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Imaging reactive oxygen species dynamics in living cells and tissues

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1. ABSTRACT

Reactive oxygen species (ROS) can mediate cellular signal transduction, destroy hazardous foreign pathogens, regulate transcriptional activities and therefore are essential in normal cellular physiology. On the other hand, inadequate control of ROS production can induce peroxidation of essential biomolecules and result in cellular dysfunction. In the worst condition even cell death ensues. Therefore, they have great implications in the initiation and progression of numerous diseases. Chemical instability of ROS limits its direct detection and the dynamics remain poorly studied. To study the ROS dynamics, the endogenous or exogenous redox-sensitive fluorophores provide a good chance to detect the real-time changes in *in vivo* or *in vitro* settings. Here these methods are reviewed and their potential applications are discussed.

2. INTRODUCTION

Reactive oxygen species (ROS) are a diverse group of oxygen-containing chemical species with higher chemical reactivity than molecular oxygen. Free radicals (such as hydroxyl radical, superoxide radical, hydroperoxyl radical, lipid-peroxyl radical, and alkoxyl radical) and non-radicals (such as oxygen singlet, hypochlorous acid and hydrogen peroxide) are all included in the category. These molecules are produced either as a byproduct of respiratory activities, by mitochondria, or by various membrane or cytosolic oxidases. ROS can mediate cellular signal transduction, destroy hazardous foreign pathogens, regulate transcriptional activities and therefore are essential in normal cellular physiology. On the other hand, inadequate control of ROS production can induce peroxidation of essential biomolecules and result in cellular dysfunction. In

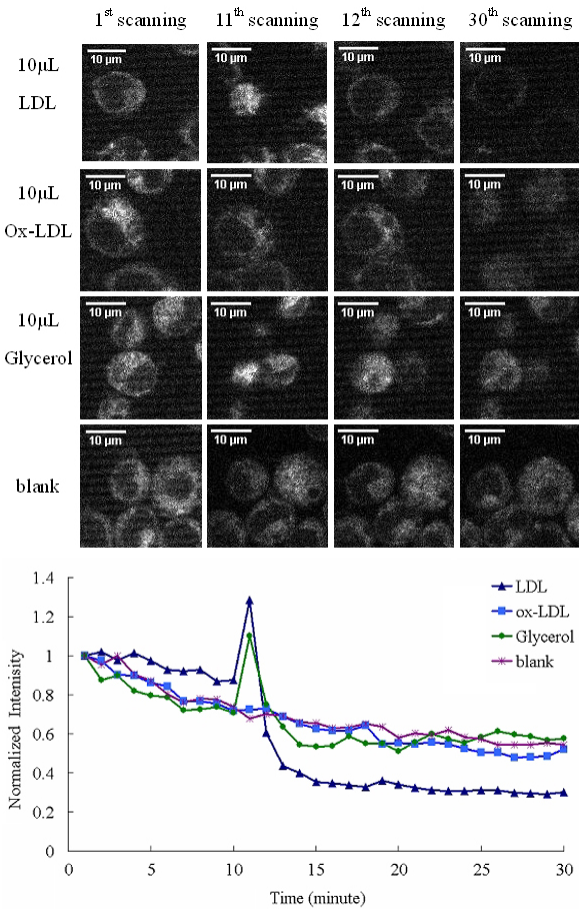

Figure 1. Cellular ROS dynamics in response to low-density lipoprotein.

