Free Rad. Res. Comms., Vols. 12–13, pp. 27–37
Reprints available directly from the publisher
Photocopying permitted by license only

SUPEROXIDE PRODUCTION BY STIMULATED NEUTROPHILS: TEMPERATURE EFFECT

CHRISTOPHER D.V. BLACK, JOHN A. COOK, ANGELO RUSSO and AMRAM SAMUNI*†

*Radiation Oncology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD. 20892 USA and the *Department of Molecular Biology, School of Medicine, Hebrew University, Jerusalem 91010, Israel*

Activation of neutrophils results in a one-electron reduction of oxygen to produce the superoxide anion and other oxygen-derived, microbicidal species. Evidence from many kinetic studies of oxygen-derived radicals generated by stimulated neutrophils *in vitro* shows that radical production is optimal at 37° C but only lasts several minutes and then rapidly subsides. These findings support the widely held perception that the neutrophil's "oxidative burst" is a transitory event that peaks within minutes of stimulation and ends shortly thereafter. However, while some studies have shown that under controlled conditions stimulated neutrophils can generate superoxide continuously for several hours, others have observed that the superoxide formation by neutrophils stimulated in buffer at 37° C does not persist. To reconcile the conflicting findings and to better understand neutrophil function, we have reinvestigated the effect of temperature on the kinetics of radical generation by PMA-stimulated cells. Electron paramagnetic resonance spectroscopy coupled with spin-trapping and SOD-inhibitable ferricytochrome c reduction were used to monitor superoxide production by neutrophils stimulated at either 25° C or 37° C in RPMI 1640 medium or in Hank's balanced salt solution. When oxygen was supplied continuously, neutrophils stimulated at 25° C in buffer or in medium generated superoxide for several hours but at 37° C, particularly in HBSS, $O_2^{\cdot-}$ formation strikingly and rapidly decreased. This cessation of superoxide generation was reversible by lowering the temperature back to 25° C. These data imply that *in vivo* neutrophils may be capable of generating oxy-radicals for prolonged periods. In part, our results may also explain the often observed termination of neutrophil-derived radical formation *in vitro* and help to dispel the perception that neutrophil-derived oxy-radical production is an ephemeral phenomenon.

KEY WORDS: Electron paramagnetic resonance, respiratory burst, cytochrome c, leukocytes.

ABBREVIATIONS: Cyt-c^{III}, ferricytochrome c; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMPO-OH, 5,5-dimethyl-2-hydroxy-1-pyrrolidinyloxyl; DMPO-OOH, 5,5-dimethyl-2-hydro-peroxy-1-pyrrolidinyloxyl; $O_2^{\cdot-}$, superoxide; DTPA, diethylenetriamine pentaacetate; MPO, myeloperoxidase, EPR, electron paramagnetic resonance; HX, hypoxanthine; PMA, phorbol-12-myristate-13-acetate; RPMI, Rosewell Park Memorial Institute medium 1640; XO, xanthine oxidase, PMN, polymorphonucleocyte; PBS, phosphate buffered saline.

INTRODUCTION

Neutrophils defend against bacterial infection by producing, among other means, a group of powerful oxidizing species (H_2O_2, ClO^- and other active species) that derive from a single, relatively less-reactive precursor: the superoxide radical ($O_2^{\cdot-}$).[1-15] Both

†Communication: Dr. Amram Samuni, Molecular Biology, School of Medicine, Hebrew University, Jerusalem 91010, Israel.

soluble and particulate agents stimulate neutrophils to produce a high flux of $O_2^{\cdot}$ through the reduction of molecular oxygen by a membrane bound NADPH-dependent oxidase. Although the initial production rate of $O_2^{\cdot}$ is known to be optimal at 37° C,[14,15] evidence acquired from various experimental methodologies indicates that neutrophils stimulated at 37°C rapidly stop producing oxy-radicals.[9-12] Recent studies using a spin trapping technique and electron paramagnetic resonance (EPR) spectrometry demonstrated that neutrophils stimulated at 25° C produce $O_2^{\cdot}$ but at 37° C, persistence of the $O_2^{\cdot}$ spin-adduct of DMPO (DMPO-OOH) was not seen.[11] Others have also only observed transient formation of DMPO-OH (a decomposition product of DMPO-OOH) by stimulated neutrophils at 37° C without a persistent DMPO-OOH signal.[12] Such results do not agree with recent findings indicating that radical production by stimulated neutrophils is prolonged for several hours.[13] In view of the physiological imoportance of neutrophil-derived $O_2^{\cdot}$ production, the failure to detect a persistent DMPO-OOH spin-adduct at body temperature seems contrary to expectations.

