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REVIEW ARTICLE

Chemistry and biochemistry of lipid peroxidation products

F. GUÉRAUD[1], M. ATALAY[2], N. BRESGEN[3], A. CIPAK[4], P. M. ECKL[3], L. HUC[1], I. JOUANIN[1], W. SIEMS[5] & K. UCHIDA[6]

[1]*UMR1089 Xénobiotiques, INRA, Toulouse, France,* [2]*Institute of Biomedicine, Physiology, University of Eastern Finland, Kuopio, Finland,* [3]*Department of Cell Biology, University of Salzburg, Salzburg, Austria,* [4]*Rudjer Boskovic Institute, Zagreb, Croatia,* [5]*KortexMed Research, Institute of Physiotherapy and Gerontology, Bad Harzburg, Germany, and* [6]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan*

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Abstract
Oxidative stress and resulting lipid peroxidation is involved in various and numerous pathological states including inflammation, atherosclerosis, neurodegenerative diseases and cancer. This review is focused on recent advances concerning the formation, metabolism and reactivity towards macromolecules of lipid peroxidation breakdown products, some of which being considered as 'second messengers' of oxidative stress. This review relates also new advances regarding apoptosis induction, survival/proliferation processes and autophagy regulated by 4-hydroxynonenal, a major product of omega-6 fatty acid peroxidation, in relationship with detoxication mechanisms. The use of these lipid peroxidation products as oxidative stress/ lipid peroxidation biomarkers is also addressed.

Keywords: *Lipid peroxidation, alkenals, 4-hydroxy-2-nonenal, glutathione, apoptosis, adducts, volatile aldehydes*

Abbreviations: *AA, Arachidonic acid; ABAD, amyloid β peptide binding-alcohol dehydrogenase; AD, Alzheimer disease; ADP, Adenosine diphosphate; AGEs, Advanced glycation end products; AKR, Aldo/keto reductase; ALDH, Aldehyde deshydrogenase; ALEs, Advanced lipid peroxidation end products; ALDH, Aldehyde deshydrogenase; AMD, Age-related macular degeneration; AMPK, AMP-activated protein kinase; ANT, Adenine nucleotide translocator; ATP, Adenosine triphosphate; CL, Cholesterol; COX, Cyclooxygenase; Cys, Cysteine; DA-GPE, Diacyl-glycero-phosphoethanolamine; DDE, 2,4-decadienal; DHA, Docosahexaenoic acid; DHN, 1,4-dihydroxy-2-nonene; DISC, Death Inducing Signalling Complex; DODE, 9,12-Dioxo-10(E)-dodecenoic acid; EDE, 4,5-Epoxy-2(E)-decenal; EGFR, Epidermal Growth Factor Receptor; Gly, Glycine; GPx, Glutathione peroxidase; GSH, Glutathione; GST, Glutathione transferase; GS-DHN, 1,4-Dihydroxynonane-glutathione conjugate; GS-HNA, 4-Hydroxynonanoic acid-glutathione; GS-HNAL, 4-Hydroxynonanoic acid-glutathione lactone; HDDE, 4-Hydroxy-2,6-dodecadienal; HHE, 4-hydroxy-2(E)-hexenal; His, Histidine; HNA, 4-Hydroxynon-2-enoic acid; HNE, 4-hydroxy-2(E)-nonenal; HPHE, 4-hydroperoxy-2(E)-hexenal; HPNE, 4-hydroperoxy-2(E)-nonenal; HPOPE, Hydroperoxy-9,11-octadecadienoic acid; HRP, Horseradish peroxidase; HSP, Heat shock protein; isoK, Isoketals; JNK, c-Jun kinase; LDL, Low-density lipoprotein; LOOH, Lipid hydroperoxides; LPO, Lipid peroxidation; LOX, lipoxygenase; Lys, Lysine; MDA, Malondialdehyde; MPO, Myeloperoxidase; MRP, Multiresistance drug associated protein; NADPH, Nicotinamide adenine dinucleotide phosphate; OHE, 4-Oxo-2(E)-hexenal; ONE, 4-Oxo-2(E)-nonenal; PDI, Protein disulphide isomerase; PE, Phosphatidylethanolamine; PDGFR, Platelet-Derived Growth Factor Receptor; PRX, Peroxiredoxin; PS, Phosphatidylserine; PUFA, Polyunsaturated fatty acid; RDH, Retinol dehydrogenase; RLIP, Ral-interacting protein; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; RPE, Retinal pigment epithelial cells; SCE, Sister chromatid exchange; SID, Streptozotocin-induced diabetic; SMC, Smooth muscle cell; SOD, Superoxide dismutase; Tcf, T-cell specific transcription factor; TNF, Tumour Necrosis Factor; TOG, Thiadiazabicyclo-ONE-GSH-adduct; TRAIL, TNF-related-apoptosis-inducing-ligand.*

Correspondence: Dr Françoise Guéraud, INRA UMR1089 Xénobiotiques, BP93173, 31027 Toulouse Cedex 3, France. Tel: + 33 561 28 50 05. Fax: +33 561 28 52 44. Email: fgueraud@toulouse.inra.fr

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Introduction

Oxidative stress and resulting lipid peroxidation is involved in various and numerous pathological states including inflammation, atherosclerosis, neurodegenerative diseases and cancer. Free radicals produced during lipid peroxidation have some very local effects, because of their short life, but the breakdown products of lipid peroxides may serve as 'oxidative stress second messengers', due to their prolonged half-life and their ability to diffuse from their site of formation, compared to free radicals. Those breakdown products, mostly aldehydes, such as malonaldehyde, hexanal, 4-hydroxynonenal or acrolein have received a lot of attention because they are most of the time reactive compounds. They have been considered for a long time as toxic end-products of lipid peroxidation. We know now that they play a real powerful biological role in cell signalling under both pathological and physiological conditions, mainly in cell cycle regulation. Due to their chemical reactivity, those breakdown products can make covalent modifications on macromolecules such as nucleic acids, protein and lipids and exert some biological effects. They also serve as biomarkers of lipid peroxidation/oxidative stress. This review aims to give recent advances concerning those aspects.

Chemistry

Lipid peroxidation (LPO) has been studied by chemists as a degradation process of natural compounds (fats, oils) and especially as a factor in the deterioration of food quality (odour, flavour, colour, texture, toxicity) [1,2].

LPO occurs *in vivo* in plants by the activation of enzymes as a result of mechanical injury or after infection by fungi, bacteria or viruses. It is also present in germinating seeds [3–6].

In mammals, LPO is related to injury and inflammation and is often the oxidative deterioration of lipids, mainly cellular membrane lipids (phospholipids, cholesterol). This can lead to changes in the permeability and fluidity of the membrane lipid bilayer and dramatically alter cell integrity [7]. Yet, LPO has been implicated as a cause and effect of cellular damage [8,9]. The relationship between LPO and diseases is increasingly mentioned but remains to be conclusively established in most cases, because the biological and chemical conditions of LPO are complex and not fully defined [7,10,11]. Cholesterol peroxidation occurring at the lipoprotein LDL has attracted attention with its implication in atherosclerosis [10,12].

Indeed, LPO refers to different mechanisms and can be classified as enzymatic, non-enzymatic non-radical peroxidation and non-enzymatic free-radical mediated peroxidation [13].

Cholesterol and the fatty acid part of phospholipids are the most commonly referred LPO substrates [14]. Peroxidation conditions also play a major role and LPO may be induced by endogenous or exogenous factors: enzymes, radical species, metal ions, UV radiations, heat, radical-initiating chemicals, drugs and a wealth of compounds referred to as 'reactive oxygen species (ROS)' and 'reactive nitrogen species (RNS)'. The free-radical non-enzymatic peroxidation of polyunsaturated fatty acids (PUFAs) has been extensively studied. Among PUFAs, the ω6 linoleic acid (18:2 n-6) is the most abundant *in vivo* in plants and is essential in mammals and arachidonic acid (20:4 n-6) plays a crucial role in inflammation response.

LPO complexity also resides in the many compounds that can result. Lipid hydroperoxides, the primary compounds, are unstable and decompose to more stable but still reactive and potentially toxic secondary compounds.

In the following parts of this review, the proposed mechanisms of LPO with focus on PUFAs peroxidation by the non-enzymatic free radical mechanism and the resulting secondary products will be discussed with references to reviews.

Proposed mechanisms of PUFA peroxidation

The non-enzymatical free radical mechanism occurs in three phases [15]:

- *Initiation*: abstraction of $H^{\cdot}$ radical from a lipid (LH) chain to give a lipid radical; an initiator is required. The formation of lipid radicals is particularly favourable when the lipid is a PUFA because the resulting radical is resonance stabilized.
- *Propagation*: the lipid radical can react with oxygen to give a lipoperoxyl radical ($LOO^{\cdot}$) which in turn reacts with a lipid to yield a lipid radical and a lipid hydroperoxyde (LOOH). LOOH are unstable: they generate new peroxyl and alcoxyl radicals and decompose to secondary products [9,16,17].
- *Termination*: It is a combination of radical species to give non-radical or non-propagating species.

Reactive oxygen species (ROS) exist under normal physiological conditions and could be produced in larger amounts *in vivo* when natural defences are overwhelmed. They could play a role in LPO initiation. The hydroxyl radical $^{\cdot}OH$ is the most reactive radical and was not clearly evidenced *in vivo*. The Fenton reaction and the Haber-Weiss reaction are both supposed to form the hydroxyl radical from hydrogen peroxide and metal species (iron, copper) [7,18,19]. The superoxide radical $O_2^{\cdot -}$ is produced

during metabolism and decreases antioxidant defence, but is unable to abstract hydrogen. Hydroperoxides could be further transformed into initiating species, namely peroxyl and alkoxyl radicals (ROO$^{\cdot}$ and RO$^{\cdot}$). The role of iron and copper in free radical mediated LPO is often mentioned [7,20,21].

Hypervalent iron complexes (ferryl and perferryl) have been suggested to be the active species in the systems generated from hydrogen peroxide and iron-containing proteins (haemoglobin, myoglobin, P450 cytochromes) [7,18,19].

Reactive nitrogen species (RNS) like nitric oxide, nitrous oxide and peroxynitrite are radical species which could also induce and, in some cases, limit LPO [22]. Recently, fatty acid dimers were suggested as new key intermediates during initiation and propagation [23,24].

LPO can be inhibited enzymatically or non-enzymatically [13,25]. Glutathione peroxidases (GPx), catalases and superoxide dismutases (SOD) are antioxidant enzymes. Vitamin E and vitamin C have radical scavenging properties and are able to inhibit the free-radical mechanism of LPO. Vitamin C is a hydrophilic antioxidant. Ascorbate, in conjunction with iron species, has been used to induce LPO in microsomes [26]. Vitamin E is a lipophilic antioxidant. It has been shown to have pro-oxidant activity *in vitro* [27,28]. Glutathione (GSH) is a major antioxidant and has also been associated with inhibition of LPO. Bilirubin could scavenge peroxyl radicals [29] and increased levels of bilirubin have been associated with inhibition of LPO [30].

The primary products hydroperoxides decompose by various mechanisms to a wealth of more stable secondary products with potential toxicity.

Secondary products

The peroxidation of PUFAs is complex because many fatty acids are present in different compartments in mammals. Esterbauer [10] estimated a range of 120–150 possible hydroperoxides. LOOHs can decompose to a variety of more stable compounds. The formation of alkoxyl radicals (LO$^{\cdot}$) prone to subsequent β-scission gives rise to short-chain products (C2 to C12, with a large range of chemical functionality) and modified chain lipid (carboxylic part). LOOH can also rearrange to hydroxyl- and epoxy-acids, dimerize or polymerize [8].

