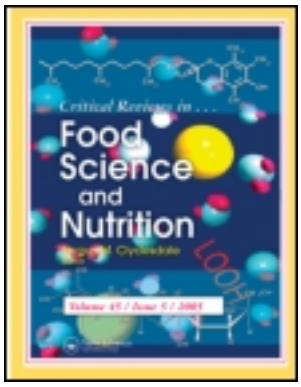

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Bioactive Lipids in Pathological Retinopathy

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Bioactive Lipids in Pathological Retinopathy

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Diabetic retinopathy is a common condition that occurs in patients with diabetes with long-standing hyperglycemia that is characterized by inappropriate angiogenesis. This pathological angiogenesis could be a sort of physiological proliferative response to injury by the endothelium. Recent studies suggested that reactive oxygen species (ROS) play a significant role in this angiogenesis. Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor that plays a significant role in diabetic retinopathy. The interaction between VEGF and ROS, and theirs in turn with pro- and anti-inflammatory cytokines and anti-inflammatory bioactive lipid molecules such as lipoxins, resolvins, protectins, and maresins is particularly relevant to understand the pathophysiology of diabetic retinopathy and develop future therapeutic interventions.

Keywords Reactive oxygen species, vascular endothelial growth factor, angiogenesis, diabetic retinopathy, lipoxins, resolvins, cytokines, inflammation

INTRODUCTION

Diabetic retinopathy is a common dreaded complication of long-standing uncontrolled hyperglycemia and is characterized by proliferative response of the endothelium to glucose-induced toxicity. Diabetic proliferative retinopathy not only involves proliferation of endothelial cells (ECs) but also includes migration of smooth muscle cells and neoangiogenesis (Brownlee, 2001; Stone and Collins, 2002). Angiogenesis, a process that involves growth of new blood vessels, is also seen in several other diseases such as cancer, atherosclerosis, rheumatoid arthritis, psoriasis, and other inflammatory conditions, in addition to its significant role in diabetic retinopathy. Large amounts of reactive oxygen species (ROS) are toxic and can cause cell death, while low concentrations produced during ischemic or anesthetic preconditioning serve as intracellular signaling molecules to induce repair mechanisms against tissue injury (Nishikawa et al., 2000; Kowluru et al., 2001; Du et al., 2003; Takaishi et al., 2003; Ushio-Fukai and Alexander, 2004; Son, 2007; Fukuoka et al., 2010). Recent studies suggested that ROS play an important role in angiogenesis.

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For neoangiogenesis to occur, elaboration of certain growth factors is necessary. One such important growth factor that plays a significant role in angiogenesis is vascular endothelial growth factor (VEGF). But, VEGF alone is not sufficient for the initiation and progression of pathological angiogenesis, since anti-VEGF antibodies are not very effective in the management of diabetic retinopathy, cancer, and other conditions wherein inappropriate angiogenesis occurs. This suggests that there could be a role for other growth factors and bioactive molecules in pathological angiogenesis. Angiogenesis is essential for the growth of the fetus and for appropriate repair process to occur in response to injury and inflammation. This physiological angiogenesis that goes awry in diseases such as rheumatoid arthritis, lupus, psoriasis, and cancer such that the repair process is impaired ultimately interferes with the structure and function of vital tissues and organs. Hence, understanding the physiological angiogenesis and various factors that regulate it is important to devise newer therapeutic strategies, both for diabetic retinopathy and other diseases in which abnormal angiogenesis occurs.

THE SOURCES OF ROS

ROS include superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$). Of all, H_2O_2 is the only one with

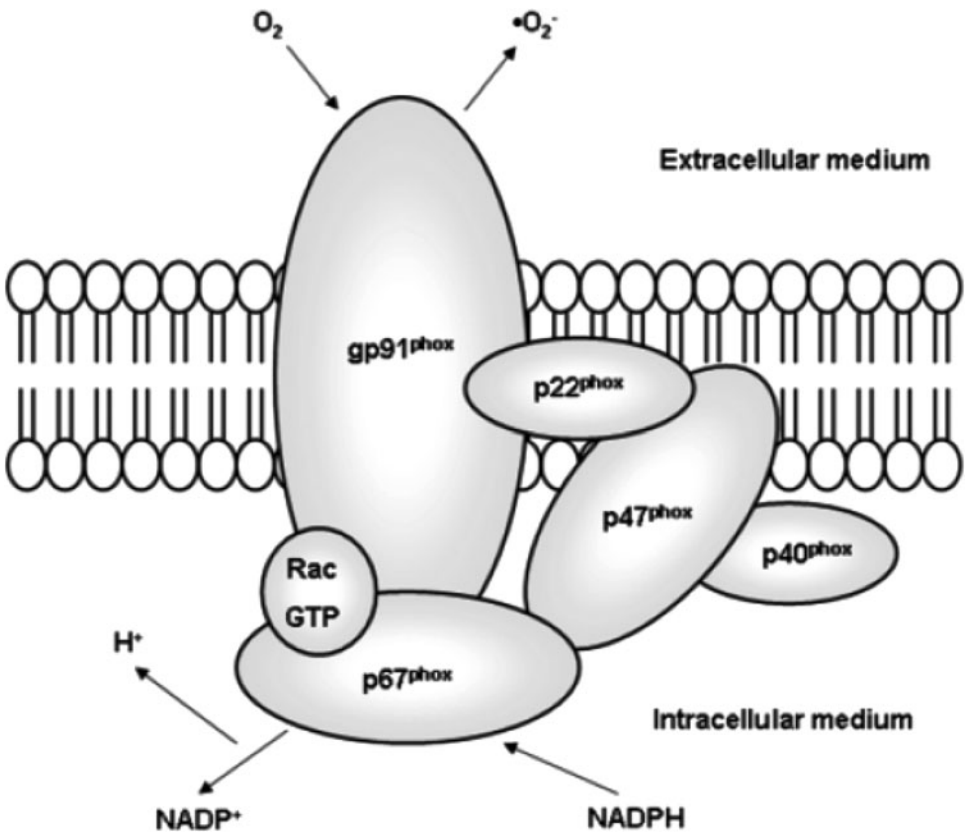

Figure 1 Structure of phagocytic NADPH oxidase. The gp91phox is the NADPH binder with electron transport function in active NADPH oxidase. The extracellular production of $O_2^{-\cdot}$ occurs by reduction of an electron of O_2 by gp91phox, using β-reduced NADPH.

the chemical stability required to establish significant steady-state concentrations in vivo. It has the added advantage of being small and uncharged, allowing it to freely diffuse across plasma membranes (Stone and Collins, 2002).

There are several enzymatic sources of ROS in mammalian cells that include: the mitochondrial electron transport system, xanthine oxidase, the cytochrome p450, the NAD(P)H oxidase(s), and nitric oxide (NO) synthase (NOS). The major source of ROS in ECs is NAD(P)H oxidase. EC express all the components of phagocytic NAD(P)H oxidase, including gp91phox, p22phox, p47phox, p67phox, and the small G protein Rac1.The subunit composition of the related phagocytic enzyme is well studied (Ushio-Fukai and Alexander, 2004) (Figure 1).

The catalytic component of this multisubunit complex is a membrane-bound heterodimeric flavohemoprotein named cytochrome b558 that is composed of a 91-kDa glycoprotein named gp91phox and a smaller 22-kDa protein named p22phox (Stone and Collins, 2002).

On stimulation, the cytosolic components translocate, guided by Rac1, to the plasma membrane to enable electron transfer from NAD(P)H to O_2, thereby generating O_2^-. The gp91phox and p22phox form the electron transfer components of the oxidase, and p47phox and p67phox are the cytosolic components that interact with these two proteins (gp91phox and p22phox) to modulate NAD(P)H oxidase activity (Ushio-Fukai and Alexander, 2004). The low-molecular-weight G protein rac also serves a regulatory function. The vascular NAD(P)H oxidase(s) continuously produce low levels of $O_2^{-\cdot}$ in unstimulated cells, yet it can be further stimulated acutely by various agonists and growth factors. $O_2^{-\cdot}$ dismutates to form H_2O_2, which can occur in a nonenzymatic manner. Dismutation can also be accomplished enzymatically by the superoxide dismutases (SODs) (Stone and Collins, 2002) (Figure 2).