In the present work the temperature effect on $O_2^{\cdot}$ formation by PMA-stimulated neutrophils has been reinvestigated using the ferricytochrome c ($Cyt\text{-}c^{III}$) reduction assay and spin-trapping coupled to EPR spectrometry. The results indicate that at 37° C when PMA-stimulated human neutrophils are maintained in RPMI medium they persistently produce $O_2^{\cdot}$ whereas, at 37° C in simple buffers, radical production only lasts a few minutes and then rapidly subsides. Although this result may explain the transient nature of oxy-radical production by neutrophils simulated in buffer at 37° C, it does not imply that *in vivo* oxy-radical production by stimulated neutrophils is ephemeral.

METHODS

Materials

Hypoxanthine was obtained from Calbiochem® Boehringer Diagnostics (San Diego, CA); phorbol-12-myristate-13-acetate (PMA) from Chemical Dynamics, Inc. (S. Plainfield, NJ), and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) from Aldrich Chemical Co. (Milwaukee, WI); superoxide dismutase (SOD), diethylenetriamine pentaacetic acid (DTPA), xanthine oxidase (EC 1.2.3.2 xanthine; oxygen oxidoreductase, grade III from buttermilk) and ferricytochrome c ($Cyt\text{-}c^{III}$) were obtained from Sigma Chemical Co (St. Louis, MO). Xanthine oxidase was further purified on a G25 sephadex® column. DMPO was purified by vacuum distillation and checked for the absence of EPR-observable contamination. The PMA was dissolved in ethyl alcohol (1 mg/ml) and diluted before use with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS). All other chemicals were prepared and used without further purification. Distilled, deionized water was used throughout all experiments. HBSS without Ca^{2+}, Mg^{2+}, or phenol red and RPMI 1640 medium without phenol red were supplied by Biofluids Inc. (Rockville, MD). To prepare inactive SOD, the enzyme was autoclaved for 60 min, checked for enzymatic activity and protein content, and stored at 4° C.

Cell Preparation

Human mononuclear cells and PMN were prepared from the blood of normal

volunteers by sedimentation on a ficoll/sodium diatriazoate gradient. Neutrophils were separated from erythrocytes by further sedimentation on 3% dextran, followed by hypotonic lysis of the remaining red cells.[16] Depending on the experiment, neutrophils 10^6–10^7 cells/ml) were stimulated in either HBSS or RPMI 1640 medium (RPMI). To avoid possible interference with spectrophotometric assays, phenol red-free RPMI and HBSS were used, although in practice the results were the same with or without phenol red.

Myeloperoxidase (MPO) Activity

To assess MPO activity of donors, blood smears were stained using the benzidine dihydrochloride method[32] and examined under light microscopy. None of the donors were found to be MPO-deficient. Blood donated by leukapheresis was routinely stained prior to donation to detect and reject abnormal leukocytes and MPO-deficient donors.

Superoxide Formation In Cell-Free System

To produce a constant flux of $O_2^{\cdot-}$, hypoxanthine (HX) 4–5 mM and xanthine oxidase (XO) 5-30 mU/ml were reacted in HBSS or RPMI at 37° C or 25° C under a constant supply of air or oxygen. The reaction mixture contained 50 μM DTPA to minimize metal-dependent XO inactivation.[17]

Electron Parmagnetic Resonance (EPR) Measurements

Samples (75–100 μl) containing 0.1 M DMPO and either the HX/XO reaction mixture or PMA-stimulated neutrophils (10^7 cells/ml) were drawn by syringe into a gas-permeable, 0.8 mm inner-diameter, teflon capillary. All reaction mixtures contained 50 μM DTPA to minimize unwanted transition metal effects. The gas-permeable capillary enabled oxygen levels to be maintained throughout the experiments. Each capillary was inserted into a 2.5 mm ID quartz tube (open at both ends) and then horizontally placed in the EPR spectrometer (to minimize effects of cells settling to the bottom of the tube during the measurements). Gases of desired compositions and temperatures were flowed around the sample within the spectrometer cavity. Without disturbing the alignment of the sample inside the EPR tube, the temperature was maintained at either 25 $\pm$ 0.3°C or 37 $\pm$ 0.3°C by using a Varian (Paulo Alto, CA) temperature control unit and a miniature thermistor inserted with the sample inside the quartz tube. Temperature changes between 25°C and 37° C were performed either abruptly (withn 2 min) or gradually over 15 min. EPR spectra were recorded on a Varian (Paulo Alto, CA) E4 or E9 X-band spectrometer, with field set at 3255 G, modulation frequency of 100 kHz, modulation amplitude of 1G, and non-saturating microwave power.