LPO-generated high-molecular weight compounds are hardly ever mentioned, probably because they are more difficult to analyse than short-chain volatiles, although they could also have deleterious effects [10,31,32].

Volatile products of PUFAs peroxidation. Volatile products formed by the peroxidation of PUFAs have attracted much attention, especially those of the ω6 and ω3 series. Complex mixtures are obtained from which the identification of compounds remains partial. Classes of compounds have been identified. Alkanes and also aldehydes, ketones, alcohols and furanes with multiple functionality were reported under various reaction conditions [8,31,33,34].

Aldehydes have received much attention because they are reactive and toxic. They are less unstable than the hydroperoxides and could diffuse from their site of formation [35].

Malondialdehyde (MDA). The enzymatic and the free-radical peroxidation of PUFAs which contain at least three double bonds, like arachidonic acid and DHA, could cleave to the bis-aldehyde malonaldehyde (MDA, Figure 1). Various mechanisms have been proposed [35] and bicyclic endoperoxides were suggested as possible intermediates [36]. MDA has been extensively mentioned in LPO studies and reported as a biomarker of the peroxidation of ω3 and ω6 fatty acids [33,35], yet with some restrictions concerning the possible existence of another biochemical origin [37].

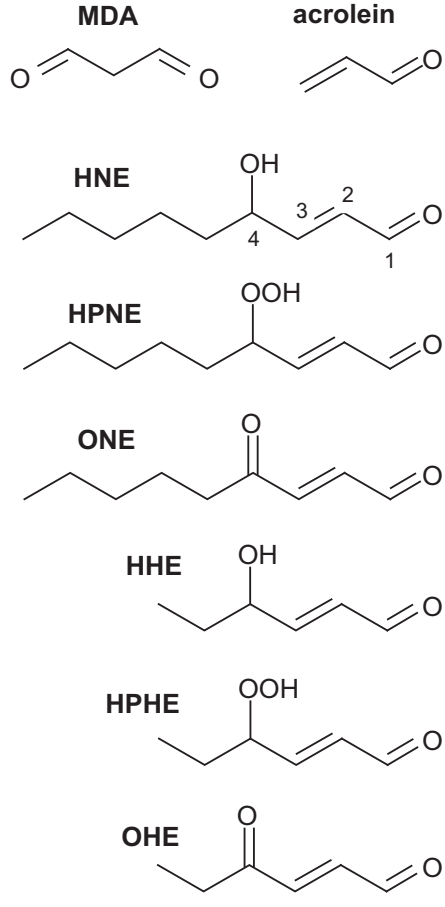

Figure 1. LPO aldehydes.

Other aldehydes. Many other aldehydes are formed during LPO: saturated, α,β-unsaturated (alkenals: acrolein, heptenal, …) [8,38], 4-hydroxy-α,β-unsaturated (4-hydroxy-alkenals), 4-hydroperoxy-α,β-unsaturated (4-hydroperoxy-alkenals), 4-oxo-α,β-unsaturated (4-oxo -alkenals), epoxy-α,β-unsaturated [16,31], conjugated dienes (2,4-dienals: 2,4-decadienal ...), ...

4-hydroxy-alkenals have three chemical functions: aldehyde (CHO), alkene (C2=C3 double bond) and secondary alcohols (OH at the chiral centre C4), which make them highly reactive. Carbon C3 is an electrophilic site for Michael-type addition, carbon C1 is also electrophilic and a redox centre (oxidation to carboxylic acid, reduction to alcohol) and carbon C4 holds the alcohol function.

4-hydroxy-2(E)-nonenal (HNE, Figure 1) is a representative compound arising from the peroxidation of ω6 fatty acids [39], while 4-hydroxy-2(E)-hexenal (HHE, Figure 1) is formed from ω3 fatty acids.

HNE was formed under various conditions like auto-oxidation and stimulated microsomal LPO [8]. LPO mechanisms of formation have been proposed [24]. HNE was found in food [40] and detected *in vivo*. Several pathologies have been associated with elevated levels of HNE. Esterbauer and Weger [41] intensively studied HNE and synthesized several 4-hydroxy-alkenals in the late 1960s. Other synthetic methods have been developed since then [35,42]. 4-hydroperoxy-2(E)-nonenal (HPNE, Figure 1) was proposed as an HNE precursor [43,44] and is also a product of plant enzymatic biotransformation [45].

HHE has been identified during the auto-oxidation of ω3 fatty acids (linolenate, DHA) [32,46] but also as a metabolite of a plant alkaloid [47]. 4-hydroperoxy-2(E)-hexenal (HPHE, Figure 1) was hypothesized as a precursor of HHE by comparison to the work on HPNE [32].

More recently, 4-oxo-2(E)-nonenal (ONE, Figure 1) was detected in the decomposition of a linoleic hydroperoxide: 13(S)-hydroperoxy-9,11-octadecadienoic acid (13(S)-HPODE). HPNE was suggested to be an intermediary compound during the *in vitro* process. ONE could be the major product of linoleic hydroperoxide decomposition [43]. ONE is electrophilic and is able to react with nucleobases [48]. It was observed *in vitro* by free radical decomposition of both (S)-regioisomers of linoleic acid hydroperoxides (9(S)-HPODE and 13(S)-HPODE) [17], but not *in vivo* in its free form. *In vivo*, only ONE metabolites have been detected [49]. This could be explained by a higher reactivity of ONE compared to hydroxyalkenals.

4-oxo-2(E)-hexenal (OHE, Figure 1) has been found in arthropods and was chemically synthesized in the late 1960s [50]. It was recently reported as a product of LPO through the identification of a deoxyguanosine adduct [51] and was detected in ω3-rich cooked food [52].

Other aldehydes with various functionalities have been mentioned as a result of linoleic acid peroxidation [17]. 4,5-epoxy-2(E)-decenal (EDE) was formed from the auto-oxidation of arachidonic acid [53] and was one of the detected odourant aldehydes [54]. 9,12-dioxo-10(E)-dodecenoic acid (DODE) was suggested as a carboxylic acid break-down product from the *in vitro* peroxidation of a linoleic hydroperoxide as the (E)-isomer [55] and was earlier reported in lentil seed as the (Z)-isomer [56].

In conclusion, lipid peroxidation represents a set of complex enzymatic and non-enzymatic suite of reactions, which can lead to a wealth of compounds with different chemical functions, molecular weight, physico-chemical properties, reactivity, toxicity, etc, …

The biological implications are of major importance and range from food quality to health concerns, with possible implications in the occurrence of various diseases. Many studies report on the formation and subsequent reactions of alkenals, as these are highly reactive compounds, which could further exert toxicity by biotransformation and adduction to biomolecules (proteins, DNA).

Biotransformations of LPO product alkenals

Biotransformations of LPO secondary products has been reported previously in the excellent review written by Esterbauer et al. [35] in 1991. More recently, reviews focused on HNE *in vitro* and *in vivo* biotransformations have been published [57,58] in the special issue of *Molecular Aspects of Medicine* on '4-hydroxynonenal: a lipid degradation product provided with cell regulatory functions'. A review on acrolein has been written in 2008 by Stevens and Maier [38]. We provide here a quick overview on biotransformations and transport of LPO secondary products, mainly 4-hydroxy- and 4-oxo-alkenals, MDA and acrolein with a specific focus on recent advances in this field.

Metabolism

LPO secondary products can, for most of them, travel across membranes by passive diffusion. Most of LPO secondary products such as HNE, MDA or acrolein are reactive compounds because they bear electrophilic properties.

4-hydroxy- and 4-oxo-alkenals. Most of them readily react with the nucleophilic tripeptide glutathione (GSH) by Michael addition to form GSH conjugates. This conjugation takes place in most cells and tissues, but it seems that liver is more efficient than other tissues. This reaction can occur spontaneously but it is several hundred times faster when catalysed by glutathione-*S*-transferases (GSTs). It seems that

some of the GST isoforms have had a specific evolution towards LPO products, as is the case for GST A4–4. GST A4–4 presents high substrate chemospecificity, but an intriguingly low stereoselectivity, as HNE is formed as a racemic mixture [59].

Michael additions can occur also to cellular proteins, cysteine sulphydryl groups being the primary soft nucleophilic targets of those compounds. This is in the case of HNE, but it is likely to occur for other 4-hydroxyalkenals, the double bond between C2 and C3 can be saturated giving 4-hydroxynonanal that further rearranges in its hemi-acetal form [60]. The enzyme involved is the NAD(P)H-dependent alkenal/one oxidoreductase, also known as leukotrieneB4 12-hydroxydehydrogenase/15-oxoprostaglandin 13-reductase.

Other modifications can concern the aldehyde function that can be either reduced into alcohol or oxidized into acid, involving alcohol dehydrogenase or aldo/keto reductases (AKR) and aldehyde dehydrogenase, respectively, for the formation of 1,4-dihydroxynonene (DHN) and 4-hydroxynonenoic acid (4-HNA) in the case of HNE. The acidic metabolite of HNE can undergo beta-oxidation. Aldose reductase was shown to reduce both HNE and its GSH conjugate [61]. Very recently, Zhong et al. [62] reported that the enzyme AKR1B10, present in the intestinal tract, was able to reduce dietary aldehydes [62]. Marchette et al. [63] reported the involvement of retinol dehydrogenase 12 for the reduction of HNE in photoreceptor inner segments of the retina. The defect of this enzyme is implicated in the pathogeny of Leber congenital amaurosis. Demozay et al. [64] reported the role of fatty aldehyde dehydrogenase in HNE-induced modifications in insulin signalling.

Metabolization of secondary lipid peroxidation products such as HNE in most cells and tissues is rapid and complete. GSH conjugation seems to be the primary and major step. If GSH is depleted by buthionine sulphoximine for instance or by a concomitant oxidative insult, there is a reduction in GS-HNE conjugate together with an increase in unmetabolized HNE and in HNE toxicity [65]. On the other hand, when the oxidative insult occurs some time before treatment of cells by HNE, preconditioned cells acquire resistance to HNE-induced apoptosis by metabolizing and excluding HNE at a higher rate when compared to non-preconditioned cells [66,67]. This primary conjugation step can be further completed by reduction of the aldehyde function by AKR, giving 1,4-dihydroxynonane-glutathione (GS-DHN) conjugate, or by oxidation of the aldehyde by aldehyde deshydrogenase (ALDH), giving 4-hydroxynonanoic acid-glutathione (GS-HNA), which can also exist in the lactone form (GS-HNAL). The oxidized derivatives GS-HNA and GS-HNAL can be further metabolized by cytochrome P450 4A, giving omega-hydroxylated and carboxylated metabolites [57,68–70]. All these metabolites can serve as biomarkers of lipid peroxidation in disease states [71]. The glutathione moiety of all these adducts is further metabolized into mercapturic acid in the kidney and the resulting metabolites are excreted into urine [38,72–74]. The reduced mercapturic acid of HNE, namely 1,4-dihydroxynonane-mercapturic acid (DHN-MA), is the major metabolite found in urine [73,75]. Those mercapturic acids can be used as biomarkers of oxidative stress/LPO available by non-invasive sampling techniques [49,76,77]. They are also used to reflect consumption of 'peroxidable' diet associated with colon cancer risk [78]. Recently, Kuiper et al. [79] reported the presence of mercapturic acids of HNE and ONE in the urine of smokers upon smoking cessation.

It is noteworthy that alkenal conjugation to GSH is a reversible reaction and that conjugates can be considered as 'transport forms' which can give back the parent compound possibly far away (other cell, other tissue) from its generation place. This reversible reaction is suppressed if the aldehyde function is reduced, underlining the importance of the enzymes involved.

