BIOCHEMICAL PHARMACOLOGY 73 (2007) 972–980

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

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

Sergey I. Dikalov [a,b,*], *Wei Li* [b,c], *Payam Mehranpour* [b,c], *Shaoshan S. Wang* [b,c], *A. Maziar Zafari* [b,c]

[a] *Free Radical in Medicine CORE, Emory University School of Medicine, Atlanta, GA, United States*
[b] *Division of Cardiology, Emory University School of Medicine, Atlanta, GA, United States*
[c] *Atlanta Veterans Affairs Medical Center, Decatur, GA, United States*

ARTICLE INFO

Article history:
Received 15 September 2006
Accepted 11 December 2006

Keywords:
Cardiovascular disease
NADPH oxidase
Superoxide
Electron spin resonance
Spin trap
Spin probe
Cytochrome *C*

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

* *Corresponding author at*: Division of Cardiology, Emory University School of Medicine, 1639 Pierce Drive, Atlanta, GA 30322, United States. Tel.: +1 404 712 9550; fax: +1 404 727 4080.
E-mail address: dikalov@emory.edu (S.I. Dikalov).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.12.012

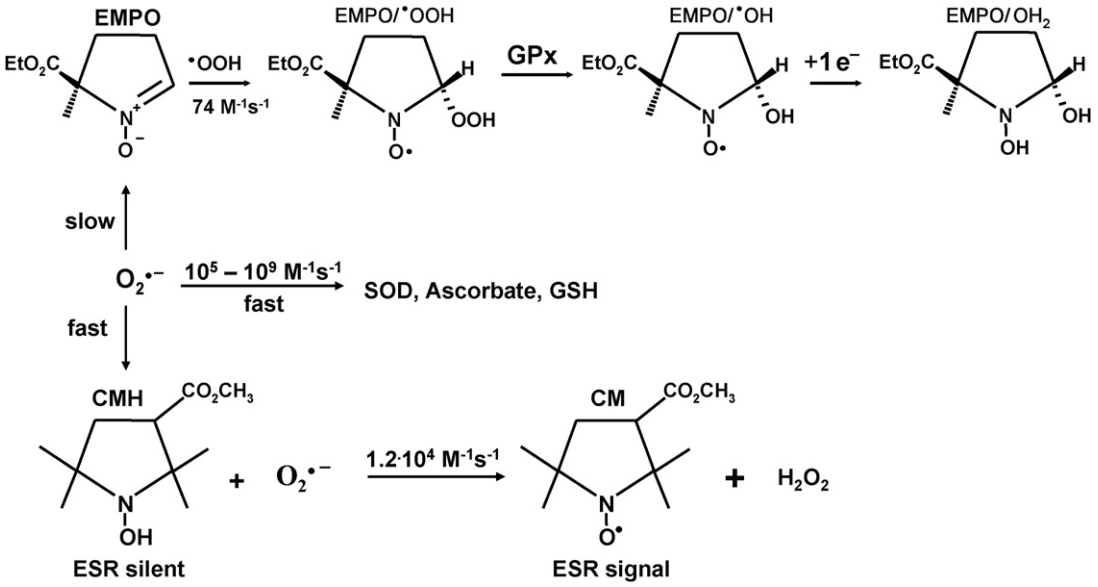

Fig. 1 – ESR detection of $O_2^{\bullet-}$. Spin trapping of $O_2^{\bullet-}$ is limited by slow reaction of $O_2^{\bullet-}$ with spin traps (EMPO) in the presence of antioxidants (SOD, ascorbate), biodegradation (glutathione peroxidase, GPx) and bioreduction of the radical adducts. Fast reaction of $O_2^{\bullet-}$ 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

on 5 ml Ficoll paque PLUS solution. After centrifugation at 500 × *g* at 20 °C for 30 min, the buffy coat was gently removed and diluted with 10 ml PBS. After low centrifugation at 200 × *g* for 7 min at room temperature, the supernatant was discarded. All subsequent steps were performed at 4 °C. The remaining red pellet underwent hypotonic lysis by the addition of 9 ml H_2O. After 20 s, osmolarity was restored with 2 ml of 10 times concentrated PIPES (piperazine-*N*,*N*′-bis-[2-ethano-sulfonic-acid]) buffer, followed by centrifugation at 200 × *g* for 7 min. The remaining pellet was resuspended in 5 ml RPMI and 10% fetal bovine serum (FBS) and incubated for 45 min in 5% CO_2 at 37 °C. The non-adherent cells were gently washed and collected. Finally, lymphocytes were pelleted after room temperature low speed centrifugation at 200 × *g* for 7 min. This procedure yielded (5–20) × 10^6 lymphocytes for each 10 ml of blood.

2.3. Cell culture

To initiate B lymphocyte cultures, lymphocytes were infected with the B95-8 strain of Epstein–Barr Virus (EBV) [12]. After EBV-transformation, B lymphocytes were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37 °C in a humid atmosphere saturated with 5% CO_2. The medium was changed twice weekly. Cell counts and viability of >95% (trypan blue stain) were monitored on a daily basis for 2 weeks until stored in liquid nitrogen for later in vitro phenotyping experiments.

2.4. Superoxide measurement with ESR

Superoxide radical was measured at room temperature by ESR using a Bruker EMX spectrometer (Bruker Biospin) using spin traps EMPO and DEPMPO, or spin probe CMH (CMH) [11]. Briefly, cells were washed with PBS, and resuspended in Krebs–Hepes buffer (KHB). Subsequently, 0.25 × 10^6 EBV-transformed lymphoblasts were placed in 0.1 ml KHB with 100 μM diethylenetriaminepentaacetic acid (DTPA) and incubated with PMA, polyethylene glycol (PEG–SOD), and PMA plus SOD. Results were expressed as pmoles of $O_2^{\bullet-}$—released per 10^6 cells/min.

2.5. Measurement of $O_2^{\bullet-}$ formation with cytochrome C assay

Superoxide formation was also determined by monitoring the SOD-inhibitable reduction of cytochrome *C* [13,14]. Cells were harvested by centrifugation and washed once in Hank's balanced salt solution (HBSS). Cells (1.25 × 10^7) were finally resuspended in 1 ml of HBSS and preincubated at 37 °C for 10 min. The reaction was started by mixing 80 μl of the cell suspension (10^6 cells in HBSS) with 20 μl of the substrate solution containing cytochrome *C* (160 μM), catalase (40 μg ml^{-1}) and PMA in HBSS; cytochrome *C* reduction was recorded at 37 °C for 10 min using a dual-wavelength ELISA-reader (at 550–540 nm) in the presence or absence of SOD. The amount of $O_2^{\bullet-}$ released was calculated using an extinction coefficient of 21 mM^{-1} cm^{-1} and expressed as the mean determination of multiple experiments ± S.E.

