Pathological retinal angiogenesis and polyunsaturated fatty acids

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ABSTRACT: Retinopathy of prematurity in children, diabetic retinopathy, and age-related macular degeneration (AMD) are characterized by vessel loss followed by hypoxia-driven destructive neovascularization. Damage to the retinal pigment epithelium and a chronic aberrant inflammatory response increase the expression of vascular endothelial growth factor (VEGF) and tumour necrosis factor-α (TNF-α) that initiate and perpetuate pathological retinal angiogenesis. Anti-VEGF approaches are of limited benefit in the treatment of AMD, diabetic retinopathy, and retinopathy of prematurity. Polyunsaturated fatty acids (PUFAs): arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), and their products lipoxins, resolvins, and protectins play an important role in the pathogenesis of pathological retinopathy in view of their anti-inflammatory; wound healing, and neuroprotective actions. Lipoxins, resolvins and protectins prevented hyperoxia-induced retinopathy in experimental animals suggesting that they are useful in the prevention and treatment of destructive angiogenesis.

INTRODUCTION

Age-related macular degeneration (AMD), diabetic retinopathy and retinopathy of prematurity in children are associated with retinal pathogenesis in which neoangiogenesis occurs. AMD, the leading cause of irreversible blindness in people 50 years of age or older, affects more than 8 million Americans.

The macula, the central, posterior portion of the retina (see Figure 1), contains the densest concentration of photoreceptors within the retina and is responsible for central high-resolution visual acuity, allowing one to see fine detail, read, and recognize faces. Retinal pigment epithelium that lies posterior to the photoreceptors forms part of the blood-ocular barrier and its functions include: photoreceptor phagocytosis, nutrient transport, and cytokine secretion. Posterior to the retinal pigment epithelium lies Bruch's membrane, a semi permeable exchange barrier that separates the retinal pigment epithelium from the choroid, which supplies blood to the outer layers of the retina.

CHANGES WITH AGE

With age, focal deposition of acellular, polymorphous debris between the retinal pigment epithelium and Bruch's membrane occurs called as drusen. On fundoscopic examination it appears as pale, yellowish lesions and are found in both the macula and peripheral retina (see Figure 2). Drusen can be categorized as small (< 63 µm) diameter, medium (63 to 124 µm) or large (> 124 µm) and are also described as hard or soft on the basis of their appearance of their margins-hard drusen have discrete margins whereas soft drusen have indistinct margins, are usually large, and can be confluent.

PATHOPHYSIOLOGY OF AMD

Age-related macular degeneration (AMD) is characterized by the presence of drusen and in most cases is present bilaterally.

However, presence of a few small, hard drusen is not considered to have AMD, since drusen are ubiquitous in people over 50 years of age as a part of normal aging process. Excess drusen, however, leads to damage to the retinal pigment epithelium and a chronic aberrant inflammatory response that causes large areas of retinal atrophy that, in turn, enhances the expression of vascular endothelial growth factor (VEGF). Excess VEGF leads to choroidal neovascularization accompanied by increased vascular permeability and fragility. Choroidal neovascularization may extend anteriorly through breaks in Bruch's membrane and lead to subretinal haemorrhage, fluid exudation, lipid deposition, detachment of the retinal

Figure 1. A) The normal retina. The area within the black circle is the macula; B) Histological section of normal retina, with photoreceptors (black arrows), retinal pigment epithelium (white arrow), and the choroid (red arrow); C) Cross-sectional image of the retina generated by optical coherence tomography, an imaging technique that allows for real-time, non-invasive visualization of retinal architecture.

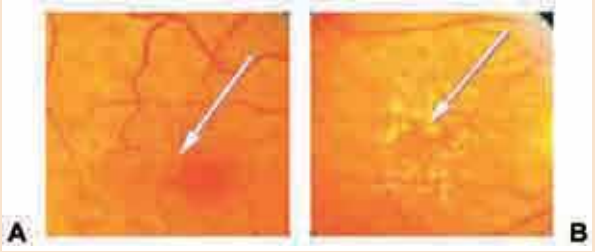

Figure 2. A) Shows medium-size drusen (arrow); B) shows a large drusen (arrow) in a patient with intermediate AMD.