In vivo biotransformation studies of dietary aldehydes such as HNE are still lacking. However, Goicoechea et al. [80] reported recently the use of an *in vitro* digestion model to study the bioaccessibility of oxygenated α,β-unsaturated aldehydes. Those compounds remained unaltered after digestion and could then be bioaccessible in the gastrointestinal tract and eventually reach the systemic circulation. This has been indirectly confirmed by the fact that enormous quantities of DHN-MA have been found in the urine of rats fed on a HNE-rich diet [78].

Conjugation of HNE to GSH is considered as a detoxication step, facilitating urinary excretion. However, Enoiu et al. [81] have reported that the metabolization of GS-HNE by gamma-glutamyl-transpeptidase giving the CysGly-HNE adduct induced a considerable increase in cytotoxicity. The authors attribute this cytotoxicity to a retro-Michael cleavage of the adduct, Cys-Gly-HNE being less stable than GS-HNE. Such a mechanism has also been reported for acrolein-mercapturic acid adducts in the kidney [82].

Acrolein. Acrolein is much more electrophilic than HNE and reacts with GSH much faster [35]. Conjugation with GSH, followed by mercapturic acid transformation of the GSH moiety, is the main pathway for its elimination. Mercapturic acids of acrolein can be reduced or oxidized, as is the case for HNE [38]. It seems that GST A4–4 can also catalyse the conjugation of GSH to acrolein [83]. Reduced mercapturic acid of acrolein is the major metabolite in urine, as is the case for HNE. As described above for other alkenals, acrolein can make adducts with proteins.

MDA. MDA is metabolized to CO_2 and water via transformation into acetaldehyde by ALDH, but it can be found unmodified in urine [77] or in plasma [84,85]. MDA can make adducts with lysine and serine residues.

Transport

Several studies have reported the transport of GS-HNE by transport proteins such as Multi Drug Resistance protein MRP1 [86] using cells over-expressing this protein or MRP2 [87] in freshly isolated hepatocytes. Recently, Miranda et al. [88] reported a protective role of ascorbate against HNE effect through the modulation of its MRP-mediated transport in human THP-1 monocytic leukaemia cells.

RLIP76 (Ral-interacting protein), an ATP dependent non-ABC multifunctional protein, is also involved in the transport of glutathione-electrophile conjugates in mammalian cells. This enzyme is believed to provide protection against different stressors including oxidant chemicals. This enzyme accounts for 80% of the GS-HNE conjugate transport, thereby counteracting the pro-apoptotic effect of HNE. This enzyme is frequently over-expressed in malignant cells [89–91]. In RLIP76 null mice, the transport of GS-electrophilic compound adducts is impaired by ~ 80%. In those mice, there is an accumulation of HNE and its GSH conjugate in tissues [91,92].

Reactivity of lipid oxidation products with macromolecules

DNA

LPO, the oxidative degradation of PUFAs of membrane lipids, leads to the formation of a variety of aldehydic breakdown products such as MDA, alkanals, 2-alkenals, 2,4 alkadienals and 4-hydroxyalkenals [8]. Since it is generated from most fatty acids with more than two double bonds [93], MDA quantitatively is the major product of lipid peroxidation, whereas the formation of other aldhydic LPO products is dependent on the parent ω-3, ω-6 and ω-9 polyunsaturated fatty acids, i.e. HHE arises from breakdown of ω-3 PUFAs, 4-hydroxynonenal (HNE) from ω-6 PUFAs and 4-hydroxyundecenal from ω-9 PUFAs [94,95]. Since ω-6 PUFAs are most abundant, HNE by far exceeds the levels of HHE and 4-hydroxyundecenal [96]. Compared to MDA, HNE is formed in up to 80-fold lower concentrations [21]. In toxicological terms, however, HNE appears to be more significant than MDA due to its higher electrophilicity [97] and its implication in pathological developments such as cancer, neurodegenerative diseases, arteriosclerosis and others (for a review see [98]). Due to their significant formation and their toxicological potential this contribution focuses on both MDA and HNE and briefly summarizes the findings on DNA adduct formation and chromosomal damage.

MDA. Mutagenicity of MDA to Salmonella typhimurium was first demonstrated in 1976 by Mukai and Goldstein [99] and later confirmed by other researchers [100,101]. The mutations observed were base pair substitutions as well as frameshift mutations [102]. By transfecting human fibroblasts with a shuttle vector, which had been allowed to react with MDA, and sequence analysis of the reporter gene after replication, Niedernhofer et al. [103] observed that the majority of mutations occurred at GC base pairs and that the most frequent mutations were large insertions and deletions and base pair substitutions.

MDA has further been shown to induce genotoxicity in mammalian cells, i.e. mutations in the mouse lymphoma assay [104], chromosomal aberrations and micronucleus formation in rat skin fibroblasts [105] and both single strand breaks and sister chromatid exchanges in CHO cells [106].

The mutational spectrum of MDA is related to its reaction with DNA at physiological pH to form guanosine, adenosine and cytidine adducts [107–109]. When reacting with guanosine both carbonyls react with nitrogen (N^2 and N1) to form pyrimido[1,2α] purin-10(3*H*)-one (M_1G), which is the most abundant MDA adduct. Adenosine and cytidine adducts arise from addition of one carbonyl with the exocyclic amino groups to form N^6-(3-oxopropenyl)deoxyadenosine (M_1A) and N^4-(3-oxopropenyl)deoxycytidine (M_1C), respectively. While M_1C is formed only in trace amounts, M_1A yields ~ 20% of M_1G [110]. M_1G adducts are detected in tissues from healthy humans [111,112], are mutagenic in bacteria and are a substrate for nucleotide excision repair [113].

HNE. After its identification as a cytotoxic product of the oxidation of liver microsomal lipids [94] a large body of evidence accumulated concerning the genotoxic action of HNE. It was demonstrated to induce significant amounts of DNA fragmentation [114], sister chromatid exchanges [114] and a dose-dependent increase in the number of mutations to 6-thioguanine resistance in Chinese hamster cells [115]. Furthermore, HNE proved to be a very potent inducer of the SOS response in the *Salmonella typhimurium* assay [116].

Further investigations carried out with metabolically competent primary hepatocytes revealed a very high sensitivity of this cell type, at least one order of magnitude higher than other mammalian cell types utilized before. The most sensitive indicator of genotoxic action was demonstrated to be sister chromatid

exchanges, followed by micronuclei and chromosomal aberrations [117]. In the same study also the genotoxic potential of 4-hydroxyhexenal and 4-hydroxyundecenal and the analogous aldehydes 2-*trans*-nonenal and nonanal were tested and turned out to be less genotoxic. Based on the structure of the aldehydes the authors concluded that the length of the lipophilic tail had no influence on chromosomal aberration induction, but appeared to influence the yield of SCE and micronucleus formation. Furthermore, the lack of the OH group (2-*trans*-nonenal) reduced the SCE-inducing potential to higher concentrations and the lack of both the OH group and the CC double bond did not result in a complete loss of SCE induction, but did not cause the formation of micronuclei and chromosomal aberrations. Based on these observations HNE appears to be the most potent mutagenic aldehydic lipid peroxidation product.

Studies with brain endothelial cell clones expressing different functions such as the blood–brain barrier resulted in a significant dose-dependent induction of micronuclei and chromosomal aberrations [118] at concentrations $\geq$ 1 μM, and a more recent study observed dose-dependent genotoxic effects in human adenoma cells as evidenced by the COMET assay [119].

Since HNE occurs in two stereoisomeric forms, (S)-HNE and (R)-HNE, and these are enantioselectively metabolized [120], both isoforms were tested in the primary rat hepatocyte assay, however, did not cause any differences of the dose response of chromosomal aberration induction [121].

When comparing the concentrations applied in the different investigations it becomes evident that the effects observed are dependent on the cell type utilized and that primary hepatocytes react most sensitively towards HNE, causing significant levels of sister chromatid exchanges, even at concentrations as low as 0.1 μM, a concentration which can be achieved under physiological conditions [96]. The reason for this high sensitivity most likely is associated with the two distinct pathways of HNE interaction with DNA.

When HNE directly interacts with the guanosine moiety of DNA, four isomeric 1,*N*-2-propano adducts are formed [122]. Second, 2,3-epoxy-4-hydroxy-nonanal can be generated from *trans*-4-hydroxy-2-nonenal by auto-oxidation or incubation with fatty acid hydroperoxides or hydrogen peroxide [123] and causes the formation of etheno adducts, i.e. 3,N^4-ethenodeoxycytidine, 1,N^6-ethenodeoxyadenosine and N^2,3-ethenodeoxyguanosine. Interestingly, 2,3-epoxy-4-hydroxy-nonanal is not a substrate of epoxide hydrolase [124] and could thus favour the formation of etheno adducts. In this respect the high sensitivity of hepatocytes may eventually be explained by the formation of 2,3-epoxy-4-hydroxy-nonanal in the course of biotransformation by cytochromes P-450 [123]. Moreover, the observation that the background level of propano adducts are particularly high in the liver [125] is in support of the high sensitivity of this organ. In fact, Hu et al. [126] demonstrated that propano adducts may even be causatively involved in hepatic carcinogenesis, since HNE preferentially forms adducts at codon 249 of the human p53 gene.

Both propano and etheno adducts of HNE appear as endogenous lesions in liver and other tissues of untreated rodents and humans [127]. In this context it appears of interest that studies by Nair et al. [128] demonstrated that the high intake of ω-6 polyunsaturated fatty acids increased malondialdehyde adducts in male and female subjects, while the levels of ethenodeoxyadenosine and ethenodeoxycytidine were not affected in male subjects; however, they were ~ 40-times higher in females.

Both MDA and HNE adducts are found in tissues of untreated rodents and humans. According to Burcham [93] the levels of these adducts can be as high or even higher than adducts induced by exogenous mutagens/carcinogens. The high sensitivity of certain cells and tissues such as liver and endothelial cells [117,118] together with the observation that there appear to be mutational hotspots for HNE adducts in the human p53 gene [126] therefore may reflect the causative contribution to the development of degenerative diseases such as cancer.

Oxidative modification of proteins with lipid peroxidation by-products

Mechanism of protein modification by reactive aldehydes. Oxidative modifications of metabolic and structural proteins play a significant role in the protein dysfunction, altered trafficking, processing of proteins, tissue damage and pathogenesis of several human diseases [129]. Protein carbonyl content is the most common biomarker of the oxidative damage of proteins [129]. Lipid peroxidation products can diffuse across membranes, allowing the reactive aldehyde-containing lipids to covalently modify proteins localized throughout the cell and relatively far away from the initial site of reactive oxygen species (ROS) formation [130–132]. Therefore, protein carbonyls are formed endogenously during lipid peroxidation and during the glycoxidation of carbohydrates, which are precursors of advanced glycation end products (AGEs) and advanced lipid peroxidation end products (ALEs) [133]. Protein-based carbonyls, key indicators of oxidative stress, reflect adduction of bifunctional lipid peroxidation-derived aldehydes, ketones and, perhaps, sugar oxidation products, rather than metal-catalysed oxidation of protein side-chains [134]. Major toxic effects of LPO-derived aldehydes are suggested to be caused by the modification and cross-linking of amino acid

proteins and peptides by these reactive aldehydes [135,136]. Mildly cross-linked, HNE-modified proteins are preferentially degraded by the proteasome, but extensive modification with this cross-linking aldehyde leads to the formation of protein aggregates. Interestingly, such cross-linked proteins are able to inhibit the proteasome and further impair cellular protein turnover [135]. The consequences of adduct formation at the protein level is associated with numerous cytotoxic consequences including the disruption of cell signalling, altered gene regulation, inhibition of enzyme activity, mitochondrial dysfunction, impaired energy metabolism, altered tertiary structure and finally loss of cytoskeletal formation [97].

