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Review

Oxidative stress, polyunsaturated fatty acids-derived oxidation products and bisretinoids as potential inducers of CNS diseases: focus on age-related macular degeneration

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Abstract:
Many pathologies of the central nervous system (CNS) originate from excess of reactive free radicals, notably reactive oxygen species (ROS), and oxidative stress. A phenomenon which usually runs in parallel with oxidative stress is unsaturated lipid peroxidation, which, *via* a chain reaction, contributes to the progression of disbalanced redox homeostasis. Among long-chain (LC) polyunsaturated fatty acids (PUFAs) abundantly occurring in the CNS, docosahexaenoic acid (DHA), a member of ω-3 LC-PUFAs, deserves special attention, as it is avidly retained and uniquely concentrated in the nervous system, particularly in retinal photoreceptors and synaptic membranes; owing to the presence of the six double bonds between carbon atoms in its polyene chain (C=C), DHA is exquisitely sensitive to oxidative damage. In addition to oxidative stress and LC-PUFAs peroxidation, other stress-related mechanisms may also contribute to the development of various CNS malfunctions, and a good example of such mechanisms is the process of lipofuscin formation occurring particularly in the retina, an integral part of the CNS. The retinal lipofuscin is formed and accumulated by the retinal pigment epithelial (RPE) cells as a consequence of both visual process taking place in photoreceptor-RPE functional complex and metabolic insufficiency of RPE lysosomal compartment. Among various retinal lipofuscin constituents, bisretinoids, originating from all-*trans* retinal substrate – a photometabolite of visual pigment cofactor 11-*cis*-retinal (responsible for photon capturing), are endowed with cytotoxic and complement-activating potential which increases upon illumination and oxidation. This survey deals with oxidative stress, PUFAs (especially DHA) peroxidation products of carboxyalkylpyrrole type and bisretinoids as potential inducers of the CNS pathology. A focus is put on vision-threatening disease, i.e., age-related macular degeneration (AMD), as an example of the CNS disorder whose pathogenesis has strong background in both oxidative stress and lipid peroxidation products.

Key words:
oxidative stress, polyunsaturated fatty acids peroxidation, docosahexaenoic acid, carboxyalkylpyrroles, bisretinoids, retina, age-related macular degeneration

Abbreviations: A2E – N-retinylidene-N-retinylethanolamine, AGE – advanced glycation endproduct, ALA – α-linolenic acid, AMD – age-related macular degeneration, ARA – arachidonic acid, AT-RAL – all-trans retinal, AT-RvD – aspirin-triggered resolvin, CAP – carboxyalkylpyrrole, CEP – 2-(ω-carboxyethyl)pyrrole, CFH – complement factor H, CHP – 2-(ω-carboxyheptyl)pyrrole, CNS – central nervous system, CNV – choroidal neovascularization, CPP – 2-(ω-carboxypropyl)pyrrole, DHA – docosahexaenoic acid, DPA – docosapentaenoic acid, EP – ethylpyrrole, EPA – eicosapentaenoic acid, FA –

fatty acid, GLA – γ-linoleic acid, HHE – 4-hydroxy-hexenal, HNE – 4-hydroxy-nonenal, HOAA – hydroxy-ω-oxoalkenoic acid, HODA – 9-hydroxy-12-oxydec-10-enoic acid, HOHA – 4-hydroxy-7-oxyhept-5-enoic acid, HOOA – 5-hydroxy-8-oxyoct-6-enoic acid, HSA – human serum albumin, LA – linoleic acid, MAC – membrane attack complex, MDA – malondialdehyde, POS – photoreceptor outer segment, LC-PUFA – long-chain polyunsaturated fatty acid, MSA – mouse serum albumin, NPD1 – neuroprotectin-1, PP – pentylpyrrole, PUFA – polyunsaturated fatty acid, RAL – retinal, REs – retinol esters, RNS – reactive nitrogen species, ROL – retinol, ROS – reactive oxygen species, RPE – retinal pigment epithelium, RvD – resolvin D, RvE – resolvin E, SOD – superoxide dismutase, TLR – toll-like receptor, TNFα – tumor necrosis factor α, VEGF – vascular endothelial growth factor

Introduction

Oxidative stress is a common phenomenon occurring in living systems. Although endowed with pathological potential, oxidative stress in fact originates from physiological reactions taking place practically inside each cell of an organism. And this is because of the fact that the production of so called free radicals, especially reactive oxygen species (ROS), accompanies, for example, oxidative phosphorylation-derived ATP-based energy metabolism which takes place in the mitochondria of a cell. ATP – originating from the mitochondrial electron transport chain – is absolutely essential for life. However, during energy transduction, some electrons "leak" to oxygen, forming the oxygen free radical superoxide, which has been implicated in the aging process (the concept known as the free radical theory of aging [19, 24, 73]), as well as in the pathophysiology of a variety of diseases [1, 77].

Under physiological conditions, free radicals (both ROS and reactive nitrogen species – RNS) are rapidly neutralized by effective enzymatic and non-enzymatic defense mechanisms, contributing to the maintenance of a proper balance – "steady state" level – between their rates of production and their rates of removal/inactivation. However, an excess of free radicals, resulting from either their overproduction or insufficient activity of antioxidant defense systems, will disturb physiological equilibrium between pro- and antioxidant systems, leading eventually to a sequence of usually non-enzymatic spontaneous reactions with possible injurious consequences, a process embraced by the term oxidative stress.

Oxidative stress and polyunsaturated fatty acids (PUFAs) are closely related, as PUFAs, occurring abundantly in the central nervous system (CNS) and possessing many "fragile" double bonds between carbon atoms (C=C), easily undergo non-enzymatic oxidation and fragmentation during lipid peroxidation and form numerous toxic products. In other words, PUFAs, especially long-, or very long-chain fatty acids, are targets of oxidative stress-mediated lipid peroxidation. Lipid peroxidation thus contributes to a chain reaction implicated in the generation of a variety of very reactive highly cytotoxic molecules.

In this article we will focus on oxidative stress, and two families of compounds strictly related to oxidative stress, as possible causes of pathologies affecting the CNS, and especially the retina. These compounds include docosahexaenoic acid (DHA)-derived oxidative protein modifications represented by carboxyethylpyrroles and lipofuscin-stored bisretinoids, whose photoexcitation/photooxidation can lead to the formation of singlet oxygen and other reactive oxygen species. The retina, being an extracranial extension of the brain, is an integral part of the CNS. The retina, similar to brain, is a tissue very rich in long-chain (LC) PUFAs, particularly in DHA, and, unlike brain (yet in agreement with its physiology), is subjected to high level exposal to light, which can produce photochemical damage. Due to its structural and functional features, the retina seems to be particularly predisposed to ROS generation. Therefore, the retina may be an excellent model tissue with which to show what may happen in the CNS tissue under condition when oxidative stress and PUFAs peroxidation meet together.

Free radicals, PUFAs and oxidative stress – short introduction

Free radicals

Free radicals are such molecules that possess one or more unpaired electrons in their outermost orbits which results in an extremely unstable configuration; they may also contain a complete set of electrons – yet occurring in an unstable state. Free radicals quickly react with other molecules or radicals to achieve the stable configuration (at a lower energy state) thereby becoming less reactive. There is a wide variety of free radicals, including RNS and oxygen-

derived free radicals, known as ROS. The former group is represented by nitric oxide radical ($NO^{\bullet}$), nitrosonium cation (NO^{+}), nitroxyl anion (NO^{-}) or extremely reactive peroxynitrite ($ONOO^{-}$); the latter group includes oxygen ions and peroxides. Superoxide anion ($O_2^{-\bullet}$), also named superoxide radical anion or hyperoxide, is considered the "primary" ROS which in biological tissues can be converted (also with the aid of superoxide dismutase – SOD) into non-radical species: hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In the presence of some transition metals, e.g., Fe^{2+}, hydrogen peroxide can easily undergo decomposition by the Fenton and Haber-Weiss reactions to hydroxyl radical ($^{\bullet}OH$) – one of the most toxic molecules, characterized by $T_{1/2}$ in the range of nanoseconds; so short biological half-life favors the oxidation of molecules just in their place of $^{\bullet}OH$ generation. Hydroxyl radical ($^{\bullet}OH$), *via* reactions with alkanes and its derivatives or fatty acids, consisting in removal of a hydrogen atom from one of the carbon atoms, forms a new, usually less active, radical and a molecule of water. On the other hand, hydrogen peroxide (H_2O_2) may be converted into water by the enzymes: catalase or glutathione peroxidase [15, 77].