Abnormal angiogenesis that occurs in patients with diabetes in the form of proliferative diabetic retinopathy and in patients with cancer that drives the growth of the tumor needs appropriate intervention. On the other hand, angiogenesis is needed for those with coronary heart disease and peripheral vascular disease to improve cardiac function and claudication. In this context, the role of free radicals and their interaction with angiogenic factors such as VEGF deserves special attention.

VEGF AND VEGFR2 (FLK1/KDR)

VEGF is a potent angiogenic factor that stimulates proliferation, migration, and tube formation of ECs and angiogenesis in vivo (Rakoczy et al., 2003; Zachary, 2003; Hoeben et al., 2004; Ray et al., 2004; Suganami et al., 2004; Takagi et al., 2004; Al-Kateb et al., 2007; Huang and Sheibani, 2008; Biscetti et al., 2008; Kakehashi et al., 2008). VEGF binds to two tyrosine kinase (TK) receptors, VEGF receptor-1 (VEGFR1, also termed

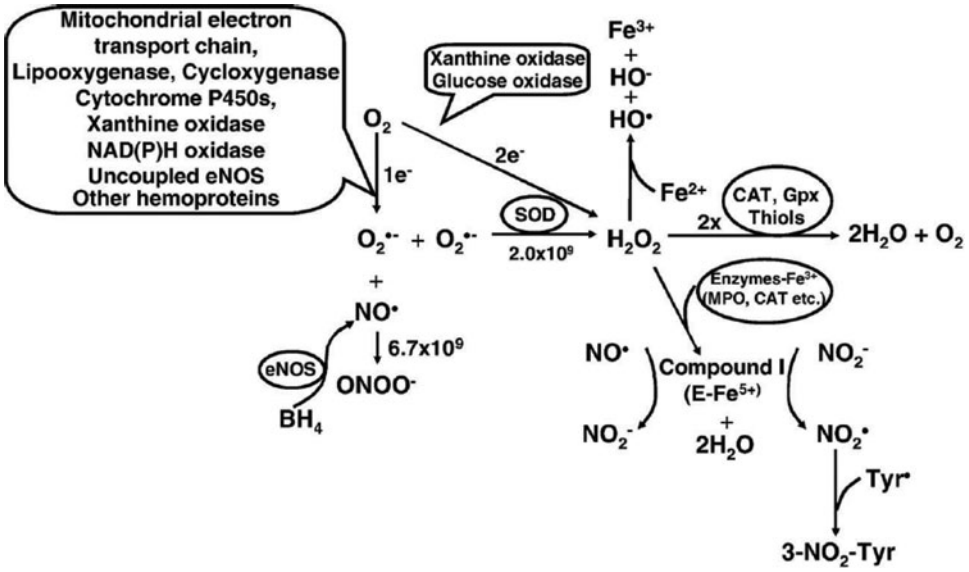

Figure 2 Superoxide dismutation and formation of H_2O_2. Spontaneous or SOD (superoxide dismutase)-catalyzed (at the reaction speed of 2.0×10^9 mol/L^{-1}.s^{-1}) dismutation of $O_2^{-\cdot}$ leads to the formation of H_2O_2. Besides directly serving as a signaling intermediate, H_2O_2 also exerts its biological effects via metabolites such as hydroxyl radical ($HO^{\cdot}$) or compound I (product of H_2O_2 oxidation of Fe^{3+}-containing enzymes such as myeloperoxidase, MPO). $O_2^{-\cdot}$ rapidly scavenges $NO^{\cdot}$ at the reaction speed of 6.7×10^9 mol/L^{-1}.s^{-1}, representing one of the mechanisms whereby bioactive $NO^{\cdot}$ diminishes independent of regulation of $NO^{\cdot}$ synthase.

Flt-1) and VEGFR2 (also termed KDR/Flk1) in ECs (Ushio-Fukai and Alexander, 2004) (Table 1). Both receptors are necessary for normal mouse development, but relevant differences have been identified in their signaling properties: stimulation of Flt-1 being predominantly linked to cell migration, while Flk-1/KDR receptor activation is associated with both EC migration and proliferation and is followed, unlike for Flt-1, by the activation of the mitogen-activated protein kinase cascade (Colavitti et al., 2002; Cross et al., 2003; Hoeben et al., 2004; Lamalice et al., 2004; Takahashi and Shibuya, 2005). VEGF binding initiates tyrosine phosphorylation of KDR, which results in activation of downstream signaling enzymes, including ERK1/2, Akt, and endothelial NOS (eNOS), which contribute to angiogenic-related responses in EC (Shu et al., 2002; Zachary, 2003; Munoz-Chapuli et al., 2004; Huang and Sheibani, 2008).

INTERACTION BETWEEN ROS AND VEGF

A strong correlation between ROS production with neovascularization and VEGF expression in the pathobiology of diabetic retinopathy has been reported (Zheng et al., 2009). VEGF stimulates ROS production through activation of an endothelial NAD(P)H oxidase, and ROS seems to be involved in VEGF signaling, linking it to angiogenic responses such as migration and proliferation of ECs (Ushio-Fukai and Alexander, 2004; Xia et al., 2007; Whiteside et al., 2009). VEGF causes translocation of p47phox to membrane ruffles through its association with WAVE1 in ECs. In HUVECs, VEGF stimulates $O_2^{-\cdot}$ production, which is inhibited to a significant degree by either overexpression of dominant-negative Rac1 and gp91phox antisense oligonucleotides. Both Rac1 and gp91phox are functional $O_2^{-\cdot}$-generating NAD(P)H oxidase components in ECs. In unstimulated ECs, NAD(P)H oxidase components exist as preassembled complexes in a predominantly perinuclear location associated with the intracellular cytoskeleton.

PROXIMAL MOLECULAR TARGETS OF VEGF

In-Vitro Studies

VEGFR2 is one of the proximal molecular targets of ROS derived from the gp91phox-containing NAD(P)H oxidase in cultured ECs. VEGF-induced VEGFR2 autophosphorylation is inhibited by thiol antioxidant NAC (N-acetylcysteine), various NAD(P)H oxidase inhibitors, and dominant-negative Rac1 and gp91phox antisense oligonucleotides. It was noted that $O_2^{-\cdot}$

Table 1 The receptors of VEGF

			Different signaling properties	
	Term	Function	Migration	Proliferation
VEGFR1	Flt-1	Necessary for normal development	√ predominantly	×
VEGFR2	KDR/Flk1		√ endothelial cell	√

radical formation in HUVECs by VEGF involves Rac1 and gp91phox. The increase in $O_2^{-\cdot}$ production that occurs as a result of VEGF stimulation is inhibited by either overexpression of dominant-negative Rac1 or gp91phox antisense oligonucleotides (Ushio-Fukai et al., 2002). These results are supported by the fact that dominant-negative mutant of the small GTPase Rac-1, a molecule crucial for both ligand-induced generation of ROS and lipoxygenase activation in several cellular models, inhibited VEGF-induced oxidative burst when overexpressed by transient transfection in PAE cells (Colavitti et al., 2002), suggesting a role for Rac-1 in transducing redox signals by KDR.

In-Vivo Studies

When the role of gp91phox-containing NAD(P)H oxidase in angiogenesis in the mouse sponge implant model in vivo was tested, it was noted that VEGF enhanced angiogenesis by a free-radical-dependent process (Ushio-Fukai et al., 2002).

FEEDBACK REGULATION BETWEEN ROS-VEGF

ROS derived from Rac1-dependent, gp91phox-containing NAD(P)H oxidase produced after VEGFR2 engagement may form a feedback loop that, through the inhibition of PTPs (protein tyrosine phosphatases), enhances and sustains VEGFR2 phosphorylation and subsequent ROS-dependent signaling to enhance angiogenesis, which includes EC proliferation and migration (Ushio-Fukai and Alexander, 2004).