Among several LPO-derived aldehydes, HNE is the most studied cytotoxic product of lipid peroxidation [137]. HNE readily modifies and causes cross-linking of proteins [138]. The chemical reactions leading to the formation of HNE-protein adducts involve Schiff base formation and Michael addition. Schiff base formation on primary amine (for example, lysine) results in the generation of more complex compounds, such as pyrrole-Lys adducts and fluorescent cross-links. Michael addition of HNE on amino groups (Lys and His) or biological thiols including cysteine, glutathione, are followed by cyclization and hemi-acetal or hemithioacetal formation [139]. Moreover, simple lysine Michael adducts are suggested to be formed reversibly [140]. Among the protein residues which react with HNE, Cys has the highest reactivity, followed by His and Lys [141], but Cys–HNE adducts are less stable than His–HNE adducts [137]. Regarding the modification of apolipoprotein B, HNE attacks mainly Lys and to a lesser extent His and Cys residues [142].

4-oxo-2-nonenal (ONE), a direct product of lipid oxidation [43], has been proposed as a more reactive protein modification and cross-linking agent than HNE [143]. The Cys-Lys and His-Lys cross-links are quite stable, although the Lys-Lys version readily suffers oxidation (require characterization by trapping with Ac_2O). Surprisingly, although ONE is more reactive than HNE towards imidazole and thiol nucleophiles, it is less reactive than HNE towards Lys/amine conjugate adduction.

MDA, one of the most abundant aldehydes formed during lipid peroxidation, reacts with Lys residues by forming Schiff bases [97] and plays a major role in low-density lipoprotein (LDL) modification and their affinity towards macrophages [144]. MDA has cross-linking activity similar to that of HNE and DDE (2,4-decadienal), it is virtually inactive at protein-carbonyl formation. It has been reported [130] that the low carbonyl incorporation at pH 7.4 increases dramatically at lower pH (pH 4–5). This property can be attributed to the character of MDA which exists predominantly at its non-electrophilic anionic conjugate base (enolate) and its carbonyl incorporation property. The accumulation of MDA adducts on proteins is involved in the formation of the fluorescent pigment lipofuscin, which accumulates progressively during ageing [145]

Other major lipid peroxidation products capable of modifying proteins are simple 2-enals, 2,4-dienals, 2-hydroxyaldehydes and 4,5-epoxy-2-enals. Acrolein is also formed during lipid peroxidation and is a strong electrophile exhibiting high reactivity with Cys, His and Lys nucleophile residues [146]. In the series of aldehydes studied at early times the rank order of carbonyl incorporation was acrolein > ONE > HNE > DDE > MDA. However, further data showed that acrolein is more potent than ONE for carbonyl incorporation and ONE has more affinity for protein cross-linking [132].

It has been recently hypothesized that the toxic effects of protein adduct not only depend on residue selectivity or affinity, but are also based on the importance of the targeted amino acid in protein function or structure [147]. Mainly acrolein and HNE, but also other lipid peroxidation by-products including ONE and HHE, are characterized by an α,β-unsaturated carbonyl structure, that is a conjugated system and contains mobile π-electrons.. The carbonyl oxygen atom is electronegative and can cause regional electron deficiency. On the basis of electron polarizability, both acrolein and HNE are considered to be soft electrophiles that form 1,4-Michael type adducts with soft nucleophilic sulphydryl thiolate groups of cysteine [148]. These anionic residues are the central nucleophilic components of catalytic sites of several key proteins of the cellular metabolism. Therefore, adduction of these regulatory thiolate groups by acrolein or HNE will disrupt redox control of protein function and, thereby, produce cytotoxicity [146,149]. Lys and His residues, main targets of nitrogen groups, are also targets for type 2 alkenes. Because these residues are relatively harder biological nucleophiles with significantly slower adduction kinetics, the toxic consequences of lysine or histidine adduction are more likely to develop during high-dose intoxication or during the late stages of chronic diseases when adduction of the cysteine thiolate pool has been saturated [146,148].

Formation of AGEs is also another type of interaction of lipid peroxidation by-products with proteins during diabetes and hyperglycaemia. A class of reactive ALE precursors, namely α-oxoaldehydes (methylglyoxal and glyoxal), are basically generated, from the Maillard reaction, resulting in the formation of a Schiff base. This reaction is followed by a non-enzymatic glycation and generates the Amadori product, where α-oxoaldehydes (methylglyoxal and glyoxal) are formed and react with Lys and arginine residues to form AGEs [150]. These α-oxoaldehydes are also produced from lipid peroxidation (glyoxal),

the catabolism of ketone bodies and the fragmentation of triosephosphates (methylglyoxal) in the early stages of glycation in hyperglycaemic conditions [151]. Glycation in *vivo* is slow and reversible at physiological glucose levels, tending to affect proteins with very slow turn-over including connective tissue collagen and lens cyrstallines. Glycation is faster at elevated glucose levels and therefore implicated with the progress and complications of diabetes. Glycated haemoglobin (HBA_{1C}) which contains a glucose Amadori product is, indeed, a marker used to monitor hyperglycaemia [150].

Recent studies provide more evidence on the mechanisms of how the modification of proteins by reactive aldehydes may lead to alterations of function of target proteins linked to disease states, signalling systems and age-related conditions. Some of the major functional protein modifications by lipid peroxidation end product are listed in this section.

Heat shock protein adducts. Heat shock protein 72 (HSP72) is the major inducible HSP found in the nucleus and cytosol [152]. Stress-induced HSP72 protects proteins against aggregation of denaturation. Moreover, HSP72 is also capable of inhibiting stress-induced apoptosis [153], even after the activation of effector caspases. Impaired synthesis of HSP72 may result in disturbances of cell proliferation [154,155]. Over-expression of HSP72 increases the survival and reduces oxidative damage and suppresses the inflammatory response [156]. On the other hand, HSP72 effect was not blunted in the absence of reactive oxygen radicals [156]. HSP72 requires ATP for its chaperone activity [157] and minimizes aggregation of newly synthesized proteins. HSP72 has an ATP-binding domain which exhibits low intrinsic ATPase activity [152,158]. Primarily ADP-bound state, but also ATP-bound state, demonstrate high affinity for the substrate [159]. Many of the molecular co-chaperones that influence the function of HSP72 do so through manipulation of the intrinsic ATPase activity. In a previous study it was tested whether major inducible stress protein HSP72 was susceptible to modification and inactivation by reactive lipid aldehydes [160]. An increase in the number and severity of HNE protein adducts was clearly demonstrated in cytosolic fractions obtained from ethanol fed animals when compared to their respective pair-fed controls [160]. Proteomic assays identified that HSP72 was one of several proteins consistently modified by HNE. Furthermore, HSP72 induction was impaired in hepatocytes isolated from ethanol-fed animals when compared to their respective control animals [160]. Interestingly, heat-denatured recombinant firefly luciferase assays demonstrated that HNE is a potent inhibitor of HSP72-mediated protein refolding. Because Cys267 adducts at ATPase domain of HSP72 were found after HNE administration, in the same set of experiments, the importance of oxidative modification on this particular residue in HNE-mediated inhibition of the chaperone was further confirmed [160]. Consistently, other studies show Cys modification and HSP72 inhibition by treatments with HNE and ONE, which are reactive to thiols, but not after MDA treatment, which does not react with thiols [35,161]. All these reports together emphasize that lipid peroxidation end products are potent inhibitors of the chaperone's refolding ability, which is possibly mediated through that Cys modification of the ATPase domain of HSP72.

Another key member of stress proteins 90-kDa heat shock protein (HSP90) accounts for nearly 2% of total protein in most unstressed cells and is involved in essential physiological processes, including protein trafficking and signal transduction, in specific steroid hormone signalling [162]. Because HSP90 together with other HSPs maintains protein homeostasis and cell survival, it was hypothesized that lipid aldehyde modification of HSP90 results in decreased chaperoning efficiency, vulnerability to cellular insults and linked to progression of disease. In a rat model of alcohol-induced oxidative stress, it was shown that HNE consistently modified HSP90, impaired the chaperoning activity through thiol modification and finally contributed to disease progression [163]. Consistent with the previous reports, in our studies we observed that pathological conditions which are linked to oxidative stress apparent with increased HNE adducts and protein carbonyl content, also impaired tissue HSP responses [164,165]. In streptozotocin-induced diabetic (SID) rats, induction of diabetes decreased HSP72 levels in liver and vastus lateralis muscle and HSP90 in liver tissue. Respectively protein carbonyl levels were increased in both tissues examined and higher levels of HNE protein adducts were observed in liver [164,165].

Cysteine adducts. Many of the LPO-derived aldehydes are capable of modifying proteins and other cellular nucleophiles through Michael addition, typically at Cys residues, causing impaired protein function [161,166, 167]. The enzyme protein disulphide isomerase (PDI) (E.C. 5.3.4.1.) is an endoplasmic reticulum protein and a molecular chaperone, which promotes the repair of incorrectly formed disulphide bonds and is thus an important component of protein maturation. PDI is one of several proteins found to be consistently modified by HNE in response to alcohol administration in the mitochondrial fractions of liver [168,169]. Two-dimensional gels assays revealed that HNE modification was occurring in the N-terminal thioredoxin-like domain, which is the active site for the repair of disulphide bonds of the damaged or newly synthesized client proteins [170]. Modification of these active N-terminal thiol sites by HNE results in impaired substrate binding and decreased enzyme

activity [170,171]. Interestingly, despite the susceptibility of PDI to inactivation by HNE, this inhibition was considerably lower compared to the inhibition of other chaperones in response to HNE modification [168,169]. Other lipid aldehydes including MDA, acrolein and ONE cause a considerable amount of inhibition of PDI upon modifying these enzymes. While PDI was almost completely resistant to MDA inhibition, acrolein results in similar PDI inhibition to that of HNE. Treatments with comparable concentrations of ONE end up with higher enzyme inactivation compared to the other aldehydes and at higher doses ONE completely inhibit PDI activity [168].

It has been recently hypothesized that biological activities of ONE, including its apoptotic and immune responses, are dependent on the ONE sulphydryl reactivity [172]. ONE-cysteine adducts, including the most prominent 2-cyclopentenone derivatives that originated from the initial Michael addition, exhibit carbonyl function [133]. Therefore, carbonyl property of ONE-cysteine adducts may explain the potent biological and toxic activities of ONE.

Another thiol protein target for HNE and ONE modification is Peroxiredoxin-6 (PRX6). Peroxiredoxins are an emerging class of thiol-specific antioxidant enzymes, exerting peroxidase activity against hydrogen peroxide, peroxynitrite, organic hydroperoxides and phospholipid hydroperoxides [173]. PRX6 is a 1-Cys class peroxiredoxin and exists as a homodimer with a critical redox-active Cys residue, which is involved in the peroxidase activity. Recent studies demonstrated that both HNE and ONE caused cross-linkage of PRX6 via Cys-Lys and Lys-Lys cross-links, resulting in the inactivation of the enzyme [174].

Mitochondrial aldehyde dehydrogenase (ALDH2; EC 1.2.1.3), an enzyme containing Cys nucleophile in its catalytic site, catalyses oxidation of the lipid aldehyde to the non-electrophilic 4-hydroxynon-2-enoic acid (HNA) in a NAD-dependent manner. HNE and ONE are both inhibitors and substrates for the enzyme. It was previously reported that the enzyme was modified reversibly by low micromolar concentrations of HNE [167]. Mass spectral analysis data demonstrated aldehyde dehydrogenase active sites were covalently modified by high concentrations of ONE [175].

