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Mitochondrial Production of Perhydroxyl Radical ($HO_2^{\bullet}$) as Inducer of Aging and Age-Related Pathologies

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Abstract

All organisms age, in the search of the universal mechanisms of aging Barja analyzed results obtained with various species of mammals and birds and concluded that only two known factors correlate in the right sense (inversely) with animal longevity in vertebrates: the long life span is associated with (a) low rates of mitochondrial reactive oxygen species production, and (b) low degree of fatty acid polyunsaturation of cellular membranes including the mitochondrial ones [Barja (2014) Prog Mol Biol Transl Sci. 127:1-27]. 20 years ago it was established that polyunsaturated fatty acids (PUFA), when still being esterified with the membrane phospholipids, undergo autoxidation with formation of products with large isomerism [Morrow *et al.* (1990). PNAS USA. 87: 9383-9387]. This process is known as the Isoprostane pathway of lipid peroxidation (IPLP), but the mechanism of IPLP initiation remained obscure. We propose that perhydroxyl radical ($HO_2^{\bullet}$), which is a protonated form of the superoxide radical ($O_2^{\bullet}$), initiates within the membrane a chain of reactions with formation of first H_2O_2, which in the hydrophobic environment undergoes homolytic fission producing two $^{\bullet}OH$ radicals, thus very rapidly abstracting three H atoms from a PUFA. As a result, the $HO_2\bullet$ molecule is converted to two molecules of water, and the molecule of a PUFA loses two double bonds, becomes highly unstable and undergoes peroxidation and random intramolecular re-arrangements causing a very large isomerism of the final products. Formation of $O_2^{\bullet}$, and thus of $HO_2^{\bullet}$ radical, are inevitable consequences of the mitochondrial aerobic respiration, and because $HO_2^{\bullet}$ has very high affinity to PUFA, even the smallest amounts of this radical will cause damages to lipids, proteins and mtDNA. Our hypothesis is fully compatible with the conclusions made by Barja, and provides reasonable explanation for one of the important aging mechanisms.

Keywords: Aging; Mitochondria; Oxidative stress; Superoxide radical; Perhydroxyl radical; Polyunsaturated fatty acids; Fatty acids autoxidation; Lipid peroxidation

List of abbreviations: AA: Arachidonic Acid (C20:4 ω6); ANT: Aadenine Nucleotide Translocase; CL: Cardiolipin; COX1 and COX2: Cyclooxygenases; DHA: Docosahexaenoic acid (C22:6 ω3); DNA: Deoxyribonucleic acid; EPA: Eicosapentaenoic acid; FRTA: Free Radical Theory of Aging; GSH: Reduced Glutathione, $HO_2^{\bullet}$: Perhydroxyl radical; IMM: Inner Mitochondrial Membrane; IsoPs: Isoprostanes; IPLP: Isoprostane pathway of lipid peroxidation, LOX: Lipoxygenases; LP: Lipid Peroxidation; MFRTA: Mitochondrial Free Radical Theory of Aging; mtDNA: mitochondrial deoxyribonucleic acid; mtROS: mitochondrial reactive oxygen species; $^{\bullet}NO$: nitric oxide radical; $^{\bullet}NO_2$: Nitric dioxide radical; $O_2^{\bullet}$: superoxide radical; $^{\bullet}OH$: hydroxyl radical; OMM: Outer mitochondrial membrane; $^{\bullet}OONO$: peroxynitrite radical; $O_2NOO^{\bullet}$: peroxynitrate radical; PEA: Phosphatidylethanolamine; PGF2: Isoprostanes containing F-type prostane rings; PGs: Prostaglandins; PLA_2: Ca-independent phospholipases A_2; PUFA: Polyunsaturated Fatty Acids; RET: Reverse Electron Transport; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; SOD1 (Cu,Zn-SOD1): Cytoplasmic SOD, SOD2 (Mn-SOD2) mitochondrial SOD

Introduction

Aging is the process of growing older or changing over time [1]. In other words, aging is the entire sequence of changes during ontogenesis of an individual organism during its lifetime. The major difference in the meanings of "ontogenesis" and "aging" is that the latter term, from the medical point of view, bears a strongly negative hint. Harman [2] defined aging as "progressive accumulation of diverse, deleterious changes with time that increase the chance of disease and death". Knight [3] observed that "although the specific biologic basis of aging remains obscure, there is general agreement that its elucidation will be at the molecular

level. Furthermore, it should be consistent, not only with the life span differences between species, but also with the fact that non-cycling cells such as neurons and myocytes undergo a relatively uniform functional decline with age".

Currently, there are numerous theories of aging, which together indicate on the complex nature of processes involved in the phenomenon, which include genetic and environmental interactions [4-8], global loss of heterochromatin [9,10], adult stem cell modification [11], hormonal regulation [12], telomere shortening [13,14], epigenetics [15], mitochondrial dysregulation [15-17], and the free radical theory [2,18-25]. These are not all examples of theories and related reviews on aging mechanisms. Many of the cited mechanisms are interconnected and reflect different aspects of the aging process. For the last four or five decades the free radical theory of aging (FRTA), first proposed by Harman [2,19,20,26], became one of the most important and controversial among the hypotheses of aging. Sometime later, the abbreviation FRTA was changed to MFRTA, to stress the leading role of mitochondria in the production of free radicals and acceleration of the aging. The mitochondrial free radical theory of aging (MFRTA) can, at present, provide the best explanation for aging and longevity in mammals, birds, and multicellular animals in general [reviewed in 24].

In the current review, we will survey the latest results and conclusions regarding the MFRTA and also offer the hypothesis, which, in our opinion, answers many unanswered questions and complement the important missing links in MFRTA. In the current version of MFRTA, the role of the isoprostane pathway of nonenzymatic lipid peroxidation has not been taken into account.

The role of mitochondria in aging

In a recent, highly informative general overview [24], Barja has stressed the complexity of the problem of aging and the longevity of an individual lifespan. He wrote: "It is known that mean lifespan or the life expectancy at birth of the individuals of a population depends more on the environment than on the genes. On the contrary, longevity and its inverse - the species aging rate - depend more than 90% on the genotype, like in the case of any other species-specific trait. Longevity and aging rate are the main parameters that matter concerning the endogenous process of aging, which is situated at the main root of all the degenerative killer diseases". However, the statement that the longevity is genetically predetermined was shaken strongly by experiments with caloric restriction [27,28]. It has been shown that caloric restriction significantly increases the life span of normal and diseased animals [29], most likely by lowering the steady-state levels of oxidative stress and damage [30].

All aging theories were based and tested on the wide types of cells, bacteria, yeast, simple organisms (*c. elegance*), birds and various species of animals [19,24]. Among the earlier indications on the possible involvement of mitochondria in aging, were experiments with yeast [reviewed in 31]. In order to test the hypothesis on the role of protein synthesis errors in aging, suggested by Orgel [32], Harrison & Holliday [33] treated the larvae of yeast with small doses of streptomycin, which suppresses the protein synthesis in bacteria [34]. The median survival for the control flies was about 66 days, whereas it was only 10 days for the treated flies, even though these flies after hatching appeared to have a completely normal phenotype. This result would be expected, if streptomycin strongly affected mitochondrial function [31,33]. On the contrary, erythromycin, which increases the accuracy of translation in bacterial ribosomes, increased by 27% the lifespan of aerobic yeast, but not of anaerobic (petite) yeast [31,35]. In many cells mitochondrial dysfunction is a frequent symptom of senescence and ageing, and is frequently considered causative (Reviewed in 36). The free radical theory suggests that the age-related increase in free-radical-damaged DNA, lipids and proteins are causal to the accumulation of dysfunctional mitochondria. However, why there should be any age-related change in generation of reactive oxygen species (ROS), or susceptibility to them, is still debated [36].