Free radicals occur widely in nature as they are formed throughout the whole life as by-products during various biochemical reactions taking place in different cellular compartments of a variety of cells. For example, hydrogen peroxide is produced under various conditions, even in normoxia, reaching constant cellular concentration between 10^{-9} and 10^{-7} M. Being products of normal cellular metabolism, free radicals may play a dual role as both deleterious and beneficial species. If endogenous antioxidative defense systems are effective (as, for example, highly active SOD, a superoxide scavenging enzyme which neutralizes $O_2^{-\bullet}$, or sufficiently active macular pigments in the retina), then the presence and actions of free radicals do not result in oxidative stress and overt pathology. If such systems fail – as it likely takes place in a senescent organism or diseased tissues/organs, prooxidative activity predominates. Consequently, an excess of ROS (resulting either from mitochondrial electron-transport chain, or excessive stimulation of NAD(P)H, or other mechanism), giving rise to oxidative stress, may lead to damage to cell structures and the development of pathological states requiring medical intervention. As far as beneficial activity of free radicals is concerned – their deployment by the immune system, and specifically macrophages and neutrophils generating ROS to kill invading microorganisms, may be a good example.

Concerning cell compartment where free radicals are formed, it is generally assumed that oxygen-derived reactive species produced in mitochondria may contribute to "physiological" aging process [78], whereas ROS of non-mitochondrial origin, produced for example in endoplasmic reticulum, cell membrane or peroxisomes, may play a role in age-dependent diseases [10, 14, 43, 48]. A mixture of ROS originating from both sources may play in concert as well [1, 15, 77, 78].

PUFAs

Fatty acids (FAs) are present in the most diverse forms of life and perform important functions as lipid components in the structure of the plasmatic/cellular membranes – being responsible for e.g., membrane fluidity, and in metabolic/signaling processes – they are important sources of energy and precursors of signaling molecules (including proinflammatory, anti-inflammatory, vasoactive, and many other mediators). The vast family of fatty acids comprise saturated and unsaturated compounds; the former lipids have no double bonds between carbon atoms (C=C) in a hydrocarbon (acyl or polyene) chain, the latter compounds possessing one C=C double bond refer to monounsaturated fatty acids; those possessing two or more double bonds refer to PUFAs. At both ends of each fatty acid there is reactive group: carboxyl group and methyl group, forming, respectively, carboxyl end and methyl end. Depending on the position of the first C=C double bond (counting from the carbon of the methyl end, designated as the first or ω carbon) PUFAs divide into two families/series: ω-3 (ω3, n-3) and ω-6 (ω6, n-6) PUFAs. The simplest FAs in each family/series are: α-linolenic acid (ALA; 18:3-ω3 or 18:3n-3) for ω-3, and linoleic acid (LA; 18:2-ω6 or 18:2n-6) for ω-6, both undergo metabolism consisting of elongation of the acyl chain and insertion of additional C=C double bonds, as depicted below.

ω-3 Family/series

α-Linolenic acid (ALA; 18:3-ω3) → octadecatetraenoic acid (18:4-ω3) → eicosatetraenoic acid (ETA; 20:4-ω3) → eicosapentaenoic acid (EPA; 20:5-ω3) → docosapentaenoic acid (DPA; 22:5-ω3) → 24:5-ω3 → 24:6-ω3 → (β-oxidation)→ docosahexaenoic acid (DHA; 22:6-ω3).

ω-6 Family/series

Linoleic acid (LA; 18:2-ω6) → γ-linoleic acid (GLA; 18:3-ω6) → dihomo-γ-linoleic acid (DGLA; 20:3-ω6) → arachidonic acid (AA or ARA; 20:4-ω6)→ docosatetraenoic acid (DTA; 22:4-ω6)→24:4-ω6 → 24:5-ω6 → (β-oxidation) → docosapentaenoic acid (DPA; 22:5-ω6).

The metabolic reactions within the ω3 and ω6 PUFA families take place in the cell endoplasmic reticulum, with the exception of the last reaction – β-oxidation which takes place in peroxisomes, requiring thus translocation of adequate substrates (i.e., 24:6-ω3 and 24:5-ω6) into this cell compartment.

PUFA residues of membrane phospholipids are very sensitive to oxidation and the action of ROS [48]. For example, hydroxyl radical ($^{\bullet}OH$) can easily abstract, or using a more colloquial expression "steal" an electron from such unsaturated fatty acids (marked as LH from lipid-H) to give rise to a carbon-centered lipid radical ($L^{\bullet}$; fatty acid radical). Lipid radical ($L^{\bullet}$) can further interact with molecular oxygen (O_2) to produce lipid peroxyl radical ($LOO^{\bullet}$; fatty acid peroxyl radical). Being an unstable species, lipid peroxyl radical interacts with another free fatty acid (LH) to produce a different fatty acid radical ($L^{\bullet}$) and lipid peroxide (LOOH). The formed $L^{\bullet}$ reacts again with molecular oxygen to produce $LOO^{\bullet}$ etc., creating the cycle which continues, as the new fatty acid radical ($L^{\bullet}$) reacts in the same way. The described process can be schematically depicted as follows:

$$\text{Lipid-H (LH)} + {}^{\bullet}OH \rightarrow L^{\bullet} \text{ (lipid/fatty acid radical)}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \text{ (lipid/fatty acid peroxyl radical)}$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \text{ (lipid peroxide)}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

The whole process is known under the term "lipid peroxidation"; being initiated by the reaction of $^{\bullet}OH$ with unsaturated lipid (initiation step), and then entering the cycle (propagation step), the lipid peroxidation leads eventually to cell damage. The harmful process can however be terminated when two radicals react and produce a non-radical species. Another possibility to speed up termination is to catch free radicals – this can be achieved by molecules with antioxidant activity, e.g., vitamin E, antioxidant enzymes (SOD, catalase and peroxidase) or xanthophyll-type macular pigments.

Peroxidation of highly unsaturated lipids, i.e., FAs that possess more than three C=C double bonds (for example: ω6 arachidonic acid, ω3 eicosapentaenoic acid, and particularly ω3 docosahexaenoic acid), leads to complex mixtures of products, including malondialdehyde (MDA), acrolein, 4-hydroxy-2-nonenal (HNE), 4-hydroxy-hexenal (HHE), as well as a number of hydroxy-ω-oxoalkenoic acids. The latter compounds, together with their derivatives of the carboxyalkylpyrrole type, are produced in the DHA-rich brain and retina, where they may contribute to, respectively, some degenerative brain disorders and vision-threatning macular pathologies.

It should be emphasized that in the CNS, including the retina – the DHA-richest CNS structure [72, 79], DHA, which is the most unsaturated FA, plays many important physiological roles which, ironically, are related just to its structural unsaturation. Structural unsaturation (six C=C double bonds) predisposes DHA for peroxidation with resultant harmful compounds. DHA is thus an example of how Nature exploits potentially dangerous molecules for physiological purposes. "Physiology" means that everything is "under control"; however, when one of many controlling factors will fail, the nicely programmed physiologic system may also fail, initiating a cascade of firstly pro-pathogenic and then true pathogenic events.

The retina – an integral part of the CNS and a suitable model tissue for brain research

The retina, together with optic nerve (which consists of axons of retinal ganglion cells), is an extension of the brain. As such, it may be considered an integral part of the CNS [53]. The retina is a thin sheet of nervous tissue (about 90 μm in the rabbit and 300 μm in the cow) situated at the back of the eye. The complex retinal processing of visual stimuli, including the detection of brightness, contrast, color and movement, is accomplished by the interaction of retinal neurons, i.e., photoreceptors (rods and cones), bipolar cells, horizontal cells, amacrine cells, interplexiform cells, and ganglion cells (whose axons form the optic nerve). In addition to these neuronal cells, glial cells, known as Müller cells, are also present in the retina. Morphologically, the retina is characterized by distinct laminar organization of its cells and processes, with two layers of synaptic connections: the outer and

inner plexiform layers. Although both electrical and chemical synaptic junctions occur in the retina, the latter predominate, and the "interplay" between retinal neurons appears to be chemically mediated in the main. Since the vertebrate/mammalian retina is derived embryologically from the brain, it would not be unreasonable to assume that substances which are proposed as chemical messengers in the brain have similar functions in the retina – what in fact appears to be the case. Similarly, long-chain PUFAs which are abundantly present in the brain also occur in high amounts in the retina, with DHA showing distinctly high levels in this ocular structure.

