Lipophilic fluorescent products of free radicals

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Background. Fluorescent pigments are the end-products of reactions involving free radical attack on biological molecules and can be formed, for example, in reactions between lipid peroxidation products, mainly unsaturated aldehydes, with free amino groups. Their characteristic emission maximum was found to be at 420-470 nm after being excited at 340-390 nm. The mechanism of their formation and chemical identity has been revealed in many in vitro studies, in which reactive aldehydes were incubated with amino group-containing molecules. Owing to their intrinsic fluorescent properties and molecular stability these products are easily measured by means of spectrofluorimetry and are used as biomarkers of oxidative stress caused by various triggers. It has been found that the fluorescent products are formed in excess in conditions linked with increased free radical production, such as atherosclerosis, Alzheimer's disease and multiple sclerosis.

Methods. We searched the literature using "MEDLINE" and "Web of Science" in order to get an overview of the state of knowledge about fluorescent products of free radicals, that is, their analysis from in vitro studies, animal and human studies and their use as markers of oxidative damage.

Conclusions. Although their chemical structure may not have been elucidated, the fluorophores formed in this way have found application as markers of oxidative stress in many animal and human studies. In vitro experiments using model reactions have given some clues as to how certain fluorescent pigments arise during oxidative reactions in vivo. Advances in analytical techniques should lead the chemical characterization of pigments of different origin to completeness.

Key words: lipofuscin-like pigments, lipid peroxidation, free radicals, fluorescence

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INTRODUCTION

Free radicals are chemical species that are, due to unpaired electrons, renowned for their extremely high reactivity. Being indiscriminate with regard what molecule they will react with, in biological environments these reactive species most often attack molecules essential for maintaining cellular homeostasis. Membrane lipids, which contain polyunsaturated fatty acids in their structure, are particularly prone to oxidative damage by free radicals. As a result of free-radical reaction, fatty acids are oxidized into lipid hydroperoxides that, because of their instability, easily break down into products capable of doing further damage to biological material. Finally, relatively stable, i.e. not as reactive as their precursors, chloroform extractable lipophilic fluorescent end-products are formed. Tappel et al[1] published the first paper claiming that the lipid peroxidation product – malondialdehyde (MDA), in reaction with free amino groups, produces fluorescent pigments. The end-products were initially called lipofuscin-like pigments (LFP) on the basis of the similarity of their fluorescence properties with those of lipofuscin - the pigment of old age[2]. However, in later studies it was documented that these blue fluorescent compounds were not directly related to yellow-brown fluorescent age pigment - intracellular and extracellular fluorescent material that accumulates in aged tissues[3]. Though other names were coined, like chromolipids[4-6], advanced lipid peroxidation end-products (ALEs) (ref.[7]) and ceroids, defined as pigments that accumulate due to various pathological processes[8,9] , the original term has survived.

These blue fluorescing pigments have specific excitation and emission maxima. Their emission maximum was found to be at 430-470 nm on being excited at 260-280 nm and 350-390 nm, indicating two excitation maxima. As it was reported, the compounds had aminoiminopropene (-N=CH-CH=CH-NH-) structure[10]. Another fluorescent group of compounds showing characteristic blue fluorescence was reported by Kikugawa et al.[11] being derivatives of 1,4-dihydropyridine-3,5-dicarbaldehyde (Fig. 1). These compounds exhibit fluorescence emission maxima at 435-465 nm when excited at 365-405 nm. Uchida and Itakura[12] argue the relevance and even contribution of products with aminoenimine structure to the fluorescence of LFP explaining that the fluorophore in these pigments is almost exclusively a dihydropyridine adduct. These fluorescent compounds all originated from reaction between MDA and amino groups of proteins, amino acids or phospholipids. Apart from MDA, another aldehyde produced during lipid peroxidation, 4-hydroxy-2-nonenal