Glutathione adducts. GSH is the most abundant intracellular thiol, present in virtually every animal cell in millimolar concentrations [176]. Directly or indirectly GSH plays a key role in many physiological functions, including antioxidant defense, storage and transport of cysteine, synthesis and degradation of proteins, transportation of amino acid, regulation of enzymes, synthesis of deoxyribonucleotide precursors of DNA, in conjugation with exogenous and endogenous compounds; detoxification of electrophilic xenobiotics, regulation of prostaglandin metabolism, modulation of redox regulated signal transduction and regulation of cell proliferation and immune functions [177,178]. GSH is a major antioxidant which provides an appropriate reducing milieu inside the cell [179]. GSH can directly scavenge ROS or enzymatically via GSH peroxidases and GSH transhydrogenases [177]. GSH-adducts of a wide range of reactive intermediates have been studied intensively *in vitro*, including Michael acceptors and HNE (from lipid hydroperoxides) [180]. HNE is more reactive towards GSH than other α,β-unsaturated aldehydes. Reaction of HNE with thiols ends up with the formation of HNE cyclic hemiacetal adducts which were observed as four isomeric adducts (H1-H4) by LC-ESI/SRM/MS analysis [181]. Recently GSH-adducts derived from ONE have been discovered as major GSH-aldehyde adducts. GSH addition to ONE leads to the formation of an unusual thiadiazabicyclo-ONE-GSH-adduct (TOG), a major endogenous GSH-adduct formed during oxidative stress [182,183]. Recent studies have also shown that TOG (as its dimethyl ester derivative) can induce endothelial cell apoptosis when intracellular GSH levels are lower than 1 mM [181]. In addition, GSH-adducts of ONE and its structural analogues are more abundant and more potent than GSH-adducts of HNE (GS-HNE) at inducing apoptosis [55]. Nevertheless, GS-HNE adducts were rapidly formed when cells were treated with HNE and resulted in an augmented apoptotic response [184]. Similarly, ít has been shown that the GS-HNE adducts rather than HNE may be involved in the signal transduction pathways that mediate increased COX-2 and lipoxygenase expression [183,185]. This evidence suggests that lipid hydroperoxide-derived endogenous GSH-adducts are involved in signal transduction pathways. Moreover, there is growing evidence that endogenous aldehyde-GSH adducts and their metabolites can be used as quantitative biomarkers of oxidative stress using high sensitivity APCI/MS systems, where TOG has a clear advantage over GS-HNE adducts [186]. In contrast to GS-HNE adducts, which are detected as four different diastereomers, in LC-MS analyses, TOG is eluted as a single isomer. Furthermore, sensitivity of TOG detection can be increased by 30-fold in LC-MS assays using PFB [181].

Interaction of LPO products with biomembranes

Biomembranes are the barrier of the cell towards its environment, but its changes in structure and function due to stress conditions are often neglected. Still, the fact is that the first contact and defence against environmental stress lies in cell membranes, together with the energy production processes which are bound to endogenous membranes. Biomembranes

are consisted mainly of lipids, which make between 30–80% by mass, proteins making 20–60% and carbohydrates 0–10% [187].

Membranes are described by Singer and Nicolson [188] with the Fluid Mosaic Model. The model presents membrane as a fluid due to hydrophobic integral components, like lipids, which move laterally or sideways through the membrane. The membrane are lipid bilayers in which protein moieties are inserted going either entirely through the membrane or just being located inside or outside the membrane. Phospholipid components of the membrane fold themselves, creating a double layer in polar surrounding like water. Membrane is the basic structure which segregates cell content from the surrounding and also surrounds cell organelles, defining their function. Alternation of all these factors, structure, content and fluidity causes abnormal function and pathological processes. Fluidity is determined by the presence of PUFAs in phospholipid molecules, in both sides of the lipid bilayer. The current lipid raft hypothesis suggests that the lipid bilayer is not structurally passive, but has 'patchy' structure with spatially organized structure and, consequently, function [189]. The model proposes the existence of lipid rafts, enriched in cholesterols, raft-associated proteins, saturated lipids (i.e. sphingolipids) and the 'non-raft' matrix. These rafts are implicated in many cellular processes, such as signal transduction, membrane trafficking and protein sorting [190]. These rafts are highly dynamic microdomains, with dimensions 20–200 nm, and lifetime from 10–2 to 10^3 s. Proteins of the microdomains are often coupled to the cytoskeleton, thereby defining the spatial distribution of the domain [190].

One of the most challenging topics in lipid peroxidation is its consequence on membrane structure and function considering lipid peroxidation products. Reactive oxygen species cause lipid peroxidation having as a consequence formation of reactive aldehydes. These reactive aldehydes are implicated in many pathological as well as physiological states. Still, their interactions with membrane structures, especially with membrane phospholipids, are not nearly understood. Major product of n-6 polyunsaturated fatty acid (arachidonic and linoleic acid, especially) peroxidation is HNE. Also, HNE is one of the reactive aldehydes with high biological significance by taking part in cell growth modulation, signal transduction, induction of apoptosis [137,191,192]. As a product of PUFA peroxidation, the first molecules to be modified with HNE are membrane molecules, phospholipids and membrane proteins. Therefore, modification of membrane lipids and proteins consequently causes modification of membrane function and fluidity [187,193]. Also, it is essential to emphasize that lipophilic properties of HNE are much more pronounced than its hydrophilic properties. Thus, HNE tends to be distributed in biomembranes rather that in aqueous compartments of the cells, which is important for its biological effects [194].

One of the important characteristics of biomembranes is distribution of phospholipids in the inner and outer leaflet of plasma membrane. Aminophospholipids, phosphatidyl ethanolamine, PE, and phosphatidyl serine, PS, are located in the inner leaflet of the cell plasma membrane. Phosphatidyl serine is kept in the inner leaflet by flippases. When the cell is designated to apoptosis, flippases are inactivated and PS could also be found in the outer leaflet. Both of these phospholipids contain a primary amino group and can therefore react with HNE [195]. Still, PS reacts poorly with HNE, possibly due to the presence of a carboxyl group in the close surrounding of amine group. On the other hand, reactions of PE with HNE seem to have biological significance.

Ethanolamine phospholipids are composed of two main sub-classes, diacyl-glycero-phosphoethanolamine (DA-GPE) and alkenyl acyl-glycero-phosphoethanolamine (plasmalogen PE). HNE binds to PE covalently, forming at least three different adducts: Michael adduct, which is the major reaction product and the result of addition of HNE via its double bond to the primary amine of PE; Schiff base adduct, the result of condensation of HNE's carbonyl group with the primary amine of PE; and a pyrrole derivative, cyclization product of the latter adduct [193,196]. The same type of reactions occurred in other α,β-aldehydes, HHE, peroxidation product of n3-PUFA, and 4-hydroxy-2,6-dodecadienal (HDDE), issued form of 12-lipoxygenase reaction with arachidonic acid (AA) [137]. Hydrophobicity of these three aldehydes, HHE, HNE and HDDE, defines their reactivity toward PE, making HDDE the most reactive, followed by HNE, and HHE being the least reactive of the three.

HNE binds to both PE sub-classes with no difference in binding capacity, but alkenyl acyl-GPE are further degraded when HNE is bound [195,196]. In addition to making covalent adducts with PE, HNE may alternate the alkenyl chain of plasmalogen PE. This alternation might be important in antioxidant potential of plasmalogen PE. Namely, studies using plasmalogen-deficient cells and *in vitro* model systems have supplied evidence that plasmalogens may have a protective role during oxidant-induced stress [194].

Biological importance of interactions between HNE and PE is easily understood when put in context of PE as a source of AA. AA metabolizes to prostaglandins, leukotriens, thormboxanes and eicosanoids. In platelets, PE appears to be a major source of AA, which is released by phospholipase A_2 (PLA_2) [197]. If bound to PE, HNE decreases 2-fold activity of PLA_2 and completely diminishes activity of phospholipase D (PLD) [194]. In this way HNE may regulate inflammation and the cell response to the same.

Another example of the importance of HNE-PE interactions is reflected in inhibition of platelet aggregation by these adducts [198], once again strengthening the bimodal role of HNE in the cell.

Michael adducts may alter membrane fluidity and HNE bound to membrane anchored proteins may alter their functions. An example of membrane protein modification is augmentation in Ca^{2+} current in dentate granulate cells exposed to HNE. In addition, the effect is specific to L-type Ca^{2+} channels, which are associated with amyloid β protein (A,β)-induced cell death [199]. The mechanism showed that HNE triggers $Ca^{2}+$ influx via L-type calcium channels by interacting with its thiol groups [200]. It is important to emphasize that HNE and other α,β-aldehydes react with proteins, including especially their lysine, cysteine and His residues. These reactions may cause protein cross-linking, leading to the formation of fluorophores [191]. Biological consequence of protein cross-linking may be either inactivation of function or activation of certain receptors with consequence in signal transduction. Physically, protein cross-linking rearranges protein moieties in membrane, thereby changing physical properties of the membrane.

One of the first described effects of lipid peroxidation on membranes was plasma membrane blebs. Blebs are plasma membrane distortions, which can rupture and discharge cell content [201]. Blebs are associated to apoptosis, together with cell shrinkage, dynamic membrane blebbing, chromatin condensation, DNA laddering, loss of plasma membrane phospholipid asymmetry, reduction of ATP, mitochondrial oxyradical generation and mild calcium overload [202]. It is generally believed that blebs appearance is associated to oxidation of cytoskeleton-associated protein thiols. Furthermore, blebs are not only consequence of apoptosis, but represent novel sources for biologically active oxidized phospholipids [199], contributing to the initiation and progression of chronic inflammatory processes in the organism [203]. Still, the exact mechanism of blebs formation is not yet undersood.

When discussing lipid peroxidation products, mostly reactive aldehydes are elaborated. Still, there are reports about isoketals (isoK), highly reactive γ-ketoaldehydes formed by rearrangement of the isoprostane pathway of lipid peroxidation [204]. IsoK are highly reactive and rapidly form protein adducts and underlie proclivity to cross-linked proteins. Indeed, IsoK form Schiff base and pyrrole adducts with PE *in vitro*. Still, there is a need to explore their formation *in vivo* and their biological relevance.

Finally, interactions of LPO products with biomembranes are a new and insufficiently explored field. The complexity of these interactions is demonstrated through reactions of LPO products with both proteins and lipids in membrane and coupling of membrane rafts to cytoskeleton and plasmatic proteins. Modulation of signal transduction and cellular processes of HNE, one of the most intriguing LPO products, may be either by interactions with PE, thereby interfering with lipid rafts, or by interactions with membrane proteins. Therefore, progress in LPO research may be achieved by interdisciplinary collaborations which comprehend complex (bio) chemistry and biology of HNE and other reactive aldehydes.

Lipid oxidation products in cell signalling

Basic principles of lipid oxidation product reactivity into cells

Basal HNE concentration in human blood and serum is ~ 0.05–0.15 μM and it increases with age [205]. Nutrition influences these values since HNE is a lipoperoxidation product derived from linoleic acid n-6 PUFA [78]. Antioxidants, intestinal flora can also affect HNE circulating concentration [206,207]. Under conditions of oxidative stress (rheumatologic diseases for example), HNE concentrations increase up to 3–10-fold of physiological concentrations [208].

In mammalian cells, HNE is highly reactive and rapidly metabolized (3–5 min) depending on the battery of detoxifying enzymes expressed in the cell type [209]: alcohol dehydrogenase, GST, ALDH, respectively, leading to DHN, GS-HNE and HNA; 1–8% of HNE is conjugated to proteins, on histidine, cystein and lysine. The metabolites have a low reactivity and the majority of the biological effects depend on HNE itself [69,98].