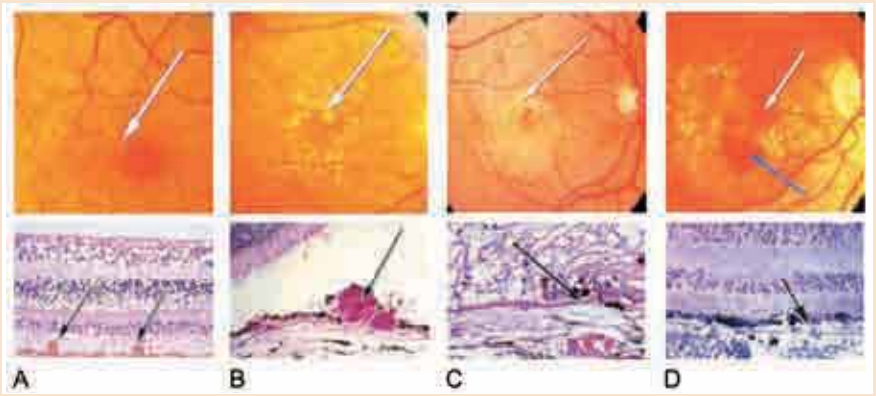

Figure 3. Upper Panel- A) Shows few medium size drusen; B) One large drusen and numerous medium-size drusen; C) Drusen and geographic atrophy extending to the center of the macula; D) Choroidal neovascularization with subretinal fluid, lipid deposition, hemorrhage, retinal pigment epithelium detachment and a fibrotic scar.
Lower Panel A-D Shows corresponding histopathological features.

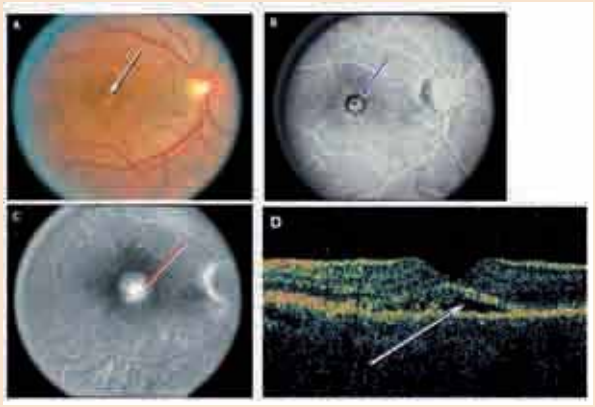

Figure 4. A) Photograph of fundus showing neovascular AMD (arrow). B) Fluorescein angiograms of the same patient with AMD show early and C) late frames reveal early hyperfluorescence (blue arrow) and late leakage (red arrow) consistent with neovascular AMD and choroidal neovascularization. D) An optical coherence tomographic image reveals subretinal fluid (arrow) in a patient with AMD with neovascular AMD.

pigment epithelium from the choroid, fibrotic scars, or a combination of these (1).
In early AMD, a few (< 20) medium-size drusen or retinal pigmentary abnormalities; in intermediate AMD at least one large druse, numerous medium-size drusen, or geographic atrophy that does not extend to the centre of the macula (see Figure 3); are seen. Advanced or late AMD is characterized by the presence of either non-neovascular (also called as dry or non-exudative or atrophic) or neovascular (also called as wet or exudative) drusen and geographic atrophy extending to the centre of the macula and choroidal neovascularization and its sequelae as shown in Figure 4. Intravenous fluorescein angiography or indocyanin green angiography can augment clinical examination by identifying and characterizing choroidal neovascular lesions as shown in Figure 4B and C. Optical coherence tomography, a non-invasive technique, helps to elucidate retinal abnormalities by creating a cross-sectional image of the retina with the use of reflecting light rays as shown in Figures 4D.
Visual loss is generally mild and often asymptomatic in early AMD. However, blurred vision, visual scotomas, decreased contrast sensitivity, and abnormal dark adaptation may occur. Over a period of time, gradual, insidious visual loss with central or peripheral visual scotomas develops. Sometimes, sudden, profound visual loss may occur as a result of subretinal haemorrhage or fluid accumulation secondary to choroidal neovascularization. Although neovascular AMD represents only 10-15 percent of the overall prevalence of AMD, it is responsible for more than 80 percent of severe visual loss or legal blindness (i.e. visual acuity of 20/200 or worse) resulting from AMD (1).