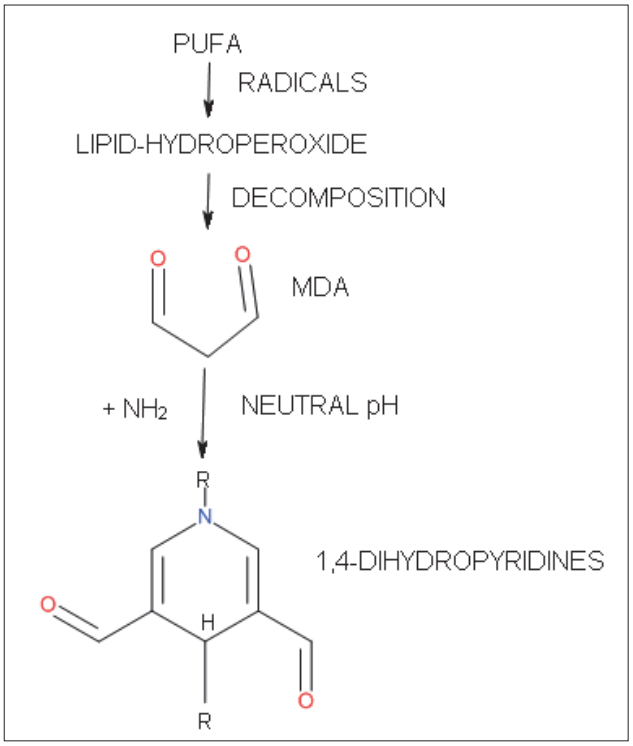

Fig. 1. Scheme of lipid peroxidation and the production of fluorescent end-products.

(4-HNE), contributes to formation of fluorescent LFP (ref.[6]). Additionally, numerous fluorescent products have been obtained following reactions between primary amines and lipid peroxidation products other than MDA and 4-HNE (ref.[13]).

It appears that LFP are generated in all biological systems. Besides several human and animal studies, they were also detected during plant ageing[14-16] and even in yeasts[17,18] and bacteria[19]. LFP were extractable to chloroform from various biological membranes. The original study, and many others, found LFP in cell membranes and mitochondria, but they were also detected in nuclei and nuclear membranes[20] and microsomes[6,21] .

LIPID PEROXIDATION

Free radicals and non-radical species of oxygen, collectively known as reactive oxygen species (ROS) are particularly important in biological systems. ROS react with various biological compounds and the consequence of these reactions is most often the damage to those molecules[22-26].

One group of compounds that are highly sensitive to damage by ROS are polyunsaturated fatty acids (PUFA) found in complex lipids e.g. phospholipids. The process where lipids, in particular PUFA, are oxidatively changed is called lipid peroxidation[27, 28]. Lipid peroxidation has been identified as a basic deteriorative process in cellular membranes[10]. Lipid peroxidation is a chain reaction in which a hydrogen atom is abstracted from allylic carbon yielding carbon-centred radical. If this radical reacts with oxygen a peroxyl radical is generated, which can easily react with neighbouring fatty acid forming a lipid-hydroperoxide, thus repeating the cycle[29].

Further decomposition of lipid-hydroperoxide gives rise to highly reactive aldehydes such as acrolein, MDA, 4-HNE (ref.[30]) and 4-hydroxyhexenal[31]. MDA and 4-HNE are widely used markers of lipid oxidation in a great variety of samples. 4-HNE is formed mainly from linoleic acid, whereas MDA arises mostly as a product of arachidonic acid oxidation[32, 33]. The α, β – unsaturated aldehydes easily react with many biomolecules (proteins, amino acids, DNA, phospholipids), which results in their impairment. Carbonyl groups of aldehydes react with amino groups of amino acids or phospholipids forming Schiff bases[34]. The products of these reactions are lipofuscin-like fluorophores[1,2,35,36]. The most studied aldehydes participating in fluorescent end-product formation are 4-HNE and MDA (ref.[37, 38]).