Light-sensitive photoreceptor cells, i.e., rods and cones, occur in different proportions among vertebrates: rods dominate, or occur even exclusively, in nocturnal animals, cones are present in retinas of daytime active species, whereas species active at night and daytime have both types of photoreceptors. Rods contain rhodopsin as a visual pigment and are very sensitive to light, being responsible for twilight and night vision, however, they do not recognize colors. In contrast, cones are generally less sensitive to light, possess at least three visual pigments, and are responsible for color and high-acuity vision. The human retina possesses both rods and cones – the latter cell type being densely packed in a small central region of the structure named the macula, or yellow macula – "yellow" refers to the color which is related to the presence in the macula of xanthophylls, known as macular pigments [34].

Depending on environmental lighting conditions, each day the human retina absorbs approximately 10^{12} to 10^{15} photons. Although such photons are informative in terms of vision, they can cause irreparable damage to the retina: brief exposure to bright light can produce immediate thermal injury, whereas exposure to light for an extended period of time may lead to photochemical damage, including retinal pigment epithelial (RPE) monolayer disruption [28]. Ambient radiation from the sun or from artificial light sources contains varying amounts of UV irradiation from A to C range (220–400 nm) and visible light (400–700 nm). The shorter the wavelength, the greater energy and therefore the greater the potential for photochemical cell/tissue damage. However, although the longer wavelengths are less energetic, they can penetrate the eye more deeply [58]. The blue region (400–500 nm) of the visible spectrum is of particular importance since it has a relatively high energy and can easily penetrate ocular tissues, including the neural retina with photoreceptors [2].

The retina is a tissue with high metabolism and the highest oxygen consumption per unit weight of all human tissues [84]. Oxygen and nutrients are supplied by two independent circulatory systems: the retina vessels and the choroid. The retina system, whose vessels penetrate as far as the outer plexiform layer, supplies oxygen and nutrients to the inner two-thirds of the retina. The outer third part of the retina physiologically remains completely avascular – yet it receives necessary nutrients and oxygen *via* the choriocapillaries of the choroidal system. High blood perfusion rate of the choroidal system delivers enough oxygen and nutrients for living and functioning of the RPE and photoreceptors (in fact, the choroidal blood flow far supersedes that required to nourish the neural retina) [84]. Thus, the high oxygen tension in this tissue, together with high levels of light exposure, and high levels of various lipid compounds, with DHA accounting for more than 80% of PUFAs in photoreceptor disk membranes, promotes the production of free radicals leading to oxidative stress, and makes the retina particularly susceptible to damage by ROS and by lipid-derived oxidative protein modifications [11, 31, 37, 58, 62].

Thus, it is not surprising that the macula (and to a lesser extent the rest of the retina) is abundantly equipped with macular pigments (i.e., lutein, zeaxanthin and lutein metabolite – meso-zeaxanthin), because, due to their specific physicochemical properties, they play an important protective role of a filter for harmful shortwave light, and of a scavenger of free radicals [34, 54, 81]. In other words, macular pigments, together with other antioxidant defense systems, protect the central retina against possible deleterious effects of blue and near-UV light entering the eye, bis-retinoids which are formed as by-products during the retinoid/visual cycle in the RPE-photoreceptor complex, and lipid (especially DHA) peroxidation-derived harmful products.

Age-related macular degeneration (AMD) – the retina disease with a strong background of oxidative stress and lipid peroxidation

As stated in preceding section, the retina, due to its structural and physiological features, is particularly predisposed to produce ROS, whose excess can generate oxidative stress, and to lipid peroxidation and

fragmentation to cytotoxic products [31, 37, 62]. All these phenomena, together with natural aging process, are implicated in the pathogenesis of age-dependent disease named AMD – one of the most common irreversible causes of severe loss of vision, including legal blindness [9, 23, 49, 50]. The disease develops slowly and insidiously, with clinically meaningful symptoms seen in the elderly (60+ years) population. Clinically, AMD is divided into two forms: atrophic – dry form, and exudative – wet or neovascular form. Despite intensive basic and clinical research, its pathogenesis remains unclear, likely due to the multifactorial and age-dependent character. Pathological changes take place in the macular region of the retina comprising choriocapillaries, Bruch's membrane, RPE cells, and photoreceptors. RPE firstly undergo degeneration, photoreceptors secondly. Clinical features common for the two types of AMD (dry and wet form) include the presence of drusen (also recently identified pseudodrusen), as well as hypo- and/or hyperpigmentation of the RPE. More than 80% of all people with intermediate and advanced AMD have the dry form, yet this form may progress to the wet form (10–15%) which leads to significantly more vision loss [4, 50]. AMD, a vision-threatening disease, is a real medical problem and this is because of its steadily increasing occurrence and the fact that it (mostly the dry form) is until now an uncurable disease [49].

The pathophysiology of AMD is complex and, in addition to genetic predispositions and environmental determinants, at least four specific processes contribute to the development of the pathology (Fig. 1):

• Lipofuscinogenesis – generation of lipofuscin in the form of aggregates which are stored in lysosomal compartment of RPE; linkage to oxidative stress and

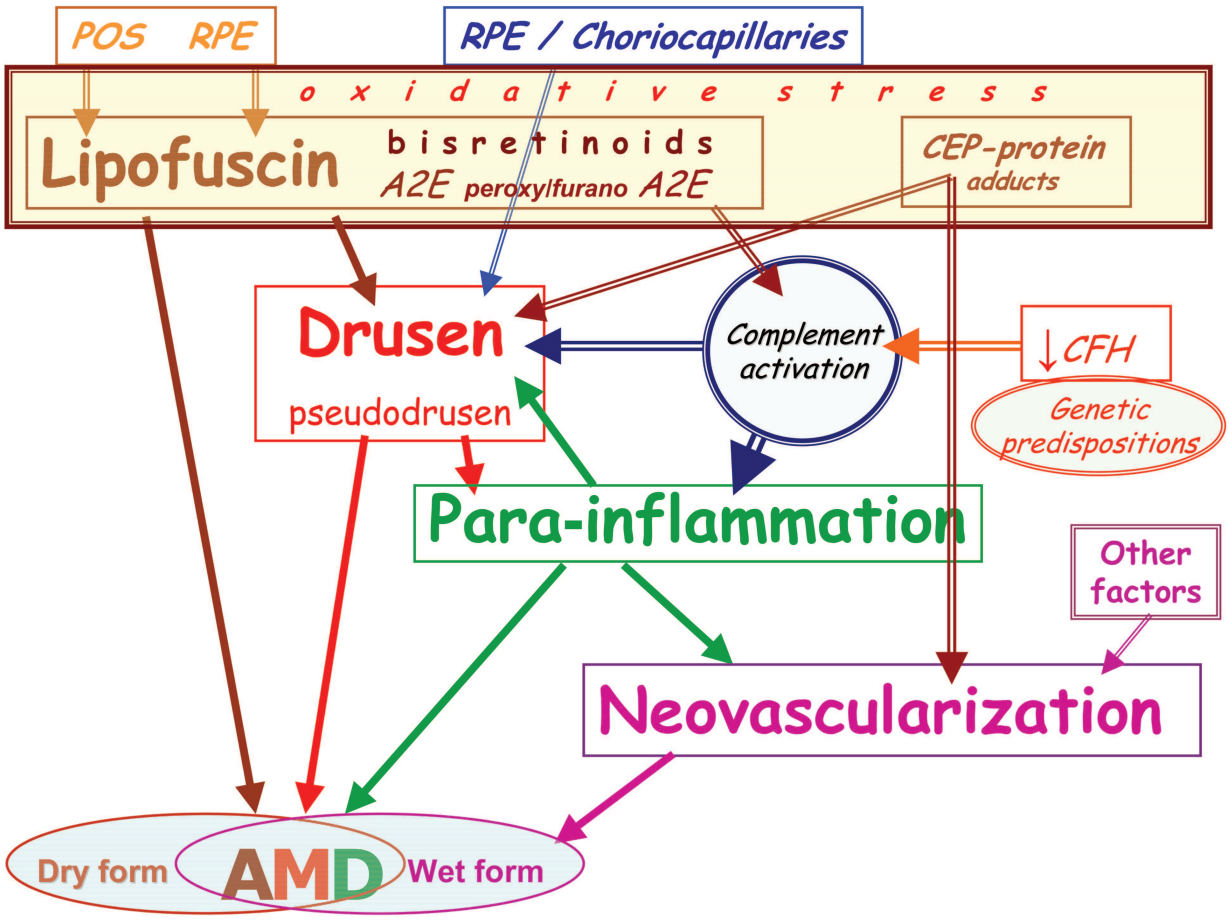

Fig. 1. Principal molecular events underlying the development of AMD pathology. Four principal processes are involved in the pathogenesis of AMD (dry and wet form): lipofuscin formation, drusen formation, local atypical inflammation (para-inflammation), and neovascularization (refers only to wet form AMD). Explanations in the text. Abbreviations: POS – photoreceptor outer segment; RPE – retinal pigment epithelium; A2E – N-retinylidene-N-retinylethanolamine; CEP – carboxyethylpyrrole; CFH – complement factor H

lipid peroxidation, which, especially in predisposed individuals, may act as a "vicious circle" contributing to the progression of the disease.