RISK FACTORS FOR AMD

Risk factors for AMD include: advancing age, genetic factors, complement factor H, Tyr402His variant, LOC387715/ARMS2, Ala69Ser variant, a history of smoking within the past 20 years, obesity, high dietary intake of vegetable fat, and low dietary intake of antioxidant and zinc. Increased consumption of n-3 polyunsaturated fatty acids reduces the risk of AMD and diabetic retinopathy.

DIABETIC RETINOPATHY

Diabetic retinopathy affects up to 80 percent of all patients who have had diabetes for 10 years or more. At least 90 percent of these patients could benefit from proper and vigilant treatment and monitoring of the eyes.
Diabetic retinopathy often has no early warning signs, even when macular edema, which can cause vision loss rapidly, is affected. New blood vessels that form a part of proliferative diabetic retinopathy (PDR) can bleed (haemorrhage) and blur vision. Large haemorrhages tend to happen more than once, often during sleep. Fundoscopic examination shows cotton-wool spots, flame haemorrhages, and dot-blot haemorrhages (see Figure 5). Figure 5A shows how the normal fundus picture of macula looks, whereas pictures Figure 5B to 5D reveal various stages of diabetic retinopathy.

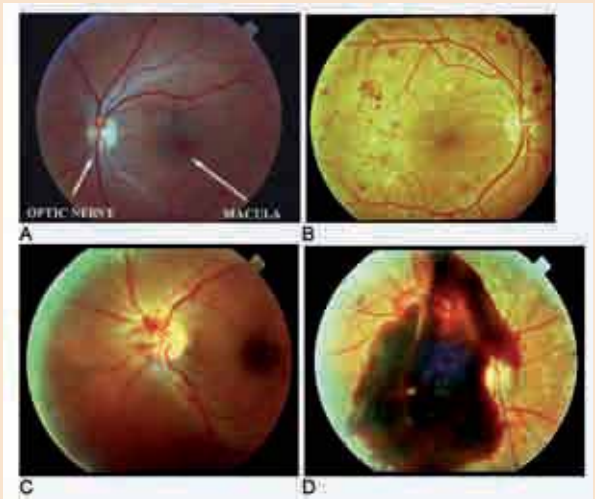

Figure 5. A) Fundus photo of normal macula;. B) Fundus photo of hemorrhages in non-proliferative diabetic retinopathy; C) Fundus photo showing new blood vessel growth (angiogenesis) around optic nerve in proliferative diabetic retinopathy; D) Fundus photo showing hemorrhage from new blood vessel growth in proliferative diabetic retinopathy.

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PATHOGENESIS OF DIABETIC RETINOPATHY

Diabetic retinopathy is the result of microvascular retinal changes. Hyperglycemia-induced pericyte death and thickening of the basement membrane lead to incompetence of the vascular walls, which breaks the blood retinal barrier and renders the retinal blood vessels more permeable. Microaneurysms and intraretinal haemorrhages are the earliest signs of diabetic retinopathy, which are present in nearly all who have had type 1 diabetes mellitus for 20 years and in nearly 80 percent of those with type 2 diabetes mellitus of this duration. As the disease progresses, patients with pre-proliferative retinopathy have an increase in the number and size of intraretinal haemorrhages that may be accompanied by cotton-wool spots, indicating regional failure of the retinal microvascular circulation that results in ischemia. Proliferative diabetic retinopathy involves the formation of new blood vessels (angiogenesis) that develop from the retinal circulation. New vessels can extend into the vitreous cavity of the eye and haemorrhage into the vitreous, resulting in visual loss, and retinal detachments. Late in the course of the disease, new blood vessels may form within the stroma of the iris and may extend, with accompanying fibrosis, into the structures that drain the anterior chamber angle of the eye. This development blocks the outflow of aqueous humour, causing neovascular glaucoma, with elevation of the intraocular pressure. Proliferative retinopathy is higher among those with type 2 diabetes who requires insulin to control their disease and is lower among those who do not. Diabetic macular edema is an important feature of diabetic retinopathy that is due to breakdown of the blood-retinal barrier, which leads to leakage of plasma from small blood vessels in the macula. This causes swelling of the central retina. Resorption of the fluid elements from plasma leads to deposition of its lipid and lipoprotein components, and the formation of hard exudates that frequently leads to severe loss of central vision.

