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# Production of extracellular superoxide by human lymphoblast cell lines: Comparison of electron spin resonance techniques and cytochrome C reduction assay

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ABSTRACT

Superoxide production by NADPH oxidases plays an important role in the development and progression of cardiovascular disease (CVD). However, measurement of superoxide ($O_2^{\bullet-}$), a marker of oxidative stress, remains a challenging task in clinical and translational studies. In this study we analyzed $O_2^{\bullet-}$ production in cultured human lymphoblast cell lines by three different methods: (a) superoxide dismutase (SOD)-inhibitable cytochrome C reduction, (b) spin trapping of superoxide with 5-(ethoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide (EMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), and (c) using electron spin resonance (ESR) with the cell-permeable spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH). Lymphocytes were isolated and immortalized by an Epstein–Barr Virus (EBV)-transformation procedure. Superoxide was measured in cultured lymphoblast cell lines at baseline and upon stimulation with phorbol 12-myristate 13-acetate (PMA). Cytochrome C and the spin traps EMPO and DEPMPO detected two to five times less superoxide compared to CMH. Thus, CMH provided the most quantitative measurement of superoxide generation in human lymphoblast cell lines. Superoxide detection with CMH was linear dependent on cell concentration and was inhibited by SOD but not by catalase. Both cell-permeable polyethylene glycol (PEG)–SOD and extracellular Cu,Zn–SOD inhibited $O_2^{\bullet-}$ detection by 90% in PMA-stimulated cells, suggesting a predominantly extracellular $O_2^{\bullet-}$ generation in human lymphoblasts. Our study describes a new technique for $O_2^{\bullet-}$ measurement in cultured human lymphoblasts using ESR and CMH. A highly sensitive in vitro measurement of $O_2^{\bullet-}$ in human cell lines would allow investigators to study genotype/phenotype interactions in translational studies.



## 1. Introduction

Superoxide ($O_2^{\bullet-}$) production plays an important role in redox cell signaling and development of pathophysiological conditions, such as hypertension, ischemia-reperfusion injury, inflammation and atherosclerosis [1]. However, detection of $O_2^{\bullet-}$ is still a challenging problem. One of the most sensitive and definitive methods of $O_2^{\bullet-}$ detection is electron spin

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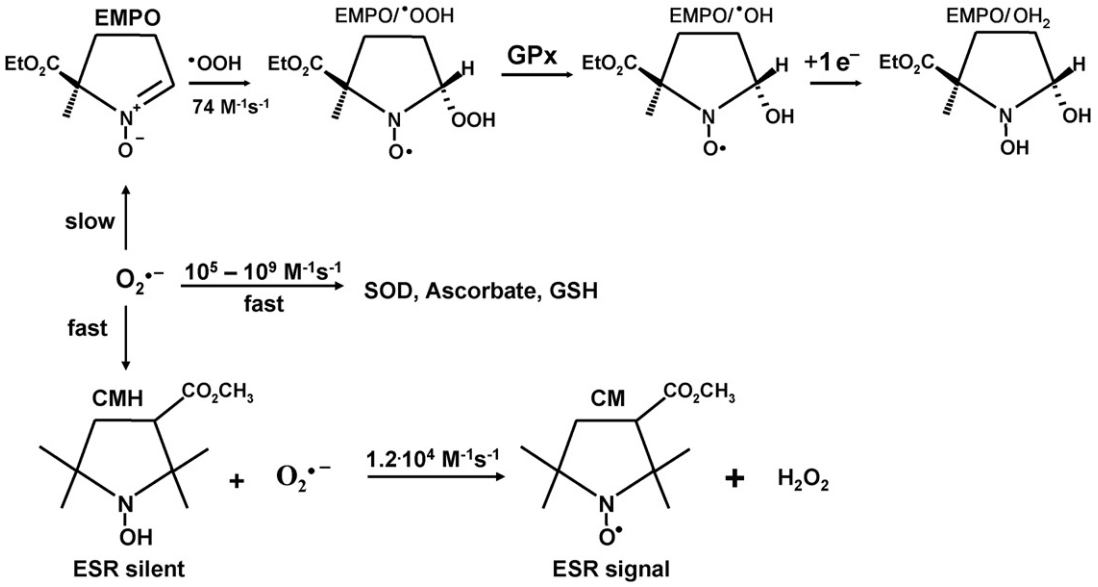


