IMPACT OF LIPID PEROXIDATION ON THE PHYSIOLOGY AND PATHOPHYSIOLOGY OF CELL MEMBRANES

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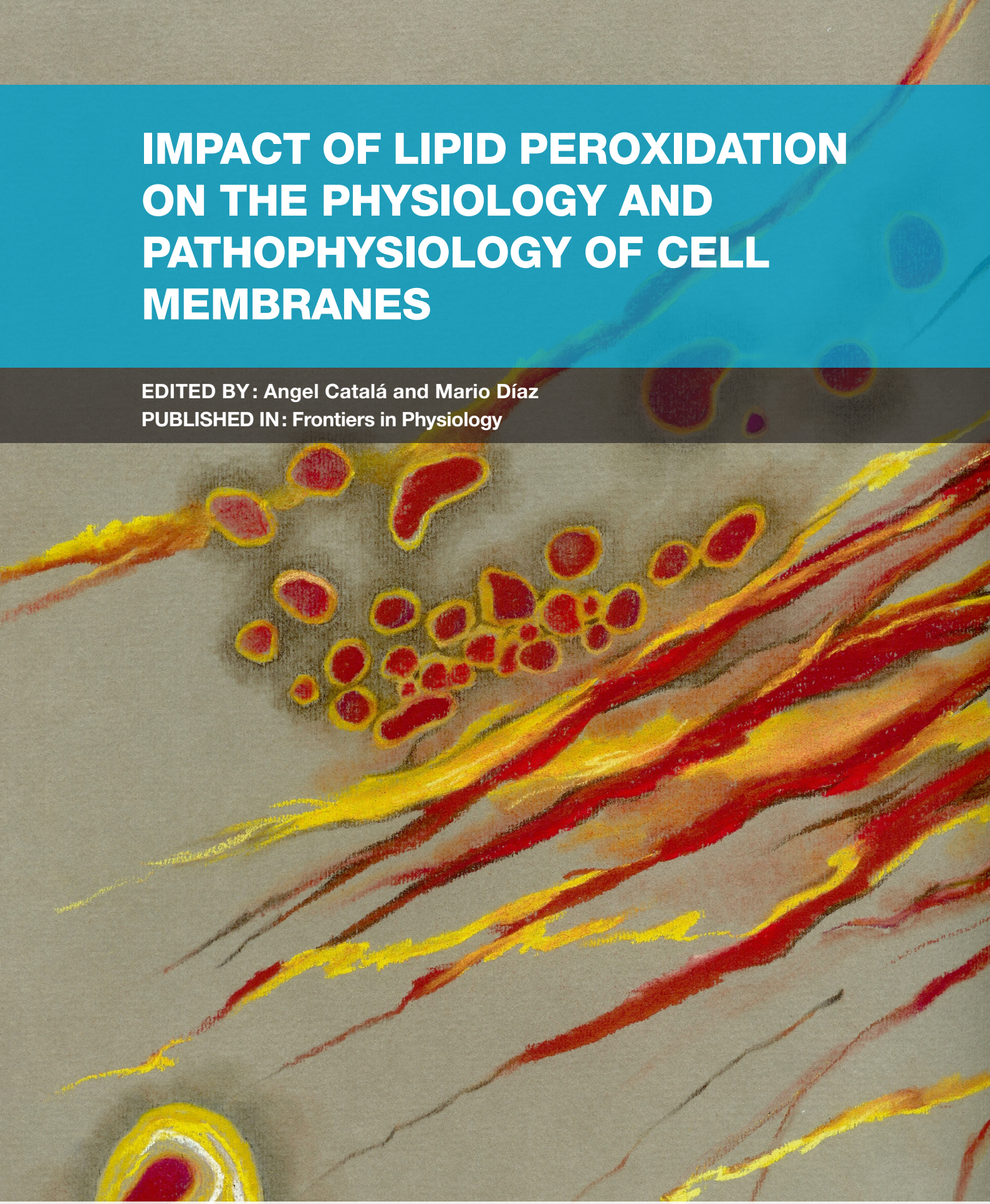

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IMPACT OF LIPID PEROXIDATION ON THE PHYSIOLOGY AND PATHOPHYSIOLOGY OF CELL MEMBRANES

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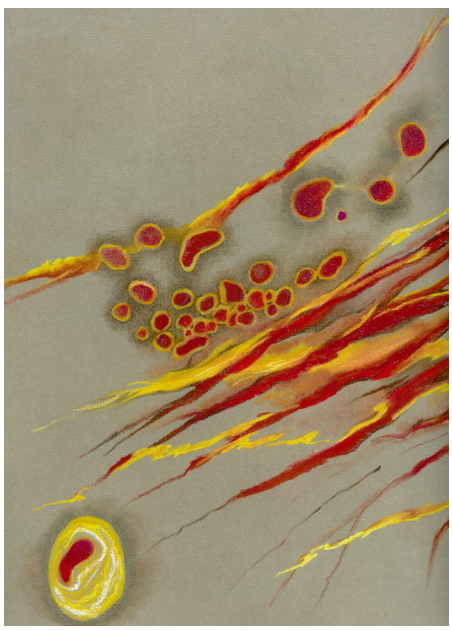

Abstraction on the generation of oxidative stress and senile plaques and neurofibrilary tangles in the hippocampus of Alzheimer´s disease brains.
Painted by Mario Díaz (2006)

The general process of lipid peroxidation consists of three stages: initiation, propagation, and termination. The initiation phase of lipid peroxidation includes hydrogen atom abstraction. Several species can abstract the first hydrogen atom and include the radicals: hydroxyl, alkoxyl, peroxyl, and possibly HO* 2. The membrane lipids, mainly phospholipids, containing polyunsaturated fatty acids are predominantly susceptible to peroxidation because abstraction from a methylene group of a hydrogen atom, which contains only one electron, leaves at the back an unpaired electron on the carbon. The initial reaction of *OH with polyunsaturated fatty acids produces a lipid radical (L*), which in turn reacts with molecular oxygen to form a lipid hydroperoxide

(LOOH). Further, the LOOH formed can suffer reductive cleavage by reduced metals, such as Fe++, producing lipid alkoxyl radical (LO*). Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes. In addition, LOOH can break down, frequently in the presence of reduced metals or ascorbate, to reactive aldehyde products, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (4-HHE) and acrolein. Lipid peroxidation is one of the major outcomes of free radical-mediated injury to tissue mainly because it can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction. In addition, a variety of lipid by-products are produced as a consequence of lipid peroxidation, some of which can exert beneficial biological effects under normal physiological conditions. Intensive research performed over the last decades have also revealed that by-products of lipid peroxidation are also involved in cellular signalling and transduction pathways under physiological conditions, and regulate a variety of cellular functions, including normal aging. In the present collection of articles, both aspects (adverse and benefitial) of lipid peroxidation are illustrated in different biological paradigms. We expect this eBook may encourage readers to expand the current knowledge on the complexity of physiological and pathophysiological roles of lipid peroxidation.

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Editorial: Impact of Lipid Peroxidation on the Physiology and Pathophysiology of Cell Membranes

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Keywords: lipid peroxidation, cell membranes

The Editorial on the Research Topic

Impact of Lipid Peroxidation on the Physiology and Pathophysiology of Cell Membranes

Oxygen-derived free radicals such as hydroxyl and hydroperoxyl species have been demonstrated to oxidize membrane lipid components, mainly phospholipids, leading to lipid peroxidation. Membrane phospholipids containing polyunsaturated fatty acids are predominantly susceptible to peroxidation. Free radical catalyzed peroxidation of long chain polyunsaturated acids (LCPUFAs) such as arachidonic acid and docosahexaenoic acid leads to generation of LCPUFA metabolites, including endoperoxides, isoprostanes, and neuroprostanes, which may further exert pharmacological/toxicological actions in many tissues (Balazy, 2000; Greco et al., 2000; Hardy et al., 2005; Bochkov et al., 2010; Davies and Guo, 2014). Eventually, peroxidation of membrane lipids can disturb the assembly of cell membranes, which inevitably will impact membrane fluidity, lipid-lipid, and lipid-protein interaction dynamics, membrane permeability, physicochemical properties, ion, and nutrient transport, membrane-initiated signaling pathways, and metabolic processes leading to cell death (Fruhwirth and Hermetter, 2008; Catalá, 2009; Adibhatla and Hatcher, 2010; Volinsky and Kinnunen, 2013). Intensive research performed over the last decades have also revealed that products of lipid peroxidation are also involved in cellular signaling and transduction pathways under physiological conditions, and regulate a variety of cellular functions, including normal aging (Bazan, 2005; Pamplona, 2008; Naudí et al.).

The present research topic might be divided in two major blocks. The first one deals on the mechanisms of lipid peroxidation under physiological and pathophysiological conditions. The second one highlight the different defenses against lipid peroxidation and oxidative stress that cells contain and the efficient and regulated mechanisms they use to buffer the noxious effects of lipid peroxidation. In the next paragraphs, we will summarize the highlights of each of these blocks and the articles included therein.

In the first block, Njie-Mbye et al. review the pathophysiological and pharmacological implications of lipid peroxidation in the eye. The eye, and particularly the retina, is the organ containing the largest amount of LCPUFAs amongst all organs in the body. Further, lipid peroxidation has been reported to be involved in degenerative ocular diseases, such as age-related macular degeneration, cataract, glaucoma, and diabetic retinopathy. Authors review the toxicological effects of hydrogen peroxide, LCPUFA-derived lipoperoxides and several synthetic peroxides in different parts of mammalian eyes, including the uvea, ganglionic retinal cells, and posterior segments in the retina. They also show that H_2O_2 and LCPUFA-derived lipoperoxides modulate excitatory and inhibitory synapses in the eye by regulating neurotransmitter metabolism, and conclude that these abilities to alter the integrity of neurotransmitter pools provide new

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potential target sites and point to novel pharmacological strategies for the treatment of degenerative ocular diseases.

Lipid peroxidation of biological membranes modifies their assembly, structure, and dynamics, both at lipid and protein levels. When the Fluid Mosaic Model (FMM) of membrane structure was introduced by Singer and Nicolson (1972), it was visualized as a basic model for cell membranes that could explain existing observations on membrane proteins and lipid structures and their dynamics. Accordingly, the membrane was topologically defined as a biological fluid of proteins and lipids oriented in two dimensions. In the FMM, amphipathic phospholipids are oriented in a lamellar mesophase organization with hydrophobic fatty acyl chains embedded within the interior of the membrane and the hydrophilic polar groups facing the aqueous environment. However, several biological processes cannot be explained on the basis of this typical phospholipid orientation but exhibit other conformations. The Lipid Whisker Model (LWM) is an extension of the FMM, introduced upon observations into the conformation of oxidized phospholipid (oxPL) species recognized by CD36 (Li et al., 2007). Thus, in the LWM, when cell membranes undergo oxidation, if not adapted by the action of phospholipases, they may "produce whiskers" including a variety of oxidized sn-2 fatty acids of diverse structures. In the (LWM), the assembly of many oxPL within cell membranes is different compared with one observed in non-oxPL described in the FMM. Indeed, biophysical evidence indicates that addition of an oxygen atom to the acyl chain produces significant changes that prevent its immersion in the interior of the membrane, because the presence of peroxyl radicals project toward the aqueous interface. However, controversies exist on the validity of the validity of this "floating peroxyl radical" hypothesis. This has been discussed in the opinion article by Dr. Catalá.

The article by Pizzimenti et al. revise the interaction of membrane proteins and aldehydes derived from lipid peroxidation under both, physiological and pathophysiological perspectives. They begin their review with a description of main aldehydes (4-HNE, acrolein, MDA, and Phosphatidyl γ-Hydroxyalkenals) protein adducts. They show that targets of lipid peroxidation-derived aldehydes are cell-type specific and dependent on both, the set of proteins expressed in the specific cell type and also by the concentration of reactive aldehydes. Not all protein-aldehyde adducts are necessarily deleterious, and the final biological effect will depend on the adduct concentration. For instance, at low concentration, HNE have an important role in signal transduction pathways, and exert anti-proliferative and anti-metastatic effects toward cancer cells, by interfering with the modulation of gene expression through generation of protein and/or DNA adducts. Under a pathological scenario, Pizzimenti and collaborators review the main evidences linking these protein-adducts with chronic neurodegenerative diseases (Alzheimer's disease), atherosclerosis and autoimmunity inflammation. In line with this, a methodological article by Wu et al. describe and validate a novel two-dimensional native SDS-PAGE that allows identification of HNE-protein adducts. The example illustrated in the article show the procedure applied to identify mitochondrial complex I (NADH-ubiquinone oxidoreductase), which are linked to diabetic kidney mitochondria.

Membrane lipid peroxidation is not limited to the generation of reactive oxygen species, but also to the production of nitrogen reactive species (RNS) from nitric oxide (NO). NO is recognized both, as a signaling molecule that regulates many enzyme activities, but also as a toxic agent in animal and plant cells. NO is able to protect photosynthetic and non-photosinthetic organims from oxidative damage resulting from $O^{\cdot -}$, H_2O_2, and alkyl peroxides by acting as a terminator of free radical chain reactions. Through the reaction of O-2 with NO, the anion peroxynitrite ($ONOO^-$) is generated, which acts as a reactive nitrating agent capable of modifying proteins (nitrotyrosine), lipids (lipid nitration), and nucleic acids (DNA nitration) (Gisone et al., 2003). These reactive effects of RNS on lipid peroxidation are commented in the article by Galatro and co-workers in the present Research Topic (Galatro et al.).

The second block of articles included this research topics are introduced by a review article by Naudí et al. related to the involvement of membrane lipid unsaturation as a physiological adaptation to animal longevity. After introducing the dual role of lipid peroxidation-derived metabolites as cell threatening or signaling molecules, authors use a comprehensive comparative study of animals along the phylogenetic tree to postulate that longevity is associated to membrane lipid unsaturation. Through a series of targeted global queries they speculate and expand on (1) The link between aging, membrane unsaturation and lipoxidation reactions, (2) Whether interspecies variations in longevity are related to differences in membrane unsaturation and lipoxidation-derived molecular damage, and (3) Whether genetic manipulations or nutritional interventions might be accompanied by attenuations of membrane unsaturation in cell membranes. Finally, authors provide a hypothesis to describe the physiological mechanisms underlying the structural adaptation of cellular membranes to oxidative stress and to review the state of the art about the link between membrane composition and longevity of animal species.

The last three articles are focused on bioactive molecules participating in cellular antioxidant strategies. The first, from the group of Dr. Reiter in Texas, is an opinion article on the role of melatonin and its metabolites, such as cyclic 3-hydroxymelatonin or N1-acetyl-N2-formyl-5-methoxykynuramine, in scavenging lipoperoxides (by acting as a direct scavenger of initiation and propagation by-products) and also on their ability to modulate the activities of a variety of antioxidant enzymes (Reiter et al.). These effects are physiologically relevant, since melatonin is produced in many cells and its synthesis may be upregulated under conditions that elevate oxidative stress in mammals, where they have been proven to exert protective actions both *in vivo* and *in vitro* and in models of numerous diseases.

Casañas-Sánchez and co-workers contribute to this topic with two articles on the mechanistic basis of action of two novel "*indirect antioxidants*" in model hippocampal cells. The term "*indirect antioxidants*" was introduced in recent years to refer to bioactive molecules that although lacking direct scavenging activities, they stimulate antioxidant activity by modulating gene expression of components of the antioxidant systems. In the first study, a hypothesis and theory, they show

that docosahexaenoic acid (DHA) is, besides a structural component of membrane phospholipids a efficient activator of specific genes belonging to the glutathione/glutaredoxin and thioredoxin/peroxiredoxin systems (Casañas-Sánchez et al.). Their hypotheses focus on the ability of DHA to regulate the expression of phospholipid-hydroperoxide glutathione peroxidase (PH-GPx/GPx4) isoforms, the main enzyme family protecting cell membranes against lipid peroxidation and capable to reduce oxidized phospholipids *in situ*, i.e., within the membrane. By doing this, DHA triggers the stimulation of genomic mechanisms leading to self-protection from oxidative damage. This is particularly relevant in neuronal cells, where the high aerobic metabolism and the presence of elevated levels of transition metals, favor the generation of reactive oxygen species and lipid peroxides from highly unsaturated fatty acids, as is the case of DHA and arachidonic acid. Authors also show that, at least in mouse hippocampal neurons (cell lines and hippocampal parenchyma), DHA upregulates a Gpx4 splicing variant (named Gpx4 CIRT), which harbors part of the first intronic region (CIRT: Cytoplasmic Intron-sequence Retaining Transcripts), which according to the "sentinel RNA hypothesis" (Buckley et al., 2014) would expand the ability of Gpx4 family of transcripts to provide neuronal antioxidant defense in cellular compartments, like dendritic zones, located away from the nucleus, independently of conventional nuclear splicing.

The second article by Casañas-Sánchez et al. is rather striking, since they show that ethanol may well be neuroprotective to hippocampal cells. Ethanol is known to cause severe systemic damage secondary to oxidative stress, and brain is particularly vulnerable to ethanol-induced reactive oxygen species, especially upon high and/or chronic exposures to ethanol. However, Casañas-Sánchez et al. demonstrate that a low and acute dose of ethanol trigger a completely opposite response in hippocampal cells, consistent with the stimulation of transcriptional expression of genes belonging to the classical, glutathione/glutaredoxin, and thioredoxin/peroxiredoxin antioxidant systems. That these genomic effects are neuroprotective is demonstrated in experiments where they show that sub-toxic ethanol exposure prevents glutamate-induced excitotoxicity in hippocampal cells, pinpointing that under this paradigm ethanol may well be neuroprotective against oxidative insults in hippocampal cells.

Overall, we believe our Research Topic will provide an exciting overview on the impact of membrane lipid peroxidation under physiological and pathophysiological scenarios. Currently, there exists an increasing appreciation on the importance of membrane lipid peroxidation in relation to health and disease, as well as longevity. We hope that this Research Topic will foster the research on the precise involvement of membrane lipid peroxidation in devastating diseases such as neurodegenerative and cardiovascular diseases, as wide spread disorders in developed societies, and on the development of pharmaceutical strategies aimed to prevent and treat them.

AUTHOR CONTRIBUTIONS

AC and MD have edited the Resarch Topic and have written and drafted the manuscript.

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Lipid peroxidation: pathophysiological and pharmacological implications in the eye

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Oxygen-derived free radicals such as hydroxyl and hydroperoxyl species have been shown to oxidize phospholipids and other membrane lipid components leading to lipid peroxidation. In the eye, lipid peroxidation has been reported to play an important role in degenerative ocular diseases (age-related macular degeneration, cataract, glaucoma, diabetic retinopathy). Indeed, ocular tissues are prone to damage from reactive oxygen species due to stress from constant exposure of the eye to sunlight, atmospheric oxygen and environmental chemicals. Furthermore, free radical catalyzed peroxidation of long chain polyunsaturated acids (LCPUFAs) such as arachidonic acid and docosahexaenoic acid leads to generation of LCPUFA metabolites including isoprostanes and neuroprostanes that may further exert pharmacological/toxicological actions in ocular tissues. Evidence from literature supports the presence of endogenous defense mechanisms against reactive oxygen species in the eye, thereby presenting new avenues for the prevention and treatment of ocular degeneration. Hydrogen peroxide (H_2O_2) and synthetic peroxides can exert pharmacological and toxicological effects on tissues of the anterior uvea of several mammalian species. There is evidence suggesting that the retina, especially retinal ganglion cells can exhibit unique characteristics of antioxidant defense mechanisms. In the posterior segment of the eye, H_2O_2 and synthetic peroxides produce an inhibitory action on glutamate release (using [^{3}H]-D-aspartate as a marker), *in vitro* and on the endogenous glutamate and glycine concentrations *in vivo*. In addition to peroxides, isoprostanes can elicit both excitatory and inhibitory effects on norepinephrine (NE) release from sympathetic nerves in isolated mammalian iris ciliary bodies. Whereas isoprostanes attenuate dopamine release from mammalian neural retina, *in vitro*, these novel arachidonic acid metabolites exhibit a biphasic regulatory effect on glutamate release from retina and can regulate amino acid neurotransmitter metabolism without inducing cell death in the retina. Furthermore, there appears to be an inhibitory role for neuroprostanes in the release of excitatory amino acid neurotransmitters in mammalian retina. The ability of peroxides and metabolites of LCPUFA to alter the integrity of neurotransmitter pools provides new potential target sites and pathways for the treatment of degenerative ocular diseases.

Keywords: lipid peroxidation, oxidative stress, peroxides, ocular diseases

INTRODUCTION

Oxidative damage is involved in the pathogenesis of a variety of chronic degenerative and neurodegenerative diseases. Increasing evidence indicates that oxidative stress (OS) plays a major role in ocular pathologies including cataract, age-related macular degeneration (ARMD), glaucoma, and diabetic retinopathy (DR). Under normal physiological states, ocular tissues possess several intrinsic antioxidant enzymes to cope with OS formed as a consequence of normal metabolism. During ocular injuries, overproduction of reactive oxygen species (ROS) and free radicals overwhelms the intrinsic antioxidant mechanisms resulting in OS and ultimately development of a pathological condition. There is evidence that products of oxygen-derived free radical pathway such as peroxides and metabolites of long chain polyunsaturated fatty acids (LCPUFAs) are involved in oxidative reactions in the eye and play major roles in the pathogenesis of most ocular diseases (Greene and Paller, 1992; van Reyk et al., 2003; Shichi, 2004; Sangiovanni and Chew, 2005). LCPUFAs are essential structural components of membrane lipids that are involved in several physiological functions in the body, including central nervous system function and development, inflammation, and immunological responses in the cardiovascular system, amongst other things (De et al., 1994; Grimble, 1998; Wu and Meydani, 1998). In the eye, deficiency of docosahexaenoic acid (DHA), the most abundant LCPUFA, is associated with abnormalities in the visual system, including retinitis pigmentosa, peroxisomal disorders and compromised growth and development in infants (Sangiovanni and Chew, 2005). Tissue damage by oxygen-derived free radicals

has been associated with a variety of pathological conditions in the eye. For instance, hydrogen peroxide (H_2O_2) a biologically derived stable oxidant intermediate, and other peroxide-induced radicals can inflict damage on ocular tissues by disrupting cellular structure and function (Wielgus and Sarna, 2008). In addition, to their patho-physiological role in the eye, oxidant-derived metabolites can exert pharmacological actions on tissues of the anterior uvea and retina. This review summarizes our current state of knowledge of the contributions of lipid peroxidation to the pathogenesis of ocular disorders as well as their pharmacological impact in the eye.

OXIDATIVE STRESS AND OCULAR DEGENERATIVE DISEASES

The eye is constantly exposed to radiation, atmospheric oxygen, environmental chemicals, and physical abrasion, thus, creating an environment that supports generation of elevated levels of ROS (Green, 1995; van Reyk et al., 2003). Evidence from literature supports a role for excess free radicals in ocular tissues serving as an underlying pathology in diseases such as cataracts, ARMD, glaucoma, and various forms of retinopathy (Green, 1995; Osborne et al., 1999; Shichi, 2004). The biological role of ROS in some ocular diseases is summarized below.

CATARACT

Cataract is an ocular disease that is characterized by increasing opacity of the lens resulting in visual impairment (Brennan and Kantorow, 2009; Sacca et al., 2009). Incidence of cataract increases with age and is one of the leading causes of blindness and visual impairment in elderly populations in developing countries (Li et al., 2009; Sacca et al., 2009). Aging is one of the greatest risk factors for non-congenital cataract formation, but other factors include gender, exposure to UV light, cigarette smoking, increase in ROS, decrease in antioxidant defense, and DNA damage (Brennan and Kantorow, 2009; Li et al., 2009; Sacca et al., 2009).

OS is a well-documented mechanism involved in cataract progression in which there is prevalent oxidation of lens DNA, proteins, and lipids (Sacca et al., 2009). There is evidence that ROS —•OH, O_2• - or H_2O_2—can damage the lens and contribute to cataract formation (Berthoud and Beyer, 2009). Moreover, there are studies in literature showing involvement of reactive nitrogen species (RNS), such as nitric oxide (NO) in cataract formation (Varma and Hegde, 2007). Furthermore, iron has the ability to catalyze free radical reactions, thereby propagating OS. Peroxidation of lipids is another cause of cataracts formation, impairing lipid-lipid and protein-lipid interactions particularly in the lenticular fiber membranes (Sacca et al., 2009). Elevated levels of hydroperoxides, diene conjugates, and oxy-derivatives of phospholipid fatty acids have been found in human lenses and aqueous humor (AH) of patients with complicated and senile cataracts (Sacca et al., 2009). Lipid peroxidation within cells decreases glutathione (GSH) and leads to DNA impairment while lipid hydroperoxidation alters membrane permeability, structure, and microviscosity of lipid-protein mediums (Sacca et al., 2009). A study by Li et al., has reported significantly higher plasma concentrations of (Z,E) isomers of hydroxyoctadecadienoic acid (HODE), oxidation products of linoleic acid, in subjects with early cataract indicating that lipid peroxidation plays a role in the progression of lens opaqueness (Li et al., 2009).

The endogenous antioxidant defense enzymes like catalase (CAT), GSH peroxidase (GPx), superoxide dismutase (SOD), and peroxiredoxins operate to balance levels of ROS in the lens. For example, GSH neutralizes H_2O_2, lipid peroxides, dihydroascorbic acid, and prevents formation of disulfide bonds by keeping thiols in the reduced state. Cysteine and methionine residues also function as antioxidants through reversible protein oxidation. Thus, under normal physiological conditions, the lens is able to protect itself from OS stimuli.

GLAUCOMA

Glaucoma refers to a diverse group of ocular disorders that are characterized by retinal neurodegeneration, visual field defects and blindness. Affecting approximately 60 million people globally, glaucoma is ranked as the second leading cause of irreversible blindness worldwide. This disease accounts for 9–12% of all cases of blindness in the US. Open angle glaucoma (POAG), the most prevalent form of this disease, affects about 95% of all glaucoma's encountered in US (Sommer et al., 1991). Although elevated intraocular pressure (IOP) is the major risk factor for POAG, a small population of patients exhibit visual defects with normal IOP—a condition termed normal-tension glaucoma. Thus, IOP elevation does not singularly account for the clinical manifestation of the POAG (Collaborative Normal-Tension Glaucoma Study Group, 1998; The AGIS Investigators, 2000). The etiology of POAG is attributed to multifactorial mechanisms, including OS, ischemia, vascular dysregulation, and altered NO metabolism (Sacca et al., 2007; Resch et al., 2009).

OS has been hypothesized to play a significant role in the pathogenesis of POAG (Alvarado et al., 1981, 1984). Indeed, both experimental and clinical studies implicate OS in the pathogenesis of POAG. Targets for OS include trabecular meshwork (TM) structures in the anterior segment and retinal ganglion cells (RGCs) and the optic nerve head (ONH) structures in the posterior segment of the eye. Although the exact outflow mechanism has not been completely elucidated, TM cells regulate AH outflow as part of the conventional outflow pathway and formation and turnover of extracellular matrix (ECM) (Yue, 1996). Physiological functions of both TM and Schlemm's canal decline with aging, a process that is characterized by an increase in oxidant stress (Grierson et al., 1984; Grierson and Howes, 1987). OS elicits TM degeneration and confers structural alterations in TM cytoskeleton, thereby compromising its physiological functions (Zhou et al., 1999). In support of this observation, POAG patients had higher concentrations of lipid peroxidation products in TM and AH, compared to control subjects (Babizhayev and Bunin, 1989). In the posterior segment, constant light-exposure of the LCPUFA-rich retina renders it highly susceptible to OS. Numerous OS biomarkers have been identified, including 4-hydroxy-2-nonenal (4-HNE), and malondialdehyde (MDA). Indeed, 4-HNE has been reported to induce apoptosis in primary cultures of normal ONH astrocytes in humans (Malone and Hernandez, 2007). Furthermore, MDA was elevated in vitreous and retina of ocular hypertensive rats, suggesting the presence of OS mechanisms in those tissues (Ko et al., 2005; Yucel et al., 2005).

Effective defense mechanisms against free radicals are present in biological systems. In the eye, ascorbic acid which is found in high concentrations in both the anterior and posterior segment of the eye, has been shown to be involved in ocular protection (Izzotti et al., 2006). Reduced GSH is another prominent antioxidant found in the eye that protects ocular tissues from damage caused by ROS (Riley, 1990; Costarides et al., 1991). High GSH concentrations can be found in the AH and TM of normal mammalian eyes (Kahn et al., 1983). There are findings that also suggest an antioxidant defense system in the aqueous outflow system (Ferreira et al., 2004; Yang, 2004; Gherghel et al., 2005). The retina possesses endogenous self-protective mechanisms such as the upregulation of pro-survival brain-derived neurotrophic factor (BDNF) and tyrosine kinase (TRK) receptors, expression of anti-apoptic Bcl-2 and BCL-x genes and increased expression of heat shock proteins (HSPs) (Gao et al., 1997; Levin et al., 1997; Chaudhary et al., 1999; Cui et al., 2002; Huang et al., 2007). The glial cells and ONH astrocytes are well-equipped with efficient antioxidant defense mechanism for self-protection against oxidative damage (Malone and Hernandez, 2007). These cells upregulate survival-promoting signals against ROS-induced OS and secrete neurotrophic factors that support RGC axonal regeneration (Qu et al., 2010). Clearly, endogenous defense mechanisms exist in both anterior and posterior segments to protect the eye against ROS induced damage associated with glaucoma.

AGE-RELATED MACULAR DEGENERATION

ARMD is characterized by irreversible loss of central vision. It is the leading cause of vision impairment and blindness in persons aged 65 and over in industrialized countries (Ethen et al., 2007; Hollyfield et al., 2008; Hollyfield, 2010). The disease affects the macula at the center of the eye and as a consequence results in loss of central vision which significantly impacts the patient's ability to read, watch television or drive (Jarrett and Boulton, 2012) Risk factors that contribute to ARMD are heterogeneous, mainly including increasing age and different genetic predispositions, together with several environmental/epigenetic factors such as cigarette smoking, dietary habits, and phototoxic exposure. In the aging retina, free radicals and oxidized lipoproteins are considered to be major causes of tissue stress resulting in local triggers for para-inflammation, a chronic status which contributes to initiation and/or progression of ARMD (Parmeggiani et al., 2012).

Although the development of ARMD is not clearly understood, OS appears to be one feasible pathway (Winkler et al., 1999; Kasahara et al., 2005; Ethen et al., 2007; Bertram et al., 2009; Bruban et al., 2009; Hollyfield, 2010). The death of retinal pigment epithelial (RPE) cells due to OS are the primary targets in the early phase of ARMD (Kasahara et al., 2005). RPE cells are transporters of selective molecules between choroidal blood and the neural retina thereby making their death a prime factor in accompanying photoreceptor damage (Kasahara et al., 2005). Indeed, OS may cause injury to the RPE cells, the Bruch's membrane, and the choroid, which are layers in the eye involved in the pathophysiology of ARMD (Yildirim et al., 2011). OS in ocular tissues and accumulation of lipofuscin in the RPE may induce production of ROS leading to lipid peroxidation and eventual RPE cell death (Winkler et al., 1999).

Multiple defense mechanisms exist in the retina to combat OS. Endogenous antioxidants, like non-enzymatic scavengers as GSH and antioxidant enzymes such as SOD, GPx, and CAT, are the first lines of endogenous defense against OS and act by scavenging potentially damaging free radical moieties (Yildirim et al., 2011). Dietary antioxidants like the carotenoids, vitamins A, C, and E, selenium, zinc, and bioflavonoids showed decreased risk of vision loss in ARMD (Winkler et al., 1999; O'Connell et al., 2008). Clearly, opportunities exist for further investigation of preventive and therapeutic measures against ARMD that reflect the pathways of disease progression and natural defense systems.

DIABETIC RETINOPATHY

DR is an ocular neuropathy associated with advanced forms of both diabetes types I and II (Kanwar et al., 2007; Madsen-Bouterse and Kowluru, 2008). In spite of advancement in research techniques, increased emphasis on diabetes prevention and therapeutic monitoring, DR remains an important complication of diabetes and a leading cause of blindness among young, working-age adults between 20 and 74 years (Centers for Disease Control and Prevention, 2013). Clinical appearance of DR is preceded by biochemical, molecular and hemodynamic alterations that begin early in the disease process. Both clinical and experimental data have confirmed the role of chronic hyperglycemia as the major initiating parameter in the pathogenesis of diabetes complications (Kaiser et al., 1993; Kuusisto et al., 1994; Ohkubo et al., 1995; Skyler, 1996). It appears that chronic hyperglycemia elicits a series of deleterious alterations that culminate in structural and functional damage to retina. Evidence suggests a prominent role for OS in hyperglycemia-induced alterations in DR patients and experimental animals (Yadav et al., 1997a,b; Gurler et al., 2000). While diabetes patients have elevated plasma OS biomarkers compared to normal subjects, DR patients exhibit higher concentrations of OS biomarkers than diabetes patients without DR, suggesting the significance of OS in the pathogenesis of DR (Gurler et al., 2000). It has been argued that not only does chronic hyperglycemia generate ROS, but it sets in motion several pathways including, activation of aldose reductase (sorbitol), protein kinase C (PKC), accumulation of advanced glycation-end-products (AGE) and release of inflammatory mediators and vascular endothelial-derived growth factor (VEGF) that further increases OS and concomitantly impairs endogenous antioxidant defense mechanisms (Al-Shabrawey and Smith, 2010; Cheung et al., 2010). Thus, OS is a vital parameter that links hyperglycemia and evolvement of DR in diabetic subjects (Kanwar et al., 2007; Madsen-Bouterse and Kowluru, 2008; Pan et al., 2008; Zheng and Kern, 2009).

Patients with diabetes also show increased production of lipid peroxidation, DNA damage, and protein oxidation due to OS. Indeed evidence shows elevated levels of OS biomarkers such as malondialdehyde,8-isoprostane (IsoP) E_2, F_2-IsoP, and lipid hydroperoxides in plasma of diabetic patients (Pan et al., 2008). Because the retina is rich in PUFAs and has increased glucose oxidation and oxygen uptake, it is susceptible to increased OS in hyperglycemia (Madsen-Bouterse and Kowluru, 2008). The observed structural and functional changes are likely to result from lipid peroxidation of the vascular endothelium.

The retina employs a complex antioxidant defense system to maintain optimal concentrations of NADPH and GSH including SOD, glucose-6-phosphate dehydrogenase, GPx, GSH reductase, and CAT (van Reyk et al., 2003; Zheng and Kern, 2009). There is evidence that DR is associated with reduced levels of GSH, SOD, CAT, and ascorbic acid (Madsen-Bouterse and Kowluru, 2008). Furthermore, there are reports that show SOD activity to be decreased in the vitreous and anterior chamber of hyperglycemic patients (Zheng and Kern, 2009).

ROLE OF OXIDANT-DERIVED METABOLITES IN PATHOGENESIS OF EYE DISEASES

Stress induced by oxygen-derived radicals such as hydroxyl radical, superoxide anion and H_2O_2 can be deleterious to cells (Gilgun-Sherki et al., 2002; Blokhina et al., 2003). In the eye, free radical-induced tissue damage has been associated with a variety of pathological conditions. H_2O_2 in particular, has been reported to be involved in oxidative reactions in ocular tissues, thus, indicting a possible role for this peroxide in ocular physiology and pathology (Ohia et al., 2005). In addition to peroxides, metabolites of LCPUFA have also been implicated in the progression of ocular neurodegenerative diseases. LCPUFAs play a significant structural and physiological role within the cell. Due to the abundance of double bonds in their structure, these fatty acids are highly susceptible to lipid peroxidation to form an array of oxidant-derived metabolites. This susceptibility is further compacted in ocular tissues due to constant exposure of the eye to light and high lipid content in some ocular tissues (Mainster, 1987; Green, 1995; Organisciak et al., 1998; van Reyk et al., 2003). In spite of evidence depicting the role of OS in ocular pathology, further research is warranted to fully delineate the physiological role and biological impact of oxidant-derived metabolites in the eye. **Table 1** provides a summary of oxidant–derived metabolites present in ocular tissues and their role in ocular pathology.

PEROXIDES

There is evidence that H_2O_2 and enzymes involved in its metabolism is present in both the anterior segment and posterior segment of the eye (Rose et al., 1998; Beatty et al., 2000). H_2O_2 is continually produced in the AH by cellular metabolism and exposure to UV light. The concentration of H_2O_2 in the AH and anterior uvea in healthy persons is in the range of 30 and 70 μM (Rose et al., 1998). In the AH and corneal epithelium, vitamin C reacts with O_2 to form H_2O_2 in protecting the eye against UV radiation (Izzotti et al., 2009). In the anterior segment, free radical-induced tissue damage has been associated with a variety of pathological conditions such as cataract and experimental autoimmune uveitis (Wu et al., 1997; Shichi, 2004). In patients with cataract, concentrations of H_2O_2 that is ten to thirty-fold higher than healthy human subjects has been reported in lens and AH (Shichi, 2004; Wielgus and Sarna, 2008). In addition, there is evidence that lens epithelial cells are exceedingly susceptible to OS generated by H_2O_2. Indeed, short term exposure of epithelial cells to 25–50 μM H_2O_2will disturb normal metabolism, especially if there is a breach in antioxidant defense (Ma et al., 2004). H_2O_2 has also been shown to cause DNA damage in lens and corneal epithelial cells (Atilano et al., 2009). Exposure of lens epithelial cells to H_2O_2 causes opacification and cataract (Jin et al., 2007). The TM of the eye is particularly sensitive to H_2O_2-induced OS; high concentrations of H_2O_2 in GSH compromised eyes leads to reduced outflow in the AH (Izzotti et al., 2009).

As opposed to the anterior segment of the eye, not much is known on the role of oxygen-derived free radicals in the posterior segment of the eye. Hence further studies are needed to understand the biological role of these metabolites in ocular tissues. In the retina, H_2O_2 has been linked to damage due to exposure to light and oxygen (Beatty et al., 2000). Both H_2O_2 and enzymes that mediate its metabolism are present in the retina (Liles et al., 1991; Beatty et al., 2000; Sandbach et al., 2001). Ascorbic acid has been reported to act as an antioxidant for the removal of H_2O_2 in ocular tissues including the retina (Shang et al., 2003). Interestingly, the concentration of ascorbic acid in the vitreous humor has been reported to be twice that found in the AH (Rose et al., 1998). The higher levels of ascorbic acid in the vitreous humor may serve to protect the retina against the damaging effects of OS and ultraviolet radiation. The concentration of H_2O_2 in the vitreous humor of human patients with cataract has been reported to be two to three times more than the amount of this oxidant found in normal patients (Shichi, 2004). It remains to be determined if such high levels of H_2O_2 in the vitreous humor can also affect normal retinal function.

Table 1 | Summary of several Oxidant-derived metabolites and their role in ocular pathology.

Oxidant-derived metabolites	Ocular tissue presence	Plausible role	Associated ocular pathology
PEROXIDES			
H_2O_2			Cataract, uveitis
Hydroperoxides	Lens, aqueous humor	Disrupt cell function and structure	
Lipid peroxides	Lens, retina		Cataract,DR
LCPUFA METABOLITES			
Hydroxyoctadecadienoic acid (HODE)	Lens	Membrane permeability alteration, cell structure changes	Cataract
4-hydroxy-2-nonenal (4-HNE)	TM, optic nerve, retina	Apoptosis, protein modification	Glaucoma, ARMD
Malondialdehyde	TM, retina, vitreous humor	Cytoskeleton alterations, functional changes	Glaucoma
ISOPs	Aqueous humor, lens, retina	Structural and functional damage to tissue	Exfoliation syndrome (XFS), cataract, DR

LCPUFA PEROXIDATION METABOLITES

The interaction of OS and LCPUFA metabolites is a less known area of research that could have significant implications in neuronal tissues. LCPUFA metabolites such as isoprostanes (IsoPs) and neuroprostanes (NeuroPs) have been shown to be present in mammalian tissues as well as human biological fluids (Reich et al., 2000; Cracowski et al., 2002; Fam et al., 2002; Shichi, 2004). IsoPs are spontaneously derived in abundance *in vivo* from the LCPUFA, arachidonic acid (AA). These prostaglandin (PG)-like compounds are produced by the free radical-catalyzed, non-enzymatic peroxidation of AA. Due to the fact that IsoPs are stable products, whose production increases with exposure to OS, they have gained acceptance as a reliable marker of oxidative injury in both *in vivo* and *in vitro* animal models (Gopaul et al., 1995; Delanty et al., 1997; Morrow, 2000; Shichi, 2004). Analogous to IsoPs, the NeuroPs are formed by spontaneous free-radical, non-enzymatic peroxidation of DHA and have been found to be applicable as diagnostic markers of oxidative injury (Fam et al., 2002; Shichi, 2004; Morrow, 2006). It has been reported that in the mammalian eye, oxidant stimuli can increase endogenous production of IsoPs in retina and AH (Nourooz-Zadeh and Pereira, 2000; Koliakos et al., 2003; LeDay et al., 2004; Yoshida et al., 2006; Dentchev et al., 2007). Furthermore, Koliakos et al. showed that the AH of patients with exfoliation syndrome (XFS) and cataract had an elevated level of 8-isoPGF$_{2\alpha}$, thus, supporting a role for IsoPs in XFS and cataract development. When used as an OS marker in ocular studies, IsoPs levels were significantly higher following tissue exposure to oxidant stress condition (Dentchev et al., 2007). To the best of our knowledge there are no reported studies on the role of neuroprotanes in ocular pathophysiology. Clearly, the need for more studies in this area of lipidomics could not be overemphasized. One can, however, speculate that since prostanoids play a role in regulation of both inflammatory and neuroprotective mechanisms in ocular tissues, both IsoPs and NeuroPs could potentially modulate disease conditions that are characterized by inflammation and neuronal degradation in the eye.