HNE has several intracellular biochemical targets: proteins, lipids and nucleic acids. When it reacts with proteins it can increase proteolysis by proteasome [58]. HNE-protein adducts impair protein function and, so, trigger signalling and enzymatic disturbances. The formation of GS-HNE is reversible; a retro Michael cleavage could occur and liberate bioactive HNE in another site, as was shown in liver and kidney [69]. HNE can also react with phospholipids of the plasma membrane, leading to potential modifications of its biophysical properties [210] (see above). HNE is genotoxic, by interacting directly with guanine or through its oxidative epoxide product [211–213]. These reactions could induce a nuclear stress and affect cell cycle and growth. Finally, HNE can also have epigenetic effects, since HNE can bind histones and, so, control chromatin condensation and gene expression [214].

As a major highly reactive aldehydic product of lipoperoxidation (LPO), HNE is able to exert cytotoxic, mutagenic and carcinogenic effects. Its appropriate and rapid catabolism could significantly contribute to modulate cellular defence system and condition cellular responses.

Cell death induced by lipid oxidation products: A stressful story

HNE is produced under stress situations, its formation is directly proportional to reactive oxygen species (ROS) production. Accumulation of reactive components leads to induction of cell death if cells cannot eliminate them quickly. So at physiological concentrations, we observe an apoptosis, mainly triggered according to intrinsic type I cell death. HNE was demonstrated to be able to alter mitochondrial respiratory complexes in PC12 cells and mediate apoptosis [215]. More precisely, cytochrome *c* oxidase and aconitase activities were strongly decreased upon HNE treatment. Moreover, HNE and HHE directly inhibit adenine nucleotide translocator (ANT), which has a key role in mitochondria-dependent apoptosis [216]. These events could amplify the oxidative stress, by induction mitochondrial ROS [217]. However, HNE can also trigger mitochondria-dependent apoptosis by targeting Bcl2 anti-apoptotic members, principally located in the external mitochondria membrane [218]. Then, in colon cancer RKO cells, activation of HSF1 (heat shock factor 1) leads to BAG3 (Bcl2-associated athanogene domain 3) upregulation, which stabilizes BclXL [219] by the formation of a complex BAG3-BclXL and HSP70. This event is crucial in survival processes in cancer cells and the silencing of HSF1 allows a decrease of many Bcl2 anti-apoptotic members like Bcl2, BclXL and Mcl1 [220]. This HSF1 inhibition would constitute a strategy to sensitize cancer cells to HNE-mediated toxicity, useful in anti-cancerous therapeutics. Mitochondria-dependent apoptosis can also be promoted by oxidative nuclear stresses. p53, as a tumour suppressor protein, is usually activated by ROS and DNA damage [221]. It can act as a transcription factor and regulate in a first step cell cycle and DNA repair enzymes. Among them, p21, GADD45 or XPC are activated to arrest cell proliferation and repair DNA damage [222]. Whether damages persist, p53 secondary induces pro-apoptotic proteins, like Bax, Fas or PUMA, and represses anti-apoptotic proteins like Bcl2 and, so, triggers apoptosis [223]. HNE, as a pro-oxidant compound, favours p53 activation, with its phosphorylation, its accumulation and its nuclear translocation [224]. In T-cell leukaemia Jurkat cell line, HNE induces p53-dependant apoptosis, with p21 and Bax induction, that finally leads to caspase activation [225]. This process was also identified in neuroblastoma SH-SY5Y cells [226], in neuronal PC12 cells [227], in human chondrocytes [228] and in retinal pigment epithelium RPE cells [229]. Thus, HNE is a major p53 activator and accumulation of this protein was correlated to an accumulation of HNE in Alzheimer's disease (AD) [230]. However, HNE can stabilize p53 by direct binding and, so, amplify neuronal apoptosis [231]. Even if HNE was described as a genotoxic compound, we have no information about the fact that p53 could be activated by HNE via DNA damage. It seems that oxidative stress and direct binding to p53 should constitute the main pathway of p53 activation by HNE. The use of antioxidants underlines this phenomenon: JNK (c-Jun kinase) appears to be upstream p53. This MAP kinase is highly sensitive to oxidative stress and more particularly to HNE. It regulates p53 expression and so triggers HNE-dependent apoptosis [225,226,229,232,233].

LPO is a process which can induce organelles stress. We have previously commented about mitochondria and nuclear stresses, but we cannot exclude lysosome stress. Actually, it was shown in hepatocytes that ferritin-induced apoptosis was mediated by an increase of LPO, which promoted lysosomal membrane permeability and allowed the release of lysosome content, like proteases. Consequently, global cell damaging was observed with DNA and protein adducts, micronuclei, p53 activation and the drop of the mitochondrial transmembrane potential $\Delta\psi M$ [234]. This cell death depends on an early lysosome disturbance, so is qualified as an apoptotic process. It is worth noting that the cross-talk between lysosome and mitochondria is relevant in the ageing field [235], a process in which HNE is strongly involved. Future studies must then focus on this axis in order to determine the HNE-related crucial events.

Extrinsic type I cell death is an apoptotic pathway dependent on death receptors, like Fas, TNF (Tumour Necrosis Factor) receptors and TRAIL (TNF-related-apoptosis-inducing-ligand) receptors [236]. Basically, this signalling is activated by cytokines (FasL for Fas ligand, TNFα, TRAIL). For example, the binding of FasL on Fas triggers the aggregation of Fas and the formation of a pro-apoptotic complex, called DISC (Death Inducing Signalling Complex). After death domain aggregation, the receptor complex is internalized via the cellular endosomal machinery. This allows the adaptor molecule FADD to bind the death domain of Fas. FADD also contains a death effector domain near its amino terminus, which facilitates binding to caspase-8. Active caspase-8 is then released from the DISC into the cytosol, where it cleaves other effector caspases, leading to major hallmarks of apoptosis. HNE was shown to induce Fas expression, which sensitizes cells to FasL-dependent apoptosis if soluble FasL is present in the extracellular medium or transmembrane FasL is expressed in neighboured cells [237]. Fas expression can also be regulated by p53 [228,234]. However, another mean to activate death receptor pathway was described: HNE can mimic FasL and activate Fas without formation of DISC [225]. So, in HNE-triggered cell death, both intrinsic and extrinsic pathways are involved, which facilitates death signal amplification [225]. However, some cells deficient for Fas could be resistant to HNE [237], meaning that, according to the cell type, extrinsic or

intrinsic pathway would be predominant and it is barely predictable.

Depending on the cell specificity, apoptosis requires particular pathways. In neurons, recent studies have demonstrated that Toll-like receptors (TLR) were induced following energy deprivation and triggered apoptosis [238]. Tang et al. [239] have shown that HNE was able to upregulate TLR4 expression and activate JNK-dependent apoptosis. Neurons mutated for TLR4 exhibit a protection regarding HNE exposure, underlying that this expression directly affects neuronal sensitivity. This discovery is relevant in terms of AD, since a decrease of TLR4-positive cells in end-stage AD patients was observed, probably due to a specific loss of TLR4 expressing neurons.

HNE induces apoptosis by disturbing organelles (mitochondria, lysosome, nucleus), by activating stress-sensors proteins (p53, JNK) and by directly binding of key regulators (Fas, p53). HNE-dependent genotoxicity was not described yet as a process involved in cell death, but the identification of caspase 2 in HNE-induced apoptosis [232,240] could put back into question the importance of DNA damage following LPO [241] (Figure 2).

Survival processed triggered by lipid oxidation products: A balanced question?

Balance survival/proliferation. HNE, by its ability to bind proteins, can activate growth factor receptors, as was shown for EGFR (Epidermal Growth Factor Receptor) [242] and PDGFR (Platelet-Derived Growth Factor Receptor) [243]. These tyrosine kinase receptors are then autophosphorylated and trigger survival pathways involved in the proliferation of smooth muscle cells (SMC) in atherogenesis [244]. In this precise disease, the induction of apoptosis by HNE could constitute a way to avoid SMC proliferation and atherome formation. Among cardiovascular diseases, cardiac hypertrophy is characterized by increased myocardial cell size, via an important protein synthesis [245]. The mammalian target of rapamycin (mTOR)/p70S6 kinase pathway is primordial in the regulation of cell growth. It is inhibited by the system LKB1/AMP-activated protein kinase (AMPK), which acts as an energy sensor, limiting anabolism [246]. A recent study has reported that HNE levels are higher in the blood and heart of hypertensive rats. In isolated cardiomyocytes, HNE forms adducts with LKB1 that inhibits the LKB/AMPK pathway and activates the mTOR pathway. Treatment with resveratrol prevents such events *in vitro* and reduces hypertrophy *in vivo*, suggesting the preponderant role of HNE in the development of cardiac hypertrophy [247]. However, the regulating role of HNE on energy sensing systems was also described in tumour growth [248], stressing the importance of LPO in anabolic homeostasis.

In other pathologies like age-related bone loss, HNE accumulation was associated with a decrease of osteoblast number. Osteoblastogenesis is related to the activation of Wnt/β catenin/Tcf (T-cell specific transcription factor) pathway, ensuring the proliferation and the differenciation of osteoblasts [249]. However, an increase of ROS associated with the ageing process favours LPO and HNE production and consecutive activation of FoxO- (Forkhead box O) mediated transcription. Since FoxO-regulated

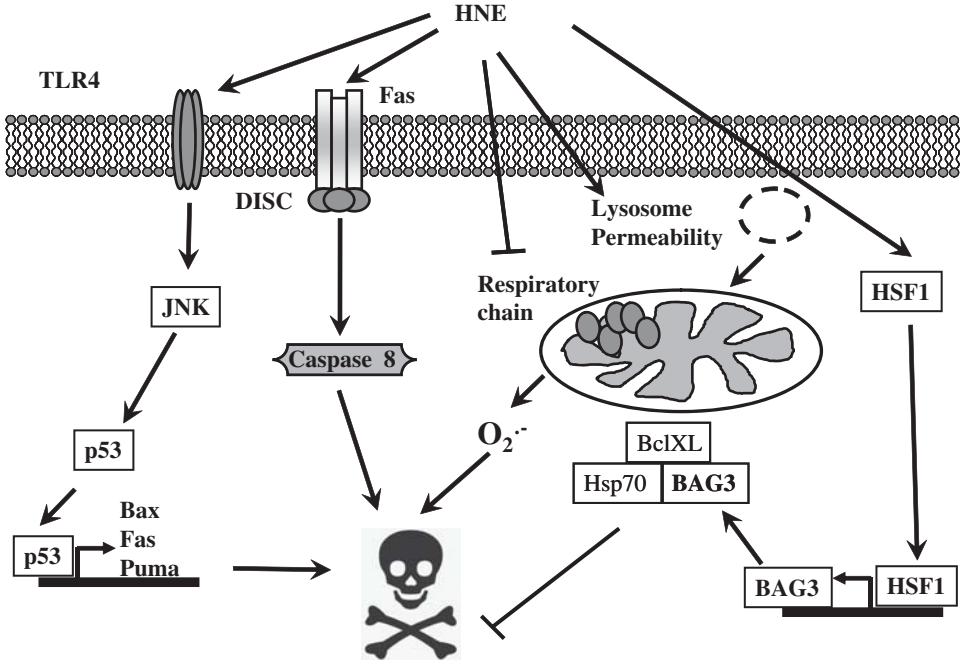

Figure 2. Main apoptotic pathways triggered by HNE.

transcription requires β catenin, the pool of β catenin acting with Tcf decreases and so attenuates Wnt-regulated gene expression [250]. Moreover, at the same time, oxidized lipids bind and activate PPARγ, which promotes β catenin degradation. According to this cascade, LPO is a major process in the decline of osteoblast number, by the extinction of Wnt/β catenin pathway required for their differentiation and survival [251].