3. Results

3.1. Measurement of $O_2^{\bullet-}$ in stimulated and unstimulated lymphoblasts using CMH

Incubation of human lymphoblasts with the spin probe CMH resulted in the generation of the ESR signal of 3-methoxycarbonyl-proxyl nitroxide (CM•) (Fig. 2A). Time scan of the low-field component of the ESR signal showed linear accumulation of CM• in unstimulated cells. Stimulation of cells by PMA (10 μM) led to several-fold increase in the slope of CM• kinetics (Fig. 2B).

CMH detects both extra- and intracellular $O_2^{\bullet-}$ [11,15]. Extracellular $O_2^{\bullet-}$ can be quantified by inhibition of the ESR signal by Cu,Zn–SOD, while supplementation of cells with cell-permeable PEG–SOD will inhibit detection of both extra- and intracellular $O_2^{\bullet-}$. Addition of Cu,Zn–SOD (50 U/ml) strongly inhibited accumulation of CM• both in PMA-stimulated and unstimulated cells to similar levels (Fig. 2B). These results confirm specific detection of $O_2^{\bullet-}$ by CMH. Furthermore, supplementation of cells with cell-permeable PEG–SOD showed inhibition of the ESR signal similar to extracellular Cu,Zn–SOD (Fig. 3). Both cell-permeable PEG–SOD and extracellular Cu,Zn–SOD inhibited $O_2^{\bullet-}$ production by 90% in PMA-stimulated cells (Fig. 3). The amount of $O_2^{\bullet-}$ generated was calculated as a difference between the cellular sample and buffer. These data imply that both PMA-stimulated and unstimulated cells produce predominantly extracellular $O_2^{\bullet-}$.

3.2. Effect of catalase on $O_2^{\bullet-}$ detection in human lymphoblasts

It has been previously shown that cyclic hydroxylamines such as CMH do not directly react with hydrogen peroxide (H_2O_2) [16,17]. However, transition metals and heme proteins may stimulate oxidation of CMH in the presence of H_2O_2 [17]. Therefore, we treated human lymphoblasts with cell-permeable PEG-catalase (100 U/ml) in order to test the role of H_2O_2 in

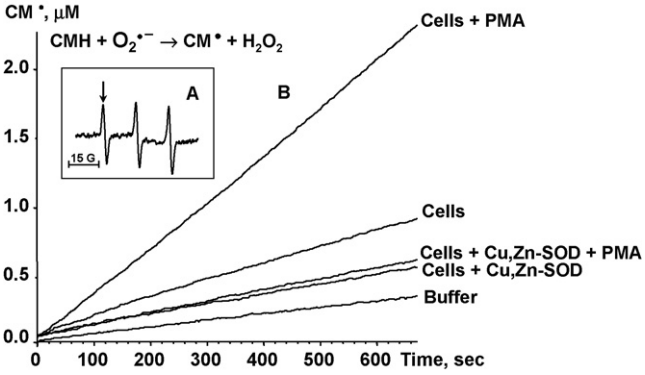

Fig. 2 – Quantification of $O_2^{\bullet-}$ in human lymphoblasts using cell-permeable CMH. (A) Typical ESR spectrum of CM-nitroxide. Arrow indicates low-field component of ESR spectrum which was used to monitor accumulation of CM-nitroxide. (B) Kinetics of CM-nitroxide accumulation in the probes with cells (2.5 × 10^3 μl^{-1}), Cu,Zn–SOD (50 U/ml) and PMA (10 μM). Cu,Zn–SOD significantly inhibits CM accumulation both in resting and PMA-stimulated cells.

