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Review article

Spatio-temporal monitoring of lipid peroxyl radicals in live cell studies combining fluorogenic antioxidants and fluorescence microscopy methods

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ABSTRACT

Lipid peroxidation of polyunsaturated fatty acids in cells may occur via their catalytic autoxidation through peroxyl radicals under oxidative stress conditions. Lipid peroxidation is related to a number of pathologies, and may be invoked in new forms of regulated cell death, yet it may also have beneficial roles in cell signaling cascades. Antioxidants are a natural line of defense against lipid peroxidation, and may accordingly impact the biological outcome associated with the redox chemistry of lipid peroxidation. Critical to unraveling the physiological and pathological role of lipid peroxidation is the development of novel probes with the partition, chemical sensitivity and more importantly, molecular specificity, enabling the spatial and temporal imaging of peroxyl radicals in the lipid membranes of live cells, reporting on the redox status of the cell membrane. This review describes our recent progress to visualize lipid peroxidation in model membrane systems and in live cell studies. Our work portrays the mechanistic insight leading to the development of a highly sensitive probe to monitor lipid peroxyl radicals (LOO•). It also describes technical aspects including reagents and fluorescence microscopy methodologies to consider in order to achieve the much sought after monitoring of rates of lipid peroxyl radical production in live cell studies, be it under oxidative stress but also under cell homeostasis. This review seeks to bring attention to the study of lipid redox reactions and to lay the groundwork for the adoption of fluorogenic antioxidant probeshancement and maximum intensity recorded in turn provide a benchmark to estimate, when compared to the control BODIPY dye lacking the intramolecular PeT based switch, the overall exte and related fluorescence microscopy methods toward gaining rich spatiotemporal information on lipid peroxidation in live cells.

1. Introduction

The term ROS (reactive oxygen species) has been coined to define an emerging class of endogenous, highly reactive, oxygen-bearing molecules. ROS are formed from the incomplete reduction of oxygen, as well as from the by-products of their reactions [1]. The most prevalent ROS found in living systems are singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\bullet-}$), hydroxyl radical (HO•), hydrogen peroxide (H_2O_2), and of relevance to this work, lipid peroxyl radicals (LOO•).

Most endogenous ROS are produced in the mitochondrion as by-products of redox reactions occurring in the electron transport chain. Indeed, 0.1–2% of oxygen is converted to superoxide radical anion ($O_2^{\bullet-}$) during respiration in vitro. Significant $O_2^{\bullet-}$ production can occur when mitochondria are not making ATP (high proton motive force) and when the ratio of reduced: oxidized electron carriers is high (e.g. NADH: NAD^+ and ubiquinol: ubiquinone) [2,3]. ROS production can also stem from exogenous sources such as UV radiation, drug metabolism, and bio-redox reactions.

The implication of ROS to various physiologies and pathologies is a complex topic, and much remains to be understood of their role in life. According to the "free radical theory of aging," advanced by Denham Harman in 1956, ROS are deleterious molecules directly responsible for aging and disease [4]. While once a long-held belief, this hypothesis is currently under revision [5,6]. Indeed, overproduction of ROS is associated with many diseases and pathologies such as neurodegenerative diseases, inflammation and cancer [7–10]. However, controlled production and release of ROS is critical for maintaining homeostasis, and enhancing the defense mechanisms of the cell [6,11].

The development of probes to monitor the effects of ROS in live cell studies and tissues will, we postulate, enable underpinning the complex physiological role they play in life. Herein, we describe recent efforts toward monitoring real-time the spatial and temporal evolution of lipid peroxyl radicals, a highly elusive, lipophilic form of ROS, in live cell studies.

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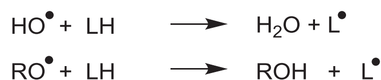
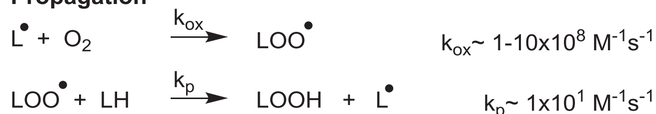
Initiation

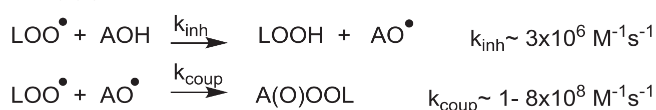


Propagation



Termination

Inhibition



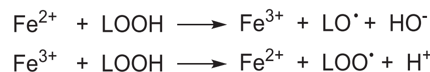
Scheme 1. Elementary reactions and their associated rate constants for the four steps of lipid peroxidation: Initiation, Propagation, Termination and Inhibition. LH represents a polyunsaturated fatty acid. (k_{ox} [14], k_p [14], k_t, [14], k_{inh} [15], k_{coup} [16]).