PHARMACOLOGICAL IMPLICATIONS

Although previously assumed to be inert, it is becoming apparent that oxidant-derived metabolites can exert pharmacological effects in biological tissues. Due to challenges involved in synthesizing and isolating pure metabolites and the lack of commercially available pure neuroPs and IsoPs, the pharmacological actions of these lipid metabolites have remained largely unexplored. The ability of these metabolites to exert pharmacological effects in the eye may underlie the mechanism whereby reactive oxygen metabolites play a role in ocular diseases such as glaucoma. Further studies are indeed needed to determine the exact pharmacological role of oxygen-derived free radicals in ocular diseases.

PEROXIDES

The anterior segment of the eye has the capacity to protect against deleterious action of oxygen-derive free radicals and is involved in the metabolism of both endogenous and exogenous H_2O_2. Exogenous application of H_2O_2 and its synthetic peroxides have been reported to exert pharmacological/toxicological actions on tissues of the anterior uvea of several mammalian species. Earlier studies have demonstrated that intracameral injection of H_2O_2 in the rabbit eye can result in lowered IOP and morphological changes in the anterior chamber (Birnbaum et al., 1987; Csukas and Green, 1988). In addition, H_2O_2 has been shown to increase sympathetic neurotransmission in iris-ciliary bodies, an action that is dependent on the generation of reactive ROS, trace amounts of extracellular calcium and the functional integrity of mitochondrial calcium stores (Birnbaum et al., 1987; Csukas and Green, 1988; Opere and Ohia, 1998). The observed increase of sympathetic activity could lead to vasoconstriction in the anterior uvea which may cause a reduction in AH production and consequently a decrease in IOP. There is evidence that PG and thromboxanes (Tx) can mediate the excitatory effects of peroxides on sympathetic neurotransmission in mammalian irides and regulate their inhibitory effects on muscarinic receptor-induced contraction of iris smooth muscle. Graham et al. demonstrated how the cyclooxygenase (COX) inhibitor flurbiprofen attenuated the increased action of H_2O_2 and cumene hydroperoxide (a synthetic peroxide) on norepinephrine (NE) release (Graham et al., 2000). The enzyme CAT which is involved in the metabolism of H_2O_2 has also been linked to neurotransmission regulation by peroxides in ocular tissues. Matutte et al. showed that the inhibition of catalase unmasked an excitatory action of H_2O_2 on evoked NE release and an inhibitory effect on muscarinic receptor-induced contraction of iris smooth muscle (Matutte et al., 2000). These studies establish that inhibiting peroxide metabolism causes both sympathetic nerves and smooth muscles of the anterior uvea to be more susceptible to the harmful effects of H_2O_2.

The retina is highly susceptible to auto-oxidation via free radical mechanism due to its high content of polyunsaturated fatty acids. Peroxides such as H_2O_2, possess the ability to impair glutamate release. LeDay et al. reported that both H_2O_2 and cumene hydroperoxide inhibited potassium-evoked release of glutamate (using [^{3}H]D-aspartate as a marker) from bovine retinae, a response prevented by the antioxidant trolox (LeDay et al., 2004). Furthermore, CAT inhibition enhanced the inhibitory action of H_2O_2 on glutamate release. Together these studies indicate that the inhibitory actions of peroxides on potassium-stimulated glutamate release in the retina is dependent on the generation of ROS and catalase. In a related study, these workers observed that peroxides such as H_2O_2 can decrease both glutamate and glycine concentrations and that catalase inhibition also caused a significant reduction in glutamate and glycine concentrations. Furthermore, LeDay et al. observed that peroxides can directly stimulate the production of COX and non-COX derived metabolites of the AA pathway and that these products are involved in mediating the effects of H_2O_2 on glutamanergic transmission in the retina (LeDay et al., 2004). These data indicate a vital role for endogenously produced peroxides in the regulation of amino acid neurotransmission in the retina.

LCPUFA PEROXIDATION METABOLITES

LCPUFA metabolites such as IsoPs and NeuroPs have been shown to exert pharmacological and toxicological effects in ocular tissues. Topical use of 8-iso-PGE$_2$ reduces IOP in normal monkey

eyes as well as those affected by glaucoma. In addition, there is evidence that IsoPs can regulate neurotransmission in human and mammalian ocular tissues. Awe et al. reported that 8-iso-$PGF_{2\alpha}$ enhances electrically evoked NE release from isolated human iris-ciliary bodies. This effect is blocked by the thromboxane receptor (TP) antagonist SQ-29548 suggesting that TP receptors mediate the mechanisms of 8-iso-$PGF_{2\alpha}$. Conversely, 8-iso-PGE_2 was found to inhibit electrically stimulated NE release from the same tissue, a response that was not affected by SQ-29548. Taken together, these studies indicate that 8-iso-$PGF_{2\alpha}$ and 8-iso-PGE_2 exert opposing effects on sympathetic neurotransmitter in human iris-ciliary bodies via different receptors or mechanisms (Awe et al., 2000). In contrast, another study by a different group showed that both 8-iso-$PGF_{2\alpha}$ and 8-iso-PGE_2 enhanced electrical-evoked NE release from bovine iris, an action that was blocked by the TP receptor antagonist SQ-29548 (Awe et al., 2000; Opere et al., 2001a). These observations support the view that a species-specific difference in response to IsoPs may exist in ocular tissues.

In addition to the anterior uvea, IsoPs also regulate neurotransmission in mammalian retina. Liu et al. demonstrated that IsoPs can attenuate dopamine release from isolated bovine retina (Liu et al., 2008). There is evidence that the enzymes COX and Tx synthase may be involved in the effects of IsoPs. The COX inhibitor, indomethacin has been shown to reduce contractile response of 8-iso-$PGF_{2\alpha}$ in the retinal vasculature of piglets and it blocked the excitatory effect of high concentration 8-iso-$PGF_{2\alpha}$ on [^{3}H]D-aspartate release from bovine retina (Lahaie et al., 1998; Opere et al., 2005). Studies also report IsoPs are potent vasoconstrictor of vessels in piglet retina (Lahaie et al., 1998; Hou et al., 2004). There is evidence that in mammalian retina, the pharmacological effect of IsoPs involve Tx receptor (TP) and prostanoid (EP) receptors. Opere et al. showed that 8-iso-$PGF_{2\alpha}$ elicited a dual effect on potassium-induced glutamate release (using [3H]D-aspartate as a marker) from bovine retina (Lahaie et al., 1998; Opere et al., 2001b; Hou et al., 2004; Opere et al., 2005, 2008). At low concentrations (1-100nM), 8-iso-$PGF_{2\alpha}$ activated the PGE receptors (EP) EP1/EP2 to produce an inhibitory response on glutamate release. Higher concentrations (100nM-30uM) of 8-iso-$PGF_{2\alpha}$ acted upon the TP receptor, promoting an excitatory effect upon glutamate release. Other studies also demonstrate that 8-iso-PGE_2 exerts its action by activation of EP receptors because this response was blocked by EP receptor antagonists SC-19220(EP1), AH-6809(EP1-3), and AH-23848(EP4) (Zhao et al., 2008, 2009). The pharmacological actions of neuroPs remain largely unexplored but in preliminary studies, neuroPs have been shown to exert an excitatory effect on potassium-evoked glutamate release from bovine retina using [^{3}H]D-aspartate as a marker. Likewise, intravitreally injected neuroP enhanced retinal glutamate and glycine levels in bovine retina (Hou et al., 2004; Opere et al., 2005, 2008; Zhao et al., 2008, 2009; Jamil et al., 2012). Glutamate is the major excitatory amino acid neurotransmitter in the retina and is involved in the transfer of visual information from the retina to the brain. A defect in glutamate synaptic transmission and uptake can result in glutamate neurotoxicity and cell death in the retina (Nucci et al., 2005; Pulido et al., 2007). Since neuronal excitotoxicity is preceded by elevation in neuronal glutamate concentrations, the ability of IsoPs and neuroPs to regulate excitatory neurotransmitter release could have significant pharmacological implications in mammalian ocular tissues.

PERSPECTIVE

Products of oxygen-derived free radical pathway such as peroxides and LCPUFA metabolites are involved in oxidative reactions in the eye and could play a major role in the pathophysiology of most ocular diseases. In addition to their pathological roles, these metabolites can exert pharmacological actions on tissues of the anterior uvea and retina. The ability of peroxides such as H_2O_2 to affect neurotransmitter pools from sympathetic and glutaminergic nerves in the eye may help provide insights on the underlying mechanism by which ROS contribute to ocular pathologies. It is well established that OS is an underlying pathology in ocular neurodegenerative diseases of the eye such as glaucoma and DR. Since LCPUFAs play a significant role in cellular structure and signaling, it is necessary to delineate the role of LCPUFA metabolites in the progression of ocular neurodegenerative diseases in the eye. In spite of current research, the role of lipid metabolites like NeuroPs and IsoPs in regulation of ocular disease is not yet clear. It is possible that addition of these lipid by-products to proteins could serve as diagnostic markers of endogenous peroxidation status. Furthermore, since numerous stereoisomers and regioisomers of IsoPs and neuroPs can be generated, *in vivo*, it is possible that the summation of their effects could significantly impact the homeostatic environment within the cell. Indeed further research is warranted to fully delineate the biological significance of these lipid mediators on biological systems like the eye.

The hypothesized role of oxidation in the development of ocular diseases has prompted research into the use of antioxidants as neuroprotective agents in ocular therapy. It is thought that people with low systemic antioxidant levels may be more prone to oxidative damage in ocular tissues. As such, substantial body of research, though controversial indicates that antioxidants can ameliorate certain common and uncommon ocular conditions. Despite inconclusive data suggesting that people who eat a diet rich in antioxidant vitamins such as carotenoids, vitamins C and E or minerals (selenium and zinc) are less likely to develop certain ocular diseases, several studies provide evidence supporting a role for these nutrients in preservation of vision. Indeed vitamins B, C and selenium have been shown to reduce the risk of cataract and prevent its development (Rapp et al., 2000; Chiu et al., 2007). In addition, vitamin E deficiency increases H_2O_2 levels and the intracellular production of ROS (Chow et al., 1999). Studies depict a synergism between vitamin E and C; vitamin C reduces oxidized vitamin E, which is crucial for protecting cell membranes from lipid peroxidation (Varma, 1991; Kang et al., 2003). Natural products such as zinc gluconate, marygold flower extract, ascorbic acid, blueberry extract and *trans*-resveratrol are also known to have antioxidant effects in the eye and have been used widely to improve vision and ocular health. Flavonoids and other antioxidants including Ginkgo biloba, Qi Ming granule have been found to be beneficial in promoting ocular health, and reduce OS in the eye (Jayle et al., 1965; Urso, 1967; Porcella et al., 2001; Rhee et al., 2001; Jarvinen et al., 2002; Majumdar and Srirangam, 2010).

Furthermore, observational and experimental data from the Age-Related Eye Disease Study (AREDS) suggest that antioxidant intake may delay progression of ARMD and vision loss (Age-Related Eye Disease Study Research Group, 2001). Although the use of antioxidants looks promising for improving outcomes in ocular disorders, more investigation is warranted in order to standardize indication for use, composition and dosing for treatment. At the present time, there remains insufficient scientific data to recommend routine antioxidant intake for primary prevention of ocular neuropathies resulting from lipid peroxidation.

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Lipid peroxidation modifies the assembly of biological membranes "The Lipid Whisker Model"

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The aim of this opinion article is to point out the basic principles that modify the assembly of biological membranes during lipid peroxidation. With this objective in mind, I describe: the structural and functional properties of membranes, the transport and diffusion of oxygen regulated by cholesterol and fatty acids; the "Lipid Whisker Model" and finally analyzed the changes induced by lipid peroxidation in membrane structure and dynamics, both at the lipid and protein level.

Several reviews have appeared in recent years related to the kinetics and biology of lipid peroxidation products (Catala, 2010; Yin et al., 2011; Pinchuk and Lichtenberg, 2014; Vigor et al., 2014; Davies and Guo, 2014). The analysis of lipid peroxidation products has been particularly important in the advancement of research in this field because of the complexity of product mixtures. I also discuss the effect of other membrane modifications, triggered by lipid peroxidation products and reducing sugars. Contrary to what is expected by the LWM, Garrec et al. (2014) have recently investigated the validity of the "floating peroxyl radical" hypothesis by means of molecular modeling and predicted that the peroxyl radical does not "float" at the surface of the membrane.

Abbreviations: 22:6 n-3, docosahexaenoic acid; ER, endoplasmic reticulum; CD36, fatty acid translocase; FMM, Fluid Mosaic Model; FRET, Förster Resonance Energy Transfer; LWM, Lipid Whisker Model; oxPL, oxidized phospholipid; 16:0, palmitic acid; PC, phosphatidylcholine; PUFAs, polyunsaturated fatty acids; 18:0, stearic acid.

INTRODUCTION

When the Fluid Mosaic Model (FMM) of biological membrane structure was introduced 42 years ago, it was visualized as a basic model for cell membranes that could explain existing data on membrane proteins and lipid structures and their dynamics. According to the (FMM), a membrane was described as a biological fluid of proteins and lipids oriented in two dimensions. The basic structure of all cell and organelle membranes is the lipid bilayer. Protein molecules are distributed in different regions of the bilayer and perform diverse functions. Cell membranes are active, fluid structures, and most of their molecules are able to travel in the plane of the membrane (Singer and Nicolson, 1972). The viscosity of a lipid membrane largely depends on whether the acyl chains attached to glycerophospholipids are grouped into a rigid state or exist in a relatively disordered, fluid state. Long chain saturated fatty acids maximize Van der Waals forces, and increase the viscosity of the membrane (Chapman and Benga, 1984). Fluidity is defined as the ease of movement and represents the reciprocal of the viscosity of the membrane (Lee, 1991). The fluid properties of biological membranes are critical for various cell functions. Still slight changes in membrane fluidity may cause unusual function and pathological processes (Garcia et al., 2005). After more than 40 years, the (FMM), described by Singer and Nicolson is still relevant to recognize the structure, function, and dynamics of biological membranes (Nicolson, 2014). However, several biological processes cannot be explained on the basis of this typical phospholipid orientation and utilize other phospholipid conformations (Catala, 2012) that are described in this article.

THE STRUCTURAL AND FUNCTIONAL PROPERTIES OF MEMBRANES ARE DETERMINED BY POLYUNSATURATED FATTY ACIDS

The fluid properties in biological membranes are recognized principally by the presence of polyunsaturated fatty acids (PUFAs) in phospholipids molecules located in both sites of the lipid bilayer. The nature and saturation of the attached fatty acid of the phospholipids generate spectacular effects on membrane packing and fluidity (Janmey and Kinnunen, 2006). The unsaturated fatty acids give a high degree of conformational flexibility to the unsaturated hydrocarbon chains in the membranes because they occupy a small wedge-shaped space. This generally results in looser packing and a more fluid membrane. In contrast, saturated fatty acids confer rigidity that results in a less fluid or more arranged membrane. The rigidity permits saturated fatty acids to group together tightly and forms a solid at inferior temperatures. When a new double bond is open in the fatty acid produces a "kink" in the molecule. For this reason unsaturated fatty acids, such as docosahexaenoic acid (22:6 n-3), assume numerous patterns since this fatty acid can turn around in the area of C–C bonds but not in the region of the rigid C=C bonds (Feller et al., 2002). Adjustments in the lipid composition modify the fluidity of

membranes. Lipid composition is modified during regulation of de novo synthesis at selected cellular places, distribution or transfer to new sites, and by specific modifying reactions. Stearyl CoA desaturase is in charge for the formation of monoethylenic fatty acids from saturated fatty acids by catalyzing the addition of a double bond into the ninth carbon of saturated C16:0 and C18:0 substrates (Enoch et al., 1976).

THE TRANSPORT AND DIFFUSION OF OXYGEN IN MEMBRANES IS REGULATED BY CHOLESTEROL AND FATTY ACIDS

Lipid peroxidation and formation of reactive oxygen species are important chemical reactions that use oxygen and occur in cell membranes. The diffusion of oxygen into synthetic membranes prepared with phosphatidylcholine and cholesterol have been investigated by Subczynski et al. (1991). Oxygen permeability through the membrane in all the membranes studied in this work was limited by the presence of cholesterol. Thus, they showed an increase in oxygen transport in the central part of the synthetic membranes because cholesterol decreases oxygen transport in and around the head group regions, where the main obstacles to the oxygen permeability exist. Based on this explanation, it can be assumed that non-raft areas rich in PUFA-phospholipids and vitamin E will be more available by oxygen than lipid rafts areas containing sphingolipids and cholesterol. This condition will make several micro areas more vulnerable to lipid peroxidation than others.

THE "LIPID WHISKER MODEL" IS SUITABLE TO EXPLAIN THE STRUCTURE OF OXIDIZED CELL MEMBRANES

The Lipid Whisker Model (LWM) (Greenberg et al., 2008) is an extension of the Fluid Mosaic Model suggested by Singer and Nicolson (1972). Recent studies into the conformation of oxidized phospholipid (oxPL) species recognized by CD36 within model membranes (Li et al., 2007) have led to the development of the LWM. A primary attribute of the (FMM) is that amphipathic phospholipids are oriented in an organization lamellar mesophase with hydrophobic fatty acyl chains embedded within the interior of the membrane and the hydrophilic polar groups facing the aqueous environment. This lipid organization accepts fast lateral circulation of lipids and membrane proteins similarly within the planar membrane surface. It also causes the water-resistant character of cell membranes to hydrophilic molecules. But, recent information proposes that in peroxidized cell membranes, numerous of the oxPL classes adopt a particular conformation. Lipid peroxidation is achieved by addition of numerous polar molecules on fatty acid chains (Catala, 2009). Consequently, when cell membranes undergo oxidation, if not adapted by the action of phospholipases, they may "produce whiskers" including a variety of oxidized sn-2 fatty acids of diverse structures. In the (LWM), the assembly of many oxPL within cell membranes is different compared with one observed in non-oxPL described in the (FMM). Biophysical studies have shown that the addition of an oxygen atom to the acyl chain produces a significant change that prevent its immersion in the interior of the membrane, however, the modified acyl chain is projected on the aqueous medium (**Figure 1**) (Subczynski et al., 1991; Greenberg et al., 2008).

As cell membranes are peroxidized "grow whiskers" because phospholipids are peroxidized, and several of its oxidized fatty acids are projected onto the surface (**Figure 1**). This change produced by the oxidation of a fatty acid chain in the membrane may be a trigger event to numerous biological activities.

THE EFFECT OF OTHER MEMBRANE MODIFICATIONS, TRIGGERED BY LIPID PEROXIDATION PRODUCTS AND REDUCING SUGARS INTRODUCES CHANGES IN CELL MEMBRANE PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

During lipid peroxidation, biomolecules such as proteins or amino lipids can be covalently modified by lipid decomposition products (Catala, 2009). For the case of aliphatic aldehydes (alkanals) such as 1-hexanal or 1-nonanal, the Nε-amino groups of the lysine residues in proteins can be modified through the formation of a Schiff base. α,β-Unsaturated aldehydes (alkenals) such as acrolein or 4-hydroxy-2-nonenal react with lysine, cysteine, and histidine through a Michael-type addition. Conversely, lipid hydroperoxide might covalently react with protein without serious decomposition of its structure. However, the mechanism of lipid hydroperoxide-derived protein modification is not so clear. Evidence for in situ ethanolamine phospholipid adducts with hydroxy-alkenals has been recently described.

Non-enzymatic modification of aminophospholipids by lipid peroxidation-derived aldehydes and reducing sugars through carbonyl-amine reactions are thought to contribute to the age-related deterioration of cellular membranes (Naudí et al., 2013). Much evidence demonstrates the modification of aminophospholipids by glycation, glycoxidation and lipoxidation reactions.

LIPID PEROXIDATION IN MEMBRANES: THE PEROXYL RADICAL DOES NOT "FLOAT"

Some key microscopic aspects of the lipid peroxidation reaction in cell membranes are still poorly studied. In particular, it is usually accepted that the propagation of the radical reaction in lipid bilayers is hampered by the rapid diffusion of peroxyl intermediates toward the water interface, that is, out of the reaction region. Contrary to what is expected by the LWM, Garrec et al. (2014) have recently investigated the validity of this "floating peroxyl radical" hypothesis by means of molecular modeling. Combining quantum calculations of model systems and atomistic simulations of lipid bilayers containing lipid oxidation products, these authors predict that the peroxyl radical does not "float" at the surface of the membrane. Instead, it remains located quite deep inside the bilayer.

THE CHANGES INDUCED BY LIPID PEROXIDATION IN MEMBRANE STRUCTURE AND DYNAMICS, BOTH AT THE LIPID AND PROTEIN LEVEL

The mechanisms and principles establishing the thousands of proteins and lipids that make up membrane bilayers in cells are still unclear. Mueller et al. reviewed the basic properties of biological membranes

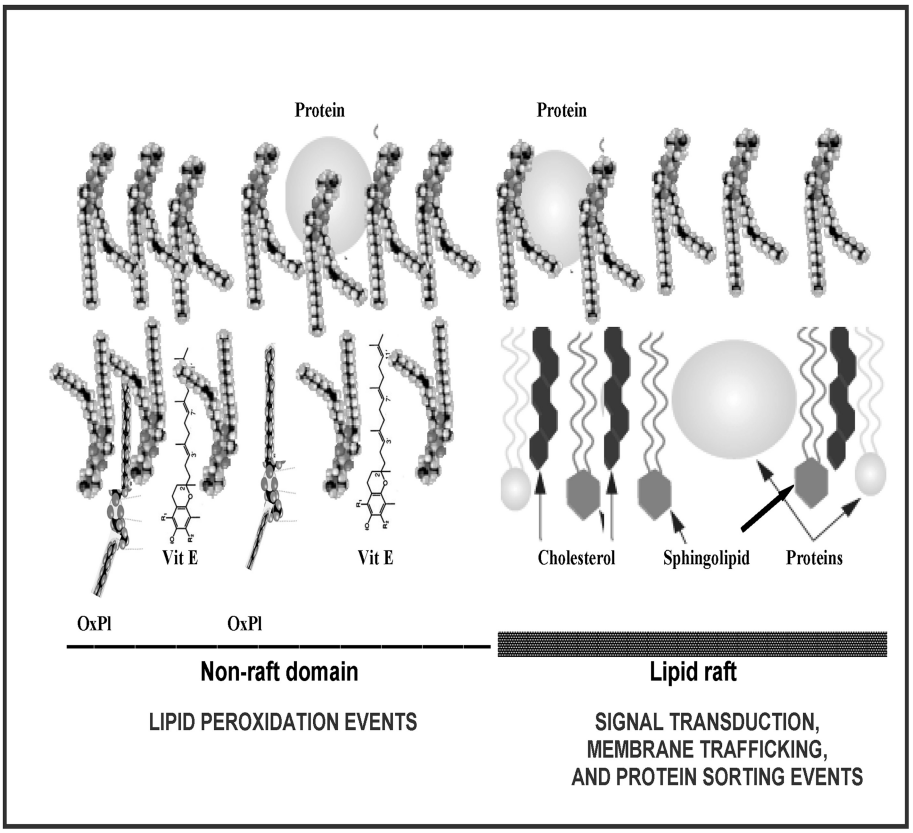

FIGURE 1 | Hypothetical model of the plasma membrane. In terms of lipids, the heterogeneous membrane is believed to consist of a mixture of a dispersed "lipid raft" phase, enriched in cholesterol, raft-associated proteins, and saturated lipids (such as sphingolipids), and the "non-raft" matrix phase enriched with phospholipids containing PUFAs and vitamin E. Vitamin E, partition into domains that are enriched in polyunsaturated phospholipids increasing the concentration of the vitamin in the place where take place the lipid peroxidation process and oxidized phospholipids (oxPl) are formed. Reproduced from Catala (2012).

and the most common theories for lateral segregation of membrane components before discussing an emerging model of a self-organized, multi-domain membrane or "patchwork membrane" (Mueller et al., 2012).

The oxidation of phospholipids has become a recent topic of interest within the field of membrane biophysics. Still, the exact mechanism of membrane injury by oxidized lipids is uncertain (Wong-Ekkabut et al., 2007).

Membrane rafts remain one of the most controversial issues in biophysics, because the methods for their detection are still far from perfect. Optical techniques rely heavily on fluorescent markers. In biological membranes, these probes are connected with lipids and thus define only the lipid part of the membrane. Therefore, they are "blind" to the presence of membrane proteins, unless FRET techniques between proteins and membrane probes are used. Therefore, the imperative future challenge for the membrane probes will be to design molecules capable of monitoring lipid surrounding specifically around a given protein of interest (Klymchenko and Kreder, 2014).

Proteins and lipids in membrane processes are reciprocally dependent on each other. While the lipid domains as organizers of proteins has attracted wide-spread consideration, the question whether proteins control lipids or lipids control proteins in cell membranes is not a simple problem to solve. The fact that proteins exploit the biophysical properties of lipids including lipid charge and phase separation for membrane function is one of the reasons why membrane biology is such an attractive area of research (Rossy et al., 2014).

To guarantee coordination of cellular activities, cells use membrane contact sites (MCSs) between the membranes of diverse organelles. MCSs are domains where two membranes come to close proximity, typically less than 30 nm, and create microdomains that favor exchange between two organelles. Since the endoplasmic reticulum (ER) is the most widespread cellular membrane network, it is thus not surprising to find the ER involved in most MCSs within the cell. The ER contacts diverse compartments such as mitochondria, lysosomes, lipid droplets, the Golgi apparatus, endosomes, and the plasma membrane. Taken into account these observations, several levels of complexity have to be considered when analyzing the changes induced by lipid peroxidation in membrane structure and dynamics, both at the lipid and protein level.

In my opinion and in the light of new investigations, several critical aspects of lipid peroxidation in biological membranes, such as their assembly

and structural organization need to be revisited.

AUTHOR NOTE

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Interaction of aldehydes derived from lipid peroxidation and membrane proteins

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A great variety of compounds are formed during lipid peroxidation of polyunsaturated fatty acids of membrane phospholipids. Among them, bioactive aldehydes, such as 4-hydroxyalkenals, malondialdehyde (MDA) and acrolein, have received particular attention since they have been considered as toxic messengers that can propagate and amplify oxidative injury. In the 4-hydroxyalkenal class, 4-hydroxy-2-nonenal (HNE) is the most intensively studied aldehyde, in relation not only to its toxic function, but also to its physiological role. Indeed, HNE can be found at low concentrations in human tissues and plasma and participates in the control of biological processes, such as signal transduction, cell proliferation, and differentiation. Moreover, at low doses, HNE exerts an anti-cancer effect, by inhibiting cell proliferation, angiogenesis, cell adhesion and by inducing differentiation and/or apoptosis in various tumor cell lines. It is very likely that a substantial fraction of the effects observed in cellular responses, induced by HNE and related aldehydes, be mediated by their interaction with proteins, resulting in the formation of covalent adducts or in the modulation of their expression and/or activity. In this review we focus on membrane proteins affected by lipid peroxidation-derived aldehydes, under physiological and pathological conditions.

Keywords: lipid peroxidation, aldehydes, membrane proteins, human diseases, signal transduction

INTRODUCTION: LIPID PEROXIDATION-DERIVED ALDEHYDES

Reactive intermediates produced under conditions of oxidative stress cause the oxidation of polyunsaturated fatty acids (PUFAs) in membrane lipid bilayers, leading eventually to the formation of aldehydes (Esterbauer et al., 1991). Among these, the most abundant aldehydes are 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), while acrolein is the most reactive one (Esterbauer et al., 1991). HNE is the lipoperoxidation product which has displayed the highest biological activity and, for this reason, has been most intensively studied. On the other hand, acrolein, which is the most electrophylic compound, has received less attention, because it is scarcely represented among lipoperoxidation products. Both acrolein and HNE are α,β-unsaturated electrophilic compounds, which preferentially form 1,4-Michael type adducts with nucleophiles, such as proteins and DNA. Even though MDA shows little reactivity under physiological conditions, at low pH its reactivity increases, when beta-hydroxyacrolein becomes the predominant species and, analogously to acrolein and HNE, it can form 1,4-Michael type adducts with nucleophiles (Esterbauer et al., 1991). Even though it was demonstrated that MDA does not react with glycine and GSH, and reacts slowly with cysteine (Esterbauer et al., 1991) under physiological conditions, cellular proteins are much more readily modified by MDA (Chio and Tappel, 1969).

Due to the high chemical reactivity of aldehydes, mammals have evolved a battery of enzymes which convert these compounds to less reactive chemical species. The main reactions of aldehydes are the adduction with glutathione (GSH), which can either occur spontaneously or be catalyzed by glutathione S-transferases (GSTs), the reduction to alcohol by aldo–keto reductases (AKRs) or alcohol dehydrogenase and the oxidation to acid by aldehyde dehydrogenases. The metabolism of aldehydes has been reviewed in excellent mode by Esterbauer and collaborators (1991). More recent reviews were focused on the biochemistry of lipid peroxidation products (Guéraud et al., 2010) and acrolein biotransformation (Stevens and Maier, 2008). The catabolic rates of the various aldehydes contribute, together with their rates of production from lipid peroxidation, in determining their steady-state intracellular concentrations. At high concentrations, all these aldehydes were found to play a role in the toxic effects of lipid peroxidation. Aldehyde toxicity is mainly due to the alterations of several cell functions, which mostly depend on the formation of covalent adducts with cellular proteins (Grimsrud et al., 2008). Due to their amphiphilic nature, aldehydes can easily diffuse across membranes and can covalently modify any protein

in the cytoplasm and nucleus, far from their site of origin (Negre-Salvayre et al., 2008). Similarly, the aldehydes formed outside the cells (i.e., in a site of inflammation or in plasma), can react with adjacent cells, even in cases when they are not primary sites of lipid peroxidation. In the latter instance, plasma membrane proteins represent the first targets for adduct formation. Exogenous or endogenous aldehydes can react also with nuclear proteins, thus modulating protein expression through their reaction with transcription factors or other regulatory elements (Jacobs and Marnett, 2010). The targets of lipid peroxidation-derived aldehydes are cell-type specific and dependent both on the pattern of proteins expressed by the cell and the aldehyde concentration. Moreover, the modification of a specific protein can have different biological consequences, in relation to its specific cell function. However, at low concentration, HNE in particular can play an important role in signal transduction and exert antiproliferative and anti-invasive actions toward cancer cells, by interfering with the modulation of gene expression via the formation of protein and/or DNA adducts (Gentile et al., 2009; Barrera, 2012).

The presence of aldehyde-protein adducts has been demonstrated in a wide range of physiological and pathological conditions. Those among the latter in which aldehyde-protein adducts, in particular HNE-protein adducts, have been most intensively studied are neurodegenerative diseases and atherosclerosis. Recently, a role has emerged for aldehyde-protein adducts in autoimmune diseases, since the covalent alteration of protein structure can bring about a sufficient modification of a self antigen for it to break the immunological tolerance of autoreactive T and/or B cells. In the following sections, we shall examine the mechanisms of formation of aldehyde-protein adducts and the main biological consequences of the formation of aldehyde adducts with membrane proteins in neurodegenerative diseases, atherosclerosis, autoimmune diseases and in relation with the functions played by cell proteins at the plasma membrane level. The chemical structures of HNE, MDA and acrolein are illustrated in **Figure 1**

CHARACTERISTICS OF ALDEHYDES AND THEIR PROTEIN ADDUCTS

4-HYDROXYNONENAL (HNE)

4-Hydroxynonenal (HNE) is an aldehyde highly represented among the products of lipid peroxidation, which displays high biological activity. This aldehyde has three main functional groups: the aldehyde group, the C=C double bond and the hydroxyl group, which can participate, alone or in sequence, in chemical reactions with other molecules (Esterbauer et al., 1991). Due to its strong hydrophobic nature, HNE is mostly associated with the membranes where it is produced, but it can also diffuse to different cellular compartments (Butterfield and Stadtman, 1997). HNE is a highly electrophilic molecule that easily reacts with glutathione, proteins and, at higher concentration, with DNA. HNE forms adducts with three different amino acyl side chains, namely of Cys, His, and Lys residues, via Michael addition either to thiol (−SH) or to amino ($-NH_2$) groups. Cys residues display the highest reactivity with HNE, even though Cys residues are not always the preferential targets of HNE, because the tertiary structure of the protein can condition their accessibility and, therefore, their reactivity toward exogenous chemicals. No reaction of HNE was detected with Glu (Doorn and Petersen, 2003). Besides by the simple formation of Michael adducts to lysyl, histidyl, and cysteinyl residues (Esterbauer et al., 1991), HNE can modify protein structure through Schiff base formation with lysyl residues, leading to pyrrole formation (Sayre et al., 1996). In addition, HNE modification can result in the cross-linking of two lysyl residues through reversibly formed Schiff base Michael adducts (Parola et al., 1999; Xu et al., 1999), as well as irreversibly formed 2-hydroxy-2-pentyl-1,2-dihydropyrrol-3-one iminium moieties (Parola et al., 1999; Dianzani, 2003; Barrera et al., 2008). The target proteins of HNE adduct formation *in vitro* and *in vivo* have been reviewed in great detail by Poli et al. (2008).

HNE has been detected *in vivo* in several pathological conditions characterized by increased lipid peroxidation, including inflammation, atherosclerosis, chronic degenerative diseases of the nervous system, and chronic liver diseases (Moreau et al., 2005).

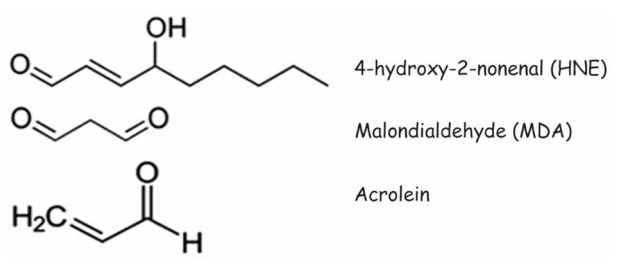

FIGURE 1 | Structures of 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and acrolein.

ACROLEIN

Acrolein is a little aldehyde with three carbon atoms and a double bond. Besides being formed endogenously during lipid peroxidation, this aldehyde is inhaled with cigarette smoke and is present in cooked oils and other foods (Stevens and Maier, 2008). Acrolein is the strongest electrophile in the α,β-unsaturated aldehyde series; its reaction with the thiol group of cysteine was about 110–150 times faster than that of HNE (Esterbauer et al., 1991; Witz, 1997). The toxicity of acrolein is related to its ability to deplete glutathione (Kehrer and Biswal, 2000), and to form DNA and protein adducts (Esterbauer et al., 1991; Sanchez et al., 2005; Feng et al., 2006). Potential targets of acrolein in proteins include the side chains of cysteinyl, histidyl, and lysyl residues, as well as free N-terminal amino groups (Cai et al., 2009). Cysteine is widely accepted as the most likely site of acrolein adduct formation. The sulfhydryl group of a cysteinyl residue is the most reactive nucleophile in proteins and the thiol adducts with acrolein are considerably more stable than the adducts formed by other α,β-unsaturated aldehydes (Esterbauer et al., 1991; Witz, 1997). Cysteinyl residues are located at the active sites of several proteins and are often involved in the catalytic activity of enzymes, thus the formation of acrolein-cysteine adducts has broad functional implications. It has been reported that the modification of cysteinyl residues by acrolein leads to the inactivation of enzymes, such as aldose reductase (Srivastava et al., 1999) and protein

tyrosine phosphatase 1B (Seiner et al., 2007). However, no cysteine adducts of acrolein have been identified *in vivo*. Other Authors have shown that acrolein generated during lipid peroxidation may primarily react with histidyl residues of proteins, to form Nτ-(3-propanal)-histidine and that acrolein-histidine is the major adduct formed with proteins in *in vitro* studies (Maeshima et al., 2012).

Elevated plasma concentrations of acrolein are detected in patients with chronic renal failure, and the abundance of the proteins adducts of acrolein is increased in tissues obtained from patients with Alzheimer's disease, Parkinson's disease, atherosclerosis and chronic obstructive lung disease (Uchida et al., 1998a; Shamoto-Nagai et al., 2007; Stevens and Maier, 2008; Moretto et al., 2012).

MALONDIALDEHYDE (MDA)

Malondialdehyde (MDA) is widely used as a marker for the peroxidation of ω3 and ω6 fatty acids, measured by the chemical determination of thiobarbituric acid reactive substances (TBARS) (Negre-Salvayre et al., 2010), although the latter provides an incomplete perspective, as MDA derives from the decomposition of only certain lipid peroxidation products and is neither the sole end product, nor one of lipid peroxidation only (Halliwell and Whiteman, 2004). At neutral pH, MDA is present as enolate anion, with low chemical reactivity (Esterbauer et al., 1991). Nevertheless, it is able to interact with nucleic acid bases to form several different adducts (Marnett, 1999). MDA has been reported to react *in vivo* with primary amines, to form Nε-(2-propenal) lysine and generate lysine-lysine cross-links with 1-amino-3-iminopropene and pyridyledihydropyridine type bridges (Uchida, 2000). These reaction products have been detected in Apo B fractions of oxidized lipoproteins (LDL) and are thought to be involved in the impaired interaction of modified lipoproteins with macrophages (Palinski et al., 1994). Mooradian and coworkers have reported that protein glycosylation and the presence of acetaldehyde enhance MDA modification of proteins (Mooradian et al., 1996, 2001). Moreover, MDA and acetaldehyde can form stable adducts (MAA) (Tuma et al., 1996) and can react covalently and synergistically with proteins, forming MAA–protein adducts. The latter can be pro-inflammatory and pro-fibrogenic and are capable of inducing strong immune responses (Tuma, 2002).

PHOSPHATIDYL γ-HYDROXYALKENALS (PC-HAs)

Phosphatidylcholine γ-hydroxyalkenals (PC-HAs) are the most abundant and biologically relevant compounds in the class of γ-hydroxyalkenal phospholipids, deriving from the peroxidation of polyunsaturated fatty acids (PUFAs) esterified to phosphoglycerides at the *sn*-2 position of phosphatidylcholine (PC). β-Scission of an alkoxyl radical derived from dihydroperoxide produces two γ-hydroxy-α,β-unsaturated aldehydes, i.e., a methyl-terminal HNE molecule and a mirror image of HNE, still esterified to PC (namely, 9-hydroxy-12-oxo-10-dodecenoic acid [HODA] or its PC ester from linoleate and 5-hydroxy-8-oxo-6-octenoic acid [HOOA] or its PC ester from arachidonate). Because they possess a γ-hydroxy-α,β-unsaturated terminal aldehyde like HNE, PC-hydroxyalkenals are expected to form Michael adducts with primary amino groups of lysyl residues and thiol groups of cysteinyl residues, as well as pentylpyrrole adducts, incorporating the ε-amino groups of lysyl residues (**Figure 2**). γ-Hydroxyalkenal phospholipids contribute strongly in the pathogenesis of the atherosclerotic disease. ω-Carboxyalkylpyrrole modifications of proteins, after lypolysis of intermediate phospholipid adducts, are of pathogenetic importance in age-related macular degeneration, autism and cancer, and promote wound healing. In regard, the reader is referred to the excellent reviews by Salomon et al. (2011), and Salomon and Gu (2011).

ALDEHYDE-PROTEIN ADDUCTS IN NEURODEGENERATIVE DISEASES

Central nervous system (CNS) is one of the major targets of lipid peroxidation. The brain is highly sensitive to oxidative stress because it consumes about 20–30% of inspired oxygen and contains high levels of PUFAs. In particular, high levels of the markers of lipid peroxidation have been found in brain tissues and body fluids in several neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), Huntington disease (HD) and Down syndrome (DS) (Sajdel-Sulkowska and Marotta, 1984; Butterfield et al., 2010; Ruiperez et al., 2010; Lee et al., 2011; Shichiri et al., 2011). We focus here on the adducts of lipid peroxidation-derived aldehydes with protein targets whose oxidative modifications might be relevant for the neuronal dysfunctions observed in Alzheimer's disease.