The free radical theory of aging was first suggested by Harman as a consequence of ionization radiation [26] and was later extended to other types of radical formation and centered on mitochondria [19,20,37,38]. In a comprehensive review [24], Barja summarized the results of experiments on various species of living organisms and selected those features, which affected the life span in all species tested. He concluded that only two known factors correlate in the right sense with animal longevity in vertebrates including mammals and birds: the long life span is associated with (a) low rates of mitochondrial reactive oxygen species production [39] and (b) low degree of fatty acid polyunsaturation of cellular membranes including the mitochondrial ones [24,40,41].

For a long time it was considered that dietary antioxidants or increases in antioxidant enzymes might increase the longevity of individual lives. However, numerous studies led to conclusion that increased antioxidant enzymes, nonenzymatic dietary antioxidants, lack the capacity to slow down aging. Independent of the way in which the antioxidants were manipulated, dietary or genetic, the result was the same: there was no effect of antioxidants on mammalian longevity [24]. To explain these data, Barja suggested that the close vicinity or even contact between the site of ROS generation and mtDNA avoids antioxidants to interfere with ROS induced final forms of irreversible damage in mtDNA [24].

Studies of many different mammals, various bird species, and some invertebrates, without finding a single exception, all led to the final conclusion that the total amount of unsaturated and saturated fatty acids does not change among species [24,41]. Instead, it is the unsaturation degree of the polyunsaturated fatty acids present what decreases from short- to long-lived animals [24]. To explain this conclusion, Barja suggested that: "since the low degree of unsaturation occurs both in mitochondrial and in total cellular membranes in long-lived animals, it can diminish lipoxidation-derived damage in various cellular compartments including the mitochondrial one where there is strong abundance of membranes" [24].

These important conclusions, presented by Barja [24], we will consider more closely because the current views on oxidative stress give no reasonable explanation to the following questions: what radical is responsible for the continuous and unescapable process of aging, why polyunsaturated fatty acids are important for the longevity, and why mitochondria are so important? We begin, for the sake of our discussion, with a brief consideration of the mitochondrial lipid composition.

Lipid composition of mitochondrial membranes

Biological membranes are made of phospholipids whose structure and properties depend on the properties of the polar head at C1 atom of the glycerol backbone, and two fatty acids at C2 and C3 carbons. In general, the phospholipid composition of the membranes is strongly associated with the membrane's functions. With the exception of phosphatidylethanolamine (PEA) and cardiolipin (CL), which cannot by themselves form the bilayer planar membrane, other phospholipids form planar membranes that surround the cell (cytoplasmic membrane) and other cellular organelles [42]. Mitochondria have two membranes: the outer mitochondrial membrane (OMM), which has pores permeable to compounds up to 1500 Da, and inside the relatively small volume limited by the OMM lies a huge "bag" limited by the inner mitochondrial membrane (IMM) with enormous surface that limits the inner space called matrix. With the exception of liver mitochondria, the matrix of mitochondria of most other organs represents a hard gel. The IMM forms numerous folds, called, cristae, and is impermeable to most low molecular weight compounds and ions, except water. The inner membrane contains very large amount of proteins inserted into the lipid phase on both outer and inner surfaces, as well as the integral proteins that penetrate through the membrane. Lipids comprise only 20-25% of the membrane's mass [42]. Because of many curves of the cristae and high concentration of protein complexes, the integrity of the inner membrane is maintained by PEA and CL, which have a conical form and can accommodate proteins into the curves [43].

In the tissues of mammals, PEA has more polyunsaturated fatty acids (PUFA), usually arachidonic acid (20:4 ω3) or docosahexaenoic acid (C22:6 ω6), in comparison with phosphatidylcholine. The amount of PEA in the mitochondrial membrane phospholipids varies strongly between the tissues: about 20% in the liver and 45% in the brain [44]. Cardiolipin is the only phospholipid that is present almost exclusively in the IMM. The "head" of the CL consists of a small glycerol molecule to which are attached two molecules of 1,2-diacyl-sn-glycero-3 phosphate (phosphatidic acid). This, seemingly simple structure of CL, provides mitochondria with the ability to fulfil its specific functions efficiently by keeping together proteins, participating in oxidative phosphorylation, as supercomplexes [44,45].

The ability of CL to accommodate negative curvatures explains why most of CL is present in the inner leaflet of the inner mitochondrial membrane (IMM) and interact non-covalently with different proteins. The list of proteins, which are associated with CL is very large, including respiratory chain complexes, ATP-synthase, ADP/ATP carrier (ANT), Pi-transporter and many other membranous proteins. In the outer leaflet of the IMM, CL is associated with cytochrome c and at the contact sites, of the IMM and OMM, with creatine kinase and ANT [46]. Cardiolipin is the major phospholipid of the IMM bearing negative charge, comprising on average 20% of all lipids [42]. However, because of the unique features of its structure, CL is concentrated in the inner leaflet of the IMM and thus concentrate the negative charges with overlapping Coulomb radii forming the so called antennae [47,48].

Recent findings revealed that mitochondrial functions strongly depend on the simultaneous presence of PEA and CL. Both phospholipids have a shape of conus and thus cannot form bilayer membranes. Therefore they easily organize into the hexagonal (HII) structures when fatty acids "look" outside. The negatively charged heads of cardiolipins strongly interact with the positively charged groups of proteins and peptides, whereas the lipid parts of PEA and CL interact with the phospholipids that form the bilayer membrane [43,49]. Mutation experiments have shown that when only one of the two phospholipids is missing, either PEA or CL, the organisms survive, although the mitochondrial functions are abnormal. The lack of CL result in disruption of interactions between complexes III and IV, which leads to slow electron transport and low membrane potential ($\Delta\psi$). The deficiency of PEA results in disrupted transport of proteins into mitochondria, slow electrons transport and low $\Delta\psi$. However, unlike the lack of CL, the deficit of PEA results in stabilization of the respiratory chain superstructures and even formation of megastructures [48,49]. It seems that the presence of PEA in the IMM is necessary for limitation of the ability of CL to organize proteins into superstructures and makes them functionally more flexible.

Polyunsaturated fatty acids (PUFA) in biological membranes

Arachidonic acid (C20:4, ω6)

Eicosatetraenoic acid, or arachidonic acid (AA), is a carboxylic polyunsaturated fatty acid with 20 carbon atoms and four double bonds. The 1st double bond is at the 6th C atom from the final (omega) $-CH_3$ group. According to the current nomenclature it is designated as C20:4, ω6 fatty acid.

Although, in principle, AA can exist in two possible configurations *cis*- and *trans*-, in the natural phospholipids AA is always present in *cis*- configuration. This means that the two hydrogen atoms at the double bonds (the allylic H atoms) stick out in the same direction. The rigidity of the double bonds freeze the conformations, and in the case of the natural *cis*- configuration the molecule of AA is strongly curved and has limited mobility (Figure 1). As a result, if a PUFA is part of a phospholipid, the molecules

of fatty acids in phospholipids cannot interact strongly, and thus cannot form a planar bilayer membrane, unlike the phospholipids containing saturated or mono unsaturated fatty acids, as in phosphatidylcholine. PEA is a typical phospholipid containing a PUFA (usually AA) at the C2 atom of the glycerol backbone. Because of the high content of PEA, the IMM is flexible and has very little cholesterol. This is because cholesterol binds tightly to the straight molecules of saturated and lightly curved monounsaturated fatty acids, such as in the cytoplasmic membrane. Cholesterol limits the mobility of fatty acids and thus makes the membrane rigid.