PATHOGENIC MECHANISMS OF DIABETIC RETINOPATHY AND THERAPIES

The exact cause and sequence of events that cause diabetic retinopathy is not clear. But several possibilities have been suggested. These include:

a) Aldose reductase: Increased activity of aldose reductase that occurs in diabetes mellitus enhances the production sorbitol (due to the reduction of glucose) causes osmotic or other cellular damage. But none of the aldose reductase inhibitors tested produced any significant benefits.

b) Inflammation: Increased adherence of leukocytes to capillary endothelium causes inflammation in diabetes mellitus, decreases blood flow and increase in hypoxia resulting in breakdown of blood-retinal barrier and results in macular edema. But, anti-inflammatory drugs such as aspirin did not yield favourable results. Intravitreal injection of triamcinolone, a corticosteroid, either alone or in combination with anti-VEGF antibody is of benefit in diabetic retinopathy but, long-term follow up studies are not yet available. Intravitreal triamcinolone acetonide reduced diabetic macular edema and improved visual acuity compared to the use of bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, suggesting that diabetic macular edema is not only due to excess VEGF but that other mechanisms also operate in this process (2, 3).

c) Protein kinase C, reactive oxygen species, and nonenzymatic glycation of proteins and advanced glycation end products are increased in diabetes mellitus. But, clinical trials with a protein kinase Cβ isoform inhibitor, anti-oxidants, and aminoguanidine, an inhibitor of formation of advanced glycation end products, failed to show favourable results.

d) Inducible form of nitric oxide synthase: Hyperglycemia causes upregulation of inducible nitric oxide synthase (iNOS) that, in turn, enhances free radical formation leading to upregulation of VEGF. Aminoguanidine that suppresses iNOS, failed to prevent diabetic retinopathy.

Vascular endothelial growth factor (VEGF): Human retinal pigment epithelial cells and choroid secrete VEGF and VEGF receptors are present on the inner choriocapillaries. Increased retinal hypoxia and other mechanisms enhance the production of VEGF that induces breakdown of the blood-retinal barrier, leading to macular edema, proliferation of retinal capillary cells and neovascularization. Blocking VEGF synthesis and intravitreal injection of anti-VEGF antibodies, a chimeric fragment of a VEGF membrane receptor bound to an IgM fragment, or antisense VEGF DNA can prevent retinal neovascularization and help in the treatment diabetic retinopathy and macular edema. VEGF binds to its receptors on endothelial cells, triggering angiogenesis. Anti-VEGF therapies for AMD, diabetic retinopathy and retinopathy of prematurity include: monoclonal antibodies such as bevacizumab (Avastin®), antibody derivatives such as ranibizumab (Lucentis®), or orally-available small molecules that inhibit the tyrosine kinases stimulated by VEGF: sunitinib (Sutent®), sorafenib (Nexavar®), axitinib, and pazopanib. Pegaptanib (Macugen®) is a 28-base ribonucleic acid aptamer covalently linked to two branched 20-kD polyethylene glycol moieties that bind and block the activity of extracellular VEGF, specifically the 165-amino acid isoform ($VEGF_{165}$). Aptamers characteristically bind with high specificity and affinity to target molecules. The binding relies on the specific three dimensional conformation of the properly folded aptamer. All these anti-VEGF strategies are of limited benefit in the treatment AMD, diabetic retinopathy and in retinopathy of prematurity (4-6).