ALZHEIMER'S DISEASE (AD)

Oxidative damage occurs in early stages of Alzheimer's disease (AD) (Butterfield et al., 2006a; Reed et al., 2008, 2009a; Mangialasche et al., 2009). Several Authors, using redox proteomic approaches, have undertook the task of compiling inventories of cellular proteins modified as a consequence of increased oxidation, glycoxidation, or lipoxidation in the course of oxidative and/or nitrosative stress (Pamplona et al., 2005; Butterfield et al., 2006b; Newman et al., 2007; Reed et al., 2008, 2009a,b; Perluigi et al., 2009). Other searches focused upon proteins modified by tyrosine nitration (Castegna et al., 2003; Sultana et al., 2006a; Reed et al., 2008) and S-glutathionylation (Newman et al., 2007). A number of comprehensive reviews are available (Mangialasche et al., 2009; Martínez et al., 2010; Reed, 2011; Sultana et al., 2012). Like in other neurodegenerative diseases with proteinaceous deposits, vulnerable proteins could be assigned to a few distinct functional groups with crucial roles in plasma membrane ion and nutrient transport, energy metabolism (glycolysis, mitochondrial electron transport and oxidative phosphorylation), cell signaling, cytoskeletal organization, antioxidant defenses, cellular stress responses, protein synthesis, signal transduction, and regulation of neurotransmission (**Table 1**).

HNE-Amyloid β peptide adducts

Amyloid β (Aβ) peptide is the major protein component of amyloid plaques and one of the main components of neurofibrillary tangles (NFTs), hallmarks of AD. This molecule is a 40-to-42-amino acid peptide derived from the integral membrane Amyloid Precursor Protein (APP), through sequential proteolytic cleavages

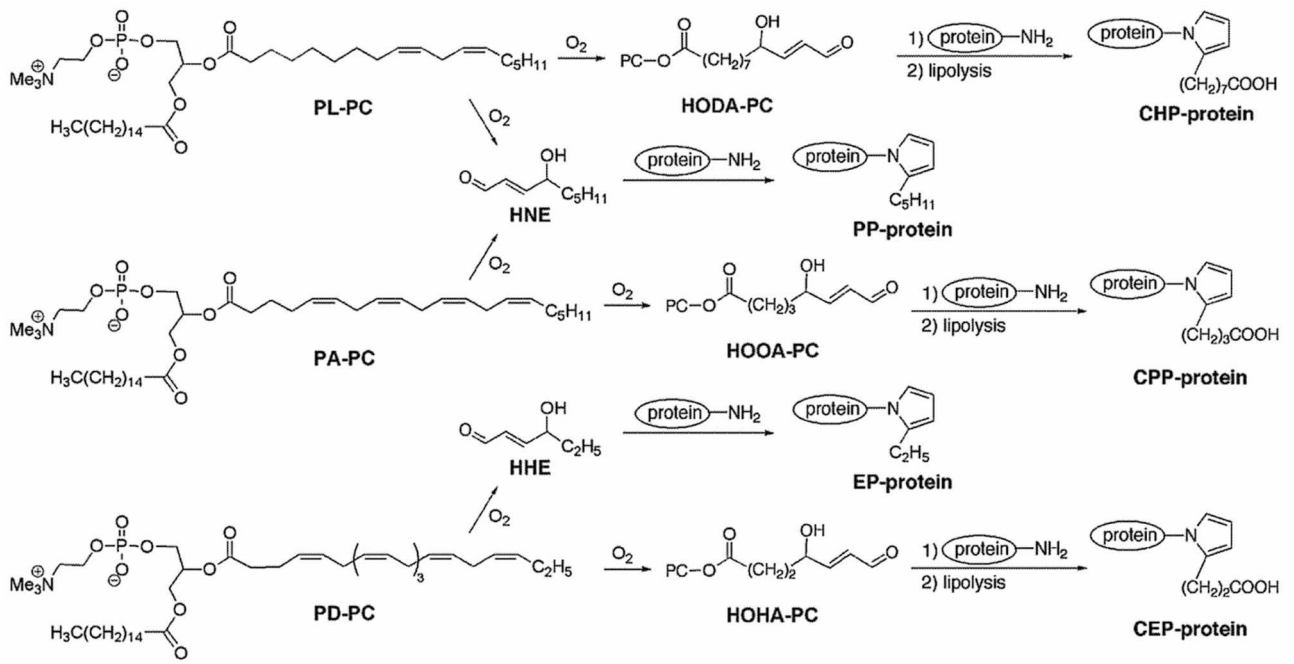

FIGURE 2 | Structures and reactions of phosphatidylcholine γ-hydroxyalkenals (PC-HAs). In addition to 2-pentylpyrrole-modification of proteins by the electrophilic addition of HNE to ε-amino lysyl groups, carboxyalkylpyrrole-modified proteins are also formed by adduct formation with other γ-hydroxyalkenals (mirror images of esterified HNE) also formed, at the time of HNE formation, from the oxidation of PUFA-containing phospholipids. Legend: HHE, 4-hydroxy-2-hexenal; HNE, 4-hydroxy-2-nonenal; PL-PC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PA-PC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PD-PC, 1-palmitoyl-2-docosahexanoyl-*sn*-glycero-3-phosphocholine; HODA-PC, 9-hydroxy-12-oxo-10-dodecenoyl-phosphatidylcholine (this compound may also derive from 1-linoleoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, LA-PC); HOOA-PC, 5-hydroxy-8-oxo-6-octenoyl-phosphatidylcholine; HOHA-PC, 4-hydroxy-7-oxo-5-heptenoyl-phosphatidylcholine; PP-protein, 2-pentylpyrrole-modified protein; EP-protein, 2-ethylpyrrole-modified protein; CPP-protein, carboxypropylpyrrole-modified protein; CEP-protein, carboxyethylpyrrole-modified protein [Reprinted with permission from Salomon et al. (2011)].

by β-secretase (BACE) and γ-secretase (Hardy and Selkoe, 2002). HNE can react directly with the Aβ peptide. This process was reported to exacerbate the formation of toxic Aβ-dependent diffusible oligomers and insoluble aggregates, which, in turn, enhanced oxidative stress, fromation of lipid peroxidation products, such as HNE, and Aβ oligomerization and toxicity (Siegel et al., 2007). HNE modified the three histidyl residues of Aβ, so that HNE-modified Aβ molecules had increased affinities for membrane lipids and adopted a similar conformation as mature amyloid fibrils (Murray et al., 2007; Liu et al., 2008).

HNE-α-enolase adducts

α-Enolase (non neural enolase, ENO1) is a multiform, multifunctional protein. In the cytoplasm, it is a 48-kDa enzyme, catalyzing 2-phospho-D-glycerate dehydration to phosphoenolpyruvate. At the cell surface of neutrophils, B and T cells, monocytes, epithelial, endothelial cells and neurons, it serves as a plasminogen receptor, involved in fibrinolysis (Pancholi, 2001) and in neutrophil and monocyte recruitment (Busuttil et al., 2004; Wygrecka et al., 2009). Binding to α-enolase protects plasmin from inactivation by α2-antiplasmin (Bergman et al., 1997). Alternative translation of α-enolase mRNA produces a 37-kDa protein, preferentially located in the nucleus (myc Binding Protein-1, MBP-1), with *c-myc* gene promoter-binding and tanscription repressing activity (Feo et al., 2000; Subramanian and Miller, 2000). In addition, α-enolase was reported to be a hypoxic stress protein (Graven and Farber, 1998). A regulatory circuit between c-myc, MBP-1, and α-enolase was described, connecting cell energy metabolism and proliferation (Sedoris et al., 2007). Redox proteomic studies identified α-enolase as a target of oxidative modification in all stages of Alzheimer's disease, undergoing the formation of carbonyl groups (Castegna et al., 2002; Butterfield et al., 2006b; Sultana et al., 2006b), MDA adducts (Pamplona et al., 2005), 4-HNE adducts (Reed et al., 2008, 2009a; Perluigi et al., 2009), tyrosine nitration (Castegna et al., 2003; Sultana et al., 2006a; Reed et al., 2009b) and S-glutathionylation (Newman et al., 2007). A typical metabolic feature of AD is the reduced rate of glucose metabolism, as seen in positron-emission tomography with 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG/PET) (de Leon et al., 2001). Despite compensatory increases of α-enolase expression in AD (Sultana et al., 2007) and even though enzymatic activity was not assayed, it was suggested that the loss of function associated with the oxidative modifications of α-enolase might render neurons prone to apoptosis, by dysrupting their energy metabolism (Sultana et al., 2012). As peptide Aβ(1-42), by aggregating in cross-β-structured fibrils, with a similar conformation as fibrin peptides, could substitute for fibrin in the activation of tissue plasminogen activator (tPA) (Kingston

Table 1 | HNE-protein adducts detected in Alzheimer's disease, in relation with disease progression[a].

Protein	AD stage[a]	Function	References
Aldolase	PAD, LAD	Energy metabolism	Perluigi et al., 2009
Triose phosphate isomerase (TPI)	EAD	Energy metabolism	Reed et al., 2009a
Phosphoglycerate kinase (PGK)	MCI	Energy metabolism	Reed et al., 2008
α-Enolase (non neural enolase, ENO1)[b]	MCI, EAD, LAD	Energy metabolism	Reed et al., 2008, 2009a; Perluigi et al., 2009
Pyruvate kinase (PK) M2 isoform	PAD, MCI	Energy metabolism	Reed et al., 2008
Lactate dehydrogenase B (LDHB)	MCI	Energy metabolism	Reed et al., 2008
Aconitase	PAD, LAD	Energy metabolism, mitochondrial function	Perluigi et al., 2009
Malate dehydrogenase, mitochondrial	EAD	Energy metabolism, mitochondrial function	Reed et al., 2009a
ATP synthase α subunit	PAD, MCI, EAD, LAD	Energy metabolism, mitochondrial function	Reed et al., 2008, 2009a; Perluigi et al., 2009
Mn Superoxide dysmutase (SOD2)	EAD, LAD	Mitochondrial function, antioxidant defense	Perluigi et al., 2009; Reed et al., 2009a
Carbonyl reductase 1	MCI	Antioxidant defense	Reed et al., 2008
Peroxiredoxin VI (Phospholipase A2)	LAD	Antioxidant defense	Perluigi et al., 2009
Heme oxygenase 1 (HO-1)	MCI, LAD	Antioxidant defense	Sultana et al., 2012
70-kDa heat shock protein (HSP70)	MCI	Stress response	Reed et al., 2008
Pleckstrin homology-like domain, family A, member 2 (IPL)		Signal transduction	Reed et al., 2008
β-Actin	MCI	Cytoskeleton	Reed et al., 2008
α-Tubulin	LAD	Cytoskeleton	Perluigi et al., 2009
Elongation factor Tu (EF-Tu)	PAD, MCI	Protein synthesis	Reed et al., 2008
Initiation Factor α (eIFα)	MCI	Protein synthesis	Reed et al., 2008
Glutamine synthetase	LAD	Excitotoxicity	Perluigi et al., 2009
Neuropolypeptide h3	PAD, MCI	Neuronal communication	Reed et al., 2008
Collapsin response mediated protein 2 (CRMP-2)[c]	EAD, LAD	Neuronal communication	Perluigi et al., 2009; Reed et al., 2009a

[a] Clinical stages of Alzheimer's disease (AD) progression, in chronological order: PAD, preclinical AD; MCI, mild cognitive impairment; EAD, early stage AD; LAD, late stage AD.

[b] Integral or perypheral membrane proteins of plasma or organelle membranes are underlined.

[c] Also known as dihydropirimidinase-related protein 2 (DRP-2).

et al., 1995), and because plasmin cleaved peptide Aβ1-40 into a truncated form, with potent stimulatory activity toward tPA (VanNostrand and Porter, 1999), it was proposed that the loss of plasminogen-binding activity of HNE-α-enolase might foster apoptosis in AD, by hindering Aβ peptide degradation (Sultana et al., 2012).

Such a scenario is supported by a functional study of HNE-α-enolase adducts in HL-60 leukemic cells (Gentile et al., 2009). α-Enolase was among a few proteins recognized by anti-histidine-HNE antibodies, after 15 min of exposure to 1–10 μM HNE. HNE–α-enolase adducts were detected early on the surface of HL-60 cells, indicating a high degree of α-enolase exposure. HNE treatment did not alter α-enolase expression or enzymatic activity. The low-level expression of an anti-α-enolase Ab-reactive 37-kDa peptide, possibly corresponding to MBP-1, did not vary after HNE treatment. The main functional alteration of HNE-α-enolase concerned its plasminogen-binding ability. Treatment with 1 μM HNE strongly inhibited plasminogen binding to α-enolase at the cell surface and consequently reduced HL-60 cell adhesion to human umbilical venous cells (HUVECs), suggesting that HNE and other inhibitors of plasminogen binding to α-enolase may be of use in the control of tumor invasion. α-Enolase emerged from this study as a protein most susceptible to HNE adduct formation, in keeping with various proteomic studies, in the context of neurodegeneration, cited in section Alzheimer's Disease (AD), which pinpointed a limited number of protein targets of oxidative modification, including α-enolase, grouped in selected functional subsets (Martínez et al., 2010; Reed, 2011; Sultana et al., 2012). Some of these proteins targets were also identified as autoantigens frequently recognized by autoantibodies in autoimmune diseases. α-Enolase, in particular, was recurrently indicated as a novel autoantigen in systemic lupus erythematosus (SLE), systemic sclerosis (SSc) (Moscato et al., 2000; Pratesi et al., 2000; Mosca et al., 2006; Bussone et al., 2011), SSc with interstitial lung fibrosis (Terrier et al., 2010), rheumatoid arthritis (Goëb et al., 2009; Saulot et al., 2002), mixed cryoglobulinemia (MC) with nephropathy (Sabbatini et al., 1997; Moscato et al., 2000; Pratesi et al., 2000), pulmonary arterial hypertension (Bussone et al., 2012), giant-cell arteritis (Régent et al., 2011), Behçet's disease (Lee et al., 2003) and inflammatory bowel disease (IBD) (Roozendaal et al., 1998). Anti-α-enolase autoantibodies isolated from patiens with SLE, SSc and MC recognized membrane-associated α-enolase and inhibited plasminogen binding to it (Moscato et al., 2000). It is tempting to speculate

that the high susceptibility of α-enolase to modification by HNE and other aldehydes might be instrumental for its involvement in autoimmunity as an oxidatively modified self antigen, capable of breaking the immunological tolerance of autoreactive T and B cells. This is in keeping with the identification of α-enolase among the proteins undergoing carbonyl addition and HNE adduction in heart homogenates and cardiomyocytes oxidized *in vitro* with 4-HNE or H_2O_2. Oxidative modifications correlated with increased recognition of α-enolase by serum antibodies of rodents and humans affected with Chagas' disease, which is characterized by increased production of ROS of inflammatory and mitochondrial origin (Dhiman et al., 2012).

HNE adducts with other neuronal enzymes, transporters, and receptors

Inducible ***heme oxygenase 1 (HO-1)*** catalyzes heme conversion to biliverdin-IXa, which is further reduced to antioxidant bilirubin-IXa (Mancuso and Barone, 2009). The expression of the *HO-1* gene is redox-regulated by an antioxidant responsive element in its promoter. Activation of HO-1 contributes to the adaptive response to oxidative stress in AD (Poon et al., 2004). Increased levels of HO-1 were observed in association with neurofibrillary tangles (NFTs) and senile plaques (Takeda et al., 2000) and in hippocampal neurons of AD patients, together with increases of serine phosphorylation, tyrosine nitration and 4-HNE modification of HO-1, as though adaptive increases in HO-1 expression and activation were counteracting the structural and functional impairment of HO-1, via tyrosine nitration and HNE-HO-1 adduct formation.

Collapsin response mediator protein 2 (CRMP2). Participates in axon guidance and synapse maturation, by mediating the transduction of reelin (Yamashita et al., 2006) and semaphorin 3A signals (Uchida et al., 2009). Sultana et al. (2012) has proposed that the HNE-CRMP2 adducts (Reed et al., 2008; Perluigi et al., 2009) might be of pathogenic importance for neurite shortening and the loss of synapses, early features of AD (Hensley et al., 2011; Scheff et al., 2011), and that Aβ peptide-induced oxidation of peptidylprolyl cis/trans isomerase (Pin1) (Butterfield et al., 2006b; Sultana et al., 2006b) may be responsible for the dysregulation of glycogen synthase kinase-3β (GSK-3β) and cyclin-dependent kinase 5 (CDK5) and for the hyperphosphorylation of tau proteins and of colocalized CRMP2 within NFTs (Williamson et al., 2011).

Reduced glucose utilization and energy production (Rhein and Eckert, 2007) are early occurrences in AD. They may be explained by the reported formation of HNE adducts with neuronal ***glucose transporter GLUT3*** in rat hippocampal neurons (Mark et al., 1997a) and with the ***mitochondrial ATP synthase α subunit*** in human AD brains (Reed et al., 2008; Perluigi et al., 2009; Terni et al., 2010). Decreased levels of ATP synthase activity were also reported in AD (Schagger and Ohm, 1995). Soluble Aβ peptide oligomers were responsible for electron-transport chain dysruption, enhanced ROS generation, mitochondrial fragmentation, and synaptic damage (Reddy et al., 2010), as well as for enhanced HNE production (Mark et al., 1997b). ATP synthase α subunit was colocalized with CRMP2 within NFTs (Sergeant et al., 2003). In AD brains, LDL receptor-related protein 1 (LRP-1), a membrane receptor involved in Aβ peptide removal, was also covalently modified by HNE, which might contribute to the extracellular deposition of amyloid substance (Owen et al., 2010).

Acrolein-protein adducts

Acrolein is neurotoxic *in vitro*. Moreover, in Alzheimer's brains, high levels of acrolein were detected in hippocampus and temporal cortex, where oxidative stress is high (Dang et al., 2010). Thus, several studies addressed the mechanism of acrolein neurotoxicity. Dang and coworkers (2010) showed that, in neuronal primary cultures of hippocampal cells, acrolein exerted more toxic effects than HNE. This might depend on the higher reactivity of acrolein, which was an initiator of oxidative stress by forming adducts with cellular nucleophilic groups in proteins, lipids, and nucleic acids. Indeed, it was documented that in synaptosomal proteins, exposed to high concentrations of acrolein, a loss of thiol group content occurred, due to Michael adduct formation between acrolein and thiol groups of proteins (LoPachin et al., 2007). Moreover, such adduct formation led also to protein cross-linking. Based on the cited evidence, LoPachin et al. (2002, 2003) proposed that nerve terminals were the primary sites of acrolein action and that synaptic dysfunction was a necessary step in the production of neurotoxicity. In support of this hypothesis, *in vivo* and *in vitro* studies showed that exposure to acrolein was associated with reduced presynaptic neurotransmitter release. This effect involved inhibition of key proteins, which regulate membrane–vesicle fusion, such as N-ethylmaleimide-sensitive fusion protein (NSF) and synaptosomal-associated protein of 25 kDa (SNAP-25) (Barber and LoPachin, 2004; LoPachin, 2004). Acrolein also inhibited presynaptic membrane neurotransmitter uptake and vesicular storage *in vivo* and *in vitro* (LoPachin et al., 2006; LoPachin, 2004). Proteomic analyses showed that these dysfunctions were associated with the formation of adducts with the ***dopamine transporter*** and ***v-ATPase***, respectively Barber and LoPachin, 2004; LoPachin, 2004; LoPachin et al., 2007; Barber et al., 2007. *In vitro* studies showed that acrolein and HNE disrupted synaptosomal membrane protein conformation and phospholipid asymmetry (Subramaniam et al., 1997; Pocernich et al., 2001; Castegna et al., 2004), reduced glutamate uptake and GLUT3-mediated glucose transport in synaptosomes and cultured nerve cells (Keller et al., 1997a,b; Lovell et al., 2000), reduced respiration and induced oxidative stress in synaptosomal mitochondria (Humphries et al., 1998; Morel et al., 1999; Picklo et al., 1999; Picklo and Montine, 2001; Luo and Shi, 2005; Raza and John, 2006), inhibited membrane Na^+ and Ca^{2+} ion pumps and dysrupted ion regulation in cultured nerve cells (Keller et al., 1997b; Mark et al., 1997b).

MDA-protein adducts

A largely coincident protein repertoire as the one delineated by anti-HNE antibodies was compiled by the immunochemical detection of Nε-MDA-lysine. It included: α- (non neural) and γ-enolase (neural), glutamic acid dehydrogenase I, creatin kinase B chain (CKB), ubiquinol-cytochrome c reductase complex core protein I, ATP synthase β subunit, glutathione synthase (GS), 60-kDa heat shock protein (HSP-60),

guanine nucleotide-binding protein G(I)/G(S)/G(T) β subunit 2 (GNB2), β- and γ-actin, α- and β-tubulin, vimentin, neurofilament L, glial fibrillar acidic protein (GFAP), collapsin response mediator protein 2, CRMP2, DRP-2) (Pamplona et al., 2005), and 14-3-3 protein ζ (HYWAZ) and γ (HYWAG) isoforms (Santpere et al., 2007).

A redox model of Alzheimer's disease pathogenesis

Far beyond individual protein dysfunctions, the generation of markers of lipid peroxidation in AD appears to be associated with the progressive endangerment of vital processes, such as energy metabolism, antioxidant defenses, signal transduction, axonal transport, and synapse conservation. A redox model of Alzheimer's disease pathogenesis (Sultana et al., 2012) is depicted in **Figure 3**.

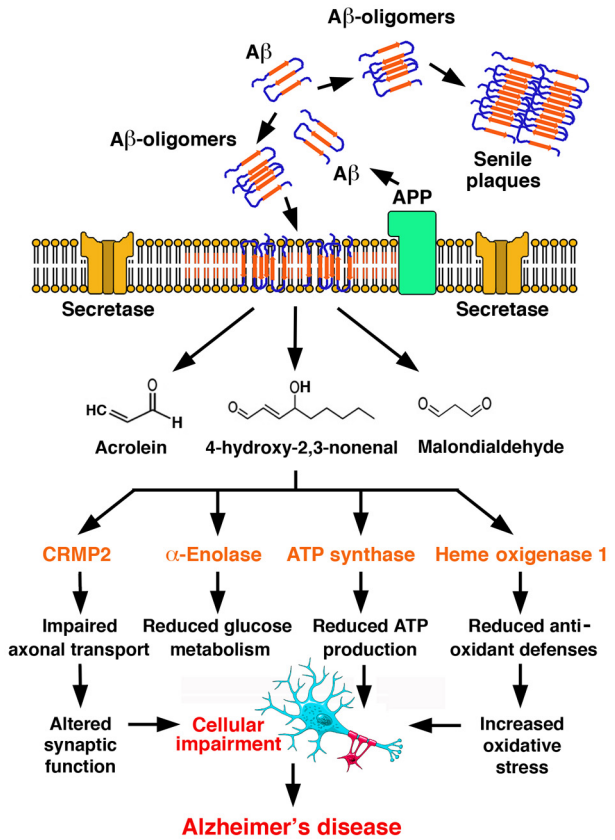

FIGURE 3 | A redox model of Alzheimer's disease pathogenesis. Amyloid β-peptide (Aβ) is generated by proteolytic cleavage of Amyloid Precursor Protein (APP) by secretases. Aβ undergoes aggregation, with the formation of oligomers, which undergo a conformational transition to β-structured diffusible oligomers and eventually deposit as amyloid plaques in the ECM. Aβ oligomers insert in the plasma membrane, where they initiate lipid peroxidation, leading to the formation of reactive aldehydes, such as acrolein, MDA and HNE. Adduct formation compromises the function of critical proteins in a number of functional subsets of neurotransmission, energetic metabolism, mitochondrial function, antioxidant defenses, represented here by collapsin response mediated protein 2 (CRMP2), α-enolase, ATP synthase α subunit and heme oxygenase 1. Such process is self-feeding and ultimately leads to Alzheimer's disease [Redrawn with permission from Sultana et al. (2012)].

ALDEHYDE-PROTEIN ADDUCTS IN ATHEROSCLEROSIS

The potential role of reactive aldehydes in the pathogenesis of atherosclerosis was suggested by their increases in plasma in association with extensive aortic atherosclerosis and the high levels of aldehydes generated during the oxidation of phospholipids in LDLs (Salomon et al., 2000). The observed consequences of LDL oxidation by aldehydes *in vitro* are described below. The formation of protein-bound lipid peroxidation products in atherosclerotic lesions was also repeatedly reported.

ALDEHYDE-LDL ADDUCTS

Early studies of the contribution of aldehyde-protein adducts to atherogenesis provided evidences that modification of LDL by aldehydes enhanced their recognition and uptake by macrophages (Hoff et al., 2003). The formation of aldehyde adducts with apolipoprotein B (Apo B) in LDL converted the latter to an atherogenic form that was taken up by macrophages, leading to the formation of foam cells (Steinberg et al., 1989; Steinberg, 1995). The adduction products detected in Apo B of oxidized LDL included: (a) acrolein derivatives, such as N-(3-methylpyridinium)lysine (MP-Lys) (Obama et al., 2007) and the 3-formyl-3,4-dehydropiperidino adduct (FDP-lysine) formed by the addition of two acrolein molecules to one lysyl side chain (Uchida et al., 1998a,b); (b) HNE adducts, such as the enaminal-type HNE-histidine and HNE-lysine adducts (Uchida et al., 1994); (c) MDA adducts, such as Nε-(2-propenal)-lysine (Uchida et al., 1997), and 1-amino-3-iminopropene-type MDA-lysine cross-links (Requena et al., 1997). The formation of aldehyde-LDL adducts could alter the binding of LDL to membrane scavenger receptors at the surface of endothelial cells and activated macrophages. The participation of reactive aldehydes in LDL-receptor interactions was documented by several immunohistochemical analyses of atherosclerotic lesions from human aorta, using antibodies against various aldehyde adducts, such as HNE-histidine (Uchida et al., 1995), Nε-MDA-lysine (Uchida et al., 1997), and Nε-acrolein-lysine (FDP-lysine) (Uchida et al., 1998a), in which intense positivities were associated with cells, primarily macrophages. It was recently reported that HNE–histidine Michael adducts had significant affinities and interacted with LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1), an important scavenger receptor mediating endothelial oxLDL uptake. HNE-modified proteins strongly inhibited the uptake of acetylated LDL (AcLDL). In human aortic endothelial cells, the binding of HNE-histidine adducts to LOX-1 stimulated ROS formation and activated extracellular signal-regulated kinase 1/2 (ERK 1/2) and NF-κB (Kumano-Kuramochi et al., 2012).

Using recombinant human Apo E (an exchangeable antiatherogenic apolipoprotein) and immunoblotting with acrolein-lysine-specific antibodies, other Authors (Tamamizu-Kato et al., 2007) showed that acrolein severely compromised the functional integrity of Apo E, as for heparin, lipid, and LDL receptor binding. These results were in agreement with previous observations of acrolein being widely present in atherosclerotic lesion, as revealed by the use of anti-acrolein antibodies (Uchida et al., 1998a). Nε-(3-methylpyridinium)-lysine (MP-Lys), an acrolein derivative, was detected in Apo B of native LDL (Obama et al., 2007). Moreover, acrolein-LDL induced foam

cell formation from macrophages, suggesting that acrolein maight contribute to LDL modification, foam cell formation and atherogenesis (Watanabe et al., 2013).

PHOSPHATIDYLCHOLINE γ-HYDROXYALKENALS AND ATHEROSCLEROSIS

Starting from the early observation that proteins modified by 2-pentylpyrrole incorporation of lysyl ε-amino groups, upon covalent addition of HNE, accumulated in the blood of individuals with atherosclerosis and in brain neurons of patients with Alzheimer's disease (Sayre et al., 1996), it became evident that γ-hydroxyalkenal phospholipids and their ω-carboxyalkylpyrrole derivatives contributed strongly in the pathogenesis of atherosclerosis. This was the subject of recent reviews (Salomon and Gu, 2011; Stemmer and Hermetter, 2012). Antibody-based studies revealed the presence of carboxyethylpyrroles (CHPs) and carboxypropylpyrroles (CPPs) in oxLDL (Kaur et al., 1997). Also the CHP immunoreactivity, reflecting the presence of protein adducts of 9-hydroxy-12-oxo-10-dodecenoic acid (HODA) or its phosphatidylcholine ester in human plasma, was significantly higher in the plasma of patients with atherosclerosis and end-stage renal disease than in healthy controls (Kaur et al., 1997). HODA-protein adducts were produced *in vivo* from 9-hydroxy-12-oxo-10-dodecenoyl-phosphatidylcholine (HODA-PC), one of the oxidized lipids derived from 1-palmityl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PA-PC), altogether referred to as oxPA-PC. Chemically synthesized 5-hydroxy-8-oxo-6-octenoyl-phosphatidylcholine (HOOA-PC) exhibited properties of a chemical mediator of chronic inflammation. It activated, in a dose-dependent manner, human aortic endothelial cells to bind monocytes and to secrete increased levels of monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8), two chemokines promoting monocyte entry into chronic lesions. It also inhibited LPS-induced expression of E-selectin, an adhesion molecule mediating endothelial-neutrophil interactions (Subbanagounder et al., 2002). HOOA-PC was found unbound and in pyrrole adducts in lipid extracts of oxLDL and human atheromas (Podrez et al., 2002; Hoff et al., 2003). A scenario emerged from these studies, in which atherogenesis might involve myeloperoxidase-initiated, free radical-induced production of oxPC, which promoted subendothelial monocyte infiltration and endocytosis of oxLDL by macrophages, accompanied by conversion into foam cells and atheroma formation (Salomon and Gu, 2011). Thereafter, it was shown that scavenger receptor CD36, another mediator of oxLDL uptake by macrophages, at variance with the LDL receptor, bound oxidized lipid derivatives within oxLDL, including the derivatives of 1-palmitoyl-2-arachidonoyl-glycerophosphocholine (oxPA-PC), such as HOOA-PC, and of 1-linoleoyl-2-arachidonoyl-glycerophosphocholine (oxLA-PC), such as HODA-PC (**Figure 2**). These γ-oxygenated-α,β-unsaturated aldehydes, collectively referred to as $oxPC_{CD36}$, were potent activators of the CD36-mediated endocytosis of oxLDL by macrophages, promoting the cytotoxic effects of the formation of protein adducts of electrophilic oxidized derivatives of cholesterol and phospholipids (Salomon and Gu, 2011). As an instance, the formation of Michael or pyrrole adducts of HOOA-PC or HODA-PC to a cysteinyl thiol group of lysosomal cathepsin B reduced the ability of mouse macrophages to degrade internalized macromolecules (Hoff et al., 2003). OxLDL and individual $oxPC_{CD36}$ also interfered with the binding of HDL to scavenger SR-B1 receptors of hepatocytes, thus inhibiting the HDL-mediated delivery of cholesteryl esthers to the liver (Ashraf et al., 2008).

HNE-SCAVENGER RECEPTOR B1 ADDUCTS AND KERATINOCYTE HDL UPTAKE

Scavenger Receptor B1 (SR-B1), also known as HDL receptor, is expressed in cells of the epidermal *stratum corneum*. In cultured human keratinocytes, exposure to cigarette smoke caused the translocation and eventual loss of SR-B1, driven by the activation of cellular NADPH oxidase (NOX) and the enhanced H_2O_2 production. Cigarette smoke also caused the formation of acrolein-SR-B1 and HNE-SR-B1 adducts and increased SR-B1 ubiquitination. It was proposed that such oxidation-dependent modifications of SR-B1 subcellular localization and stability might affect the physiological uptake of cholesterol by SC epidermal cells, which, in turn, might compromise their lipid composition and barrier function in the course of oxidative stress (Sticozzi et al., 2012).

ALDEHYDE-PROTEIN ADDUCTS IN AUTOIMMUNITY

Modification of self antigens in the course of oxidative stress, by adduct formation with reactive products of lipid peroxidation, HNE being one of the most commonly involved, is generally regarded to as a mechanism by which concomitant modification of self and neoantigen formation may lead to the breaking of tolerance to self antigens and, thus, to the pathogenesis of autoimmune disease. Indeed, it was known for a long time that abnormally high levels of HNE-protein adducts can be detected in the sera of children affected by autoimmune diseases (Grune et al., 1997). According to this view, cross-linking of HNE with self antigens would be instrumental in creating neoantigens from formerly tolerated autoantigens and, thus, initiating autoimmunity.

HNE-PROTEIN ADDUCTS IN SJÖGREN'S SYNDROME (SS) AND SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Sjögren syndrome (SS) is an autoimmunity-driven chronic infammatory disorder, characterized by infiltration and destruction of lacrimal and salivary glands by effector $CD4^+$ and $CD8^+$ T cells and activated macrophages, resulting in keratoconjunctivitis with dry eyes and xerostomia (dry mouth). Secondary SS can also add to the clinical picture of other autoimmune diseases, such as systemic lupus erythematosus (SLE). Among autoimmune diseases, SS is second only to rheumatoid arthritis (RA) in prevalence (1%), with affected females outnumbering males by 9–1. Antibodies to self antigens, such as anti-nuclear antibodies (ANA), are characteristically found in SS, some of them being in common with other autoimmune diseases, such as SLE, RA, and systemic sclerosis (SSc). Typical ANA targets in SS include the SS-A/Ro and SS-B/La proteins. The former include a 52-kDa form, located in nucleus and in cytoplasm, (SS-A1/Ro52; TRIM21) and a 60-kDa cytoplasmic form (SS-A2/Ro60; TROVE2). Both are components of Ro ribonucleoprotein (RNP) particles, in which they associate with short non-coding, histidine-rich RNAs

(hY-RNAs). The 48-kDa SS-B/La antigen is a transcription termination factor for RNA Polymerase III, transiently associated with hY-RNAs in ribonucleoprotein particles involved in tRNA processing and histonic mRNA stabilization. Systemic lupus erythematosus (SLE) is a multisystemic disease characterized by a polyclonal B cell activation, leading to the differentiation of plasma cells producing autoantibodies toward a broad range of autoantigens. ANA are found in 95% of patients with SLE, as well as in patients with other autoimmune diseases. They are heterogeneous and include antibodies toward: double stranded (ds) DNA; histones; ribonucleoproteins (RNP), such as the Smith (Sm) antigen (corresponding to the common core proteins of spliceosomal small nuclear RNPs), and the SS-A/Ro and SS-B/La antigens. The formation and deposition of immune complexes and complement in the wall of small arteries, at the dermo-epidermal junction and in the glomerular basal membrane (GBM) is responsible, respectively, for the diffuse necrotizing vasculitis, the cutaneous lesions of erythematous, bullous, and ulcerative kind, and the nephritis associated with SLE.

Notwithstanding their nuclear and/or cytoplasmic location, Ro and La antigens appeared to become exposed at the cell surface in the course of apoptosis. Epitopes expressed at the surface of apoptotic cells are named "apotopes." After the first observations of the clustering of cytoplasmic and nuclear antigens, including SS-Ro and SS-La antigens, in two types of blebs at the surface of apoptotic cells (Casciola-Rosen et al., 1994), the accessibility of SS-A/Ro and SS-B/La antigens at the surface of apoptotic cells was further confirmed (Miranda-Carús et al., 2000; Ohlsson et al., 2002). In SLE and SS, both the number of circulating apoptotic leukocytes and the susceptibility of lymphocytes to activation-induced apoptosis *in vitro* increased (Emlen et al., 1994; Georgescu et al., 1997; Zeher et al., 1999; Ren et al., 2003). Impaired efferocytosis (clearance of apoptotic cells) by macrophages also contributed to the higher degree of exposure to autoantigens determined by the increased rate of apoptosis in SLE (Ren et al., 2003). It was speculated that both factors may trigger autoimmunity (Savill et al., 2002). It was proposed (Casciola-Rosen et al., 1994) that the breaking of tolerance to autoantigens at the surface of apoptotic cells might be favored by oxidative modifications occurring as a result of the oxidative stress that characterizes apoptosis (Hockenberry et al., 1993).

The contribution of the formation of HNE adducts to the modification of self antigens, such as SS-A2/Ro60, in Sjögren's syndrome was explored by Scofield and coworkers. They hypothesized that modification of SS-A2/Ro60 with HNE might facilitate the breaking of tolerance to the self antigen. After immunizing rabbits with either HNE-modified or unmodified SS-A2/Ro60, they observed that autoimmunity was established faster and more strongly in animals immunized with HNE-modified SS-A2/Ro60 (Scofield et al., 2005). In an extension of this model, an SS-like condition, with anti-SS-A2/Ro60 antibodies, decreased salivary flow and salivary gland mononuclear infiltrates, could be induced in BALB/c mice by immunization with a peptide of SS-A2/Ro60 (Kurien et al., 2011). Efficient production of anti-SS-A2/Ro60 and anti-SS-B/La autoantibodies ensued immunization with SS-A2/Ro60, both as such and modified with increasing concentrations of HNE (0.4, 2, or 10 mM). However, antibody production was faster after low- and medium-level modification of SS-A2/Ro60 with HNE. Differential use of unmodified or HNE-modified SS-A2/Ro60 as the solid-phase substrate in ELISAs for autoantibodies revealed, among the antibodies produced by mice immunized with HNE-modified SS-A2/Ro60, an additional subpopulation of antibodies, which recognized HNE or HNE-SS-A2/Ro60, but not unmodified SS-A2/Ro60. Most interestingly, immunization with medium-level HNE-modified SS-A2/Ro60 was accompanied by the appearance of anti-dsDNA autoantibodies, which induced the Authors to imply a SLE-like disease, although they did not provide pathological evidence of it. Together with the already mentioned appearance of anti-SS-B/La antibodies, following immunization with SS-A2/Ro60, the occurrence of anti-dsDNA antibodies represented an example of intermolecular epitope spreading. In turn, the ability of HNE to form adducts with a large number of biological macromolecules could be of help in understanding the broad range of autoantibody responses in SLE and SS. Moreover, immunization with high-level HNE-modified SS-A2/Ro60 was associated with weaker antibody responses to unmodified SS-A2/Ro60 and SS-B/La, reduction of salivary flow and lymphocytic infiltration of salivary glands, suggesting a Sjögren's syndrome-like condition. Notably, high-level HNE modification of SS-A2/Ro60 was accompanied by aggregation, which prompted the Authors to interpret the results as due to increasing bifunctional cross-linking of SS-A2/Ro60 and diminished exposure of HNE at the surface of SS-A2/Ro60 molecules (Kurien et al., 2011). A more likely interpretation could be that large, particulate immunocomplexes of aggregated HNE-SS-A2/Ro60 and autoantibodies stimulated the phagocytic and antigen-presenting activity of macrophages, which skewed the autoimmune response toward a prevailingly cytotoxic cell-mediated mechanism.

The molecular mimicry between the adducts of lipid peroxidation products with proteins and nucleic acids, as a possible mechanism initiating the production of anti-DNA autoantibodies, in response to some other modified self antigen, was the subject of interesting studies by Uchida and coworkers. After raising an anti-HNE monoclonal antibody (anti-*R* mAb 310), recognizing enantioselectively (*R*)-HNE-histidine Michael adducts (Hashimoto et al., 2003), they unexpectedly found that the sequence of this anti-HNE mAb was highly similar to those of various clonally related anti-DNA antibodies. Despite these structural similarities, the cross-reactivity of mAb R310 with native dsDNA was limited, but was strongly enhanced by treating DNA with 4-oxo-2-nonenal (ONE), a HNE analog. The 7-(2-oxo-heptyl)-substituted 1,N^2-etheno-type ONE-2'-deoxynucleoside adducts were identified as alternative epitopes of mAb R310 in ONE-modified DNA. On these grounds, these Authors hypothesized that endogenous reactive electrophiles, like HNE, might function as immunologic triggers for human autoimmunity (Akagawa et al., 2006). These Authors further investigated the possible involvement of HNE-modified proteins as the endogenous source of anti-DNA antibodies. They found HNE-specific epitopes in the epidermis and dermis of patients with SLE, pemphigus vulgaris and contact dermatitis, as well as antibodies against HNE-modified bovine serum albumin (BSA) in the sera of patients affected with

SLE, Siögren's syndrome, rheumatoid arthritis, systemic sclerosis and idiopathic inflammatory miopathies, and also in the sera of diseased MRL/*lpr* mice. Upon repeated immunization with HNE-modified KLH, mice developed also a subpopulation of B cell clones recognizing native DNA, but not HNE-BSA. In agreement with previous results, the reactivity of anti-HNE B cell clones toward DNA was greatly enhanced by DNA modification with ONE. On the other hand, anti-DNA mAbs cross-reacted with ONE-modified BSA. These data suggested that HNE-specific epitopes produced upon physiological generation of HNE in cells might serve as triggering antigens for the development of bispecific antibodies against native DNA and ONE-modified proteins. On the whole, these findings strongly supported the pathogenic role of lipid peroxidation products in autoimmune disease (Toyoda et al., 2007). The pathogenic role of lipid peroxidation in SLE and the potential usefulness of anti-MDA and anti-HNE antibody titers in predicting its progression was underscored also by a report showing that the prevalences and serum levels of MDA- and HNE-protein adducts, as well as of MDA- and HNE-specific antibodies, were significantly higher in SLE patients than in healthy controls, and were in correlation with the SLE Disease Activity Index. The levels of each aldehyde-protein adduct were also in correlation with the titers of the respective antibodies (Wang et al., 2010).