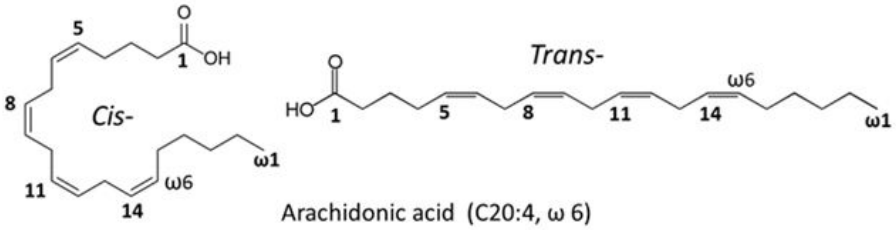

Figure 1: *Cis*- and *trans*- configurations of arachidonic acid.

Docosahexaenoic acid (C22:6 ω3)

Systemic name: all-cis-docosa-4,7,10,13,16,19-hexa-enoic acid. Docosahexaenoic acid (DHA) is a carboxylic polyunsaturated fatty acid with 22 carbon atoms and six double bonds. The 1st double bond is at the 3d C-atom from the omega C atom (-CH_3), that is C22:6, ω3. With six double bonds, DHA is curved into almost full ring. In humans, DHEA is an important structural component of mitochondria in brain, retina, skin, spermatozoa and ovum [52].

It should be noted that both AA and DHA normally are not present in cardiolipin, which has 4 fatty acids, usually linoleic acid (C18:2). However, recently it has been shown that during aging and experimental diabetes in animals the contents of AA and DHA in the mitochondrial CL may increase several-fold [5,53]. It is not yet clear, whether this remodeling of cardiolipin results from pathology, or is an adaptive mechanism against the pathological changes. In the next sections we explain the significance of PEA, CL and the PUFA for aging and pathology.

The special roles of PUFA

Due to the distinguishing features of their structure, polyunsaturated fatty acids, especially AA and DHA, have important regulatory functions in addition to their structural properties in the membrane phospholipids. The metabolic fates of PUFA in a cell strongly depend on whether they originated from exogenous sources (with food) or were released inside the cell by specific Ca-independent phospholipases A_2 (PLA_2) [55,56]. In humans, AA and DHA are synthesized from dietary precursors linoleic (C18:2 ω6) and α-linolenic (C18:3 ω3) acids correspondingly in a highly controlled way, and peroxisomes may be the primary site for degradation of PUFA when they are supplied in excess from the diet [56,57]. The signal-dependent release of AA and DHA from phospholipids by PLA_2 is usually associated with their metabolic transformations by specific enzymes to a number of biologically active molecules, such as prostaglandins, prostacyclins, tromboxanes, leukotrienes and others, which are important intracellular and extracellular local signalling molecules. The specific enzymatic transformations of PUFA are catalyzed by cyclooxygenases (COX1 and COX2), lipoxygenases (LOX) and epoxygenases, which produce from each molecule of a PUFA a single regulatory molecule with the certain structure and function. There is a large body of literature on numerous specific functions of eicosanoids as exemplified by recent reviewing articles [58-61].

In the middle of 70s of the last century, it was observed that during autoxidation of linolenic acid or prolonged storage of the human blood plasma at -20 °C, there were formed products similar to prostaglandins H and F_2. Pryor *et al.* [62] suggested that some of the complex symptoms of lipid peroxidation *in vivo* could be due to nonenzymatically produced prostaglandins or their stereoisomers. Almost 20 years later, Roberts and Morrow, from the Vanderbilt University, begun to explore a possibility of the nonspecific auto-oxidative formation of prostaglandins *in vivo* [63-66] and discovered the new nonenzymatic pathway of PUFA lipid peroxidation, which results in the formation of prostaglandin-like compounds with enormous variations in molecular positional- and stereo-isomerism in structure and biological activities.

A significant number of products of this type of autoxidation of PUFA have very high reactivity with lipids and proteins and the resulting products can be determined in the body's tissues and fluids as one of the most reliable and sensitive early markers of oxidative damages of lipids and proteins [67-69]. So far, however, there is no reasonable explanation of what causes this type of lipid peroxidation of PUFA. Here we discuss observations, that taken together suggested a new model of initiation of this type of lipid peroxidation with involvement of the perhydroxyl radical ($HO_2^{\bullet}$).

The isoprostane pathway of lipid peroxidation

The sequence of events, which led to discovery of the unusual lipid peroxidation, later designated as the isoprostane pathway of lipid peroxidation (IPLP), were described in a number of publications [63-66]. As the result of IPLP, one class of oxidation products formed in abundance *in vitro* and *in vivo* is the isoprostanes (IsoPs), which were named so because the first class of IsoPs

discovered contained F-type prostane rings analogous to PGF_2 [70].

There are several key distinctions between IsoPs and the cyclooxygenase derived prostaglandins (PGs). First, PGs are generated with side chains that are predominantly oriented *trans* to the prostane ring whereas IsoPs are formed almost exclusively with *cis* side chains, a more thermodynamically favorable configuration [64,71]. Although in some cases smaller amounts of PGs containing *trans* side chains can be formed via the IsoP pathway both *in vitro* and *in vivo* [72], however, PGs derived via the IsoP pathway are generated as a racemic mixture, whereas those formed by COX are enantiomerically pure [73]. The second, very important difference is that IsoPs are formed *in situ* from arachidonic and docosahexaenoic acids, which are esterified to phospholipids, and are only subsequently released by PLA_2, whereas PGs are generated only from free AA and DHA [74]. Molecular modeling of the IsoP-containing phospholipids reveals them as remarkably distorted molecules [65]. The third, most important difference is that IPLP greatly increases the number of possible stereo- and positional isomers of the resulting molecules (Figure 2).

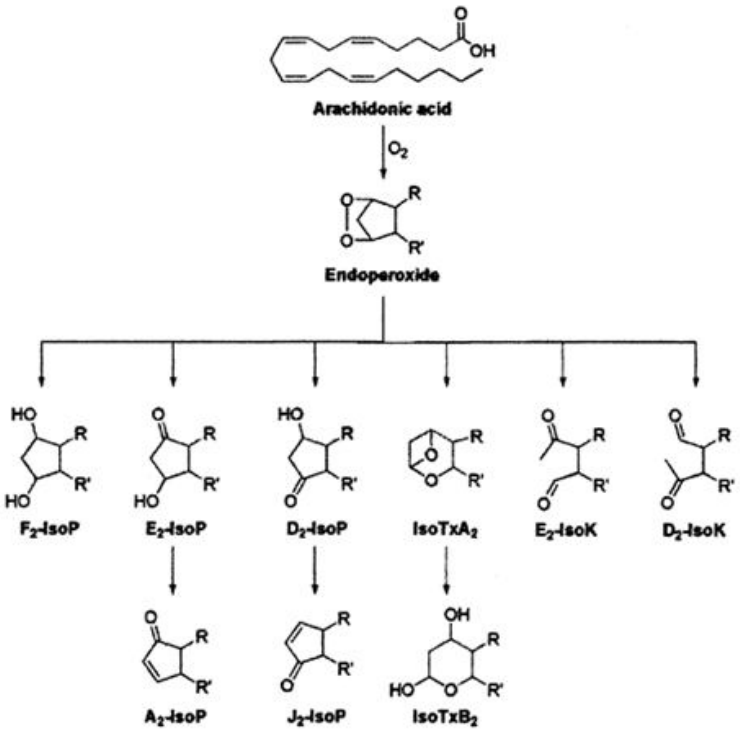

Figure 2: Autooxidation of arachidonic acid with rearrangement into different ring structures. Abbreviations: F_2-IsoP, E2-IsoP, D_2-IsoP are Isoprostanes with rings correspondingly F_2, E_2, D_2 or A_2 and J_2; $IsoTxA_2$ and $IsoTxB_2$ formed from Prostanglandin-H_2 (PGH_2); E_2-IsoK and D_2-IsoK are Isoketals with rings correspondingly E_2 and D_2 [71].