• Drusogenesis – formation of extracellular deposits of insoluble material in the form of drusen localized between RPE and Bruch's membrane; pseudodrusen are located between photoreceptors and RPE monolayer. Taking the RPE monolayer as a reference structure it can be said that drusen are situated beneath RPE, whereas pseudodrusen are above RPE. Small and non-numerous drusen (so called hard drusen) may occur in healthy eyes, yet their numerous population and especially their bigger size or confluent character, is a hallmark of AMD.

• Chronic inflammation – named para-inflammation, as there are no signs of typical inflammatory process.

• Choroidal neovascularization (CNV) – the choriocapillaries-derived pathological new vessels are formed which break through the Bruch's membrane and direct toward the RPE-photoreceptor complex; the process contributes to the wet – neovascular (exudative) form of AMD.

It is generally accepted that the impairment of RPE cell function is an early and crucial event in the molecular pathways leading to photoreceptor degeneration and clinically relevant AMD [36, 38, 44, 49, 50, 74]. Such view has its rationale in the fact that RPE serves a variety of metabolic and supportive functions that are of vital importance for retinal photoreceptors, including maintenance of the blood-retina barrier, participation in the visual cycle (uptake, processing, transport, release of vitamin A derivatives), and phagocytic uptake and degradation of constantly shed apical photoreceptor outer segments (POS). One of driving forces of the RPE dysfunction is an age-dependent phagocytic and metabolic inefficiency of postmitotic RPE cells (lysosomal failure hypothesis). This leads to progressive accumulation of lipofuscin (or "age pigment") granules/aggregates composed mostly of lipids (≈50%) and proteins (≈44%) of phagosomal, lysosomal, and photoreceptor origin, modified to varying degree by oxidative processes as a result of both exposure to visible and UVA light and high oxygen levels in the eye [69].

Although lipofuscin accumulates in RPE of healthy eyes – what can be imaged ophthalmoscopically as the retina fundus autofluorescence (the natural fluorescence of retina) [69, 70], an excess of accumulating age-pigment, as a highly reactive material, may lead to harmful consequences. And this is because of the presence of bisretinoid compounds which, upon illumination, are capable of taking up oxygen, the process increasing with age, and producing ROS, including singlet oxygen [20, 60, 61]. Photooxidation of bisretinoids results in the formation of epoxides, furanoid moieties and endoperoxides [35]. It has been demonstrated that photoexposure of RPE containing bisretinoid A2E led to cell damage and death [65, 71]. In addition to bisretinoid fluorophores, lipofuscin constituents include also DHA-derived carboxyethylpyrrole (CEP) modifications of proteins known as CEP-protein adducts [62]. Due to its specific, above mentioned ingredients (bisretinoids in particular) that are absent from non-retinal lipofuscins, the term "retinal lipofuscin" or "RPE lipofuscin" is widely used to stress peculiarity of the age pigment accumulated in the RPE cells.

Bisretinoid fluorophores of the retinal lipofuscin

Formation of bisretinoids

At least 25 bisretinoid fluorophores originating in photoreceptor cells and resulting from reactions of all-*trans*-retinal (AT-RAL) have been identified until now [47, 70]. Well characterized members of such bisretinoid family include: A2-PE, A2E, A2-DHP-PE, A2-GPE, AT-RAL-dimer. A2-PE refers to the compound possessing two AT-RAL molecules, i.e., A2, conjugated with phosphatidylethanolamine (PE); A2E consists of two AT-RAL-derived arms connected through a pyridinium ring (in other words, hydrolysis of the phosphate ester of A2-PE yields A2E); A2-DHP-PE – A2-dihydropyridine-PE; A2-GPE – A2-glycerophosphoethanolamine. It is noted that the bisretinoid-starting compound, i.e., AT-RAL, is a physiological molecule that is formed in the visual cycle *via* conformational change – photoconversion – of photon-absorbing visual pigment co-factor, i.e., 11-*cis*-retinal [11-*cis*-RAL] (Fig. 2). A proposed synthesis of some bisretinoids is depicted below [70]:

11-*cis* retinal + *hv* → *all-**trans*** **retinal** (AT-RAL) + phosphatidylethanolamine (PE)

→ N-retinylidene-PE Schiff base (NRPE) → // [1,6] H-shift → tautomer x // + AT-RAL

→ bisretinoid phosphatidyl-dihydropyridinium (dihydropyridinium-A2PE)

