antagonist via induction of metallothionein, an antioxidant protein that alleviates cadmium toxicity by sequestering it, the favorable impact of high-dose zinc (80 mg daily) on progression of AMD in the Age-Related Eye Disease Study 1 (AREDS1) might be attributable in part to cadmium antagonism. (Of particular interest is the fact that subjects enrolled in the zinc-supplementation arm of this study also enjoyed a significant 27% reduction in total mortality rate, relative to subjects not receiving zinc, during the median follow-up of 6.5 years; could this reflect the broader pathogenic role of cadmium?)

**Complement Cascade** - Recent research may help to rationalize the high risk for AMD in people who express alleles of genes for complement factors or complement regulatory factors that would tend to up-regulate activation of the alternative complement pathway. Oxidative stress in RPE cells suppresses their production of cell-surface complement inhibitors, increasing risk for alternative
pathway activation on the membranes of these cells;\(^8\) such activation should be most intense in those with low activity alleles of complement factor H, an inhibitor of the alternative pathway. Activation of the alternative complement cascade in the microenvironment of the RPE likely explains why complement metabolites are found in drusen.\(^5\) Moreover, ARPE-19 cells have been found to express receptors for the pro-inflammatory complement metabolites C3a and C5a; activation of the C5a receptor promotes production of the key angiogenic factor VEGF.\(^8\) When RPE cells are exposed simultaneously to oxidative stress and serum, they produce VEGF; this effect is blocked if the cells are concurrently exposed to an alternative pathway inhibitor.\(^8\) Exposure of oxidatively stressed RPE cells to serum likewise potentiates their loss of tight junctions – an effect that again is suppressed by an alternative pathway inhibitor.\(^8\) Although the impact of C5a on NADPH oxidase activity in RPE cells has not yet been reported, C5a has been shown to stimulate this activity, in macrophages, neutrophils, and eosinophils.\(^89\)\(^-\)\(^92\)

**All-Trans-Retinal** - The extent to which impaired metabolism of all-trans-retinal (atRAL) might contribute to the pathogenesis of AMD remains unclear. atRAL is capable of activating NADPH oxidase in RPE cells as well as in photoreceptors by triggering the activation of receptors coupled to heterotrimeric G proteins.\(^93\)\(^-\)\(^95\) Indeed, atRAL may mediate the toxicity of severe light exposure to photoreceptors by activating NADPH oxidase:photoreceptor-specific knock-out of Rac1 in rod photoreceptors markedly protects them from light toxicity.\(^96\) The NADPH oxidase inhibitor apocynin is likewise protective in this regard.\(^96\) After atRAL is generated by rhodopsin light absorption within photoreceptor discs, the Abca4 ATP-binding cassette transporter is required for atRAL removal from photoreceptor discs and its subsequent reductive metabolism; genetic variants of this transporter have been linked to increased risk for AMD, suggesting that increased delivery of atRAL to RPE cells via phagocytosis of photoreceptor outer segments can exacerbate the AMD syndrome.\(^97\)

**Down-Regulating NADPH Oxidase Activity as a Strategy for Preventing/Controlling AMD**

High-dose lipophilic statins have the potential to down-regulate NADPH oxidase activity by suppressing isoprenylation of Rac1, a step required for full activation of certain isoforms of NADPH oxidase.\(^98\)\(^-\)\(^101\) Hence, if NADPH oxidase activity in RPE cells plays a key role in the pathogenesis of AMD, one would expect that clinical or epidemiological studies would observe lower risk for AMD, or slower progression of the disorder, in patients receiving high-dose statins for vascular protection. Studies examining risk for or progression of AMD in statin users have observed inconsistent results, but arguably this could reflect the fact that statin doses achieving adequate control of blood lipids may be insufficient to influence NADPH oxidase activity.\(^102\)\(^-\)\(^104\) An open pilot trial of high-dose atorvastatin (80 mg daily) in AMD was initiated following the observation that, within 6 months of increasing his atorvastatin dose to 80 mg, an AMD patient experienced complete disappearance of drusen and an improvement in visual acuity by 12 letters.\(^105\) Twenty-three patients with prominent drusen deposits whose AMD had not yet progressed to geographic atrophy or choroidal neovascularization were recruited and treated for one year. In ten of the patients, drusen deposits decreased, and in 8 of these drusen virtually disappeared. These responding patients averaged a 3 letter gain in visual acuity. None of the patients in the trial progressed to neovascular AMD. Response did not correlate with extent of cholesterol reduction. These findings suggest that a properly controlled study of this regimen would be warranted.
An alternative possibility for down-regulating NADPH oxidase activity is suggested by the discoveries that intracellular free bilirubin functions intracellularly as an inhibitor of NAPDH oxidase\textsuperscript{106-108} (thereby rationalizing the antioxidant activity of heme oxygenase), and that phycocyanobilin (PhyCB), a bilirubin homolog that functions as a chromophore in spirulina and other microalgae, can mimic this activity.\textsuperscript{109, 110} Since orally administered spirulina or phycocyanin – the spirulina protein which contains PhyCB as a chromophore – exerts neuroprotective effects in rodent studies, and parenterally administered phycocyanin or PhyCB is highly protective in rodent stroke models, there is reason to suspect that spirulina or PhyCB-enriched spirulina extracts may have the potential to confer antioxidant protection to the retina.\textsuperscript{111-114} So far, however, no studies have examined the impact of spirulina or phycocyanin on retinal function or pathology. It would be of particular interest to determine whether individuals with Gilbert syndrome - an innocuous genetic variant associated with chronically elevated plasma levels of unconjugated bilirubin and reduced risk for cardiovascular disease\textsuperscript{115} – are at decreased risk for AMD. More generally, do plasma levels of unconjugated bilirubin correlate inversely with risk for AMD in prospective cohorts?

**Overview**

Current nutraceutical/pharmaceutical strategies for preventing or impeding the progression of AMD, as suggested by controlled clinical trials – high-dose zinc, antioxidant vitamins, xanthophylls, DHA, folate/B12 – are worthwhile, but fail to stop progression of the syndrome in a high proportion of patients. The evidence presented above strongly suggests that activation of NADPH oxidase in RPE cells may play a key pathogenic role in AMD, and hence that effective targeting of NADPH oxidase might enable a further advance in management of this disorder. Further controlled evaluation of high-dose lipophilic statins in AMD appears warranted. In regard to the possible utility of spirulina in this regard, the absence of a rodent model of AMD makes it difficult to evaluate this potential pre-clinically. Once PhyCB-enriched spirulina extracts are available as nutraceuticals, open label pilot studies could be attempted to ascertain whether these extracts appeared to have any evident impact on progression of AMD. If these preliminary efforts yielded encouraging observations, double-blind trials would then be appropriate.
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