ALDEHYDE-PROTEIN ADDUCTS AND STRUCTURAL INTEGRITY, ION TRANSPORT, AND SIGNAL TRANSDUCTION AT THE PLASMA MEMBRANE LEVEL

HNE is the product of lipid peroxidation which has been shown to be mostly involved in the control of cell functions. Under physiological conditions, HNE can be found at low concentrations in human tissues and plasma (Parola et al., 1998; Okada et al., 1999; Ji et al., 2001; Siems and Grune, 2003), where it participates in the control of signal transduction, cell proliferation and differentiation (Parola et al., 1998). HNE-protein adducts in peripheral blood primarily involve albumin, transferrin and immunoglobins (Barrera et al., 1996). Adducts between HNE and proteins have been detected *in vitro* in various mammalian cell types (Parola et al., 1998; Okada et al., 1999; Ji et al., 2001; Siems and Grune, 2003), in which the percent of total added HNE in HNE-protein adducts was between 1 and 5% (Rinaldi et al., 2001). Some adducts of HNE with cell proteins involved in specific functions at the plasma membrane level were characterized in detail.

HNE-SPECTRIN ADDUCTS AND RED CELL MEMBRANE INTEGRITY

Spectrin is the main component of the submembranous cytoskeleton lining the intracellular side of the plasma membrane of red blood cells, playing a fundamental role in maintaining its stability and strength, via direct interactions with membrane lipids and the actin cytoskeleton. Immunoblotting and mass spectrometric analyses revealed that, in human red cells, α- and β-spectrin were the primary targets of HNE adduction. Exposure of intact red cells to HNE resulted in selective HNE-spectrin adduct formation, with preferential β-spectrin modification and cross-linking of HNE-modified spectrin molecules. The Authors speculated that local spectrin aggregation, by freeing the lipid bilayer from the underlying spectrin-actin cytoskeleton, might lead to membrane surface area loss by extrusion (Arashiki et al., 2010). Together with the reported accumulation of HNE in aging circulating red blood cells (Ando et al., 1995), these observations may be of relevance not only for the physiological destruction of aged red cells, but also for the immune-mediated hemolysis of red blood cells under conditions of enhanced production of lipid hydroperoxides.

HNE-Na^+-K^+-ATPase ADDUCTS

Na^+-K^+-ATPase is an integral plasma membrane protein of great functional importance. Its primary functions are the maintenance of intracellular K^+ ion levels and the excretion of Na^+ ions. It contains 70 cysteinyl residues per molecule. The binding of HNE at 1–10 μmolar concentration to Na^+-K^+-ATPase was rapid and was accompanied by a decrease in measurable SH-groups and an irreversible loss of enzyme activity (Siems et al., 1996). Na^+-K^+-ATPase could be attacked by HNE formed both intra- and extracellularly, due to the free access of HNE to integral plasma membrane proteins. These Authors suggested that the reduction of Na^+-K^+-ATPase activity upon covalent HNE binding might represent an important form of secondary oxidative cell damage. Their findings were confirmed by the demonstration that in cultured hippocampal neurons HNE impaired Na^+-K^+-ATPase activity and induced increases of intracellular Ca^{2+} ion concentration (Mark et al., 1997b).

HNE ADDUCTS WITH TYROSINE KINASE RECEPTORS

Tyrosine kinase receptors (RTKs), such as the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR), are transmembrane glycoproteins, displaying tyrosine kinase activity in their cytoplasmic domains. Stimulation of RTKs by ligand-dependent or -independent mechanisms (radiation, metal ions, ROS) induces receptor dimerization and autophosphorylation of tyrosyl residues, followed by catalytic activation, whereas downregulation of RTKs is mediated by internalization and dephosphorylation (Pawson and Scott, 1997). Oxidized LDL (but not native LDL) and free HNE induced in living cells the formation of HNE-EGFR and HNE–PDGFR adducts, evidenced by the binding of anti-HNE-protein antibodies and by the loss in free $-NH_2$ group content (Suc et al., 1998; Hubbard and Till, 2000; Escargueil-Blanc et al., 2001). At physiological or moderate HNE concentrations (0.1 μM and 1–10 μM), the formation of HNE-EGFR and HNE-PDGFR adducts resulted in sustained RTK activation (Suc et al., 1998; Escargueil-Blanc et al., 2001). A short incubation of vascular smooth muscle cells (SMCs) with a low concentration of HNE (0.1–1 μM) induced the derivatization and autophosphorylation of RTKs, with the consequential activation of the phosphatidylinositol 3-kinase (PI3K)/Akt-mediated survival pathway and of the mitogenic response of SMCs (Auge et al., 2002). On the other hand, high concentrations of HNE, for longer incubation times, inhibited EGFR- and PDGFR-mediated cell proliferation (Liu et al., 1999; Vindis et al., 2006), through inhibitory effects on RTK signaling (Negre-Salvayre et al., 2003). High doses of HNE exerted similar negative effects on proteasomes (Okada et al., 1999; Vieira et al., 2000), mitochondrial transition pores (Irwin et al., 2002), glyceraldehyde-3-phosphate dehydrogenase

(Uchida and Stadtman, 1993) and cathepsin B activities (Crabb et al., 2002). The inhibitory effect of 4-HNE on growth factor-dependent cell proliferation was in agreement with the progressive desensitization of PDGFR β subunit to its ligand PDGF B-chain in SMCs (Vindis et al., 2006). In other cell types, low HNE concentrations (1 μM) did not cause RKT activation. In human hepatic stellate cells (hHSC), 1 μM HNE rather inhibited tyrosine autophosphorylation of PDGFR β induced by the PDGF BB isoform, which resulted in the inhibition of the mitogen-activated protein kinase (MAPK) and PI3K cascades and a consequential decrease of PDGF-dependent DNA synthesis (Robino et al., 2000). Acrolein was also shown to be a potent inactivator of protein tyrosine phosphatase 1B (PTP1B), a member of an important class of cysteine-dependent enzymes, working in tandem with protein tyrosine kinases in the regulation of a number of signal transduction pathways (Seiner et al., 2007).

HNE ADDUCTS WITH PROTEINS IN THE INSULIN SIGNALING CASCADES

The regulation of insulin signaling starts with the binding of insulin to its receptor, whose tyrosyl residues are rapidly phosphorylated. This permits the recruitment of adaptor proteins, such as insulin receptor substrates (IRSs) and Src homology-2-containing (Shc) proteins, which transmit the insulin signal down the PI3K cascade for glucose, lipid, and protein metabolism and the MAPK cascade for cell proliferation and differentiation (Saltiel and Kahn, 2001; Van Obberghen et al., 2001; White, 2002; Taniguchi et al., 2006). Reductions in the levels of IRSs and insulin-induced IRSs and a decrease in insulin receptor β phosphorylation were observed upon exposure to HNE at non-toxic concentrations (Demozay et al., 2008). Such effects could be due to the formation of HNE-IRS adducts, likely impairing IRS function and favoring IRS degradation. The downstream signaling cascades, involving PI3K and protein kinase B (PKB), were also down-regulated upon exposure to HNE, which resulted in blunted metabolic responses. The Authors of this study hypothesized that HNE build-up in diabetic rats (due to increased lipid peroxidation and altered clearance of its products by detoxifying enzymes) might be a cause of signaling dysfunction, hindering insulin action.

CONCLUSIONS

The adducts of reactive aldehydes with membrane proteins participate in physiological, as well as pathological processes and can determine variable functional consequences, in relation with the protein targets of adduction and their functional roles. Polyclonal and monoclonal antibodies directed against protein-bound aldehyde adducts have been of great help in exploring the aldehyde-related modifications of the cell proteome, while mass spectrometry-based techniques have been playing a key role in elucidating the stoichiometry and sites of covalent protein modification with reactive aldehydes. Nonetheless, the inventory of aldehyde-modified membrane proteins detected so far is probably still largely incomplete, when compared with the plethora of biological effects displayed by these molecules. Quantitative technical limitations in the individuation of aldehyde-protein adducts are being gradually overcome by the increases in sensitivity, molecular specificity and tolerance to impurities of spectrometric instrumentation and techniques (Wu and Vogt, 2012). Current challenges include: (1) characterizing the functional consequences of cell protein modification with aldehydes, which was not addressed by most redox proteomic studies published until now. This may involve major efforts of expression, reconstitution, modification and activity/interactivity assays of protein targets of aldehyde modification *in vitro*, as well as innovative approaches of protein-specific tracking and functional characterization at the cellular level; (2) clarifying the sources, sites and circumstances of increased lipid peroxidation in cells and the topological/functional relationships (e.g., in terms of subcellular compartmentalization and regulation of gene expression and gene product activity) linking the increased generation of reactive aldehydes with the modifications of specific cell membrane proteins.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Membrane_Physiology_and_Membrane_Biophysics/10.3389/fphys.2013.00242/abstract

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Nitric oxide and membrane lipid peroxidation in photosynthetic and non-photosynthetic organisms under several stress conditions

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INTRODUCTION

Oxidative damage to lipids was characterized in terms of the nature of the oxidant, the type of lipid, and the severity of the oxidation (Simontacchi et al., 2011). Even though malondialdehyde detection with the thiobarbituric acid reactive substances test (TBARS) is the most currently used assay for the determination of lipid oxidation, it is unspecific since the reaction can be reproduced by other biological compounds (Simontacchi et al., 2011). On the other hand, electron paramagnetic resonance (EPR) spectroscopy showed the capacity of detecting the presence of the lipid radicals ($LR^{\bullet}$) formed during peroxidation, by yielding unique and stable products with spin traps (Malanga and Puntarulo, 2012). Nitric oxide (NO) is recognized both, as a signaling molecule that regulates many enzyme activities, but as a toxic agent as well. It has been found that NO is able to protect animal and plant cell types from oxidative damage resulting from superoxide (O_2^-), hydrogen peroxide (H_2O_2) and alkyl peroxides by acting as a terminator of free radical chain reactions (Wink et al., 1995, 1996; Yalowich et al., 1999; Beligni and Lamattina, 2002; Sharpe et al., 2003). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) interact through the reaction of O_2^- with NO, to generate peroxynitrite ($ONOO^-$) at a rate close to diffusion. $ONOO^-$ acts as both, a nitrating agent and a powerful oxidant capable of modifying proteins (formation of nitrotyrosine), lipids (lipid oxidation, lipid nitration), and nucleic acids (DNA oxidation and DNA nitration) (Gisone et al., 2004).

The purpose of this commentary is to point out that NO complex interactions with other cellular components lead to a wide range of effects depending on the biological system under study and the oxidative stress condition.

LIPID PEROXIDATION AND NO IN PHOTOSYNTHETIC ORGANISMS

In cultures of the green algae *Chlorella vulgaris* no significant changes were observed in either of the parameters showed in **Table 1**, in the stationary phase as compared to the log phase of growth. However, Qian et al. (2009) demonstrated in *Chlorella vulgaris* that, depending on its concentration, NO increased the activity of antioxidant enzymes to protect against the oxidative damage caused by herbicide stress. Data from Simontachi et al. (2004) showed that the NO steady state concentration in homogenates from sorghum embryonic axes reached a maximum at 24–30 h after the starting of imbibition, in coincidence with the initiation of an active germination. The generation of $LR^{\bullet}$ in sorghum axes was drastically increased at 36 h of imbibition from non-detectable values at 24 h, coincidentally with the significant decline in the content of cellular NO (**Table 1**).

To analyze the role of NO during senescence, Jasid et al. (2009) sprayed daily soybean cotyledons with an NO donor solution (sodium nitroprusside, SNP), from day 5 to 25. Differently from naturally senescent cotyledons, where NO content reached a maximum at day 10 of seedling development and declined, in SNP-treated cotyledons NO content was higher, and remained unchanged from day 10 to 25 after germination, as compared to control values. While naturally senescent cotyledons experimented no change in the content of $LR^{\bullet}$ during the studied period, SNP-treated cotyledons showed a decrease as time progressed (**Table 1**).

Shi et al. (2005) suggested that the protective effect of NO on the oxidative damage of thylakoid membrane proteins in *Phaseolus vulgaris* beans under UV-B radiation may be mediated by increasing the level of expression of genes encoding ROS-scavenging enzymes. Moreover, Jasid et al. (2006) showed that isolated chloroplasts from soybean leaves exposed to 2 μM NO for 30 min, decreased the rate of $LR^{\bullet}$ generation (**Table 1**). Also, Jasid et al. (2008) reported that axes from sorghum seeds incubated 24 h in the presence of SNP, showed a significantly higher NO content as compared to control axes, and $LR^{\bullet}$ content evaluated in the microsomal fractions was significantly lower, as compared to control membranes (**Table 1**).

LIPID PEROXIDATION AND NO IN NON-PHOTOSYNTHETIC ORGANISMS

Data in **Table 1** show that seasonality affects oxidative metabolism in digestive glands (DG) from *Nacella magellanica* limpets isolated from the Beagle Channel, Tierra del Fuego, Argentina. A significant increase in both $LR^{\bullet}$ and NO content in summer, as compared to winter,

Table 1 | Nitric oxide and lipid peroxidation under stress conditions in photosynthetic and non-photosynthetic organisms.

Biological system	Stress condition	Lipid peroxidation		NO	References
		TBARS	LR•		
PHOTOSYNTHETIC ORGANISMS					
Intact cells					
Chlorella cells	Development	No change day 12–18	No change day 12–18	No change day 12–18	Malanga and Puntarulo, 1995; Estevez et al., 2001; Estévez and Puntarulo, 2005
Homogenates					
Sorghum embryonic axes	Development	nd	Increased from 36 to 48	Decreased from 36 to 48 h	Simontachi et al., 2004
Soybean cotyledons	Senescence	nd	No change day 10–25	Non-detectable at day 25	Jasid et al., 2009
	+SNP	nd	Decrease 58% day 10–25	No change day 10–25	
Sub-cellular structures					
Chloroplasts from soybean leaves	+GSNO 250 μM	nd	Decrease 29%	2 μM NO (supplementation)	Jasid et al., 2006
Microsomes from sorghum embryonic axes	+SNP 1 mM	nd	Decrease 43%	Increase 140%	Jasid et al., 2008
NON-PHOTOSYNTHETIC ORGANISMS					
Invertebrates					
Nacella magellanica	Summer vs. winter	nd	1.7-fold increase	1.6-fold increase	Malanga et al., 2007
Mya arenaria	Fe 500 μM	4-fold increase day 0–17	nd	4-fold decrease day 0–17	González et al., 2010
Mammals					
Fetus rat brain	γ radiation 2 h	No change	No change	6-fold increase	Gisone et al., 2003
	γ radiation 4 h	Increase 51%	nd	No change	Gisone et al., 2003
Rat liver	Fe 500 mg/kg	2.7-fold increase	nd	No change	Galleano and Puntarulo, 1992; Rousseau et al., 2011

nd stands for non-determined.

was reported (Malanga et al., 2007). However, studies on toxicological effects of Fe exposure under laboratory conditions showed that significant increases in lipid peroxidation were temporarily associated to decreases in NO content in DG from the bivalve *Mya arenaria* after 17 days of treatment (González et al., 2010) (**Table 1**).

In vivo γ irradiation of rat fetuses did not significantly affect neither the content of LR• nor the content of TBARS in the brain up to 2 h post-irradiation (pi). However, 4 h after the exposure, a significant increase in the TBARS content was measured. These results are consistent with the hypothesis that changes could be produced in the brain at the early stages after exposure to γ radiation to limit free radical-dependent damage, since increased lipid peroxidation was only detected after 4 h pi. Gisone et al. (2003) showed that total NO synthase activity was increased after 30 and 60 min pi, and returned to control values after 2 h pi, and accordingly NO content was significantly increased (**Table 1**).

Galleano and Puntarulo (1992) showed that liver homogenates from Fe-dextran overloaded male Wistar rats showed a significant increase in TBARS 6 h post-injection, as compared to control rats (**Table 1**). Later, Galleano et al. (2001) pointed out that the significant increase in NO, assayed as $DETC_2$–Fe–NO adducts 5 h after Fe administration could be an artifact due to the excess of Fe during the measurement. Recently, Rousseau et al. (2011) showed that one of the molecular footprints left by the reactions of ROS with biomolecules, the level of protein 3-nitrotyrosines, was not increased by Fe-dextran administration, suggesting that Fe overload in liver did not change NO cellular content (**Table 1**).

CONCLUDING REMARKS

The results summarized here implied the existence of a very complex regulatory interplay between NO and ROS. The multiple effects of NO on the process of lipid peroxidation imply that the net result will depend on the balance of competing factors. The rate and location of NO formation, and also the rate of formation of O_2^-, or other mitigating factors, will all contribute to the degree and the nature of the effect on lipid oxidation in a particular system. Detailed analysis of the molecular mechanisms in each condition is required. In this regard, no yet deeply studied NO reactions, such as NO binding to Fe and

endogenous thiols and other nitrosyl–Fe complexes that seems to favor Fe release from the cell avoiding its accumulation, could reveal to be a key factor in NO cellular interactions and should be further characterized.

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METHODS
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Two dimensional blue native/SDS-PAGE to identify mitochondrial complex I subunits modified by 4-hydroxynonenal (HNE)

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The lipid peroxidation product 4-hydroxynonenal (HNE) can form protein-linked HNE adducts, thereby impacting protein structure and function. Mitochondrial complex I (NADH-ubiquinone oxidoreductase), containing at least 45 subunits in mammalian cells, sits in a lipid-rich environment and is thus very susceptible to HNE modifications. In this paper, a procedure for the identification of HNE-modified complex I subunits is described. Complex I was isolated by first dimensional non-gradient blue native polyacrylamide gel electrophoresis (BN-PAGE). The isolated complex I band, visualized by either Coomassie blue staining or silver staining, was further analyzed by second dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). HNE-modified proteins were visualized by Western blotting probed with anti-HNE antibodies. HNE-positive bands were then excised and the proteins contained in them were identified by mass spectrometric peptide sequencing. The method was successfully applied for the identification of two complex I subunits that showed enhanced HNE-modifications in diabetic kidney mitochondria.

Keywords: blue native/SDS-PAGE, diabetes, 4-hydroxynonenal, mitochondria, reactive oxygen species, streptozotocin

Introduction

Lipids are targets of oxidative damage induced by reactive oxygen species (ROS). 4-hydroxynonenal (HNE) is a lipid peroxidation byproduct derived from membrane lipid oxidation by ROS (Dalleau et al., 2013). It is a signaling molecule (Vatsyayan et al., 2011) and can react with certain protein amino acid residues such as lysine, histidine, and cysteine (Uchida and Stadtman, 1992a,b), leading to changes in protein structure and function. Indeed, HNE and HNE -modified proteins have been suggested to be involved in the pathogenesis of many age-related diseases such as Alzheimer's (Gwon et al., 2012) and Parkinson's disease (Farooqui and Farooqui, 2011), cardiovascular disease (Anderson et al., 2012), diabetes (Cohen et al., 2013), and cancer (Warnakulasuriya et al., 2008). Therefore, studying HNE modified proteins

Abbreviations: CBB, Coomassie brilliant blue; DDM, n-dodecyl-β-D-maltoside; HNE, 4-hydroxynonenal; NADH, nicotinamide adenine dinucleotide; LC-MS, liquid chromatography-mass spectrometry; NBT, nitro blue tetrazolium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ROS, reactive oxygen species; STZ, streptozotocin.

may help understand the mechanisms of cell death in a given pathophysiological condition.

Mitochondrial complex I (NADH-ubiquinone oxidoreductase) is the first complex in the mitochondrial electron transport chain (Vinothkumar et al., 2014). It takes electrons from nicotinamide adenine dinucleotide (NADH) and passes them to coenzyme Q (Vinothkumar et al., 2014). During this NADH oxidation process, protons are pumped via complex I into the intermembrane space, forming a proton gradient across the inner membrane that drives the synthesis of ATP. In the meantime, NADH oxidation also drives complex I production of superoxide anion (Hirst et al., 2008; Treberg et al., 2011), the precursor of other ROS such as hydrogen peroxide, hydroxyl radical, and peroxynitrite when nitric oxide is available (Yan, 2014). As proteins are susceptible to oxidative damage, complex I is thus both a source and target of ROS. It has been established that complex I dysfunction is linked to numerous aging-related diseases such as Parkinson's disease and diabetes (Cooper et al., 1992; Schapira, 1998; Fassone and Rahman, 2012).

Complex I is a multisubunit complex consisting of at least 45 subunits in mammalian cells (Carroll et al., 2006). Many of the subunits are redox-sensitive proteins that are susceptible to attacks by ROS and lipid peroxidation productions such as HNE (Hattori et al., 1991; Yoritaka et al., 1996; Rafique et al., 2001), which may lead to impairment of complex I function. In this article, we present a gel-based method for isolation and resolution of complex I subunits and identification of HNE-modified complex I proteins. The method involves isolation of whole complex I using first dimensional blue native polyacrylamide gel electrophoresis (BN-PAGE) followed by second dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to resolve individual complex I subunits. HNE modified proteins are identified by Western blotting analysis using anti-HNE antibodies. Protein bands showing positive HNE immuno-staining are then excised for identification by mass spectrometric peptide sequencing. It should be noted that we took the BN-/SDS-PAGE approach for our study because the conventional IEF/SDS-PAGE would disrupt complex I association and does not yield an intact complex for further in-gel activity measurement and complex I subunit analysis.

Materials and Methods

Chemicals and Reagents

Sucrose and mannitol were purchased from BDH Chemicals and Mallickrodt Chemicals, respectively. Bis-Tris, tricine, and amino-caproic acid were purchased from MB Biochemicals (Irvine, CA). Pre-stained SDS-PAGE markers were purchased from Thermo Scientific (Pittsburgh, PA). Bradford protein assay solution and Coomassie brilliant blue (CBB) R-250 were from Bio-Rad laboratories (Richmond, CA). Silver nitrate, streptozotocin (STZ), sodium citrate, NADH, EDTA, n-dodecyl-β-D-maltoside (DDM), and nitro blue tetrazolium (NBT) chloride tablets were obtained from Sigma (St. Louis, MO, USA). Serva Blue G was purchased from Serva (Heidelberg, Germany). Rabbit anti-HNE polyclonal antibodies (IgG) and goat anti-rabbit IgG conjugated with horseradish peroxidase were purchased from US Biological (Salem, MA) and Invitrogen (San Diego, CA), respectively. Hybond-C membrane and a Western blot detection kit were obtained from GE Healthcare (Piscataway, NJ).

Animals and Induction of Diabetes

Young adult male Sprague Dawley rats (2–6 months old) purchased from Charles River were used in this study. Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg body weight) after overnight fasting (Gajdosik et al., 1999). STZ was prepared fresh by dissolving in 0.1 M citrate buffer pH 4.5 and control animals received citrate buffer only. Blood glucose levels were monitored once a week using blood glucose test strips (FreeStyle lite from Abbott Diabetes Care Inc., Alameda, California). Animals with blood glucose levels exceeding 200 mg/dl were deemed to be diabetic. Four weeks after STZ injections, animals were sacrificed and tissues were collected.

Isolation of Tissue Mitochondria

Mitochondria from either heart or kidney were used in this study. The procedures for mitochondria preparations from both tissues were essentially the same as previously described (Navarro et al., 2004). Briefly, tissues were homogenized (1 g tissue per 10 ml isolation buffer) in mitochondrial isolation buffer containing 70 mM sucrose, 230 mM mannitol, 15 mM MOPS (pH 7.2), and 1 mM potassium EDTA. The homogenates were then centrifuged at 800 g for 10 min at 4°C. The supernatant was kept and further centrifuged at 8000 g also for 10 min at 4°C. The resulting pellet, containing mitochondria, was washed once with 10 ml isolation buffer and centrifuged again under the same conditions. The obtained mitochondrial pellet was either stored at -80°C or used immediately. For preparation of mitochondrial membrane proteins, mitochondrial pellet was resuspended in 30 mM potassium phosphate and sonicated four times 30 s with 1 min interval. The resuspension was then centrifuged at 80,000 × g for 30 min and the resulting pellet contained mitochondrial membranes (Yan et al., 1997).

First Dimensional Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) and In-gel Complex I Activity Staining

A non-gradient BN-PAGE was employed as previously described (Yan and Forster, 2009). Mitochondrial pellet was solubilized in a BN-PAGE sample buffer containing 0.75 M aminocaproic acid, 75 mM Bis-Tris, and 1% DDM, pH 7.0. After brief sonication and further incubation on ice for 60 min, the solution was centrifuged at 8000 g and the resulting supernatant was used for BN-PAGE. Protein concentrations were determined by the Bradford assay (Bradford, 1976). Gel buffer contained 500 mM aminocaproic acid, 50 mM Bis-Tris. The cathode buffer contained 50 mM tricine, 15 mM Bis-Tris pH 7.0 with or without 0.02% CBB. The anode buffer contained 50 mM Bis-Tris, pH 7.0. Gel was run at 150 voltages using CBB-containing cathode buffer until the front reached at one-third of the gel where the cathode buffer was replaced with the one that didn't have CBB. Gel running was resumed at 200 voltages until complete. After gel electrophoresis, the gel was stained either by CBB staining or by complex I activity staining. Activity staining was achieved at room temperature by

incubating the gels or gel strips in 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mg/ml NADH and 0.2 mg/ml NBT (Yan and Forster, 2009).

Second Dimensional SDS-PAGE and Western Blotting

For second dimensional SDS-PAGE, a 10% resolving gel was usually performed. Gel strips derived from the first dimensional blue native gel was equilibrated in 5% 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 10 mM glycerol for 20 min (Yan et al., 1998). The strip or complex I band was then placed onto the second dimensional gel for electrophoresis as previously described (Yan et al., 2007). Usually two gels were run simultaneously. One gel was for CBB or silver staining, and the other gel was used for membrane transfer and Western blotting according to standard procedures. HNE signals were revealed by anti-HNE antibodies. All densitometric quantifications were performed using AlphaEaseFC image analysis software (Alpha Innotech, SanLeandro, CA). Statistical analysis was performed using GraphPad software. $^{*}P < 0.05$ indicates significant differences between control and STZ groups.

Silver Staining

A procedure of silver staining (Yan et al., 2000) was modified and used in this study. Following electrophoresis, gels were fixed in 50% methanol, 12% acetic acid containing 0.05% formalin for 1 h. The gels were then washed with double-distilled (dd) water for three times with each time gently shaking for 5 min. This was followed by sensitization in 0.02% sodium thiosulfate for 2 min. The gels were again washed with dd water for three times with each time also gently shaking for 5 min. Silver staining was performed by incubating the gels in a silver nitrate (0.2%) solution for 20 min. After washing twice in water for 1 min each, gel bands were developed in an ice-cold 6% sodium bicarbonate. Staining was stopped by a solution containing 50% methanol and 12% acetic acid. The gels were kept in a solution containing 10% methanol and 8% acetic acid. This method is compatible with mass spectrometric peptide sequencing (Yan et al., 2000).

Mass Spectrometric Peptide Sequencing

Protein identification via mass spectrometric peptide sequencing was conducted at ProtTech (Phoneixville, PA) using NanoLC-MS/MS peptide sequencing technology. Briefly, the silver-stained gel band was excised, cleaned, and digested with sequencing grade trypsin. The resulting protein mixture was analyzed by an LC-MS/MS equipment. The collected mass spectrometric data were used to search the protein database using ProtTech's software suite.

Results and Discussion

First Dimensional BN-PAGE

Cardiac mitochondria were used in our condition set-up experiments (**Figures 1**–**3**). For complex I isolation, we used a non-gradient blue native PAGE method as previously described (Yan and Forster, 2009). **Figure 1A** shows complex I band localization after gel electrophoresis. It was not necessary to further stain the gel after electrophoresis as complex I was always visible due to its pre-binding of Coomassie blue achieved during sample preparation. **Figure 1B** shows in-gel complex I activity staining by incubating the gels strips in a solution containing NADH and NBT.

Second Dimensional SDS-PAGE

For the resolution of each individual complex I subunit, we initially took two approaches. The first one was to turn the whole gel strip 90 degrees counter clockwise and layered it onto the second dimensional gel. As shown in **Figure 2**, this approach separated not only complex I subunits, but also those of complexes V and III, among others. The second approach was to excise the complex I band from the blue native gel, and placed this band onto a second dimensional gel, resulting in the resolution of only complex I subunits as shown in **Figure 3** whereby both CBB staining and silver staining of individual complex I proteins were demonstrated. This approach was preferred because it would

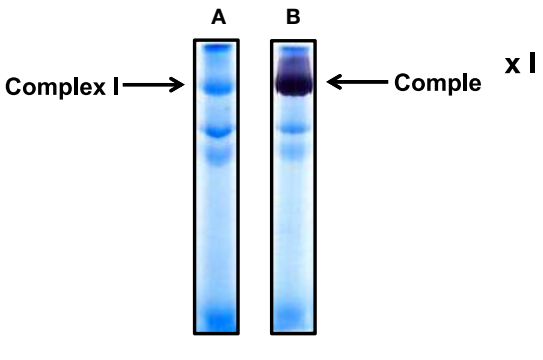

FIGURE 1 | Non-gradient blue native gel electrophoresis of cardiac mitochondria isolated from rat heart. (A) Gel image after electrophoresis without further CBB staining; **(B)** complex I activity staining by NADH and NBT. An 8% resolving gel was performed.

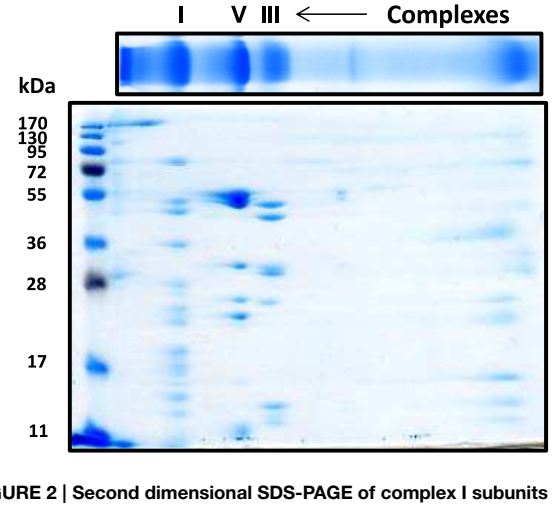

FIGURE 2 | Second dimensional SDS-PAGE of complex I subunits together with those of complex V and complex III. The blue native gel strip was placed on top of the second dimensional SDS gel (10% resolving). Gel was stained by CBB staining.

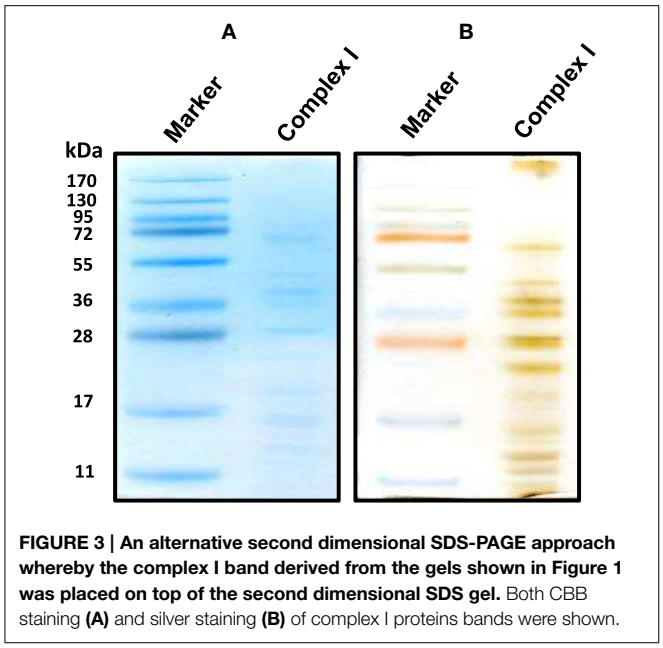

FIGURE 3 | An alternative second dimensional SDS-PAGE approach whereby the complex I band derived from the gels shown in Figure 1 was placed on top of the second dimensional SDS gel. Both CBB staining **(A)** and silver staining **(B)** of complex I proteins bands were shown.

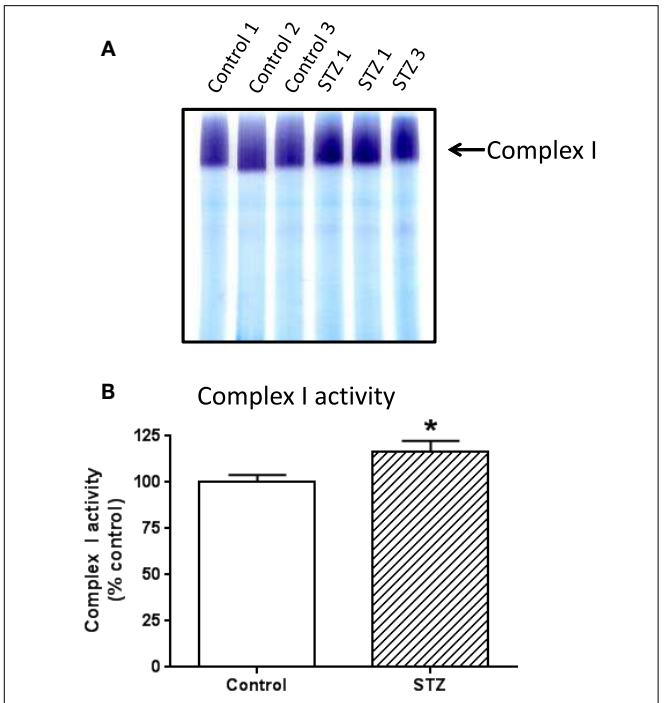

FIGURE 4 | In-gel complex I activity staining and quantitation of complex I activity in each group of rats. Shown was comparison between control and diabetic (STZ) mitochondria isolated from rat kidney; **(A)** BN-PAGE activity staining, **(B)** densitometric quantification of complex I activity based on **(A)**. $P < 0.05$ indicates significant difference between control and STZ groups.

facilitate band matching and excision between stained gels and Western blot membranes as no other complexes were present.

Western Blot and Identification of Complex I Subunits modified by HNE

To investigate which complex I subunits could undergo HNE modifications, we then switched to kidney mitochondria isolated from STZ diabetic rats. This switch was due to our observation that there were no detectable changes in cardiac complex I activity in STZ diabetic rats 4 weeks post STZ injection. The idea was to explore which subunits underwent enhanced HNE modifications under diabetic conditions as it is possible that kidney mitochondrial proteins may exhibit enhanced HNE modifications in diabetes (Sivitz and Yorek, 2010). Results are shown in **Figures 4**, **5**. **Figure 4A** shows a first dimensional in-gel complex I activity staining between control and diabetes. **Figure 4B** shows densitometric quantification of complex I activity in each group whereby complex I activity in STZ diabetes was higher than that in the control group. **Figure 5A** shows anti-HNE immunostaining of complex I subunits, in which two prominent bands could be visualized to show a basal level of HNE modification that was enhanced by STZ-induced diabetes. HNE content in each band was also higher in STZ diabetes group than in control group (**Figures 5B,C**). These two bands were excised and subjected to mass spectrometric peptide sequencing. As shown in **Figure 6**, there were 21 peptides in band 1 (**Figure 6A**) that matched to the NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1) and 17 peptides in band 2 (**Figure 6B**) that matched to the NADH dehydrogenase iron–sulfur protein 2 (NDUFS2). Hence, the two proteins identified were NDUFS1 (75 kDa) and NDUFS2 (53 kDa), respectively (**Figure 6C**). Both have been reported to undergo HNE modifications under different experimental conditions (Choksi et al., 2008; Zhao et al., 2014). It should be noted that the reason that these two proteins could be positively identified is likely due to the fact that they are the most abundant subunits in complex I, which is the drawback of all gel-based proteomic approaches for identification of posttranslationally modified proteins.

For the first dimensional blue native gel analysis, complex I from tissue mitochondria are always well-resolved. The band can be clearly observed after gel electrophoresis without further CBB staining. Therefore, either the CBB staining band or the activity staining band can be excised for second dimensional SDS-PAGE followed by Western blotting. However, for cultured cells, we have found that a clear complex I band usually failed to be observed even after further CBB staining because of low complex I levels. In this situation, the existence of a well-resolved complex I band could only be visualized by in-gel activity staining which is more sensitive than that of CBB staining. Therefore, for analysis of complex I from cultured cells, the activity containing band should be excised for further analysis.

It should be noted that when whole mitochondrial preparations are loaded for the isolation of complex I in the first dimensional blue native gel analysis, certain non-complex I proteins can co-migrate with complex I, resulting in contamination of complex I by other proteins. Therefore, the use of mitochondrial membrane preparations that contain all complex I subunits should be preferred for identification of complex I subunits.

It should also be noted that the method described in this article may also be used for the analysis of other types of

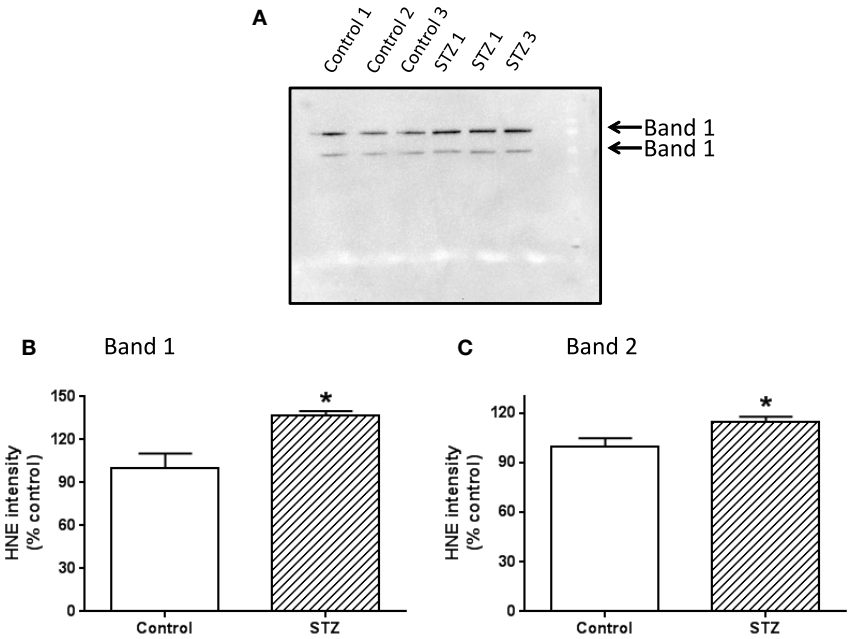

FIGURE 5 | Western blot detection of HNE modification of complex I subunits. Two bands showing HNE modification were indicated in **(A)**. **(B,C)** Show the densitometric quantification of HNE content in bands 1 and 2, respectively. $P < 0.05$ indicates significant difference between control and STZ groups.

A Band 1 peptides obtained (21 peptides)

Scan No.	Peptide Mass	Peptide Sequence
2193	1030.47	YDHLGDSPK
2205	1096.51	SATYVNTEGR
2209	1030.47	YDHLGDSPK
2271	988.50	VASGAAAEWK
2345	979.58	VAVTPPGLAR
2369	1082.61	LGEVSPNLVR
2375	1353.62	GNDMQVGTYIEK
2393	1384.79	KPMVVLGSSALQR
2419	1233.53	DFYMTDSISR
2438	1165.58	VAGMLQSFEGK
2477	2070.15	IASQVAALDLGYKPGVEAIR
2489	2118.94	YDDVEEANYFQQASELAK
2501	1402.78	VALIGSPVDLTYR
2577	1610.79	GLLTYTSWEDALSR
2593	1528.81	DDGAAILAAVSSIAQK
2599	2074.09	ALSEIAGITLPYDTLDQVR
2621	2135.19	LVDQEFLADPLVPPQLTIK
2637	1751.02	AVAAIAGGLVDAEALVALK
2765	2757.45	SNYLLNTTIAGVEEADVVLLVGTNPR
3042	2120.08	KTESIDVMDAVGSNIVVSTR
3187	1751.02	AVAAIAGGLVDAEALVALK

B Band 2 peptides obtained (17 peptides)

Scan No.	Peptide Mass	Peptide Sequence
2176	770.40	MPPGEIK
2369	1046.62	LLNIQPPPR
2387	1046.62	LLNIQPPPR
2416	1195.63	APGFAHLAGLDK
2421	1195.63	APGFAHLAGLDK
2432	933.53	VLFGEITR
2435	1328.65	TSMESLIHHFK
2437	1030.52	GSGIQWDLR
2459	2268.13	LYTEGYQVPPGATYTAIEAPK
2468	1471.69	GEFGVYLVSDGSSR
2491	2268.13	LYTEGYQVPPGATYTAIEAPK
2507	2268.13	LYTEGYQVPPGATYTAIEAPK
2515	2031.02	ETAHWKPPPWNDVDVLK
2516	2031.02	ETAHWKPPPWNDVDVLK
2544	1385.70	TYLQALPYFDR
2547	2227.04	TQPYDVYDQVEFDVPIGSR
2655	2368.08	WQPDIEWAEQFSGAVMYPSK

C *Protein identity:*

Band 1: NDUFS1, NADH-ubiquinone oxidoreductase, 75 kDa

Band 2: NDUFS2, NADH dehydrogenase iron-sulfur protein 2, 53 kDa

FIGURE 6 | Mass spectrometric peptide sequencing of band 1 and band 2. Shown are **(A)** 21 peptides that matched to NDUFS1, **(B)** 17 peptides that matched NDUFS2, and **(C)** the identity of each protein indicated in **Figure 5A**.

posttranslational modifications such as acetylation (Fritz et al., 2012) and carbonylation (Yan, 2009). For carbonylation analysis, however, the first dimensional gel strips will need to be incubated with carbonyl probes such as 2,4-dinitrophenylhydrazine or biotin-containing probes (Yan and Forster, 2011) before second dimensional gel electrophoresis.