the worst condition even cell death ensues. For the past decades an enormous body of evidence has demonstrated the role of ROS in initiation and progression of aging, cancers, chronic inflammation, neurodegenerative diseases as well as cardiovascular diseases. Since the half-life of ROS is extremely short, it is difficult to quantify directly the amount of ROS in living cells or tissues. Instead, most common methods only detect stable modified products (1). For example, superoxide dismutase-sensitive cytochrome C reduction and lucigenin-enhanced chemiluminence are two widely accepted methods to detect the reactive oxygen radicals. The former relies on the presence of reduced cytochrome C, which is formed by accepting an electron from oxygen radicals. The latter depends on electron transfer from oxygen radical to lucigenin and formation of a lucigenin-derived high energy intermediate, which spontaneously emits photon for detection. These methods, despite widely used and well accepted, usually require destructive preparation and as a result can not demonstrate spatial and temporal dynamics of ROS in a living system. Recently the advance of technology has made real-time ROS monitoring as a potentially feasible goal. Application of paramagnetic species in modified electron paramagnetic resonance imaging and Overhauser-enhanced magnetic resonance imaging techniques have successfully mapped redox status of an implanted tumor (2). However, the facilities are too expensive. Interested readers should be referred to more detailed reviews (3,4). Detecting ROS dynamics with redox-sensitive fluorophores is another potential and cheaper option. For example, the endogenous nicotinamide adenine dinucleotide phosphate (NADPH) as well as the exogenous dihydroethidium (DHE) could be used to monitor the ROS dynamics in both cells and tissues. Some research results and concerns regarding this interesting category of methods are discussed in the following sections.

3. NADPH-DERIVED AUTOFLUORESCENCE

NADPH is a major endogenous fluorophore in cells and is responsible for the green autofluorescence upon UV excitation. Its excitational spectrum peaks at 390 nm and emission spectrum peaks at 530 nm, which can be slightly modified by adjacent microenvironment. NADPH is produced during respiratory chain reactions, and is an essential cofactor for many enzymes. During intracellular oxidative stress, NADPH is oxidized to NADP and lost its fluorescent properties. Therefore NADPH fluorimetry has been applied to explore cellular metabolism activity as well as an indicator of intracellular ROS (5,6). Despite its abundance, the low quantum yield and strong photobleaching effects of NADPH makes it difficult to record fluorescence dynamics with traditional fluorescence microscopy. In addition, the requirement of UV excitation means energy accumulation during recording and therefore light-cell interaction can be a significant issue. The appearance of two-photon excitation microscopy provides a solution to these concerns. Two-photon excitation refers to a nonlinear optical phenomenon in which two low-energy photons work in concert to cross the energy gap of a specific molecule and bring it to a high energy intermediate, which may spontaneously return to the basal state and release energy in the form of emitted photons. Two-photon excitation microscopy is especially useful in imaging biological samples. It usually requires a light source between 700-1000 nm, which allows a deeper penetration and avoid water absorption. In addition, extremely position-sensitivity of two-photon excitation results in spontaneous optical sectioning and significantly decreases the photobleaching of fluorophores and light-tissue interaction. These properties greatly increased the feasibility of continual NADPH fluorimetric monitoring. It has been shown that intracellular NADPH abundance in response to different pharmacological stimulations can be dynamically recorded with two-photon excitation microscopy (6). We also applied two-photon excitation microscopy on J744 macrophages to study the effects of low-density lipoprotein on cellular NADPH abundance (7). A Ti:sapphire laser provided 780 nm incident light at 3 mWatts, and the scanning speed was 0.3 frame per second. Each scan was spaced 1 minute apart, and the reagents were applied after the 10th scan. Results and representative images are shown in Figure 1. First of all, over 1 hour period a trend of slightly decreased signal intensity can be discerned in all groups. Secondly, immediately after addition of native low-density lipoprotein (LDL), which was mixed with 50% glycerol to allow freeze preservation