Superoxide Assay

Superoxide formation rate was determined by following SOD-inhibitable Cyt-c^{III} reduction. Neutrophils were stimulated by PMA (0.5 μg/ml) and maintained at 25° C in either HBSS, PBS containing 0.1% glucose, or RPMI. The media were generally

supplemented with 50 μM DTPA and 25 mM HEPES buffer. At timed intervals following adding PMA an aliquot of the stimulated cells was added to each of the cuvettes (0.5–1 × 10^6 cells/ml). The assay was started by the addition of 100 μM Cyt-c^{III} and 65 U/ml catalase to both sample and reference cuvettes and the OD change at 550 nm was followed using an SLM DW®-2C dual beam spectrophotometer.[18] The reference cuvette contained, in addition, 90 U/ml superoxide dismutase (SOD). A similar procedure was used to determine $O_2^{\cdot}$ produced by hypoxanthine and xanthine oxidase.

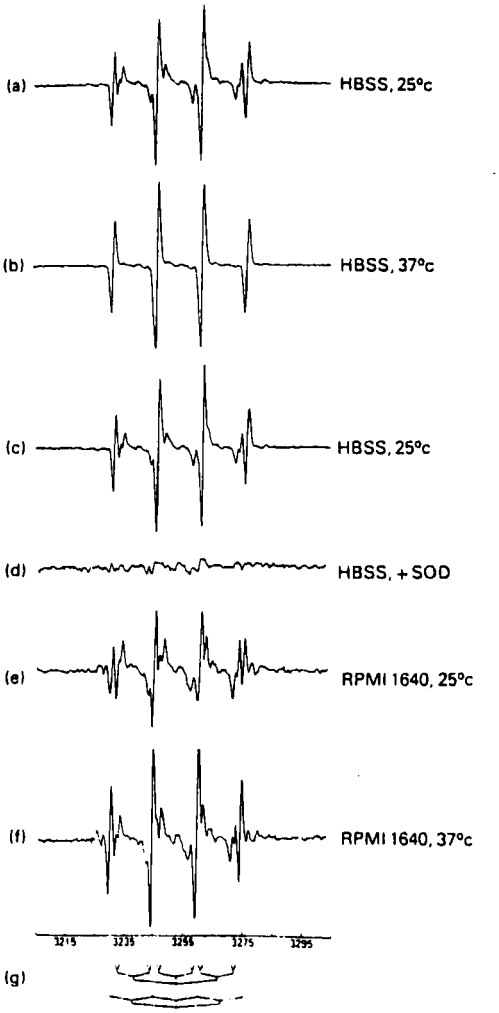


FIGURE 1 EPR spectra of DMPO spin-adducts detected following neutrophil stimulation in buffer or medium at 25° C and 37° C: EPR spectra of DMPO spin adducts formed upon stimulating 10^7 neutrophils/ml with 1 μg/ml PMA in the presence of 0.1 M DMPO, scanned 45–60 min after stimulation. Spectra were recorded at: a) 25° C in HBSS; b) 37° C in HBSS after warming a; c) 25° C in HBSS after cooling b; d) 25° C in HBSS in the presence of 14 U/ml SOD; e) 25°C in RPMI medium; f) 37°C in RPMI medium after warming e; g). stick diagrams denoting the EPR lines of DMPO–OOH (upper) and DMPO–OH (lower).