Intravitreal ranibizumab ((Lucentis®), a recombinant, humanized, monoclonal antibody Fab that neutralizes all active forms of VEGF-A, showed improvement in visual acuity by 15 or more letters in 24.8 percent of those who received 0.3 mg and 33.8 percent of the 0.5 mg group compared with 5.0 percent of the sham-injection group in a multicenter 2-year, double-blind, sham-controlled study in patients with AMD (8). In a prospective, randomized, double-blind, multicenter, dose-ranging, controlled study in which intravitreal pegaptanib (Macugen®), a 28-base ribonucleic acid aptamer, was used maintained patients visual acuity or gained acuity (33 percent vs. 23 percent) (9). Despite these encouraging results, still ~ 66 percent of patients are in need of better drugs.

Pigment epithelium derived factor (PEDF): PEDF, not only present in retinal pigment epithelial cells but also in several other cells, inhibits neovascularization. In diabetic retinopathy, the levels of PEDF are decreased whereas those of VEGF are increased. Normally, a balance is maintained between VEGF and PEDF that is critical for maintaining the normal anatomy and function of the retinal and choroidal blood vessels. PEDF is important for maintaining the neural architecture of the retina. Intravitreal PEDF and PEDF gene inhibit retinal and choroidal neovascularization.

Growth hormone and insulin-like growth factor-1 (IGF-1): Excess growth hormone may worsen diabetic retinopathy. But, clinical trials with pegvisomant, which blocks growth hormone receptors, did not cause regression of the new

retinal vessels in patients with proliferative diabetic retinopathy, despite a decrease in the plasma levels of IGF-1by 50 percent.

RETINOPATHY OF PREMATURITY

When an infant is born prematurely, the relatively hyperoxic environment the baby is introduced to due to oxygenation therapy shuts down the production of VEGF that delays retinal maturation. Subsequently, at a time when intraocular VEGF levels would normally be declining, abnormally high levels of VEGF are seen due to large areas of avascular retina and associated tissue hypoxia. Hence, anti-VEGF treatment could be of benefit in retinopathy of prematurity (5, 6). Retinopathy of prematurity in children is somewhat similar to AMD and diabetic retinopathy.

Though, the currently available anti-angiogenic therapies are reasonably effective in pathological retinopathy, their use is associated with substantial side effects, in addition to the fact that ~ 60-70 percent is not benefited from their use (5, 6). The side effects include: endophthalmitis, retinal detachment, and when they pass into the systemic circulation could result in hypertension, proteinuria, and increased cardiovascular events and impaired wound healing. Hence, more effective therapeutic measures are needed to treat AMD, diabetic retinopathy and retinopathy of prematurity. It is noteworthy that lipids play a significant role in pathological retinal angiogenesis.

POLYUNSATURATED FATTY ACIDS

Linoleic acid (LA) and α-linolenic acid (ALA) are essential fatty acids (EFAs) that can be converted to their respective metabolites: gamma-linolenic acid (GLA, 18:3, n-6), dihomo-GLA (DGLA, 20:3, n-6) and arachidonic acid (AA, 20:4, n-6); and eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3) respectively by Δ^6 and Δ^5 desaturases. DGLA is the precursor of 1 series of prostaglandins (PGs); AA is the precursor of 2 series of PGs and 4 series of leukotrienes (LTs); and EPA forms the precursor of the 3 series of PGs and the 5 series of LTs. PUFAs, PGs, TXs, and LTs are biologically active and play a role in many diseases (8).

AA, EPA and DHA also give rise to anti-inflammatory molecules: lipoxins (LXs), resolvins, and protectins. Thus, PUFAs form precursors to both pro- and anti-inflammatory molecules and the balance between these mutually antagonistic compounds could determine the final outcome of the disease process (see Figure 6 for metabolism of essential fatty acids).