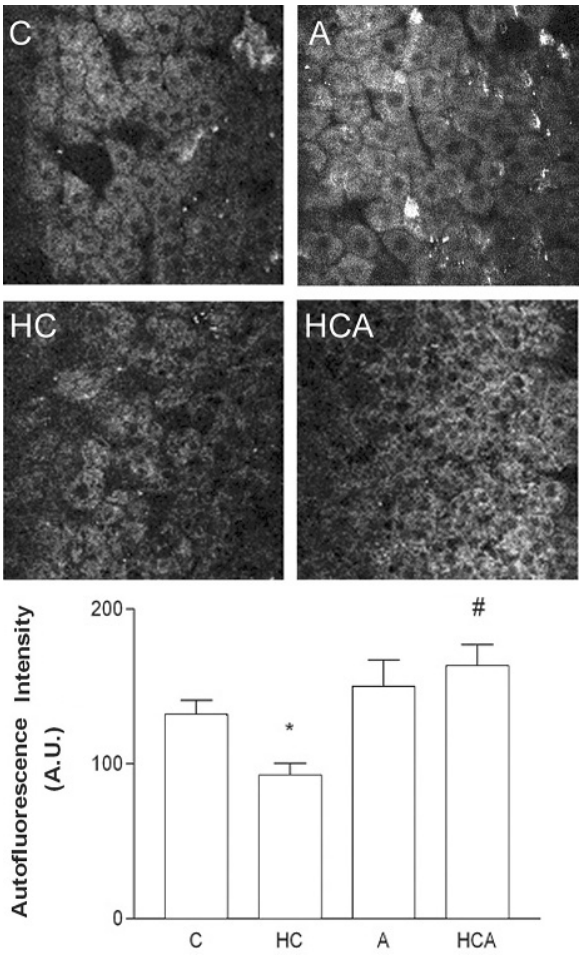

Figure 2. NADPH-derived autofluorescence reflects excessive free radical production in hepatocytes of hypercholesterolemic rats.

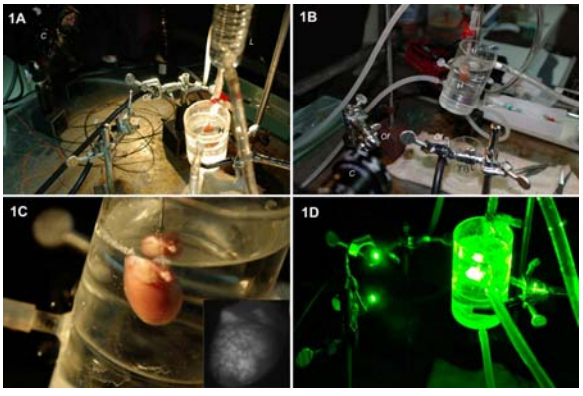

Figure 3. Setup for optical mapping of ROS in isolated perfused rat heart.

in -20^{o}C, there was a transient signal peak. Similar phenomenon was reproduced if glycerol was applied alone. Although the actual mechanism was not well known, the effects of glycerol on increased fluorescence intensity during two-photon excitation microscopy might be due to its higher viscosity (8). Glycerol itself is not reported to influence the cellular metabolism as well as ROS balance, and in our experiment the signal curve of the glycerol group did not significantly differ from that of saline-treated group later on when the applied glycerol was supposed to be equally dispersed in the aqueous medium. Thirdly, exposure to native LDL but not oxidized LDL resulted in a quick and persistent drop in NADPH fluorescence intensity. Because both native and oxidized LDL did not interfere with Kreb's cycle as well as oxidative phosphorylation, increased ROS production and subsequently NADPH oxidation is a possible underlying mechanism. In fact, we also found similar phenomenon in isolated rat monocytes with flow cytometry, using DCFDA as an intracellular ROS indicator (7). We proposed that native LDL acted through actin polymerization and intracellular calcium mobilization to induce NADPH oxidase activity. Increased NADPH oxidase activity not only elicited intracellular oxidative stress but also consumed cellular NADPH, both of which would result in depletion of cellular NADPH and decrease NADPH derived autofluorescence. While oxidized LDL has long been known to trigger inflammatory response and produce an pro-oxidative microenvironment in the vascular wall, our studies focused on the immediate redox response to native LDL in monocytes/macrophages. Such dynamic process might not be accurately displayed with time-consuming destructive techniques such as concentrations of conjugated diene and thiobarbituric acid reactive substances (TBARS). An immediate deduction from our observations is that besides prolonged subintimal retention and subsequent modification by vascular cells, incubating moncytes/macrophages in postprandial concentrations of native LDL in the plasma leads to intracellular oxidative signals and release of oxidants, which may also contribute to the formation of oxidized LDL. A recent revisit of antioxidative defense brought about by LDL-receptor mediated native LDL clearance also supported the notion of pro-oxidative properties of native LDL (9, 10).