RESULTS

Superoxide Generation by Stimulated Neutrophils

Human neutrophils (10^7 cells/ml) at 25° C in HBSS containing 0.1 M DMPO and 50–100 μM DTPA were stimulated with 1 μg/ml PMA, placed in the EPR cavity, and scanned for EPR spectra while being maintained under air. Resting neutrophils did not produce any EPR signal, but stimulated neutrophils gave rise to the 12-lined spectrum as seen in Figure 1a, (a_N = 14.2 G, a_{H_β} = 11.3 G, a_{H_γ} = 1.3 G) typical of DMPO-OOH, and a 4-lined DMPO-OH signal (a_N = 14.9 G, a_{H_β} = 14.9 G). Similar EPR spectra of DMPO-OOH and DMPO-OH were obtained when the neutrophils were stimulated and maintained in RPMI at 25° C (Figure 1e). In both cases the DMPO-OOH spectrum persisted for several hours which indicates continuous $O_2^{\cdot-}$ production. Changing the gas from air to argon, without disturbing the alignment of the sample inside the EPR tube, eliminated (within 7–9 min) the DMPO-OOH, though not the DMPO-OH signal. Upon re-oxygenation, the DMPO-OOH signal was fully restored within 1.5 min (data not shown). In the presence of 14 U/ml SOD, (Figure 1d) neither DMPO-OOH nor DMPO-OH were observed. Inactivated SOD had no effect on the EPR signal. This supports previous conclusions that the DMPO-OH spin-adduct derives from $O_2^{\cdot-}$ (through decomposition of DMPO-OOH) and not from spin-trapping of any authentic ·OH radicals.[19]

Temperature Effect

When neutrophils were stimulated and maintained in RPMI at 37° C, similar EPR spectra to those observed at 25° C were obtained, although the DMPO-OOH signal was smaller (compare Figure 1e with 1f). Nevertheless, the DMPO-OOH signal persisted for about an hour, indicating that in RPMI, $O_2^{\cdot-}$ production continued, though to a lesser extent, at 37° C. In contrast, when neutrophils were stimulated and maintained at 37° C in HBSS, the DMPO-OOH signal lasted only a few minutes and then disappeared (compare Figure 1a with 1b and 1f). Furthermore, when the cells were stimulated at 25° C in HBSS and then 30 min later warmed to 37° C, the DMPO-OOH signal rapidly disappeared. This happened irrespective of the rate of temperature change (2 min vs. 15 min). The DMPO-OOH signal arising from stimulated cells incubated in HBSS did not persist at 37° C even when the HBSS was supplemented with 10% fetal calf serum; yet, restoration of the signal was observed within 30 min after cooling the HBSS cell suspension back to 25°C.

Temperature Effect on DMPO-OOH in Cell-Free System

Failure to record the DMPO-OOH signal produced by stimulated neutrophils in buffer at 37° C has been recently attributed to temperature-facilitated decay of the DMPO-OOH spin-adduct.[11] To check this possibility and to ensure a steady formation rate of $O_2^{\cdot-}$, the radicals were generated by the hypoxanthine/xanthine oxidase (0.03 U/ml) system in HBSS. Oxygen supply was kept constant by flowing air around the sample and the effect of substrate depletion was minimized by using a large excess of HX (5 mM). The spin trap DMPO 0.1 M was added to the reaction mixture to scavenge $O_2^{\cdot-}$ for EPR detection. Under such conditions the EPR signals of both DMPO-OOH and DMPO-OH appeared and persisted for about 2 h (Figure 2)