VEGF binding to VEGFR2 leads to the activation and translocation of the small GTPase Rac1 (Lamalice et al., 2004) into the plasma membrane, which, in turn, stimulates gp91phox-containing NAD(P)H oxidase in ECs. It is likely that ROS derived from NAD(P)H oxidase may oxidize and inactivate PTPs, which may negatively regulate VEGFR2, thereby enhancing and sustaining VEGFR2 phosphorylation and subsequent ROS-dependent signaling linked to angiogenesis. Several PTPs have been shown to be associated with or inhibit VEGFR2 action that may directly target ROS induced by VEGF. This leads to the activation of diverse downstream signaling pathways such as mitogen-activated protein kinases (MAPKs), Akt/protein kinase B, and eNOS, which are essential for VEGF-induced EC migration and proliferation (Shu et al., 2002; Zachary, 2003; Munoz-Chapuli et al., 2004; Huang and Sheibani, 2008).

ANTI-VEGF THERAPIES FOR PATHOLOGICAL RETINAL ANGIOGENESIS

It is evident from the preceding discussion that VEGF plays a significant role in diabetic retinopathy, age-related macular degeneration (AMD), and retinopathy of prematurity. These evidences led to the employment of anti-VEGF antibodies such as bevacizumab (Avastin®), antibody derivatives such as ranibizumab (Lucentis®), and orally active small molecules that inhibit TKs stimulated by VEGF such as sunitinib (Sutent®), sorafenib (Nexavar®), axitinib, and pazopanib in diabetic retinopathy and AMD (Moshfeghi et al. 2002; Goldberg et al. 2005; Jorge et al., 2006; Antoniak and Nowak, 2007; Cohen et al. 2007).

Despite the use of these monoclonal antibodies for diabetic retinopathy and AMD, the outcomes of treatment of these conditions have been less than satisfactory. For example, ranibizumab (Lucentis®) is a recombinant, humanized, monoclonal antibody Fab that neutralizes all active forms of VEGF-A which showed improvement in visual acuity by 15 or more letters when given intravitreally in 24.8% of those who received 0.3 mg and 33.8% of the 0.5-mg group compared with 5.0% of the sham-injected group in a multicenter two-year, double-blind, sham-controlled study in patients with AMD (Rosenfeld et al., 2006). A prospective, randomized, double-blind, multicenter, dose-ranging, controlled study in which pegaptanib (Macugen®), a 28-base ribonucleic acid aptamer, was injected intravitreally maintained their visual acuity or gained acuity (33% vs. 23%) (Gragoudas et al., 2004). Despite these encouraging results, a substantial number of patients (∼66%) remain without any benefit.

Intravitreal anti-VEGF therapy is currently being used in the treatment of proliferative diabetic retinopathy and diabetic macular edema in clinical practice. Intravitreal injection is an effective means of delivering anti-VEGF drugs to the retina, though it is an invasive procedure associated with potentially serious complications, such as endophthalmitis or retinal detachment. In addition, although delivered within the vitreous, anti-VEGF drugs could pass into the systemic circulation, which could potentially result in hypertension, proteinuria, increased cardiovascular events, and impaired wound healing. Pegaptanib, ranibizumab, and bevacizumab are being tried in diabetic retinopathy. These drugs not only have limited efficacy, but also could have systemic adverse effects in a high-risk population such as diabetic patients. This suggests that better strategies and/or drugs are needed both in the prevention and treatment of AMD, diabetic retinopathy, and other related conditions such as retinopathy of prematurity in children.

Retinopathy of prematurity is initiated by hyperoxia-induced obliteration of newly formed blood vessels in the retina of the premature newborn as a consequence of hyperoxia-induced shutoff of VEGF production by neuroglial cells (Alon et al., 1995) that leads to selective apoptosis of ECs. This premature apoptosis of ECs leads to hypoxia of the developing retina, which, in turn, leads to the production of abnormally high levels of VEGF due to large areas of avascular retina and associated tissue hypoxia. Hence, anti-VEGF therapy is useful in the treatment of retinopathy of prematurity (Hellstrom et al., 2001).

CURRENT ANTIANGIOGENIC THERAPIES ARE NOT EFFECTIVE FOR PATHOLOGICAL RETINOPATHY

Though antiangiogenic drugs such as Macugen® or Lucentis® and Avastin® are reasonably effective in the

treatment of AMD, diabetic retinopathy, and retinopathy of prematurity, there could occur significant side effects, in addition to the fact that substantial number of patients (∼60–70%) are not benefited from their use (Gragoudas et al., 2004; Rosenfeld et al., 2006). These side effects due to antiangiogenic drugs: Macugen®, Lucentis®, and Avastin® include endophthalmitis and retinal detachment, and when they pass into the systemic circulation, could result in hypertension, proteinuria, increased cardiovascular events, and impaired wound healing. Hence, newer therapeutic strategies are needed for the management of AMD, diabetic retinopathy, and retinopathy of prematurity. In this context, it is noteworthy that lipids play a significant role in pathological retinal angiogenesis.

METABOLISM OF ESSENTIAL FATTY ACIDS

Mammals cannot synthesize essential fatty acids (EFAs): linoleic acid (LA, 18:2, n–6) and α-linolenic acid (ALA, 18:3, n–3) and hence, have to be obtained from diet. Both LA and ALA are metabolized by the same set of enzymes. LA is converted to γ-linolenic acid (GLA, 18:3, n–6) by the action of the enzyme Δ^6 desaturase (d-6-d) and GLA is elongated to form dihomo-GLA (DGLA, 20:3, n–6), the precursor of the 1 series of prostaglandins (PGs). DGLA can also be converted to arachidonic acid (AA, 20:4, n–6) by the action of the enzyme Δ^5 desaturase (d-5-d). AA forms the precursor of the two series of PGs and thromboxanes, and the four series of leukotrienes (LTs). ALA is converted to eicosapentaenoic acid (EPA, 20:5, n–3) by Δ^6 and Δ^5 desaturases. EPA forms the precursor of the three series of PGs and the five series of LTs (see Figure 7 for metabolism of EFAs). AA and EPA also are converted to their respective LTs. EPA can give rise to docosahexaenoic acid (DHA, 22:6 n–3), while DHA can be retroconverted to EPA. PGs, TXs, and LTs are biologically active and play a significant role in inflammatory diseases such as atherosclerosis, bronchial asthma, inflammatory bowel disease, rheumatoid arthritis, lupus, and other conditions (Das, 2002; Das, 2006a, 2006b, 2010, 2011).

AA, EPA and DHA give rise to anti-inflammatory molecules: lipoxins (LXs), resolvins (RSVs), protectins (PTs), and maresins (MSs) that suppress inflammation, and enhance wound healing and repair process. 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 3 for the metabolism of essential fatty acids).

Saturated fats, cholesterol, trans-fatty acids, alcohol, adrenaline, glucocorticoids, pyridoxine, zinc, nicotinic acid, magnesium, insulin, ageing, oncogenic viruses, radiation, fasting, protein deficiency, glucose-rich diet, and partial caloric restriction are some of the factors that modulate the activity of enzymes Δ^6 and Δ^5 desaturases (reviewed in Das, 2002, 2006a, 2006b, 2010, 2011). Activities of Δ^6 and Δ^5 desaturases are decreased in insulin resistance, diabetes mellitus, hypertension, hyperlipidemia, metabolic syndrome, Alzheimer's disease, and ageing, which may be responsible for the reduced formation of anti-inflammatory LXs, RSVs, PTs, and MSs in these diseases. Trans-fats interfere with the metabolism of EFAs and decrease the formation of LXs, RSVs, PTs, and MSs and thus promote inflammation, atherosclerosis, coronary heart disease, and pathological retinopathy (Brenner, 1982; Das, 2002; Seddon et al., 2003; Mozaffarian et al., 2004; Kermonant-Duchemin, 2005; Lopez-Garcia et al., 2005; Das, 2006a, 2006b, 2010, 2011). Several PUFAs, especially EPA and DHA and LXs, RSVs, and PTs inhibit the production of proinflammatory cytokines: interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-1, and IL-2 (Das, 2002; Das, 2006a, 2006b; Das, 2010; Das, 2011). These data indicate that trans-fats, saturated fats, and cholesterol have proinflammatory actions, whereas PUFAs possess anti-inflammatory properties.