We also explored the possibility to apply this system to monitor ROS dynamics in anaesthetized animals. For this purpose, we investigated the NADPH-derived autofluorescence in the liver of rats receiving normal chow and cholesterol-enriched diet (11). The experimental setup was basically the same with that in the previous section, except the power of laser was decreased to 3 mWatts. Under adequate anaethesia, left lobe of rat liver was exposed and scanned, and the representative images and quantitative analysis are shown in Figure 2. Autofluorescence imaging provided sharp tissue contrast and the parenchymal architecture as well as vascular spaces could be clearly identified. In normal hepatocytes, autofluorescence homogenously distributed in cytosol but not in the nuclei. High cholesterol diet resulted in cholesteryl ester and triglyceride accumulation in the intracellular vesicles, which was not fluorescent upon two-photon excitation. Comparing cytosolic autofluorescence intensity we found a significantly decreased intensity in the hepatocytes of hypercholesterolemic animals. Administration of apocynin, a NADPH oxidase inhibitor, resulted in significantly increased fluorescence intensity

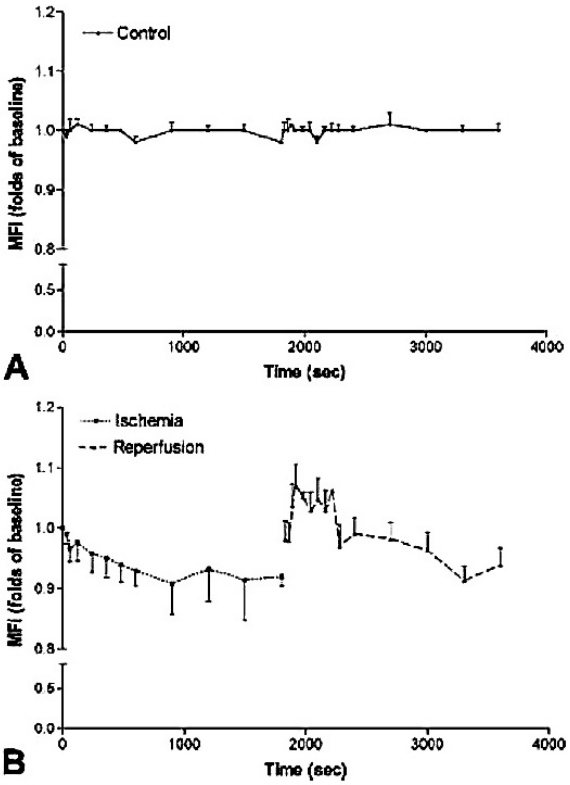

Figure 4. Fluorometric determination of myocardial ROS throughout global ischemia-reperfusion.

both in control and hypercholesterolemic animals. These changes also paralleled the biochemical oxidative burden as measured by TBARS, reduced glutathione and oxidized glutathione. All these facts pointed out that ROS overproduction contributed to decreased NADPH-derived autofluorescence during hypercholesterolemia.

The two examples from our lab addressed the potential of real time ROS imaging with NADPH-derived autofluorescence. However we have to point out that the altered NADPH fluorescence alone is sensitive but not specific to indicate intracellular oxidative stress. Cellular ROS is not the only factor to modulate NADPH abundance. Ischemia or hypoxia will reduce cellular oxidative phosphorylation and therefore markedly decrease the biosynthesis of NADPH (12). In this case decreased autofluorescence intensity does not correlate with the status of intracellular ROS but reflects a disturbed electron transfer chain. Therefore it is important to rule out the involvement of altered mitochondrial energy metabolism in a specific experiment design before applying this technique. It is even better if results from NADPH fluorescence measurements can be reproduced with other standard methods such as electro-spin resonance measurement.

