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Review

Evaluation of methods of detecting cell reactive oxygen species production for drug screening and cell cycle studies

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ABSTRACT

Intracellular reactive oxygen species (ROS) production is essential to normal cell function. However, excessive ROS production causes oxidative damage and cell death. Many pharmacological compounds exert their effects on cell cycle progression by changing intracellular redox state and in many cases cause oxidative damage leading to drug cytotoxicity. Appropriate measurement of intracellular ROS levels during cell cycle progression is therefore crucial in understanding redox-regulation of cell function and drug toxicity and for the development of new drugs. However, due to the extremely short half-life of ROS, measuring the changes in intracellular ROS levels during a particular phase of cell cycle for drug intervention can be challenging. In this article, we have provided updated information on the rationale, the applications, the advantages and limitations of common methods for screening drug effects on intracellular ROS production linked to cell cycle study. Our aim is to facilitate biomedical scientists and researchers in the pharmaceutical industry in choosing or developing specific experimental regimens to suit their research needs.

Contents

1. Introduction

Reactive oxygen species (ROS) are a group of chemically reactive molecules and free radicals formed by incomplete single-electron reduction of oxygen as by-products of cellular aerobic metabolic processes (Valko et al., 2007). These molecules readily react with other biological products and influence the cell function in response to intracellular and extracellular stimuli such as drug intervention. ROS are produced by virtually every mammalian cell type from a variety of different sources such as mitochondrial electron transport chain, ionizing radiation, NADPH oxidase, cytochrome P450 reductase, xanthine oxidase, and nitric oxide synthase (Dröge, 2002; Finkel, 2011; Gutteridge & Halliwell, 2010; Li & Shah, 2004; Valko et al., 2007). Common ROS include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{\cdot-}$), reactive aldehydes, nitric oxide (NO) and other hydroxyl radicals. Among them, the $O_2^{\cdot-}$ radical has several unique effects on

Abbreviations: ROS, reactive oxygen species; DHE, dihydroethidium; 2-OH-E^+, 2-hydroxyethidium; SOD, superoxide dismutase; HPLC, high-performance liquid chromatography; CM-DCF-DA, 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorodihydrofluorescein; PCNA, proliferating cell nuclear antigen.

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other ROS i.e. rapid inactivation of NO while generating $ONOO^{\cdot-}$, and it serves as the precursor of other ROS such as H_2O_2 and $OH^{\cdot}$ (Ardanaz & Pagano, 2006). In general ROS affect cellular functions mainly through (a) acting as regulatory mediators in signalling processes that lead to altered gene transcription, and protein and enzyme activities (so-called "redox signalling") (Biswas, Chida, & Rahman, 2006; Dröge, 2002; Finkel, 2011), or (b) oxidative damage to cellular proteins, nucleic acids and lipids resulting in cell apoptosis and death (Circu & Aw, 2010; Dröge, 2002; Whiteman, Hong, Jenner, & Halliwell, 2002).

Cells have also antioxidant defence systems which can be divided into non-enzymatic molecules and antioxidant enzymes. Non-enzymatic molecules are ROS scavengers such as uric acid, ascorbic acid, α-tocopherol, and sulfhydryl-containing molecules such as glutathione. Antioxidant enzymes include catalase, glutathione peroxidase and particularly superoxide dismutases (SODs) (Verbon, Post, & Boonstra, 2012; Li & Shah, 2004). The overall balance between oxidative (ROS generating) and reductive (ROS scavenging) processes in the cell constitutes the cellular "redox state". When the redox homeostasis of a cell is interrupted and the rate of ROS formation exceeds the capacity of the antioxidant defence systems, the condition of oxidative stress occurs (Görlach et al., 2002; Vokurkova, Xu, & Touyz, 2007; Vurusaner, Poli, & Basaga, 2012). Redox homeostasis and oxidant signalling are essential elements that maintain cell function and regeneration, whereas oxidative stress or excessive production of ROS in response to environmental challenges causes genetic and epigenetic changes and may lead to cell dysfunction (Verbon et al., 2012; Vurusaner et al., 2012).

The concept of redox regulation of cell cycle progression represents an important mechanism linking the oxidative metabolic processes to the cell cycle regulatory machinery (Li, Fan, George, & Brooks, 2007; Menon & Goswami, 2007; Venkatachalam et al., 2008; Vokurkova et al., 2007). ROS are short-lived and certain types of ROS i.e. NO and H_2O_2, can diffuse within and between cells to interact with redox-sensitive signalling molecules that in turn regulate cell cycle progression. The direction of cell cycle progression is determined by both the upstream ligand-dependent stimulation of ROS production and the downstream ROS targets (Burch & Heintz, 2005; Burhans & Heintz, 2009; Menon & Goswami, 2007). It is well known that ROS can modulate cellular signalling pathways at multiple levels from membrane receptors and ion channels to various intracellular protein kinases, phosphatases and nuclear transcription factors (Li & Shah, 2004). Many important cell signalling molecules, such as the mitogen-activated protein kinases, nuclear factor-κB and the tumour suppressor protein p53 have redox-sensitive motifs (cysteine residues or metal co-factors) (Menon & Goswami, 2007; Vurusaner et al., 2012). Several key cell cycle components such as cyclins (i.e. cyclin D1 and E), cyclin dependent kinases (i.e. CDK2 and 4) and cell cycle check point proteins (i.e. Chk1, Chk2) are also redox sensitive (Maryanovich & Gross, 2013; Verbon et al., 2012; Vokurkova et al., 2007). Their functions and activities are influenced by fluctuations in the intracellular ROS levels which in turn direct the cell to either progress through, withdraw from the cell cycle or undergo apoptosis. There is a close link between the redox cycle and cell cycle in mammalian cells and the threshold of ROS levels required to promote or to inhibit cell cycle progression may vary according to the type of cell and the extracellular environment.