CHARACTERIZATION OF FLUORESCENT END-PRODUCTS

Fluorophores, produced by cross-linking amino groups with carbonyl moieties of reactive aldehydes, represent a mixture of numerous compounds of known and unknown chemical structure. The fluorescent pigments showing representative lipofuscin-like fluorescence of known structure or at least of known origin have been best characterized and described in experiments where they were formed in vitro by incubating aldehydes derived from lipid oxidation (MDA, 4-HNE) with compounds having a free amino group. Similarly, compounds with a primary amino group can be incubated with PUFA itself or PUFA containing phospholipids in order to analyse arising fluorophores[39]. Fluorescent compounds produced in model reactions in vitro with known and unknown chemical structure have been analysed in order to gain insight into the mechanism of their formation. Trombly and Tappel prepared such fluorescent products in reaction between arachidonic acid and a synthetic phospholipid - dipalmitoyl phosphatidyl etahanolamine (PE), irradiated with UV light to initiate free radical formation, as well as by mixing MDA and PE. They determined excitation maxima of the products, which were at wavelengths 260 nm and 365 nm, and one emission maximum at 430 nm. The fluorescence observed here lies within the interval typical for lipid peroxidation end-products[10]. In similar in vitro experiments Deng and colleagues synthesized fluorescent products in direct reaction of MDA with biological amines e.g. gamma-aminobutyric acid (GABA) and taurine. In addition, they confirmed the structure of a lipofuscin-like fluorescent product – 1,4-dihydropyridine, which was detected by means of HPLC. Besides this, these findings also show the scavenging properties of various biological amines on reactive aldehydes formed during lipid peroxidation. The amines can attenuate reactive aldehydes' harmful effect as they are able to compete for aldehyde carbonyl groups with amino groups of important macromolecules, thus preventing protein adduct and cross-link formation[40, 41].

In another experiment, MDA was incubated with pyridoxamine (PM) and this yielded three different products analysed by HPLC and LC-MS: 1-amino-3-iminopropene, 1-pyridoxamino-propenal and a dihydropyridine-pyridinium complex. Dihydropyridine-pyridinium derivative and dihydropyridine are end-products showing typical lipofuscin-like fluorescence. The same authors incubated bovine serum albumin (BSA) with MDA, which resulted in formation of fluorescent products with fluorescence similar to dihydropyridines, and whose intensity was decreased when PM was added, clearly indicating that PM can inhibit, to some extent, the reaction of MDA with proteins[42].

Slatter et al. found that the principal product of the reaction of MDA and propylamine is a stable dihydropyridine derivative, namely *N*-propyl-4-methyl 2,6-dihydropyridine 3,5-dicarbaldehyde. Likewise, if a protein lysine residue, whose amino group is most reactive towards aldehydes, reacts with MDA the product is *N*-lysyl-4-methyl 2,6-dihydropyridine 3,5-dicarbaldehyde. Additionally, the aldehyde side-chains on the dihydropyridine ring can further react with other amino groups to form protein cross-links. Similarly, protein amino groups other than those of lysine can directly react with MDA to form adducts[33, 43].

Another reactive aldehyde formed during lipid peroxidation, 4-HNE, is also responsible for production of fluorescent compounds in reactions with amino groups. It has been found that HNE forms a fluorescent hydroxyiminodihydropyrrole derivative with the amino group of lysine residue in oxidized low-density lipoprotein[44]. In another study, where in a model reaction, lysine derivative was exposed to autoxidation of linoleic acid, a fluorophore with hydroxyiminodihydropyrrole structure was produced. The fluorophore was analysed by HPLC with fluorescence detection (360/430 nm excitation/emission). HPLC analysis revealed two different products, one being lysine derivative-HNE adduct, while the second adduct was made of another lipid peroxidation product, 9-hydroxy-12-oxo-10-dodecanoic acid, and the lysine derivative. This finding was confirmed by LC-MS analysis[45]. Riazy et al. described lipid peroxidation end-products formed by addition of unfragmented oxidation products of arachidonic and linoleic acid, probably unfragmented aldehydic products, hydroperoxides or endoperoxides, onto amino groups of proteins and phospholipids. On analysing these adducts by MS/MS, the authors found that some of the arachidonic acid-derived lysine-adducts were isolevuglandins containing lactam and hydroxylactam rings. The fluorescence maxima for these products were 360 nm for excitation and 430 nm for emission[46]. These findings show that not only decomposition products of lipid-hydroperoxides (mainly aldehydes), but also unfragmented oxidized lipids, can form LFP fluorophores.