During the IPLP of AA, four variants of F_2-IsoP regioisomers can be generated; compounds are denoted as 5-, 8-, 12-, or 15-series regioisomers, depending on the carbon atom to which the side chain hydroxyl group is attached [73]. However, later it was shown that IsoPs possessing an F-type prostane ring (F_2-IsoPs) represent only a small part of possible variants of isoprostanes, when IsoP endoperoxide intermediates undergo reduction, which to a large degree depends on the presence of GSH (reduced glutathione) [75]. In the absence of conditions for reduction, which typically occurs in brain, the IsoP endoperoxide intermediates undergo isomerization to form D/E-type (D_2/E_2-IsoPs) and other types of prostane rings, as shown on Figure 2 [73,75]. D_2 and E_2 rings also have four types of regioisomers of isoprostanes each consisting of 8 diastereomers. Moreover, E_2 and D_2 rings can be transformed to A_2 and J_2 ring isoprostanes, and both E_2 and D_2 prostane rings can be opened to form correspondingly E_2 and D_2 γ-ketoaldehydes, which were named correspondingly E_2- and D_2-isoketals (Figure 2). Thus for each of the four series of regioisomers can be formed 64 variants of isoprostanes or isoketals because the starting molecules have several stereologically active centers.

γ-Ketoaldehydes, one of the products of IPLP, are highly reactive molecules that, still being part of the intramembrane phospholipid, can form adducts with primary amines of the lysine-containing proteins and phosphatidylethanolamine. The most active among γ-ketoaldehydes formed from AA via the IPLP are isolevuglandins (IsoLG). From four IsoP bicyclic endoperoxide regioisomers can be formed four E_2-IsoLG and four D_2-IsoLG regioisomers. Each E_2-IsoLG and D_2-IsoLG regioisomer is theoretically comprised of four racemic diastereomers [76]. IsoLGs are so reactive, that were revealed only as adducts with proteins. A number of IsoPs have also been found to possess potent biological activity and thus likely are also mediators of the oxidant injury, and some may perform abnormal cellular signaling [70].

In recent years, additional related compounds, derived from various PUFA such as eicosapentaenoic acid (EPA) [77] and DHA [74], have been discovered as products of the IPLP [73]. Because DHA is present in a larger quantity in neurons, the products of IPLP were correspondingly named neuroprostanes and neuroketals. However, since DHA contains 6 unsaturated bonds, they form 6 regioisomers of endoperoxides and the number of possible stereoisomers for each regioisomer increases up to 254.

These unusual features of IPLP, listed above, raise a number of questions: First, what are exactly the differences between IPLP and the "classical" lipid peroxidation described in the literature? Second, what mechanism is responsible for such enormous diversity of stereo- and positional isomers of the final product of PUFA during the IPLP? Third, what radical (or radicals) is (are) responsible for initiation of IPLP?

Differences between the "classical" lipid peroxidation and the isoprostane type lipid peroxidation

The well-known lipid peroxidation (LP), which we designate here as "classical", was described in the literature, for example in a recent review by Repetto *et al.* [78]. A comparison of this "classical" LP with the established properties of the IPLP reveal several major differences. 1) The autoxidation of AA during IPLP occurs while AA and DHA are esterified to phospholipids, that are inside the hydrophobic milieu [66], whereas "classical" LP occurs either at the border of the hydrophilic and hydrophobic phases (membranes or micelles), or in the water/ethanol mixture [79-81]; 2) The "classical" LP includes fatty acids with one or two unsaturated bonds, whereas IPLP oxidizes only PUFA with three or more double bonds; 3) The products of the IPLP have extremely high positional and stereo isomerism in their structure, whereas products of the "classical" LP are rather limited in their structure; 4) Unlike the "classical" peroxides, the products of IPLP are not prone to induce chain reactions in peroxidation of other lipids, but react highly specifically with proteins and phosphatidylethanolamine; and 5) LP initiated by hydroxyl radicals and/or H_2O_2 (in the presence of xanthine oxidase) is inhibited by superoxide dismutase or catalase. When added to the above system, pure AA is co-oxidized, which is not stimulated by the presence of hydroperoxides [80]. On the contrary, the IPLP is highly activated in the presence hydroperoxides [62,70]. A thorough discussion of differences between the two types of lipid peroxidation can be found in [82].

What radicals are responsible for initiation of the IPLP?

The question of the radical(s) responsible for initiation of the IPLP is not usually discussed in the papers on isoprostanes. Potentially, there are several powerful oxidants, such as hydroxyl radical ($^{\bullet}OH$), perhydroxyl radical ($HO_2^{\bullet}$), nitrogen dioxide ($^{\bullet}NO_2$), peroxynitrite radical ($^{\bullet}OONO$) and peroxynitrate radical ($O_2NOO^{\bullet}$) that can initiate lipid peroxidation by abstraction of hydrogen atoms in PUFA, such as AA and DHA.

The fact that IPLP oxidizes AA and DHA when they are still esterified with phospholipids, that is in the fully hydrophobic environment, imposes restrictions on most radicals, because they are charged and thus insoluble in lipids, or not sufficiently active in reaction with PUFA, like nitric oxide radical ($^{\bullet}NO$) [79]. Superoxide radical ($O_2^{\bullet}$), when formed in the mitochondrial membrane, is rapidly removed into the matrix or intermembrane space [80]. In addition, $O_2^{\bullet}$ very poorly interacts with PUFA and amino acids [80,85]. Peroxynitrite, as well as peroxynitrate, react relatively slowly with most, but not all, biological molecules, making these two radicals rather selective oxidants. Both $^{\bullet}OONO$ and $O_2NOO^{\bullet}$ modify tyrosine in proteins to create nitrotyrosines, leaving a footprint detectable *in vivo* [83,86,87]. The most active radical in initiation of LP, at least in the in vitro system, the $^{\bullet}OH$, indeed is so active, that it reacts within 1 to 5 molecular diameters of their site of formation [88]. Pryor [88] have pointed out, that $^{\bullet}OH$ reacts with free linoleate with rate constants that are nearly diffusion controlled. Therefore, the lifetime of $^{\bullet}OH$ radicals have been estimated 10^{-9} sec. It should be noted that $^{\bullet}OH$ radical would react with any reactive species in its neighborhood with this rate constant, so the choice of substrate is not critical. Water is present at high concentration in all compartments of a cell, but radicals do not, in general, react with water [88]. The above considerations leave us with the only plausible candidate for initiation of the IPLP, the perhydroxyl radical ($HO_2^{\bullet}$).

Properties of the perhydroxyl radical ($HO_2^{\bullet}$)

Perhydroxyl radical is a protonated form of superoxide radical and has molecular formula $HO_2^{\bullet}$, and it is much more active chemically than $O_2^{\bullet}$, and is always present in the cell due to reversible reaction $O_2^{\bullet} + H^+ \rightarrow HO_2^{\bullet}$ with pKa = 4.88. Thus it was widely accepted that at pH in the cytoplasm of 7.2, much less than 1% of [$O_2^{\bullet}$] is present as $HO_2^{\bullet}$ [89]. Probably for this reason, many researchers assumed that $HO_2^{\bullet}$ has little or no role in initiation of LP [90]. However, as was stressed by several authors, the pH values in the microvolumes very close to the charged membranes may be several units lower than in the bulk volume of a cell [85,92]. This may occur particularly around the negatively charged heads of phospholipids, such as cardiolipin, phosphatidylserine and phosphatidylinositol, which may retain protons both at the matrix side, which has a negative charge, and at the inter-membrane side, to where protons are released by the respiratory proton pumps. Therefore, $HO_2^{\bullet}$ can come into being on both sides of the inner membrane, and because $HO_2^{\bullet}$ has no charge, it can easily go back into the lipid core of the membrane and even cross it [92].