Balance survival/cell death. HNE is able to repress the survival process and induce apoptosis by acting on the cell death/survival balance. The TrkA receptor is a high affinity receptor for nerve growth factor (NGF) and is located in lipid rafts [252]. In cholinergic neurons, which express TrkA receptors, HNE favours the oxidation of lipid rafts and, so, impairs internalization and transport of signalling endosomes. Moreover, it prevents retrograde transport, which finally leads to a decrease of TrkA receptors at the plasma membrane and anneals the pro-survival signal. This mechanism could explain the loss of a particular neuron population implicated in memory and attention function in neurodegenerative diseases [253].

HNE also deregulates cell death/survival balance by altering the apoptosis pathway. In the previous part was described the activation of Fas by HNE, via its direct interaction with Fas, mimicking FasL. However, Awasthi et al. [224] have demonstrated an inhibitory loop allowing the self-limitation of the pro-apoptotic signal mediated by HNE. This process involves Daxx, a nuclear protein which is associated to different DNA binding transcription factors and represses their activities. During stress, Daxx translocates to the cytoplasm and acts as a death receptor adaptor at the cell surface [254]. Its role as a pro-survival or pro-apoptotic mediator is still debated. Some studies suggest that Daxx binds Fas and activates apoptosis with the involvement of ASK1 (Apoptosis Signal Regulating Kinase 1) which activates JNK. This process is independent of the DISC formation. In CRL2571 cells, HNE induces Daxx translocation and binding to Fas, with an activation of ASK1 and JNK. However, experiments on Daxx-deficient cells reveal a potentialization of HNE-induced apoptosis, with a higher ASK1/JNK activation and caspase 3 cleavage. So, in this system, Daxx exerts a brake in Fas-dependent apoptosis following HNE treatment. The authors have also demonstrated that this inhibitory action of Daxx was also relevant in the case of classical activation of Fas by Fas antibodies [225]. The protective role of Daxx is extended to the regulation of HSF1. Actually, the release of Daxx from a nuclear compartment allows the upregulation of HSF1, which induces the expression of HSF1-target genes, like Hsp70, involved in cell defence against oxidative stress [219]. This self-limiting signal could have a physiological role, because HNE is highly diffusible. Its spreading among tissue could have detrimental consequences on tissue homeostasis. With this process, apoptosis would stay limited, without affecting neighboured cells.

Balance proliferation/differentiation. The Notch signalling pathway has been implicated in the development of several leukaemia and lymphoma. It is commonly mutated or its ligand Jagged is frequently over-expressed in acute myelogenous leukaemia, leading to a persistent activation [255]. The inhibition of this pathway is a strategy to prevent cell proliferation and promote cell differentiation. In HL60 cells (human promyelocytic leukaemia cells), HNE induces a down-regulation of Notch1. Thus, Notch target genes, like Hes1, are repressed and inhibit cell proliferation [256]. No apoptosis induction was observed but an induction of differentiation: this suggests that HNE acts in that case on the balance proliferation/differentiation. This work also underlines Notch as a molecular target of HNE and knowing the multiple roles of Notch in carcinogenesis [257], development [258], neuronal diseases [259], etc…, it can open new perspectives of studies, in relationship with LPO.

Balance survival/autophagy. Autophagy is a major process regulating catabolic reaction. This pathway is involved in the destruction of dysfunctional organelles and is essential for cell growth and development to balance protein synthesis, organelle biogenesis and degradation. The two major protein degradation and recycling pathways are the Ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway. The UPS is responsible for the degradation of short-lived proteins, whereas autophagy regulates long-lived proteins and organelles. Macroautophagy implies the engulfment of cytoplasmic constituents within cytoplasmic vacuoles and their delivery to lysosomes for degradation. This phenomenon provides substrates for energy metabolism and recycles amino-acids, fatty acids and nucleotides [260]. It is a low level but constitutive process which could be increased upon nutrient deprivation, growth factor withdrawal and other stresses like protein aggregation. mTOR is a negative regulator of autophagy and its inhibition by rapamycin activates autophagy [261]. Autophagy is associated to diseases occurrence, like ageing, cancer and neurodegenerative disorders and can mainly be detected by microscopy analysis of the vacuolization, phagophores and multilamellar vesicles [262]. HNE, as a major cellular stressor, could act on this process; however, the number of studies is quite limited. Relative to cardiovascular diseases,

HNE and acrolein are involved in the activation of autophagy in SMC *in vitro* [263]. HNE modifies proteins that are gradually removed from cells and this process is amplified by cotreatment with rapamycin. HNE, nonenal and acrolein increase the formation of LC3II, a protein located at the membrane of vacuoles. Electron micrographs, with characteristic vacuolization, confirm the induction of autophagy by aldehyde-modified proteins. This process is relative to survival because, when inhibited by 3-methyladenine, it is an apoptotic cell death that is triggered: the accumulation of proteins modified by LPO is deleterious for cell life. The ability for aldehydes to induce autophagy appears dependent on their electrophilicity: acrolein is the more potent inducer, followed by HNE and nonenal. Saturated aldehyde nonanal is inactive, whereas phospholipid aldehyde is barely effective. Finally, it is not the simple protein modification by HNE that promotes deleterious effects, but the capacity to form protein cross-linking that strongly affects cell homeostasis

Autophagy is a primordial process in very specialized cell types, like retinal pigment epithelial cells (RPE). They are non-dividing long-lived cells that require continuous renewal. They are at the interface of photoreceptor layer and choriocapillaris and ensure the photoreceptor outer segment (POS) phagocytosis to maintain the visual cycle. Progressive dysfunction, accelerated in age-related macular degeneration (AMD), leads to accumulation of imperfectly degraded material (called lipofuscin) in acidic vacuoles [264]. This process of lipofuscinogenesis implies HNE and MDA modifications of POS and lysosomal degradation becomes harder in the long-term [265]. In a recent study, Krohne et al. [266] demonstrate that HNE- and MDA-modified POS strongly reduce autophagy in RPE cells. Consequently, they are then more susceptible to cell death regarding any other stresses and the accumulation of this undigested material could contribute to cell ageing and degeneration.

Importance of detoxication/metabolism of lipid oxidation product in cell signalling

We have described non-exhaustively some relevant signalling processes targeted by HNE. Some studies underline the fact that detoxification mechanisms are major in their occurrence. Thus, GSTs, like GSTA4–4 and GST5.8, are key enzymes controlling free HNE concentration. Their over-expressions decrease intracellular free HNE and abrogate HNE-induced effects [225,228,267,268]. When HNE is conjugated to GSH, its reactivity becomes low. In some cells, HNE induces a depletion of GSH. However, some others enzymes like aldehyde dehydrogenase and aldose reductase would also play a role in detoxification. For example, in neurons, ABAD (amyloid β peptide binding-alcohol dehydrogenase) detoxifies HNE when it is over-expressed in SH-SY5Y [269]. This mitochondrial enzyme is inhibited by amyloid β peptide and the absence of HNE catabolism renders it toxic. In the retina, photoreceptor retinol dehydrogenase 12 (RDH12) mutations cause retinal dystrophy. *In vivo* experiments demonstrate that RDH12 prevents light-induced apoptosis of photoreceptors, by limiting the formation of HNE-adducts. The biotransformation of HNE into non-toxic alcohol protects cells from macromolecules modifications [63].

The basal level of intracellular GSH has an importance in HNE toxicity. If GSH is low, it would increase the rate of HNE metabolization. However, basal cellular levels of GSH are physiologically 3-fold higher that HNE concentration and HNE is able to rapidly upregulate glutamate cystein ligase to promote GSH neosynthesis [224,270].

Elimination of HNE as GS-HNE is an efficient mechanism to decrease HNE bioavailability and limit its toxicity. RLIP76 is a Ral binding GTPase-activating protein which transports GS-HNE and metabolite conjugates according to an ATP-dependent manner. Its inhibition strongly triggers HNE accumulation and could lead to apoptosis [271–273], even in the absence of any stressor [184]. Ascorbic acid was shown to promote GS-HNE efflux, via MRP and, hence, prevent apoptosis [88].

Regarding signalling processes mediated by HNE, it appears primordial to consider the molecular events in their whole (metabolism, stress origin, balance survival/proliferation/autophagy/cell death) in a cell type context, to really evaluate the consequences of the products of lipoperoxidation.

Aldehydes as bioactive markers of LPO

The most sensitive cellular target of free radical reactions may represent PUFAs. Lipid peroxidation leads to the formation of a broad array of different products with diverse and powerful biological activities. Among them are a variety of different aldehydes. The primary products of lipid peroxidation, lipid hydroperoxides, can undergo carbon–carbon bond cleavage via alkoxyl radicals in the presence of transition metals, giving rise to the formation of short-chain, unesterified aldehydes of 3–9 carbons in length and a second class of aldehydes still esterified to the parent lipid [35]. The important agents that give rise to the modification of a protein may be represented by reactive aldehydic intermediates, such as 2-alkenals and 4-hydroxy-2-alkenals (Figure 3) [35,39]. These reactive aldehydes are considered important mediators of cell damage due to their ability to covalently modify biomolecules, which can disrupt important

cellular functions and can cause mutations [35]. Furthermore, the adduction of aldehydes to apolipoprotein B in LDL has been strongly implicated in the mechanism by which LDL is converted to an atherogenic form that is taken up by macrophages, leading to the formation of foam cells.

Excellent review papers have been published on the use of HNE-protein adducts as biomarkers of lipid peroxidation [274] and on the methodological aspect of their measurement [275], so we will focus on the saturated aldehydes, such as hexanal, that are most abundantly formed [276,277]. Upon reaction with proteins, these aldehydes react with lysine residues to form an imine or Schiff base adduct [278]. Due to the reversible nature of such unconjugated Schiff bases, these aldehydes have received relatively little attention as the causative agent for modification of nucleophilic biomolecules. However, Ishino et al. [279] have recently established a novel mechanism of irreversible covalent protein modification by aldehydes, in which H_2O_2 and alkyl hydroperoxides mediate the binding of saturated aldehydes to the lysine residues of protein to generate structurally unusual *N*-acylation products.

Here I provide an overview of studies on lipid peroxidation-specific adduction of proteins by the most reactive aldehydes 2-alkenals. In addition, the latest finding on the involvement of H_2O_2 and hydroperoxides in covalent binding of n-alkanals to protein generating *N*-acylation products is also described.

Covalent protein modification by 2-alkenals

2-Alkenals represent a group of highly reactive aldehydes containing two electrophilic reaction centres. A partially positive carbon 1 or 3 in such molecules can attack nucleophiles, such as protein. Acrolein and its methyl derivative, crotonaldehyde, represent the most potent electrophilic 2-alkenals commonly detected in mobile source emissions, cigarette smoke and other products of thermal degradation [280]. Thus, they had been considered as the 'unnatural' environmental pollutants; however, recent studies revealed that these aldehydes were endogenously produced under oxidative stress [281–284].