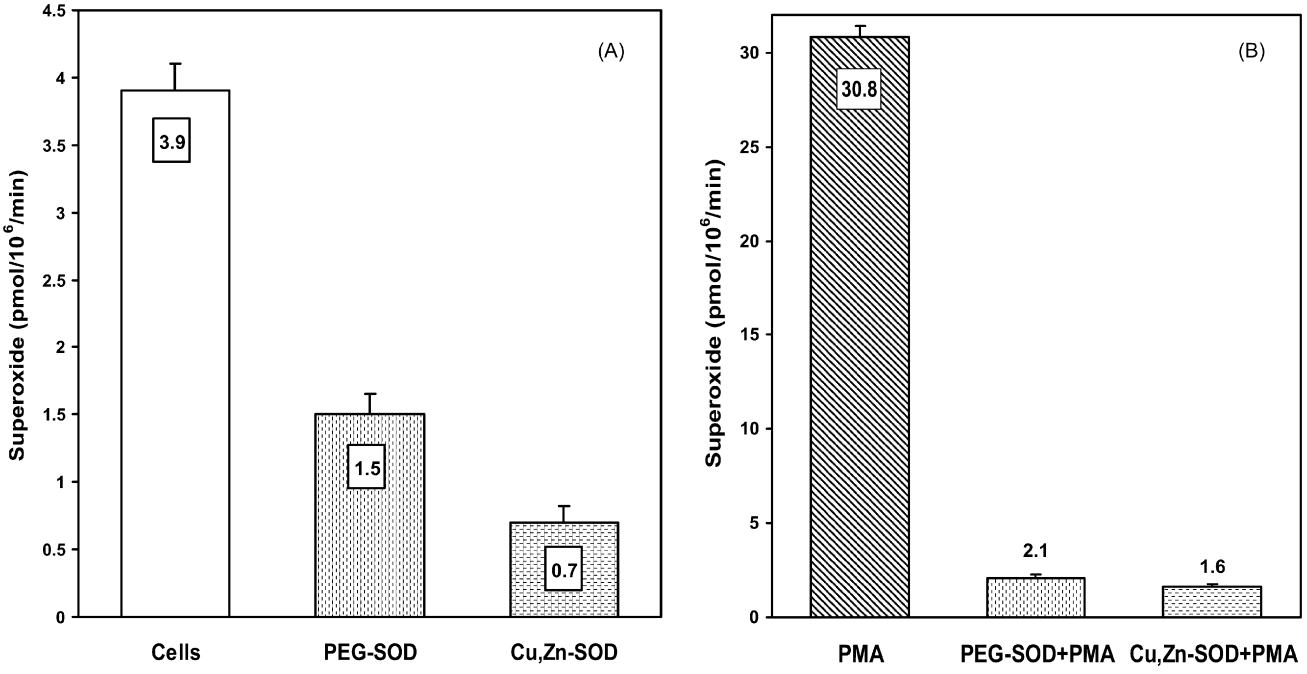

Fig. 3 – Inhibition of $O_2^{\bullet-}$ detection in human lymphoblasts treated by PEG–SOD or Cu,Zn–SOD. Superoxide is measured in unstimulated (A) or PMA-stimulated cells (B) in the presence of cell-permeable PEG–SOD or extracellular Cu,Zn–SOD (50 U/ml). Both PEG–SOD and Cu,Zn–SOD inhibit $O_2^{\bullet-}$ detection to similar levels.

CMH oxidation in lymphoblast suspensions. Treatment of lymphoblasts with PEG-catalase did not affect basal $O_2^{\bullet-}$ production but slightly decreased $O_2^{\bullet-}$ generation in PMA-stimulated cells (Fig. 4). It is important to note that in the presence of SOD, CMH is exposed to H_2O_2. Meanwhile, addition of catalase to cells and SOD did not change $CM^{\bullet}$ formation (Fig. 4C). Inhibition of $O_2^{\bullet-}$ generation by catalase in PMA-stimulated cells (30.8 pmol/10^6/min versus 19.8 pmol/10^6/min in the presence of catalase) can be explained by redox regulation of phagocytic NADPH oxidase in PMA-stimulated lymphoblasts rather than presence of SOD activity because catalase did not affect $O_2^{\bullet-}$ detection in the xanthine oxidase system (data not shown). Thus, the CMH signal was not dependent on cellular H_2O_2, but scavenging of H_2O_2 attenuated PMA-mediated stimulation of NADPH oxidase.

3.3. *Optimization of $O_2^{\bullet-}$ detection in human lymphoblasts*

Superoxide production was proportional to PMA concentration, and duration of PMA treatment (Fig. 5). Five-minute incubation with 10 μM PMA provided optimal condition for cell stimulation.

Superoxide detection with CMH was linear dependent on cell concentration, and was inhibited by SOD but not by catalase (Fig. 6A). In order to have sufficient amount of cells and linear dependence of ESR signal on cell number we selected 2.5×10^6 cells/ml as the optimal cell concentration. Furthermore, analysis of $O_2^{\bullet-}$ production at various concentrations of CMH did not show significant increase in $O_2^{\bullet-}$ detection at concentrations of CMH higher than 1 mM (Fig. 6B). Therefore, 1 mM CMH scavenges most of the cellular $O_2^{\bullet-}$. It was previously shown that CMH allows high sensitivity detection of both extra- and intracellular $O_2^{\bullet-}$. The fact that further increase in CMH concentration did not result in substantial increase in detected $O_2^{\bullet-}$ supports our observation that human lymphoblasts generate predominantly extracellular $O_2^{\bullet-}$.

3.4. *Comparison of $O_2^{\bullet-}$ detection by CMH with spin traps DEPMPO and EMPO*

Despite unambiguous detection of $O_2^{\bullet-}$ by the spin trap DEPMPO its application to cells is limited by bioreduction of the DEPMPO/$O_2^{\bullet-}$ radical adduct. Indeed, in human lymphoblasts treated with DEPMPO (Fig. 7, DEPMPO: cells) we did not observe a spectrum for the $O_2^{\bullet-}$ radical adduct. The eight-line ESR spectra correspond to DEPMPO/$^{\bullet}$OH radical adduct produced by GSH peroxidase-mediated decomposition of DEPMPO/$^{\bullet}$OOH adduct (Fig. 1). Formation of DEPMPO/$^{\bullet}$OH was significantly inhibited by Cu,Zn–SOD. Spin trapping of $O_2^{\bullet-}$ in cells stimulated with PMA resulted in formation of a mixture of DEPMPO/$^{\bullet}$OH and DEPMPO/$^{\bullet}$OOH adducts (Fig. 7). Cu,Zn–SOD inhibited formation of both radical adducts, which supports the detection of extracellular $O_2^{\bullet-}$ followed by cell-mediated decomposition of DEPMPO/$^{\bullet}$OOH to DEPMPO/$^{\bullet}$OH. Meanwhile, accumulation of radical adducts (followed by intensity of low-field ESR component) was not linear and flattened after a few minutes, suggesting bioreduction of the radical adducts.

In comparison, ESR spectra of CMH plus $O_2^{\bullet-}$ is linear and consistent (Fig. 7). All samples containing CMH showed less

