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Review

Membrane phospholipids, lipoxidative damage and molecular integrity: A causal role in aging and longevity

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ABSTRACT

Nonenzymatic molecular modifications induced by reactive carbonyl species (RCS) generated by peroxidation of membrane phospholipids acyl chains play a causal role in the aging process. Most of the biological effects of RCS, mainly α,β-unsaturated aldehydes, di-aldehydes, and keto-aldehydes, are due to their capacity to react with cellular constituents, forming advanced lipoxidation end-products (ALEs). Compared to reactive oxygen and nitrogen species, lipid-derived RCS are stable and can diffuse within or even escape from the cell and attack targets far from the site of formation. Therefore, these soluble reactive intermediates, precursors of ALEs, are not only cytotoxic per se, but they also behave as mediators and propagators of oxidative stress and cellular and tissue damage. The consequent loss-of-function and structural integrity of modified biomolecules can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage. The causal role of ALEs in aging and longevity is inferred from the findings that follow: a) its accumulation with aging in several tissues and species; b) physiological interventions (dietary restriction) that increase longevity, decrease ALEs content; c) the longer the longevity of a species, the lower is the lipoxidation-derived molecular damage; and finally d) exacerbated levels of ALEs are associated with pathological states.

1. Introduction

Aging causes a multitude of detrimental changes in all animal species at all levels of biological organization, decreases maximum functional capacities and homeostasis, and increases the probability of suffering degenerative processes and finally death. All these changes probably originate from a small number of basic causes that continuously operate throughout the lifespan. The basic chemical and biological processes underlying aging and longevity can be explained and predicted by the mitochondrial oxidative stress theory of aging [1–3]: the reaction of reactive oxygen species (ROS), physiologically generated by mitochondrial electron transport chain, with cellular constituents' initiates the changes associated with aging and determines the maximum longevity.

The involvement of oxidative stress in aging is intrinsically related to their key role in the origin and evolution of life. Oxidative stress is not only a natural consequence of aerobic life, but it has also been a determinant factor which demanded structural and functional adaptations to the cells and organisms that, in turn, determined their longevities [4]. Available evidences suggest that aerobic life, and long-lived species, evolved by reducing the relative abundance of those structural components that are highly susceptible to oxidative damage, but without renouncing them, thus conferring to the cellular

Abbreviations: AA, arachidonic acid; AKR, aldo-keto reductase; ALDH, aldehyde dehydrogenase; ALEs, advanced lipoxidation end-product; ARE, antioxidant response element; CBP, CREB-binding protein; CR, caloric restriction; CREB, cAMP-responsive element-binding factor; DBI, double bond index; 2-DE-WB-MALDI-TOF, two dimensional electrophoresis-western blot-matrix assisted laser desorption ionization time of flight; DHA, docosahexaenoic acid; EGFR, epidermal growth factor receptor; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; GPX, glutathione peroxidase; GSH, glutathione; GST, glutathione-*S*-transferase; 2-HH, 2-hydroxyheptanal; 4-HHE, 4-hydroxyhexenal; 4-HNE, 4-hydroxy-*trans*-2-nonenal; HSFs, heat-shock transcriptional factors; HSPs, heat-shock proteins; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1; LA, linoleic acid; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; M1dG, malondialdehyde-deoxyguanosine; NF-kB, nuclear factor-kB; Nrf2, nuclear factor-erythroid 2 (NF-E2)-related factor 2; ONE, 4-oxo-*trans*-2-nonenal; PDGFR, platelet-derived growth factor receptor; PHGPX, phospholipids hydroperoxide glutathione peroxidase; PI, peroxidizability index; PUFA, polyunsaturated fatty acid; RCS, reactive carbonyl species; RONS, reactive nitrogen species; ROS, reactive oxygen species; SAM, senescent accelerated mouse; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; TGFbeta-1, tissue growth factor beta1; TKR, tyrosine kinase receptors; UCPs, uncoupling proteins

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constituents a higher structural stability and lower susceptibility to oxidative damage [4]. In addition, long-lived species also show low rates of mitochondrial ROS generation and oxidative molecular damage [4]. In this scenario, membrane phospholipids play a causal role in aging and longevity by modulating oxidative stress and molecular integrity.

In this critical review and hypothesis paper, a causal role for membrane phospholipids unsaturation in the aging process is proposed based on the available evidence relating membrane fatty acid composition and lipoxidation-derived molecular damage with the mitochondrial oxidative stress theory of aging and focused on comparative and nutritional intervention models and the possible underlying mechanisms involved.

2. Biological membranes and unsaturated fatty acids

Biological membranes are dynamic structures that generally consist of bilayers of amphipathic molecules held together by non-covalent bonds [5,6]. In eukaryotic cells, phospholipids are the predominant membrane lipids and are, from a topographic point of view, asymmetrically distributed across the bilayer [7–9]. Phospholipids consist of a hydrophilic head group with attached hydrophobic acyl chains. The variation in headgroups and aliphatic chains allows the existence of more than 100 different phospholipids species in any eukaryotic cell. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cardiolipin, as well as sphingomyelin and glycosphingolipids are the major phospholipids [5,6]. In most eukaryotic membranes, phosphatidylcholine and phosphatidylethanolamine represent together around 60–85% of the phospholipid fraction, while for the other phospholipids small but significant differences can be found depending of the cell membrane and even animal species [5,6,10–12]. Phospholipids play multiple roles. They constitute a permeability barrier, modulate the functional properties of membrane-associated activities, provide a matrix for the assembly and function of a wide variety of catalytic processes, and act as donors during the synthesis of macromolecules. The wide range of processes in which phospholipids are specifically involved explains the need for diversity in phospholipid structures and fatty acid composition [13]. This diversity requires complex metabolic and regulatory pathways [5,6]. In fact, for example, eukaryotic cells invest around 5% of their genes to synthesize all of these lipids [10]. The membrane phospholipid composition is maintained primarily by feedback regulation of phospholipid biosynthesis. Recent insights have emerged from the study of membrane-bound transcription factors called sterol regulatory element-binding protein (SREBP) that seem to monitor cell membrane composition and to adjust lipid synthesis accordingly [14].

The acyl chains are either saturated, monounsaturated or polyunsaturated hydrocarbon chains that normally vary from 14 to 22 carbons in length [15]. In eukaryotic cells from vertebrate species, the average chain length of a biological membrane is strictly maintained around 18 carbon atoms, and the relative distribution between saturated and unsaturated fatty acids follow a ratio 40:60, respectively. Polyunsaturated fatty acids (PUFAs) are essential components of cellular membranes in higher eukaryotes that strongly affect their fluidity, flexibility and selective permeability. Additionally, PUFAs affect many cellular and physiological processes in animals, including modulation of ion channels and carriers, activities of membrane-associated enzymes, regulation of gene expression, endocytosis/exocytosis, cold adaptation and survival, and pathogen defence [15]. In vertebrates, C20 PUFAs are metabolized by oxygenases and other enzymes to produce short-lived prostaglandins, leukotrienes and thromboxanes that bind to specific G-protein-coupled receptors and signal cellular responses that mediate vasodilation, blood pressure, pain, fever, and inflammation, among others [6]. Polyunsaturated fatty acids are generally synthesized by the modification of saturated fatty acid precursors that are products of fatty acid synthase. The desaturase enzymes, which are conserved across kingdoms, insert double bonds at specific carbon atoms in the fatty acid chain and the fatty acid elongation system elongates the precursors in two-carbon increments [16]. The pathways for the synthesis of arachidonic acid (20:4n-6, AA) and eicosapentaenoic acid (20:5n-3, EPA) involve alternating fatty acid desaturation and elongation reactions that have been characterized biochemically and are supported by the cloning and characterization of desaturase and elongase genes. The pathway to docosahexaenoic acid (22:6n-3, DHA) involves synthesis and desaturation of 24:5n-3 followed by one cycle of β-oxidation in the peroxisome [15,16]. The fatty acid desaturation pathway and the deacylation–reacylation cycle are the mechanisms responsible for the particular fatty acid composition of cell membranes.

In summary, life demands membranes. Membrane composition (phospholipids classes' distribution and fatty acid profile) is strictly and dynamically regulated. The mechanisms of the homeostatic regulation of the membrane composition, the mechanisms that create lipid asymmetry and their functional implications, and the full definition of the utility of the eukaryotic lipid repertoire are beginning to be understood, being an exciting and rapidly expanding field.

3. Membrane unsaturation and lipid peroxidation

As a principle, chemical reactions in living cells are under strict enzyme control and conform to a tightly regulated metabolic program. One of the attractors involved in biomolecular evolution is the minimizing of unnecessary side reactions. Nevertheless, uncontrolled and potentially deleterious reactions occur, even under physiological conditions.