There are many ways to detect ROS species generated by cells or tissues, for example electrochemical quantification (Borgmann, 2009; Dikalov, Griendling, & Harrison, 2007; Halliwell & Whiteman, 2004; Tarpey, Wink, & Grisham, 2004), fluorescent probe techniques (Dikalov et al., 2007; Halliwell & Whiteman, 2004; Kalyanaraman et al., 2012; Tarpey et al., 2004) and electron spin resonance (Dikalov et al., 2007; Halliwell & Whiteman, 2004; Tarpey et al., 2004) and their applications have been extensively reviewed previously. However, in terms of cell cycle study and screening drug compound cytotoxicity, the techniques of detecting intracellular ROS have not yet been evaluated. Moreover, due to the large variety, low quantity, high reactivity and the extremely short half-life of ROS generated during each cell cycle, accurate measurement of intracellular ROS level is difficult. Additional complementary approaches of ROS measurement are necessary in order to accurately reflect the intracellular oxidative changes. In this article, we have focused on easily applicable screening techniques using commonly available research laboratory instruments for the detection of intracellular ROS production, in particular $O_2^{\cdot-}$ by living cells and homogenates of cells/tissue. We have provided brief descriptions for the rationale of using these methods based on our experience and illustrated with results generated using settings in our laboratories. We have also given an updated overview of the applications, advantages and limitations of these techniques in relationship to pharmacological and toxicological studies of cell cycle control, cell proliferation and cell death.

2. Monitoring intracellular $O_2^{\cdot-}$ generation by lucigenin (N,N′-dimethyl-9,9′-biacridinium dinitrate)-chemiluminescence

Chemiluminescence is one of the most widely used methods for detecting ROS production by mammalian cells and tissues. Several luminescence probes are available on the market and each of them has advantages and limitations depending on experimental settings. Technical details and applications of these probes can be found in previous reviews (Freitas, Porto, Lima, & Fernandes, 2009; Maghzal, Krause, Stocker, & Jaquet, 2012; Mahfouz, Sharma, Lackner, Aziz, & Agarwal, 2009; Nauseef, 2014; Tarpey & Fridovich, 2001; Tarpey et al., 2004). In this article we will focus on lucigenin-chemiluminescence for $O_2^{\cdot-}$ detection. Lucigenin-chemiluminescence has been a popular method for measuring intracellular $O_2^{\cdot-}$ production by non-phagocytic cells due to the alleged advantages of lucigenin i.e. great cell membrane permeability, minimal cellular toxicity, high sensitivity and specificity of reaction with $O_2^{\cdot-}$. The rate constant for the reaction between $O_2^{\cdot-}$ and lucigenin is three times higher than that for $O_2^{\cdot-}$ with cytochrome c (Afanas'ev, Ostrakhovitch, Mikhal'chik, & Korkina, 2001), which makes lucigenin-chemiluminescence particularly useful in the detection of low levels of $O_2^{\cdot-}$ production by vascular endothelial cells (Fan, Teng, & Li, 2009; Li & Shah, 2001; Li et al., 2007; Teng, Fan, Meijles, & Li, 2012), vascular smooth muscle cells (Brandes et al., 2002; Chamseddine & Miller, 2003; Menshikov et al., 2006), fibroblasts (Chamseddine & Miller, 2003; Venkatachalam et al., 2008); kidney cells (Berasi, Xiu, Yee, & Paulson, 2004; Hannken, Schroeder, Stahl, & Wolf, 1998); lymphocytes (Berasi et al., 2004) lung alveolar epithelial cells (Tickner, Fan, Du, Meijles, & Li, 2011) and human spermatozoa (Mahfouz et al., 2009). The reaction is fast and can be monitored in real-time ranging from seconds to minutes which provides a kinetic reading of $O_2^{\cdot-}$ generation by living cells under experimental conditions. The assay can be performed in a 96 well microplate where lucigenin is injected in the dark by an auto-dispenser in the chemiluminescence microplate reader. The entire system can be pre-warmed to 37 °C, and the reading is obtained instantly and monitored for hours. A diagram of the technique is shown in Fig. 1A, and Fig. 1B shows an example of the kinetic measurement of $O_2^{\cdot-}$ production by mouse primary coronary microvascular endothelial cells. For vascular endothelial cells, there was no difference in the basal (without adding NADPH) levels of $O_2^{\cdot-}$ production between cells cultured under 3 different conditions. However there was a significant difference in NADPH-dependent $O_2^{\cdot-}$ production between cells. Compared to cells cultured in 10% FCS growth medium, quiescent cells (0% FCS for 24 h) produced less ROS, and TNFα-stimulated cells produced much more ROS.

It had been reported previously that lucigenin could be reduced univalently by diverse enzymes including xanthine oxidase, glucose oxidase, and might react with NADH to mediate $O_2^{\cdot-}$ production in a system where lucigenin was mixed with high concentration of xanthine oxidase (25–100 units/mL) plus NADH (Liochev & Fridovich, 1997). However, such artificial conditions would not exist in mammalian cell biology. In fact, another report found that when lucigenin was used at a lower dose of 80 μmol/L, the direct enzymatic reduction of lucigenin decreased