ANTI-INFLAMMATORY LXs, RSVs, AND PTs, AND THEIR ROLE IN PATHOLOGICAL RETINOPATHY

Aspirin converts AA, EPA, and DHA to form aspirin-triggered 15 epimer LXs (ATLs) that are potent inhibitors of acute inflammation (Brenner, 1982; Claria and Serhan, 1995; Chiang et al., 1999; Serhan et al., 2000b; Das, 2002; Mozaffarian et al., 2004; Lopez-Garcia et al., 2005; Das, 2006a, 2006b; Das, 2010; Das, 2011). Acetylation of COX-2 by aspirin prevents the formation of prostanoids, but the acetylated enzyme remains active in situ to generate 15*R*-hydroxy-eicosatetraenoic acid (15R-HETE) from AA, which is released and converted by activated PMNs to the 15-epimeric LXs (Claria and Serhan, 1995; Chiang et al., 1999; Serhan et al., 2000a). This interaction between ECs and PMNs leads to the formation of 15R-HETE and its subsequent conversion to 15-epimeric LXs by aspirin-acetylated COX-2 (Claria and Serhan, 1995; Chiang et al., 1999; Serhan et al., 2000a). ECs oxidize AA (and possibly EPA and DHA) via P450 enzyme system to form 11,12-epoxy-eicosatetraenoic acid(s) that blocks EC activation, suggesting that COX-2 enzyme is essential for the formation of LXs. Deficiency or absence of LXs leads to interaction between PMN and ECs that results in the initiation, progression, and persistence of inflammation.

Compounds similar to 15R-HETE and 15-epimeric LXs are also formed from EPA and DHA. Thus, EPA is converted to 18R-HEPE (18R- hydroxy-eicosapentaenoic acid), 18-HEPE, and 15R-HEPE. Activated human PMNs, in turn, convert 18R-HEPE to 5,12,18R-triHEPE and 15R-HEPE to 15-epi-LXA_5 by 5-lipoxygenase. Both 18R-HEPE and 5,12,18R-triHEPE inhibited LTB_4-stimulated PMN transendothelial migration similar to 15-epiLXA_4. 5,12,18R-triHEPE competed with LTB_4 for its receptors and inhibited PMN infiltration, and thus, 5,12,18R-triHEPE suppresses LT-mediated responses when present at the sites of inflammation (Brenner, 1982; Claria and Serhan,

1995; Chiang et al., 1999; Serhan et al., 2000a; Das, 2002; Mozaffarian et al., 2004; Lopez-Garcia et al., 2005; Das, 2006a, 2006b, 2010, 2011).

Murine brain cells transform enzymatically DHA to 17*R* series of hydroxy DHAs (HDHAs), which, in turn, is converted enzymatically by PMNs to di- and tri-hydroxy-containing docosanoids (Hong et al. 2003b; Marcheselli et al. 2003; Mukherjee et al., 2004). Similar small-molecular-weight compounds (similar to HDHAs) are generated from AA and EPA. Thus, 15*R*-hydroxy-containing compounds are formed from AA, 18*R* series from EPA, and 17*R*-hydroxy series from DHA that have potent anti-inflammatory actions and induce resolution of the inflammatory process and hence are called "resolvins." Resolvins (RSVs) inhibit cytokine generation, leukocyte recruitment, leukocyte diapedesis, and exudate formation. AA-, EPA-, and DHA-derived RSVs from acetylated COX-2 are formed due to communication between ECs and PMNs. RSVs inhibit brain ischemia–reperfusion injury (Hong et al., 2003b). Thus, LXs and RSVs have cytoprotective actions.

Of the several 17-hydroxy-containing bioactive mediators derived from DHA that were termed docosatrienes and 17*S* series RSVs, 10,17*S*-dihydroxydocosatriene, termed as neuroprotectin D1 (NPD1) (nowadays, this and other similar compounds are also termed as "protectins") that reduced infiltration of PMNs, showed anti-inflammatory and neuroprotective properties (Claria and Serhan, 1995; Chiang et al., 1999; Serhan et al., 2000a). NPD1 inhibited oxidative stress-induced apoptosis of human retinal pigment epithelial cells (Mukherjee et al., 2004). Both LXs and NPD1 enhanced wound healing (Gronert et al., 2005) and promoted brain cell survival via the induction of antiapoptotic and neuroprotective gene expression programs (Calon et al., 2004; Lukiw et al., 2005; Das, 2008a).

VEGF AND PUFAS

PUFAs are present in large amounts in the retina and other ocular tissues and the brain. It is known that EPA inhibits the abnormal gap junctional intercellular communication (GJIC) induced by hypoxia/reoxygenation (H/R) via suppressing TK activation (Zhang et al. 1999). EPA protected against VEGF-induced reduction in GJIC and phosphorylation of connexin (Zhang et al., 2002). EPA attenuated VEGF-induced proliferation of ECs (Yang et al., 1998) and improved H/R-induced endothelial dysfunction through inhibition of TK activation (Morita et al., 2001), a mechanism by which PUFAs could protect against the development and progression of diabetic retinopathy. Aspirin-triggered LX stable analog 15-epi-16-(para-fluoro)-phenoxy-lipoxin A_4 is a potent inhibitor of VEGF-induced angiogenesis (Fierro et al., 2002), and LXs prevent hypoxia-induced proliferative retinopathy in experimental animals (Connor et al., 2007). These results suggest that increased intake of PUFAs prevents the development and progression of diabetic retinopathy, AMD, and retinopathy of prematurity by enhancing the formation of LXs, RSVs, and PTs in the retinal vasculature (see Figure 3).

INFLAMMATION PLAYS A ROLE IN PATHOLOGICAL RETINAL ANGIOGENESIS

Complement activation and inflammation have a significant role in the pathogenesis of AMD and, possibly, diabetic retinopathy and retinopathy of prematurity (Edwards et al., 2005; Haines et al., 2005; Haines et al., 2006). Since complement pathway is involved in inflammation and complement activation occurs in AMD, it is likely that inflammation is involved in the pathogenesis of AMD. This is supported by the observation that patients with AMD had elevated levels of C-reactive protein (Seddon et al., 2004). The physicians' health study found that subjects who took aspirin (325 mg) every other day had a 23% reduced risk of AMD over five years (Christen et al., 2001).

Mice deficient in copper-zinc superoxide dismutase (SOD1) have features of AMD (Imamura et al., 2006). The retinal pigment epithelial cells of $Sod1^{-/-}$ mice showed oxidative damage, and their β-catenin-mediated cellular integrity was disrupted, suggesting that oxidative stress plays a role in AMD. Even SOD2 knockdown mouse showed several of the features seen in AMD (Justilien et al., 2007). Oxidized lipoproteins were immunohistochemically detected in the choroidal neovascular membranes, macrophages and retinal pigment epithelial cells in the choroidal neovascular membranes-expressed cell surface scavenger receptors for oxidized lipoproteins, suggesting that oxidative stress plays a significant role in AMD (Kamei et al., 2007). In addition, the concentrations of antioxidant enzymes that facilitate refolding or degradation of oxidatively damaged proteins such as copper-zinc superoxide dismutase (Cu-Zn-SOD), manganese superoxide dismutase (Mn-SOD), catalase, heat shock protein (HSP) 27, HSP 90, and proteasome increased significantly in AMD, lending support to the role of oxidative mechanisms in the pathogenesis and progression of AMD (Decanini et al., 2007).