Reactive oxygen species express a variety of molecules and free radicals (chemical species with one unpaired electron) physiologically generated from the metabolism of molecular oxygen [17]. They are extremely reactive and have damaging effects. Superoxide anion, the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions. Dismutation of superoxide anion (either spontaneously or through a reaction catalyzed by superoxide dismutase enzymes) produces hydrogen peroxide, which in turn may be fully reduced to water or in the presence of ferrous or cuprous ions forms the highly reactive hydroxyl radical. In addition, superoxide anion may react nonenzymatically with other radicals including nitric oxide in a reaction controlled by the rate of diffusion of both radicals. The product, peroxynitrite, is also a very powerful oxidant. The oxidants derived from nitric oxide have been called reactive nitrogen species (RONS). In living cells, the major sites of physiological ROS generation are the complexes I and III of the mitochondrial electron transport chain, which contains several redox centers (flavins, iron–sulfur clusters, and ubisemiquinone) capable of transferring one electron to oxygen to form superoxide anion [3,4,18–22]. Oxidative damage is a broad term used to cover the attack upon biological molecules by free radicals. Reactive oxygen species attack/damage all cellular constituents [17].

The susceptibility of membrane phospholipids to oxidative alterations is related to two inherent traits, the physico-chemical properties of the membrane bilayer and the chemical reactivity of the fatty acids composing the membrane [23]. The first property is related with the fact that oxygen and free radicals are more soluble in the fluid lipid bilayer than in the aqueous solution. Thus, membranes contain an interior organic phase, in which oxygen may tend to concentrate. Therefore, these differences in solubility are important when considering the availability of oxygen/free radicals for chemical reactions inside living systems: organic regions may contain more free radicals than aqueous regions [24,25] and, consequently, membrane lipids become primary targets of oxidative damage.

The second property is related to the fact that PUFA residues of phospholipids are extremely sensitive to oxidation. Every membrane phospholipid contains an unsaturated fatty acid residue esterified to

the 2-hydroxyl group of its glycerol moiety. Many of these are polyunsaturated and the presence of a methylene group between two double bonds renders the fatty acid sensitive to ROS-induced damage, their sensitivity to oxidation increasing exponentially as a function of the number of double bonds per fatty acid molecule [26,27] (Fig. 1). Consequently, the high concentration of PUFAs in phospholipids not only makes them prime targets for reaction with oxidizing agents but also enables them to participate in long free radical chain reactions. Reactive free radicals can pull off hydrogen atoms from PUFA side chains. A hydrogen atom (H.) has only one electron. This hydrogen is bonded to a carbon in the fatty acid backbone by a covalent bond. Hence, the carbon from which H. is abstracted now has an unpaired electron (i.e it is a free radical). Polyunsaturated fatty acid side chains (two or more double bonds) are much more easily attacked by radicals than are saturated (no double bonds) or monounsaturated (one double bond) side chains. When C. radicals are generated in the hydrophobic interior of membranes, their most likely fate is combination with oxygen dissolved in the membrane. The resulting peroxyl radical is highly reactive: it can attack membrane proteins and oxidize adjacent polyunsaturated fatty acid side chains. So, the reaction is repeated and the whole process continues in a free radical chain reaction, generating lipid hydroperoxides [17,28,29]. Lipid hydroperoxides are more hydrophilic than unperoxidized fatty acid side chains. They try to migrate to the membrane surface to interact with water, thus disrupting the membrane structure, altering fluidity and other functional properties and making the membrane leaky.

Lipid peroxidation generates hydroperoxides as well as endoperoxides, which undergo fragmentation to produce a broad range of reactive intermediates called reactive carbonyl species (RCS) with three to nine carbons in length, the most reactive being α,β-unsaturated aldehydes [4-hydroxy-*trans*-2-nonenal (HNE) and acrolein], di-aldehydes [malondialdehyde (MDA) and glyoxal], and keto-aldehydes [4-oxo-*trans*-2-nonenal (ONE) and isoketals] [29,30]. 2-Hydroxyheptanal (2-HH) is another major aldehydic product of lipid peroxidation of PUFAn-6, while 4-hydroxyhexenal (4-HHE) is generated in a lower yield. Additionally, a number of other short chain aldehydes are produced during lipid peroxidation through poorly understood mechanisms. These carbonyl compounds, ubiquitously generated in biological systems, have unique properties contrasted with free radicals. For instance, compared with both ROS and RONS, reactive aldehydes have a much longer half-life (i.e., minutes to hours instead of microseconds to nanoseconds for most free radicals). Further, the non-charged structure of aldehydes allows them to migrate with relative ease through hydrophobic membranes and hydrophilic cytosolic media, thereby extending the migration distance far from the production site. Based on these features alone, these carbonyl compounds can be more destructive than ROS/RONS and may have far-reaching damaging effects on target sites within or outside membranes.

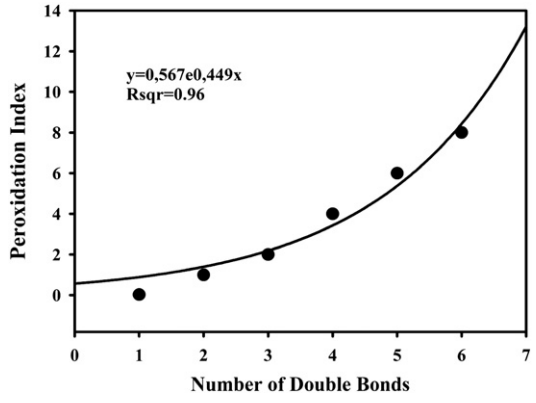

Fig. 1. The relative susceptibilities of selected unsaturated fatty acids to peroxidation. Data are from [26], and all were empirically determined as rates of oxygen consumption. They are expressed relative to the rate for linoleic acid (18:2n-6) which is arbitrarily given a value of 1.

In this scenario, and with these premises, biological membranes – in a clear adaptive response to the oxidative conditions inherent to aerobic life – evolved by lessening the relative abundance of PUFAs highly susceptible to oxidative damage — highly unsaturated fatty acids (higher than 2 double bonds) are less abundant compared with saturated, mono- and di-unsaturated fatty acids, obtaining a higher structural stability and a lower susceptibility to oxidative damage [4,23].

In summary, highly unsaturated fatty acids of cellular membranes are the macromolecules most susceptible to oxidative damage in cells, and this sensitivity increases as a function of their number of double bonds. In addition, the carbonyl compounds generated as lipid peroxidation-derived end-products extend the membrane damage to other cellular constituents.

4. Lipid peroxidation and lipoxidation-derived molecular damage: targets

Carbonyl compounds react with nucleophilic groups in macromolecules like proteins, DNA, and aminophospholipids, among others, resulting in their chemical, nonenzymatic, and irreversible modification and formation of a variety of adducts and cross-links collectively named advanced lipoxidation end-products (ALEs) [31]. Thus, by reacting with nucleophilic sites in proteins (belonging basically to Cys, Lys, Arg, and His residues), carbonyl compounds generate ALE adducts such as MDA-Lys, HNE-Lys, FDP-Lys, and *S*-carboxymethyl–cysteine; and the cross-links glyoxal–lysine dimmer, and lysine–MDA–lysine, among several others. The accumulation of MDA adducts on proteins is also involved in the formation of lipofuscin. Thus, lipofuscin becomes a nondegradable intralysosomal fluorescent pigment formed through lipoxidative reactions [32].

Lipid peroxidation-derived end-products can also react at the exocyclic amino groups of deoxyguanosine, deoxyadenosine, and deoxycytosine to form various alkylated products [33]. Guanine is, however, the most commonly modified DNA base because of its high nucleophilicity. Some common enals that cause DNA damage, analogously to proteins, are MDA, HNE, and acrolein, among others. Thus, the most common adducts arising from enals are exocyclic adducts such as etheno adducts, and MDA-deoxyguanosine (M1dG). Finally, the amino group of aminophospholipids can also react with carbonyl compounds and to initiate some of the reactions occurring in proteins and DNA, leading to the formation of adducts like MDA-phosphatidylethanolamine, and carboxymethyl-phosphatidylethanolamine [34].

In summary, lipoxidation-derived molecular damage is a natural consequence of aerobic life (Fig. 2). ALEs induce the chemical, nonenzymatic and irreversible modification of cellular constituents, and they are the evidence for the existence of a 'lipoxidative stress' in vivo. Molecular modification by lipoxidation-derived reactive carbonyl species is little known. Current challenge is to establish the chemical structure of these modifications and the mechanisms for their formation, and to identify what factors control the nature, selectivity, extent, and irreversibility of the molecular modification occurring in vivo.