Among all the α, β-unsaturated aldehydes, acrolein shows the greatest reactivity with proteins. Upon reaction with protein, acrolein selectively reacts with the side-chains of the cysteine, histidine and lysine residues. Of these, lysine generates the most stable product. The β-substituted propanals (R-NH-CH_2-CH_2-CHO) and Schiff's base cross-links (R-NH-CH_2-CH_2-CH=N-R) had been suggested as the predominant adduct; however, the major adduct formed upon the reaction of acrolein with protein was identified as a novel lysine product, N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) (Figure 4), which requires the attachment of two acrolein molecules to one lysine side chain [283, 284]. The formation of a similar FDxP-type adduct (dimethyl-FDP-lysine) has been reported in the lysine modification with the acrolein analogue, crotonaldehyde [281]. In addition, these FDP-type adducts have also been detected in the reaction of other 2-alkenals, such as 2-pentenal and 2-hexenal, with the lysine derivative, suggesting that the condensation reaction via formation of the Michael addition-derived imine derivatives is characteristic of the reaction of 2-alkenals with primary amines. Due to the fact that the core structure of the FDP-lysine is resistant to the conventional acid hydrolysis of proteins even without reduction by pre-treatment with sodium borohydride, the FDP adducts of acrolein and crotonaldehyde have been successfully detected not only in the acrolein-treated LDL but also in LDL exposed to metal-catalysed oxidation [283]. Furthermore, by use of a monoclonal antibody, the detection of the FDP-lysine has so far been reported in

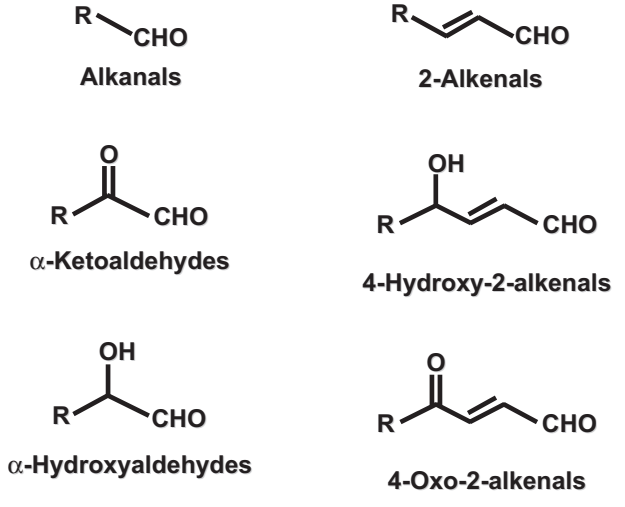

Figure 3. Reactive aldehydic intermediates.

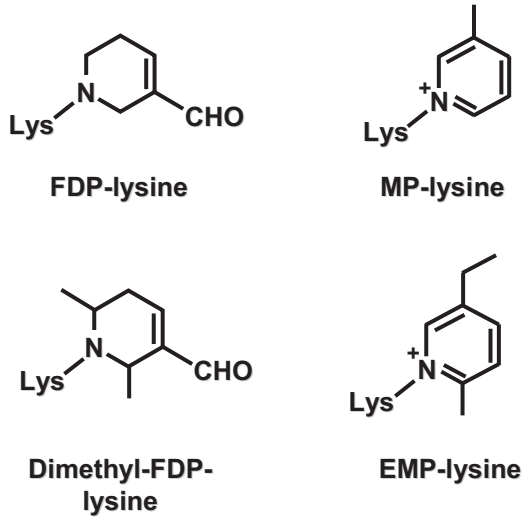

Figure 4. Lysine adducts.

plaque deposits of atherosclerotic lesions [284] and neurofibrillary tangles and plaque neuritic elements in Alzheimer's disease [285]. These observations are in line with the accumulating body of literature supporting the role of oxidative stress in the pathogenesis of these disorders. In a later study, Furuhata et al. [286] also revealed the electrophilic potential of FDP-lysine and established a novel mechanism of protein thiolation in which the FDP-lysine generated in the acrolein-modified protein reacts with sulphydryl groups to form thioether adducts.

On the other hand, it was also shown that acrolein modification of lysine generates an alternative acrolein-lysine adduct, N^{ε}-(3-methylpyridinium)lysine (MP-lysine) [287]. The formation of MP-lysine can be reasonably explained by the mechanism involving the formation of a Schiff base derivative as the first intermediate. The Schiff base further reacts with a second acrolein molecule via a Michael addition to generate an imine derivative. The subsequent conversion of this imine derivative to the final product (MP-lysine) obviously requires two oxidation steps and intramolecular cyclization. The formation of these lysine-pyridinium species in proteins results in the placement of a fixed, positive charge on the e-amino group. Moreover, in contrast to the fact that the FDP-type adducts are unstable intermediates against nucleophilic addition, the pyridinium adducts are highly stable end products. The formation of the pyridinium adducts is also a dominant pathway for the modification of the primary amine with 2-alkenals, such as crotonaldehyde, 2-hexenal and 2-octenal [281]. Based on the identification of a novel acrolein-lysine adduct (MP-lysine), Furuhata et al. [287] re-examined the specificity of the monoclonal antibody raised against acrolein-modified protein and found that the antibody recognized MP-lysine far more efficiently than FDP-lysine. The preferential recognition of the antibody to MP-lysine has been explained by the structural characteristics in the side chain of these adducts. In contrast to FDP-lysine, MP-lysine contains a more fixed, positive charge on the pyridinium side chain, which may represent important immunological epitopes. Indeed, a monoclonal antibody raised against crotonaldehyde-modified proteins recognized a similar pyridinium adduct, N^{ε}-(5-ethyl-2-methylpyridinium) lysine (EMP-lysine), as the major epitope [281]. In addition, Nagai et al. [288] have raised a monoclonal antibody against glycolaldehyde-modified protein and found that a lysine pyridinium adduct constitutes an epitope of the antibody.

Among the 2-alkenals, 2-nonenal is probably the most well recognized substance due to its relevance to ageing. It has a characteristically unpleasant greasy and grassy odour. It is also a major contributor to the unpleasant cardboard flavour in aged beer. It was previously shown that 2-nonenal could be formed through lipid peroxidation as a product in peroxide-mediated oxidation of high concentrations of linoleic acid hydroperoxide or from liver microsomes treated with ADP/iron *in vitro* [35]. Toyokuni et al. [289] also reported the production of C2–C12 saturated and unsaturated aldehydes, including 2-nonenal, in the kidney of rats exposed to ferric nitrilotriacetate (Fe^{3+}-NTA), an iron chelate that induces acute renal proximal tubular necrosis, a consequence of free radical-mediated oxidative tissue damage, eventually leading to a high incidence of renal adenocarcinoma in rodents. More recently, Haze et al. [290] analysed the body odour components that adhered to the subjects' shirts by GC/MS and demonstrated that 2-nonenal is present in increasing amounts in the body odours of persons 40 years or older. They also suggested that *cis*-2-nonenal and *trans*-2-nonenal are formed from the oxidative degradation of polyunsaturated fatty acids, such as palmitoleic acid. A monoclonal antibody against protein-bound 2-nonenal was recently developed and the epitope structure recognized by the antibody was identified to be novel 2-nonenal-lysine adducts possessing a pyridinium structure [279]. The immunohistochemical studies also demonstrated the formation of the immunoreactive materials in the kidney of rats exposed to Fe^{3+}-NTA. Furthermore, using high performance liquid chromatography with on-line electrospray ionization tandem mass spectrometry, the formation of the 2-nonenal-lysine pyridinium adducts during the lipid peroxidation-mediated modification of protein has been confirmed *in vitro* and *in vivo*.

H_2O_2-mediated protein modification by n-alkanals

Among the variety of lipid peroxidation-derived aldehydes, the saturated aldehydes, such as hexanal, are most abundantly formed [276,277]. Hexanal, an aldehyde produced in high quantity during lipid peroxidation, shows metabolic, genotoxic and mutagenic effects, as well as inhibitory effects on cell proliferation [35]. It has also been shown that hexanal is by far the major aldehyde and that its production correlates well with the oxidation of PUFAs in LDL and reflects the degree of LDL oxidation *in vitro* [291–294]. Moreover, a remarkable over-production of this aldehyde was shown in skin fibroblasts from a patient with cardiomyopathy and cataracts both under basal conditions and after menadione or doxorubicin treatment *in vivo* [295]. Other short chain n-alkanals, such as acetaldehyde, have also been detected in micromolar amounts in the effluent perfusates of hearts perfused with a free radical-generating system and are proposed to be useful markers for monitoring oxidative stress during reperfusion of ischemic myocardium [296].

It is well recognized that saturated aldehydes mainly form Schiff bases through the formation of unstable

carbinolamine intermediates. However, due to the reversibility of this reaction, the simple aldehydes have received relatively little attention as protein-modifying reagents. Ishino et al. [279] recently discovered that H_2O_2 and to a lesser extent alkyl hydroperoxides are capable of mediating covalent modification of proteins by saturated aldehydes (Figure 5). This finding suggests the possibility that saturated aldehydes, in combination with H_2O_2 or ROOH, may contribute to the modification of nucleophilic biomolecules and the development of tissue damage under oxidative stress. A probable mechanism for the reaction has been suggested to be the imine analogue of the Baeyer-Villiger reaction of ketones with peroxides to give esters, which also pertains to the mechanism of oxidation of aldehydes to carboxylic acids by ROOH. The reaction would proceed by addition of ROOH (R=H, alkyl) to the Schiff base, followed by 1,2-migration of hydride and expulsion of H_2O or alkyl-OH, respectively. The reaction may be acid-catalysed (to create a better leaving group) and would be more efficient for the latter reason using a peracid rather than ROOH. At the same time, however, it is well known that potent oxene donors like peracids react with imines (usually in organic solvent) directly to generate oxaziridines, semi-stable species that decompose to amides only upon heating or in the presence of transition metal catalysts [297]. Although the Schiff base-derived peroxycarbinolamine could decompose to oxaziridine in competition with 1,2-hydride migration, the distinction of the Baeyer-Villiger as opposed to oxaziridine pathway has been pointed out in the literature [298]. In the reaction using 3-chloroperoxybenzoic acid at low concentration, no oxaziridine product was observed, although it is unclear whether such species would survive the ionization conditions. In any event, since the generation of circulating peracids in physiological oxidative stress is unlikely, it seems unnecessary at this time to invoke a competing oxaziridine pathway for amide formation.

N^{ε}-Hexanoyllysine formed upon the reaction of a lysine derivative with hexanal in the presence of H_2O_2 was previously identified as a product of the reaction of the lysine residue with the oxidized linoleate, 13-hydroperoxyoctadecadienoic acid [299]. The authors speculated the direct interaction between the lipid hydroperoxide and the lysine residues of protein as an underlying mechanism. Metz et al. [300] also isolated a similar *N*-hexanoylated derivative of pyridoxamine and proposed a mechanism in which the conjugated diene hydroperoxides oxidatively decompose to ketoaldehydes, which then react with the primary amine to form the *N*-acylation product through the formation of a hemiacetal derivative. Based on the fact that lipid peroxidation generates a great number of oxidized products, including aldehydes and reactive oxygen species, the combined action of saturated aldehydes and either H_2O_2 or ROOH is likely to occur. Thus, N^{ε}-hexanoyllysine, which has been considered to be one of the earlier and stable markers for lipid peroxidation-derived protein modification compared to the aldehyde-derived protein adducts [301], may actually be the product of the lysine modification by hexanal, originating from the lipid hydroperoxide, and either H_2O_2 or the circulating lipid hydroperoxide directly as the oxidant.

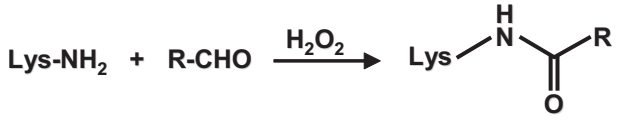

Figure 5. Reaction of saturated aldehydes with lysine.

In conclusion, this part of the review summarized the protein adduction chemistry with volatile aldehydes, such as 2-alkenals and n-alkanals, generated from the peroxidation of polyunsaturated fatty acids. The knowledge of the protein reactivity of these volatile aldehydes provides an under-pinning for the eventual interpretation of various types of biological activities that are being observed for these important exogenously and endogenously formed molecules. Furthermore, it has been shown that the protein bound to aldehydes could be an excellent immunogen that is capable of stimulating an adaptive immune response. Of interest, a monoclonal antibody, showing recognition specificity toward DNA, has been shown to bind protein-bound aldehydes. These findings suggest the connection between modification of proteins by aldehydes and autoimmune response. Further studies are required to understand the biological consequences of the production of aldehydes under oxidative stress.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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