In contrast to the expectation, it was noted that in a mouse model of choroidal neovascularization, $IL\text{-}10^{-/-}$ mice, which have increased inflammation in response to diverse stimuli, showed significantly reduced choroidal neovascularization with increased macrophage infiltration compared with the wild type. The choroidal neovascularization inhibition by macrophages was found to be mediated by the TNF family death molecule Fas ligand (CD95-ligand). These results suggest that immune–vascular interactions are complex and that macrophages may have both positive and negative action on neovascularization (Apte et al., 2006). Despite the fact that IL-10 is an anti-inflammatory molecule, IL-10 was found to enhance choroidal neovascularization, suggesting that under some specific conditions macrophages may be antiangiogenic. Since the absence of IL-10 resulted in increased macrophage influx and reduced neovascularization, local IL-10 function could play an important role in the pathogenesis of AMD, diabetic retinopathy, and retinopathy of prematurity. It is also likely that some degree of inflammation is necessary, which, in turn, could trigger the production of anti-inflammatory cytokines and bioactive lipids such as LXs, RSVs, PTs, and nitrolipids at the target site to

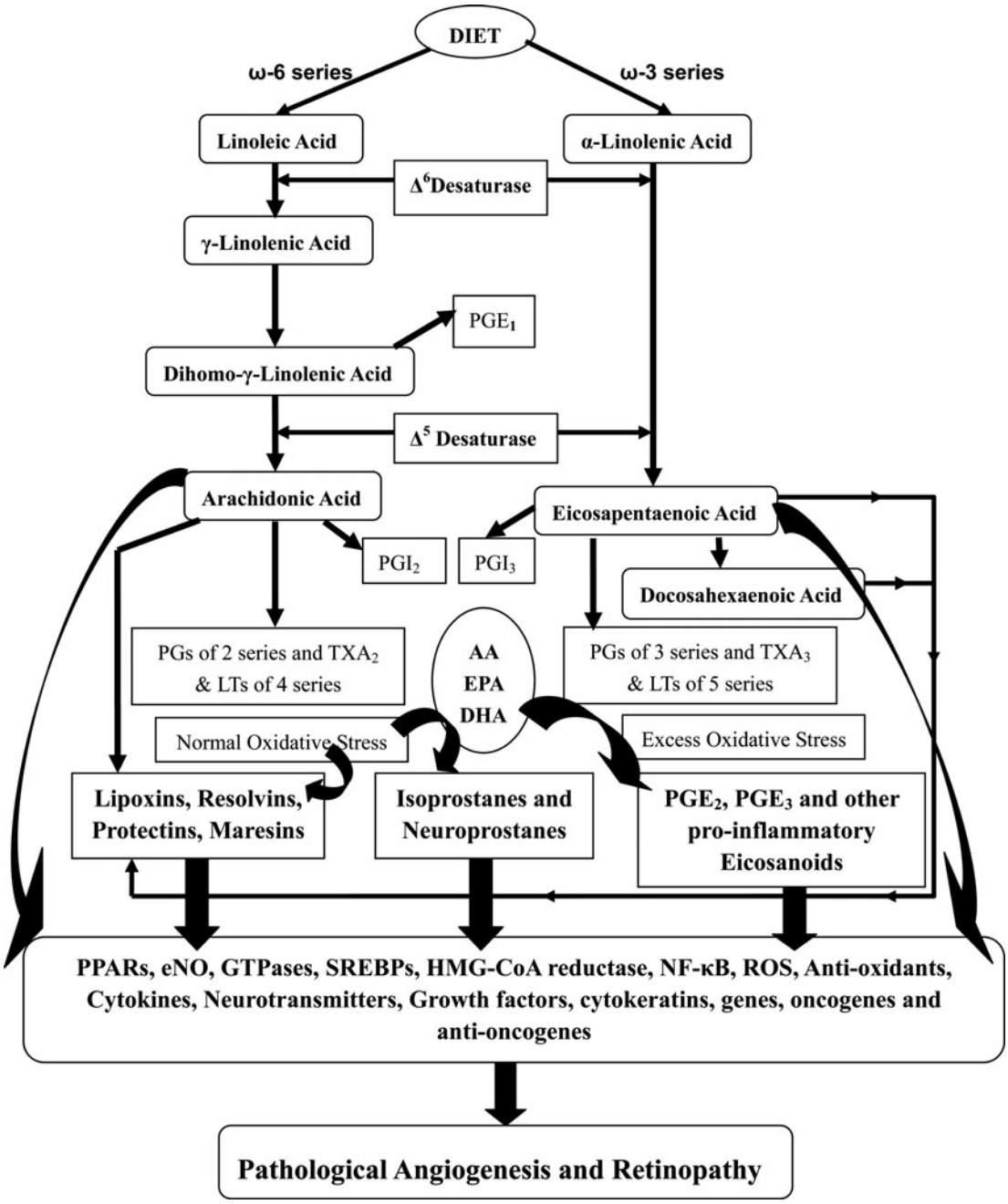

Figure 3 Scheme showing possible interaction(s) and feedback regulation among various factors involved in the pathobiology of diabetic retinopathy, AMD, and retinopathy of prematurity. (+) indicates increase in the synthesis/action/enhancement of retinopathy; (−) indicates decrease in the synthesis/action/block or reversal of retinopathy. ROS = reactive oxygen species, NO = nitric oxide including NO_2, LXs = lipoxins, RSVs = resolvins, PTs = protectins, MSs = maresins, Cis-PUFAs = cis-polyunsaturated fatty acids, Trans-PUFAs = trans-polyunsaturated fatty acids such as trans-arachidonic acid. Hyperoxia, as it happens in retinopathy of prematurity, triggers increase in the generation of free radicals and NO that leads to the formation of trans-AAs, trans-EPAs, trans-DHAs (by converting cell membrane cis-fatty acids to trans-fatty acids). TAAs increase the production of thmrombospondin-1, an antiangiogenic molecule that leads to apoptosis of endothelial cells. This causes hypoxia that triggers increase in the production of VEGF, a proangiogenic molecule. High VEGF causes neovascularization and development of pathological retinopathy. Hyperglycemia decreases NO generation but enhances the synthesis and release of proinflammatory cytokines, superoxide anion, and other reactive oxygen species, and decreases the formation of cis-PUFAs (due to decrease in the activity of Δ^6 and Δ^5 desaturases—see Ghiso et al., 1999; Fierro et al., 2002; Goldberg et al., 2005; Gao et al., 2007; Fukuoka et al., 2010) that may lead to increase in the formation of TAAs, TEPAs, and TDHAs and consequent fall in the formation of lipoxins, resolvins, protectins, and maresins (due to a deficiency of cis-PUFAs). Deficiency of PUFAs and lipoxins, resolvins, protectins, and maresins (and possibly, nitrolipids) enhances the formation of proinflammatory cytokines due to the absence of their feedback regulation on these cytokines and decreases the formation of NO. This causes retinal hypoxia due to low NO (a vasodilator) and low lipoxins, resolvins, protectins, and maresins that have anti-inflammatory, vasodilator, and retinal cell protective actions. The resultant hypoxia and inflammation enhances the production of VEGF and other proangiogenic factors, leading to the initiation and progression of pathological retinopathy and AMD. It is predicted that a close interaction exists among semaphorins, ephrins, Slit proteins, Notch signaling pathway, anti- and proangiogenic factors, pro- and anti-inflammatory cytokines, cis-PUFAs, trans-PUFAs, lipoxins, resolvins, protectins, maresins, and Robo4-Slit2.

initiate repair process and abrogate pathological angiogenesis seen in AMD, diabetic retinopathy, and retinopathy of prematurity.

FREE-RADICAL-MODIFIED PUFAS AND THEIR ROLE IN ANGIOGENESIS

Regulated development of vasculature in the retina is important for the preservation of vision. On the other hand, retinal neovascularization, resulting in hemorrhage and retinal detachment, causes blindness in diabetic retinopathy, AMD, and retinopathy of prematurity. Paradoxically, the growth of new blood vessels results from vascular regression, which is the primary pathology in diabetic retinopathy, AMD and retinopathy of prematurity, since this inappropriate vascular regression leads to hypoxia, which prompts neovascularization.

In patients with retinopathy of prematurity, the premature infant exposed to oxygen therapy initiates loss of retinal vessels and subsequent development of retinopathy. After birth, the infant is exposed to higher than normal concentration of oxygen in utero that causes the retina to "believe" that oxygen delivery is excessive, creating a shift from a pro- to antiangiogenic cellular environment. This leads to disintegration or regression of the developing retinal vessels, decreasing oxygen delivery. At this instance, as the infant is growing and the retina is maturing, it becomes metabolically more active. As a result of the prematurely regressed blood vessels, the retina is unable to receive adequate blood supply and, hence, becomes hypoxic. This leads to stimulation of the proangiogenic factors in response to the hypoxic environment and, thus, retinal neovascularization sets in.