5. Physiological steady-state levels of ALEs: cellular defence mechanisms

Since cells continuously produce free radicals, oxidative stress homeostasis can only be maintained if endogenous cellular antioxidants are present. A large battery of antioxidant defences, both enzymatic and nonenzymatic, has been selected and conserved during animal evolution [17,35]. An antioxidant can be defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". An antioxidant reacts with and neutralizes an oxidant, or regenerates other molecules capable of reacting with the oxidant.

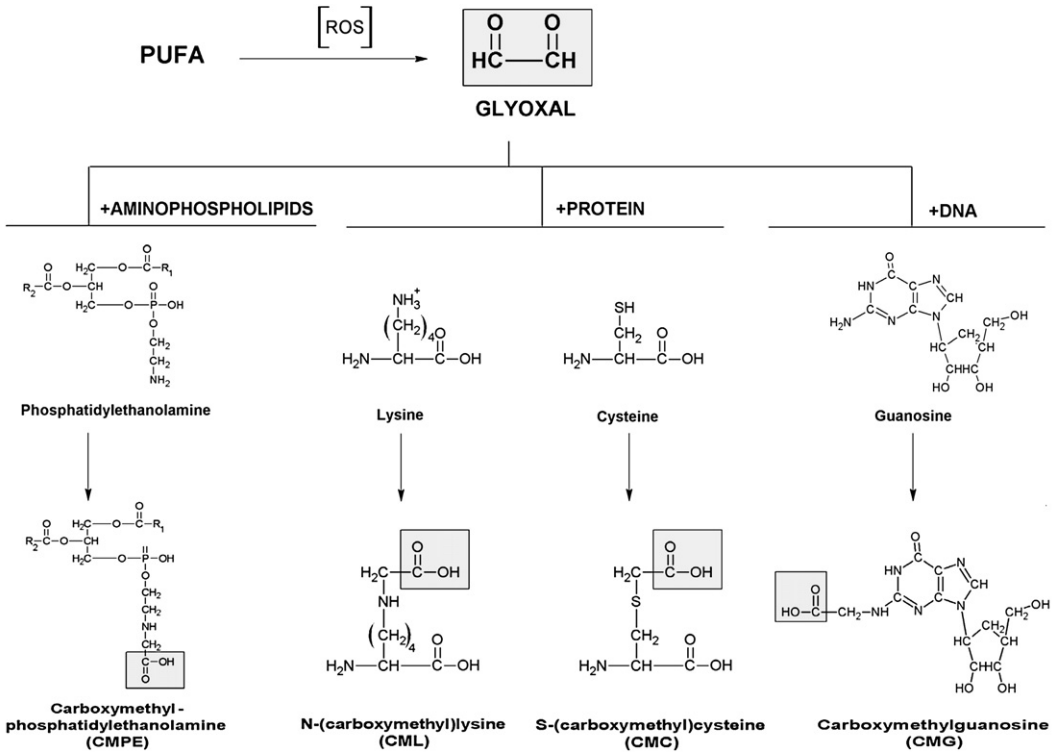

Fig. 2. Aminophospholipid, protein and DNA damage resulting from aldehydic products of lipid peroxidation. Shown are examples of molecular adducts (advanced lipoxidation end-products, ALEs) generated by the reactive carbonyl compound glyoxal.

In this context, tocopherols and carotenoids are the main radical scavenger antioxidants that act in lipophilic environments of cells [36]. The major scavenger inside membranes is D-α-tocopherol (vitamin E). Most membranes are thought to contain approximately one tocopherol molecule per thousand lipid molecules [37]. Vitamin E acts on lipid peroxyl groups inside membrane bilayers, reducing them to hydroperoxides, and thus inhibiting the propagation of the peroxidative chain reaction [29]. It breaks the chain reaction of lipid peroxidation but is itself converted to a radical during the process. Vitamin E also reduces lipid alkoxyl radicals to lipid alcohols. Oxidized vitamin E can be recycled back to its reduced form by ascorbate or ubiquinone (coenzyme Q). Carotenoids quench singlet oxygen, and interact with other ROS at physiological tissue oxygen partial pressures. Ubiquinol, the reduced form of coenzyme Q, is an important antioxidant [38]. It is a hydroquinone that is synthesized and present in all cellular membranes and its antioxidant activity is exhibited through scavenging of lipids radicals or reduction of vitamin E radical. Regeneration of coenzyme Q is performed by reductases that use NADPH or NADH as cofactors.

Protection of membranes is also achieved by additional complex system that involves several mechanisms: lipid repair, lipid replacement, scavenging of lipoperoxidation-derived carbonyl compounds, and degradation-removal of ALE-modified molecules [23]. The action of chain-breaking antioxidants can result in the production of lipid hydroperoxides. Some of these are metabolized by glutathione peroxidase (GPX) antioxidant enzymes, which act on H_2O_2 and also on free fatty acid hydroperoxides, reducing them to fatty acid alcohols. However, the peroxidized fatty acids must be first released from the membrane lipids. A phospholipid hydroperoxide glutathione peroxidase (PHGPX) has also been described in some mammalian tissues and is capable of acting on peroxidized fatty acid chains still esterified to membrane phospholipids and is thus a very important antioxidant defence for membranes in situ [39]. An important mechanism for removing peroxidized lipids is membrane lipid remodelling. While the enzyme PHGPX is important in removing peroxidized acyl chains from phospholipids other enzymes are also involved in the continual deacylation/reacylation of phospholipids and this turnover of membrane acyl chains is very rapid. Phospholipase A_2 is an important enzyme for removing acyl chains from phospholipids while acyltransferase and transacylase enzymes are responsible for reacylation of phospholipids [40]. Thus, for example, when isolated rat liver cells are subjected to oxidative stress, lipid peroxidation is increased, however there is no change in either the fatty acid composition of, or in the rate of acyl turnover of membrane phospholipids. There is however a decrease in the PUFA content of cellular triacylglycerols. It is suggested that rapid constitutive recycling of membrane phospholipids rather than selective in situ repair is responsible for eliminating peroxidized phospholipids, with triacylglycerols providing a dynamic pool of undamaged PUFA for phospholipid resynthesis [41]. Carbonyl compounds are, however, generated during the lipid peroxidation cascade.

Most reactive carbonyl species are detoxified in multiple ways, including, conjugation to glutathione (GSH), oxidation by aldehyde dehydrogenases, or reduction by aldo-keto reductases [17,23,29,30,33]. Strongly electrophilic carbonyl compounds induce rapid and significant depletion of intracellular glutathione content, suggesting that this is an important detoxification mechanism. Once GSH is depleted, cells exhibit an intracellular change in redox status and propagate an oxidative stress response following their production. Reaction of α,β-unsaturated aldehydes with GSH can proceed in one of two ways-either by nonenzymatic conjugation or through GSH transferase (GST)-mediated conjugation to form Michael adducts. Glutathione-*S*-transferases (GSTs) are particularly involved in the detoxification of highly reactive intermediate aldehydes. GSTs belong to a supergene family of multifunctional enzymes [42] and among the eight main classes of human GSTs, those involved in carbonyl detoxification include the Alpha, Mu and Pi classes (GSTA, GSTM, and GSTP) being active against a wide variety of carbonyl compounds and being their expression tissue specific. GSTA4-4 is the principal GST responsible for metabolism of most long chain α,β-unsaturated aldehydes, although many GSTs may be involved in the metabolism of these molecules. Several

oxidoreductases also inactivate HNE and related aldehydes. Particularly, detoxification of HNE in this manner is accomplished by oxidation or reduction of the aldehyde or by reduction of the 2,3-double bond. Aldehyde dehydrogenase 3 (ALDH3) is responsible for the conversion of HNE to 4-hydroxy-2-nonenoic acid, a noncytotoxic molecule. Aldose reductase and aldo-keto reductase AKR1C1 efficiently reduce HNE to 1,4-dihydroxy-2-nonene. Reduction of the 2,3-double bond of HNE is carried out by 15-oxoprostaglandin oxidoreductase, forming 4-hydroxynonanal. Recent reports suggest that related molecules (e.g., ONE) are metabolized in a similar fashion. Finally, natural detoxifying mechanisms to protect tissues from RCS damage also include the cytosolic GSH-dependent glyoxalases, thiol- and histidine-containing dipeptides, presumably acting as trapping agents, and ascorbic acid.