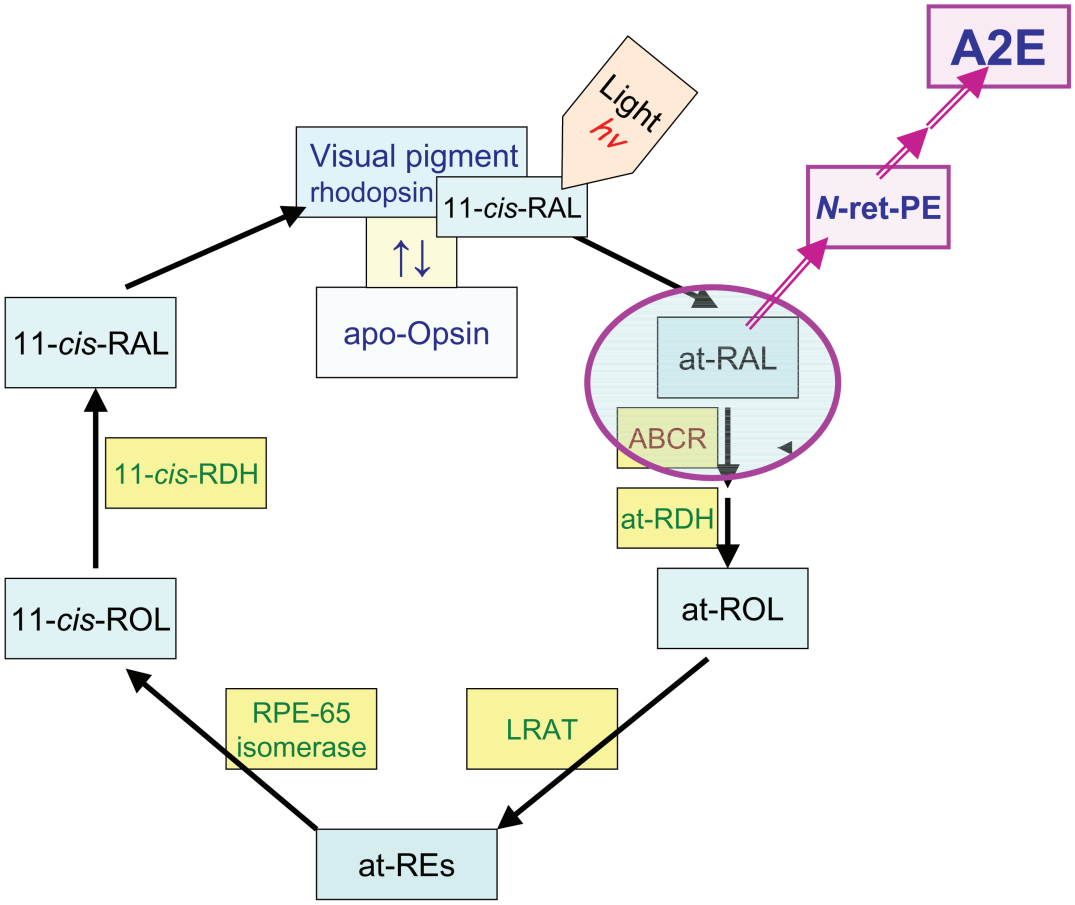

Fig. 2. Retinoid cycle of the visual cascade and generation of bisretinoid A2E. Explanations in the text. In short: visual pigment consists of protein opsin and photosensitive cofactor 11-*cis*-RAL. Following light absorption, 11-*cis*-RAL undergoes conformational change into at-RAL, a physiological substrate for transporter ABCR (ABCA4) and retinal dehydrogenase (at-RDH). Further physiological changes within the retinoid cycle are: at-ROL → at-REs → 11-*cis*-ROL →11-*cis*-RAL → 11-*cis*-RAL/apo-opsin (regenerated visual pigment capable of capturing photons of light). Lack or functional inefficiency of ABCR makes that at-RAL spontaneously enters the pathway leading to A2E formation (as described in the text). Abbreviations: 11-*cis*-RAL – 11-*cis*-retinal; hv – photon of light absorbed by visual pigment; at-RAL – all-*trans*-retinal; ABCR – ATP-binding cassette transporter; at-RDH – all-*trans* retinal dehydrogenase; at-ROL – all-trans-retinol; LRAT – lecithin : retinol acyl transferase; RBP – retinal binding protein; at-REs – all-trans-retinal esters; N-ret-PE – N-retinylidene-phosphatidylethanolamine; other explanations as in Figure 1

→ //automatic loss of 1 H^+//→
A2-dihydropyridine-PE (A2-DHP-PE);

→ //automatic loss of 2 H^+// →
phosphatidyl-pyridinium bisretinoid (A2PE)

→ // phosphate hydrolysis // → **A2E** or A2-GPE

Another pathway:

11-*cis* retinal + *hv* → *all-**trans*** **retinal** (AT-RAL) + phosphatidylethanolamine (PE)

→ N-retinylidene-PE Schiff base (NRPE) → // [1,6] H-shift → tautomer x // + AT-RAL

→ all-*trans*-retinal-dimer + PE →
all-*trans*-retinal-dimer-PE

Biological activity of bisretinoids

A2E (N-retinylidene-N-retinylethanolamine) – the first RPE lipofuscin constituent to be described – was thoroughly analyzed for its biological activity in general and for its harmful effects in particular. Keeping in mind that A2E, a lipofuscin constituent, is formed practically in each human retina as a by-product of the visual cycle, the experimental findings are really impressive and simultaneously worrying. For example, A2E accumulates in mitochondrial membranes of cultured RPE cells becoming a proapoptotic molecule acting *via* a mitochondrial pathway. A possible explanation of this observation is that upon reaching a critical intralysosomal concentration, A2E is released

from the lysosome and then specifically targets the outer mitochondrial membrane thereby initiating apoptosis of the RPE cell [64]. Furthermore, A2E absorbs visible blue light and in consequence is able to generate singlet oxygen, superoxide anion, hydrogen peroxide, or lipid hydroperoxides – all of which may act as an injury stimulus to RPE cells [8, 82, 85]. Other bisretinoids are also toxic. Interestingly, unconjugated all-*trans*-retinal-dimer was shown to be even more potent generator of singlet oxygen than A2E, and photooxidized A2E derivatives (resulting from spontaneous photooxidation at C=C double bonds), especially furano- and peroxy-photoproducts, appeared to be capable of effectively activating complement system [30, 68], which is an effector mechanism of the innate immune system and plays a major role in shaping the adaptive immune response [75]. Thus, the photoreceptor-derived lipofuscin-stored bisretinoids predispose or sensitize the macula to pathologic reactions, as generation of the low grade complement activation may lead to chronic inflammatory processes in the form of so called para-inflammation [4, 33, 52,

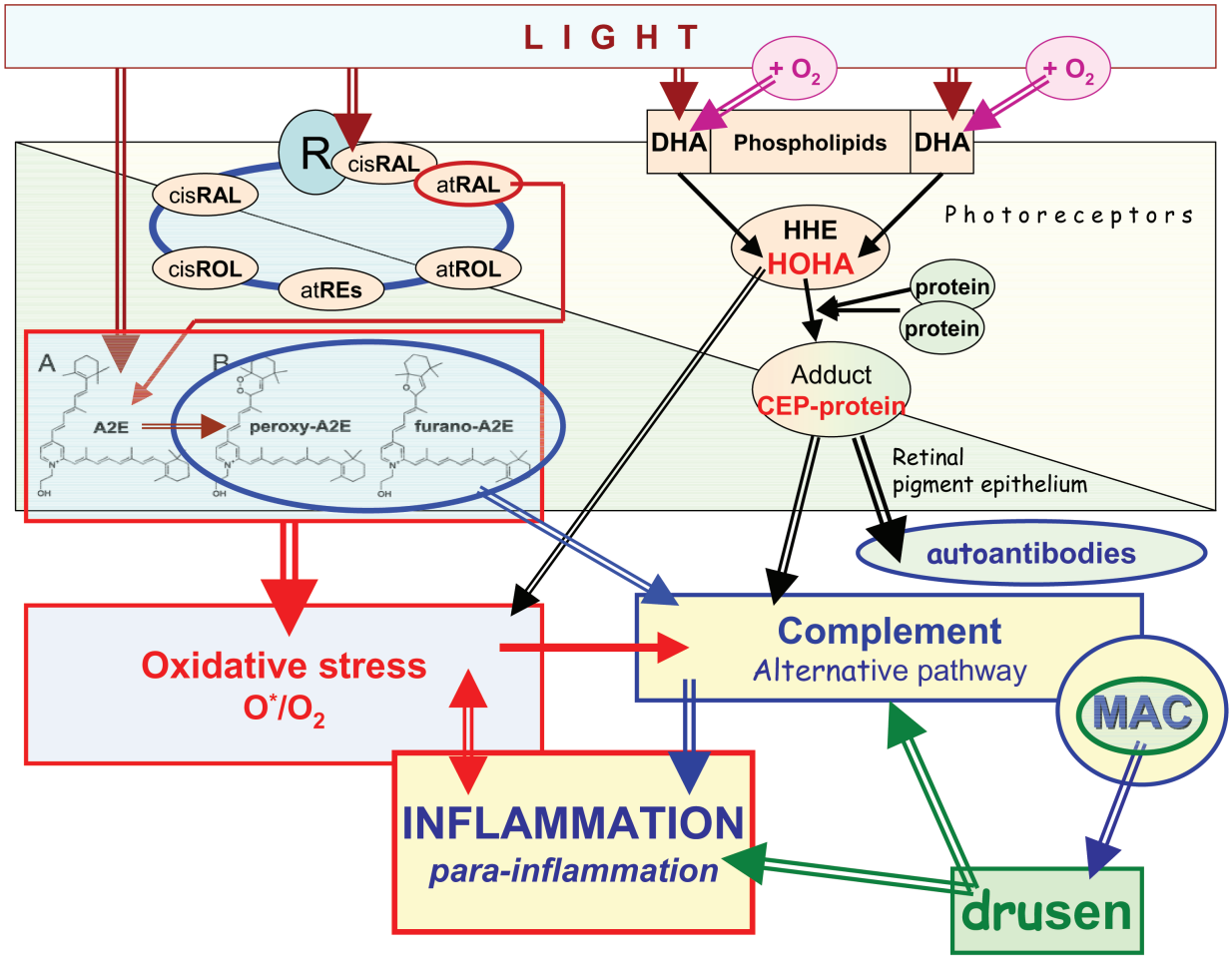

Fig. 3. Interrelations between molecular and cellular processes occurring at early stages of AMD pathogenesis. Explanations in the text. In short: due to very high oxygen tension and high levels of light exposure (especially of short wavelengths, i.e., blue region and UV-A) the retina is particularly susceptible to damage by reactive oxygen species (ROS). Oxidative stress results from both the retinoid (visual) cycle-derived atRAL being converted to photocytotoxic bisretinoid A2E and its metabolites, and DHA peroxidation- derived reactive fragments (HHE, HOHA). A2E, oxidative stress, and HOHA-driven immunogenic CEP-protein adducts, all lead to stimulation of complement (alternative pathway) activity. In consequence, oxidative stress, hyperactive complement and accompanying chronic atypical inflammation (para-inflammation) interact to form a kind of "vicious circle" driving an array of molecular and cellular reactions from physiology to pathology. In addition, the AMD hallmark drusen, containing various chemical constituents, including cytotoxic complement-derived MAC, additionally contribute to persistency of para-inflammation and complement activity. The mentioned changes, together with individual genetic predispositions and environmental determinants, all may contribute to the development of AMD pathology. Abbreviations: HHE – 4-hydroxyhexenal; HOHA – 4-hydroxy-7-oxyhept-5-enoic acid; MAC – membrane attack complex; other explanations as in Figure 2