METHODOLOGICAL APPROACHES TO LFP ANALYSIS

To quantify the LFP concentration in a given biological sample, the sample is usually extracted to chloroform-methanol mixture (2:1, v/v) which is then washed with water. The chloroform phase is separated and used for fluorescence measurements. This first step in analysis is the most important and potentially flawed by inappropriate ratio of analysed tissue to the volume of the extraction mixture. The intensity of fluorescence is proportional to the concentration of the fluorophores in a reasonable concentration range. However, at high concentrations of the fluorophores proportionality is no longer satisfied, because significant collisional quenching between the molecules of the fluorophore themselves appears. Therefore, if the concentration of LFP fluorophores in the sample is higher than the concentration, above which the fluorescence is no longer proportional to the concentration and can be termed threshold, LFP can produce internal quenching of fluorescence. This concentration, up to which the linear dose-response is obtained, differs in individual samples. As LFP are not chemically characterized, it is not possible to express their concentration in absolute units. LFP in different tissues too have different composition. Thus, the concentration of LFP can be expressed in arbitrary units only and comparison between different tissues is not possible. Generally, if we extract less than 15 mg of tissue in 1 mL of solvent, we obtain LFP at a concentration that is within linear dose-response range. However, before LFP assay in a new tissue, the range of linear dose-response must be assessed.

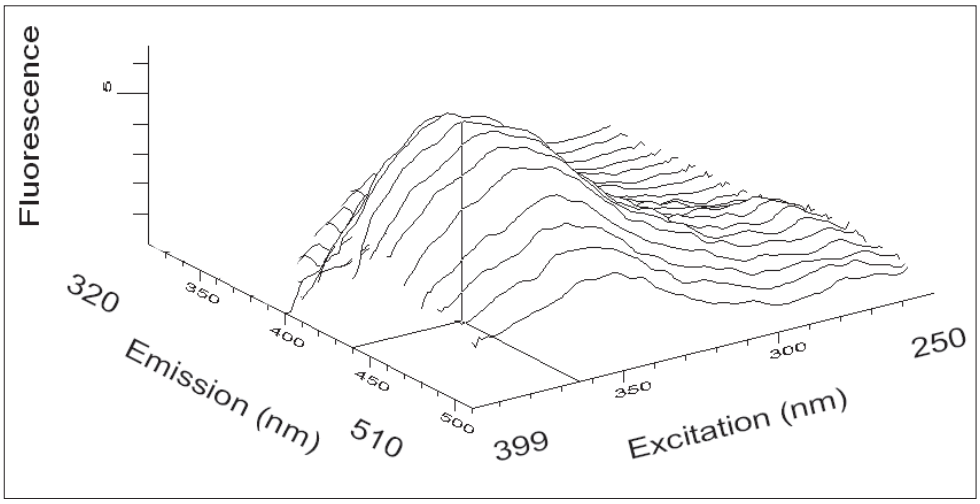

Fig. 2. Fluorescence tridimensional spectral arrays of extracts from isolated mitochondria incubated in vitro with MDA at 37°C for 48 hours. Fluorescence intensity is expressed in relative fluorescence units.

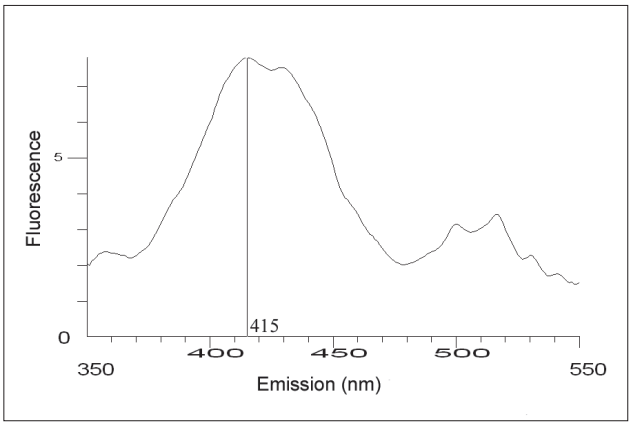

Fig. 3. Fluorescence synchronous spectral arrays of extracts from isolated mitochondria incubated in vitro with MDA at 37°C for 48 h. The difference between excitation and emission is 50 nm. Fluorescence intensity is expressed in relative fluorescence units.