Of the several pro- and antiangiogenic factors that regulate normal and abnormal angiogenesis, the most important proangiogenic factor is VEGF, which is an oxygen-regulated protein, and the most important antiangiogenic factor is thrombospondin-1 (TSP-1). Under hyperoxic conditions, VEGF production is shut down, whereas hypoxia stimulates its production and much of the mechanism of oxygen-induced vessel loss has been attributed to VEGF (Chan-Ling et al., 1997; Okamoto et al., 1997; Miller, 1997; Yancoupoulos et al., 2000; Ferrari et al., 2009). In addition, free radicals, nitric oxide (NO), and PUFAs also regulate vessel loss and proliferation. Targeted disruption of eNOS or pharmacological inhibition of NO protects against oxygen-induced loss of retinal vessels in the mouse model of retinopathy of prematurity (Brooks et al., 2001; Keshet, 2001). This is interesting since a close interaction exists between VEGF and NO. NO production is induced by hypoxia in an HIF-1-dependent fashion. Both VEGF and NO are detectable in the vitreous of proliferative diabetic retinopathy patients, and both are co-induced in the same ischemic regions of the retina. NO is required downstream of VEGF for endothelial proliferation and angiogenesis (Papapetropoulos et al., 1997; Ziche et al., 1997). On the other hand, VEGF stimulates production of NO in ECs (Papapetropoulos et al., 1997; Hood et al., 1998), and, in turn, NO blocks hypoxia-induced production of VEGF (Tsurumi et al., 1997; Ghiso et al., 1999). In this context, it is noteworthy that the early step of hyperoxia-induced vessel obliteration was not inhibited in iNOS-null mice (Sennlaub et al., 2001), suggesting that NO may have diametrically different actions on neovascularization depending on the amount of NO released and the stage of neovascularization. This, in part, may also depend on the modulatory effect of NO on lipids and the byproducts formed.

It is now known that modification of lipids by ROS and NO have specific function in the regulation of vascular regeneration. This is supported by the observation that COX-dependent metabolism of AA produces PGs that regulate inflammatory responses, cell survival, vascular function, and angiogenesis. Oxidative stress leads to the formation of trans-AA metabolites (TAAs) (these include: *5E*-, *8E*-, *11E*-, and *14E*-AA; see Figure 4) that mediate apoptosis of microvascular cells, resulting in retinal microvascular degeneration in vivo (Kermorvant-Duchemin et al., 2005). These trans-AAs are major products of $NO_2^{\bullet}$-mediated isomerization products of AA within the cell membrane (Balazy, 2000; Balazy and Lopez-Fernandez, 2003; Roy et al., 2004). TAAs have a variety of biological actions (Balazy et al., 2001; Jain et al., 2005; Kooli et al., 2008) and can be found in the human blood plasma (Zghibeh et al., 2004). Trans-AAs formed under conditions of hyperoxia lead to EC apoptosis and microvascular degeneration, which ultimately contributes to oxygen-induced retinopathy (Smith and Connor, 2005). In experimental animals, injection of exogenous TAAs into the eye correlated with increased loss of retinal vessels. Furthermore, TAAs induce the formation of thrombospondin-1, which binds to its receptor CD36 on vascular ECs to initiate apoptosis of ECs and regression of blood vessels (Smith and Connor, 2005; Long et al., 2008). These results explain how 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 significant role in these conditions. It is likely that ROS and nitrosative stress induce pathological retinopathy, whereas TAAs inhibit such retinopathy due to their ability to initiate apoptosis of ECs and regression of blood vessels (Smith and Connor, 2005). Thus, TAAs may serve as negative feedback regulators of oxidative stress-induced pathological retinopathy and neoangiogenesis. It is possible that similar to TAAs, trans-EPAs and trans-DHAs are also formed due to $NO_2^{\bullet}$- mediated isomerization, which may also have biological activity, especially in the pathophysiology of diabetic retinopathy, AMD, and retinopathy of prematurity (Wilson et al., 2006; Gao et al., 2007; Gridley, 2007; Acevedo et al., 2008; Jones et al., 2008; Long et al., 2008; Das, 2009).

One product of DHA oxidation is trans-4-hydroxy-2-hexenal (HHE), a six carbon analog of the n–6 fatty acid-derived trans-4-hydroxy-2-nonenal (HNE). HHE and HNE have been reported to be toxic to primary cultures of cerebral cortical neurons that were prevented by the addition of thiol scavengers (Long et al., 2008). EPA and DHA subjected to free radical oxidation process form J_3-isoprostanes, which induced Nrf2-directed

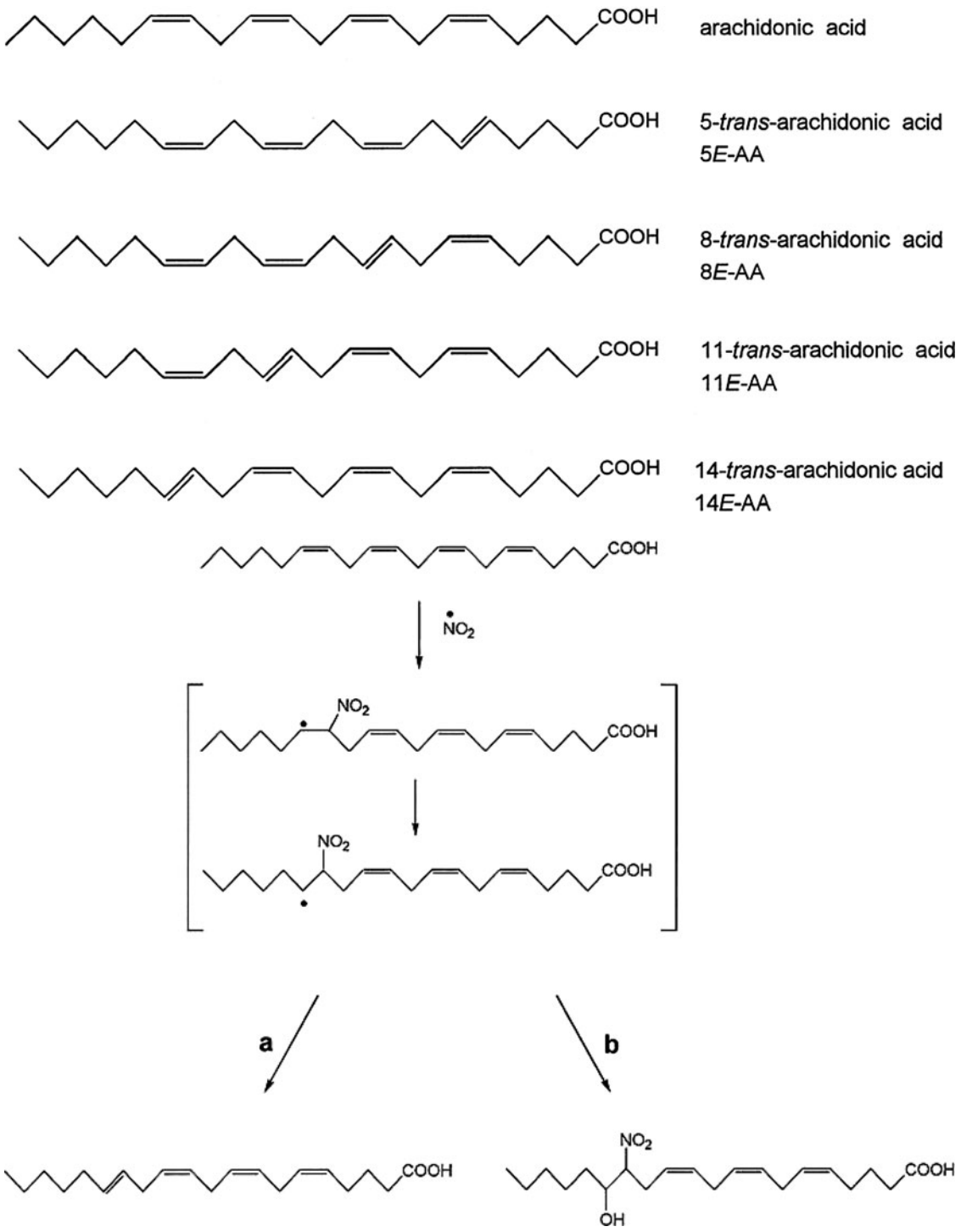

Figure 4 (A) Structure of arachidonic acid and its trans-isomers. (B) Mechanism for a nitrogen dioxide-mediated formation of 14-trans-arachidonic acid (a) and 14-nitro, 15-hydroxyeicosatrienoic acid (b).

gene expression (Gao et al., 2007). These evidences suggest that TEPAs and TDHAs compounds could form in vivo similar to TAAs due to $NO_2^{\bullet}$-mediated isomerization.