It should be also kept in mind, that on both sides of the IMM protons have much higher mobility by the Grotthuss mechanism in the few layers of structured water molecules close to the charged surfaces of the membrane [93,94]. All events happen at the interfaces of the IMM in matrix and cytosol.

In sharp contrast to the reductant superoxide ($O_2^{\bullet}$), $HO_2^{\bullet}$ is a powerful oxidant. This follows from their redox potentials. For reaction of $O_2^{\bullet}$ formation: $O_2 + e^- \rightarrow O_2^{\bullet}$, $E^0 = -0.33$ V; whereas for the reaction of $HO_2^{\bullet}$ formation: $H+ + O_2^{\bullet} + e \rightarrow HO_2^{\bullet}$, $E^0 = 1.0$ V

[91]. Therefore, in comparison with other oxygen radicals, $HO_2^{\bullet}$ shows very high specificity in reaction with PUFA. Bielski *et al.* [80] studied reaction of $HO_2^{\bullet}$ with linoleic (C18:2), linolenic (C18:3), and AA (C20:4) in water-alcohol solutions by the stopped flow technique. The corresponding rate constants (k) were: 1.2 x 10^3, 1.7 x 10^3, and 3.0 x 10^3 M/sec. The kinetic parameters of reactions showed that $HO_2^{\bullet}$ reacts with a double allylic H atom of the PUFA with rate constants proportional to the number of double bonds. Thermodynamic approximations indicated that the reaction is exothermic by approximately 10 kcal/mol. [80]. This indicates that when $HO_2^{\bullet}$ encounters PUFA it reacts with it irreversibly, with very high probability and very fast.

The suggested mechanism of initiation of the isoprostane pathway lipid peroxidation by $^{\bullet}HO_2$

Figure 3 presents schematically the currently proposed mechanism of IPLP of AA induced by $^{\bullet}OH$ or unknown radical. The figure was acquired from the Internet articles "Isoprostanes" by W. Christie [96,97]. In the original figures of both articles (in 2016), the first H atom was shown as being abstracted from AA by $^{\bullet}OH$ radical, and the mechanism of abstraction of other two hydrogen atoms was not designated. In 2017 the "$^{\bullet}OH$" radical was removed from one of the figures [96], but not from the other [97]. The problem is that hydroxyl radical ($^{\bullet}OH$) is not hydrophobic, therefore it cannot by itself appear near arachidonic acid inside the phospholipid core.

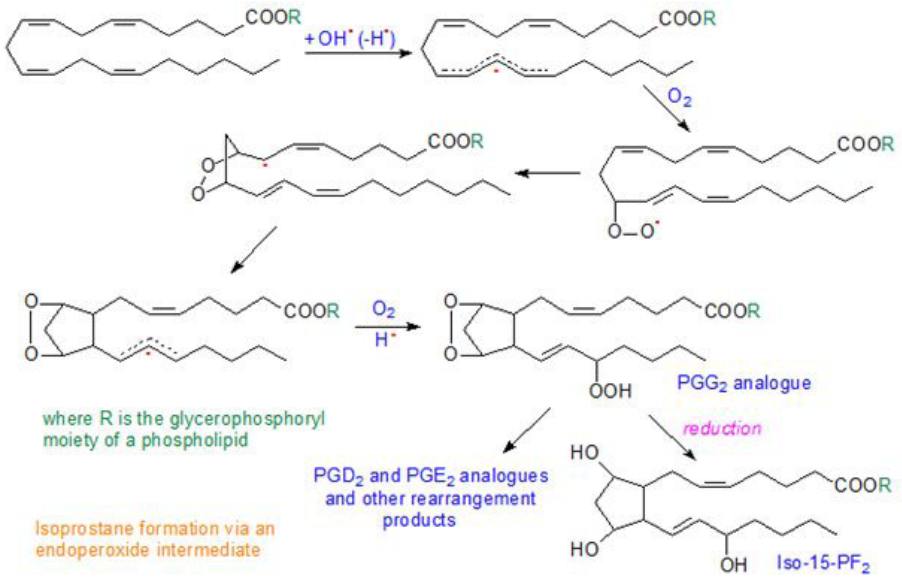

Figure 3: Biosynthesis of isoprostanes: Synthesis of isoprostanes in animal tissues *in vivo* is brought about by a series of free radical-catalyzed reactions, most of which do not involve enzymes, and any fatty acid with three or more double bonds can be a substrate. The main route via an endoperoxide intermediate is illustrated below with the synthesis of 15-F_2-IsoP isomers as the example. The figure was acquired from [96,97].

We suggest that the first hydrogen from AA is abstracted by the perhydroxyl radical, which is highly hydrophobic and stable enough to reach a polyunsaturated fatty acid inside the membrane's phospholipid core [85, 99]. Abstraction of the first allylic (or vinylic) hydrogen atom from AA [80] in the hydrophobic environment results in the conversion of $HO_2^{\bullet}$ to hydrogen peroxide. However, in the hydrophobic milieu, the relatively large molecule of H_2O_2, with the stretched -O—O- bond is highly unstable, therefore it undergoes homolytic cleavage to two $^{\bullet}OH$ radicals [99,100], which instantly and randomly abstract another two hydrogen atoms from the AA, producing two molecules of water (Figure 4).

$$HO_2 + (H)_1 \rightarrow [H_2O_2] \rightarrow 2\,[OH^*] + (H)_2 + (H)_3 \rightarrow 2\,H_2O$$

Figure 4: The suggested sequence of transformations of perhydroxyl radical during its reaction with a PUFA molecule inside the lipid phase of the membrane: The abstracted H atoms are shown in red. The square brackets indicate that the compound is highly unstable. The abstraction by $HO_2^{\bullet}$ of the first (presumably allylic) hydrogen (H_1) from the double bond results in the formation of H_2O_2, that instantly undergoes homolytic cleavage to two molecules of $OH^{\bullet}$ radicals that instantly abstract two hydrogens (H_2 and H_3) from the double bonds resulting in formation of two molecules of H_2O. The extremely fast abstraction of three hydrogen atoms from any two double bonds creates a highly unstable molecule, which very rapidly and randomly reacts with two molecules of O_2 and undergoes intramolecular rearrangements, which results in a large number of positional- and stereoisomers. The more PUFA has double bonds, the larger is the number of positional- and stereoisomers. The figure was acquired from [98].

In the reaction shown above, the abstraction of all hydrogen atoms more likely occurs extremely fast, almost simultaneously, as one chain reaction, before two O_2 molecules join the remaining skeleton of AA as shown in Figure 3. Because rapid abstractions of the three H atoms occur randomly at any double bond, this make the molecule of fatty acid highly unstable, and thus O_2 molecules bind randomly with formation of variants of regioisomers in accordance with the number of double bonds in the parent PUFA,

and the following intramolecular rearrangements also occur randomly with formation of different variations of the final product. Thus, initiation of lipid peroxidation of PUFA by $HO_2^{\bullet}$ in the hydrophobic milieu of the membrane results in abstraction of three H atoms and the loss by a PUFA of two double bonds. Figure 5 shows a comparison of parent molecules of AA and DHA with the structures of resulting correspondingly an isoprostane and a neuroprostane.