83]. In fact, all stages of AMD of both dry and wet form are associated with inflammatory cells, notably macrophages, neutrophils or dendritic cells [4, 23, 29, 83]. In addition, bisretinoid photodegradation may also lead to generation of highly cytotoxic dicarbonyl molecules responsible for advanced glycation endproducts (AGE)-modification of proteins, which are present in drusen [82], and which may contribute to age-related pathologies not only in the retina, but also in other parts of the CNS (notably the brain), as well as in peripheral tissues [76]. Taken collectively, the mentioned mechanisms may all contribute to the development of pathological states in various body organs, including ocular pathology such as AMD (Fig. 3).

Tab. 1. Polyunsaturated fatty acids (PUFAs) peroxidation leads to the generation of carboxyalkylpyrrole-protein adducts

PUFA	Hydroxy-ω-oxoalkenoic acid	Adduct carboxyalkylpyrrole-protein
• LA, ALA	→ HODA + protein	→ CHP-protein
• GLA, ARA, EPA	→ HOOA + protein	→ CPP-protein
• DHA	→ HOHA + protein	→ CEP-protein

LA – linoleic acid, ALA – α-linolenic acid, GLA – γ-linolenic acid, ARA – arachidonic acid, EPA – eicosapentaenoic acid, DHA – docosahexaenoic acid; HODA – 9-hydroxy-12-oxodec-10-enoic acid, HOOA – 5-hydroxy-8-oxooct-6-enoic acid, HOHA – *(E)*-4-hydroxy-7-oxohept-5-enoic acid, CHP – 2-(ω-carboxyheptyl)pyrrole, CPP – 2-(ω-carboxypropyl)pyrrole, CEP – 2-(ω-carboxyethyl)pyrrole

PUFAs-derived carboxyalkylpyrroles (CAPs)

CAP-protein adducts derive from PUFAs subjected to peroxidation [40, 41]. Upon oxidation, PUFAs firstly undergo fragmentation to smaller molecules, such as: 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE), malondialdehyde (MDA), or an array of hydroxy-ω-oxoalkenoic acids, including HODA, HOOA, and HOHA. The generated PUFAs truncated molecules can next form covalent adduction with proteins resulting in alkyl- or carboxyalkyl-pyrrole modifications [41]. Alkylpyrrole modifications include such adducts as: pentylpyrrole (PP)-protein or ethylpyrrole (EP)- protein, whereas CAP modifications are: carboxyheptylpyrrole (CHP)-protein, CPP-protein, CEP-protein (Tab. 1).

The idea that CAP modifications of proteins may contribute to the development of some CNS diseases has its roots in observations made at the end of last century [3, 32, 63, 79]. It has been shown that a γ-hydroxyalkenal product of phospholipid oxidation, i.e., HNE, can form covalent adducts that incorporate the γ-amino group of protein lysyl residues in PP modifications that accumulate in neurons of patients with Alzheimer's disease and in the blood of individuals with atherosclerosis. Other studies have shown the formation *in vivo* of CHP-, CPP-, and CEP-protein modifications from oxidized PUFA (linolenic, arachidonic, and docosahexaenoic acid, respectively)-containing phospholipids. Based on these observations it was suggested that the formation of CAP-protein modifications should be greater in tissues containing high levels of respective PUFAs.

As mentioned, the retina is the DHA-richest tissue in human body. The distribution of this fatty acid is however uneven throughout the tissue, showing highest concentrations in membranes of the photoreceptor outer segments and the RPE [3, 79]. Thus, high amounts of DHA, together with lipofuscin-stored bisretinoids and high supply of oxygen, makes this retina region (which is responsible for capturing photons, being often endangered from intensive light irradiation) particularly suitable for generation of ROS and oxidation-forced DHA metabolites, both endowed with pathogenic potential capable of inducing oxidative stress, complement activation (with its terminal product responsible for cell lysis, i.e., membrane attack complex – MAC), and then inflammation – three interrelated phenomena that work as self-driving mechanism with damaging effects on cells and tissues (Fig. 3).

Coming back to DHA peroxidation-driven changes, it is interesting to mention that the formation of 4-hydroxy-7-oxyhept-5-enoic acid (HOHA), and then CEP-protein adducts, can take place in many tissues, including melanoma, aging vasculature or healing wounds, as well as autistic brain [18, 62, 80], however, their generation in the retina, just due to high levels of DHA, is exceptionally prominent.

CEP-protein adducts in AMD patients

Using rabbit polyclonal anti-CEP antibody, the presence of intense CEP immunoreactivity was found in the POS and RPE, and lighter immunoreactivity in the inner plexiform layer of mouse retina. Similar find-

ings were obtained in studies on whole human retina and samples of drusen-containing RPE/Bruch's membrane/choroid tissue, yet consistently more CEP immunoreactivity was present in the AMD tissue than in the normal (healthy) retina [13, 21].

Since CEP-protein adducts were shown to be formed more abundant in ocular tissues (drusen, Bruch's membrane) from AMD patients than from normal human donors, it has been hypothesized that they may be involved in the pathogenesis of this ocular disease [13, 27] (see Fig. 3).

CEP immunoreactivity was detected not only in human retina, but also in human plasma, with values being again significantly higher in the plasma of AMD donors than in the plasma samples of both younger and older healthy donors [22]. Interestingly, the plasma CEP immunoreactivity positively correlated with CEP autoantibody titer [21], indicating that CEP behaves as an antigen which generates production of specific anti-CEP antibodies. The immune-mediated events related to immunogenic CEP-protein adducts, which in AMD patients are probably generated through many decades, may contribute as one of many molecular links to the development of AMD pathology (Fig. 3).

Recent experiments carried out on mice immunized with CEP-modified mouse serum albumin (CEP-MSA) and Freund's adjuvant (in an attempt to rise the level of sensitivity to endogenously generated CEP) have shown that the retinas of such animals produced changes similar to those seen in retinas of AMD-suffering peoples [25, 26]. The observed changes included: accumulation of drusen below the RPE monolayer, swollen Bruch's membrane, fixation of complement-C3d product in Bruch's membrane, lesions in the RPE cells, decreased electrophysiological responses to light. In addition, in mice with laser-induced rupture of Bruch's membrane, subretinal injection of CEP-MSA significantly augmented CNV, the effect being similar to that produced by injections of vascular endothelial growth factor (VEGF), a major proangiogenic factor [16]. The *in vivo* angiogenic properties of the "human" adduct CEP-HSA were demonstrated in two widely used angiogenesis model