LIPOXINS, RESOLVINS, AND PROTECTINS

Aspirin converts AA, EPA and DHA to form aspirin-triggered 15 epimer LXs (ATLs) that are potent inhibitors of acute inflammation (9, 10). Deficiency or absence of LXs leads to interaction between PMN and endothelial cells as a result of which endothelial damage occurs. Compounds similar to 15R-HETE and 15-epimeric LXs are also formed from EPA and DHA.

DHA can be converted to 17R series of hydroxy DHAs (HDHAs) that, in turn, is converted enzymatically by PMNs to di- and tri-hydroxy containing docosanoids (15). Similar compounds (similar to HDHAs) are generated from AA and EPA. These compounds have potent anti-inflammatory actions and induce resolution of the inflammatory process and hence are called “resolvins”. Resolvins inhibited cytokine generation, leukocyte recruitment, leukocyte

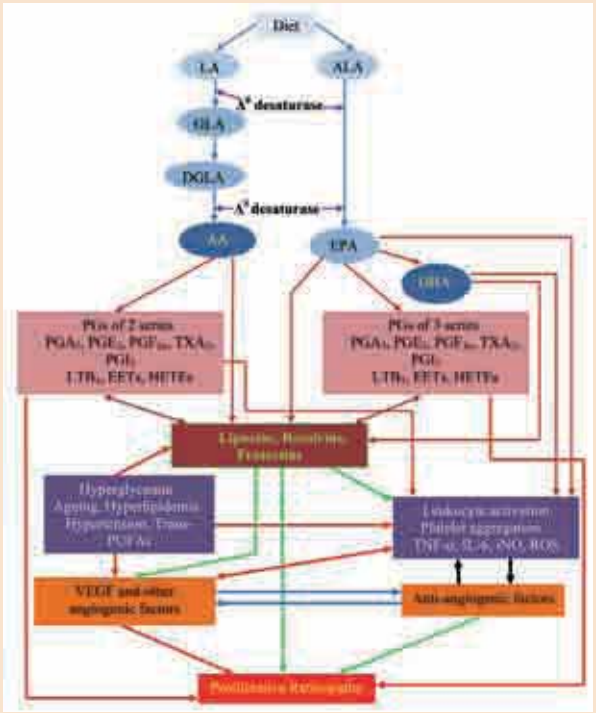

Figure 6. Metabolism of essential fatty acids and their role in proliferative retinopathy

diapedesis, exudate formation, and inhibited brain ischemia-reperfusion injury (9, 10).

Of the several 17-hydroxy-containing bioactive mediators derived from DHA, 17S series resolvins, 10,17S-dihydroxydocosatriene is termed as neuroprotectin D1 (NPD1) that reduced infiltration of PMNs, showed anti-inflammatory and neuroprotective properties. NPD1 inhibited oxidative stress-induced apoptosis of human retinal pigment epithelial cells (10). LXs and NPD1 enhanced wound healing, and promoted brain cell survival via the induction of antiapoptotic and neuroprotective gene-expression programs. Since lipoxins, resolvins and protectins (NPD1 is one of the protectins) have neuroprotective actions, it is likely that these compounds may also prevent pathological retinopathy (see Figure 7 for the metabolism of lipoxins, resolvins and protectins).

PUFAs AND RETINA

Human retina is rich in DHA, EPA, and AA. In the retina, phospholipids account for almost 80 percent of total lipids. DHA represents ~50 percent of total fatty acids in the photoreceptor outer segments, and plays a major role in membrane function, in visual process by affecting permeability, fluidity, thickness and the activation of membrane bound proteins. PUFAs of n-3 series protect retinal vascular and immuno-regulatory processes, maintain the physiologic redox balance, and facilitate cell survival. AA, EPA, DHA and their products regulate the production and action of VEGF and other angiogenic factors, matrix