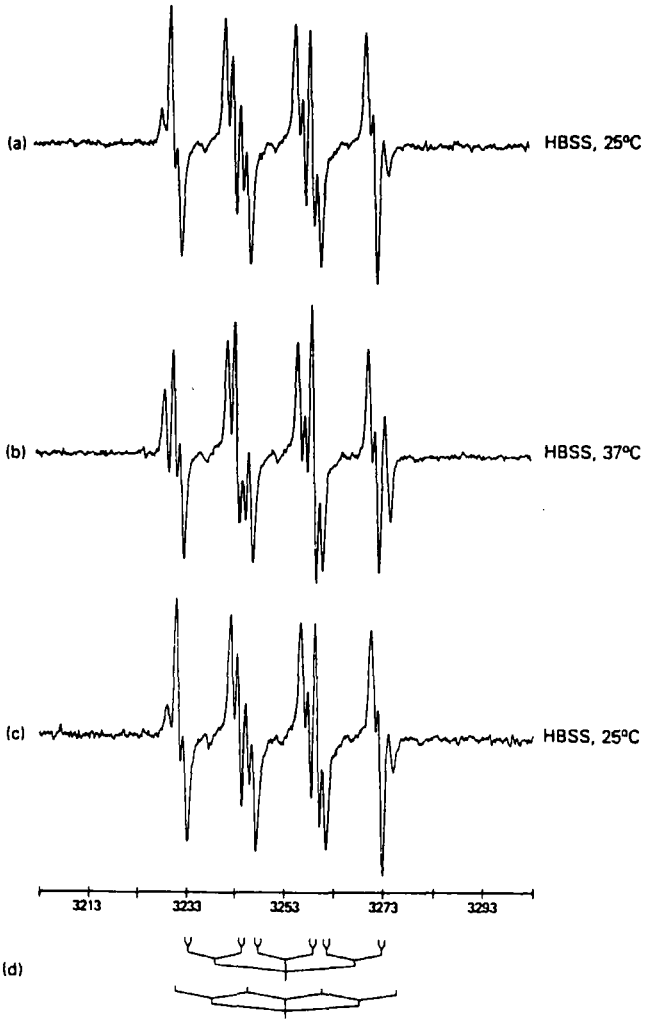


FIGURE 2. Temperature effect on the EPR spectra of DMPO spin-adducts produced by HX/XO reaction system: Experimental conditions: 5 mM hypoxanthine, 0.033 U/ml xanthine oxidase, 100 μM DTPA and 0.1 M DMPO in 50 mM phosphate buffer pH 7.3 with constant air supply. EPR spectra were scanned at: a) 25° C; b) 37° C after warming a; c) 25° C after re-cooling b; d) stick diagrams denoting the EPR lines of DMPO-OOH (upper) and DMPO-OH (lower).

implying a constant flux of $O_2^{\cdot-}$, as previously found.[20] Elevating the temperature from 25° C to 37° C hardly affected the intensity of DMPO-OOH signal (compare Figure 2a, 2b). Similarly no appreciable change was seen when the sample was cooled back to 25° C (Figure 2c).

Temperature Effect on $O_2^{\cdot-}$ Production Rate

Failure to observe a persistent DMPO-OOH signal might be expected if $O_2^{\cdot-}$ production by stimulated cells in buffer at 37° C is not continuous but rapidly diminishes or

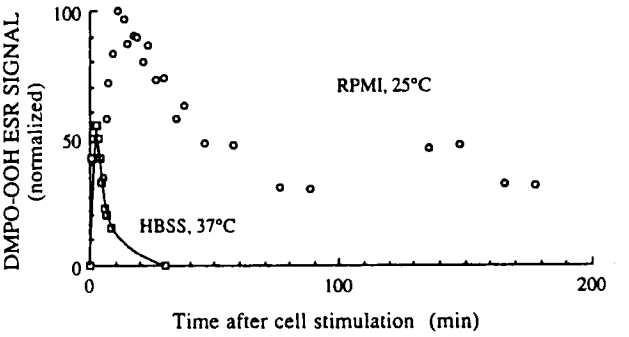


FIGURE 3. Time dependence of the rate of $O_2^{\cdot}$ production by stimulated neutrophils assayed by EPR: The rate of $O_2^{\cdot}$ production by neutrophils stimulated with 1 μg/ml PMA either in RPMI at 25° C (circles) or in HBSS at 37° C (squares) was investigated by EPR using DMPO spin trap, by monitoring the time dependence of the EPR signal of $[DMPO\text{-}OOH]_{steady\ state}$ obtained using 10^7 PMNs/ml and 0.1 M DMPO under constant oxygen supply. Signal intensity was measured at various time points following cell stimulation and all values were normalized to the maximal peak height which was taken as 100.

ceases. Such a possibility apparently contrasted with previous reports that $O_2^{\cdot}$ flux produced by stimulated neutrophils is higher at 37° C than at 25° C.[14,15] Nevertheless, to study the temperature effect on the kinetics of $O_2^{\cdot}$ formation by neutrophils, the full time-course of DMPO-OOH steady state signal was studied. Neutrophils (10^7 cells/ml) in RPMI were maintained in the absence of DMPO and stimulated by 1 μg/ml PMA at 25° C, 100 μl aliquots were removed at various time points, mixed with 5 μl DMPO 2 M and scanned for EPR signal of DMPO-OOH. The intensity of the highest-field line of DMPO-OOH, which is least affected by the DMPO-OH signal, was determined and the results of a typical experiment are presented in Figure 3 (circles). Similar time-course was observed for cells maintained in HBSS or PBS with