If these metabolic pathways are compromised or overcome by excessive aldehyde generation, reaction with DNA, proteins, and aminophospholipids occurs, potentially leading to adverse alterations in cellular homeostasis. Additional defences able to cope with the modifications derived from the interaction between RCS and macromolecules have been developed by biological systems. Among them the following ones may be mentioned: systems that degrade lipoxidatively damaged proteins (e.g. the proteasome), and DNA repair enzymes [17,33,43].

In summary, aerobic life demands antioxidant defences. Physiological steady-state level of both RCS and ALEs results from the balance of several cellular mechanisms and is species-tissue–cell type specific. Variations in the steady-state levels of lipoxidatively-modified cellular constituents in vivo are due to corresponding differences in rates of oxidant generation, local RCS concentration, antioxidant defences, removal and repair capacity, and susceptibility to oxidative modifications, among others (Fig. 3).

6. Molecular and cellular effects and signaling properties of RCS and ALEs

Reactive carbonyl species (RCS) generated during the lipid peroxidation reactions exhibit a wide range of molecular and biological effects, ranging from protein, DNA, and phospholipid damage to signaling pathway activation and/or alteration. The detailed mechanisms of 'toxicity' are, however, mostly unknown.

6.1. ALEs and molecular integrity

Lipoxidation reactions lead to structural and functional changes on proteins [31,44] such as i) alterations in physico-chemical properties (conformation, charge, hydrophobicity, elasticity, solubility, and electrophoretic mobility, among others); ii) formation of intra- and inter-molecular protein cross-links and aggregates; iii) decrease/inhibition in enzyme activity; iv) alteration of protein degradation; v) altered trafficking and processing of proteins; vi) modification of extracellular matrix properties and cell–matrix interactions; and vii) stimulation of autoimmune response, among others.

These nonenzymatic protein modifications are spontaneous, random, unprogrammed and uncatalyzed chemical reactions. In addition, as a general rule, all proteins can be the target of reactive carbonyl species. However, whether some key cellular or extracellular molecules are preferential targets of these chemical modifications and whether the extent of their modification is sufficient to explain impaired cellular and tissue function are key questions. In this context, the determination of the steady-state levels for given ALEs does not allow us to discern if specific proteins are damaged. Is there pattern selectivity in the main targets?, What are the specifically damaged proteins?, Why are this protein pool (if any) modified? Few studies have addressed these questions, although various specific proteins have been detected by using 2DE-WB-MALDI-TOF analysis that are modified by ALEs [44] such as aconitase, actin, adenine nucleotide translocase, albumin, aldolase A, apolipoproteins, aspartate aminotransferase, ATPsynthase, carbonic anhydrase II, collagen, alpha-crystallin, alpha-enolase, GFAP, glyceraldehyde-3-phosphate dehydrogenase, HSP27, HSP60, phosphoglycerate kinase 1, proteasome, SOD and other antioxidant enzymes, succinate dehydrogenase, alpha-synuclein, and thioredoxin-1. Obviously, more studies are needed to obtain a scenic view by tissues, organs, systems, species and markers of the specifically affected proteins that allow us to evaluate structural/functional factors shared by these proteins (if any) in order to explain this "specificity". RCS probably act in a random fashion; however, the sensitivities and proximities of potential targets differ. The factors that can affect selectivity of lipoxidative damage to proteins could include the presence of a metal-binding site, molecular conformation, rate of proteolysis, and relative abundance of amino acid residues susceptible to lipoxidative reactions, among others.

The degradation of modified proteins by RCS deserves special mention. Though proteasomal degradation is activated by oxidative stress [43], ALE-modified proteins are poorly degraded by proteasome and tend to inhibit the proteolytic activity [43]. In fact, the extensive modification of cellular proteins by 4-HNE and by the related carbonyl compounds leads to the formation of protein aggregates that accumulate in cells and are not degraded by the proteasome, likely due to a direct inhibition of proteasome by oxidized and cross-linked proteins, and by 4-HNE-modified proteins. Though the modification of proteasome is not observed at low or moderate 4-HNE concentrations, higher concentrations may directly form adducts which readily inhibits the enzymatic activity of proteasome and contributes to the accumulation of modified proteins [43].

DNA lipoxidative damage is present in the genome of healthy humans and other animal species at biologically significant levels similar or even higher that oxidation markers sensu stricto. DNA damage is mutagenic, carcinogenic, and with powerful effects on signal transduction pathways [33,45]. Furthermore, they are efficient premutagenic lesions that induce mutations frequently detected in

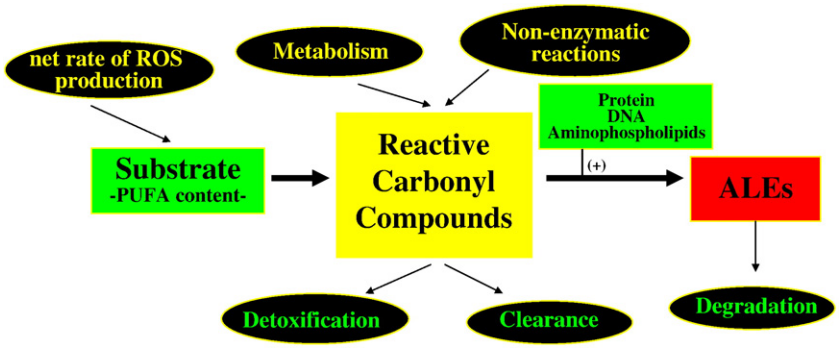

Fig. 3. Physiological steady-state level of ALEs results from the balance of several cellular mechanisms and is species-tissue-cell type specific.

oncogenes or tumor suppressor genes from human tumors, and correlate to alterations in cell cycle control and gene expression in cultured cells. Thus, lipid peroxidation must be considered a significant endogenous source of DNA damage and mutations.

Finally, the amino group of aminophospholipids can also react with carbonyl compounds and to initiate some of the reactions occurring in proteins, expanding the negative biological effects of this non-enzymatic modification [34]. Biological processes involving aminophospholipids could be potentially affected by this process. Among these processes, it may be highlighted i) asymmetrical distribution of aminophospholipids in cellular and different subcellular membranes; ii) translocation between and lateral diffusion in the membrane; iii) membrane physical properties; iv) biosynthesis and turnover of membrane phospholipids; and v) activity of membrane-bound proteins that require aminophospholipids for their function [34].

In summary, the molecular integrity of proteins, DNA and aminophospholipids can be altered as a consequence of a lipoxidative stress that, in turn, can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damages.

6.2. Cellular adaptive response signaling to RCS

As mentioned above, the peroxidation of the PUFA chains of phospholipids generates a complex mixture of carbonyl compounds. Initially, these aldehydes were believed to produce only "cytotoxic" effects associated with oxidative stress, but evidence is increasing that these compounds can also have specific signaling roles inducing adaptive responses driven to decrease oxidative damage and improve antioxidant defences.

6.2.1. Cardiolipin oxidation, RCSs and uncoupling proteins: regulatory role in mitochondrial free radical production

Cardiolipin, a phospholipid of unusual structure localized almost exclusively within the inner mitochondrial membrane, is particularly rich in unsaturated fatty acids. This phospholipid plays an important role in mitochondrial bioenergetics by influencing the activity of key mitochondrial inner membrane proteins, including several anion carriers and electron transport complexes I, III and IV [46]. Mitochondrial cardiolipin molecules are possible targets of oxygen free radical attack, due to their high content of polyunsaturated fatty acids and because of their location in the inner mitochondrial membrane near to the site of ROS production. In this regard, it has been recently demonstrated that mitochondrial-mediated ROS generation affects the activity of complex I, as well as complexes III and IV, via peroxidation of cardiolipin following oxyradical attack to its fatty acid constituents [47]. These findings might explain, at least partially, the decline in respiratory chain complexes observed in mitochondria isolated from aged animals and in pathophysiological conditions that are characterized by an increase in the basal rate of the ROS production. So, cardiolipin integrity plays an important role determining the rate of ROS generation by mitochondrial complexes I and III.