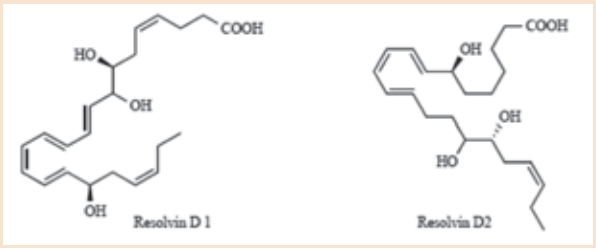

Figure 7a. Structure of resolvins

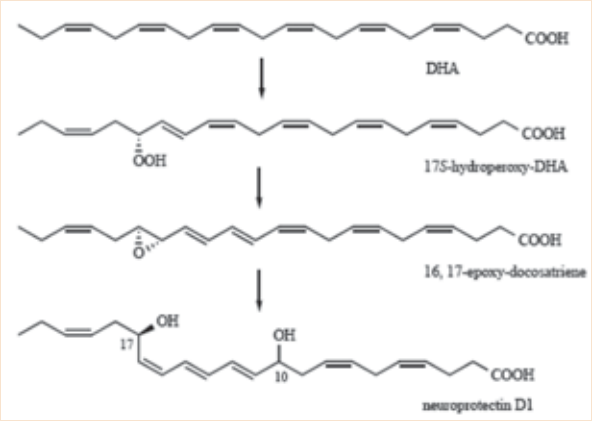

Figure 7b. Formation of neuroprotectin D1 from DHA

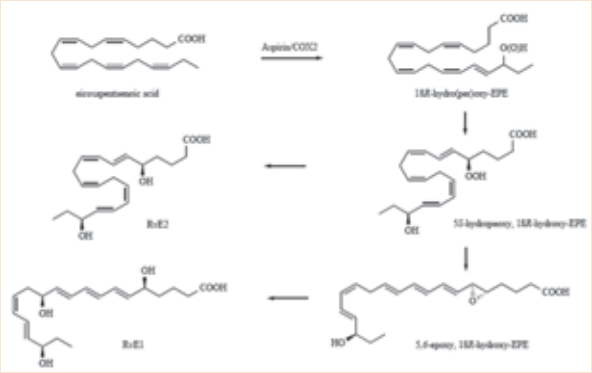

Figure 7c. Formation of resolvin E1 from EPA

metalloproteinases, reactive oxygen species, neurotransmitters and pro-inflammatory cytokines.

EPA attenuated VEGF- induced proliferation of endothelial cells and improved hypoxia-reoxygenation-induced endothelial dysfunction, a mechanism by which PUFAs could prevent diabetic retinopathy (11). Lipoxin A_4 is a potent inhibitor of VEGF-induced angiogenesis and lipoxins prevented hypoxia-induced proliferative retinopathy in experimental animals (11). These results suggest that lipoxins, resolvins and protectins in the retinal vasculature prevent diabetic retinopathy (see Figure 6). It is likely that when the levels of EPA/DHA/AA in the retina and vascular endothelial cells are low, formation of lipoxins, resolvins and protectins could be inadequate to prevent proliferative diabetic retinopathy. Hence, supplementation of adequate amounts of PUFAs could increase the formation of lipoxins, resolvins and protectins that, in turn, inhibit the formation of VEGF and suppress or arrest of proliferative retinopathy in age-related macular degeneration (AMD), retinopathy of premature and diabetic retinopathy. This implies that one of the natural functions of PUFAs and their products lipoxins, resolvins and protectins is to prevent retinal cell damage, regulate endothelial proliferation and the expression of VEGF and other growth factors to prevent inappropriate vascular development and thus, prevent pathological retinal angiogenesis.