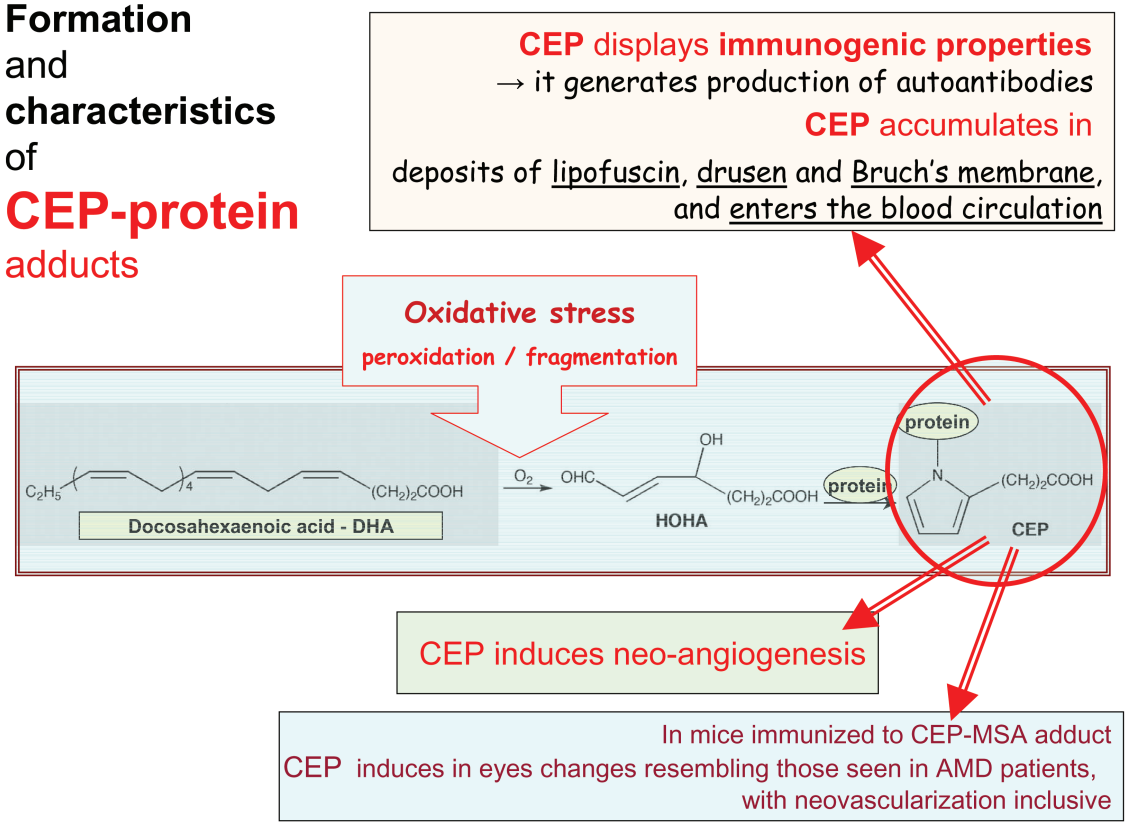

Fig. 4. Formation and characteristics of carboxyethylpyrrole (CEP)-protein adduct

systems: the chick chorioallantoic membrane and rat corneal micropocket assay. The results showed CEP-HSA to be highly potent (active at low picomolar amounts) inducer of neovascularization that utilized VEGF-independent pathways [16]. In conclusion, CEP-MSA has a potential to produce in mice a full spectrum AMD, including both dry (degenerative-atrophic) and wet (neovascular) form, rising a possibility that the human analog (CEP-HSA), formed endogenously from oxidation of DHA-containing lipids, will display similar profile of activity in humans (Fig. 4), being able to produce pro-AMD changes, together with CNV at least in some patients (Fig. 1).

CEP as an inducer of angiogenesis

The ability of CEP-protein adducts to induce angiogenesis is not restricted to ocular neovascularization, but seems to be a phenomenon of general importance. Experiments carried out on different model systems, such as tumor (melanoma) implantation and growth, hind limb ischemia model (ligature of femoral artery), wound healing model, as well as tube formation assay and matrigel plug assay, clearly demonstrated the angiogenic potential of CEP-protein adducts, both in a positive (physiologic or therapeutic) and negative (pathologic) sense, similar to angiogenic profile of VEGF. The mechanisms underlying VEGF- and CEP-driven angiogenesis are different – VEGF realizes its action *via* specific VEGF receptor-mediated signaling pathway, whereas CEP (and also CPP)-induced angiogenesis involves activation of toll-like receptor type 2 (TLR2), but not TLR4 or scavenger receptors on endothelial cells [80]. These observations may have important practical consequences, also in ophthalmology (AMD), since CNV occurring in wet form AMD is currently treated with anti-VEGF drugs: pegaptanib (aptamer), ranibizumab and bevacizumab (monoclonal antibodies), or aflibercept (soluble decoy receptor) [49]. Yet, CNV resistant to anti-VEGF therapy is not unusual in AMD patients, indicating in such cases the role of VEGF-independent mechanism(s). Therefore, it is not unlikely that in such VEGF-independent CNV in AMD patients, CEP oxidative protein modifications and TLR2-directed signaling pathway may operate – a suggestion that is possible (based on animals' data), yet requiring experimental support for its validity in humans.

Concluding remarks and important questions

Numerous experimental and clinical data gathered over many decades indicate that oxidative stress may contribute to various human pathologies, including CNS malfunctions and diseases, especially those with degenerative and/or inflammatory background [12]. In addition to oxidative stress, other molecular risk factors, e.g., lipofuscin-stored photoreactive bisretinoids and PUFAs-derived metabolites and protein modifications, may also be of importance, as already demonstrated in some human diseases [31, 62, 71]. The mentioned phenomena usually play in concert to induce various pathological situations, including ocular disease – AMD (Fig. 5).

However, although both lipofuscin and oxidation fragments of long-chain PUFAs can be formed in different tissues even in physiology (without obvious pathological symptoms), their pathogenic potential reveals when they are formed in excess, or in the presence of some additional factors favoring or strengthening pro-pathogenic mechanisms. Substantial amounts of such potentially pathogenic compounds depend on cell/tissue levels of respective substrates which should assure an intense formation of given products. The CNS tissue offers, and under specific conditions creates a suitable milieu for generation of both oxidative stress and the end products of lipid oxidation.

The retina lipofuscin varies from lipofuscin formed and accumulated in neurons, cardiomyocytes or skeletal muscle cells, and the difference lies in that the RPE age pigment contains an array of retinoids originating from the visual/retinoid cycle which are absent from other age pigments. Retinoids, being highly reactive molecules, can spontaneously fuse together to form various bisretinoids (e.g., A2E) endowed with photocytotoxic potential. Concerning PUFAs-derived oxidative protein modifications, some tissues due to distinctly high levels of a given fatty acid substrate will generate more oxidatively truncated potentially harmful compounds than other tissues. In this respect, the retina is an exception among different CNS structures in that its photoreceptors contain comparatively huge amounts of DHA. This, together with high oxygen supply and high levels of light exposure, results in its natural predisposition to ROS generation and PUFAs peroxidation. For these reasons, the retina can be con-