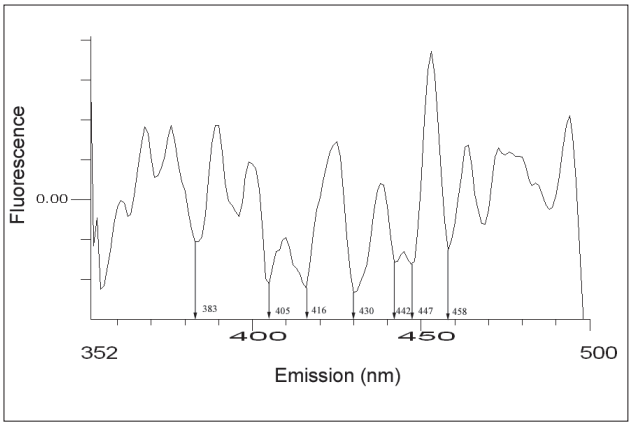

Fig. 4. Second derivatives of synchronous spectral arrays of extracts from isolated mitochondria incubated in vitro with MDA at 37°C for 48 h. The vertical arrows indicate the emission maxima of major peaks. Fluorescence intensity is expressed in relative fluorescence units.

The next step usually consists in finding excitation and emission maxima. This is most easily done by measuring 3D spectral arrays of fluorescence spectra. In this way we find even minor fluorescence peaks. For illustration see Fig. 2 which depicts 3D spectral arrays of extracts from isolated mitochondria incubated in vitro with MDA at 37 °C for 48 h.

LFP is a mixture of several fluorophores that can be characterized by differential synchronous fluorescence spectra. This method has been developed for the analysis of complex mixtures and represents a "fingerprint" of a given sample. This method is especially advantageous if we study changes during certain biological processes (e.g. development of a pathology, or ageing of a cell culture). An example of this spectrum is shown in Fig. 3 (LFP from isolated mitochondria incubated in vitro with MDA at 37 °C for 48 h with the constant difference of 50 nm between excitation and emission wavelengths). Finer resolution of the fluorophores into individual species is obtained by analysing 2nd derivatives of synchronous spectra as seen in Fig. 4 using the same sample as Fig. 2 and 3.

By applying these procedures, we obtain spectroscopically characterized fluorophores that can be quantified in relation to a given standard. We can further resolve the mixture by HPLC with a fluorescence detector. Using HPLC we can resolve spectrally characterized fluorophore into several species. This technique with online mass spectroscopy could lead to final characterization of LFP fluorophores. However, this is a quite complicated task due to the number of fluorescent species and their low concentration. So far this goal has not been achieved.

LFP AS MARKERS OF OXIDATIVE STRESS IN MODEL SYSTEMS

LFP are currently mainly used as indicators of oxidative stress. As relatively stable end-products of lipid peroxidation, LFP are good markers of free radical production and of consequent damage to lipids. Moreover, they are used not only as markers of lipid degradation but also to estimate amino acid and protein loss due to cross-linking. So far, LFP have been mostly used as robust markers of oxidative damage without defining the specific chemical identity of compounds representing these pigments. In such cases, the fluorescent pigments are simply markers of free radical production under different circumstances.