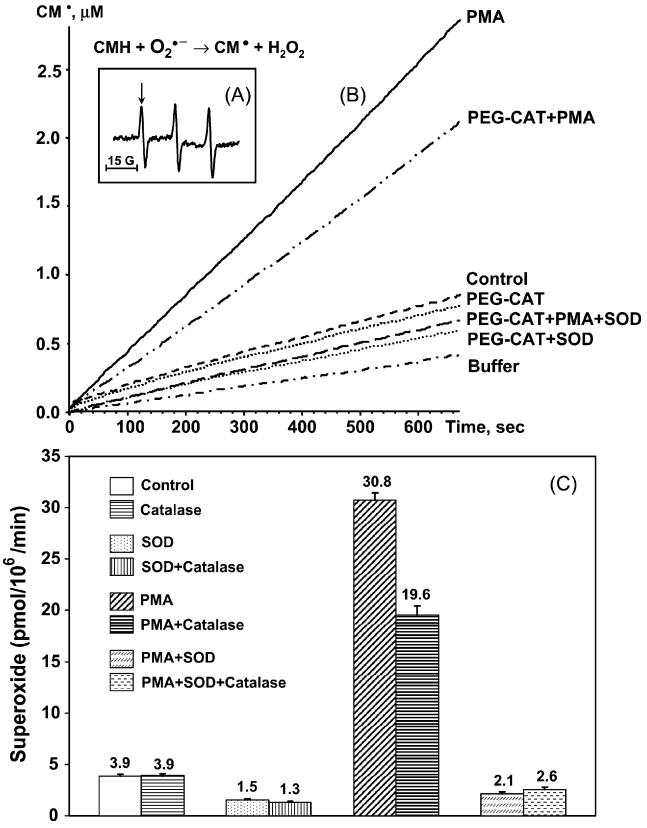

Fig. 4 – Superoxide detection in human lymphoblasts treated with PEG-catalase. (A) Typical ESR spectrum of CM-nitroxide. (B) Accumulation of CM-nitroxide in the cellular probes (2.5×10^3 μl^{-1}) containing PEG-catalase (100 U/ml), Cu,Zn–SOD (50 U/ml) and PMA (10 μM). (C) Production of $O_2^{\bullet-}$ calculated as a difference between CM-accumulation in the cellular sample and buffer. PEG-catalase does not affect basal $O_2^{\bullet-}$ production but attenuates stimulation of cells by PMA.

noise in their ESR spectra due to higher intensity of the 3-line spectrum compared to the 12-line spectrum of DEPMPO radical adducts. Finally, accumulation of ESR signal in CMH containing samples was linear in time and the amount of detected $O_2^{\bullet-}$ with CMH was five-fold higher than with the spin trap DEPMPO (Fig. 7). Of note, the presence of small initial ESR spectra in CMH samples did not interfere with the measurements because time scans show only the increase in ESR amplitude.

Spin trapping of $O_2^{\bullet-}$ with EMPO produced only the EMPO/•OH adduct, which was inhibited by Cu,Zn–SOD (Fig. 8A). These data suggest that cells transformed EMPO/•OOH into EMPO/•OH much faster than DEPMPO/•OOH OOH into DEPMPO/•OH. Incubation of cells with the spin probe CMH resulted in accumulation of CM• nitroxide, which was also inhibited by Cu,Zn–SOD (Fig. 8B). In addition, ESR spectra of CMH containing samples did have much better signal to noise ratio compared to EMPO samples. Interestingly, accumulation of EMPO/•OH radical adducts in unstimulated cells was not linear, while ESR kinetics in CMH containing samples was linear and the amount of detected $O_2^{\bullet-}$ with CMH was three-fold higher than with EMPO (Fig. 8C and D).

3.5. *Comparison of $O_2^{\bullet-}$ detection by CMH with cytochrome C*

It has been previously reported that cyclic hydroxylamine probes, such as CPH and CMH, detect more cellular $O_2^{\bullet-}$ than cytochrome C [10]. This difference was particularly larger in PMA-stimulated neutrophils, potentially due to oxidation of the ferrous cytochrome by H_2O_2. Indeed, SOD-inhibitable reduction of cytochrome C was twice lower than the amount of $O_2^{\bullet-}$ detected by CMH in unstimulated cells (Table 1). Moreover, the amount of $O_2^{\bullet-}$ detected by cytochrome C was 4.3-times lower than the amount of $O_2^{\bullet-}$ detected by CMH in PMA-stimulated cells (Table 1).

3.6. *Production of $O_2^{\bullet-}$ by human lymphoblasts from subjects with and without cardiovascular disease*

We have analyzed $O_2^{\bullet-}$ production in cultured lymphoblast cell lines obtained from subjects with and without CVD. We

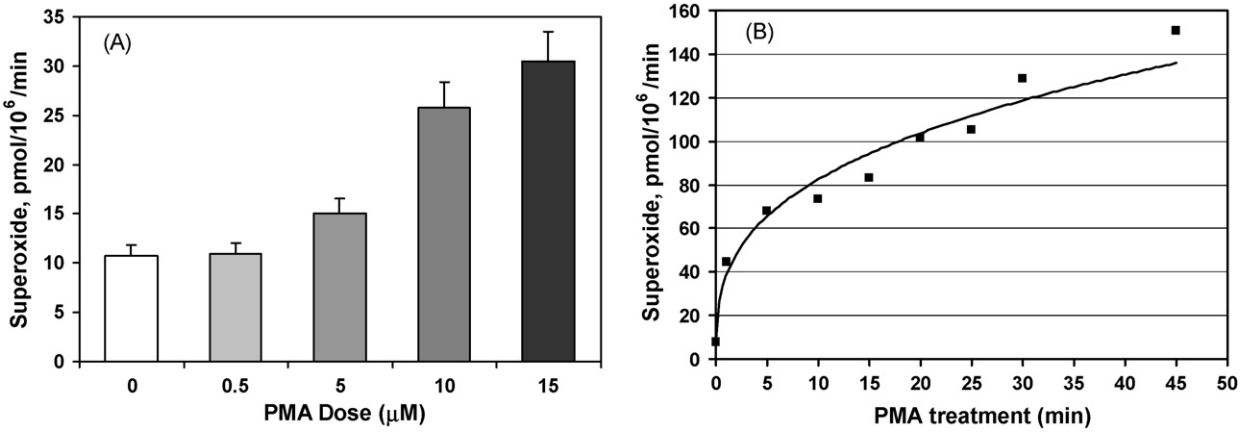

Fig. 5 – Dependence of $O_2^{\bullet-}$ production on the dose and duration of PMA treatment. (A) Suspension of human lymphoblasts (2.5×10^3 μl^{-1}) were treated for 5 min with different doses of PMA (0–15 μM) before addition of CMH ($n = 3$). (B) Time course of cell activation was tested in cells incubated with 10 μM PMA for various time periods before addition of CMH ($n = 3$).