TRANS-PUFAS IN PATHOLOGICAL RETINOPATHY

It is believed that modification of lipids by reactive oxygen species and NO are byproducts of cellular respiratory metabolism. But, it is now evident that these modified lipids have specific function in the regulation of vascular regeneration. For instance, under oxidative stress, trans-AA metabolites (these include: 5E-, 8E-, 11E-, and 14E-AA, see Figure 8) are formed that mediate apoptosis of microvascular cells resulting in retinal microvascular degeneration in vivo (12). These trans-AAs (trans-arachidonic acids) are major products of $NO_2^{\bullet}$- mediated isomerization of AA within the cell membrane. Trans-AA metabolites have a variety of biological actions and can be measured in the human plasma. In experimental animals, injection of exogenous TAAs (trans-AAs) into the eye correlated with increased loss of retinal vessels. TAAs induce the formation of thrombospondin-1, which binds to its receptor CD36 on vascular endothelial cells to initiate apoptosis of endothelial cells and subsequent regression of blood vessels. Thus, nitrosative stress and formation of TAAs contribute to oxygen-induced retinopathy. Since increased generation of free radicals and nitrosative stress occurs in diabetic retinopathy and retinopathy of prematurity, it is likely that TAAs play a role in these conditions. It is not known whether trans-EPAs and trans-DHAs are formed due to $NO_2^{\bullet}$- mediated isomerization, and if so what is their biological activity.

Several other proteins such as semaphorins, ephrins, Slit proteins, and Notch signalling pathway also participate in vessel formation. Of all, Roundabout 4 (Robo4)-Slit2 signalling axis is interesting since it counteracts the effects of VEGF (13). It is not known whether TAAs, PUFAs, and lipoxins, resolvins, and protectins have a regulatory role in the expression and function of these proteins and thus, modulate angiogenesis. The possibility is that they do. But this remains to be established.

CONCLUSIONS

Though the currently available anti-angiogenic therapies such as pegaptanib sodium (Macugen®), or ranibizumab (Lucentis®) and bevacizumab (Avastin®) are reasonably effective in the treatment of AMD, diabetic retinopathy and retinopathy of prematurity, their use is associated with substantial side effects, in addition to the fact that significant number of patients are not benefited from their use (14, 15).

As discussed above, PUFAs, lipoxins, resolvins and protectins play a major role in pathological retinopathy (see Figures 6). Under oxidative stress, trans-AA metabolites and possibly, trans-EPA and trans-DHA metabolites are formed that contribute to oxygen-induced retinopathy. These TAAs, trans-EPAs and trans-DHAs are formed from cis-PUFAs present in the cell membranes implying that their increased formation results in deficiency of cis-PUFAs that, in turn, could lead a deficiency of lipoxins, resolvins and protectins. These lipids have a modulatory influence on the production and actions of VEGF, TNF (tumour necrosis

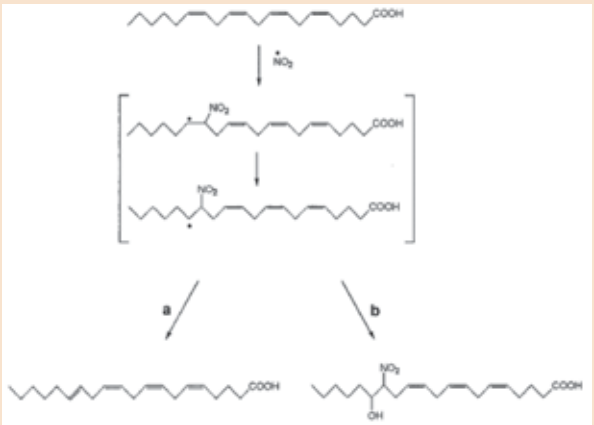

Figure 8. Scheme showing the mechanism for a nitrogen dioxide-mediated formation of 14-trans-arachidonic acid (a) and 14-nitro, 15-hydroxyeicosatrienoic acid (b) from cis-arachidonic acid

factor), NO, angiogenesis, and wound healing, and thus, play a role in pathological retinopathy. Lipoxins, resolvins and protectins have potent actions at pico and nanomolar concentrations and can be injected intravitreally to study their beneficial actions in various forms of retinopathy. It will be interesting to study the effect of various PUFAs, lipoxins, resolvins, and protectins on gene expression profiling focusing on growth factors (including VEGF), their receptors and genes that are participating in inflammatory and oxidative stress responses that play a role in pathological retinal angiogenesis. Such studies could give valuable information that may lead to the development of specific thrapeutic strategies in various retinpathies.

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