Free radical production and oxidative damage in biological systems, caused by various triggers, were assayed by measuring LFP in many studies. Hypoxia, hyperoxia and ischemia/reperfusion are accompanied by increased production of free radicals and LFP were used as markers of oxidative damage[36, 47-49]. Elevated LFP concentrations were found in erythrocytes and spleen of rats exposed to hypoxia for different time periods compared to animals that had been kept in normoxic conditions[36]. Measurement of LFP concentration proved to be useful when assessing the antioxidative property of different potential antioxidants[49,50]. Further, LFP were used as indicators of oxidative stress induced by physical activity[51], phagocytosis of oxidized proteins[52] and ionizing radiation[53-60]. LFP were analysed in various tissues and cell compartments of rats exposed to radiation such as liver homogenates and mitochondria[53,56,58], skeletal muscles[55], erythrocytes[54], spleen[54] and adipose tissue[59,60]. There are also studies where, for example, erythrocyte ghosts or mitochondria were peroxidized in vitro using different triggers of free radical production (ferrous ions and ascorbic acid) or incubated with lipid peroxidation intermediates (MDA), in order to study the mechanisms of LFP formation or to assess the extent of radical-mediated damage[50,61-63]. Low-density lipoproteins (LDL), which play a very important role in atherosclerotic lesion formation, have been also extensively studied as a model protein, with regard to its oxidation and forming fluorescent adduct on the protein amino groups[44, 46] As mentioned, lipofuscin is the ageing pigment and it accumulates as a normal part of senescence. On the other hand, LFP are produced as a result of oxidative stress, which is connected to pathological processes. LFP are known to be involved in ageing and diseases related to

it e.g. neurodegenerative and heart diseases. Hence, LFP are a potential marker of oxidative damage to biological material due to increased ROS production in aged subjects. In one such study, LFP were used as a tool to assess the extent of ROS formation in brain cortex of rats during early postnatal development. The highest accumulation was found immediately after birth and the levels were falling up to three months from birth, which is believed to be a period when ageing starts in rats[64]. LFP were also used in an experiment conducted on dogs diagnosed with canine counterpart of senile dementia of Alzheimer's type as one of the markers of lipid peroxidation, which has been proposed to take part in the pathogenesis of this condition[65]. Sen et al. measured fluorescent lipid peroxidation end-products as markers of ageing in rat brains. Aged rat brains had considerably increased accumulation of end-products compared to those from young animals[66]. Davydov and Shvets analysed lipid peroxidation products, with LFP being one of them, in the heart of adult and old rats exposed to stress to assess whether myocardium becomes less resistant against stress at old age[67]. LFP were also measured in brain of rats exposed to lead in order to investigate the possible mechanism of lead toxicity and its role in oxidative stress in brain[68].

APPLICATION OF LFP ANALYSIS IN BIOMEDICINE

Since LFP are markers of oxidative damage of lipids and other molecules caused by ROS, their measurement may be a useful tool for monitoring pathological processes linked with oxidative stress. ROS overproduction, which leads to impaired biological molecules, underlies the pathophysiological mechanism of many diseases. The most common disorders, whose developments ROS contribute to, are neurodegenerative diseases e.g. Alzheimer's disease[69], Parkinson's disease[70] and multiple sclerosis[69], atherosclerosis and other vascular diseases[25], cancer[71] and diabetes[72].

The role of ROS in the pathogenesis of Alzheimer's disease (AD) has been widely accepted as there is a strong evidence for it in scientific literature. ROS production exceeding its removal by antioxidant mechanisms leads to oxidative injury in the AD brain. PUFA from brain phospholipids are notably prone to oxidation and there are several studies showing that lipid peroxidation is increased in the AD patient compared with healthy subjects[73,74]. Fluorophores have been described, and identified as HNE-adducts with a pyrrole structure, in the neurons and lesions of AD subjects[75]. Inasmuch as LFP are end-products of lipid peroxidation, they might be used as specific markers of AD. In a study by Skoumalova and colleagues LFP extracted from red blood cells of patients with AD and their age-matched controls were analysed in order to find a diagnostic marker easy to measure since blood samples can be routinely taken. They found an increase in LFP formation, measured by means of fluorescence, in patients diagnosed with AD when compared with controls. As particular fluorophores, obtained by fluorescence measurements, represent a mixture of different compounds they were then resolved into distinctive fractions by HPLC. Chromatograms from these two groups revealed a difference in LFP composition[76]. Another neurodegenerative disease linked with oxidative stress is multiple sclerosis (MS). Yet the relation between oxidative stress and the progression of disability in MS is still unclear. Therefore, Koch and colleagues attempted to assess this relation using LFP as a marker of oxidative stress. LFP was measured in plasma from patients with a different MS disease course and healthy controls. LFP levels were increased in MS patients, compared to controls, but the authors failed to confirm the relation between oxidative stress and disease course and progression[77].