Available studies support the notion that superoxide radical produced by the electron transport chain can cause mild uncoupling of mitochondria by activating the membrane proton conductance by uncoupling proteins (UCPs; [19,22]). Insight into the mechanism by which superoxide radical activates UCPs comes from the finding that the lipid peroxidation product 4-HNE and its homologs induce uncoupling of mitochondria through UCP1, UCP2, and UCP3 and also through the adenine nucleotide translocase [48]. This and other observations support a model in which endogenous superoxide production generates carbon-centered radicals that initiate lipid peroxidation, producing alkenals like 4-HNE that may activate UCPs and adenine nucleotide translocase. While the thermogenic function of UCP1 has been well characterized, a function for its homologs (UCP2, UCP3, avian UCP, and plant UCP) has yet to be unambiguously defined. A possible physiological function for UCPs has been proposed [48]. In this model, UCPs respond to overproduction of matrix superoxide by catalyzing mild uncoupling, which lowers proton motive force and would decrease superoxide production by the electron transport chain. This will attenuate superoxide-mediated molecular damage [49], at the cost of a slightly lowered efficiency of oxidative phosphorylation. It was hypothesized that this negative feedback loop will protect cells from ROS-induced damage and might represent the ancestral function of all UCPs [19].

6.2.2. Antioxidant response signaling

Reactive carbonyl species can activate the 'antioxidant response' likely to prevent their accumulation to toxic levels [50,51]. This signaling cascade culminates in the nuclear translocation of and transactivation by the transcription factor Nrf2, the master regulator of the response [52]. Nrf2 activity is repressed by an inhibitory binding protein, Keap1. Keap1 retains Nrf2 in the cytosol, closely associated with the actin cytoskeleton, and promotes proteasomal degradation of Nrf2 through Cullin3-dependent polyubiquitination. Following exposure to RCS, Keap1 can be directly modified on several cysteine residues, and this modification can promote release of Nrf2. Intracellular modification of Keap1 similarly allows for increased stabilization and nuclear localization of Nrf2, potentially through direct modification of Keap1 coupled with Keap1 ubiquitination and degradation. Nrf2 contains a C-terminal basic leucine zipper structure that facilitates dimerisation and DNA binding, specifically to the antioxidant response element (ARE). The binding of Nrf2 to the ARE, which requires heterodimerisation with small Maf proteins, stimulates transcription of downstream genes, in part, by recruiting transcriptional co-activators, particularly CREB-binding protein (CBP) through the Neh4 and Neh5 domains of the transcriptional factor. Nrf2 activity is also enhanced through phosphorylation by several kinases, including protein kinase C isoforms and the ER-stress responsive kinase PERK. The Nrf2-regulated cytoprotective genes are [52]: aldo-keto reductases (AKR), glutamate cysteine ligase [catalytic subunit (GCLc), and regulatory subunit (GCLm)], glutathione-*S*-transferase (GST), glutathione synthetase (GS), heme-oxygenase 1 (HO-1), metallothionein, microsomal epoxide hydrolase (mEH), NAD(P)H:quinine oxireductases (NQO), peroxiredoxin 1 (Prx1), superoxide dismutases (SOD), thioredoxin reductases (TrxR), thioredoxins (Trx), and UDP-glucuronosyltransferases (UGT). The mechanism(s) by which carbonyl compounds activate the antioxidant response element (ARE) through the Nrf2 transcription factor is currently under investigation.

6.2.3. Heat-shock response signaling

Exposure of cells to environmental and physiological stress leads to an imbalance in protein metabolism, which challenges the cell to respond rapidly and precisely to the deleterious effects of stress on protein homoeostasis. The heat-shock response, through activation of heat-shock transcription factors (HSFs) and the elevated expression of heat-shock proteins and molecular chaperones, protects the cell against the accumulation of non-native proteins [53].The heat-shock response is stimulated by endogenous oxidants and lipid peroxidation products alike [53,54]. Activation of heat-shock factor-1 (HSF1) during this process subsequently induces the expression of a variety of heat-shock proteins (Hsps). HSF1, like Nrf2, resides predominantly in the cytosol under basal conditions, where it is bound and inhibited by various Hsps, including Hsp90, Hsp70, and Hsp40. Following extensive protein damage by RCS, Hsps are recruited to sites of protein damage and therefore release HSF1, thereby promoting its activation. This sequence of events allows HSF1 to migrate into the nucleus, and stimulate transcription of Hsp genes. HSF1 regulation is also redox sensitive, with its activation potentially requiring formation of a disulfide bond. When active, trimeric HSF1 binds to DNA in the promoters of numerous Hsp genes, giving rise to downstream effects that influence both protein folding homeostasis and cell death.

Of the molecular chaperones regulated by HSF1, Hsp70 is currently the best understood regarding its regulation of signaling and cell death pathways [53]. In addition to its function of promoting refolding or clearance of misfolded or aggregated proteins, Hsp70 prevents apoptosis through disruption of apoptosome formation, potentially through directly binding Apaf1. An additional mode through which Hsp70 potentially inhibits cell death is by blocking stress-responsive mitogen-activated protein kinase (MAPK) signaling. Therefore, specific Hsp70 isoforms induced in stressed cells following HSF1 activation may function as negative regulators of apoptotic signaling in multiple ways.

6.3. Cellular damaging effects of RCS

6.3.1. Modification of tyrosine kinase receptors and cell cycle

4-HNE added to cultured cells exhibits a dose-dependent effect. So, physiological concentration of 4-HNE has growth-regulating effect, whereas higher concentration is primarily cytotoxic [55,56]. 4-HNE added to the culture medium induces both modification and dysfunction of tyrosine kinase receptors (TKRs) such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) in a biphasic manner. 4-HNE, at physiological and moderate concentrations (<1 μM and 1–10 μM, respectively) triggers a sustained activation of TKRs EGFR and PDGFR [57,58]. The mechanism of TKRs activation involves the formation of 4-HNE adducts on the receptor, which triggers TKR autophosphorylation and the activation of the downstream signaling pathway, extracellular signal-regulated kinase (ERK)1/2 phosphorylation and cell cycle progression [57,58]. So, at physiological concentrations, 4-HNE can act as a growth factor and promotes cell proliferation. However, high concentrations of 4-HNE inhibit cell proliferation mediated by growth factor receptors EGFR and PDGFR, though a two-fold increase of the c-*fos* mRNA level was observed as a protective but abortive stress-induced response. This inhibitory effect of 4-HNE on growth factor-mediated cell proliferation is in agreement with the progressive desensitization of PDGFR to its own ligand PDGF-BB, observed in smooth muscle cell in prolonged contact with 4-HNE (at concentrations >10 μM). Inhibition of cell cycle progression, reported in leukaemic cells, is mediated by a decrease in expression of cyclin D1, D2 and A and an increase in the expression of the cycline kinase inhibitor p21, thereby inducing an accumulation of cells in the G0/G1 phase of the cell cycle [59]. 4-HNE induces an accumulation of the hypophosphorylated form of the retinoblastoma tumor suppressor gene product hyperphosphorylated retinoblastoma, which binds and inactivates the E2F transcription factors, represses the transcription and induces cell cycle arrest. Moreover, 4-HNE inhibits the expression of the protooncogene c-myc in HL-60, without affecting the expression of c-*fos*. Higher toxic concentrations of 4-HNE (50 μM) enhanced c-*fos* transcription while cell proliferation is inhibited. These biological effects are not restricted to 4-HNE, since other carbonyl compounds such as methylglyoxal and glyoxal are also able to inhibit various RTKs, including EGFR, PDGFR and insulin receptor [60,61].

6.3.2. NF-κB

The proinflammatory transcription factor NF-κB (nuclear factor-κB) is a direct regulator of proinflammatory and antiinflammatory genes, cell survival and proliferation [62]. The activation of NF-κB requires the phosphorylation of its inhibitor κB (IκB), which is necessary for its degradation by the ubiquitin–proteasome pathway. 4-HNE and acrolein inhibit the activation of NF-κB, either via a direct inhibitory effect on proteasome or through the inhibition of an upstream step required for the phosphorylation of IκB as reported in human monocytic cells, in which 4-HNE inhibits the activation of NF-κB induced by lipopolysaccharide, interleukin-1β and phorbol ester [63]. Conversely, 4-HNE prevents the activation of NF-κB elicited by *Chlamydia pneumoniae* by inhibiting the phosphorylation of IκB and its subsequent proteolysis [64]. A potential mechanism has been proposed recently [65] by reporting that the inhibition of interferon-α (TNF α)-induced activation by acrolein could be due to the modification of IKK-subunit by acrolein. In contrast, aldehydes may induce inflammation via an activation of NF-κB, as shown for 4-HHE that activates NF-κB via the IκB kinase/NF-κB inducing kinase pathway. This mechanism involves an upstream activation of p38 MAPK and ERK1/2 kinase [66]. Finally, it has recently been described a clear proinflammatory effect for ALEs in monocytes [67].