**Fig. 1 – ESR detection of $O_2^{\bullet-}$. Spin trapping of $O_2^{\bullet-}$ is limited by slow reaction of $O_2^{\bullet-}$ with spin traps (EMPO) in the presence of antioxidants (SOD, ascorbate), biodegradation (glutathione peroxidase, GPx) and bioreduction of the radical adducts. Fast reaction of $O_2^{\bullet-}$ with spin probe CMH permits detection of cellular $O_2^{\bullet-}$. CMH produces stable CM-nitroxide, which can be quantified by ESR.**

resonance (ESR) [2,3]. The ESR spin-trapping technique has been used to detect $O_2^{\bullet-}$ radicals induced by inflammation via neutrophil NADPH oxidase in cellular systems in vitro [4]. However, the commonly used nitrone spin traps have a very low efficacy for trapping of $O_2^{\bullet-}$ radicals (Fig. 1) [5]. Thus, formation of the radical adduct is limited by slow kinetics of $O_2^{\bullet-}$ trapping and obstruction by antioxidants. Furthermore, superoxide radical adducts suffer from decomposition to hydroxyl ($^{\bullet}OH$)-radical adducts by glutathione (GSH) peroxidase [6]. Finally, both $O_2^{\bullet-}$ and $^{\bullet}OH$ radical adducts can be reduced to ESR silent hydroxylamines by ascorbate, transition metals, or flavin enzymes (Fig. 1) [7].

Recently, cyclic hydroxylamines were found to be effective scavengers of $O_2^{\bullet-}$ radicals [8,9]. Hydroxylamine probes 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine (PPH) and 1-hydroxy-3-carboxy-pyrrolidine (CPH) have been previously used for quantitative detection of extracellular $O_2^{\bullet-}$. The advantage of hydroxylamine probes is that they are effective scavengers of $O_2^{\bullet-}$ and produce a stable nitroxide radical [8]. Previously, we reported the activity of the phagocytic NADPH oxidase in neutrophils from healthy subjects using CPH, and measuring $O_2^{\bullet-}$ as SOD-inhibitable formation of 3-carboxyproxyl [10].

In the current investigation, we studied superoxide production in cultured lymphoblast cell lines at baseline and upon stimulation with phorbol 12-myristate 13-acetate (PMA) by three methods: (a) superoxide dismutase (SOD)-inhibitable cytochrome *C* reduction, (b) spin trapping of superoxide with 5-(ethoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide (EMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), and (c) using ESR with the cell-permeable spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (Fig. 1) [11]. Reaction of $O_2^{\bullet-}$ with CMH is much faster ($1.2 \times 10^4\ M^{-1}\ s^{-1}$) than with nitrone spin traps, thereby enabling the hydroxylamines to compete with cellular antioxidants and react with both extra- and intracellular $O_2^{\bullet-}$.

Our study describes a new technique for $O_2^{\bullet-}$ measurement in cultured human lymphoblasts using ESR and CMH.

## 2. Methods and materials

### 2.1. Reagents

Spin traps 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), 5-(ethoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide (EMPO) and spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) were purchased from Alexis Corporation (San Diego, USA). Polyethylene-glycol-conjugated superoxide dismutase (PEG–SOD), and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma–Aldrich (St. Louis, MO). All other reagents were obtained from Sigma–Aldrich.

### 2.2. Establishment of immortalized cell lines

In collaboration with the Emory University General Clinical Research Center human immortalized lymphoblast cell lines were developed from peripheral blood mononuclear cells of subjects with and without CVD at the Atlanta Veterans Affairs Medical Center (AVAMC). The study was approved by the Institutional Review Board of Emory University and the AVAMC's Research and Development Committee. All subjects provided informed consent.

Lymphocytes were isolated from whole blood by Ficoll density gradient centrifugation [12]. After low speed centrifugation of freshly sampled venous blood (10 ml), the buffy coat and red blood cells (4 ml) were diluted with 4 ml phosphate buffered saline (PBS). The diluted blood was layered