2. Lipid peroxidation via free radical chain autoxidation reactions

Following formation, ROS may react with vital bio-molecules including proteins, DNA, and lipids, effectively altering their structure and hence their function [1,12,13]. Lipids, specifically polyunsaturated fatty acids (PUFA) present in cellular membranes, are particularly susceptible to ROS elicited damage due to the relatively weak C–H bond adjacent to the unsaturation [14,15]. PUFA are readily oxidized to lipid hydroperoxides (peroxidation) in a reaction that is initiated by a free radical and that takes place through a free radical chain reaction. The autoxidation of PUFA thus occurs via four steps: 1) initiation, 2) propagation, 3) termination, and, in the presence of a radical trapping antioxidant (RTA, AOH in Scheme 1), 4) inhibition, see Scheme 1 below [14].

2.1. Initiation and propagation

Lipid peroxidation via autoxidation is initiated by an H-atom abstraction at the bis-allylic position of a polyunsaturated acid by a source of ROS, such as hydroxyl (HO•), alkoxyl (LO•), or peroxyl radicals (LOO•), to give a carbon centered radical L•. The relatively weak bond dissociation energy (BDE) of the bis-allylic H-C bond (estimated at 80 kcal/mol) [17] makes it particularly vulnerable to abstraction compared to H-C bonds of saturated lipids (100 kcal/mol) [17], where the low BDE is due to delocalization and thus stabilization of the resulting radical with the adjacent unsaturations. Once formed, L• may propagate the free radical chain reaction. Molecular oxygen first reacts at diffusion controlled rates with L• to give a lipid peroxyl radical, LOO• [14,18]. The newly formed lipid peroxyl radical may next abstract a hydrogen atom from another lipid, LH, to give L• and LOOH, a lipid hydroperoxide. H-atom abstraction by LOO• is relatively slow and consequently the rate determining step of the chain reaction [14]. Lipid peroxyl radicals, of particular interest to this review, are thus the effective chain carriers in the free radical chain autoxidation of PUFA. Importantly, newly formed LOOH may subsequently decompose in the presence of free iron, or copper, via Fenton-chemistry, to yield LO• and/or LOO• further initiating new chain reactions, see Scheme 2 below.



Scheme 2. Lipid hydroperoxides and Fenton chemistry in the presence of Fe [19].

2.2. Termination and inhibition

Newly formed LOO• may undergo chain termination reactions that are second order in LOO•, a highly unlikely event given their low concentration [20]. In turn, inhibition in the presence of RTAs (AOH in Scheme 1) plays a key role preventing chain autoxidation of PUFA. Phenolic compounds inhibit lipid peroxidation by scavenging peroxyl radicals via H-atom abstraction of the phenolic group, resulting in a phenoxyl radical (AO•). The phenoxyl radical then may couple to a second peroxyl radical to form a non-radical product. When the rate of initiation of lipid peroxidation (R_i) is constant, the consumption of AOH is zeroth order in antioxidant concentration and is dependent on R_i and the stoichiometric number of peroxyl radicals scavenged by the antioxidant, n (Eq. (1)). In homogeneous non-polar solvents, phenolic RTAs associated with Vitamin E family of compounds typically scavenge two peroxyl radicals (i.e. $n = 2$) [15].

$$\frac{d[AOH]}{dt} = -\frac{R_i}{n} \quad (1)$$

A member of the vitamin E family of compounds, α-tocopherol (TOH) has long been recognized as the most active naturally occurring lipid soluble antioxidant (Fig. 1) [15]. In a first elementary step, TOH reacts with a peroxyl radical (ROO• or LOO•) via H-atom transfer to yield a tocopheroxyl radical (TO•) and a hydroperoxide ROOH/LOOH. The analysis of the rate law for the overall process shows that the antioxidant activity of TOH is given by the rate constant for this second order reaction (k_{inh}, the rate constant of H-atom abstraction). In the next elementary step, the TO• initially formed rapidly scavenges a second ROO•/LOO• to yield addition products (e.g. tocopherones) [15,21–23]. In the presence of initiators and in homogeneous solution TOH thus scavenges two peroxyl radicals, where the rate of generation R_g of peroxyl radicals may be equaled to the rate of initiation R_i of lipid peroxidation and is calculated by measuring the rate of consumption of TOH (see Eq. (2)) [15].

$$-\frac{d[TOH]}{dt} = k_{inh}^{TOH} \times [LOO^{\bullet}] \times [TOH] = \frac{R_g}{2} \quad (2)$$

3. Lipid hydroperoxides: physiological and pathological role

Lipid peroxidation may occur at the cellular level via free radical chain autoxidation as described above, but also, in a controlled manner, via enzymatic pathways upon reaction with cyclooxygenases (COXs) and lipooxygenases (LOXs) [24,25]. Yet another biologically relevant mechanism yielding lipid peroxidation occurs upon reaction of singlet oxygen ($^1O_2$, another form of ROS) with PUFA [26–28]. This mechanism is particularly relevant to mechanisms of photodynamic therapy (PDT) [29]. In the latter case, upon sensitization, singlet oxygen may directly react with lipids via the ene reaction, forming lipid hydroperoxides.

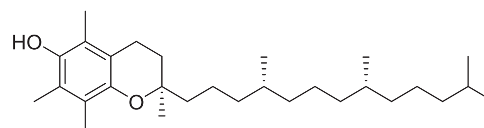


Fig. 1. Structure of α-tocopherol.

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