6.3.3. Apoptosis signaling

High concentrations of 4-HNE or acrolein (>20 μM) are highly toxic for most cell types (reviewed in [33,56]). The mechanism of apoptosis elicited by RCS involves various effects, including signaling or protein modification. The activation of JNK has been particularly investigated in the antiproliferative and apoptotic effect of 4HNE [68]. This JNK pathway plays a major role in the cooperative apoptotic effect of tissue growth factor β-1 (TGFβ-1) and 4-HNE on colon cancer cell lines. Both acrolein and 4-HNE increase the levels of the phosphorylated form of transcription factors c-*jun* (which promotes apoptosis) and CRE-binding protein (CREB) (involved in survival), but decrease the activity of the CREB-responsive promoters (while increasing c-*jun* responsive promoter), which contributes to neuron degeneration and apoptosis. Methylglyoxal and glyoxal are pro-apoptotic through mechanisms involving calcium deregulation, GSH depletion, oxidative stress, and the activation of stress kinases p38 and JNK. 4-HNE increases the mRNA and protein expression of the pro-apoptotic adaptors/regulators FasR, FasL, Bax, and caspases-1, -2, -3 and -8. In human lens cultured cells (HLE B-3), 4-HNE adducts are correlated with the induction of Fas, the activation of JNK and caspase 3, while the transfection of the α-class GST mGSTA4a (which neutralizes 4-HNE) inhibits Fas expression. The mechanism of cell death evoked by these aldehydes could also involve the generation of peroxynitrite, as reported for 4-HHE and for methylglyoxal.

4-HNE impairs the mitochondrial function, via the alteration of GSH metabolism and the induction of massive mitochondrial oxidative stress. More specifically, moderately elevated concentrations of 4-HNE or very low doses of 4-HHE trigger a calcium-mediated induction of the mitochondrial transition pore. In addition, in vitro experiments on isolated mitochondria or reconstituted models for the adenine nucleotide translocator pretreated with 4-HNE or 4-HHE indicate that the modification of ANT by these aldehydes impairs its function and activity. Lastly, 4-HNE alters mitochondrial calcium uptake and cytosolic calcium homoeostasis, which results in necrosis or apoptosis. This mechanism is involved in neuronal cell death.

7. Membrane unsaturation, RCS, ALEs, and the physiological aging process

The loss-of-function and structural integrity of ALE-modified biomolecules have a wide range of downstream functional consequences and is the cause of subsequent cellular dysfunctions and tissue damage. Many evidence suggests a role for membrane unsaturation, RCS generation, and ALEs formation in aging and longevity based on: i) its accumulation with aging in several tissues and species; ii) physiological interventions (dietary restriction) that increase longevity, attenuate age-related changes in membrane unsaturation and lipoxidative damage; iii) the longer the longevity of a species, the lower is the membrane unsaturation and lipoxidation-derived molecular damage; and finally iv) exacerbated levels of ALEs are associated with pathological states. Comprehensive reviews on this last point have recently been published [30,33,56].

7.1. Membranes and aging

The singular importance of membrane unsaturation in the aging process is highlighted by studies showing i) Age-related changes in

Table 1
Effect of aging on membrane lipid parameters in tissues from different animal species

Marker/parameter	Tissue	Species	Change with aging	References
Membrane fatty acid composition				
PI*/PUFA content	Whole	*Drosophila*	↑	#
	Liver	Rat	↑	[71–73]
	Liver microsomes and mitochondria	Rat	↑ or =	[74–78]
	Kidney microsomes and mitochondria	Rat	↑	[78]
	Heart	Rat	↑	[73]
	Heart mitochondria	Rat	↑ or =	[79,80]
	Brain synaptosomes	Rat	↓	[81]
	Cerebral cortex	Rat	↓	[82]
Membrane lipid peroxidation				
Lipofuscin	Liver	Mouse, human	↑	[83–85]
	Brain	Mouse, rat	↑	[84–88]
	Testis	Mouse, rat	↑	[88,89]
	Heart	Mouse, rat, dog	↑	[84,87–92]
	Adrenal cortex	Rat	↑	[88]
	Kidney	Rat	↑	[88]
MDA-TBARS	Liver	Mouse, rat	↑	[72,84,93,94]
	Liver microsomes and mitochondria	Rat	↑	[76,94–96]
	Heart	Rat	↑	[94]
	Heart mitochondria	Rat	↑	[97]
	Brain and Brain regions	Rat	↑	[94,98]
	Plasma, splenic lymphocytes	Rat	↑	[99]
Lipid hydroperoxides	Liver	Rat	↑	[72]
	Liver microsomes and mitochondria	Rat	↑	[76]
Conjugated double bonds	Heart mitochondria	Rat	↑	[97]
Expired hydrocarbons[1]	Whole animal	Rat, human	↑	[100–102]
Membrane order parameters				
Membrane fluidity	Liver	Rat	↓	[94,107]
	Liver mitochondria and microsomes	Rat	↓	[103,104]
	Brain	Rat	↓	[82,94,105,107]
	Brain mitochondria and synaptosomes	Rat	↓	[105,106]
	Heart	Rat	↓	[94,107]
	Heart mitochondria	Rat	↓	[79]

*PI, Peroxidizability index: calculated from fatty acid compositional analysis; PUFA: Polyunsaturated fatty acid content.; MDA-TBARS, malondialdehyde determined by thiobarbituric acid reactive substances method; #, unpublished results; [1], expired hydrocarbons: pentane and others.

physico-chemical and physiological properties (Table 1); ii) Increases in double bond and peroxidizability indexes and in vivo and in vitro lipid peroxidation during aging in an organ-dependent way (table 1). This is mainly due to decreases in the less unsaturated linoleic and linolenic acids and to increases in the highly unsaturated arachidonic acid, and docosotetra- penta- and -hexaenoic acids. An important exception, in the contrary sense, is the brain; iii) Increases in the amount ethane and pentane in exhaled air (Table 1); iv) Increases in the lipoxidation-derived protein and DNA damage with age (Table 2); and v) The senescent accelerated prone mouse (SAM-P) has higher levels of the very unsaturated arachidonic (AA) and docosahexaenoic (DHA) acids and peroxidizability index and lower levels of linoleic acid than SAM-resistant controls [69,70].

Additional observations suggesting that membrane unsaturation is related to aging include data showing that strongly unsaturated fatty acids like AA and DHA can have detrimental effects in vivo (reviewed in [3,4]). Examples of this include decreases in respiratory control and increases in proton leak in mitochondria, increased mitochondrial breakage and dysfunction, peroxisome proliferation, fatal ventricular fibrillation in rats, neurological damage, increased lipid peroxidation in association with various diseases, increased incidence of death from apoplexy, or sudden cardiac death in humans induced by AA or DHA. Increases of more than one order of magnitude in AA (to 500 μM) occur in the brain during ischemia and even concentrations of AA and eicosapentaenoic acid (20:5n-3) in the much lower 20–40 μM range uncouple mitochondria and cause tissue edema. Hypermetabolic uncoupling effects of thyroid hormones on rat liver mitochondria are due to a great extent to increased AA/LA ratios caused by increases in desaturase activities induced by the hormone, whereas LA is considered a “proton plug” or coupler. Furthermore, the largest amounts of unsaturated fats in the healthy human diet must be present as fatty acids with low degrees of unsaturation like oleic acid and LA, whereas beneficial levels of dietary n-3 PUFAs (the n-3 “paradox”) occur only at the low (1%) optimum dietary levels recommended by the World Health Organization. These beneficial effects are probably observed because the conversion of dietary linolenic acid to highly unsaturated fatty acids like DHA is strongly limited thanks to the constitutively low delta-5/-6 desaturase activities of humans. In this context, two studies deserve special attention: a) Inuits are human populations showing unusually low incidence of coronary heart disease, psoriasis, rheumatoid arthritis and asthma and have very low levels of AA in plasma phospholipids due to a genetic abnormality in essential fatty acid desaturation which persists even after changing them to a LA-rich diet [123]; and b) in a recent prospective study on old healthy subjects, it was found that a higher monounsaturated fatty acid intake (Mediterranean diet) increased survival, while a higher unsaturated/saturated fatty acid ratio increased total mortality [124].