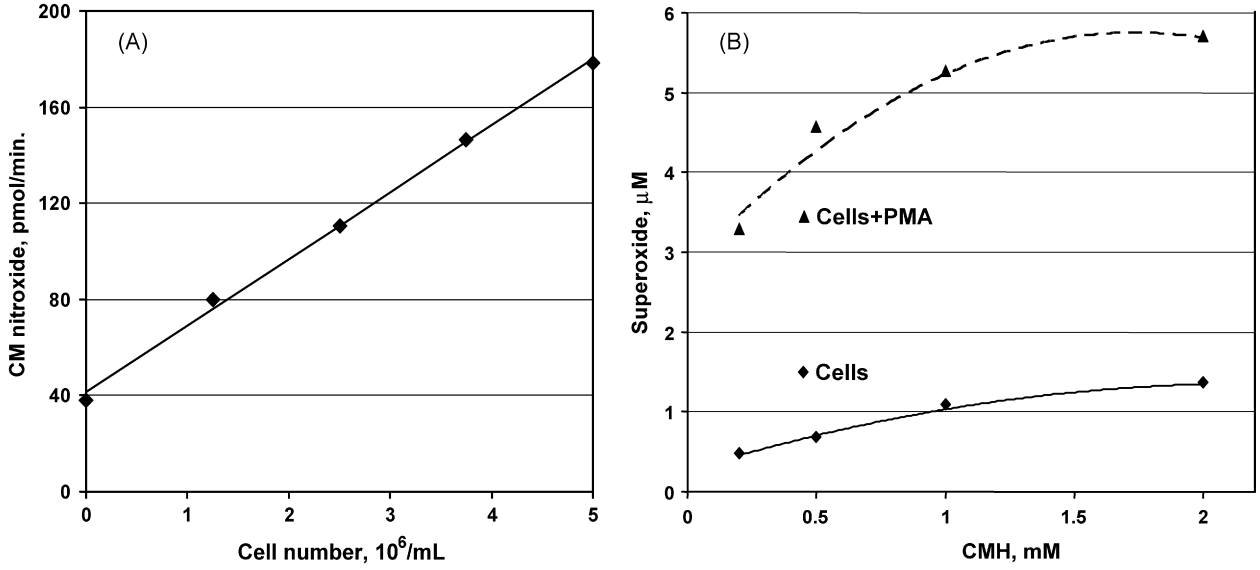

Fig. 6 – Dependence of ESR signal on concentrations of cells and CMH. Accumulation of CM-nitroxide was linearly proportional to concentrations of cells (A). Superoxide measurements were performed at different CMH concentrations (B). Amount of $O_2^{\bullet-}$ was not increased at CMH concentrations above 1 mM. Graphs show average of three independent measurements.

found that basal $O_2^{\bullet-}$ production in lymphoblast cell lines of CVD patients ($n = 8$) compared to non-CVD subjects ($n = 8$) was 10.9 ± 1.5 pmol $O_2^{\bullet-}/10^6$ cells/min versus 7.6 ± 0.9 pmol $O_2^{\bullet-}/10^6$ cells/min, respectively ($p = 0.04$). In addition, PMA-stimulated $O_2^{\bullet-}$ production in lymphoblast cell lines of CVD patients compared to non-CVD subjects were not statistically significantly different (data not shown). This preliminary data demonstrate that CMH was successfully used for $O_2^{\bullet-}$ measurements in cultured lymphoblast cell lines obtained from subjects with and without CVD. Larger clinical association studies with more characterized phenotypes are required to confirm these observations.

4. Discussion

In the present study, we observed that the cell-permeable spin probe CMH provided the most quantitative measurement of $O_2^{\bullet-}$ generation in human lymphoblast cell lines. As shown in Table 1, cytochrome C and the spin traps EMPO and DEPMPO detected two to four times less $O_2^{\bullet-}$ compared to CMH. Higher reactivity of CMH with $O_2^{\bullet-}$ and the stability of the CM-nitroxide (product of CMH and $O_2^{\bullet-}$ reaction) are likely responsible for higher $O_2^{\bullet-}$ detection by CMH. In addition, we show that human lymphoblasts predominantly produce extracellular $O_2^{\bullet-}$, confirming the phagocytic NADPH oxidase as the main source of $O_2^{\bullet-}$ in human lymphoblasts. Recently, a new cell-impermeable spin probe CAT1H has been suggested for quantification of extracellular $O_2^{\bullet-}$ [18]. Our preliminary data (data not shown) suggest that CAT1H could be used for measurements of NADPH oxidase activity in human lymphoblasts due to specific detection of extracellular $O_2^{\bullet-}$.

Table 1 – Comparison of $O_2^{\bullet-}$ detected with cytochrome C, DEPMPO, EMPO, CMH

	Cytochrome C	DEPMPO	EMPO	CMH
Cells	2.4 ± 0.2	2.3 ± 0.3	2.7 ± 0.4	$5.5 \pm 0.5^{*}$
Cells + PMA	11.2 ± 0.9	9.4 ± 0.9	$16 \pm 2.1^{**}$	$48.6 \pm 8.2^{*}$

Data are average of four independent experiments expressed as ±S.E. pmol $O_2^{\bullet-}/10^6$ cells/min ($n = 4$, $^{*}p < 0.01$; $^{**}p < 0.05$).

Measurements of $O_2^{\bullet-}$ by reduction of cytochrome C is a reproducible and cost-efficient assay. However, the cytochrome C assay has several limitations: (a) it detects only extracellular $O_2^{\bullet-}$; (b) has low sensitivity; and (c) is not specific and can be reduced by ascorbate and flavin enzymes [2,3]. In addition, it has been previously suggested that the cytochrome C assay underestimates $O_2^{\bullet-}$ values due to oxidation of cytochrome C by cellular H_2O_2, which may account for significant differences between CMH and cytochrome C in PMA-stimulated $O_2^{\bullet-}$ generation (Table 1).

Recently, it has been shown that spin trapping detects mainly extracellular but not intracellular $O_2^{\bullet-}$ [19] due to low efficacy for $O_2^{\bullet-}$ trapping [5]. This issue, however, is not the major limiting factor here due to the extracellular $O_2^{\bullet-}$ production by human lymphoblasts. By examining the ESR spectra of DEPMPO and EMPO radical adducts, we show that biodegradation and bioreduction of the radical adducts are the major problems regarding spin trapping of $O_2^{\bullet-}$ produced by human lymphoblasts. Non-linear kinetics is likely associated with decay of the EMPO and DEPMPO superoxide adducts. In comparison, linear CMH kinetics supports resistance of the CM-nitroxide to bioreduction. Meanwhile, new analogs of EMPO may be more resistant to bioreduction [20].