In summary, we present in this paper a method for identification of complex I subunits that can be modified by the lipid peroxidation product HNE. The procedure involves complex I isolation by BN-PAGE, subunit resolution by SDS-PAGE, Western blot detection of HNE-conjugated proteins using anti-HNE antibodies, and protein identification by mass spectrometric peptide sequencing. The procedure may also find applications in identifying complex I subunits undergoing other types of posttranslational modifications.

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frontiers in PHYSIOLOGY

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Membrane lipid unsaturation as physiological adaptation to animal longevity

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The appearance of oxygen in the terrestrial atmosphere represented an important selective pressure for ancestral living organisms and contributed toward setting up the pace of evolutionary changes in structural and functional systems. The evolution of using oxygen for efficient energy production served as a driving force for the evolution of complex organisms. The redox reactions associated with its use were, however, responsible for the production of reactive species (derived from oxygen and lipids) with damaging effects due to oxidative chemical modifications of essential cellular components. Consequently, aerobic life required the emergence and selection of antioxidant defense systems. As a result, a high diversity in molecular and structural antioxidant defenses evolved. In the following paragraphs, we analyze the adaptation of biological membranes as a dynamic structural defense against reactive species evolved by animals. In particular, our goal is to describe the physiological mechanisms underlying the structural adaptation of cellular membranes to oxidative stress and to explain the meaning of this adaptive mechanism, and to review the state of the art about the link between membrane composition and longevity of animal species.

Keywords: fatty acid biosynthesis, membrane unsaturation, oxidative damage, peroxidizability index, phylogenomic analysis, rate of aging, reactive carbonyl species

INTRODUCTION

The appearance of oxygen in the terrestrial atmosphere represented an important selective pressure for ancestral living organisms and contributed toward setting up the pace of evolutionary changes in structural and functional systems (McCord, 2000; Lane, 2002; Embley and Martin, 2006; Pamplona and Costantini, 2011). The evolution of using oxygen for efficient energy production served as a driving force for the evolution of complex organisms (Lane, 2002; Schirrmeister et al., 2013). The redox reactions associated with its use were, however, responsible for the production of reactive species [reactive oxygen species (ROS) and reactive carbonyl species (RCS)] with damaging effects due, basically, to oxidative chemical modifications of essential cellular components (Halliwell and Gutteridge, 2007). Consequently, aerobic life required the emergence and selection of antioxidant defense systems (Halliwell, 1999). As a result, a high diversity in molecular and structural antioxidant defenses evolved (Pamplona and Costantini, 2011). Nevertheless, the balance between oxidant production systems and antioxidant defenses is adjusted in a species-specific way to generate a net flux of oxidant (see **Figure 1**) for maintaining antioxidant responses through redox signaling pathways perfectly integrated in the cellular metabolic machinery. This oxidative stress has become a universal constraint of life-history evolution in animals and a modulator of phenotypic development (Dowling and Simmons, 2009; Pamplona and Costantini, 2011).

Living organisms on the Earth which are divided into three major domains—Archaea, Bacteria, and Eucarya—, probably came from a common ancestral cell. The cell membrane is a key dynamic structural component of a cell, and lipid molecules are essential for cell membranes (Itoh et al., 2001; Lombard et al., 2012). All cell membranes are composed of glycerol phosphate phospholipids, and this commonality argues for the presence of such phospholipids in the last common ancestor (Lombard et al., 2012). Therefore, all living organisms have lipid membranes.

Biological membranes are dynamic structures that generally consist of bilayers of amphipathic molecules held together by non-covalent bonds (Yeagle, 1993; Vance and Vance, 1996). Phospholipids, the predominant membrane lipids in eukaryotic cells, are made up of a glycerol backbone with a hydrophilic headgroup bound to carbon 3, and fatty acids to C1 and C2. Phospholipids are a large group of diverse molecules that participate in a wide range of biological processes (Dowhan, 1997). This diversity requires complex metabolic and regulatory pathways (Yeagle, 1993; Vance and Vance, 1996). Indeed eukaryotic cells, to monitor cell membrane composition and to adjust lipid synthesis accordingly (Dobrosotskaya et al., 2002), invest around 5% of their genes to synthesize all of these lipids (Van Meer et al., 2008).

The phospholipid acyl chains are saturated (SFA), monounsaturated (MUFA), or polyunsaturated (PUFA) hydrocarbon chains that normally vary from 14 to 22 carbons in length (Wallis et al., 2002). In eukaryotic cells from vertebrate species, for example,

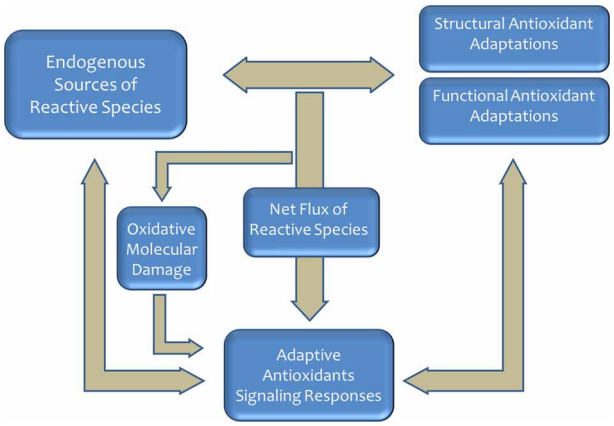

FIGURE 1 | Aerobic life produces reactive species that required the emergence and selection of antioxidant defense systems. Redox state is an important selective pressure faced by most organisms, and a myriad of mechanisms have evolved to regulate and adjust this process. In homeostatic conditions, the sytem play a key role in the aging process and the determination of the longevity.

the average chain length of a biological membrane is strictly maintained around 18 carbon atoms, and the relative distribution between SFAs and UFAs follows a ratio of 40:60 (Pamplona, 2008). Substrate precursors for UFA biosynthesis are generally SFA that are products of fatty acid synthase (FAS), as well as essential fatty acids from dietary sources. The desaturase and elongase enzymes, which are conserved across kingdoms, as well as the peroxisomal beta-oxidation pathway, will allow cells to obtain all the diversity of fatty acids present in a cellular membrane (Nakamura and Nara, 2004; Guillou et al., 2010) (see **Figure 2**). Finally, the deacylation-reacylation cycle will be the mechanism responsible for the particular fatty acid composition of cell membranes.

In the following paragraphs, we analyze the adaptation of biological membranes as dynamic structural defense against reactive species evolved by animals. In particular, our goal is to describe the physiological mechanisms underlying the structural adaptation of cellular membranes to oxidative stress and to explain the meaning of this adaptive mechanism, and to review the state of the art about the link between membrane composition and longevity of animal species.

LIPID PEROXIDATION OF BIOLOGICAL MEMBRANES: SIGNALING vs. CITOTOXICITY

The physico-chemical properties of the membrane bilayer and the chemical reactivity of the fatty acids that compose the membrane are two inherent traits of the membrane phospholipids that determine their susceptibility to oxidative damage (Pamplona et al., 2002a,b,c; Hulbert et al., 2007; Pamplona, 2008). The first property is related to the fact that oxygen and reactive species are more soluble in the fluid lipid bilayer than in the aqueous solution (Moller et al., 2005; Gamliel et al., 2008). Consequently, membrane lipids become primary targets of oxidative damage. The second and more significant property is related to the fact that PUFA residues of phospholipids are extremely sensitive to oxidation, their sensitivity increasing exponentially as a function of the number of double bonds per fatty acid molecule (Holman, 1954; Bielski et al., 1983). Consequently, PUFA side chains (with two or more double bonds) are much more easily attacked by radicals than are SFA (no double bonds) or MUFA (one double bond) side chains. In this scenario, from a given membrane fatty acid profile it is possible to calculate its peroxidizability index (PI) by combining this composition with the relative susceptibility of individual fatty acids to peroxidation. So, PI is an approach to the relative susceptibility of a given membrane fatty acid composition to peroxidative damage. The higher the number the more susceptible, the lower the value of PI, the more resistant to lipid peroxidation is the membrane bilayer (Pamplona et al., 2002a,b,c; Hulbert et al., 2007).

Lipid peroxidation generates hydroperoxides as well as endoperoxides, which undergo fragmentation to produce a broad range of reactive intermediates called 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] (Esterbauer et al., 1991; Catalá, 2009; Zimniak, 2011; Fritz and Petersen, 2013). 2-Hydroxyheptanal and 4-hydroxyhexenal are other significant aldehydic products of lipid peroxidation of PUFAs. These carbonyl compounds, ubiquitously generated in biological systems, have unique properties contrasted with other reactive species. For instance, compared with ROS, reactive aldehydes have a much greater half-life (i.e., minutes to hours instead of nanoseconds to microseconds for most ROS). Further, the non-charged structure of RCS allows them to migrate easily through hydrophobic membranes and hydrophilic cytosolic media, thereby extending the migration distance far from the generation site (Pamplona, 2008).

These compounds have specific physiological signaling roles inducing adaptive responses driven to decrease oxidative damage and improve antioxidant defenses (Pamplona, 2008, 2011; Higdon et al., 2012). Two of these mechanisms involved in the prevention of oxidative damage effects are: (i) the regulation of uncoupling protein activity (Echtay et al., 2003; Brand et al., 2004), and (ii) the activation of the antioxidant response signaling pathway that includes the expression of enzymes such as glutathione-S-transferase (GST) specifically designed to detoxify reactive carbonyl compounds (Wakabayashi et al., 2004; Copple et al., 2008; Giles, 2009; Maher and Yamamoto, 2010). As important as GST is for these adaptive mechanisms, is the role GP × 4 (phospholipid hydroperoxide glutathione peroxidase) has in restoring reduced state of membrane fatty acids from phospholipids to ensure membrane lipid homeostasis (Imai and Nakagawa, 2003; Brigelius-Flohé, 2006; Conrad et al., 2007). GP × 4 gene structure, expression and activity is likely to have evolved in a coherent fashion to cope with prooxidant conditions.

Based on the features mentioned above these carbonyl compounds can be, however, more destructive than ROS and may have far-reaching damaging effects on target sites within or outside membranes. Carbonyl compounds react with nucleophilic groups in macromolecules (lipoxidation reactions) like proteins (Thorpe and Baynes, 2003), DNA (West and Marnett, 2006), and aminophospholipids (Naudi et al., 2013), among others, resulting

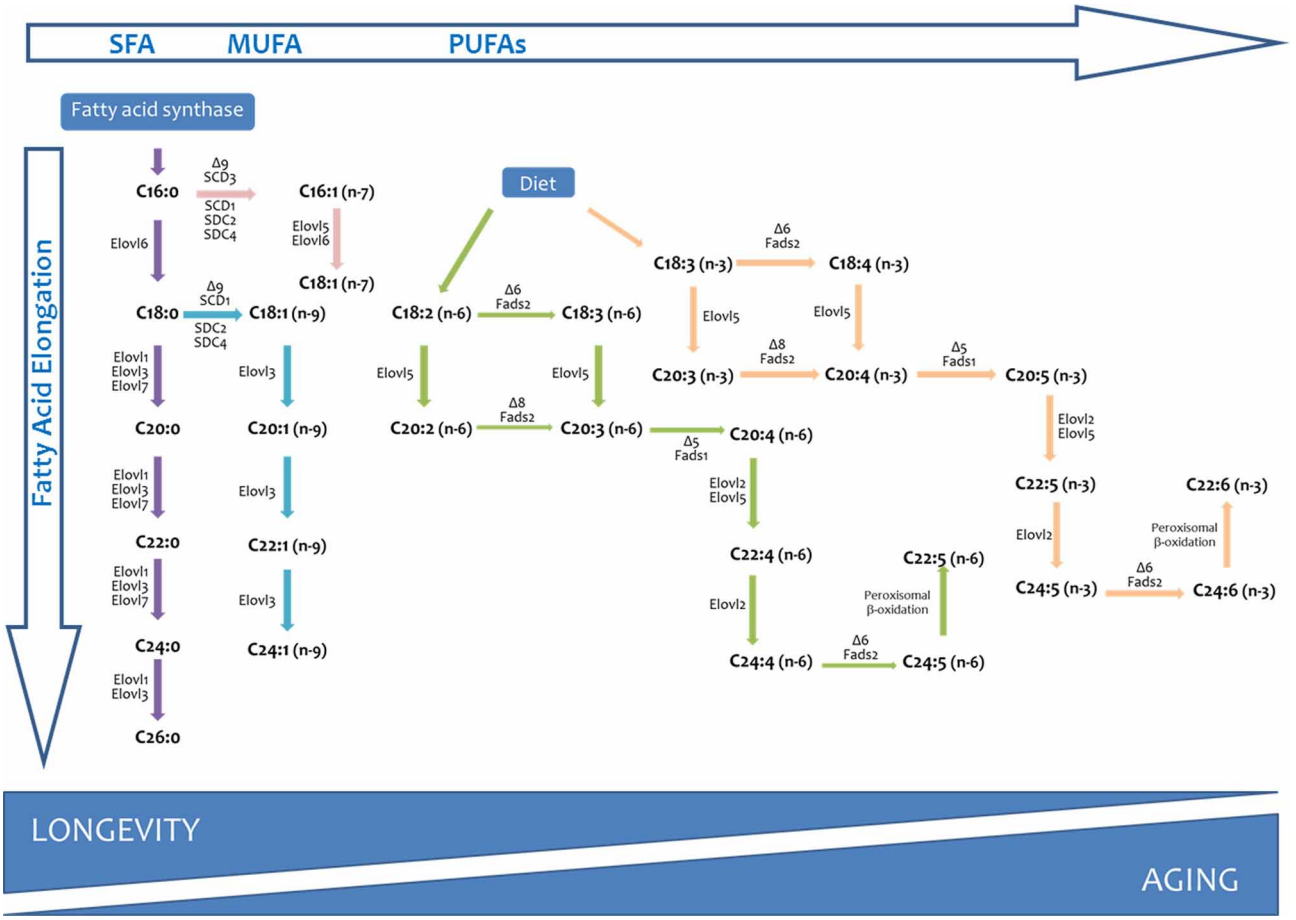

FIGURE 2 | Long chain and very long-chain fatty acid biosynthesis in vertebrates. The long chain saturated fatty acids and unsaturated fatty acids of the *n*-7 and *n*-9 series can be synthesized from palmitic acid (C16:0) produced by the fatty acid synthase (FAS). Long-chain fatty acids of the *n*-6 and *n*-3 series can only be synthesized from precursors obtained from dietary precursors (DIET). *Elovl*, elongation of very long chain fatty acids (fatty acid elongase); Fads, fatty acid desaturase.

in their chemical, nonenzymatic, and irreversible modification and formation of a variety of adducts and crosslinks collectively named Advanced Lipoxidation Endproducts (ALEs) (Thorpe and Baynes, 2003; Pamplona, 2011).

Consequently, 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. The regulatory function and cytotoxicity of the lipid peroxidation-derived aldehydes hinges on its abundance, reactivity, and half-life. It is plausible to postulate that a low degree of fatty acid unsaturation in cellular membranes could be advantageous by decreasing their sensitivity to lipid peroxidation. This would also protect other molecules against lipoxidation-derived damage. In other words, it is proposed that membrane unsaturation acts as a structural adaptive system and it is related to the animal longevity.

MEMBRANE UNSATURATION AND ANIMAL LONGEVITY

Findings from different experimental paradigms recently link membrane unsaturation and lipoxidation-derived molecular damage to longevity: (i) changes that occur in individuals during aging; (ii) different maximum longevity that are characteristic of species and the different longevity of strains and specific mutants within species; and (iii) physiological treatments that alter rate of aging and thus longevity.

IS AGING DUE TO INCREASE OF MEMBRANE UNSATURATION AND LIPOXIDATION REACTIONS?

It has now been documented that there is an age-associated increase in membrane PI and lipoxidation-derived molecular damage (see **Table 1**). In general, PI increases during aging in an organ-dependent way. This is mainly due to decreases in the less unsaturated linoleic (LA, 18:2*n*-6) and linolenic (LNA, 18:3*n*-3) acids and to increases in the highly unsaturated arachidonic acid (AA, 20:4*n*-6), and docosotetra- penta- and –hexaenoic [22:4*n*-3, 22:5*n*-3, and 22:6*n*-3 (DHA), respectively] acids. In a similar way, the lipoxidative damage, which has been measured in tissue homogenates and mitochondria, also increases with age. Tissues that are composed of long-lived, postmitotic cells, such as the brain, heart, and skeletal muscle, tend to accrue

Table 1 | Effect of aging on membrane peroxidizability index and lipoxidation-derived molecular damage in tissues from different species.

Tissue	Species	Age(s)	Change with aging in Peroxidizability Index (PI)	Change with aging in Lipoxidation-derived molecular damage	References
Whole	*Drosophila*	From 10 to 50 days	Increase	Increase	Magwere et al., 2006
Whole	*Drosophila*	From 5 to 40 days	Increase	Increase	Jacobson et al., 2010
Brain	Mouse	6 vs. 24 months	Increase	Increase	Arranz et al., 2013
Spleen	Mouse	6 vs. 24 months	Increase	Increase	Arranz et al., 2013
Heart	Rat	8 vs. 30 months	Increase	Increase	Ayala et al., 2006
Liver	Rat	8 vs. 30 months	Increase	Increase	Ayala et al., 2006
Liver mitochondria	Rat	6, 18, 28 months	Increase	Increase	Lambert et al., 2004
Liver microsomes and mitochondria	Rat	6, 12, 24 months	Increase	n.d.	Laganiere and Yu, 1993
Erythrocyte membranes	Human	From 20 to 90 years	Increase	n.d.	Rabini et al., 2002

n.d., not determined.

relatively greater amounts of damage than those composed of short-lived non-mitotic cells. In this line, lipofuscin, a complex age-pigment derived from lipoxidation reactions considered a hallmark of aging, also shows an accumulation that correlates with age (Tsuchida et al., 1987; Terman and Brunk, 2004).

The singular importance of membrane unsaturation in the aging process is highlighted by studies showing age-related changes in membrane physico-chemical properties. During aging, the membrane fatty acid profile changes with increased peroxidizable PUFAs (see **Table 1**). PUFAs have lower melting points than SFAs, and consequently, a relative increase in the PUFA content of a membrane would be expected to render the membrane more fluid. Based solely on the fatty acid composition, membranes from older animals should exhibit greater fluidity than those from younger individuals. Paradoxically, the opposite is systematicaly reported; membrane fluidity decreases with age (reviewed in Hulbert et al., 2007). The reasons for the changes of membrane fluidity can be explained in two ways: (1) a change caused by fatty acid chain composition and (2) a change in the cholesterol content of the membrane. The paradox described above is explained by considering that cellular components undergo increased oxidative damage with time. Because PUFAs are more vulnerable to oxidative attack, they experience greater lipid oxidative damage, and the resulting RCSs have been shown to contribute significantly to membrane rigidity and loss of its function (Laganiere and Yu, 1987, 1993; Yu et al., 1992; Yu, 2005; Naudi et al., 2013). A special mention to the recent indications that membrane microdomains (i.e., rafts) also undergo an aging process not necessarily identical to that in the bulk membrane (given the differential biochemical composition between raft and non-raft domains) (Tomoiu et al., 2007; Ohno-Iwashita et al., 2010; Fabelo et al., 2012). These observations apparently correlate with increased membrane microviscosity that parallels changes in both PUFA and cholesterol (which would have opposed effects), and very interestingly, they appear to be accelerated by neurodegenerative processes.

Studies on possible causes of age-associated increase in membrane unsaturation (see **Table 1**) indicated that activities of some of the desaturase and elongase enzymes that participate in the PUFA biosynthesis pathway, as well as the peroxisomal beta-oxidation pathway increase with age suggesting the presence of a generalizable pattern. The reasons (dysfunctionality or adaptation?) for the increase in these enzymatic activities during aging remain unknown. In any case, this pattern favors a high age-associated membrane unsaturation that, in turn, is highly susceptible to lipid peroxidation (independently of the presence or absence of an increase in age-associated ROS production, or defects in molecular removal or repair) and to the induction of a high steady-state level of lipoxidation derived molecular damage. Therefore, the available data tend to favor the view that the increase in membrane unsaturation is a key factor responsible for age-related accrual of molecular lipoxidative damage.

ARE INTERSPECIES VARIATIONS IN LONGEVITY RELATED TO CORRESPONDING DIFFERENCES IN MEMBRANE UNSATURATION AND LIPOXIDATION-DERIVED MOLECULAR DAMAGE?

The first indication of a connection between membrane fatty acid composition and maximum longevity was the report by Pamplona et al. (1996) which demonstrated that the PI of liver mitochondria from rats, pigeons and humans was strongly correlated with their respective longevity. Later, it was shown that this was the case for a wide range of tissues and animal species including mammals, birds, insects and crustaceans. Unfortunately, no data are currenly available for reptiles, amphibians, fishes, and many invertebrates.

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 and PI than short-lived ones (**Table 2**). In agreement with this, it was demonstrated that in long-lived animal species a low degree of total tissue and mitochondrial fatty acid unsaturation was 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 skeletal muscle, heart, liver, and brain (Pamplona et al., 2002a,b,c; Pamplona, 2008; Pamplona and Barja, 2011). Reinforcing this idea of low lipoxidative damage in long-lived species, lipofuscin also showed an accumulation rate that inversely correlates with longevity (Terman and Brunk, 2004). These findings were consistent with the negative correlation previously observed between longevity

Table 2 | Comparative studies between membrane unsaturation (peroxidizability index, PI) and longevity in animal species (by chronological order).

Species compared	Organ	PI long-lived species	References
Rat-Pigeon-Human	Liver mitochondria	Lower	Pamplona et al., 1996
SAM-R/1 vs. SAM-P/1 mice	Liver	Lower	Park et al., 1996
8 mammalian species	Liver mitochondria	Lower	Pamplona et al., 1998
Rat vs. pigeon	Heart mitochondria	Lower	Pamplona et al., 1999a,b
Mouse vs. canary	Heart	Lower	Pamplona et al., 1999a,b
Mouse vs. parakeet	Heart	Lower	Pamplona et al., 1999a,b
Rat vs. pigeon	Liver mitochondria	Lower	Gutiérrez et al., 2000
Rat vs. pigeon	Heart mitochondria and microsomes	Lower	Gutiérrez et al., 2000
8 mammalian species	Heart	Lower	Pamplona et al., 2000a,b
7 mammalian species	Liver	Lower	Pamplona et al., 2000a,b
8 mammalian species	Liver mitochondria	Lower	Herrero et al., 2001
Rat vs. pigeon	Skeletal muscle	Lower	Portero-Otín et al., 2004
Mouse, parakeet, canary	Brain	Lower	Pamplona et al., 2005
8 mammalian species	Heart	Lower	Ruiz et al., 2005
Strains of mice (Idaho, Majuro, and WT)	Skeletal muscle and liver	Lower	Hulbert et al., 2006a,b
Naked-mole rat vs. mouse	Skeletal muscle mitochondria and Liver mitochondria	Lower	Hulbert et al., 2006a,b
12 mammalian species and 9 bird species	Skeletal muscle	Lower	Hulbert et al., 2007
10 mammalian species and 8 bird species	Liver mitochondria	Lower	Hulbert et al., 2007
Queen honey bees vs. workers	Head, thorax, abdomen	Lower	Haddad et al., 2007
42 mammalian species	Skeletal muscle	Lower*	Valencak and Ruf, 2007
13 bird species	Heart	Lower	Buttemer et al., 2008
Echidna vs. mammals	Liver, liver mitochondria, and Skeletal muscle	Lower	Hulbert et al., 2008
Humans (offspring of nonagenarians vs. control)	Erythrocytes	Lower	Puca et al., 2008
D. melanogaster (long-lived mutant strains)	Whole organism and mitochondria	Lower	Sanz et al., 2010
C. elegans (long-lived mutant strains)	Whole organism	Lower	Shmookler Reis et al., 2011
Rat vs. pigeon	Erythrocytes, heart, kidney, liver, skeletal muscle (whole tissue and mitochondria)	Lower	Montgomery et al., 2011
5 marine molluscs species	Whole mitochondria	Lower	Munro and Blier, 2012
Exceptionally-old mice	Brain, spleen	Lower	Arranz et al., 2013
Humans (Middle aged offspring of nonagenarians vs. control group)	Plasma	Lower	Gonzalez-Covarrubias et al., 2013
Long-lived vs. short-live mouse (*P. leucopus* vs. *M. musculus*)	Skeletal muscle mitochondria	Lower	Shi et al., 2013
Wild-type vs. long-lived Ames dwarf mice	Skeletal muscle, heart, liver, mtLiver, brain	Lower**	Valencak and Ruf, 2013
11 mammalian species	Plasma	Lower	Jové et al., 2013a,b
D. melanogaster (wild-type strains)	Whole organism and mitochondria	Lower	Naudi et al., 2013

*Results obtained after correction for body weight and phylogeny showed that longevity decreases as the ratio of n-3 to n-6 PUFAs increases. No relation between longevity and PI was found; **No significant differences were observed for brain.

and the sensitivity to lipid autoxidation of mammalian kidney and brain homogenates (Cutler, 1985).

All these observations made at cell-tissue level can be interestingly extended to plasma lipids. Thus, in a recent study (Jové et al., 2013a,b) the plasma lipidomic profile by using high-throughput lipidome profiling technologies of 11 mammalian species ranging in maximum longevity from 3.5 to 120 years was determined. Using a non-targeted approach about 14,000 lipid species in plasma was detected, and the multivariate analyses separated perfectly 11 groups, indicating a specific signature for each animal

species which accurately predicts animal longevity. Regression analysis between lipid species and longevity revealed that long-chain free fatty acid concentrations, PI, and lipid peroxidation-derived products correlated in a specific and significant way. Thus, the greater the longevity of a species, the lower is its plasma long-chain free fatty acid concentration, peroxidizability, and lipid peroxidation-derived products content, suggesting that the lipidomic signature is an optimized feature associated with animal longevity.

While longevity can differ dramatically between mammal and bird species, there can also be significant longevity differences within a species. Thus, 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 (Miller et al., 2002). The PI of both skeletal muscle and liver phospholipids of the two wild-type mice strains with the extended longevity was significantly smaller than that of the laboratory mice (Hulbert et al., 2006a,b). This is notable because, since the different mice strains were fed the identical diet, it shows that the differences in membrane fatty acid composition between species are not determined by dietary differences but is genetically regulated. In a similar way, in the senescence-accelerated mouse (SAM) strain, the SAM-prone mice had greater levels of the highly polyunsaturated peroxidation-prone fatty acids 22:6 *n*-3 and 20:4 *n*-6 and lower levels of the more peroxidation-resistant 18:2 *n*-6 PUFA in their membranes, and consequently had a greater PI than the SAM-resistant mice (Park et al., 1996). SAM-prone mice also showed greater degrees of lipid peroxides in their tissues than do SAM-resistant mice.

In this context, it is also of great interest to know and discern the degree of membrane unsaturation and the steady-state levels of lipoxidative damage in physiological systems from exceptionally long-lived specimens. Thus, in a recent work (Arranz et al., 2013), adult (28 weeks), old (76 weeks), and exceptionally old (128 weeks) BALB/c female mice were used. Brain and spleen were analyzed for membrane fatty acid composition and markers of lipoxidative molecular damage. The results showed significantly lower PI and lipoxidation-derived protein damage in brain and spleen from exceptionally old animals when compared to old specimens, and in a range analogous to adult animals. Therefore, low susceptibility to lipid peroxidation and maintenance of adult-like molecular lipoxidative damage could be key factors for longevity achievement.

Comparing biological processes in closely-related species with divergent longevity can also be a powerful approach to study mechanisms of longevity. Thus, the skeletal muscle mitochondria from long-lived white-footed mouse *Peromyscus leucopus* (MLSP = 8 years) display lower levels of isoprostanes (lipid peroxidation-derived compounds) than the common laboratory mouse, *Mus musculus* (MLSP = 3.5 years) (Shi et al., 2013).

Two exceptionally long-living mammalian species (naked mole-rats and echidnas) also have membrane fatty acid profiles that are resistant to lipid peroxidation as one would predict from their longevities. Thus, when membrane fatty acid composition was measured in tissues from naked mole-rats, the longest-living rodents known with a recorded longevity exceeding 28 years (Buffenstein, 2005), it was found that they have very low levels of 22:6*n*-3 in their tissue phospholipids compared to mice. Although both mice and naked mole-rats have similar levels of total UFAs in their tissue phospholipids, the low 22:6*n*-3 levels of the naked mole-rats result in lower PI and more peroxidation-resistant membranes in skeletal muscle and liver mitochondria (Hulbert et al., 2006a,b; Mitchell et al., 2007). In a similar way, the echidna *Tachyglossus aculeatus*, a monotreme mammal from Australia that is exceptionally long-living with a documented longevity of 50 years, also had a membrane composition resistant to lipid peroxidation (Hulbert et al., 2008). Accordingly, membrane lipids of echidna tissues (skeletal muscle, liver, and liver mitochondria) were found to have a lower content of PUFAs and a higher content of MUFAs, resulting in a low PI and, consequently, indicating that the cellular membranes of echidnas is peroxidation-resistant (Hulbert et al., 2008).

Honeybees (*Apis mellifera*) and flyes (*D. melanogaster*) provide another example of variation in longevity within a species that extend previous findings in vertebrates to invertebrates. In the honey bee, depending on what they are fed, female eggs become either workers or queens (Winston, 1987). Hence, queens and workers share a common genome. However, the longevity of queens is an order-of-magnitude greater than that of workers. In order to test if differences in membrane composition could be involved the fatty acid composition of phospholipids of queen and worker honey bees were compared (Haddad et al., 2007). The cell membranes of both young and old honey bee queens were highly monounsaturated with very low content of PUFAs. Newly emerged workers show a similar membrane fatty acid composition to queens but within the first week of hive life, they increase the polyunsaturate and decrease the monounsaturate lipid content of their membranes, possibly due to the activation of a genetic program and metabolic reprogrammation resulting from pollen consumption. This means that their membranes likely become more susceptible to lipid peroxidation in this first week of hive life. So, the results again support the suggestion that membrane composition might be an important factor in the determination of longevity. In another approach, these predictions have also been tested in a comparison among three wild type strains of *D. melanogaster* differing in their longevities (a long-lived strain: Oregon R, and two short-lived strains: Canton S and Dahomey). The results also confirm the presence of an inverse correlation between membrane unsaturation and lipoxidation-derived molecular damage and longevity. So, the greater the longevity of the *Drosophila* strain, the lower is the membrane unsaturation (Naudi et al., 2013).

Recent studies show that bivalves are excellent models for longevity research (Abele et al., 2009; Philipp and Abele, 2010). This taxonomic group includes the longest-living non-colonial metazoan (the Iceland clam *Arctica islandica*, MLSP = 507 years; Wanamaker et al., 2008), as well as surf clams (= Family Donacidae) with species of no more than 1 year longevity. Two traits make bivalves excellent models for longevity research: first, bivalves from temperate and cold-water environments can be accurately aged by counting their annual shell growth rings. This makes it possible to relate physiological state to chronological age in wild populations. Secondly, bivalve molluscs are genetically

intermediate to classical invertebrate models of longevity (e.g., worms and flies) and mammals. This provides an opportunity to study the evolution of oxidative stress response pathways and animal longevity (Austad, 2009; Philipp and Abele, 2010). In this scenario, a recent study (Munro and Blier, 2012) analyzed the possible existence of a PI vs. longevity relationship by comparing the phospholipid fatty acid composition from mitochondrial membranes and other cell membranes of the longest-living metazoan species (*Arctica islandica*, 507 years) to four other sympatric bivalve molluscs also differing in their longevities (28, 37, 92, and 106 years, respectively). The results certified that long-lived marine bivalves possess peroxidation-resistant membranes. Indeed there is a significant inverse correlation between PI and longevity, analogously to the described findings from vertebrates (mammals and birds) (Hulbert et al., 2007).

A final specific comment concerning humans, as exceptionally long-lived species, is also pertinent. The findings from a recent work centered in offspring of long-lived individuals again seem to reinforce the association between membrane unsaturation and longevity. Thus, the fatty acid composition and PI of erythrocyte membranes from 41 nonagenarian offspring were compared with 30 matched controls (Puca et al., 2008). The results of this study demonstrated a lower PI in the lipid composition of erythrocyte membranes derived from nonagenarian offspring versus matched controls. This is indicative of reduced susceptibility to oxidative stress and increased membrane integrity at the cellular level for nonagenarian offspring compared with the general population under investigation. In this context, it should be plausible to infer that lipid composition of erythrocyte membranes could represent a useful biomarker of longevity. Finally, with the idea to investigate which specific lipids associate with familial longevity, another study (Gonzalez-Covarrubias et al., 2013) has explored the plasma lipidome in 1526 middle-aged offspring of nonagenarians (59 years) and 675 (59 years) controls from the Leiden Longevity Study. In men, no significant differences were observed between offspring and controls; whereas in women, 19 lipid species were related to familial longevity. More interestingly, the longevity-linked lipidomic profile showed by female offspring expressed a higher ratio of MUFA over PUFA lipid species revealing a plasma lipidome more resistant to oxidative stress.

Overall, all these comparisons (a) support an important role for membrane fatty acid composition in the determination of longevity, (b) reinforce the idea that the connection between membrane unsaturation and longevity is not restricted to vertebrates, and (c) suggest that membrane composition is regulated in a species-specific way.

ARE EXPERIMENTAL EXTENSIONS IN LONGEVITY BY GENETIC MANIPULATIONS ACCOMPANIED BY ATTENUATIONS OF MEMBRANE UNSATURATION AND LIPOXIDATION-DERIVED MOLECULAR DAMAGE?

The relevance of membrane unsaturation in determining longevity has also been recently reinforced by using Drosophila as an experimental model (Sanz et al., 2010). In this study, transgenic strains of Drosophila that express yeast NDI1ubiquitously were created (in yeast, the single-subunit NADH dehydrogenase Ndi1 serves as a non-proton-translocating alternative enzyme that replaces complex I, bringing about the reoxidation of intramitochondrial NADH). NDI1 expression bring about a decreased accumulation of lipoxidation-derived damage markers, accompanied by a reduced rate of ROS production and restored bioenergetic state, resulting in an increased longevity. This lower lipoxidation-derived damage in the long-lived strains is also linked to an adaptive response with a low degree in membrane unsaturation (Naudi et al., 2013).

In *C. elegans* as experimental model (Hulbert, 2011; Shmookler Reis et al., 2011), it was analyzed the fatty acid profile of lipids extracted from strains of *C. elegans* that vary in longevity by ∼10-fold, and display several significant log-linear correlations between longevity and fatty acid composition. The results—strongly influenced by two mutant strains (daf-2 and age-1, both long-lived mutant strains linked to a dysruption of the insulin like-signaling pathway) that showed the greatest longevities—demonstrated that comparing the shortest-living with longest-living strains total MUFAs increased from 34 to 48%, total PUFAs decreased from 37 to 26%, and PI decreased from 141 to 81. All together, these findings suggest that mutations leading to higher longevities require of, at least, an adaptation of the degree of membrane unsaturation.

Functional assays, using RNAi to attenuate gene expression, might provide evidence that such genes (e.g., for desaturase or detoxifying enzymes) play causal roles in enhancing longevity. Longevity extensions permit stronger inferences than its decrease, due to the many ways longevity could be shortened upon disruption of any pathway that contributes to survival. In this context, two studies made on *C. elegans* related to the PUFA biosynthesis and detoxifiying enzymes for RCS deserve a special mention.

As mentioned above, RCS are compounds produced under oxidative stress conditions. These compounds are detoxified in multiple ways, including conjugation to glutathione, oxidation by aldehyde dehydrogenases, or reduction by aldoketoreductases (Pamplona, 2008). Reaction of RCS with gluthathione can proceed in one of two ways: by nonenzymatic conjugation or through glutathione transferase (GST)-mediated conjugation to form Michael adducts. GSTs belong to a supergene family of multifunctional enzymes, which are particularly involved in the detoxification of highly reactive aldehydes (Sheehan et al., 2001). Interestingly, protection against oxidative stress is the major driver of positive selection in mammalian GSTs, explaining the overall expansion pattern of this enzymes' family. The biological relevance of this protein-enzyme family is highlighted by studies in *C. elegans* demonstrating that interference with the expression of these enzymes significantly shortens the longevity of the organism and increases the formation of lipoxidation-derived protein adducts (Ayyadevara et al., 2007), whereas the overexpression of GSTs increases the longevity (Ayyadevara et al., 2005). Consequently, in this approach, the relationship between longevity and the expression of the detoxifiying enzymes indicates that the substrate of that enzyme, reactive carbonyl compounds, may be causally involved in limiting longevity. In the other approach, *C. elegans* benefits from RNAi suppression of genes encoding either of two elongases or a delta-5 desaturase, fat-4, whereas knochdowns of delta-9

desaturase genes can slightly reduce longevity (Shmookler Reis et al., 2011). Taken together, these functional data imply that the modulation of fatty acid composition to increase resistance to lipid peroxidation is one of the mechanisms for longevity extension.

ARE EXPERIMENTAL EXTENSIONS IN LONGEVITY BY NUTRITIONAL INTERVENTIONS 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 the determination of longevity. In order to clarify whether the low membrane unsaturation of long-lived animals protects their cellular components from lipid oxidation and lipoxidation-derived molecular damage, studies of experimental dietary modification of *in vivo* membrane fatty acid unsaturation have been performed (Herrero et al., 2001; Portero-Otin et al., 2003; Pamplona et al., 2004). 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 demonstrate that lowering the membrane unsaturation of cellular membranes protects tissues against lipid peroxidation and lipoxidation-derived macromolecular damage.

Available evidences in favor for a relationship between membrane unsaturation and longevity proceed from nutritional interventions that extend longevity in experimental models. So, caloric (CR), as well as protein (PR) and methionine (MetR) restriction attenuates age-related changes in the degree of membrane unsaturation and the level of lipoxidation products in a variety of tissues and animal species (Yu, 2005; Pamplona and Barja, 2006, 2011; Jové et al., 2013a,b). Thus, a decrease in membrane unsaturation, lipid peroxidation and lipoxidation-derived damage has been reported in tissues like liver, heart, and brain from these dietary restrictions in rats and mice (Laganiere and Yu, 1987; Pamplona et al., 2002a,b,c; Lambert et al., 2004; Sanz et al., 2005, 2006; Ayala et al., 2007; Gómez et al., 2007; Naudí et al., 2007; Caro et al., 2008, 2009; Jové et al., 2013a,b). CR has also been shown to reduce levels of lipofuscin in tissues of rodents and *C. elegans* (Enesco and Kruk, 1981; De et al., 1983; Rao et al., 1990; Terman and Brunk, 2004; Gerstbrein et al., 2005).

From these studies it can be inferred that the magnitude of the change is modest for membrane unsaturation (between 2.5–10%) compared to that for the lipoxidation-derived molecular damage (between 20–40%) likely due to the added effect of the lower mitochondrial ROS generation also induced by these nutritional interventions. In addition to the moderate but significant effect on membrane unsaturation, these nutritional interventions show an effect directly related to the intensity of the dietary restriction applied, being both PR and MetR even more intense and effective that CR. It is suggested from available data that 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 PI 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, PI and lipoxidation-derived 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.

Very valuable information could be also obtained from studies designed to modify membrane fatty acid composition in order to increase membrane unsaturation and to evaluate its impact in animal longevity. Thus, in a recent study (Tsuduki et al., 2011) the influence of long-term ingestion of fish oil (a PUFAn-3 rich oil) on lipid oxidation and longevity was examined in senescence-accelerated (SAMP8) mice. Male mice were fed a fish oil diet (5% fish oil and 5% safflower oil) or a safflower oil diet (10% safflower oil) from 12 weeks of age. The SAMP8 mice fed fish oil showed a significantly reduced longevity, in association with a higher lipid peroxidation, when compared to mice fed safflower oil.