7.2. Membranes and aging: effects of dietary and pharmacological interventions, and genetic manipulations

Are experimental extensions in mean and maximum longevity accompanied by attenuations of membrane unsaturation and lipoxidation-derived molecular damage? This question is a key issue that goes beyond correlation to establish a causative role for membranes and lipoxidative stress in aging. In the performed studies, investigators either fed animals antioxidants or genetically altered the expression of antioxidant enzymes to modify the flux of free radicals in cells thereby altering oxidative damage to biomolecules. Unfortunately, in most

Table 2
Effect of aging on steady-state levels of lipoxidative protein and DNA modifications in tissues from different species

Compound (units)	Tissue	Species	Steady-state levels	References
CML	Skin collagen	Human, rat	↑	[108–111]
	Articular cartilage collagen	Human	↑	[110,112]
	Lens protein	Human	↑	[113,114]
	Urine	Human	↑	[115]
	Liver mitochondria	Rat	↑	[75,116]
	Serum	Rat	↑	[117]
	Aorta	Rat	↑	[117]
	Heart	Rat	↑	[117]
GOLD	Lens protein	Human	↑	[118,119]
MDAL	Liver	Rat	↑	[73]
	Liver mitochondria	Rat	↑	[75]
	Heart	Rat	↑	[73]
	Cerebral cortex	Human	↑	(#)
HNE-protein	Heart	Rat	↑	[120]
MDA-DNA	Liver	Rat	↑	[45,121]
	Kidney	Rat	↑	[121]
	Testes	Rat	=	[121]
	Brain	Rat	↑	[121,122]

CML, Carboxymethyl-lysine; GOLD, Glyoxal lysine dimer; HNE-protein, hydroxynonenal-protein (cysteine, lysine, or histidine); MDAL, Malondialdehyde-lysine; MDA-DNA, malondialdehyde-DNA. (#) Unpublished results.

cases, and with the exception of caloric restriction approach, it was not determined whether the manipulation, either pharmacological or genetic, altered the levels of lipoxidatively damaged proteins and DNA that accumulate with age.

Caloric restricted (CR) rodents were the only experimental manipulation known to increase longevity, until 1987 when it was observed that mutations in *Caenorhabditis elegans* and later mutations in *Drosophila melanogaster* could give rise to long-lived mutants [125–127]. CR, in which rodents are fed 60–70% of the food consumed by pair-fed rodents fed ad libitum, has been shown to delay or reduce the onset of most age-related diseases and alter most physiological processes that change with age [128]. Because the increase in longevity (both mean and maximum longevity) occurs with retardation in disease and physiological decline, it has been argued that CR increases the survival by modulating the basic mechanisms underlying the aging process. At the present time, several evidences seem to suggest that CR might delay aging and extend longevity through mechanisms that involve changes in the lipoxidative status.

Caloric, as well as protein and methionine restriction – nutritional interventions that increase longevity – attenuates age-related changes in the degree of membrane unsaturation and the level of lipoxidation-derived protein damage in a variety of tissues and animal species [96,129] (Table 3). Thus, a decrease in lipid peroxidation and lipoxidation-derived protein damage has been reported in CR flies (*Drosophila*; Pamplona et al., unpublished results), and tissues (liver and heart) from rats and mice [75,76,80,96,130–136]. CR has also been shown to reduce levels of lipofuscin in tissues of rodents and *C. elegans* [23,32,84,87,137,138], as well as to decrease oxidative damage to mitochondrial DNA as measured by the levels of oxo8dG (reviewed in [3]). No data are available for DNA damaged by carbonyl compounds. The magnitude of the change is lower for membrane unsaturation (between 2.5 and 10%) than that for the lipoxidation-derived molecular damage (between 20 and 40%) likely due to the added effect of the lower mitochondrial free radical generation also induced by these nutritional interventions. In addition to the moderate but significant effect on membrane unsaturation, these nutritional interventions show an effect that is directly related to the percent of the dietary restriction applied, being both protein and methionine restriction even more intense and effective that caloric restriction. The effects of CR on membrane unsaturation could be divided in three stages depending of CR duration in rats. During short-term CR periods, decreases in the rate of mitochondrial ROS production and lipoxidation-derived protein damage are observed in some tissues together with minor changes in membrane fatty acid composition. If CR is applied for several weeks–months, changes in particular fatty acids with moderate or no changes in double bond content occur, although the magnitude of the changes depends on the organ and the intensity of the restriction. Finally, in long-term CR, the beneficial effects on ROS production, DBI-fatty acid composition, and lipoxidation-derived protein damage are evident. In fact, CR diminishes the slope of the relationship between age and age-related lipid peroxidation. Thus, the CR manipulation seems to trigger an adaptive response protecting the most basic requirements of membrane integrity.

Antioxidants, although possibly involved in protection against various age-related diseases, do not seem to control the rate of aging [3,4,20,22]. There are four lines of evidence for this. First, contrary to early hypotheses, it is now well known that the endogenous levels of antioxidants in tissues including the brain do not decrease during aging. Second, it is possible that the slow rate of aging of long-lived animals could be due to a constitutively higher antioxidant defence system. Surprisingly, when this was analyzed the reverse was found. Most studies showed that the levels of antioxidant enzymes and low-molecular-weight antioxidants in tissues correlate inversely with the species-specific longevity of vertebrates. Thus, it may be inferred that a low antioxidant levels of long-lived animals predict that their rate of ROS generation in vivo must also be low (and lower than that of short-lived animals), otherwise they could not maintain a level of oxidative stress homeostasis compatible with its rate of aging. The third source of information is studies in mammals in which levels of antioxidants are experimentally increased through dietary supplementation, pharmacological induction or transgenic techniques. The outcome of almost all such investigations is that maximum longevity remains unaffected. This is consistent with investigations performed in invertebrate models. Finally, the fourth line of evidence comes from studies in which genes encoding particular antioxidants are knocked

Table 3
Effect of caloric- protein- and methionine restriction on membrane unsaturation and advanced lipoxidation end-products (ALEs) of different rat tissues

Specie	Tissue	DR type (%)	DR duration	Effect on membrane unsaturation (PI)	ALEs	References
Rat	Liver mitochondria	8.5% CR	7 weeks	↓	↓	[133]
Rat	Liver mitochondria	25% CR	7 weeks	↓	↓	[133]
Rat	Heart mitochondria	40% CR	4 months	↓	↓	[130]
Rat	Heart mitochondria	40% CR	1 year	↓	↓	[80]
Rat	Liver mitochondria	40% CR	4–24 months	↓	↓	[75]
Rat	Liver	40% CR	6 weeks	↓	↓	[136]
Rat	Liver	40% PR	7 weeks	↓	↓	[132]
Rat	Liver mitochondria	40% MetR	7 weeks	↓	↓	[135]
Rat	Liver mitochondria	80% MetR	7 weeks	↓	↓	[131,135]
Rat	Heart mitochondria	80% MetR	7 weeks	↓	↓	[131]
Rat	Brain	80% MetR	7 weeks	↓	↓	[134]

out: the resulting animals can show different pathologies but their aging rates do not seem to be affected. Increased mean longevity is a much more frequent finding than increased maximum longevity in antioxidant treated or antioxidant-induced animals. Consequently, the broad lack of effect of antioxidants on longevity indicates that they do not slow down the endogenous aging process.

Finally, one of essential tools in studying the biological mechanism (s) underlying aging are animal models that show alterations in aging, most importantly, models that show retarded aging. The discovery in 1987 that chemical induced mutations in *C. elegans* lead to a mutant, *age-1*, with increased longevity [125] has led to the discovery of a large number of loss-of-function mutants in *C. elegans* and *Drosophila* that are shown to increase longevity [127]. The studies with mutant invertebrates have led investigators to question whether similar mutations in mammalian systems would also lead to retarded aging and increased longevity. Until recently, the only mammalian model of retarded aging was caloric restriction. However, over the past ten years, mutant models of mice have been reported that have a significant increase in longevity compared to wild-type controls. Globally, the studies with *S. cerevisiae*, like the studies with *C. elegans*, *Drosophila* and mice, indicate a link between increased longevity and resistance to oxidative stress [3,22,44]. However, the physiological mechanism whereby increased resistance to stress, including oxidative stress, leads to increased longevity has not been identified. It is generally assumed that the increased resistance to oxidative stress would lead to a reduced age-related accumulation in oxidative damage. Therefore, it is too early to conclude that most of these manipulations have increased longevity by retarding the accumulation of lipo- and oxidative damage during aging.

In summary, studies comparing constitutive antioxidant levels in both vertebrates and invertebrates' species, and experiments increasing or decreasing their tissue antioxidant concentrations in different ways, consistently indicate that antioxidants do not seem control aging rate, although they can protect against different pathologies and early death. The discovery and design of new mitochondrial antioxidant defences and new strategies of antioxidant over-expression will offer us more solid and definitive conclusions.