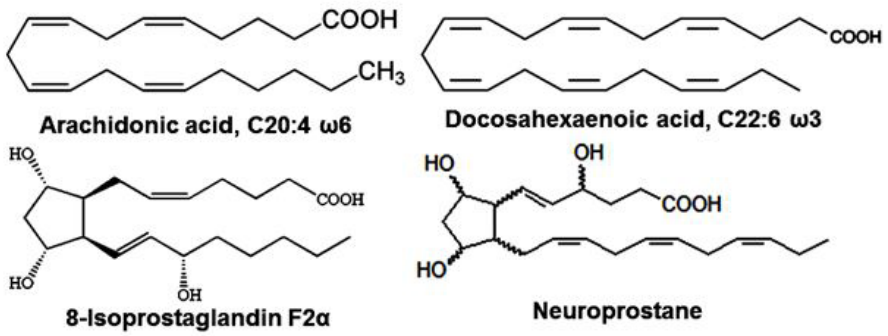

Figure 5: Examples of the parent and the corresponding product molecules resulting in the loss of two double bonds during nonenzymatic IPLP. During IPLP, the parent arachidonic and docosahexaenoic acids lose two unsaturated bonds. Images of the parent and one of the product molecules were taken from references [66,73].

All radicals have *cis* configuration relatively to the cyclopentane ring. Evidently, the direction of intramolecular transformations will depend on the surrounding local conditions in the membrane. For example, higher O_2 content in the lipid phase of the membrane results in the enhanced formation of Isofurans, whereas availability of reduced glutathione will produce more $IsoPF_2$ and so on [71].

The principal distinction between the *in vitro* conditions described for $^{\bullet}OH$ and $HO_2^{\bullet}$ radicals in water-ethanol solution of AA [10,39], and the interactions of $HO_2^{\bullet}$ with AA inside the membrane, shown in Figures 3 and 4, is that in the membrane reactions proceed in the completely hydrophobic environment. According to Gebicki & Bielski [85], in the water-ethanol medium the reaction proceeds in consent with the reaction sequence of the "classical" LP, and abstraction of the first hydrogen atom from linoleic acid results in formation of hydrogen peroxide: $LH + {}^{\bullet}HO_2 \rightarrow L^{\bullet} + H_2O_2$, which undergoes heterolytic cleavage with the final formation of the stable end product linoleic hydroperoxide: $LOO^- + LH^{\bullet} \rightarrow LOOH + L^{\bullet}$ [88]. Similar abstraction of hydrogen atom from linoleic acid by $^{\bullet}OH$ results in the conversion of the $^{\bullet}OH$ radical to water: $LH + {}^{\bullet}OH \rightarrow L^{\bullet} + H_2O$.

Bielski *et al.* [80] have shown that reaction of $HO_2^{\bullet}$ radical with a double allylic H atom of a PUFA is exothermic by approximately 10 kcal/mol, and the reaction is directly proportional to the number of double allylic H atoms. The latter appear to be the targets for $HO_2^{\bullet}$ attack, which makes PUFAs highly specific targets with high affinity for perhydroxyl radicals [80,85]. Such selectivity was not observed in similar studies with hydroxyl radicals, which abstract H atoms randomly. Following the reaction of $HO_2^{\bullet}$ with linoleic, linolenic, and arachidonic acids in water-ethanol solution, the chain formation of a stable product was observed on a relatively slower time scale. In the earlier study on linoleic acid, this product was identified by thin layer chromatography as the corresponding hydroperoxide [80]. Studies on IPLP suggest that in a fully hydrophobic environment reaction of $HO_2^{\bullet}$ with PUFA is extremely fast and produces a racemic mixture of a large number of positional and stereoisomers [73,76].

Factors promoting formation of perhydroxyl radical in mitochondria

From the reversible reaction $O_2^{\bullet} + H+ \rightarrow HO_2^{\bullet}$ (pKa = 4.88) it is clear that formation of $HO_2^{\bullet}$ is primarily dependent on the amount of the superoxide radical in mitochondria. The secondary determinants are pH and mobility of protons at the sites of $O_2^{\bullet}$ release from the membrane, which determine availability of H+ for the above reaction. This is important because both cytosol and matrix, in particular, are highly structured and interactions between $O_2^{\bullet}$ and H+ occur locally at the membrane's surface.

According to the currently accepted view, at physiological pH of 7.2 in the bulk of the cytosol, less than 1% of any $[O_2^{\bullet}]$ formed will be present as $HO_2^{\bullet}$ [101], and close to zero in the matrix with pH about 8 or higher. However, during the last three decades it was clearly shown that after proton release by an integral membrane protein, long-range proton transfer along the membrane surface is faster than proton exchange with the bulk water phase, and that protons can efficiently diffuse along the membrane surface between a source and a sink (for example ATP synthase) without dissipation losses into the aqueous bulk [102-105]. The electrostatic barrier is higher for monovalent anions moving toward the surface of IMM than for monovalent cations [106]. It was suggested that water structuring at the interface seems to be mandatory for both providing the high H+ conductivity and for generating the energy of the kinetic barrier opposing equilibration with bulk pH [107].

Formation of the pH gradient between the bulk of a compartment and the IMM surface, due to the kinetic barrier for proton transfer from the respiratory proton pump vicinity to the bulk phase [102,103,105], can cause an elevation of the proton concentration at the interface [85,103,106]. Taking typical values for the density of proton pumps and their turnover rate, Cherepanov *et al.* [106] calculated that a potential barrier of 0.12 eV yielded at the surface of the IMM a steady-state pH of approximately 6.0 units, and this value of pH was independent of pH in the bulk water phase under neutral and alkaline conditions. The efficiency of the

anomalously fast lateral diffusion decreased gradually with an increase in mobile buffer concentration suggesting that structural diffusion is physiologically important for distances of approximately 10 nm [93].

Upon its formation inside the inner membrane, $O_2^{\bullet}$ leaves the membrane, and immediately, at the membrane's interface, where pH is lower and proton conductivity is high, [85,106,108], the concentration of $HO_2^{\bullet}$ may be much higher than suggested earlier [85,108]. Evidently, the concentration of protons at the IMM surface would be particularly high where the negative charges are concentrated. In most biological membranes about 10%–20% of the lipids bear a net negative charge, whereas positively charged lipids are extremely rare [109]. In mitochondria the major negatively charged phospholipid is cardiolipin [110,111]. Cardiolipin constitutes up to 20% of all phospholipids of the IMM, and 80% is concentrated in the inner leaflet of the IMM [42,111]. Cardiolipins normally contain less PUFA and form rafts with imbedded large proteins, such as ATP-synthase-ANT complexes and respiratory complexes, allowing rearrangements of the superstructures consisting of protein complexes participating in oxidative phosphorylation [109,112,113].

Because cardiolipin specifically interacts with the respiratory complexes of mitochondria, there are areas within the membrane with high contents of cardiolipin. The Coulomb radii of the negatively charged phosphates of the cardiolipins can overlap creating a stable negative charge over some areas of the inner membrane, the so called antennae [113,114]. At these areas on both sides of the inner membrane the concentration of protons can be significantly higher and independent of pH in the bulk of the compartment [102-106]. In addition to extending the capture radius for protons in solution, the antenna also facilitates the protonation rates of components located at the membrane surface close to the antenna [115,116]. Evidently, the chance of increased formation of $HO_2^{\bullet}$ at the rafts supporting respirosomes is much higher than it was assumed. Release or diffusion of $O_2^{\bullet}$ close to the membrane interface, where the negatively charged antenna is located and thus pH may be lower up to 3 pH units, results in the higher local concentration of $HO_2^{\bullet}$ that may return back to the lipid phase of the membrane [85,106,113].

The suggested chemical reactions of $HO_2^{\bullet}$ and PUFA, shown in Figure 4 belong to a class of multi-stage homogenous-heterophase chain reactions when reagents and products belong to different phases, but the reaction occurs in one phase [117]. This type of reactions with $HO_2^{\bullet}$ are currently investigated by researchers working with chain reactions in organic chemistry of polymers [118] and combustion of fuels [100,119,120].