Reinforcing this outcome, there is also a reduction in *C. elegans* longevity with the addition of PUFAs to their diet (Shmookler Reis et al., 2011). Thus, adult worms were maintained on agar plates spotted with *E. coli* (strain OP50). Control plates were unsupplemented, while treatment plates contained either palmitic acid (16:0) or eicosapentaenoic acid (20:5*n*-3). The results showed that longevity in the presence of 20:5*n*-3 was reduced 20% relative to unaugmented controls, and by 16% relative to worms supplemented with 16:0. In an independent experiment, supplementation with 22:6*n*-3 produced similar reductions in survival, 24% relative to untreated controls and 15% relative to palmitic acid. In contrast to these results, in another study (Hillyard and German, 2009) it is described a beneficial effect of these molecules (20:5*n*-3 and 22:6*n*-3) when added to the short-lived mutant *fat-3*, which lacks a functional delta-6 desaturase, and thus PUFAs including 20:5*n*-3.

MECHANISM RESPONSIBLE FOR THE LONGEVITY-RELATED DIFFERENCES IN MEMBRANE UNSATURATION

The low PI observed in long-lived species are due to changes in the type of unsaturated fatty acid that participates in membrane composition (Pamplona et al., 2002a,b,c; Hulbert et al., 2007). Globally, there is a systematic redistribution between the types of PUFAs present from highly unsaturated fatty acids such as 22:6*n*-3, 20:5*n*-3, and 20:4*n*-6 in short-lived animals to the less unsaturated 18:3*n*-3, 18:2*n*-6, and 18:1 in the long-lived ones, at mitochondrial and tissue level. Furthermore, the PI of the respective diets did not correlate with longevity. This indicates again that the contribution of the variations in the degree of unsaturation of dietary fats to the inter-species differences is, if any, very modest.

The mechanisms responsible for the longevity-related differences in fatty acid profile can be related, in principle, to the PUFAs biosynthesis pathway (including desaturases, elongases, and peroxisomal beta-oxidation; see **Figure 1**), and the deacylation-reacylation cycle. The available estimates of delta-5 and delta-6

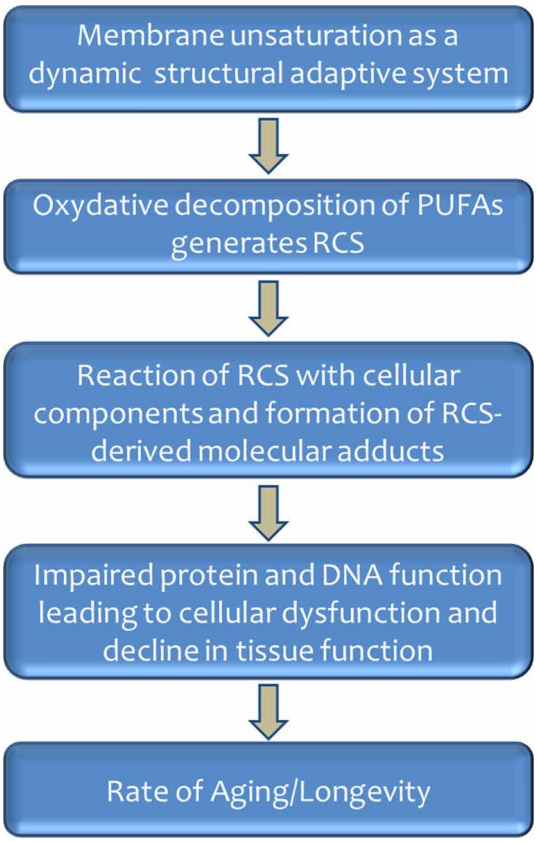

FIGURE 3 | Role of membrane unsaturation and lipoxidation-derived molecular damage in aging and longevity.

desaturase activities, as well as different elongase activities, indicate that they are several folds lower in long-lived species than in short-lived ones (Pamplona et al., 2002a,b,c; Pamplona and Barja, 2003; Hulbert et al., 2007; Shmookler Reis et al., 2011). This can explain why e.g., 22:6*n*-3 and 20:4*n*-6 decreases and 18:2*n*-6 and 18:3*n*-3 increases, from short-to long-lived animals, since elongases/desaturases are the rate-limiting enzymes of the *n*-3 and *n*-6 pathways synthesizing the highly unsaturated PUFAs 20:4*n*-6 and 22:6*n*-3 from their dietary precursors, 18:2*n*-6 and 18:3*n*-3, respectively. Thus, elongation-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. In addition, a relevant role for peroxisomal beta-oxidation, a metabolic pathway key for the obtention of 20:5*n*-6 and 22:6*n*-3 should be also considered to the light of recent results.

In accordance with this interpretation, a recent study (Jobson et al., 2010) with a phylogenomic approach to identify the genetic targets of natural selection for extended longevity in mammals has been published. The premise of this work is that genes preventing high rates of aging should be under stronger selective pressure in long-lived species, relative to short-lived ones. In other words, long-lived species should allow identifying a stronger level of amino acid conservation from specific genes than short-lived ones. The results obtained by comparing the nonsynonymous and synonymous evolution of 5.7 million codon sites across 25 species proved that genes involved in fatty acid biosynthesis (elongases, desaturases, and lipoxidation repair), as well as extracellular collagen composition, have collectively undergone increased selective pressure in long-lived species, whereas genes involved in DNA replication/repair or antioxidants, among others, have not.

More investigations are, however, needed to confirm the role of these metabolic pathways in aging and longevity, and to extend them to the potential role of regulatory and transcriptional factors involved in PUFA metabolism.

THE BIOLOGICAL MEMBRANE AS DYNAMIC STRUCTURAL ADAPTIVE SYSTEM

Animals with a high longevity have a low degree of membrane fatty acid unsaturation based on the redistribution between types of PUFAs. 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 e.g., 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 (Brenner, 1984). This is so because the kink has a larger impact on fluidity when the double bond is situated near the center 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, PI increase irrespective of the double bond location at the center or laterally on the fatty acids (Holman, 1954). Thus, by switching fatty acids with four or six double bonds by those having only one, two (or even 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 termed *homeoviscous longevity adaptation* (Pamplona et al., 2002a,b,c), and displays the biological membranes as a dynamic structural adaptive system (see **Figure 3**).

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Melatonin reduces lipid peroxidation and membrane viscosity

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INTRODUCTION

Lipid peroxidation (LPO) occurs as a result of the oxidative deterioration of polyunsaturated fatty acids (PUFA), i.e., those that contain two or more carbon–carbon double bonds. The most apparent feature of the oxidative breakdown of lipids is rancidity, a problem that was recognized centuries ago during the storage of fats and oils. Rancidity persists as a widespread problem in today's society because of the common use of polyunsaturated fats and oils.

The outer limiting membrane of cells and membranes of subcellular organelles, e.g., mitochondria, liposomes, peroxisomes, etc., are generally rich in PUFA and their protection from oxidation is essential for the optimal function and survival of the cell. In addition to lipids, cell membranes also contain proteins in varying amounts depending on the unique physiology of the membrane. Thus, the inner mitochondrial membrane, because of its high density of respiratory complex proteins, contains only 20% lipids; this is also the case with chloroplast thylakoid membranes. In contrast, the myelin sheath surrounding axons are up to 80% lipid. Due to the differences in the percentage of lipids in membranes, they are subjected to different degrees of peroxidation.

Membranes are fluid structures and optimal membrane fluidity is required for their proper function. When membrane fatty acids are oxidized, cell membranes become viscous (more rigid). Many factors contribute to the oxidation of membrane lipids and, during aging, cell membranes become progressively more rancid and rigid; this contributes to the degenerative signs of aging.

The oxidation of lipids is a highly complex process that is initiated when a hydrogen atom is abstracted from a methylene ($–CH_2–$) group by a free radical (**Figure 1**). PUFA are particularly susceptible to peroxidative initiation because of their numerous carbon–carbon double bonds. Of the free radicals and other reactive oxygen (ROS) and reactive nitrogen species (RNS) generated within cells, the hydroxyl radical ($•OH$) is easily capable of initiating LPO. In contrast, the superoxide anion radical ($O_2•–$) is not sufficiently reactive to abstract a hydrogen atom from a lipid molecule. As a consequence of the initiation of lipid breakdown, a lipid peroxyl radical ($ROO•$) is eventually generated. $ROO•$ are highly reactive and are capable of abstracting a hydrogen atom from a neighboring lipid (causing another initiation event). This is referred to as the propagation phase of LPO. Due to this auto-oxidative chain reaction, a single initiation event could theoretically lead to the oxidation of all lipids in a cellular organelle, or in a cell. Other reactive species which initiate LPO include peroxynitrite anion ($ONOO^-$) and singlet oxygen ($^1O_2$). Because of the highly destructive structural and functional nature of LPO, there is great interest in identifying molecules which reduce the initiation and/or progression of the denaturation of PUFA.

MELATONIN AND ITS DERIVATIVES AS ANTIOXIDANTS

What has come to be known as melatonin's antioxidant cascade accounts, presumably in large part, for its ability to reduce oxidative damage, including that to PUFA (Tan et al., 2007). When melatonin functions in the detoxification of radicals, the metabolites that are formed are also radical scavengers. The initial derivative that is produced is cyclic 3-hydroxymelatonin (c3OHM) (**Figure 1**). This derivative functions as a radical scavenger to generate N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) which, like its predecessor, neutralizes toxic ROS/RNS. In doing so AFMK is metabolized to N1-acetyl-5-methoxykynuramine (AMK). AMK likewise is capable of defeating radicals and beyond this there may yet be other derivatives that function as antioxidants.

Via this cascade of reactions, each molecule of melatonin is predicted to scavenge up to 10 ROS/RNS. This unique property of melatonin makes it highly effective in combatting oxidative stress and LPO. Melatonin is produced in many cells and its synthesis may be upregulated under conditions that elevate oxidative stress in mammals, as happens in plants (Arnao and Hernandez-Ruiz, 2013).

Besides its direct actions as a scavenger, melatonin also stimulates the activities of a variety of antioxidative enzymes including manganese and copper-zinc superoxide dismutase, glutathione peroxidase and reductase and glutamylcysteine ligase (Rodriguez et al., 2004). The combined

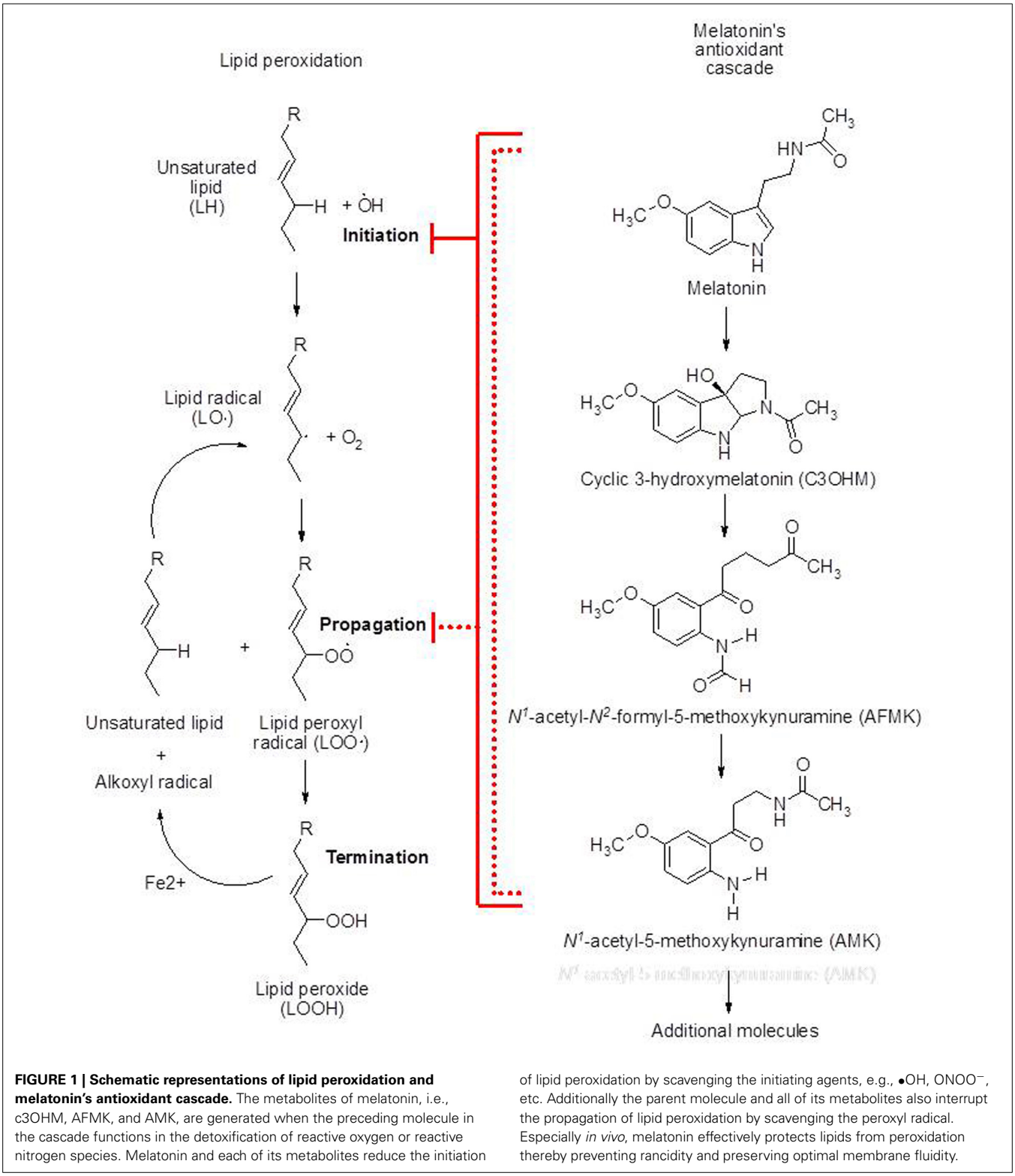

FIGURE 1 | Schematic representations of lipid peroxidation and melatonin's antioxidant cascade. The metabolites of melatonin, i.e., c3OHM, AFMK, and AMK, are generated when the preceding molecule in the cascade functions in the detoxification of reactive oxygen or reactive nitrogen species. Melatonin and each of its metabolites reduce the initiation of lipid peroxidation by scavenging the initiating agents, e.g., •OH, $ONOO^-$, etc. Additionally the parent molecule and all of its metabolites also interrupt the propagation of lipid peroxidation by scavenging the peroxyl radical. Especially *in vivo*, melatonin effectively protects lipids from peroxidation thereby preventing rancidity and preserving optimal membrane fluidity.

actions as well as its function at the inner mitochondrial membrane where it limits electron leakage (called radical avoidance) makes melatonin exceptionally effective in reducing oxidative stress.

MELATONIN: REDUCING LIPID PEROXIDATION

The ability of melatonin to protect against LPO has been repeatedly documented in many animal and plant tissues under numerous oxidizing conditions, e.g., ionizing radiation, heavy metal toxicity, drug metabolism, intense exercise, etc. (Garcia et al., 2014). The precise mechanisms by which melatonin and/or its metabolites

function to limit LPO is not yet established. The mechanistic information that is available has come primarily from studies on lipid vesicles (micells) containing with one or several phospholipids. Data indicate that melatonin is embedded preferentially in a superficial location in membrane lipid layers near the polar heads of these molecules (Ceraulo et al., 1999). Its juxtaposition to the lipid molecules allows melatonin to protect them from the onslaught of free radicals. The lipid protective actions of melatonin have been proven, both *in vitro* and *in vivo*, and in models of numerous diseases. Among subcellular organelles, membranes and mitochondria have the highest intrinsic levels of melatonin and these concentrations are not diminished when blood levels of the indoleamine are depleted (Venegas et al., 2012). Its small molecular size and its amphiphilic properties facilitate melatonin's penetration into subcellular compartments.

While melatonin reduces the initiation of LPO, according to Marshall et al. (1996), it is not considered to be highly effective as a chain breaking antioxidant. This finding is disputed, however, by the data of Mekhloufi et al. (2007) and of Marchetti et al. (2011) who observed melatonin is in fact highly efficient as a LOO• scavenger. Recently, c3OHM and AMK were proposed as highly effective LOO• scavengers. Hence, melatonin as well as its metabolites function as chain breaking antioxidants (**Figure 1**).

In plants, the chloroplast envelope as well as their thylakoids possess a high percentage of PUFA; thus, like mitochondria, they are also readily susceptible to LPO. Moreover, like the inner mitochondrial membrane, the electron transport chain in the thylakoid of chloroplasts leak electrons on to O_2 to generate radical products. Melatonin has been identified in plants where it functions in protecting against oxidative damage (Arnao and Hernández-Ruiz, 2014). The ability of melatonin to protect plant cells from LPO is of special interest since recent data suggests the chloroplasts, like mitochondria, likely produce melatonin (J. Kong et al., unpublished).

Another major contributor to LPO is ONOO–. Like the •OH, it is a powerful initiator of lipid breakdown. Since melatonin also neutralizes the ONOO–, this is another means whereby melatonin may alleviate the decomposition of membrane lipids (Cuzzocrea et al., 1997).

Melatonin also directly scavenges the alkoxyl radical, a product resulting from the transition metal-catalyzed degradation of lipid peroxides (Zavodnik et al., 2006) (**Figure 1**). This is important for the control of LPO since the alkoxyl radical can abstract a hydrogen atom from a PUFA (**Figure 1**); the resulting LOO• can obviously continue the propagation of lipid degradation.

MELATONIN'S DERIVATIVES: REDUCING LIPID PEROXIDATION

Galano et al. (2014) examined the reaction of c3OHM with the •OH and LOO• in both a lipid and aqueous environment by means of Functional Density Theory considering three potential mechanisms of action: radical adduct formation, hydrogen transfer and single electron transfer. Regardless of the polarity of the environment, c3OHM reacted with the •OH at a diffusion controlled rate which was slightly better than that of either melatonin, AFMK or AMK. Against the LOO•, c3OHM was orders of magnitude better than AFMK and AMK and roughly 100-fold better than vitamin E. Although melatonin and its metabolites, AFMK and AMK, are LOO• scavengers, the findings of Galano et al. (2014) indicate that melatonin's ability to resist LPO may also involve its metabolite, c3OHM.

A more direct approach to test c3OHM as a scavenger was taken by Tan et al. (2014). Their results support the conclusion that c3OHM is a highly effective radical scavenger and has particularly high efficacy in protecting molecules that contain haemprotein, e.g., hemoglobin and cytochrome c, from degradation; c3OHM functions by donating a single electron thereby recovering oxidized horseradish peroxidase to its ground state. c3OHM was also found to be a better scavenger of the •OH than vitamin C in recovering oxidized horseradish peroxidase.

Although AFMK provides protection against oxidative stress, there are few studies related to its ability to limit LPO (Galano et al., 2013). AFMK reduces free radical damage to lipids in rat liver homogenates (Tan et al., 2001) while in an *in vivo* study, Manda et al. (2007) reported that the oxidative modification of brain lipids was reduced when mice were treated with melatonin prior to their exposure to ionizing radiation.

Cyclic voltammetry studies found that AFMK is capable of donating two electrons as evidence of its reductive potential. With the aid of electron spin resonance spectroscopy, it was shown that AFMK readily scavenges the •OH which could account for its ability to control LPO. The high efficiency by which AFMK neutralizes the •OH has been confirmed while the data related to the ability of this melatonin metabolite to directly detoxify the LOO• is more limited (Galano, 2011).

While AMK reduces LPO, whether this is due to its direct scavenging ability or a result of its stimulation of antioxidant enzymes has not been determined (Ressmeyer et al., 2003). AMK reportedly scavenges singlet oxygen ($^1O_2$) and nitric oxide (•NO) (Schaffer and Hardeland, 2009). $^1O_2$ can directly react with carbon–carbon lipid double bonds to yield peroxides (Xia et al., 2012). Besides scavenging •NO, melatonin also inhibits its production (Leon et al., 2006), both of which would control LPO, since •NO couples with O_2•– to produce $ONOO^-$, a proven initiator of LPO.

MELATONIN AND MEMBRANE FLUIDITY

Since the degree of lipid breakdown in cell membranes generally correlates with the fluidity of these organelles, it is predicted that melatonin would also reduce membrane rigidity. This has been amply demonstrated. Such findings have significant functional relevance, since limiting the movement of molecules in cell membranes, which increased viscosity does do negatively impacts cellular physiology. Aging is characteristically associated with elevated cell membrane rigidity.

Depressed levels of melatonin naturally occur with aging or as a consequence of pinealectomy leads to elevated and levels of LPO and more viscous cellular membranes (Reiter et al., 1999; Hardeland, 2013). Likewise, treatment of senescence-accelerated prone mice (SAMP8) with melatonin preserves mitochondrial membranes in a more fluid state (Garcia et al., 2014). Membrane fluidity relates to the

degree of LPO; thus, when the fluidity of membranes is reduced, the amount of oxidized lipids in membranes is increased.

CONCLUDING REMARKS

Melatonin is a highly evolutionarily conserved molecule that both directly and indirectly markedly reduces the breakdown of lipids in both animals and plants, especially *in vivo*. What is difficult to determine is whether this protective action is exclusively attributable to its radical scavenging ability or whether it is a consequence of this action by its metabolites c3OHM, AFMK or AMK. It has been difficult to unravel the mechanisms behind melatonin's LPO inhibitory effects since all the metabolites mentioned are formed during melatonin's antioxidant cascade. Finally, melatonin is a well-known stimulator of antioxidative enzymes which would indirectly reduce LPO. Currently, what is known is that both endogenously-generated and exogenously-administered melatonin has an important role in restricting lipid rancidity and preserving optimal membrane fluidity. Also of importance is that neither melatonin nor its metabolites have revealed any pro-oxidant activity in normal cells, a feature that occurs with some classic antioxidants.

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Docosahexaenoic (DHA) modulates phospholipid-hydroperoxide glutathione peroxidase (*Gpx4*) gene expression to ensure self-protection from oxidative damage in hippocampal cells

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Casañas-Sánchez V, Pérez JA, Fabelo N, Quinto-Alemany D and Díaz ML (2015) Docosahexaenoic (DHA) modulates phospholipid-hydroperoxide glutathione peroxidase (Gpx4) gene expression to ensure self-protection from oxidative damage in hippocampal cells. Front. Physiol. 6:203. doi: 10.3389/fphys.2015.00203

Docosahexaenoic acid (DHA, 22:6n-3) is a unique polyunsaturated fatty acid particularly abundant in nerve cell membrane phospholipids. DHA is a pleiotropic molecule that, not only modulates the physicochemical properties and architecture of neuronal plasma membrane, but it is also involved in multiple facets of neuronal biology, from regulation of synaptic function to neuroprotection and modulation of gene expression. As a highly unsaturated fatty acid due to the presence of six double bonds, DHA is susceptible for oxidation, especially in the highly pro-oxidant environment of brain parenchyma. We have recently reported the ability of DHA to regulate the transcriptional program controlling neuronal antioxidant defenses in a hippocampal cell line, especially the glutathione/glutaredoxin system. Within this antioxidant system, DHA was particularly efficient in triggering the upregulation of *Gpx4* gene, which encodes for the nuclear, cytosolic, and mitochondrial isoforms of phospholipid-hydroperoxide glutathione peroxidase (PH-GPx/GPx4), the main enzyme protecting cell membranes against lipid peroxidation and capable to reduce oxidized phospholipids *in situ*. We show here that this novel property of DHA is also significant in the hippocampus of wild-type mice and, to a lesser extent in APP/PS1 transgenic mice, a familial model of Alzheimer's disease. By doing this, DHA stimulates a mechanism to self-protect from oxidative damage even in the neuronal scenario of high aerobic metabolism and in the presence of elevated levels of transition metals, which inevitably favor the generation of reactive oxygen species. Noticeably, DHA also upregulated a *Gpx4* CIRT (Cytoplasmic Intron-sequence Retaining Transcripts), a novel *Gpx4* splicing variant, harboring part of the first intronic region, which according to the "*sentinel RNA hypothesis*" would expand the ability of *Gpx4* (and DHA) to provide neuronal antioxidant defense independently of conventional nuclear splicing in cellular compartments, like dendritic zones, located away from nuclear compartment. We discuss here, the crucial role of this novel transcriptional regulation triggered by DHA in the context of normal and pathological hippocampal cell.

Keywords: docosahexaenoic acid, hippocampal cells, glutathione peroxidase 4, phospholipid-hydroperoxide glutathione peroxidase, transcriptional regulation, intron retention, neuroprotection

Introduction

Docosahexaenoic acid (DHA) is the most abundant n-3 long-chain polyunsaturated fatty acid (LCPUFA) in nerve cells. In fact, brain is the organ containing the largest amount of DHA in the whole organism, and it seems that it was selected soon in the evolution of the cephalization process of vertebrates to provide a special biochemical microenvironment to nerve cell membranes, especially during the massive accretion in the evolution of primate brains (Crawford et al., 1999, 2013; Simopoulos, 2011). DHA is a pleiotropic molecule; it is an essential component of nerve cells membranes where it esterify *sn-2* position of glycerophospholipids (mainly phosphatidylethanolamine and phosphatidylserine, the most abundant phospholipids in nerve cells) and is largely determinant of the structural and physicochemical properties of plasma membrane. Indeed, properties like membrane viscosity, lateral mobility, phase separation and microdomain segregation, conformational transitions and stability of membrane proteins, lipid-protein and protein-protein interactions, all have been shown to be modulated by DHA (Uauy et al., 2001; Stillwell and Wassal, 2003; Díaz et al., 2012, 2015). Besides its structural role, DHA participates in the modulation of neurogenesis, synaptogenesis and neurite outgrowth, refinement of synaptic connectivity, neurotransmitter release, and in memory consolidation processes (Alessandri et al., 2004; Calderon and Kim, 2004; Innis, 2007; Cao et al., 2009; Moriguchi et al., 2013), but also in the activation of signaling pathways for neuronal survival against oxidative and inflammatory cascades (Oster and Pillot, 2010; Bazinet and Layé, 2014). The importance of DHA for brain health is highlighted by the extensive epidemiological and experimental evidence linking its depletion with the development of neurodegenerative diseases (Huang, 2010; Díaz and Marín, 2013).

The Pro-oxidant Environment of Brain and Lipid Peroxidation

Chemically, DHA is a 22 carbon atoms fatty acid containing six double bonds. The presence of a double bond in the fatty acid weakens the C–H bonds on the carbon atom nearby the double bond and thus facilitates $H^{\bullet}$ abstraction from a methylene group, giving rise to an unpaired electron on the carbon ($-^{\bullet}CH-$) susceptible for oxidation. This circumstance is likely to be favored in the brain parenchyma given its high metabolic rate and elevated oxygen consumption, which inevitably will produce significant amounts of reactive oxygen species as by-products, including the highly reactive superoxide anion O_2^- that is converted to H_2O_2 (Dröge, 2002). In addition, brain is rich in redox transition metals, particularly iron, which by virtue of Fenton reaction with endogenous H_2O_2 produce iron(III) and generate the highly reactive hydroxyl radical $OH^{\bullet}$ at the expense of endogenous reducing agents, i.e., polyunsaturated fatty acids like DHA or arachidonic acid, generating lipoperoxyl radicals. Lipid peroxidation generates hydroperoxides as well as endoperoxides, which undergo fragmentation to produce a broad range of reactive intermediates called reactive carbonyl species (RCS) such as isoprostanes (IsoPs), neuroprotanes, malondialdehyde, unsaturated aldehydes including 4-hydroxy-2-*trans*-nonenal (HNE), 4-hydroxy-2-*trans*-hexenal (HHE), and 2-propenal (acrolein), with different degrees of reactivity (Porter et al., 1995; Niki et al., 2005; Catalá, 2009; Fritz and Petersen, 2013; Naudí et al., 2013).

Clearly, the concurrency of these factors in the presence of high amounts of polyunsaturated fatty acids, mainly DHA, is expected to favor the free radical-induced peroxidation of DHA in the brain parenchyma (Van Kuijk et al., 1990; Dröge, 2002; Valko et al., 2007). An important aspect of lipid peroxidation is its self-propagating nature and fundamentally different from other forms of free radical injury in that it is a self-sustaining process capable to provoke extensive brain tissue damage (Catalá, 2009; Singh et al., 2010). As most lipids in the brain are contained in the membrane phospholipids, the main outcome of lipid peroxidation is the structural damage of membranes, causing structural changes that impact membrane fluidity and permeability, neurotransmission, signaling, ion transport, and impaired electrical conduction.

Nerve cells are endowed with different antioxidant systems that render them protected from oxidative damage caused by lipid peroxides. This protection is mainly accomplished by phase II detoxifying enzymes belonging to two antioxidant systems, namely thioredoxin and glutathione systems, which use hydrophilic thiol-containing molecules (thioredoxin and glutathione, respectively), as electron donors to generate conjugated metabolites (Arnér and Holmgren, 2000; Imai and Nakagawa, 2003). However, although members of the thioredoxin system (mammalian thioredoxin reductases) are capable to reduce some non-disulfide-containing molecules, including lipid hydroperoxides independently of thioredoxin (Björnstedt et al., 1995), it is the only within the glutathione system where enzymes exist that are capable to recover oxidized membrane lipids (Imai and Nakagawa, 2003).

Phospholipid-hydroperoxide Glutathione Peroxidase and Membrane Protection

Phospholipid hydroperoxide glutathione peroxidase, glutathione peroxidase 4, or GPx4, is a member of glutathione peroxidases family of selenoproteins, most of which bear a selenocystein as catalytically active amino acid, which confers a more efficient reaction with peroxide substrates (Nauser et al., 2006). GPx4 uses preferentially glutathione as electron donors as long as cellular concentrations of the reduced form (GSH) are not limiting, but may accept other thiol groups in proteins as reducing equivalent (Godeas et al., 1997). Therefore, GPx4 can either act as a GSH peroxidase or a thiol peroxidase depending on the availability of GSH (Brigelius-Flohé and Maiorino, 2013). However, unlike other glutathione peroxidases, GPx4 is capable of reducing complex lipid peroxides, like phospholipid hydroperoxides, even when integrated in highly structured lipid-protein assemblies such as lipoproteins and membranes (Imai and Nakagawa, 2003). X-ray data indicated that, in contrast to other GPx isoforms, the active site of GPx4 lacks a surface-exposed loop domain which appears to limit the accessibility of large oxidized substrates, but instead contains a large hydrophobic surface

that allows GPx4 to closely associate with membranes and lipoproteins (Scheerer et al., 2007). Its ability to directly reduce phospholipid hydroperoxides in membranes without prior action of phospholipase A2 membrane makes GPx4 unique amongst antioxidative enzymes (Imai et al., 2003; Savaskan et al., 2007). Further, unlike other glutathione peroxidases (GPx1, GPx2, GPx3, GPx5, and GPx6) which exist as homo(tetra)-oligomeric proteins, GPx4 is a monomeric protein of about 20 kDa and misses the tetramer interfaces for oligomer formation (Scheerer et al., 2007; Brigelius-Flohé and Maiorino, 2013).

GPx4 is also peculiar amongst glutathione peroxidases because of its additional regulatory functions. Thus, GPx4 is considered not only a phase II antioxidant enzyme but is also endowed with functions associated with the regulation of apoptosis, gene expression, eicosanoid biosynthesis, and embryo development (Imai and Nakagawa, 2003; Savaskan et al., 2007; Ufer and Wang, 2011). The essential nature of GPX4 is demonstrated by the fact that genetic disruption of the entire *Gpx4* gene in homozygous *Gpx4* knockout causes *in utero* fetal death by midgestation (Imai et al., 2003; Yant et al., 2003). This intrauterine lethality has been related to increased apoptosis and cell death leading to malformation of embryonic structures and major defects in brain development (Imai et al., 2003; Yant et al., 2003). This contrasts profoundly with the knockouts on *Gpx1–3* genes, which are viable though more sensitive to stressors (Brigelius-Flohé and Maiorino, 2013), indicating a degree of redundancy of these genes for at least some of their biochemical functions, i.e., oxidant scavengers, *in vivo*.

In mammalian cells GPx4 is found in the cytoplasm, nucleus, mitochondria, and also in the endoplasmic reticulum (Imai and Nakagawa, 2003). From the molecular point of view, GPX4 is also singular because the three isoenzymes, namely cytosolic GPx4 (c-GPx4), mitochondrial GPx4 (m-GPx4), and nuclear GPx4 (n-GPx4), all derive from a single gene (**Figure 1**), but that can be distinguished by their N-terminal sequences (Imai and Nakagawa, 2003; Savaskan et al., 2007). The *Gpx4* gene comprises 7 exons, of which exons 2–7 encoding for the functional enzyme are shared by the three isoforms. The differential N-terminal sequences are attained by three major start sites for translation (**Figure 1**) that reside in two alternative exons 1 (E1a and E1b). Thus, exon 1a contains two in-frame translational start sites (5′AUG and 3′AUG) separated by a sequence that encode for a mitochondrial leader peptide. Translation initiation at the 5′AUG results in the generation of m-GPx4 isoenzyme, while translation from the 3′AUG, that lacks this leader signal, yields c-GPx4. Because the mitochondrial leader peptide is cleaved off after import into mitochondria, c-GPx4 and m-GPx4 cannot be differentiated on the basis of their primary structure. Finally, the alternative first exon (E1b) encodes the N-terminal part of the nuclear isoform, n-GPx4, and contains a nuclear targeting sequence which is apparently retained after nuclear import (Pfeifer et al., 2001), and makes this isoform distinguishable from m/c-GPx4 at protein level.

Unexpected DHA-induced Transcriptional Regulation of *Gpx4* Gene

We have recently reported that DHA upregulates several members of both glutathione/glutaredoxin and thioredoxin/peroxiredoxin antioxidant systems in mouse hippocampal HT22 cells (Casañas-Sánchez et al., 2014). Noticeably, within the glutathione/glutaredoxin system, largest

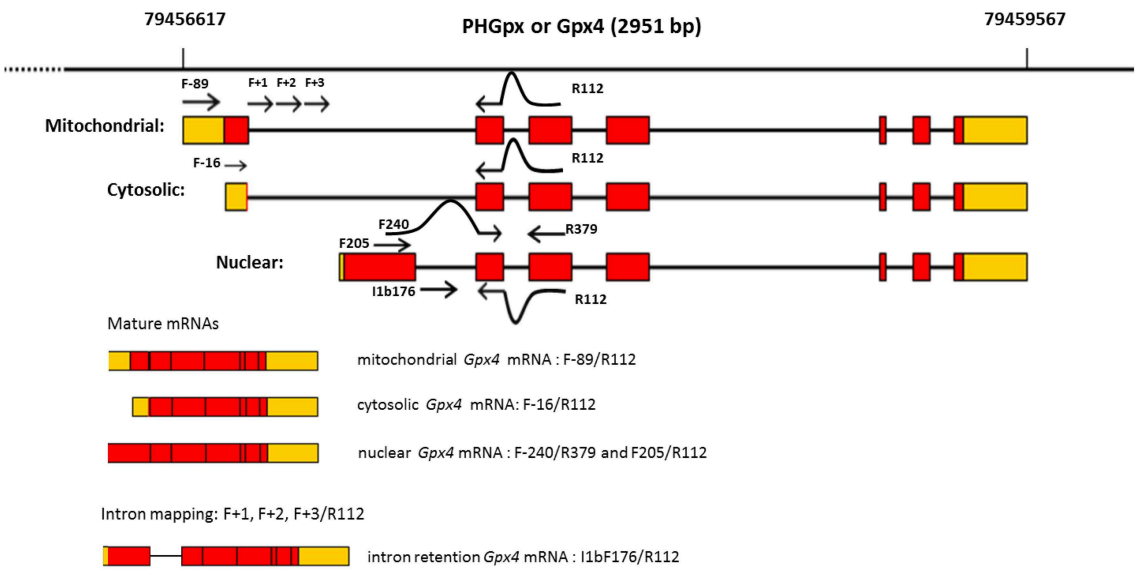

FIGURE 1 | Structural organization of mouse Gpx4 gene. Boxes indicate coding exons (red) and UTRs (orange). Bold lines represent intronic sequences. Sites targeted by amplification primers are shown as arrowed lines.

changes in gene expression were observed for the *Gpx4* isoforms, around 150%, and affecting all transcripts analyzed, including the mitochondrial, cytosolic, and nuclear isoforms. The change induced by DHA on glutathione peroxidase genes was specific for *Gpx4* since no variation was observed for the cytosolic *Gpx1* gene (Casañas-Sánchez et al., 2014). Paralleling these changes, DHA treatment significantly increased GPx4 (and total GPx) activities, following a time-course that was compatible with the necessary delay for *Gpx4* mRNA translation. Importantly, these effects of DHA were specific for DHA and were not be detected when DHA was replaced by arachidonic acid, another highly abundant polyunsaturated fatty acid in nerve cells, under identical experimental conditions (Casañas-Sánchez et al., 2014).

Absolute quantification of *Gpx4* isoform expression in unstimulated HT22 cells revealed that the largest expression values corresponded to the cytoplasmic variant and the lowest for the nuclear mRNA variant, with a difference of about 3 orders of magnitude (**Figure 2**). Furthermore, in absolute terms, we observed that DHA treatment increases nearly 1.5-fold the expression of m/c-*Gpx4*, which must be attributable to the enhancement of the mRNA coding for the cytoplasmic isoform, whose abundance is about one order of magnitude higher than for m-*Gpx4*. The nuclear isoform was also up-regulated by DHA, but its absolute magnitude was negligible compared to m/c-*Gpx4* (**Figure 2**).

Using identical amplicons (**Table 1**), we further explored for the presence of these different *Gpx4* isoforms in the hippocampus of C57BL/6 mice. The results revealed the presence of all different isoforms, and noticeably, following a similar expression pattern, with m/c-*Gpx4* being the most abundant transcripts and n-*Gpx4* showing lowest expression. Interestingly, animals exposed to High or Low-DHA diets also exhibited differential *Gpx4* transcriptional regulation in response to DHA-containing or DHA-impoverished diets and differentially affected between wild-type and APP/PS1, a familial model of Alzheimer's disease (**Figure 3**). Thus, Absolute quantification of *Gpx4* isoforms in WT animals, revealed that Low-DHA diets leads to the stimulation of gene expression of all isoforms (between 1.43 and 1.99 times), being the largest stimulation observed for the cytosolic isoform, which, in turn is the most abundant *Gpx4* isoform under any circumstance (**Figure 3**). These observations strongly suggest a compensatory genetic strategy aimed to ensure protection of membrane DHA from oxidative damage under conditions of limited DHA availability. Overall these data indicates that the lack of sufficient DHA or its deficient supply is accompanied by increased *Gpx4* mRNAs expression and GPx4 protein synthesis, which consequently augment cellular resistance to oxidative damage of DHA-containing phospholipids. A different behavior was observed in APP/PS1 animals, where the high-DHA containing diet stimulated the expression of cytoplasmic and mitochondrial *Gpx4* mRNAs. The different expression levels observed in APP/PS1 animals, especially for the m-*Gpx4* isoform, suggest a genotype-related transcriptional regulation, which fits well with the increased levels of hippocampal oxidative stress demonstrated in transgenic animals (Aso et al., 2012) and also the lower levels of hippocampal DHA compared to WT animals (Fabelo et al., 2012).

Finally, using appropriate amplicons (**Table 2**) we tested for the presence and absolute abundance of the different *GPX4* transcripts in the human cell line SHSY-5Y. The results revealed that all *GPX4* isoforms are expressed in this cell line and, more interestingly, that their absolute abundance followed the same sequence observed in HT22 cells, though in this case the amount of the most prominent isoform, namely c-*GPX4*, was notably higher (about four orders of magnitude) than any other isoform.

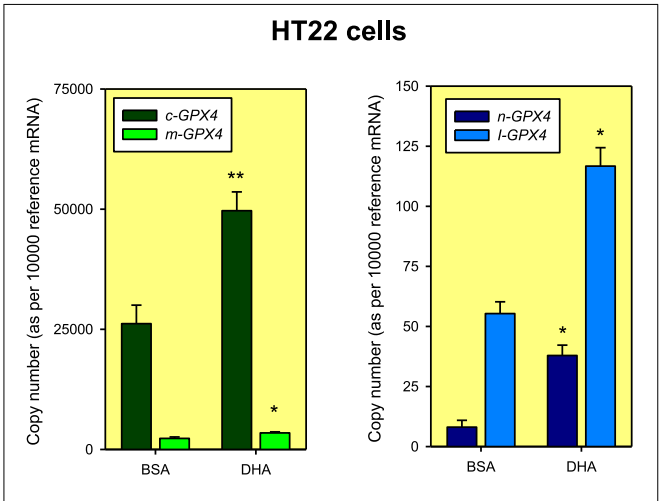

FIGURE 2 | Absolute quantification of *Gpx4* mRNA isoforms in HT22 cells exposed to BSA (Vehicle: Bovine serum albumin) or DHA for 48 h. The normalization factor (reference mRNA) for each cDNA sample was calculated as the geometric mean of the expression values of reference genes *Hprt1* and *Tbp* genes using standard curves generated from purified amplicons as described in Expósito-Rodríguez et al. (2011). Data are expressed as mean ± SEM from four different experiments. Statistical comparisons were assessed using Mann-Whitney *U*-test for independent samples. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to BSA.