Several other proteins also participate in vessel development and some of them include: semaphorins, ephrins, Slit proteins, and Notch signaling pathway (Wilson et al., 2006; Gridley, 2007; Acevedo et al., 2008; Jones et al., 2008; Das, 2009). Of all, the role of Roundabout 4 (Robo4)–Slit2 signaling axis is interesting since it counteracts the effects of VEGF. It is likely that TAAs, PUFAs, and LXs, RSVs, and PTs have a regulatory role in the expression and function of these proteins that modulate angiogenesis.

ANTI-INFLAMMATORY LXs, RSVs, AND PTs MAY PLAY A ROLE IN RETINOPATHY

PUFAs (especially AA, EPA, and DHA) are present in large amounts in the retina. EPA inhibits the abnormal gap junctional intercellular communication (GJIC) induced by H/R via suppressing TK activation (Zhang et al., 1999) and protected against VEGF-induced reduction in GJIC and phosphorylation of connexin (Zhang et al., 2002). EPA attenuated VEGF-induced proliferation of ECs (Yang et al., 1998) and improved H/R-induced endothelial dysfunction through inhibition of TK activation (Morita et al., 2001), a mechanism by which PUFAs protect against the development and progression of diabetic retinopathy. Aspirin-triggered LX stable analog 15-epi-16-(para-fluoro)-phenoxy-lipoxin A_4 is a potent inhibitor of VEGF-induced angiogenesis (Fierro et al., 2002), and LXs prevented hypoxia-induced proliferative retinopathy in experimental animals (Connor et al., 2007).

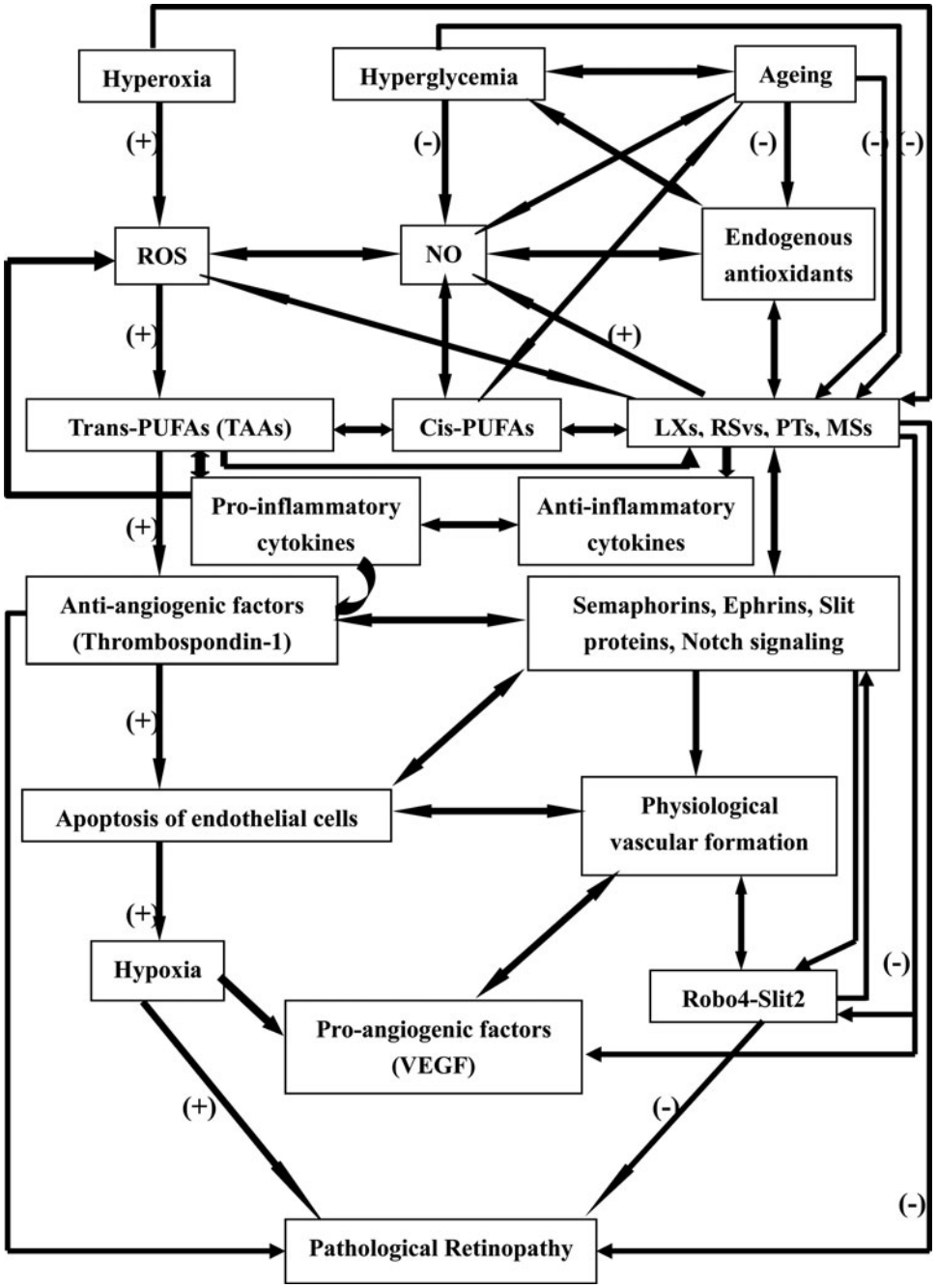

Figure 5 Scheme showing the metabolism of essential fatty acids and their relationship to pathological angiogenesis.

These results suggest that LXs, RSVs, PTs, and MSs prevent the development and progression of diabetic retinopathy. It is likely that a similar mechanism operates even in AMD and retinopathy of prematurity, which are also characterized by proliferative retinopathy.

It is noteworthy that AA, EPA, and DHA augment eNO generation; LXs, RSVs, and PTs that possess anti-inflammatory actions also enhance NO production, inhibit leukocyte activation, facilitate wound healing, inhibit TNF-α production and NF-κB activation, antagonize LTB_4 actions, and protect human retinal pigment epithelial cells from oxidative stress. Even neurotrophins-promoted retinal epithelial cell survival is mediated through neuroprotectin D1 signaling (Claria and Serhan, 1995; Gronert et al., 1998; Chiang et al., 1999; Serhan et al., 2000a; 2000b; Das, 2002; Jozsef et al., 2002; Levy et al., 2002; Hong et al., 2003a, 2003b; Marcheselli et al., 2003; Bannenberg et al., 2004; Calon et al., 2004; Mukherjee et al., 2004; Paul-Clark et al., 2004; Akbar et al., 2005; Arita et al., 2005; Bannenberg et al., 2005; Gronert et al., 2005; Lukiw et al., 2005; Arita et al., 2006; Das, 2006a; Mukherjee et al., 2007; Schwab et al., 2007; Das, 2008a, 2008b, 2009; Niemoller et al., 2009; Das, 2010, 2011). It is important to note here that these lipids (PUFAs such as AA, EPA, and DHA) are present in large amounts in the retina and brain and they can give rise to the anti-inflammatory bioactive molecules such as LXs, RSVs, PTs, and MSs that can suppress inflammation and VEGF-mediated events that lead to the initiation and progression of pathological retinopathy. Since TAAs also have a role in pathological retinopathy, it is likely that the local balance between pro- and antiangiogenic factors, various PUFAs, their cis- and trans-products, LXs, RSVs, PTs, and MSs, and ROS, NO, and pro- and anti-inflammatory cytokines will ultimately determine the degree of vascularization, neoangiogenesis, and degree of retinopathy or relief or prevention of retinopathy. Under conditions of hyperoxia, excess of TAAs and TEPAs and TDHAs are formed that induce the formation of thrombospondin-1 to initiate apoptosis of ECs and, thus, contribute to oxygen-induced retinopathy. Both in diabetic retinopathy and AMD, deficiency of PUFAs (especially of AA, EPA, and DHA) occurs, which leads to decreased formation of LXs, RSVs, and PTs, which, in turn, enhances VEGF production that leads to the onset of retinopathy. In retinopathy of prematurity, oxygen therapy causes generation of excess of ROS that attack the cell membrane cis-PUFAs to convert them to TAAs, TEPAs, and TDHAs, leading to the depletion of cis-PUFAs. Thus, in retinopathy of prematurity, diabetic retinopathy, and AMD, deficiency of cis-PUFAs could occur, which, in turn, leads to reduced formation of LXs, RSVs, PTs, and MSs (and possibly that of nitrolipids). Thus, the contribution and involvement of various PUFAs and their products such as LXs, RSVs, PTs, and MSs, and proangiogenic factors, pro- and anti-inflammatory cytokines, ROS, NO, and TAAs, TEPAs, and TDHAs in the pathogenesis of AMD, diabetic retinopathy, and retinopathy of prematurity depends on the initiating event and the positive and negative feedback regulation among these factors (see Figure 3 and 5).