7.3. Membranes and longevity

Lipid oxidation is one of the natural consequences of aerobic life. The high concentration of PUFAs in cellular membrane phospholipids not only makes them prime targets for reaction with oxidizing agents but also enables them to participate in long free radical chain reactions. With these premises, and maintaining other physiological properties, a low degree of fatty acid unsaturation in cellular membranes may be advantageous by decreasing their sensitivity to lipid peroxidation. This would also protect other molecules against lipoxidation-derived damage. Thus, it has been found that long-lived animals (birds and mammals, including humans) have a lower degree of total tissue and mitochondrial fatty acid unsaturation (low Double Bond Index, DBI, and Peroxidizability Index, PI) than short-lived ones [3,4,18,20,22,23,35,129] (Table 4). In agreement with this, it has been demonstrated that in long-lived animal species a low degree of total tissue and mitochondrial fatty acid unsaturation is accompanied by a low sensitivity to in vivo and in vitro lipid peroxidation and a low steady-state level of lipoxidation-derived adducts in both tissue and mitochondrial proteins from organs like the skeletal muscle, heart, liver, and brain (Table 4). In this line, lipofuscin, often considered a hallmark of aging also shows an accumulation rate that inversely correlates with longevity [32]. These findings were consistent with the negative correlation observed between maximum longevity and the sensitivity to lipid autoxidation of mammalian kidney and brain homogenates [156]. While maximum longevity can differ dramatically between mammal and bird species, there can also be significant lifespan differences within a species. For example, populations of two wild-derived strains of mice display extended longevity (both mean and maximum longevity) compared to genetically heterogenous laboratory mice when kept under identical conditions [157]. The PI of skeletal muscle phospholipids and liver phospholipids of the two wild-type mice strains with the extended longevity was significantly smaller than that of the laboratory mice [153]. This is interesting because, since the different mice strains were fed the same diet, it shows that the differences in membrane composition between species are not determined by dietary differences but is genetically controlled. It is also interesting that in the senescence-accelerated mouse (SAM) strain, the SAM-prone mice have greater levels of the highly polyunsaturated peroxidation-prone fatty acids 22:6n-3 and 20:4n-6 and lower levels of the less peroxidation-prone 18:2n-6 PUFA in their membranes, and consequently have a greater PI than the SAM-resistant mice [69]. SAM-prone mice also show greater degrees of lipid peroxides in their tissues than do SAM-resistant mice. Finally, naked mole-rats are mouse-sized and are the longest-living rodents known, with a recorded MLSP exceeding 28 years [158]. When membrane fatty acid composition was measured in tissues from naked mole-rats, it was found that they have very low levels of 22:6n-3 in their tissue phospholipids compared to mice. Although both mice and naked mole-rats have similar levels of total unsaturated fatty acids in their tissue phospholipids, the low 22:6n-3 levels of the naked mole-rats result in lower PI and more peroxidation-resistant membranes in

Table 4
Comparative studies of membrane unsaturation in animal species with different maximum longevities

Species compared	Maximum longevity (years)	Organ	Correlation with maximum longevity	References
Rat–pigeon–human	4–120	mtLiver	Negative	[139]
Rat vs pigeon	4, 35	mtLiver	Negative	[140]
Rat vs pigeon	4, 35	mtHeart and microsomes	Negative	[140]
8 mammals	3.5–46	mtLiver	Negative	[141]
Rat vs pigeon	4, 35	mtHeart	Negative	[142]
8 mammals	3.5–46	Heart	Negative	[143]
Mouse vs canary	3.5, 24	Heart	Negative	[144]
Mouse vs parakeet	3.5, 21	Heart	Negative	[144]
7 mammals	3.5–46	Liver	Negative	[145]
8 mammals	3.5, 46	mtLiver	Negative	[11]
11 mammals+9 birds	3.5–120	Skeletal muscle	Negative	[146,147]
9 mammals+8 birds	3.5–120	mtLiver	Negative	[148,149]
Rat vs pigeon	4, 35	Skeletal muscle	Negative	[150]
Mouse, parakeet, canary	3.5, 21, 24	Brain	Negative	[151]
8 mammals	3.5–46	Heart	Negative	[152]
SAM-R/1 vs SAM-P/1 mice	1.8, 1.2	Liver	Negative	[69]
Strains of mice (Idaho, Majuro and WT)	3.97, 3.58, 3.35	Skeletal muscle and liver	Negative	[153]
Mice vs naked mole-rats	3–4, 28	mtSkeletal muscle and mtLiver	Negative	[154]
Queen honey bees vs workers	2–5, 75–135 days	Head, thorax, abdomen	Negative	[155]

skeletal muscle and liver mitochondria [154]. In summary, a low degree of unsaturation of cellular membranes is a general characteristic of long-lived vertebrate homeotherms, both birds and mammals.

The occurrence of correlation does not necessarily mean that a cause-effect relationship is operative. In order to clarify whether the low membrane unsaturation of long-lived animals protects their mitochondria from lipid oxidation and lipoxidation-derived protein modification, studies of experimental dietary modification of in vivo membrane fatty acid unsaturation have been performed. These studies were specially designed to partially circumvent the homeostatic system of compensation of dietary-induced changes in membrane unsaturation which operates at tissue level. The obtained findings suggest that lowering the membrane unsaturation of cellular membranes protects post-mitotic tissues against lipid peroxidation and lipoxidation-derived macromolecular damage [159–161].

The membrane acyl composition of the mammals and birds studied indicates that their biological membranes maintain an identical fatty acid average chain length (18 carbon atoms), a similar ratio of saturated versus unsaturated fatty acids (ratio 40:60), and a similar phospholipid distribution irrespective of animal longevity. The low DBI and PI observed in long-lived species are due to changes in the type of unsaturated fatty acid that participates in membrane composition. So, there is a systematic redistribution between the types of PUFAs present from the highly unsaturated 22:6n-3 and sometimes 20:4n-6 in short-lived animals to the less unsaturated 18:2n-6, and, in some cases 18:3n-3 in the long-lived ones, at mitochondrial and tissue level. Furthermore, the DBI of the respective diets did not correlate with maximum longevity. This indicates again that the contribution of the variations in the degree of unsaturation of dietary fats to the interspecies differences is, if any, very modest.

What are the mechanisms responsible for these longevity-related differences in fatty acid profile? They can be related, in principle, to the fatty acid desaturation pathway, and the deacylation–reacylation cycle. The available estimates of delta-5 and delta-6 desaturase activities indicate that they are several folds lower in long-lived species than in short-lived ones [18,35]. This can explain why 22:6n-3 and 20:4n-6 decreases, and 18:2n-6 and 18:3n-3 increases, from short- to long-lived animals, since desaturases are the rate-limiting enzymes of the n-3 and n-6 pathways synthesizing the highly unsaturated PUFAs 20:4n-6 and 22:6n-3 from their dietary precursors, 18:2n-6 and 18:3n-3, respectively. Thus, desaturation pathways would make available in situ the n-6 and n-3 fatty acids to phospholipid acyltransferases in order to remodel the phospholipid acyl groups. The fact that acyltransferase/n-6 desaturase activity ratio is about 10:1 in tissues [162] reinforces the idea that regulation of desaturases can be the main limiting factor responsible for the observed membrane unsaturation-longevity relationship. However, a role for a phospholipid-specific deacylation–reacylation system cannot be discarded since it has been observed that the longevity-related redistribution particularly affects the phosphatidylcholine and phosphatidyletanolamine fractions in liver mitochondria, and does not modify cardiolipin [11].

Animals with a high maximum longevity have a low degree of membrane fatty acid desaturation based in the redistribution between types of PUFAs without any alteration in the total (%) PUFA content, average chain length, and phospholipid distribution. This may be viewed as an elegant evolutionary strategy, because it decreases the sensitivity to lipid peroxidation and lipoxidation-derived damage to cellular macromolecules without strongly altering fluidity/microviscosity, a fundamental property of cellular membranes for the proper function of receptors, ion pumps, and transport of metabolites. This would occur because membrane fluidity increases acutely with the introduction of the first and less with the second double bond (due to their introduction of "kinks" in the fatty acid molecule), whereas additional (the third and following) double bonds cause few further variations in fluidity [163]. This is so because the kink has a larger impact on fluidity when the double bond is situated near the centre of the fatty acid chain (first double bond) than when it is situated progressively nearer to its extremes (next double bond additions). In the case of the sensitivity to lipid peroxidation, however, double bonds increase it irrespective of their location at the centre or laterally on the fatty acids. Thus, by substituting fatty acids with four or six double bonds by those having only two (or sometimes three) double bonds, the sensitivity to lipid peroxidation is strongly decreased in long-lived animals, whereas the fluidity of the membrane would be essentially maintained. This hypothesis, reminiscent of membrane acclimation to different environments at PUFA level in poikilotherms and bacteria, has been denominated homeoviscous longevity adaptation [18].

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