Defining a Unified Rationale for DHA-induced Regulation of *Gpx4* Gene Expression

Our initial observations in HT22 indicated that the upregulation of *Gpx4* expression occurred with a significant delay form the initial exposure to DHA. Thus, it was necessary to expose cells for 48 h to detect the significant change in the expression levels of all *Gpx4* isoforms (Casañas-Sánchez et al., 2014). Our interpretation of these findings was related to the fact that control unstimulated HT22 cells (as most neuronal cell lines studied so far) contain extremely low levels of DHA in their membrane phospholipids (Martín et al., 2006). Supplementation of culture medium with DHA causes a nearly immediate incorporation by deacylation-reacylation mechanisms, whereby some monounsaturated fatty acids (mainly oleic acid) are readily replaced with DHA in membrane phospholipids (Farooqui et al., 2000; Martín et al., 2006). The activation of the transcriptional process occurred only after membrane phospholipids were replenished, because only then enough DHA remained unesterified in the cytoplasm

TABLE 1 | Oligonucleotides used as primers for quantification of different *Gpx4* mRNA isoforms and for mapping 5′ end of *l-Gpx4* mRNA in HT22 cells and mouse hippocampus.

Targeted gene	Primers	Exon/intron	Amplicon size (bp)	gDNA discrimination
m-Gpx4	F-89: CCgCCgAgATgAgCTgg	E1a	128	No
	R112: TgCACACgAAACCCCTgTACT	E2-E3		
m/c-Gpx4	F-16: TggTCTggCAggCACCAT	E1a	201	No
	R112: TgCACACgAAACCCCTgTACT	E2-E3		
n-Gpx4	F240: gTTCCTgggCTTgTgTgCAT	E1b-E2	140	No
	R379: AggCCACgTTggTgACgAT	E3		
n/l-Gpx4	F205: CTgCAAgAgCCTCCCCAgT	E1b	157/370	Yes
	R112: TgCACACgAAACCCCTgTACT	E2-E3		
l-Gpx4	Fl1b176: ggACCTgggTTAggACACTCA	l1b	147	Yes
	R112: TgCACACgAAACCCCTgTACT	E2-E3		
Mapping (+1)	F+1: gTgggCTACTggGAACTTgg	l1a	907	
	R112: TgCACACgAAACCCCTgTACT	E2-E3		
Mapping (+2)	F+2: gggAAAgCggAgCCTgATAg	l1a	787	
	R112:TgCACACgAAACCCCTgTACT	E2-E3		
Mapping (+3)	F+3: CTTggCTACCggCTCTTTg	l1a	630	
	R112: TgCACACgAAACCCCTgTACT	E2-E3		
Hprt1	F: TCAgACTgAAgAgCTACTgTAATgA	E3-E4	136	Yes
	R: AAgTTTgCATTgTTTTACCAgTg	E6		
Tbp	F: gACCCACCAgCAgTTCAgTAg	E6	136	Yes
	R: CTCTgCTCTAACTTTAgCACCTgT	E7-E8		

Determination of absolute mRNA levels by real-time quantitative PCR (RT-qPCR) were performed as described in Expósito-Rodríguez et al. (2011). Hprt1, Hypoxanthine guanine phosphoribosyl transferase 1; Tbp, TATA binding protein.

of neuronal cells, being then susceptible for non-enzymatic oxidation in the prooxidant cellular environment (**Figure 4**). Indeed we could only detect significant levels of the specific DHA-derived lipoperoxide 4-hydroxy-2-hexenal (HHE) after 36 h exposure to DHA, just before upregulation of *Gpx4* expression occurred. These findings were interpreted as HHE providing the signal to trigger transcriptional regulation, likely through activation of Nrf2 transcription factor. Indeed, it is known that Nrf2 is a master factor for the regulation of antioxidant response elements (ARE) in several antioxidant and phase II detoxifying proteins in different cell types, including nerve cells (Kobayashi and Yamamoto, 2005; Zhang et al., 2013), and several reports have shown that although DHA itself does not bind Nrf2, it may stimulate transcriptional activity of ARE-containing genes through activation of Nrf2 upon generation of 4-hydroxy-2-hexenal, which indeed activates Nrf2 (Ishikado et al., 2013; Kusunoki et al., 2013; Yang et al., 2013).

The results obtained in the hippocampus of WT mice were particularly interesting, since transcriptional activation of all *Gpx4* isoforms was significantly higher under the Low-DHA dietary condition compared to the High-DHA diet. Though apparently these results might contradict the results obtained in HT22 cells, the outcomes suggest that it is under a low DHA diet with limited availability of n-3 precursors, when it is especially important to activate mechanisms (like upregulation of *Gpx4* isoform expression) that may ensure the preservation of DHA in brain membranes. In fact, analyses of hippocampal lipid composition in WT and APP/PS1 animals revealed a significant reduction of DHA contents in Low-DHA diet compared to the High-DHA diet, yet the amount of DHA in the Low-DHA condition was surprisingly higher than expected from the dietary supply (**Table 3**), which reflects an extremely efficient ability to preserve brain DHA even when subjected to negligible levels in the diet (Díaz et al., accepted). This exceptional capability is mainly attributable to the favorable gradient for fatty acids to cross the blood-brain barrier, either passively or via fatty acid binding proteins (FATPs), since once in the brain, unesterified fatty acids are converted to CoA thioesters by the long-chain-fatty-acid-CoA synthase (ACSL) family of proteins, therefore maintaining a continuous concentration gradient (see (Bazinet and Layé, 2014) for comprehensive review).

As mentioned before, APP/PS1 animals also responded to the different diets with changes in the transcriptional levels of *Gpx4* expression. However, we only observed increased expression of c-*Gpx4* isoform, and in the High-DHA diet. The apparent discrepancy between WT and APP/PS1 animals is likely related to the involvement of constitutive oxidative stress associated to amyloid generation in the brain of transgenic animals (Aso et al., 2012; Fabelo et al., 2012). Indeed, it is now widely accepted that amyloid peptide induces oxidative stress, and inflammation and oxidative stress generate more Aβ, giving rise to a vicious cycle between Aβ and free radical formation/lipid peroxidation in the presence of transition metals (Behl et al., 1994; Pamplona et al., 2005; Solfrizzi et al., 2006). In line with this matter, increased levels of LCPUFA–derived lipid hydroperoxides like HHE, HNE, and

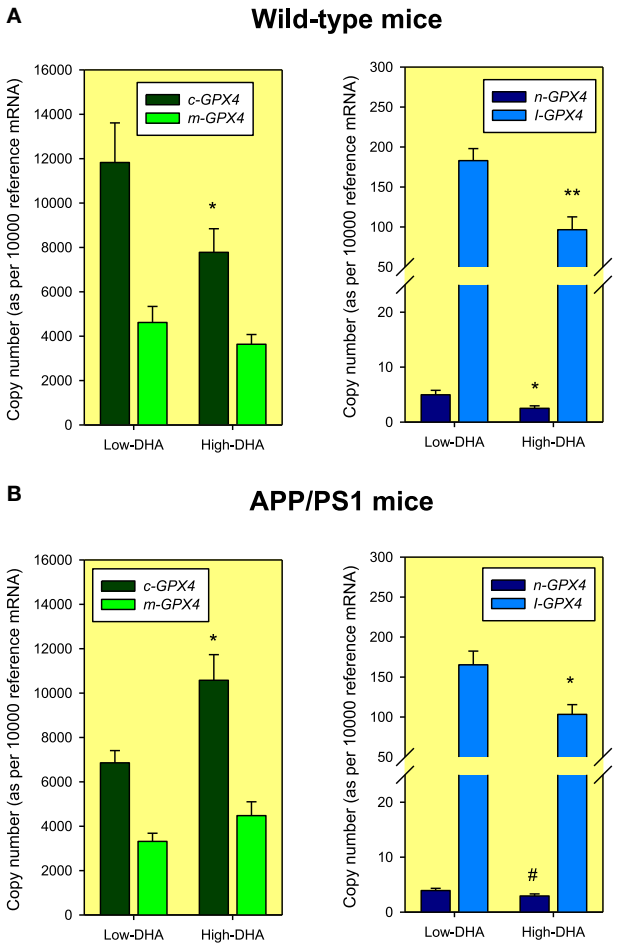

FIGURE 3 | Absolute quantification of *Gpx4* mRNA isoforms in 6 months old Wild-type (A) and APP/PS1 (B) mice fed Low-DHA or High-DHA diets for 3 months. Data are expressed as mean ± SEM from four different animals under each dietary condition and genotype. The normalization factor (reference mRNA) for each cDNA sample was calculated as the geometric mean of the expression values of reference genes *Hprt1* and *Tbp* genes using standard curves generated from purified amplicons as described in Expósito-Rodríguez et al. (2011). Statistical comparisons were assessed using Mann-Whitney *U*-test for independent samples. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{\#}p < 0.1$ compared to Low-DHA.

other endoperoxides like isoprostanes have been observed in the brain of transgenic models of Alzheimer's disease, including 3xTgAD and APP/PS1 animals, and more relevantly, in different areas of human brain and cerebrospinal fluid of AD patients (Marcus et al., 1998; Markesbery and Lovell, 1998; Praticò et al., 1998; Valko et al., 2007; Gwon et al., 2012). In this order of ideas, it seems plausible that in the high-DHA diet, lipid-derived hydroperoxides must be elevated in the more prooxidant environment of brain parenchyma in transgenic animals, including DHA-related HHE, which may trigger the stimulation of Nrf2 transcription factor, which eventually would favor the activation of a transcriptional program to increase *Gpx4* isoforms expression, especially the cytoplasmic isoform. In agreement with our findings, recent genomic gene expression profiles by microarray analyses in a "gene dose-response" model of Nrf2-null model have revealed that graded activation of Nrf2 increased the transcription of a large number of genes involved in Nrf2-mediated oxidative stress response, glutathione metabolism, and xenobiotic metabolism, including several genes responsible for the reduction of superoxide ions using GSH, i.e., glutathione peroxidases *Gpx2* and *Gpx4*, which augmented by 2260 and 105%, respectively, in Keap1-HKO mice compared to WT mice (Wu et al., 2011).

Alternatively, altered genetic regulation of *Gpx4* expression caused by overexpression of mutated APP and PS1 in transgenic animals, may have disturbed the normal gene expression pattern, as it has been recently demonstrated to occur in the frontal cortex and hippocampus of transgenic mice carrying the Δ E9 mutant hPS1 gene (Unger et al., 2005). Likewise, recent comparative studies in sporadic (icv-STZ Mouse) and familial (3xTg-AD mouse) mice models of AD have revealed the transcriptional alteration of more than 80 genes related to synapse function, apoptosis and autophagy, AD-related protein kinases, glucose metabolism, insulin signaling, and mTOR pathway in the hippocampus and the cerebral cortex, not only when compared to WT animals, but also between both AD models (Chen et al., 2012).

In summary, we hypothesize that by upregulating *Gpx4* gene expression and increasing GPx4 protein levels and enzyme activity, DHA initiates a self-protective biochemical strategy in the hippocampal neuron to control non-enzymatic peroxidation of DHA-containing phospholipids in the membrane from cellular and subcellular compartments. Given the extremely low capacity of hippocampal cells to biosynthesize DHA (reviewed in Plourde and Cunnane, 2007; Bazinet and Layé, 2014), and the essential nature of this fatty acid in the nervous system, this strategy provides an adaptive mechanism to keep the high DHA levels in hippocampal phospholipids.

Retained Intronic Sequences in *Gpx4* mRNA

In initial experimental strategy used for the detection of the different *Gpx4* mRNAs, we designed a couple of primers targeted to exons E1b (forward primer) and the boundary between exons E2/E3 (reverse primer) for the specific detection of nuclear *Gpx4* mRNA. Unexpectedly, in these experiments we detected the presence of two amplicons: one of the expected molecular size (157 bp) for a fully processed n-*Gpx4* mRNA, and another fragment 370 bp. Once sequenced, we observed that this later amplicon contained an extra 213 bp sequence corresponding to intron I1b located between exons E1b and E2. This unexpected sequence (named here I-*Gpx4*) was detected in control HT22 cells (treated with vehicle), but more interestingly, that its expression level was subjected to significant upregulation by DHA treatment (near 100% compared to control cells), which was in the range observed for the changes in expression for the set of other *Gpx4* mRNA variants (**Figure 2**). In absolute terms, I-*Gpx4* quantification revealed that this isoform was nearly 3 times more abundant than n-*Gpx4* under unstimulated conditions, and about

TABLE 2 | Oligonucleotides used as primers for quantification of different *GPX4* mRNA isoforms and for mapping 5′ end of l-*GPX4* mRNA in SHSY-5Y cells.

Targeted gene	Primers	Exon/intron	Amplicon Size (bp)	gDNA discrimination
m-GPX4	F-E1m: CATTggTCggCTggACgAg	E1a	242	Yes
	R-E34: CACACgAAgCCCCggTACT	E2-E3		
m/c-GPX4	F-E1c: CCTggCCgggACCATg	E1a	123	Yes
	R-E34: CACACgAAgCCCCggTACT	E2-E3		
n- GPX4	F-E2si: CAggCAgCggTgCCAgAg	E1b	170	Yes
	R-E23: gggACgCgCACgggTC	E1b-E2		
n/l- GPX4	F-E2$_N$: gATCCACgAATgTCCCAAgTC	E1b	138/394	Yes
	R-E34: CACACgAAgCCCCggTACT	E2-E3		
l- GPX4	F-l1b: gAggAgCgTTCAggTCTTCAg	l1b	242	No
	R-E34: CACACgAAgCCCCggTACT	E2-E3		
Mapping (+1)	F+1: gTgAgCTAgCgCCgCg	l1a	1165	
	R-E34: CACACgAAgCCCCggTACT	E2-E3		
Mapping (+2)	F+2: CCCTCCAggCCgTTgTAgg	l1a	978	
	R-E34: CACACgAAgCCCCggTACT	E2-E3		
Mapping (+3)	F+3: CggAgggCTggAAATCCC	l1a	730	
	R-E34: CACACgAAgCCCCggTACT	E2-E3		
HPRT1	F: gACCAgTCAACAggggACAT		173	Yes
	R:AACACTTCgTggggTCCTTTTC			
RPL32	F: CATCTCCTTCTCggCATCA		153	Yes
	R:AACCCTgTTgTCAATgCCTC			

Determination of absolute mRNA levels by real-time quantitative PCR (RT-qPCR) were performed as described in Expósito-Rodríguez et al. (2011). HPRT1, Hypoxanthine guanine phosphoribosyl transferase 1; RPL32, Ribosomal protein L32.

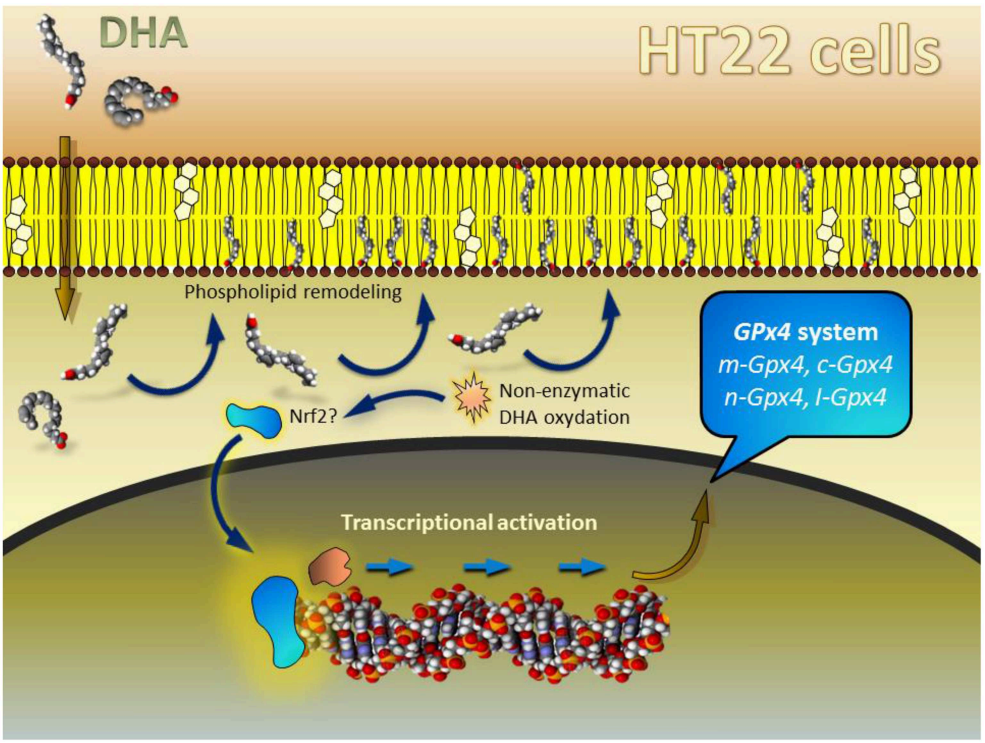

FIGURE 4 | Schematic model depicting the hypothetical mechanism of DHA-mediated regulation of *Gpx4* in HT22 cells. For details see "*Defining a unified rationale for DHA-induced regulation of Gpx4 gene expression.*" Modified from Casañas-Sánchez et al. (2014).

TABLE 3 | Fatty acid composition of total lipids in the hippocampus of 6 months old WT and APP/PS1 mice, and fed for 3 months with either Low-DHA or High-DHA diets.

Fatty acid	WT		APP/PS1	
	Low-DHA	High-DHA	Low-DHA	High-DHA
14: 0	0.10 ± 0.00*#	0.12 ± 0.00#	0.12 ± 0.01	0.13 ± 0.00
16: 0 DMA	2.16 ± 0.01	2.12 ± 0.01	2.11 ± 0.04	2.13 ± 0.04
16: 0	18.41 ± 0.06#	18.58 ± 0.16	17.80 ± 0.16	18.25 ± 0.20
18: 0 DMA	4.07 ± 0.04	4.09 ± 0.06	4.01 ± 0.15	4.04 ± 0.05
18:1 n-9 DMA	1.51 ± 0.04	1.54 ± 0.03	1.52 ± 0.08	1.52 ± 0.06
18:1 n-7 DMA	1.94 ± 0.05	2.01 ± 0.04	2.09 ± 0.05	1.99 ± 0.08
18: 0	19.60 ± 0.10*	19.05 ± 0.01	19.54 ± 0.12	19.14 ± 0.30
18: 1 n-9	15.81 ± 0.06*#	17.04 ± 0.11	16.32 ± 0.16	17.00 ± 0.25
18: 1 n-7	3.59 ± 0.06*	3.40 ± 0.01	4.18 ± 0.60	4.11 ± 0.56
18: 2 n-6	0.49 ± 0.01	0.48 ± 0.02	0.50 ± 0.02	0.48 ± 0.03
20: 0	0.28 ± 0.01	0.26 ± 0.01	0.29 ± 0.01	0.26 ± 0.02
20: 1 n-9	1.76 ± 0.05	1.80 ± 0.03	1.96 ± 0.07	1.74 ± 0.11
20: 1 n-7	0.34 ± 0.01	0.36 ± 0.00	0.37 ± 0.02	0.36 ± 0.02
20: 3 n-6	0.28 ± 0.01*	0.57 ± 0.00#	0.28 ± 0.01*	0.54 ± 0.01
21: 0	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20: 4 n-6	9.87 ± 0.16*#	7.64 ± 0.02	9.34 ± 0.07*	7.54 ± 0.19
20:5 n-3	0.00 ± 0.00*	0.19 ± 0.01	0.00 ± 0.00*	0.21 ± 0.01
22:0	0.22 ± 0.01	0.21 ± 0.00	0.24 ± 0.01	0.21 ± 0.02
22: 4 n-6	3.05 ± 0.06*	1.85 ± 0.02	3.14 ± 0.10*	1.82 ± 0.07
22: 5 n-6	1.19 ± 0.02*#	0.09 ± 0.00	0.87 ± 0.04*	0.09 ± 0.01
22: 5 n-3	0.03 ± 0.01*	0.39 ± 0.01	0.01 ± 0.01*	0.39 ± 0.01
24: 0	0.26 ± 0.01	0.26 ± 0.01	0.29 ± 0.02	0.27 ± 0.03
22: 6 n-3	12.69 ± 0.21*#	16.66 ± 0.10Δ	11.19 ± 0.11*	15.04 ± 0.55
24: 1 n-9	0.85 ± 0.03#	0.83 ± 0.04#	1.19 ± 0.09*	0.70 ± 0.01
TOTALS AND INDEXES				
Saturates	45.35 ± 0.05*	44.84 ± 0.12	44.61 ± 0.38	44.59 ± 0.49
Unsaturated	54.54 ± 0.05*	55.05 ± 0.12	55.27 ± 0.39	55.28 ± 0.50
DMAs	9.67 ± 0.08	9.76 ± 0.12	9.74 ± 0.31	9.68 ± 0.15
n-9	20.34 ± 0.18*#	21.62 ± 0.16	21.53 ± 0.15	21.43 ± 0.56
n-3	12.80 ± 0.21*	16.31 ± 0.09	11.27 ± 0.12*	15.16 ± 0.55
n-6	14.99 ± 0.22*#	10.70 ± 0.04	14.23 ± 0.12*	10.53 ± 0.27
n-3/n-6	0.85 ± 0.02*	1.52 ± 0.01	0.86 ± 0.01*	1.53 ± 0.03
18:1 n-9/n-3 H	1.24 ± 0.02*#	1.06 ± 0.01	1.34 ± 0.02*	1.07 ± 0.06
Unsaturation Index	163.12 ± 0.99*#	166.48 ± 0.30	158.64 ± 0.53	165.50 ± 2.94
saturates/n-3	3.55 ± 0.05*	2.75 ± 0.01	3.64 ± 0.03*	2.77 ± 0.07
saturates/n-9	2.23 ± 0.02*#	2.07 ± 0.02	2.07 ± 0.02	2.09 ± 0.07

*Results are expressed as mole % and represent means ± SEM of four different animals under each dietary condition and genotype. DMA: Dimethyl acetals. *$p < 0.05$ compared to High-DHA. #, Δ: $p < 0.05$ and $p < 0.1$ compared to APP/PS1. Fatty acid extraction and determination was performed as described in Fabelo et al. (2012) and Martín et al. (2006). Data were analysed by One-Way ANOVA followed by Tukey's multiple comparison test. Comparisons between Low-DHA and High-DHA treatments were performed using Student's t-test or Mann-Whitney U-test where appropriate. Main monoenoic (oleic acid, 18:1n9), n-6 LCPUFA (arachidonic acid, 20:4n-6), and n-3 LCPUFA (DHA, 22:6n-3) are highlighted on pink background.*

7 times higher after DHA treatment, pointing to a significant biological role of this intron-retaining isoform in hippocampal cells. Nonetheless, I-*Gpx4* was expressed a much lower amount than c-*Gpx4*, even after DHA treatment.

In order to gain a deeper insight on the transcription start site for the intron-retained transcript, we designed several amplification primers directed to intron I1a (**Figure 1** and **Table 1**). While the primers targeted to E1b exon and intron I1b amplified the expected amplicons, all other primers targeting E1b exon upstream sequences were unsuccessful in these PCR assays. These results strongly indicate that a *Gpx4* CIRT transcript represents an unprocessed variant of the n-*Gpx4* mRNA isoform.

In order to assess for the biological relevance of this intron-retaining *Gpx4* isoform, we used these specific amplicons to unveil the presence in the hippocampus of C57Bl/6 mice. We observed that not only the amplicon was detected, but also that

its expression was modulated by the contents of DHA in the diet. Thus, levels of I-*Gpx4* isoform were significantly higher (around 2 times) in animals receiving Low-DHA diet compared to animals receiving a High-DHA diet. A similar result was obtained in transgenic APP/PS1 animals, though in this case the increase in I-*Gpx4* gene expression was around 1.6 times higher in animals maintained in a Low-DHA diet (**Figure 3**). Absolute comparisons between WT and APP/PS1 animals revealed that I-*Gpx4* was higher in WT animals than in the APP/PS1 genotype. Overall these data indicates that the lack of sufficient DHA or its deficient supply is accompanied by an increase in I-*Gpx4*. Again, these higher expression levels observed in APP/PS1 animals suggests a genotype-related transcriptional regulation, which may be related to increased hippocampal oxidative stress in transgenic animals (Aso et al., 2012).

Whether this regulated *Gpx4* mRNA variant constitutes an intermediate processing stage of *Gpx4* splicing or represents a different level of transcriptional regulation by intron retention remains unrevealed. However, the notion of intron retention has been gaining consistency in the last decade, as an alternative processing of conventional (nuclear) mRNA, adding a novel level of complexity in gene expression regulation. These sequences, named as CIRTs (Cytoplasmic Intron-sequence Retaining Transcripts), comprise only few introns and are limited to small fractions of introns within the gene (Buckley et al., 2014). Indeed, in recent years, a number of transcripts containing intronic sequences and subjected to cytoplasmic splicing have been unveiled across a number of cell types and species (Buckley et al., 2014), and the list keeps growing steadily. Noticeably, a recent study performed in mouse brains have revealed the broad presence of cytoplasmic intron-sequence retaining transcripts in hippocampal neurons, with 59.4% of total gene transcripts in dendrites containing intronic reads (Khaladkar et al., 2013). Examples of retained introns from individual transcripts of relevance in neuronal cells include the large-conductance calcium-dependent potassium channel (BK) encoded by *KCNMA1* gene (Bell et al., 2008, 2010), γ-subunit of GABA-A receptor (encoded by *GABRG3*) (Buckley et al., 2011), ionotropic Glutamate receptor (encoded by *GRIK1* gene) and type 1 NMDA receptor (encoded by GRIN1) (Buckley et al., 2011) or the α-1 subunit of T type, voltage-dependent Calcium channel (encoded by *CACNA1H*) (Zhong et al., 2006). Though the biological relevance of CIRTs is still largely unknown, their abundance in neuronal dendrites (and also in enucleated platelets) suggest that endogenous cytoplasmic transcripts harboring retained intronic substrates could be subject to extranuclear splicing, thereby obviating the need for shuttling molecules to and from the nucleus for processing (Buckley et al., 2014). In this manner, dendritic splicing could represent a novel method of post-transcriptional gene regulation.

In this order of ideas, it is tempting to speculate that CIRT in *Gpx4-I* gene may be part of the normal endogenous post-transcriptional regulation pathway. Such regulated extranuclear splicing variants may be part of what Buckley et al. (2014) designed as "sentinel RNA hypothesis," according to which a master transcript harboring CIRTs would serve to generate transcript variants within the cytoplasm in response to specific stimulation, or under conditions in which a rapid processing is required and adaptive. A plausible scenario for this stimulus-dependent cytoplasmic splicing of I-*Gpx4* in hippocampal cells, is that this transcript processing in cellular domains distant from cellular nucleus, as is the case of dendrites, would enhance the antioxidant buffering capacity against the acute generation of lipid peroxides by oxidative insults, which would commit the integrity of membrane unsaturated phospholipids, especially those enriched in DHA.

Conclusions and Hypothesis

In summary, current available data indicate that *Gpx4* gene is subjected to a complex transcriptional regulation that renders nuclear, cytoplasmic, and mitochondrial isoforms, but also generate an intron-retaining variant, likely corresponding to a CIRT, all derived from the same gene. At least in hippocampal HT22 cells and in the hippocampus of wild type mice, the transcriptional control of most of these *Gpx4* isoforms is modulated by DHA following an unprecedented homeostatic strategy aimed to buffer its potential oxidation and to ensure its preservation in the hippocampal neuronal membrane. The observations in transgenic APP/PS1 mice, suggest that such homeostatic mechanism is disturbed by the presence of mutated APP and/or PS1, and may be relevant in Alzheimer's disease, as suggested by the generalized observation that DHA contents are reduced in the hippocampus of AD brains.

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Sub-toxic Ethanol Exposure Modulates Gene Expression and Enzyme Activity of Antioxidant Systems to Provide Neuroprotection in Hippocampal HT22 Cells

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Ethanol is known to cause severe systemic damage often explained as secondary to oxidative stress. Brain is particularly vulnerable to ethanol-induced reactive oxygen species (ROS) because the high amounts of lipids, and because nerve cell membranes contain high amounts of peroxidable fatty acids. Usually these effects of ethanol are associated to high and/or chronic exposure to ethanol. However, as we show in this manuscript, a low and acute dose of ethanol trigger a completely different response in hippocampal cells. Thus, we have observed that 0.1% ethanol exposure to HT22 cells, a murine hippocampal-derived cell line, increases the transcriptional expression of different genes belonging to the classical, glutathione/glutaredoxin and thioredoxin/peroxiredoxin antioxidant systems, these including *Sod1, Sod2, Gpx1, Gclc,* and *Txnrd1*. Paralleling these changes, enzyme activities of total superoxide dismutase (tSOD), catalase, total glutathione peroxidase (tGPx), glutathione-*S*-reductase (GSR), and total thioredoxin reductase (tTXNRD), were all increased, while the generation of thiobarbituric acid reactive substances (TBARS), as indicators of lipid peroxidation, and glutathione levels remained unaltered. Ethanol exposure did not affect cell viability or cell growing as assessed by real-time cell culture monitoring, indicating that low ethanol doses are not deleterious for hippocampal cells, but rather prevented glutamate-induced excitotoxicity. In summary, we conclude that sub-toxic exposure to ethanol may well be neuroprotective against oxidative insults in hippocampal cells.

Keywords: ethanol, antioxidant systems, superoxide dismutases, glutathione, thioredoxins, hippocampal cells, HT22 cells

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INTRODUCTION

Ethanol is known to induce neurocognitive deficits and to provoke neuronal injuries associated with neuronal degeneration (Givens et al., 2000). Although oxidative stress and mitochondrial damage are implicated in nerve tissue injury (Suh et al., 2004; Das and Vasudevan, 2007) the precise mechanisms underlying ethanol-induced neurological disorders remain unclear. Ethanol-induced

oxidative stress is linked to its metabolism in both microsomal and mitochondrial systems, which is directly involved in the generation of reactive oxygen species (ROS) and reactive nitrogen species (Das and Vasudevan, 2007). It is known that high or chronic ethanol exposure results in the depletion of reduced glutathione (GSH) levels, decreases antioxidant activity, and elevates malondialdehyde and hydroxynonenal protein adducts (Das and Vasudevan, 2007). These increased levels of oxidative stress (and secondary membrane lipid peroxidation, in particular) may disrupt neuronal energy metabolism and ion homeostasis by impairing the function of membrane ion-motive ATPases and glucose and glutamate transporters. Such oxidative and metabolic disturbances may thereby render neurons vulnerable to excitotoxicity and apoptosis. Indeed, it is known that acute alcohol exposure inhibits cognitive functions, including learning, and memory in humans (Givens et al., 2000), and that ethanol-induced memory impairments are mainly due to deficits in processing new memories, rather than retrieval of consolidated memories (White et al., 2000). The hippocampus is the main locus for ethanol-induced alterations of cognitive functions. Compelling evidence have demonstrated that acute ethanol exposure inhibits long-term potentiation (LTP) in the CA1 region of the hippocampus both *in vivo* and *in vitro* (Givens and McMahon, 1995; White et al., 2000; Ramachandran et al., 2015). Apparently, the primary mechanism by which ethanol inhibits LTP is the increase in GABAergic transmission, which, in turn, inhibits the depolarization phase required for N-methyl-D-aspartate (NMDA) receptor activation (White et al., 2000; Schummers and Browning, 2001).

Apart from these deleterious effects, several lines of evidence have shown that consumption of low doses of alcohol may provide neuroprotective effects against Alzheimers disease (AD). Indeed, Belmadani et al. (2004) observed that acute administration of ethanol was able to prevent the toxic effects of amyloid beta peptides (Aβ), the main protein aggregates in Alzheimers disease, in brain slices. Pre-treatment with physiologically relevant concentrations of ethanol (0.02–0.08%) protected neurons against Aβ-induced synapse damage, and recovered levels of synaptophysin, an indicator of synapse density in cortical and hippocampal neurons (Bate and Williams, 2011). Further, it has been reported that moderate wine consumption reduced neuropathologic traits (decreased amyloid plaques and reduced spatial memory impairment), in Tg2576 transgenic mice which overexpress amyloid pre-cursor protein (Wang et al., 2006). Moreover, epidemiological studies in a large cohort of subjects (>3600) have pointed out that low or moderate alcohol consumption is associated with lower risk of incident dementia among older adults, and these individuals are less likely to develop phenotypic symptoms of Alzheimers disease (Mukamal et al., 2003). Interestingly, ethanol also protected neurons against synapse damage induced by pre-synaptic aggregates of α-synuclein, which are characteristic of Parkinson's disease and dementia with Lewy bodies (Bate and Williams, 2011).

The potential neuroprotective mechanism(s) of action of ethanol remain unknown. However, given that amyloid beta peptides and α-synuclein toxicities have been linked to increased oxidative stress (Simonian and Coyle, 1996; Mattson et al., 1999; Bossy-Wetzel et al., 2004; Mancuso et al., 2006), in the present study we have examined a possible mechanism by which moderate ethanol concentrations might exert a neuroprotective effect on hippocampal cells through modification of cellular antioxidant capabilities. We demonstrate for the first time, that sub-toxic ethanol exposure triggers the activation of cellular antioxidant systems and that its effects may be unraveled both at transcriptional and enzymatic levels. Our results demonstrate that, at low doses, ethanol exerts a role as an "*Indirect Antioxidant,*" at least in hippocampal cells.

MATERIALS AND METHODS

Cell Culture Conditions, Ethanol Supplementation and Preparation of Cell Extracts

The immortalized mouse hippocampal cell line, HT22, was cultured in standard Dulbecco's modified Eagle's medium (DMEM) as described in Martín et al. (2006). Culture medium was changed every 2 days and, when reached 90% confluence, cells were subcultured after treatment with 0.25% trypsin-EDTA mixture, at a density of 4×10^5/ml on T25 flasks (for gene expression analyses) and T75 flasks (for enzyme activity studies).

After allowing 24 h for attachment, culture media were replaced with standard medium supplemented with ethanol in a 0.1% final concentration or vehicle phosphate buffered saline (PBS). Every 24 h of incubation the medium was replaced with fresh medium containing ethanol in PBS or PBS. Cell cultures were collected at 6, 24, 30, and 48 h, and immediately processed for either total RNA extraction or preparation of total extracts required for determination of enzyme activities, and glutathione and TBARs levels.

Cell extracts were prepared by homogenization in cold hypotonic buffer (Tris-HCl 20 mM, pH = 7.6) containing 1X protease inhibitors cocktail (Roche Diagnostics, Barcelona, Spain), then centrifuged at 900 g for 10 min, and the supernatants collected and stored at −80°C until analyses. For glutathione measurements, cell extracts were homogenized in cold hypotonic buffer containing 5% TCA and centrifuged at 10,000 g for 10 min at 4°C to isolate the post-mitochondrial supernatant. Supernatants were stored in 150 μl aliquots at −80°C. Protein determination was carried out using the Bradford assay (Bradford, 1976).

RNA Purification, cDNA Synthesis and Relative Quantification of Gene Expression

Total RNA was purified from 3×10^6 HT22 cells using a commercial kit and following the manufacturer's recommendations (RNeasy® Protect Minikit, Qiagen), and on-colum DNase I digestion to remove genomic DNA (gDNA). The integrity of purified RNA was estimated through the 3′:5′ assay (Nolan et al., 2006).

cDNA samples for real-time reverse transcription quantitative PCR (RT-qPCR) experiments were obtained with the Transcriptor First Strand cDNA Synthesis Kit (Roche), using 6 μg total RNA as template and anchored oligo$(dT)^{18}$ primers.

A mixture of the 33 diluted cDNA samples was used for the selection of the optimal concentration of each PCR primer pair (see **Table 1**), based on the lowest quantification cycle (Cq) values. Resulting amplicons for each primer pair from the cDNA pool were checked by electrophoresis on 3% agarose gels and sequenced. The possibility of gDNA contamination in the RT-qPCR assays was controlled in several ways. First, amplification primers were targeted to different exons, often spanning an exon/exon boundary (see **Table 1**). Next, each primer pair was tested by real-time qPCR using 1 ng genomic DNA as template. The level of gDNA contamination in each of the 32 RNA samples was assessed, assaying a quantity equivalent to the cDNA used in the amplification reactions (i.e., 40 ng of total RNA) was amplified by real-time qPCR using primers targeted to alpha-tubulin sequences.

Real-time amplifications were run in triplicate using SYBR Green detection on a LightCycler 480 platform (Roche). Relative quantities of the targeted mRNAs were calculated from Cq data following an efficiency-correction model implemented in the REST software (Pfaffl, 2001). The normalization factor for each cDNA sample was calculated as the geometric mean of the expression values of reference genes *Hprt1, Polr2f,* and *Tbp* genes.

Antioxidant Enzyme Activities and Levels of Glutathione and Thiobarbituric Acid Reacting Substances (TBARS)

Superoxide dismutase (SOD) activity was measured using the pyrogallol method following Marklund and Marklund (1974). One unit was defined as 50% inhibition of the rate of autoxidation of pyrogallol. The activity of SOD is expressed as units/mg protein. Catalase (CAT) activity was determined as described previously (Sani et al., 2006), by following the rate of decomposition of H_2O_2 in 10 mM potassium phosphate buffer at 240 nm. One CAT unit was defined as the decomposition of 1 mmol H_2O_2/min, and was expressed as units/mg protein.

Total glutathione peroxidase (GPX) and phospholipid-hydroperoxide glutathione peroxidase (GPX4) activities were measured using the glutathione reductase-NADPH methods described by Lawrence and Burk (1976) and Scheerer et al. (2007), respectively, by monitoring the rate of decrease in the concentration of NADPH as recorded at 340 nm. Glutathione reductase (GSR) was analyzed by determining the reduction of oxidized glutathione (GSSG) at the expenses of NADPH oxidation and monitored at 340 nm, according to the method of Carlberg and Mannervik (1985). GPX, GPX4 and GSR activities were expressed as nmol NADPH oxidized/min.mg protein.

Glutathione-*S*-transferase (GST) activity was determined following the conjugation of GSH with CDNB (1-chloro-2,4-dinitrobenzene) at 340 nm (Habig et al., 1974) and expressed as nmol GS-DNB/min.mg protein.

Thioredoxin reductase (TXNR) activity was measured using the DTNB-NADPH assay described by Arnér et al. (1999), in which the generation of thionitrobenzoate ion (TNB) catalyzed by TXNR upon oxidation of NADPH is monitored at 412 nm. TXNR activity was expressed as nmol TNB/min.mg protein.

Glutathione levels were determined fluorimetrically using excitation/emission wavelengths of 355 nm/420 nm according to Hissin and Hilf (1976). GSH, GSSG, and total glutathione (GSH+2GSSG) levels were determined against proper calibration curves and expressed as nmol/mg protein. Lipid peroxidation was determined by the thiobarbituric acid reacting substances (TBARs) method (Ohkawa et al., 1979), using TMP (1,1,3,3-tetramethoxypropane) as standard for calibration curves. TBARS were measured fluorimetrically with 485 nm (excitation)/ 535 nm (emission) wavelengths. TBARS contents were expressed as nmol/mg protein.

Real-time Cell Proliferation Assays

Real-time cell proliferation studies were performed using the xCELLigence biosensor technology (Roche), an electrical impedance-based system that allows for the measurement of real-time cell proliferation (Ke et al., 2011). Briefly, HT22 cells were trypsinized, and seeded at a density of 3.5×10^3 cells/well into 3 independent E16—xCELLigence plates. After an initial stabilization period, the impedance was recorded at 15 min intervals along the experiment, and the values converted to Cell Index (CI), a measure of the degree of cellular adhesion to the multi-electrode array. Generally, cell number directly correlates with output CI reading until confluency is achieved (CI_{max}). 48 h after seeding, 10 μl of vehicle (PBS), or ethanol (final concentration 0.1%) were added to each well, and incubated for additional 24 h before replacing the incubating solutions with either DMEM, DMEM+0.1% EtOH, or DMEM+1% EtOH (see **Figure 5A**). In some experiments, glutamate (20 mM final concentration) was added during the last medium replacement. The concentration of glutamate chosen for these experiments was calculated as the IC50 obtained in the dose-response analyses performed using this same device (**Figure 5B**). For this purpose 24 h after initial attachment, cells were exposed to single doses of glutamate, ranging from 3 to 30 mM, while CI was continuously monitored. 24 h after exposure CI_{max} was obtained and used for logistic analyses.

Statistics

Gene expression data were processed following an efficiency-corrected model for relative quantification (Pfaffl, 2001) and normalization with multiple internal controls as implemented in the qBASE software (Hellemans et al., 2007). Four genes showing high expression stability in relative expression analysis were tested as potential reference genes according to qBASE normalization tools, three of which were finally selected. A relative expression software tool (REST 2008, Pfaffl et al., 2002) was used to obtain the corresponding significance levels for each individual change in gene expression. Comparisons of gene expression levels between PBS and ethanol and expressed as fold values. *P*-values below 0.05 were considered statistically different.