Consequences of the $^{\bullet}HO_2$ activated IPLP

It is known that a correct hypothesis must give answers to a number of important related questions. Pryor *et al.* [62] stressed that any suggested mechanism, regarding autoxidation of PUFA must explain the fact that only lipids containing three or more double bonds not only undergo autoxidation, but is also accompanied with formation physiologically active prostaglandins, along with many other stereo- and positional isomers. Our hypothesis, presented in this paper and elsewhere [98], just gives such an explanation and also answers some other questions. To our opinion, the HO_2• hypothesis gives explanation to the Barja's conclusion that only two known factors correlate inversely with animal longevity in vertebrates including mammals and birds: (a) the rate of mitochondrial reactive oxygen species production [39], and (b) the degree of fatty acid unsaturation of tissue cellular membranes including the mitochondrial ones. [24,40,41,121]. Andreev *et al.* [38] raised another important question: what radical or radicals is/are responsible for the persistent and inevitable process of age related accumulation of hazardous changes in cells and organs. And still, another question also requires explanation: why our body ages unevenly, some organs, such as brain, heart and skeletal muscles, age faster and are more prone to the age related diseases than, say, liver or kidney. So, the correct hypothesis must answer

At the present, the problem with the MFRTA is that none of the suspected radicals could answer all the above questions. The limitations of many free oxygen radicals: superoxide radical, hydrogen peroxide, hydroxyl radical, nitric oxide, peroxynitrite and peroxynitrate, as well as ozone, triplet and singlet oxygen, were thoroughly discussed in a number of reviews, [86,87,122]. Every of the listed radicals under certain conditions, or in a specific tissue, may have hazardous effects and contribute to aging and disease. Some radicals cause damages in a rather specific manner. For example, nitric oxide and peroxynitrite have hazardous effects, which are mostly limited to endothelium of blood vessels and tissues with high blood supply. This is because formation of one molecule of $^{\bullet}NO$ requires two oxygens, and the half-life of $^{\bullet}NO$ in vivo is about 7seconds. Therefore in order to maintain the steady-state [$^{\bullet}NO$] of 1 μM, it is required 120 nmol O_2 per 1 gram of tissue per 1 minute, which is a very large amount of oxygen [123]. That is why $^{\bullet}NO$ is a specific signaling molecule in endothelial cells and is a powerful vasodilator of arterial blood vessels rich with oxygen. Atherosclerosis is the major consequence of this tissue specificity of nitric oxide and peroxynitrite radicals, when damaged endothelial membranes begin accumulating cholesterol. Other examples of tissue specificity caused by oxygen are the biological effects of singlet oxygen and ozone. Although singlet oxygen ($O_2(a^1\Delta_g)$) is not a radical, it is usually regarded as one, because it has damaging effects on some biological pigments: rhodopsin, flavins and porphyrins, that being exposed to bright light in the presence of O_2 may effectively promote formation of the excited singlet oxygen and thus cause eye problems and accelerate skin aging [99]. Ozone at high concentrations also may stimulate lipid peroxidation and formation of ozonides at the surface membranes of skin and lungs [62].

Other known oxygen and nitrogen radicals are either not active enough ($O_2^{\bullet}$), or too active and have very short lifetime ($^{\bullet}OH$), or circumstantial ($O_2NO^{\bullet}$). All charged radicals are instantly expelled from the membranes. This leaves perhydroxyl radical ($HO_2^{\bullet}$) as the only suspect capable of activating IPLP.

The possibility that $HO_2^{\bullet}$ may be considered as the major damaging radical in oxidative stress has been raised by several authors during the last three decades [79,80,87,106,120,123-126]. The issue was thoroughly discussed in a critical review by De Grey [108]. It should be mentioned also that, until relatively recently, the isoprostane pathway of lipid peroxidation was also not widely appreciated by researchers studying oxidative stress. However, for some reason, even at the present, the role of $HO_2^{\bullet}$ in development of oxidative stress still remains beyond consideration by most researchers working in the field and has not even been mentioned in recent reviews [38,84]. To our opinion, this may be associated with the fact that the existing explanations of the mechanism of $HO_2^{\bullet}$ actions as a radical did not added something principally new, as compared with other, much more common, oxygen radicals that also initiate lipid peroxidation.

The leading role of IPLP as the universal aging mechanism

New studies have demonstrated that IsoPs are the most early and reliable among available markers of lipid peroxidation *in vivo*, and recent studies examining IsoP formation provided valuable information about participation of IPLP in pathogenesis of numerous human diseases [67-69,81,127]. According to our model of IPLP initiation by $HO_2^{\bullet}$, the perhydroxyl radical upon encounter with a PUFA very rapidly reacts with it and practically stoichiometrically produces one of many variants of isoPG, iso-γ-ketoacids or iso-levuglandins. Basing on our model, it becomes clear that regardless how small is production of $HO_2^{\bullet}$, it will cause some damages to the mitochondria or other cellular membranes either via impairing the cell's signalling, or directly via formation of adducts with phosphatidylethanolamine or lysine-containing proteins. Even though the level of $HO_2^{\bullet}$ formation may be very low, it is, probably, the major mechanism of small, but persistent, accumulation of damages and regulatory noises, which we designate as aging. Initially, the damages evoked by $HO_2^{\bullet}$ evidently stimulate a series of hormetic mechanisms that for some time protect the organism from severe dysfunctions [16].

The rate of aging is proportional to the rate of ROS production

Since mitochondria in cells are the major producers of superoxide radical, and thus also perhydroxyl radical, they play the most important role in aging and aging-dependent diseases. It is known that maximal mitochondrial oxidative capacity declines with age, while reactive oxygen species production increases [16,128,129]. In this respect, however, in the literature there are some misconceptions, which have to be clarified. For example, in a recent review, Gonzalez-Freire *et al.* [16] stated a rather common view that progressive mitochondrial dysfunction is considered as a hallmark of aging, and impaired mitochondrial function causes an accelerated aging phenotype, which is particularly evident in high energy demanding tissues such as brain, heart, and skeletal muscle, and in kidney and liver, two organs with essential metabolic roles. Here we see some hidden controversy between "progressive mitochondrial dysfunction" and "accelerated aging phenotype", which presumes the higher rate of ROS production by dysfunctional mitochondria. To illustrate the above notion, the authors also provided an interesting figure (see Figure 6). The legend to the original figure 1 stated: "Tissue-specific oxygen consumption rate. Impaired mitochondrial function may cause an accelerated aging phenotype mainly in high energy demanding tissues such as brain, heart, and skeletal muscle, and in kidney and liver, two organs with essential metabolic roles" [16].

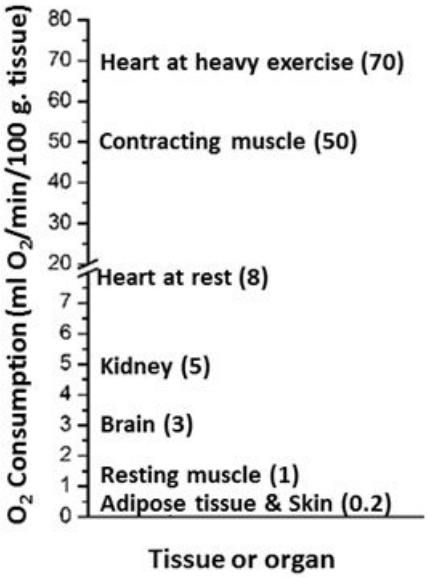

Figure 6: Tissue-specific oxygen consumption rates at various functional states. The figure was adapted from [16]

For the sake of comparison and discussion, we present also data from the classical review by Rolfe & Brown [130], which is very often referenced with a phrase: "although brain comprises only 2% of the body's mass (human), it consumes 20% of the body's total O_2 consumption at rest". As we see, both from Figure 6 and Table 1, in the resting human or rat, the liver, kidney heart and brain have the highest rates of respiratory activity, while resting skeletal muscles have low O_2 consumption per mass unit. For our

discussion of aging, of particular importance is the following metabolic property of the heart, skeletal muscle and brain: upon functional loads, these organs can increase the respiratory rates several fold, particularly the skeletal muscle. Although liver and kidney have high rates of respiration, the functional activities of these organs remain relatively constant at all times. It is not the respiratory activity *per se*, that influences aging, but the difference in oxygen consumption between the resting and functionally active states.