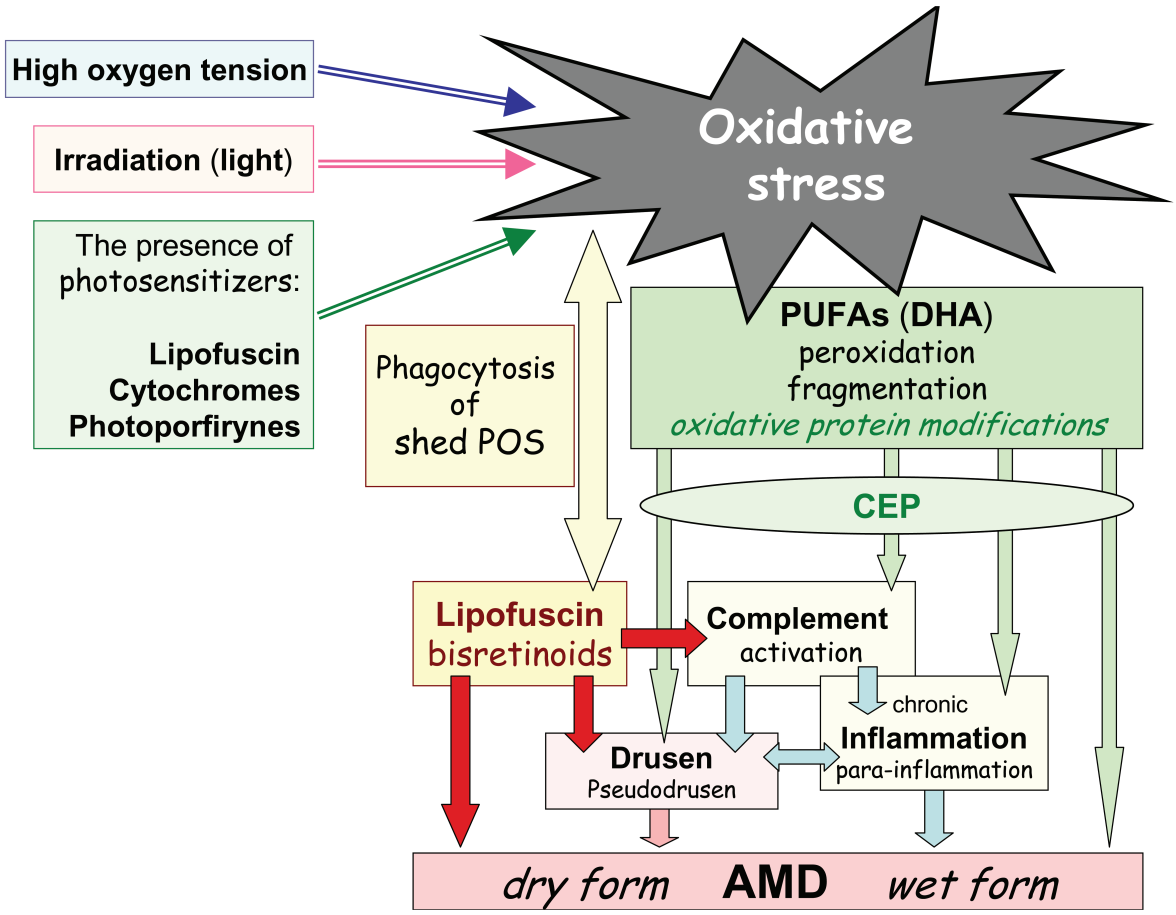

Fig. 5. Key role of oxidative stress in AMD pathogenesis

sidered an excellent CNS model tissue with which to show what may happen in the CNS under stressful conditions.

In this article, a focus has been made on vision-threatening pathology such as AMD, which, in its molecular background, has many similarities to age-dependent brain malfunctions or disorders. One of such common features may be generation of PUFAs-derived oxidative protein modifications, with CEP-protein adducts being the best example. In AMD patients, the serum levels of CEP-protein adducts and anti-CEP-autoantibodies were so pronounced that they were proposed to serve an early biomarker of this retina disease [13, 27, 62]. Perhaps such biomarkers will help to substantiate early diagnosis of developing AMD, since at early stages of the disease clinical symptoms are either absent or unclear or non-unequivocal. However, due to a multifactorial and complex nature of AMD (with genetic, immunological and environmental determinants), there is still an open question whether CEP-protein adducts, as well as cytotoxic bisretinoids, are really the primary cause of the disease. Current views seem to favor the concept implicating their role in the pathogenesis in AMD, but direct evidence supporting their role as major causative factors is lacking, and one cannot exclude a possibility that these molecules simply accompany the disease – evidently the problem awaits elucidation [9, 31, 44, 56, 71].

There are several unanswered issues/questions concerning oxidative stress and lipid peroxidation-driven compounds. Firstly, it is not clear whether under *in vivo* conditions oxidative stress results from decreased activity of endogenous antioxidative defense systems or is simply a manifestation of accelerated aging process. Secondly, potentially harmful bisretinoids are formed in photoreceptors as by-products in the visual cycle and are stored in lipofuscin granules

accumulating in lysosomal compartment of RPE cells. As lipofuscin (age pigment) and its formation, i.e., lipofuscinogenesis, age-dependently occur in each retina (including healthy eyes), should they be considered a strictly pathological process? Thirdly, long-chain PUFAs, particularly DHA in the brain and retina, are physiologically indispensable constituents of all plasma membranes in living organisms; however, based on experimental findings discussed in this survey, one could formulate a conclusion: the more unsaturated fatty acid(s) the more problems in terms of possible pathology, of which the retinal disease – AMD may be a good example. Of many PUFAs, DHA is the most complex compound not only in its chemical structure, but also, or first of all, in its biological activity. DHA is a multifunctional molecule, and this aspect deserves short comment.

DHA can be readily oxidized, but simultaneously DHA is a substrate for neuroprotectin D1 and an array of anti-inflammatory proresolving mediators

In this review, long-chain PUFAs were presented as substrates for generation of oxidative products such as hydroxy-ω-oxoalkenoic acids (HOAAs) and CAPs, the latter being endowed with mainly pathogenic potential (though its role as an angiogenic factor in wound healing and tissue recovery should be considered a beneficial activity [80]). Concerning DHA, the respective compounds are: HOHA and CEP, the latter being likely involved in the pathogenesis of several disorders both in periphery, e.g., artheriosclerosis [32], and in the CNS, e.g., Alzheimer's disease, autism, AMD [62]. Connecting CEP-protein adducts and AMD, a logical relationship can be suggested: the more CEP-protein adducts generated in the photoreceptor-RPE complex the faster AMD progression and the worse clinical prognosis.

Yet, the retinal DHA obviously has two opposite faces. A negative face was discussed above. A positive face is connected firstly – to its role as a plasma/cell membrane constituent, and secondly – to the fact that just this fatty acid is a substrate for neuroprotectin D1 (NPD1) and an array of anti-inflammatory proresolving mediators [5, 51, 52, 66, 67]. The former links DHA mainly to endowing brain/photoreceptor membrane domains with physical properties that positively contribute to functional modulation (e.g., optimization of membrane fluidity or maintenance of retinal integrity), while the latter refers to DHA-derived products such as resolvins (RvD1–RvD4, AT-RvD1-AT-RvD4; resolvins can also originate from EPA forming E-series resolvins: RvE1 and RvE2), maresins and already mentioned NPD1. Resolvins and maresins are endogenous regulators of inflammatory process which physiologically extinguish or resolve acute phase of inflammation – hence their name: proresolving mediators [66]. NPD1 was originally discovered as a neuroprotective lipid mediator acting against harmful factors/situations such as H_2O_2/TNFα oxidative stress-triggered apoptotic RPE DNA damage [46]. The biosynthetic pathway for NPD1 is as follows:

DHA → [15-lipoxygenase + O_2] → 17*S*-HpDHA → [–H_2O] → 16*S*,17*S*-epoxide intermediate → [hydrolase + H_2O] → 10*R*,17*S*-dihydrodocosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid (neuroprotectin D1/protectin D1, depending on the site of formation).

NPD1 displays an array of biological effects. In addition to the effect mentioned above, it appeared also to up-regulate antiapoptotic proteins (e.g., Bcl-2) and to decrease proapoptotic Bax and Bad expression [46]. Furthermore, DHA-derived NPD1 appeared to counteract proinflammatory cell-damaging events triggered by multiple factors not only in the diseased retina but also in the Alzheimer brain [6, 7, 41, 42, 45].

It has been demonstrated that in the AMD-afflicted retina and RPE/choroid the levels of very long-chain PUFAs, including DHA, were significantly decreased [40], an observation which is in line with current clinical trends to recommend DHA-rich dietary supplements to AMD patients [49, 57]; such a trend also applies to an array of psychiatric diseases [39, 55, 59]. In consequence, a plethora of various dietary supplements containing ω-3 long-chain PUFAs (DHA, EPA, DPA) are commercially available, of which many are specifically recommended for patients with AMD and various brain diseases. Taking the above into consideration, and keeping in mind experimental data discussed in this survey, as well as those presented in, for example, recently published article under meaningful title: "High levels of retinal membrane docosahexaenoic acid increase susceptibility to stress-induced degeneration" [72], physicians can experience a kind of frustration dealing with DHA. An important (from practical/therapeutic point of view) and unanswered until now question is whether patients with AMD

(and also with brain diseases, including Alzheimer's disease or autism) should or should not supplement DHA? Producers of such PUFAs-rich dietary supplements encourage people to buy and take them (as they are freely available, i.e., without presctiption), physicians say to their patients that such supplements may help, but there is no certainty they really will help; only few physicians consider possible unwanted effects related to DHA overdosage [49]. Interestingly, the experts of the European Food Safety Authority (EFSA) Panel recently announced their opinion regarding LC ω-3 PUFAs supplementation: "The Panel concludes that the available data are not sufficient to establish a tolerable upper intake level for n-3 LC PUFA (DHA, EPA and DPA, individually or combined) for any population group. The Panel considers that supplemental intakes of EPA and DHA combined at doses up to 5 g/day, and supplemental intakes of EPA alone up to 1.8 g/day, do not raise safety concerns for the adult population. The Panel also considers supplemental intakes of DHA alone up to 1 g/day do not raise safety concerns for the general population" [17].

Thus, is DHA good or bad? Should people supplement it or should they look for a more detailed information on pros and cons regarding LC-PUFAs intake? Such questions can be multiplied – yet, they are of vital importance. They deserve prompt answering since they touch an existing dichotomy between everyday practice and scientific data [49].

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