The fact that CMH detected more $O_2^{\bullet-}$ than spin traps or cytochrome C may suggest that CMH is the source of

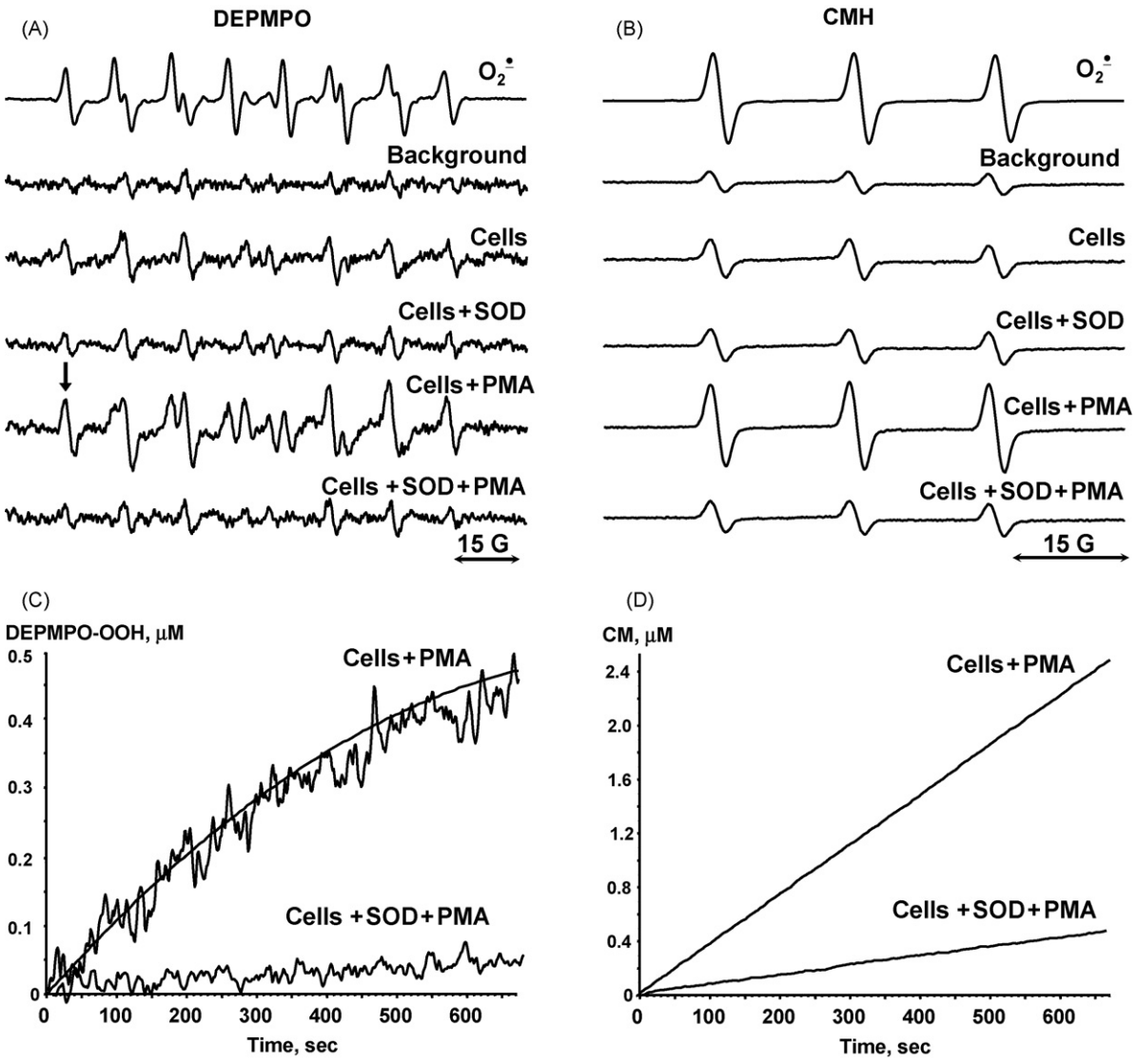

Fig. 7 – Detection of $O_2^{\bullet-}$ by DEPMPO and CMH in human lymphoblasts. Typical ESR spectra of 50 mM DEPMPO (A) or 1 mM CMH (B) with xanthine oxidase $O_2^{\bullet-}$ generating system (0.1 mM xanthine + 10 U/ml xanthine oxidase); in the absence of cells; in the presence of 2.5×10^3 μl^{-1} cells; cells plus Cu,Zn–SOD (50 U/ml); cells plus PMA (10 μM); cells plus PMA (10 μM) and Cu,Zn–SOD (50 U/ml). Accumulations of the DEPMPO radical adducts (C) or CM-nitroxide (D) in the probes with resting and PMA-stimulated cells in the presence or absence of Cu,Zn–SOD (50 U/ml). Figure shows typical ESR data representative of multiple measurements (n = 3).

additional $O_2^{\bullet-}$ production. Our data does not support such a conclusion because (a) amount of $O_2^{\bullet-}$ was proportional to the cell number (Fig. 6A); (b) amount of detected $O_2^{\bullet-}$ was not increased above 1 mM CMH (Fig. 6B); (c) amount of $O_2^{\bullet-}$ detected in the xanthine oxidase system was 25% of oxidized xanthine determined spectrophotometrically, which corresponds to normal $O_2^{\bullet-}/H_2O_2$ ratio for xanthine oxidase; (d) the background oxidation of CMH was rather minimal (Fig. 2B: buffer); and (e) PMA-stimulation of $O_2^{\bullet-}$ production was similar for CMH (8.8-fold) and spin trap EMPO (6-fold). Thus, our data support a higher efficiency of $O_2^{\bullet-}$ detection by CMH compared with spin traps.

The phagocytic NADPH oxidase of neutrophils and lymphoblasts has been extensively studied in chronic granulomatous disease (CGD), where defects caused by genetic variations such as deletions, insertions, and single nucleotide polymorphisms (SNP) in various enzyme subunits lead to impairment in respiratory burst and $O_2^{\bullet-}$ production [21–25]. More recently, lymphoblasts have been used in studies of NADPH oxidase in relation to hypertension and pre-eclampsia. An increase in $p22^{phox}$ expression and $O_2^{\bullet-}$ production was reported in hypertensive patients, and there was an enhanced agonist-stimulated NADPH oxidase-mediated $O_2^{\bullet-}$ production in pre-eclampsia, which may be important in mediating the endothelial dysfunction seen in this disease [26,27]. More recently, we have shown that the presence of the CC genotype of the *CYBA* C242T SNP is associated with significantly increased basal NADPH oxidase activity in human lymphoblasts of patients with CVD, independent of risk factors for atherosclerosis [28].