CONCLUSIONS AND CLINICAL IMPLICATIONS

It is evident from the preceding discussion that VEGF, NO, and lipids play a significant role in retinopathy of prematurity, diabetic retinopathy, and AMD, which are characterized by neovascularization, increase in local concentrations of VEGF, retinal hypoxia, an imbalance in the anti- and proangiogenic molecules, enhanced levels of TNF, and decreased content of PUFAs in the retinal cell membranes and their products such as LXs, RSVs, PTs, and MSs. Under oxidative stress, TAAs (these include: *5E*-, *8E*-, *11E*-, and *14E*-AA), and TEPAs and TDHAs could be formed that cause apoptosis of microvascular cells, resulting in retinal microvascular degeneration, which contributes to oxygen-induced retinopathy (Kermorvant-Duchemin et al., 2005). Since LXs, RSVs, PTs, and MSs modulate the production and actions of VEGF, NO, angiogenesis, and wound healing, it is proposed that these lipid molecules play a significant role in pathological retinopathy. LXs, RSVs, PTs, and MSs and their precursors AA, EPA, and DHA are small molecules (molecular weight $\sim$280–320), have potent actions at pico and nanomolar concentrations, and can be injected intravitreally to produce local beneficial actions, either alone or in combination with anti-VEGF antibodies for retinopathy. It will be interesting to study the sequence of formation of TAAs, TEPAs, and TDHAs, and LXs, RSVs, PTs and MSs in animal models of different types of retinopathy and subjects with diabetic retinopathy, AMD, and retinopathy of prematurity and correlate their formation to VEGF, NO, and other anti- and proangiogenic molecules as the disease is initiated and progresses. Such measurement of these biomolecules could be done in the vitreal fluid and plasma of these patients. In such study, information about the exact timing of formation of TAAs and other trans-PUFAs from cis-PUFAs and how this conversion of cis- to trans-PUFAs influences the synthesis and action of LXs, RSVs, PTs, and MSs, VEGF, NO, cytokines, and other pro- and antiangiogenic molecules need to be assessed. This would give an idea as to when intravitreal injection of TAAs, TEPAs, TDHAs, LXs, RSVs, PTs, and MSs is needed to prevent not only initiation but also the progression of retinopathy. It is likely that in the early stages of retinopathy of prematurity LXs, RSVs, PTs, and MSs are more useful, whereas TAAs, TEPAs, and TDHAs are useful in the late stages of diabetic retinopathy and AMD when neovascularization is dominant.

In summary, it is evident that both hypoxia and hyperoxia can trigger pathological retinal angiogenesis in which VEGF, NO, pro- and anti-inflammatory cytokines, and bioactive lipids play a significant role. Though a dominant role for VEGF has been demonstrated in AMD, retinopathy of prematurity, and diabetic retinopathy, anti-VEGF therapies cannot completely suppress pathological angiogenesis. Bioactive lipid molecules such as LXs, RSVs, PTs, MSs, nitrolipids, and trans-AAs, EPAs, and DHAs regulate the production of VEGF, NO, ROS, and cytokines and, thus, influence both physiological and pathological angiogenesis and pathological retinopathy. Hence, it is necessary to study the role of bioactive lipids in pathological

retinopathy and evaluate their possible therapeutic use in these conditions.

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ABBREVIATIONS

AMD = Age-related macular degeneration
ROS = Reactive oxygen species
VEGF = Vascular endothelial growth factor
H_2O_2 = Hydrogen peroxide
O_2^{-}. = Superoxide anion
OH = Hydroxyl radical
NAD(P)H oxidase = Nicotinamide adenine dinucleotide phosphate oxidase
EC = Endothelial cells
NO = Nitric oxide
SOD = Superoxide dismutase
Flt-1 = FMS-like tyrosine kinase-1
KDR = Kinase insert domain receptor [(KDR), a Type III receptor tyrosine kinase)], also known as vascular endothelial growth factor receptor 2 (VEGFR-2), is a VEGF receptor. *KDR* is the human gene encoding it. KDR has also been designated as CD309 (cluster of differentiation 309).
Flk1 = Fetal liver kinase 1
Akt = Akt/PKB is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription, and cell migration.
v-Akt = Murine thymoma viral oncogene homolog 1. In humans, there are three genes in the "Akt family": Akt1, Akt2, and Akt3. These genes code for enzymes that are members of the nonspecific serine/threonine protein kinase family (EC 2.7.11.1)
Akt1 is involved in cellular survival pathways.
Akt2 is an important signaling molecule in the insulin signaling pathway.
The role of Akt3 is less clear, though it appears to be predominantly expressed in the brain. It has been reported that mice lacking Akt3 have small brains.
eNOS = Endothelial nitric oxide synthase
HUVECs = Human umbilical vascular endothelial cells
Rac1 = Ras-related C3 botulinum toxin substrate 1
PTPs = Protein tyrosine phosphatases
PAE cells = Porcine aortic endothelial (PAE) cells
GTPase = Guanosine triphosphatases are a large family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP).
MAPKs = Mitogen-activated protein kinases
LA = Cis-linoleic acid
GLA = Gamma-linolenic acid
DGLA = Dihomo-GLA
AA = Arachidonic acid
ALA = Alpha-linolenic acid
EPA = Eicosapentaenoic acid
DHA = Docosahexaenoic acid
LXs = Lipoxins
RSVs = Resolvins
PTs = Protectins
MSs = Maresins
EFAs = Essential fatty acids
PUFAs = Polyunsaturated fatty acids
PGs = Prostaglandins
ATLs = Aspirin-triggered lipoxins
15R-HETE = 15*R*-hydroxy-eicosatetraenoic acid
18R-HEPE = 18R- hydroxy-eicosapentaenoic acid
COX = Cyclo-oxygenase
LOX = Lipoxygenase
TXs = Thromboxanes
LTs = Leukotrienes
GJIC = Gap junctional intercellular communication
H/R = Hypoxia/reoxygenation
TK = Tyrosine kinase
IL-6 = Interleukin-6 T
TNF-α = Tumor necrosis factor-α
IL-1 = Interleukin-1
IL-2 = Interleukin-2
HDHAs = Hydroxy DHAs
TAAs = Trans-arachidonic acid metabolites
TEPAs = Trans-eicosapentaenoic acid metabolites
TDHAs = Trans-docosahexaenoic acid metabolites
NPD1 = Neuroprotectin D1
TSP-1 = Thrombospondin-1
HIF-1 = Hypoxia-inducible factor-1
iNOS = Inducible nitric oxide synthase
HHE = Trans-4-hydroxy-2-hexenal
HNE = Trans-4-hydroxy-2-nonenal
Nrf1 = Nuclear respiratory factor 1
Robo4 = Roundabout 4

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