Vascular diseases characterized by formation of atherosclerotic lesions are associated with oxidative stress. ROS overproduction leads to increased oxidation of low-density lipoprotein, endothelial dysfunction and vascular smooth muscle cell proliferation, which promotes progression of atherosclerotic events[78,79]. Coronary heart disease (CHD) is characterized by accumulation of atherosclerotic plaques and their subsequent rupture in the coronary arteries that supply the myocardium with oxygen and nutrients. Among other parameters that predict disease development and progression, lipid peroxidation products malondialdehyde and fluorescent end-products are ones that are frequently utilized[80]. Wu et al. carried out clinical studies using fluorescent LFP as potential markers of oxidative stress. LFP were measured in plasma from individuals participating in the epidemiological studies to evaluate their ability to predict development of CHD as well as to assess their potential as a global marker of oxidative stress in this type of study. It was found that high levels of fluorescent products were significantly associated with incidence of CHD among individuals without previous cardiovascular events. Further, these markers were found to be an independent risk factor for CHD. The same authors concluded that LFP measured in plasma could be a useful marker of oxidative stress for large epidemiological studies[81,82]. Tertov et al. measured LFP in normal and atherosclerotic areas of the human aorta to deepen our knowledge of atherogenesis and the development of advanced lesions in arterial walls. The level of fluorescent LFP, measured at 360/430 nm (excitation/emission), did not differ markedly between normal intima and initial lesions. Nevertheless, there was a significant rise in LFP level from atherosclerotic plaques compared to unaffected intima. In addition, it was found that LFP content in lipid extracts from the media underlying normal intima and initial lesions was similar, whereas it was significantly higher in extracts from the media underlying advanced atherosclerotic lesions[83]. Gu et al. stressed the potential usefulness of a specific lipid peroxidation product, omega-(2-carboxyethyl) pyrrole, as a marker for predicting the development of age-related macular degeneration. This protein adduct is formed from a product of free-radical induced oxidation of docosahexaenoic acid[84].

CONCLUSION

LFP are formed as the products of reactions between carbonyls and amino group of biological material. There are many sources of reactive carbonyls. Free radical-mediated peroxidation of lipids from cell membranes contributes substantially to the formation of these very reactive compounds. LFP are a group of compounds that are relatively stable and have intrinsic fluorescence, which makes their analysis easy. Therefore, fluorophores produced in the final stage of the oxidation reactions have great potential as a reliable marker of these processes. To date, these vague fluorophores are still poorly defined and just few of them have been chemically characterized. Nevertheless, LFP have proven to be very useful as indicators of oxidative stress and the pathologies related to it in many animal and some human studies. Improvements in analytical techniques and methods would enable easier characterization and identification of hundreds of fluorophores formed either as consequence or at the onset of various pathological conditions. This is particularly the case with biological samples in which fluorophores, produced as a result of ROS attack on biomolecules, are present in complex mixtures. What complicates understanding of these pigments is that, once formed, they can produce further adducts and cross-links with other molecules. In vitro studies have elucidated some of the mechanisms of LFP formation and have revealed their complete characterization i.e. chemical structure of some fluorophores. This could aid in better understanding the metabolism of all fluorophores formed as products of side reactions in various tissues undergoing pathological processes in vivo.

ABBREVIATIONS

4-HNE, 4-hydroxy-2-nonenal; AD, Alzheimer's disease; ALEs, Advanced lipid peroxidation end-products; BSA, Bovine serum albumin; CHD, Coronary heart disease; GABA, Gamma-aminobutyric acid; HPLC, High-performance liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; LDL, Low-density lipoprotein; LFP, Lipofuscin-like pigments; MDA, Malondialdehyde; MS, Multiple sclerosis; MS/MS, Tandem mass spectrometry; PE, Dipalmitoyl phosphatidyl etahanolamine; PM, Pyridoxamine; PUFA, Polyunsaturated fatty acid; ROS, Reactive oxygen species; UV, Ultraviolet.

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Authorship contributions: JI, JW: literature search, manuscript writing, study design, data collection, analysis and interpretation; JI: statistical analysis, figures; JW: final approval.

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