4. ETHIDIUM-DERIVED FLUORESCENCE

Although NADPH-derived autofluorescence is a sensitive and dye-free method with superb spatial resolution to monitor the intracellular ROS, there are several technical considerations even when the involvement of altered oxidative phosphorylation can be reasonably excluded. First, in tissues with abundant extracellular matrix (for examples, collagen and elastin bundles) and contractile proteins, these structures are strongly fluorescent and therefore might overshadow the fluorescence derived from NADPH. Secondly, So far the scanning speed for this kind of equipment is still below the requirement of real time imaging. As a result these images are highly motion-sensitive and can only be applied on static subjects such as cells in a culture dish or organs whose primary function are not related to movement. Obviously ROS dynamics in the intact heart is not a suitable target to be evaluated with this method. To investigate myocardial ROS dynamics throughout global ischemia reperfusion, we made use of a high-speed fluorescence imaging technique and applied dihydroethidium as the exogenous ROS indicator (13). Dihydroethidium readily penetrates cell membrane and is distributed in cytosol with a later concentration in membranous compartment. It is converted to ethidium by intracellular ROS. Ethidium is a DNA minor groove binder with an approximate Kd of 2.3 μM (14). It will become fluorescent upon DNA binding and the peaks of excitation and emission are 530 and 620 nm respectively. The setup is shown in Figure 3. The rat heart was quickly removed and mounted on a Langendorf perfusion apparatus, in which constant flow of oxygenated Tyrode's solution provided adequate perfusion. Dihydroethidium was loaded at 3 μM through the perfusion apparatus and a 0.5 mWatt Verdi Laser deliver excitation light at 532 nm through a dispersion optic fiber to homogenously shine the epicardial surface. In contrast to previous microscopy approach, here a high speed charge-coupled device camera, equipped with a long-pass filter, was used to collect epifluorescence with wavelength longer than 600 nm. For each recording, an image stream (containing 1000 images) was obtained at 400 frames per second and mean fluorescence intensity were calculated to represent that stream. The result is shown in Figure 4. During ischemia, there was a gradual decrease in myocardial ROS production. Immediately upon reperfusion a surge of ROS appeared, and this surge subsided after 10 minutes reperfusion together with partial recovery of sinus rhythm and ventricular contractility. While this result demonstrated the feasibility of ROS mapping in isolated perfused heart, still there are some potential concerns. Reperfusion has been known to associate with tremendous amount of ROS release. Zweier estimated reperfusion associated ROS production with electron spin resonance technique and found that maximal ROS surge after reperfusion can be as high as 6 folds of baseline (15). Kevin *et al* also found 4 folds increased ROS associated with reperfusion with dihydroethidium and a fiber-based detection system (16). Therefore the sensitivity of our system is waiting to be improved. Secondly, the open system design makes comparison between different preparations very difficult. Thirdly, although applying a high-speed CCD camera overcomes the problem of motion artifact, the price is decreased spatial resolution. If the system can be moderated to a reflected confocal/two photon microscope, and myocardial contraction stopped

with cytochalasin D, ROS dynamics in delicate structure such as coronary microvasculature should provide us more insight of how vascular component weigh compared with myocardial component in reperfusion-induced ROS overproduction. Last but not the least, ethidium is a potential carcinogen and therefore its application should never extend to human subjects.

5. SUMMARY AND PERSPECTIVE

Fluorophore-based detection facilitates *in vivo* recording of ROS in a living system. So far these methods provide clear dynamic images but still remain semiquantitative. In addition, the chemical properties, radical species targets as well as the cellular distribution of ROS indicators differ widely, and careful choice according to the specific experimental context will greatly improve the quality of experimental results. In addition to spectroscopic analysis of fluorescence signals, recently it has been proposed that time-domain analysis of fluorescence signal (also known as fluorescence lifetime analysis) may yield information regarding tissue redox status with a better penetration (17). The development of these non-invasive or minimally-invasive techniques may aid in studying genetically modified animals and clarify the knowledge regarding ROS in disease pathogenesis and progression. One of these very interesting topics would be the myth of antioxidants. Despite dysregulated ROS has been established as an important pathogenic mechanism, so far it is not yet clear why most major randomized clinical trials of antioxidants failed to demonstrate health benefits. One of the possibilities is that antioxidants do not penetrate cell membranes and therefore are ineffective to reduce cellular oxidative burden. Another possibility is that pharmacokinetics of these antioxidants does not allow a stable and persistent radical scavenging effects. Progress of novel imaging techniques might enable continuous ROS monitoring in model organisms and the above mentioned hypotheses can be examined with objective and convincing evidences.

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Abbreviations: ROS: reactive oxygen species; NADPH: nicotinamide adenine dinucleotide phosphate, reduced form; LDL: low-density lipoprotein

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