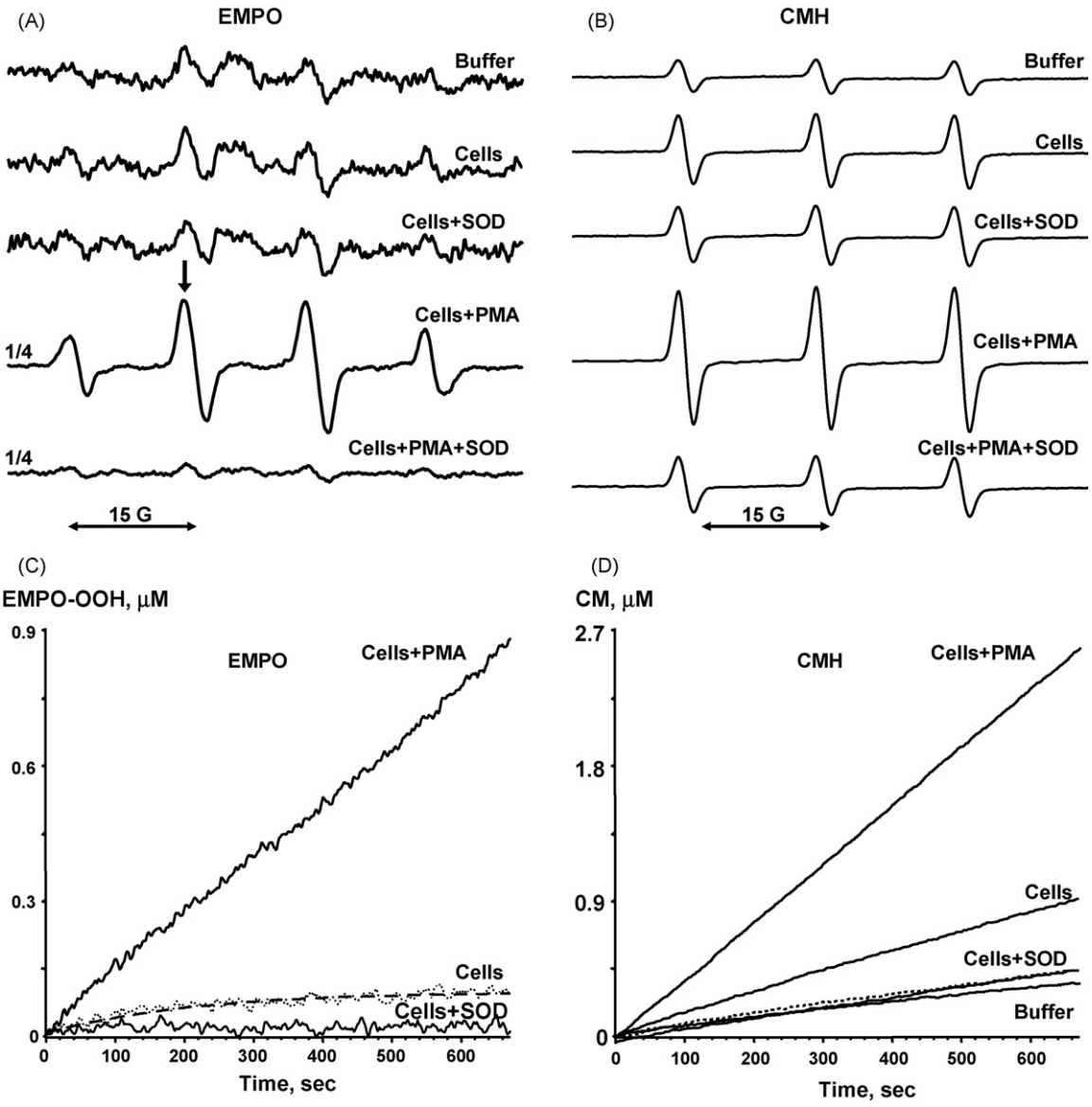

Fig. 8 – Detection of $O_2^{\bullet-}$ by EMPO and CMH in lymphoblasts. Typical ESR spectra of 60 mM EMPO (A) or CMH (B) in the presence or absence of cells (2.5×10^3 μl^{-1}); cells plus Cu,Zn–SOD (50 U/ml); cells plus PMA (10 μM); cells plus PMA (10 μM) and Cu,Zn–SOD (50 U/ml). Accumulations of the EMPO radical adducts (C) or CM-nitroxide (D) in the probes with resting and PMA-stimulated cells in the presence or absence of Cu,Zn–SOD (50 U/ml). Figure shows typical ESR data representative of multiple measurements ($n = 3$).

Previously, we showed that NADPH oxidase activity was genetically determined as measured in freshly isolated neutrophils using CPH [10]. In addition, environmental factors such as dietary supplementation with antioxidants, i.e. ascorbate, are of great interest in the prevention and management of many chronic diseases. However, antioxidants may interfere with detection of $O_2^{\bullet-}$ by cytochrome *C* or spin traps because of direct reduction of ferricytochrome and reduction of radical adducts, but not with probes such as CPH and CMH due to their resistance to bioreduction [7]. Thus, detection of $O_2^{\bullet-}$ by CMH may be advantageous in the assessment of antioxidant drugs and compounds.

In conclusion, we describe a new technique for $O_2^{\bullet-}$ measurement in cultured human lymphoblast cell lines using ESR and the spin probe CMH. We observed that cultured human lymphoblasts generate predominantly extracellular $O_2^{\bullet-}$. Finally, we believe that this highly sensitive and quantitative $O_2^{\bullet-}$ measurement in human cell lines will be useful to investigators who study genotype/phenotype interactions in translational studies of diseases related to oxidative stress.

Acknowledgements

This research was supported by National Institutes of Health grants PO-1 HL058000-05 and PO-1 HL075209, the Emory University Research Committee, the Emory University General

Clinical Research Center (M01-RR00039), Atlanta, GA, and by National Institutes of Health cardiovascular training grant T-32 HL07745 to PM and SSW.

REFERENCES

[1] Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 2000;86:494–501.

[2] Zhang H, Joseph J, Vasquez-Vivar J, Karoui H, Nsanzumuhire C, Martasek P, et al. Detection of superoxide anion using an isotopically labeled nitrone spin trap: potential biological applications. FEBS Lett 2000;473:58–62.