Dose-response curves for glutamate were obtained from real-time cell proliferation assays. Data were fitted to a four-parameter logistic equation to obtain the IC50 value, using the software implemented in XCELLigence device.

Data from enzyme activity assays and glutathione/TBARs levels are expressed as mean ± SEM, and were analyzed by

TABLE 1 | Gene names, cellular locations, and oligonucleotides used as primers in amplification reactions.

Gene		mRNA	Comments* cellular location	Amplification primers		Amplicon size (bp)	
Product	**Symbol**	**Reference sequence**		**Forward/Reverse**	**Targeted exons**	**cDNA**	**gDNA****
Hypoxanthine guanine phosphoribosyl transferase	*Hprt*	NM_013556	IS	TCAGACTGAAGAGCTACTGTAATGA/AAGTTTGCATTGTTTTACCAGTG	3rd/4th 6th	136	N/A
TATA binding protein	*Tbp*	NM_013684	IS RIA (3′)	GACCCACCAGCAGTTCAGTAG/CTCTGCTCTAACTTTAGCACCTGT	6th 7th/8th	136	N/A
TATA binding protein	*Tbp*	NM_013684	RIA (5′)	CGCAGTGCCCAGCATCA/GCATAAGGTGGAAGGCTGTTG	1st/2nd 2nd	154	N/A
α-Tubulin	Tuba1	NM_011653	gDNA contamination control	GGATTCGCAAGCTGGCTG/GGGCTGGGTAAATGGAGAAC	3rd/4th 4 h	162	162
RNA Polimerase II (subunit f)	Polr2f	NM_027231	IS	GTCAGACAACGAGGACAATTTC/ATACTTGGTCATGTAAGGAGTGGT	1st/2nd 3rd	178	N/A
Superoxide dismutase 1	*Sod1*	NM_011434	Cytosolic	CGATGAAAGCGGTGTGCG/GCACTGGTACAGCCTTGTGTATTG	1st2nd/3rd	178	N/A
Superoxide dismutase 2	*Sod2*	NM_013671	Mitochondrial	GGTGGAGAACCCAAAGGAGA/TAAGCGACCTTGCTCCTTATT	3rd/4th 4 h	150	N/A
Catalase	*Cat*	NM_009804	Peroxisomal	AGAGGAAACGCCTGTGTGAG/GTAGGTGTGAATTGCGTTCTTAG	11th12th/13th	173	N/A
Thioredoxin 1	*Txn1*	NM_011660	Cytosolic	AGCCCTTCTTCCATTCCCT/GAACTCCCCCACCTTTTGAC	2nd73rd 4th/5th	152	152
Thioredoxin 2	*Txn2*	NM_019913	Mitochondrial	GACACCAGTTGTTGTGGACTTTC/TAGGCACAGCTGACACCTCATA	1st 2nd/3rd	170	N/A
Thioredoxin interacting protein	*Txnip*	NM_023719	Cytosolic	GTCGAATACTCCTTGCTGATCTA/TCTGGGGTATCTGGGATGTT	5th/6th 6th	173	N/A
Thioredoxin reductase 1	*Txnrd1*	NM_001042523	Cytosolic	GGGGAAGAAAATATTGAAGTTTACC/TGGAAGCCCACGACACGTT	13th/14th 15th	134	N/A
Thioredoxin reductase 2	*Txnrd2*	NM_013711	Mitochondrial	TCACAGTGCTACATAAAGATGGTA/CAGCTTGACCACCTCCTCAG	15th/16th 17th	192	N/A
Thioredoxin reductase 3	*Txnrd3*	NM_153162	Endoplasmic reticulum	GACTCTTTGGGGTCTCTTTAGAA/CAAAACAAGGTGTGATACACTTCC	12th 13th/14th	154	N/A
Peroxiredoxin 2	*Prdx2*	NM_011563	Cytosolic	GGCATTGCTTACAGGGGTC/CCACATTGGGCTTGATGGT	4th/5th 6th	196	196
Peroxiredoxin 3	*Prdx3*	NM_007452	Mitochondrial	GGATCAACACACCAAGAAAGAAT/CGGAAGGTCGTTGACACTCAG	4th/5th 6th	185	N/A
Peroxiredoxin 4	*Prdx4*	NM_016764	Cytosolic/Nuclear Secreted	GTATACCTTGAAGACTCAGGACATAC/CCAGCAGGGCAGACTTCTC	4th5th/6th	176	N/A
Peroxiredoxin 5	*Prdx5*	NM_012021	Cytosolic/Mitochondrial/ peroxisomal	CACCTGGCTGTTCTAAGACCC/AGACACCAAAGAATCATCCAATAA	2nd/3rd 5th	224	N/A
Glutamate-cysteine ligase, catalytic subunit	*Gclc*	NM_010295	Cytosolic	GCCTCCTCCTCCAAACTCAGA/ATCCCCTGCAAGACAGCATC	11th 13th	213	N/A
Glutathione reductase	*Gsr*	NM_010344	Cytosolic/Mitochondrial	CCAATGTCAAAGGCGTCTATG/AGACCACAGTAGGGATGTTGTCA	10th 11th	153	N/A
Glutaredoxin 1	*Glrx1*	NM_053108	Cytosolic	AGCTCACCGGAGCGAGAAC/ATCTGCTTCAGCCGAGTCATC	1st 2nd	118	N/A
Glutaredoxin 2	*Glrx2*	NM_001038592	Cytosolic/Mitochondrial	GTCGTTTTGGGGGAAGTCTA/GGTTGCCATATTCCAGCATAT	2nd 3rd	182	N/A
Glutathione peroxidase 1	*Gpx1*	NM_008160	Cytosolic	CGGCACAGTCCACCGTGTAT/ATTCTTGCCATTCTCCTGGTGT	1st 1st/2nd	236	236
Glutathione peroxidase 4	*Gpx4*	NM_008162	Cytosolic/Mitochondrial	TGGTCTGGCAGGCACCAT/TGCACACGAAACCCCTGTACT	1st 3rd/4th	128	128
Sulfiredoxin 1	*Srxn1*	NM_029688	Cytosolic	AGAGCCTGGTGGACACGAT/AGCTTGGCAGGAATGGTCT	1st 2nd	163	N/A

** IS, internal standard for normalization; RIA, RNA integrity assessment by 3′:5′ assay. Subcellular localization of protein encoded by the detected mRNA variant.*

***N/A: the primer pair does not amplify with gDNA as template, at least with a short extension time amplification reactions.*

one-way ANOVA followed by Tukey's multiple comparison test. Comparisons between PBS and ethanol treatments at each time were performed using Student's *t*-test or Mann-Whitney *U*-test where appropriate.

RESULTS

Effects of Sub-toxic Exposure to Ethanol on the Transcriptional Activity of Genes Encoding for Antioxidant Systems

First, we analyzed the time-course of the effects of sub-toxic ethanol exposure (0.1%) on the expression of different genes encoding for antioxidant enzymes with relevant activity in neuronal cells. We explored key enzymes from the three major antioxidant systems, namely classical, thioredoxin/peroxyredoxin, and glutathione/glutaredoxin systems. Target genes analyzed are detailed in **Table 1**, together with the information on the primers used for PCR amplification.

Results in **Table 2** show that superoxide dismutase encoding genes were up-regulated yet with different time-courses. Thus, while *Sod1* expression was stimulated just 6 h after each exposure to ethanol (0 and 24 h), *Sod2* was up-regulated after 30 h exposure to ethanol and remained so until the end of the experiment. The other gene relevant in the classical system, *Cat*, encoding for catalase, remained unaltered, or was down-regulated (at 24 h). Within the thioredoxin-peroxiredoxin system, *Txnrd1* gene, encoding for cytosolic thioredoxin reductase was soon up-regulated and its expression kept stimulated throughout the experiment. Paradoxically, *Txnrd2,* and *Txnrd3* genes, encoding for thioredoxin reductases from mitochondrial and endoplasmic reticulum isoforms, respectively, were down-regulated throughout the experiment, although only significantly for *Txnrd3*. A similar set of changes were observed for genes encoding for peroxyredoxins 2–5, all of which were significantly down-regulated yet with different time patterns. Finally, *Txnip* gene, encoding for the inhibitory thioredoxin interacting protein, was highly down-regulated, particularly 24 h after each addition of ethanol. No changes in the expression patterns were observed for either thioredoxin-encoding genes.

Lastly, within the glutathione/glutaredoxin system, all affected genes were up-regulated. The most important changes were observed for *Gpx1,* encoding for cytosolic glutathione peroxidase 1, which remained stimulated throughout the experiment, and

TABLE 2 | Gene expression of antioxidant systems in HT22 cells exposed to ethanol (0.1%) or PBS.

Ethanol vs. PBS

	Ethanol 6 h		Ethanol 24 h		Ethanol 30 h		Ethanol 48 h	
Gene	**Expression**	***p***	**Expression**	***p***	**Expression**	***p***	**Expression**	***p***
CLASSICAL SYSTEM								
Sod1	**1.23***	**0.028**	1.09	0.300	**1.26***	**0.009**	1.19	0.054
Sod2	1.15	0.275	1.18	0.070	**1.41***	**0.004**	**1.46***	**0.026**
Cat	0.98	0.722	**0.74***	**0.014**	0.94	0.255	0.88	0.134
THIOREDOXIN/PEROXIREDOXIN SYSTEM								
Txn1	1.09	0.486	1.01	0.990	1.26	0.119	1.08	0.469
Txn2	0.80	0.180	1.08	0.404	1.02	0.935	0.96	0.560
Txnip	1.02	0.892	**0.47***	**0.008**	0.86	0.137	**0.30***	**0.017**
Txnrd1	**1.16***	**0.050**	**1.27***	**0.010**	**1.53***	**0.005**	**1.32***	**0.018**
Txnrd2	**0.74***	**0.032**	0.85	0.250	0.84	0.161	0.76	0.094
Txnrd3	**0.71***	**0.027**	**0.70***	**0.044**	**0.68***	**0.021**	**0.65***	**0.004**
Prdx2	**0.75***	**0.046**	**0.71***	**0.015**	**0.82***	**0.015**	**0.73***	**0.015**
Prdx3	0.76	0.078	**0.68***	**0.036**	0.84	0.310	**0.73***	**0.029**
Prdx4	**0.75***	**0.012**	**0.67***	**0.039**	**0.78***	**0.008**	**0.67***	**0.013**
Prdx5	**0.73***	**0.046**	**0.69***	**0.007**	**0.72***	**0.011**	**0.61***	**0.019**
Srxn1	1.00	0.949	1.26	0.163	1.15	0.439	1.13	0.052
GLUTATHIONE/GLUTAREDOXIN SYSTEM								
Gclc	1.13	0.135	1.07	0.700	**1.26***	**0.013**	**1.25***	**0.008**
Gsr	1.00	0.971	1.02	0.694	**1.14***	**0.007**	1.09	0.136
Glrx1	**1.25***	**0.017**	1.01	0.795	1.21	0.208	0.93	0.619
Glrx2	0.94	0.475	0.87	0.408	1.03	0.687	0.87	0.069
Gpx1	**1.70***	**0.011**	**1.84***	**0.015**	**2.04***	**0.018**	**2.20***	**0.025**
Gpx4	0.96	0.799	0.97	0.699	0.94	0.864	0.85	0.394

*Results correspond to the mean of four different experiments. PBS, phosphate buffer saline; p, probability value; *Significant differences.*
Bold values indicate those genes whose expression was altered at some stage in the experiments.

reached the maximal transcriptional stimulation amongst all genes studied at 48 h. These changes were specific for *Gpx1,* and were not observed for *Gpx4* gene which encodes the membrane-associated isoform. Also, significant changes were detected for *Gclc* gene, which encodes for the catalytic subunit of glutathione-cysteine ligase.

Effects of Sub-toxic Exposure to Ethanol on Key Antioxidant Enzyme Activities and Antioxidant Metabolites

Next, we assessed the activities of most antioxidant enzymes encoded by genes that were affected by ethanol treatment and following the same time-course used to explore changes in transcriptional activity. Results shown in **Figure 1A** shows that total superoxide dismutase activity was increased by ethanol treatment from 24 h after exposure, being the maximal activity reached at 48 h. Surprisingly, catalase activity remained similar to vehicle (PBS) until the end of the experiment, where at 48 h a significant increase (40%) was observed (**Figure 1B**).

Within the thioredoxin system, we explored the time-course of total thioredoxin reductase (tTXNRD) activity (**Figure 2**). We observed that, in line with the results of *Txnrd1* up-regulation, TXNRD activity was stimulated from 6 h, and that maximal activation was observed at 48 h.

With regards to the glutathione/glutaredoxin system, we assessed the enzyme activities of total glutathione peroxidase, glutathione peroxidase 4, glutathione-*S*-reductase, and glutathione-*S*-transferase (**Figure 3**). When compared to PBS, total glutathione peroxidase (tGPx) was found to be significantly increased from the begin of the experiment (**Figure 3A**), reaching nearly a 200% increase at 48 h (similar to what was observed for *Gpx1* gene expression). This increase is likely attributable to stimulation of the GPX1 isoform, since GPX4 activity remained unaltered all along the experiment (**Figure 3B**). Glutahione-*S*-reductase (**Figure 3C**) was only affected at 48 h, which is compatible with the significant increase in *Gsr* gene expression detected at 30 h, and agrees with the expected delay in *Gsr* mRNA translation. On the other hand, glutathione-*S*-transferase was completely unaffected by ethanol treatment (**Figure 3D**).

We also determined cellular levels of total glutathione, reduced glutathione, oxidized glutathione (**Figure 4**). None of these oligopeptides appeared to be affected by ethanol treatment throughout the experiment, when compared to PBS-treated cells. These observations on glutathione species are in contrast to the expression levels of *Gclc* gene, which were significantly increased by the end of the experiment (**Table 2**). Several possible explanations are that even at 48 h, (1) *Gclc* mRNAs has not been fully translated, (2) that newly-synthetized GCLC protein might not be physiologically active or, (3) that expression of the regulatory subunit (GCLM) might be limiting, as demonstrated for HepG2/C3A cells and murine embryonic fibroblasts cultured in cysteine-deficient medium (Sikalidis et al., 2014). Regarding TBARs, it is noticeable that their levels did not increase at any point of the experiment, indicating that potential lipid peroxidation induced by ethanol is buffered by concerted activation of antioxidant systems. Indeed, although not significantly, there appear to occur a time-dependent reduction of TBARs levels in ethanol-treated cells (**Figure 4D**).

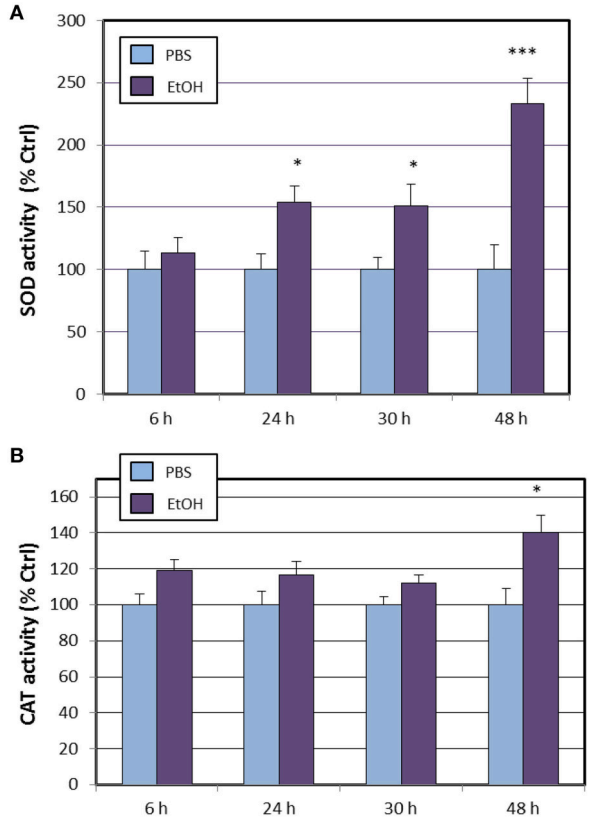

FIGURE 1 | Effects of ethanol (EtOH, 0.1%) on total SOD (A) and catalase (B) activities in HT22 cells at different times after first exposure to ethanol. Results correspond to the mean ± SEM of four different experiments. *, and ***$p < 0.05$ and $p < 0.005$ compared to vehicle (PBS), respectively.

Effects of Ethanol on the Time-course of HT22 Cell Proliferation and Resistance to Excitotoxicity

We finally analyzed the effects of different doses of ethanol treatment on HT22 cells proliferation. Results summarized in **Figure 5** shows that HT22 cells reach the maximal cell index (CI_{max}) after 120–160 h of seeding in the conditions of the present experiments (**Figure 5A**). In the presence of 0.1% ethanol, maximal CI values were similar between PBS- and ethanol-treated cells. However, when challenged with a 10-times higher dose of ethanol, a significant reduction of cell proliferation was observed, indicating an important degree of toxicity by this dose of ethanol (**Figure 5A**), likely caused by an excessive oxidative stress, which overcame the cytoprotective effects of endogenous antioxidant defense induced by low ethanol exposure.

In order to assess whether changes in antioxidant gene expression might confer resistance to toxic oxidative insults, we used glutamate as it has been reported to cause excitotoxicity in HT22 cells (He et al., 2013) and oxidative stress secondary

to cysteine depletion (Li et al., 1998). First, using non-linear regression to a four parameter logistic equation, we determined the IC50 value for glutamate toxicity against cell index, and we found a value of 19.5 mM (**Figure 5B**). Then, we assayed a glutamate concentration of 20 mM in ethanol (0.1%)-treated cells, while monitoring real time cell proliferation. We observed that pre-exposure to 0.1% ethanol significantly prevented glutamate-induced cell death to about 80% of ethanol-treated cells, compared to around 90% cell death in the absence of EtOH. Therefore, we concluded that ethanol efficiently reduced cell death caused by glutamate-induced excitotoxicity.

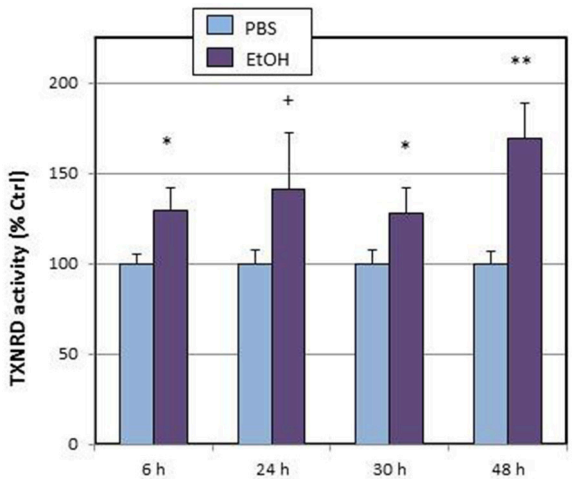

FIGURE 2 | Effects of ethanol (EtOH, 0.1%) on total TXNRD activity in HT22 cells at different times after first exposure to ethanol. Results correspond to the mean ± SEM of four different experiments. $^{+}$, *, **$p < 0.1$, $p < 0.05$, and $p < 0.01$ compared to vehicle (PBS), respectively.

DISCUSSION

Our present results demonstrate that treatment of hippocampal HT22 cells with sub-toxic doses of ethanol modifies the expression of specific genes within the classical, thioredoxin, and glutathione systems. We also show that these transcriptional changes are accompanied by consistent modifications in enzyme activities of the three systems and modulation of cellular antioxidant status. First, we observed that ethanol exposure stimulated gene expression of both superoxide dismutase genes (*Sod1* and *Sod2*), and soon increased total SOD activity. The effects of ethanol exposure on the activity of superoxide dismutase are controversial, with reports of an increase (Somani et al., 1996; Enache et al., 2008), no change (Gonenc et al., 2005), or a decrease (Ledig et al., 1981), depending on the brain region, the dose and the duration of ethanol exposure. However, our results are in agreement with previous results showing significant increases in superoxide dismutase and catalase activities in the hippocampus of rats receiving acute intraperitoneal injections

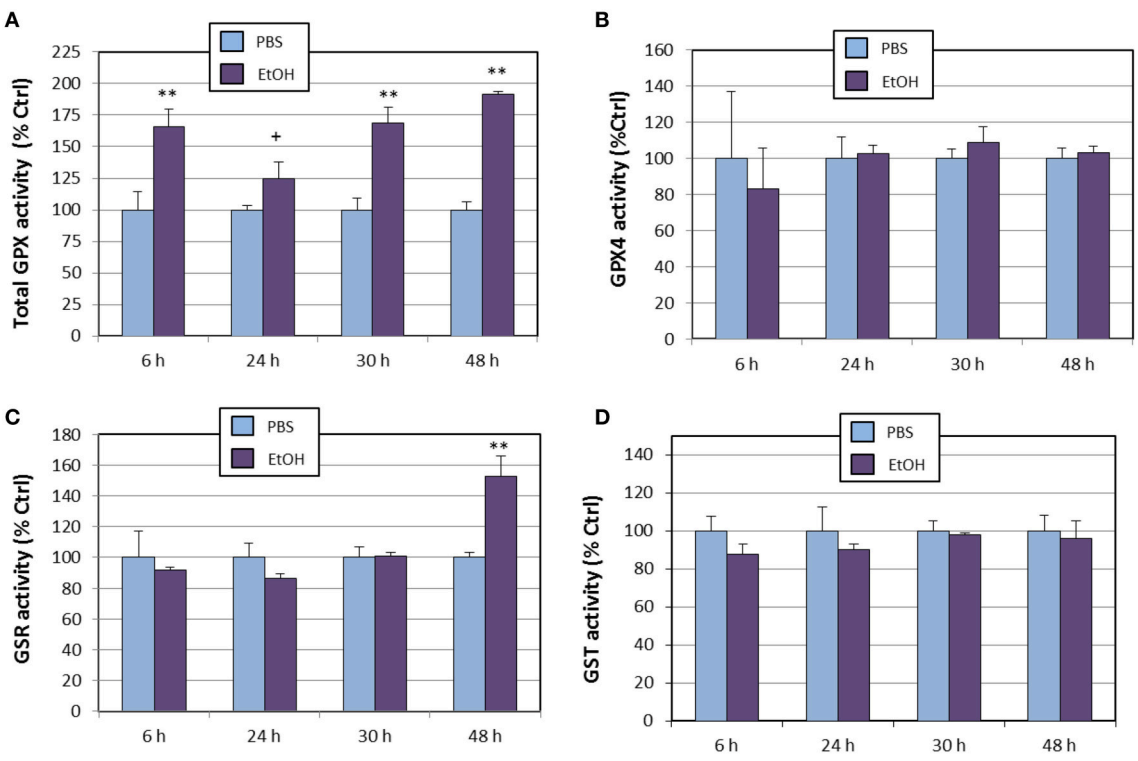

FIGURE 3 | Effects of ethanol (EtOH, 0.1%) on total GPX (A), GPX4 (B), GSR (C), and GST (D) activities in HT22 cells at different times after first exposure to ethanol. Results correspond to the mean ± SEM of four different experiments. $^{+}$, **$p < 0.1$ and $p < 0.01$ compared to vehicle (PBS), respectively.

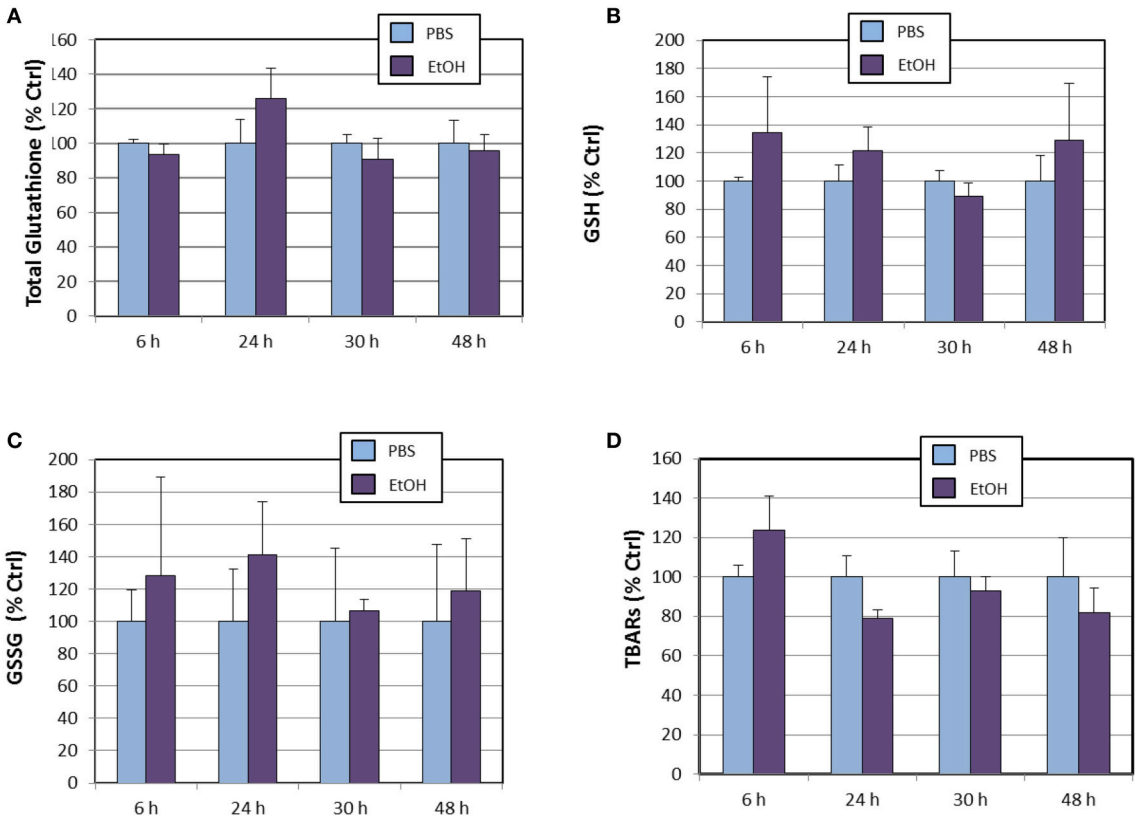

FIGURE 4 | Effects of ethanol (EtOH, 0.1%) on total glutathione (A), reduced glutathione (B), oxidized glutathione (C), and TBARs (D) levels in HT22 cells at different times after first exposure to ethanol. Results correspond to the mean ± SEM of four different experiments.

of ethanol (1.5 g/kg) (Enache et al., 2008). Similar results were observed in the rat cortex in response to acute ethanol administration (Somani et al., 1996). Interestingly, in our present study, although *Cat* gene expression was not altered by ethanol, a significant increase in enzyme activity was observed by the end of the experiment, which suggests a post-translational modulation of catalase, perhaps associated to increase H_2O_2 as a result of SOD activation. The existence of factors modulating catalase activity in response to oxidative stress has been demonstrated in different cell lines (Uenoyama and Ono, 1973; Cao et al., 2003). Several studies have shown that catalase interacts with c-Abl and Arg non-receptor tyrosine kinases, upon activation by H_2O_2through a mechanism dependent on protein kinase Cδ (Sun et al., 2000; Cao et al., 2003). The functional significance of these interactions are supported by the demonstration that cells deficient in both c-Abl and Arg exhibit substantial increases in H_2O_2 levels and a marked increase in H_2O_2-induced apoptosis (Cao et al., 2003).

Within the thioredoxin system we observed that the expression of the gene encoding for cytoplasmic thioredoxin reductase (*Txnrd1*) was significantly increased by ethanol from 6 h, while thioredoxin reductases of mitochondrial and endoplasmic reticulum origins were unaffected or down-regulated. Paralleling these observations, an equivalent increase in tTXNRD activity was observed shortly after the upregulation of *Txnrd1* expression was observed. The finding that tTXNRD activity was augmented is consistent with the fact that cytosolic thioredoxin reductase (encoded by *Txnrd1* gene) is the most abundant isoform in nerve cells (Arnér and Holmgren, 2000; Turanov et al., 2010). Another interesting observation was the dramatic down-regulation of *Txnip* gene expression in response to ethanol. It is known that *Txnip* gene encodes for the thioredoxin interacting protein, a 55 kDa protein that stabilizes reduced thioredoxin and keeps it inactive, thereby functioning as an endogenous inhibitor (Yoshihara et al., 2014). Despite ethanol does not alter thioredoxin genes (*Txn1* and *Txn2*) expression in HT22 cells, reduction in the amount of TXNIP would obviously increase free thioredoxin (Trx) proteins, which would enhance its ROS buffering capacity. This effect is physiologically relevant since mammalian TRXRD reduces oxidized substrates, such as Trx and H_2O_2, but also other non-disulfide-containing molecules, such lipid hydroperoxides and other hydroperoxides even independently of Trx, but coupled to selenocysteine or selenodiglutathione reduction (Björnstedt et al., 1995; Arnér and Holmgren, 2000), which notably increases the antioxidant spectrum of TRXR.

A family of proteins related to Trx and regulated by ethanol is peroxiredoxins. Peroxiredoxins contain two conserved cysteines in their active site and utilize Trx as reductant (Rhee et al., 2005), therefore, tightly linked to Trx oxidative status (Hawkes

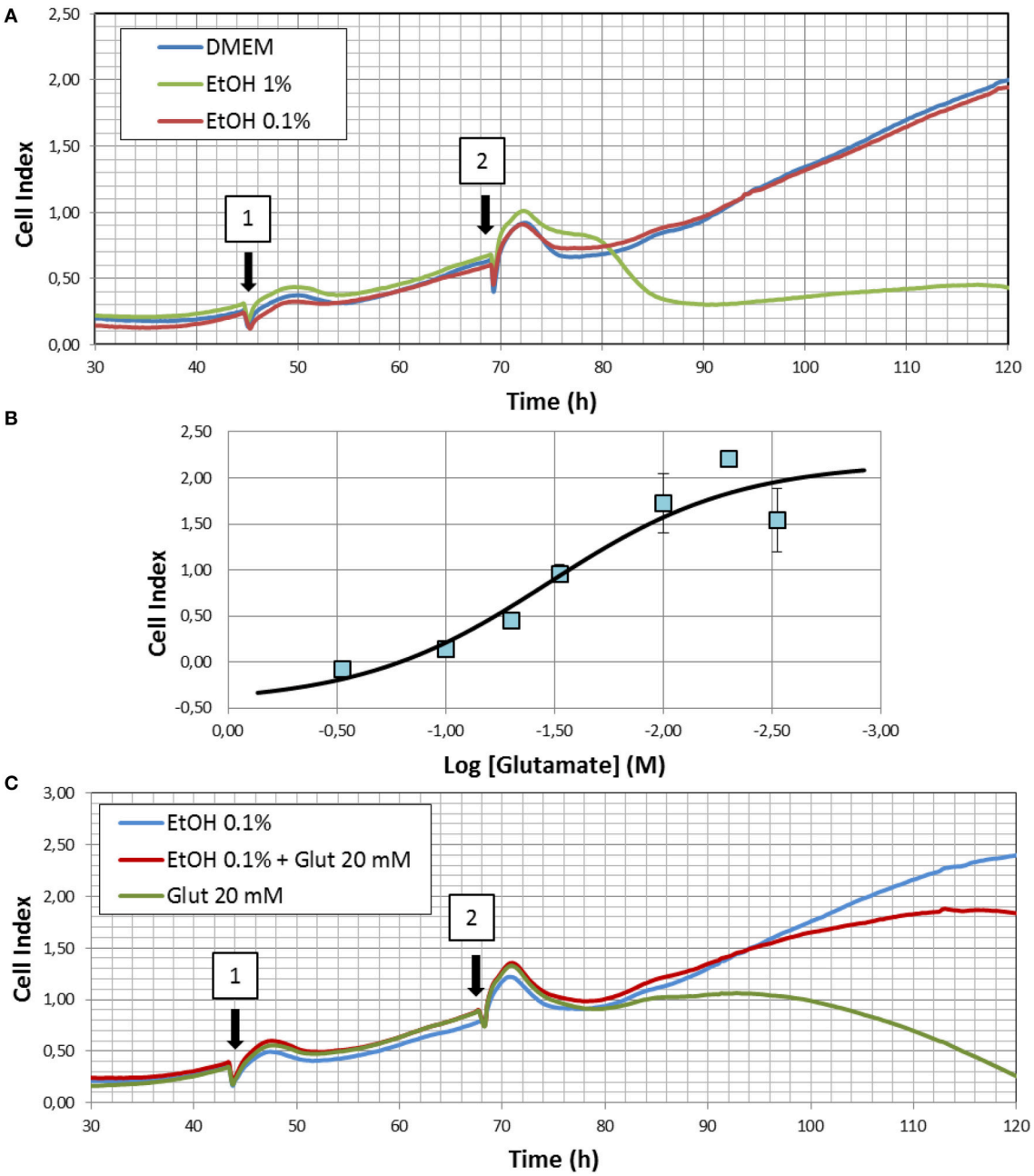

FIGURE 5 | (A) Representative experiment showing the effects of 0.1 and 1% ethanol (EtOH) on real time cell proliferation. Arrows indicate the time where media were replaced by either DMEM or EtOH 0.1% in 1 or DMEM, EtOH 0.1, or EtOH 1% in 2. **(B)** Dose-response curve for glutamate toxicity in HT22 cells. Results summarize data from three different experiments and are indicated as mean ± SEM. **(C)** Neuroprotective effects of 0.1% ethanol. Illustrated correspond to a representative experiment of glutamate excitotoxicity in the presence or absence of ethanol. Arrows indicate the time where cells were exposed to either DMEM, EtOH 0.1, or EtOH 0.1% + glutamate in 1, or EtOH 0.1%, EtOH 0.1% + glutamate, or glutamate alone in 2. Three different replicates were performed for experiments illustrated in **(A,C)**.

et al., 2013). Currently six peroxiredoxins genes have been described in mammals, with *Prdx2–5* genes being expressed in brain (Hattori et al., 2003; Rhee et al., 2005). We have observed that ethanol treatment brings about a generalized and significant down-regulation (18–39%) of all peroxyredoxin genes. These transcriptional alterations occurred soon after ethanol exposure (from 6 h) and lasted until 48 h. Noticeably, *Srxn1* gene expression (which encodes for sulfiredoxin, the main protein responsible for reactivation of oxidized peroxiredoxins) remained unaffected in response to ethanol. At present, we have no explanation for this concerted reduction of peroxiredoxins but a plausible hypothesis is that by down-regulating their expression (at the same time *Txnrd1* expression increases and *Txnip* expression decreases), reduction of Trx is mostly under

the control of thioredoxin reductase activity, with lower amounts of reduced thioredoxin being used to reduce peroxiredoxins. As mentioned before, peroxiredoxins become oxidized upon ROS attack, and their reduction is coupled to oxidation of thioredoxin (Rhee et al., 2005). Holistically, such a mechanism will help to maintain higher cytosolic levels of reduced thioredoxin. Additional experiments, including determination of cytosolic levels of oxidized and reduced thioredoxin in response to ethanol, will help to unravel the physiological significance of these findings.

The last antioxidant system modulated by ethanol in HT22 cells is glutathione/glutaredoxin system. We observed that soon after ethanol exposure, expression levels of *Gpx1* increased, and remained higher than in PBS throughout the experiment. Paralleling these findings, tGPx activity was higher in ethanol-treated cells from 6 h, therefore, correlating changes in expression levels. The increase in tGPx activity was likely attributable to GPx1 activity, since GPx4 activity (and gene expression) remained unaffected. *Gclc* gene expression also exhibited a significant increase in ethanol-treated cells, though in this case the response was delayed compared to *Gpx1* gene, and was observed only after 30 h ethanol treatment. Clearly, this modulation of the glutathione/glutaredoxin system enhances the cellular antioxidant potential attributable to glutathione in neuronal cells, at the same time that provides an efficient strategy to ensure the reduction of oxidized glutathione. Indeed, in the present study, we have observed that levels of reduced (and also oxidized) glutathione remained unchanged in spite of the two ethanol challenges and also that TBARs levels were not different from those observed in PBS-treated cells throughout the experiment. These observations pinpoint to an efficient ROS-buffering capacity in HT22 cells in response to sub-toxic ethanol exposure. Interestingly, in agreement with our results, *in vivo* studies performed in the rat cerebral cortex and corpus striatum have shown that ethanol (1.6 g/kg) significantly increases total GPx (as well as SOD) activity (Somani et al., 1996).

It is well-known that an important effect of ethanol is to increase the generation of ROS, including superoxide and the hydroxyethyl radical (Das and Vasudevan, 2007). Generation of ethanol-derived ROS is expected to interact with cellular targets, particularly with membrane lipids, giving rise to lipid hydroperoxides, which, in turn, initiate a self-propagating oxidative damage (Niki et al., 2005). Therefore, it is expected that levels of lipid-related oxidative species progressively augment as time progresses. Although ROS are generally considered to exert deleterious effects, it is becoming increasingly evident that ROS may serve second messengers implicated in signaling processes, and participate in a number of normal physiological phenomena (Dröge, 2002). Thus, it is likely that ethanol-derived oxidized metabolites may be responsible for triggering transcriptional signals to boost the expression of components of cellular antioxidant systems, as we have previously observed for lipoperoxides derived from docosahexaenoic acid (Casañas-Sánchez et al., 2014). Indeed, It is known that some of the genes studied here, and upregulated by ethanol, contain "antioxidant response elements" (ARE) in their promoter regions (Kaspar et al., 2009; Hawkes et al., 2013). In this sense, recent studies have demonstrated that NF-E2-related factor 2 (Nrf2) is the master transcription factor for the regulation of ARE in different tissues, including the brain (Kobayashi and Yamamoto, 2005; Singh et al., 2010; Zhang et al., 2013).

Overall, we may conclude that sub-toxic ethanol exposure enhances global antioxidant capacity of hippocampal neurons by at least three mechanisms: (1) by enhancing the expression and activity of the generic system through up-regulating superoxide dismutase expression (2) by increasing thioredoxin reductase 1 expression, the most abundant isoform in neuronal tissue, at the same time that down-regulates *Txnip* and peroxiredoxins expression, and (3) by upregulating glutathione peroxidase 1 and glutathione-*S*-reductase genes expression. Taken together these observations led us to envisage that hippocampal cells become more resistant to oxidative insults after being exposed to acute sub-lethal ethanol. Indeed, recent *in vivo* evidence have shown that ethanol pre-conditioning render brain tissue more resistant to oxidative damage in animal models such as the ischemia-reperfusion injury in gerbil and rat models (Liao et al., 2003; Wang et al., 2007), pro-inflammatory lipopolysaccharide-injected rats (Singh et al., 2007), or even mice models of Alzheimers disease (Wang et al., 2006).

Further, in brain cultures, non-neurotoxic alcohol exposure blocks excitotoxic receptor-mediated neurodegeneration triggered by NMDA exposure (Chandler et al., 1993; Cebere and Liljequist, 2003; Belmadani et al., 2004). Effects of alcohol pre-conditioning on inflammatory protein ($gp120_{IIIB}$)-induced neurotoxicity have also been explored in organotypic slices of rat hippocampus-entorhinal cortex, two brain regions significantly impacted in Alzheimers disease and other dementias (Collins et al., 2000, 2010). More recently, Muñoz and coworkers have shown that low concentrations of ethanol protect against synaptotoxicity induced by Aβ in hippocampal neurons (Muñoz et al., 2015). In line with this, we show here that ethanol (0.1%) can prevent excitotoxicity induced by glutamate exposure, which is in consonance with the results reported for NMDA exposure in rat primary cultured cells (Chandler et al., 1993) or in rat cerebellar granular cells (Cebere and Liljequist, 2003) in a similar range of concentrations and time-course as used here.

Different mechanisms have been proposed to underlie the neuroprotective effects of sub-lethal ethanol exposure. Overall, an emerging hypothesis is that alcohol pre-conditioning-induced neuronal survival mechanisms involve induction of heat-shock proteins (HSP27 and/or HSP70) upon ROS generation, and that HSP-dependent protection is intimately associated to selective protein kinase C (PKCα and PKCδ) and FAK (focal adhesion kinase) activation, NOS (nitric oxide synthase), the focal adhesion complex, and stabilization of the cytoskeleton (Collins et al., 2009). However, the present study is the first demonstrating that ethanol provides resistance to oxidative insults through mechanisms directly linked to transcriptional modulation of specific components within the set of antioxidant systems. Therefore, under this paradigm, ethanol may be considered an "*Indirect Antioxidant,*" as it has been coined for molecules which although lacking antioxidant activity *per se*, are capable to potentiate cellular antioxidant capacity by enhancing gene expression (Jung and Kwak, 2010). This newly identified neuroprotective (and perhaps anti-excitotoxic) effect of ethanol *in vitro* is clearly hormetic and might be clinically relevant

(Rattan, 2004), but certainly it requires further studies before its significance and window of application is completely understood.

AUTHOR CONTRIBUTIONS

VC and JP performed genetic analyses. DQ and VC performed analyses of enzyme activities. MD designed the study, analyzed the data and drafted the manuscript.

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