Organ	Human			Rat		
	% Body mass	% O_2 use	OCI	% Body mass	% O_2 use	OCI
Liver	2	17	**8.5**	5	20	**4**
Kidney	0.5	6	**12**	0.9	7	**7.8**
Heart	0.4	11	**27.5**	0.5	3	**6**
Brain	2	20	**10**	1.5	3	**2**
Skeletal Muscle	42	20	**0.48**	42	30	**0.7**

OCI (Oxygen consumption index) = % O_2 consumption / % Body mass.
Table 1: Contribution of the major oxygen-consuming organs of the body to body mass and standard metabolic rate [126]

At old age, liver and kidney (in the absence of non-aging diseases or intoxications) are usually not the prime targets for medical attention, as compared with the heart, brain and skeletal muscle. Let us look more closely at the mechanisms of $O_2^{\bullet}$ production, which is the source of $HO_2^{\bullet}$, that causes different organs age at different rates.

Evidently, formation of $HO_2^{\bullet}$ is proportional to the level of $O_2^{\bullet}$, which at any moment is determined by the rates of its production and elimination [84]. Taking into consideration that $HO_2^{\bullet}$ is extremely reactive and dangerous, it is understandable that removal of superoxide radicals is of paramount importance for defending cells from the damaging effects of its protonated form - $HO_2^{\bullet}$. Therefore the activities of Mn-SOD2 and Cu,Zn-SOD1 are of most importance for the heart and the central nerve system, where alternative antioxidant systems are relatively weak, whereas the contents of AA and DHA are at the highest [111]. Because $HO_2^{\bullet}$ interacts with PUFA inside the membranes, it is clear that any type of antioxidants will have no effect on the aging caused by $HO_2^{\bullet}$, but will have effects on aging processes caused by other radicals

Because local cellular conditions determine what type of the final IPLP products (isoprostanes, isofurans or isoketals, etc.) will predominate, together with different rates of superoxide and $HO_2^{\bullet}$ production, the IPLP might also explain why different organs and tissues have different rates of aging. For example, accumulation of lactic acid during high physical loads and mild hypoxia may increase oxidative damages to skeletal muscle cells due to acidification-induced higher levels of $HO_2^{\bullet}$ production [132].

Experiments with isolated brain mitochondria have shown, that during oxidation by complex I of NADH, which is generated by various dehydrogenases, the rate of ROS production (as superoxide radical) is slow and occurs at the FMN and FAD of complex I and complex II (SDH) respectively, and does not depend on the energy state of mitochondria [132]. Generation of ROS increases several fold in resting mitochondria (State 4) oxidizing glutamate + rotenone [132-134]. Increased $O_2^{\bullet}$ production is also observed during oxidation by brain mitochondria of succinate together with glutamate or pyruvate [132]. This is because at high membrane potential ($\Delta\Psi$) the reverse electron transport (RET) reduces the ROS producing sites [132,133]. Upon addition of ADP or uncoupler, or otherwise activation of dissipation of mitochondrial membrane potential, production of ROS becomes decreased to the basic level with NADH. To stop production of ROS on complex I, it is sufficient to decrease $\Delta\Psi$ by 20-30 mV [132-134]. That is why at all times actively functioning mitochondria in the liver and kidney produce less ROS, and thus much less $HO_2^{\bullet}$, which induces IPLP. In the brain, most mitochondria are localized at the synaptic junctions and do not have other functions except providing ATP for restoration of ion gradients across the postsynaptic membrane disturbed during neuronal activation. Therefore, in the resting brain mitochondria become hyperpolarized and increase production of ROS via RET [132]. This was particularly evident in experiments with transgenic rats expressing mutated SOD1 gene [135,136].

In the heart and skeletal muscle mitochondria, the capacity for maximal oxidative phosphorylation manifold exceeds the rates of the resting respiration, and thus also tends to stimulate the RET and the associated ROS production at low functional loads. Skeletal muscle mitochondria are much less studied in this respect, as compared with the brain and heart mitochondria, but having the 50-fold difference between the resting and maximal respiratory rates, they more likely behave similar to the heart mitochondria, which have a 9-fold difference (Figure 6). In our studies, we have found that brain and heart mitochondria have a shutter mechanism, which reduces or even prevents the RET-dependent ROS production, when mitochondria are at rest. The mechanism consists in the strong inhibition of succinate dehydrogenase (SDH), which is also known as Complex II, by oxaloacetate [133,137]. We have recently described this mechanism in detail [138], and it strongly varies between various organs and animal species [136,137]. What is important, that upon activation of the specific function: the work load in the heart and neuronal activation, the increased inflow of alternative fuel substrates, such as activated fatty acids, glutamate or pyruvate, the SDH inhibition is instantly released and oxidative phosphorylation is activated [132,136,139].

During aging, mitochondrial dysfunctions, that could lower membrane potential and thus diminish production of $O_2^{\bullet}$ and $HO_0^{\bullet}$ develop gradually, and of much more importance for the age-dependent activation of IPLP is the fact that among humans many individuals also lower their physical and often also mental activities. This results in the average increase of the membrane potential in the heart, skeletal muscle and brain mitochondria, which promote further ROS-dependent aging and associated pathologies. This is the reason why moderate physical activities at any age helps not only maintain fitness and health, but evidently also slows down aging at the organ's level. Maintenance of mental activities is also important for prevention of Alzheimer's disease.

Metabolic changes during ontogenesis and aging

There is, however, another highly important factor, which also affects the age-dependent increase of ROS production during aging of humans. At certain age, the energy metabolism begins to utilize more and more fatty acids as substrates for mitochondrial respiration. This is particularly evident in women at the post menopause period. Many elderly people develop characteristic changes in body structure and metabolism, which is designated as the metabolic syndrome. [140]. Mitochondria at all ages generally utilize mixtures of substrates specific for every organ, and the composition of the substrates depends on the age-dependent hormonal pattern [132,139] and the mitochondrial haplotype [141,142]. The metabolic syndrome is characterized by increased utilization of fatty acids, which in the presence of other mitochondrial substrates: glutamate, pyruvate or succinate dramatically increases generation of ROS by brain and heart mitochondria [133,139].

Conclusions

The hypothesis on the mechanism of initiation of IPLP by perhydroxyl radical, presented in this review and elsewhere [98], provides reasonable explanation for the Barja's conclusion that the rate of ROS production and the amount of PUFA in animal tissues are the only parameters that inversly correlate with the longevity in various species of animals, and birds [24]. Inclusion of the IPLP into the mitochondrial free radical theory of aging (MFRTA) grants additional strength to the theory and opens new possibilities for understanding mechanisms of aging. However, it was unclear what mechanism drives IPLP. We describe the mechanism of initiation of IPLP, which involves the reaction of $HO_2^{\bullet}$ radicals with PUFA in the hydrophobic milieu inside of the mitochondrial membrane, which belongs to a class of multi-stage homogenous-heterophase chain reactions when reagents and products belong to different phases, but the reaction occurs in one phase [117]. The extremely high reactivity of $HO_2^{\bullet}$ with PUFA is the cause of slow and persistent accumulation of damages in the membranes and the membrane associated proteins, even though the concentration of $HO_2^{\bullet}$, relative to superoxide radical, may be very low. Of course, $HO_2^{\bullet}$ is not the only radical, which causes aging and oxidative stress, however, it provides a new look at the problems of oxidative stress that could not be explained by researchers without considering $HO_2^{\bullet}$ and IPLP.

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