[3] Frejaville C, Karoui H, Tuccio B, Le Moigne F, Culcasi M, Pietri S, et al. 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide: a new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals. J Med Chem 1995;38:258–65.

[4] Tanigawa T, Kotake Y, Reinke LA. Spin trapping of superoxide from glass adherent polymorphonuclear leukocytes induced by *N*-formylmethionyl-leucyl-phenylalanine. Free Radic Res Commun 1993;19:101–10.

[5] Dikalov SI, Dikalova AE, Mason RP. Noninvasive diagnostic tool for inflammation-induced oxidative stress using electron spin resonance spectroscopy and an extracellular cyclic hydroxylamine. Arch Biochem Biophys 2002;402:218–26.

[6] Dikalov S, Landmesser U, Harrison DG. Geldanamycin leads to superoxide formation by enzymatic and non-enzymatic redox cycling. Implications for studies of Hsp90 and endothelial cell nitric-oxide synthase. J Biol Chem 2002;277:25480–5.

[7] Zwicker K, Dikalov S, Matuschka S, Mainka L, Hofmann M, Khramtsov V, et al. Oxygen radical generation and enzymatic properties of mitochondria in hypoxia/reoxygenation. Arzneimittelforschung 1998;48:629–36.

[8] Dikalov S, Skatchkov M, Bassenge E. Spin trapping of superoxide radicals and peroxynitrite by 1-hydroxy-3-carboxy-pyrrolidine and 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine and the stability of corresponding nitroxyl radicals towards biological reductants. Biochem Biophys Res Commun 1997;231:701–4.

[9] Dikalov S, Grigor'ev IA, Voinov M, Bassenge E. Detection of superoxide radicals and peroxynitrite by 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine: quantification of extracellular superoxide radicals formation. Biochem Biophys Res Commun 1998;248:211–5.

[10] Wyche KE, Wang SS, Griendling KK, Dikalov SI, Austin H, Rao S, et al. C242T CYBA polymorphism of the NADPH oxidase is associated with reduced respiratory burst in human neutrophils. Hypertension 2004;43:1246–51.

[11] Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J Biol Chem 2003;278:22546–54.

[12] Seigneurin JM, Guilbert B, Bourgeat MJ, Avrameas S. Polyspecific natural antibodies and autoantibodies secreted by human lymphocytes immortalized with Epstein–Barr virus. Blood 1988;71:581–5.

[13] Lambeth JD, Burnham DN, Tyagi SR. Sphinganine effects on chemoattractant-induced diacylglycerol generation, calcium fluxes, superoxide production, and on cell viability in the human neutrophil. Delivery of sphinganine with bovine serum albumin minimizes cytotoxicity without affecting inhibition of the respiratory burst. J Biol Chem 1988;263:3818–22.

[14] Clifford DP, Repine JE. Measurement of oxidizing radicals by polymorphonuclear leukocytes. Meth Enzymol 1984;105:393–8.

[15] Dikalova A, Clempus R, Lassegue B, Cheng G, McCoy J, Dikalov S, et al. Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. Circulation 2005;112:2668–76.

[16] Khatri JJ, Johnson C, Magid R, Lessner SM, Laude KM, Dikalov SI, et al. Vascular oxidant stress enhances progression and angiogenesis of experimental atheroma. Circulation 2004;109:520–5.

[17] Dikalov SI, Vitek MP, Mason RP. Cupric-amyloid beta peptide complex stimulates oxidation of ascorbate and generation of hydroxyl radical. Free Radic Biol Med 2004;36:340–7.

[18] Dikalov S, Kiriluk I, Grigor'ev I. Quantitative detection of extra- and intracellular superoxide by cyclic hydroxylamines with various charges and cell permeability. Comparison with nitrone spin traps. In: Abstracts of 8th international EPR spin trapping conference. 2005. p. 40.

[19] Zhao H, Joseph J, Fales HM, Sokoloski EA, Levine RL, Vasquez-Vivar J, et al. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. Proc Natl Acad Sci USA 2005;102:5727–32.

[20] Stolze K, Rohr-Udilova N, Rosenau T, Stadtmuller R, Nohl H. Very stable superoxide radical adducts of 5-ethoxycarbonyl-3,5-dimethyl-pyrroline *N*-oxide (3,5-EDPO) and its derivatives. Biochem Pharmacol 2005;69:1351–61.

[21] Maly FE, Schuerer-Maly CC, Quilliam L, Cochrane CG, Newburger PE, Curnutte JT, et al. Restitution of superoxide generation in autosomal cytochrome-negative chronic granulomatous disease (A220 CGD)-derived B lymphocyte cell lines by transfection with p22phox cDNA. J Exp Med 1993;178:2047–53.

[22] Condino-Neto A, Newburger PE. NADPH oxidase activity and cytochrome b558 content of human Epstein–Barr-Virus-transformed B lymphocytes correlate with expression of genes encoding components of the oxidase system. Arch Biochem Biophys 1998;360:158–64.

[23] Thrasher A, Chetty M, Casimir C, Segal AW. Restoration of superoxide generation to a chronic granulomatous disease-derived B-cell line by retrovirus mediated gene transfer. Blood 1992;80:1125–9.

[24] Cobbs CS, Malech HL, Leto TL, Freeman SM, Blaese RM, Gallin JI, et al. Retroviral expression of recombinant p47phox protein by Epstein–Barr Virus-transformed B lymphocytes from a patient with autosomal chronic granulomatous disease. Blood 1992;79:1829–35.

[25] Hancock JT, Henderson LM, Jones OTG. Superoxide generation by EBV-transformed B-lymphocytes. Immunology 1990;71:213–7.

[26] Pettit AI, Wong RKM, Lee VM, Jennings SC, Quinn PA, Ng LL. Increased free radical production in hypertension due to increased expression of the NADPH oxidase subunit p22phox in lymphoblast cell lines. J Hypertens 2002;20:677–83.

[27] Lee VM, Quinn PA, Jennings SC, Ng LL. NADPH oxidase activity in preeclampsia with immortalized lymphoblasts used as models. Hypertension 2003;41:925–31.

[28] Wang SS, Mehranpour P, Li W, Dikalov S, Austin H, Zafari AM. The CYBA genotype is associated with altered NADPH oxidase activity in patients with cardiovascular disease. J Am Coll Cardiol 2006;47:340A–1A.