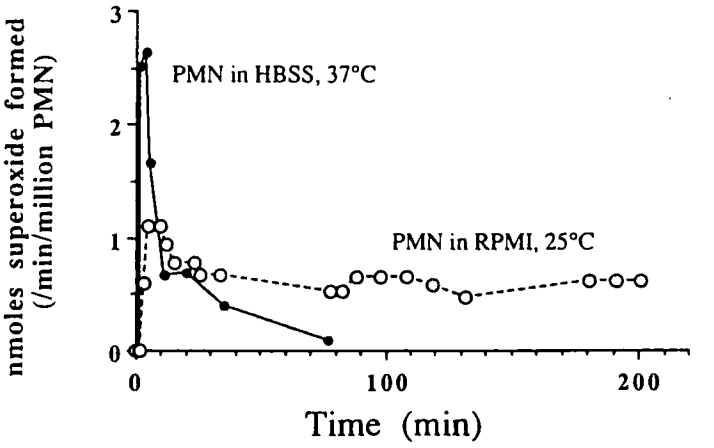


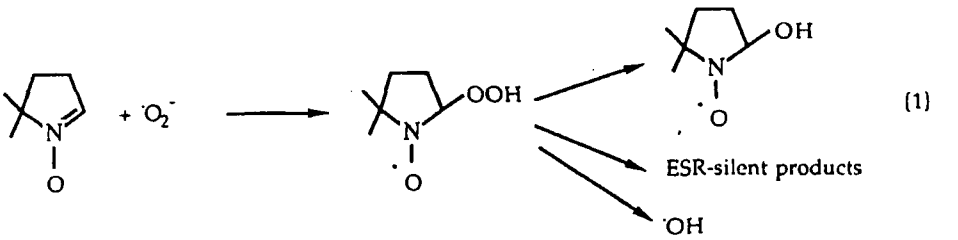
FIGURE 4 The kinetics of $O_2^{\cdot}$ production by PMA-stimulated neutrophils assayed by Cty-c^{III} reduction: The production rate of $O_2^{\cdot}$ was determined using the SOD-inhibitable Cyt-c^{III} reduction assay. PMNs were incubated in RPMI at 25° C (open symbols) or in HBSS at 37° C (solid symbols) and stimulated by 0.5 μg/ml PMA. At various time points aliquots of 100 μl were sampled into 2 ml RPMI containing 100 μM Cyt-c^{III} and 65 U/ml catalase. The reference cuvette contained, in addition, 90 U/ml SOD. Cyt-c^{III} reduction was followed at 550 nm and rates of reduction were determined from the initial slopes.

0.1% glucose at 25° C, indicating a continuous $O_2^{\cdot}$ production (data not shown). When, however the cells were maintained and stimulated in HBSS at 37° C the DMPO–OOH signal was observed only for a short time as seen in Figure 3 (squares). As a further check the full time-course of cell-derived $O_2^{\cdot}$ production was determined by following the SOD-inhibitable Cyt-c^{III} reduction.[17,18] The results, seen in Figure 4, showed that neutrophils stimulated at 37° C in HBSS manifested higher initial rates of $O_2^{\cdot}$ production, but rapidly lost most of their ability to generate $O_2^{\cdot}$ (Figure 4, solid circles). It should be noted that these cells remained fully viable as judged by exclusion of trypan blue. In contrast, cells stimulated and maintained in RPMI at 25° C continued to release $O_2^{\cdot}$ for hours (Figure 4, open circles).

DISCUSSION

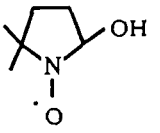
Examination of the literature indicates that most studies on the kinetics of $O_2^{\cdot}$ formation were performed using simple buffers at 37° C with or without glucose and/or serum.[9–12,24–30] The initial production rate of superoxide was found to be maximal, although transitory, at 37° C.[14,15] The present results (Figure 4) support the conclusion that **initially,** the production rate of $O_2^{\cdot}$ by neutrophils maintained in RPMI or HBSS and stimulated with PMA is higher at 37° C than at 25° C.[14,15] Most important, however, is the observation evident from Figures 1, 3, and, 4 that at 25° C radical production is persistent for several hours, whereas it is greatly lost if the cells are maintained in buffer at 37° C. Our findings were obtained with two independent methodologies; and, while Cyt-c^{III} reduction assay measures $O_2^{\cdot}$ production rate indirectly, the spin-trapping technique is a powerful tool for direct detection and identification of free radicals; however, interpretation of the resulting EPR spectra might be extremely complex and filled with pitfalls.[13,23]

A major problem in analyzing EPR results is the correlation of the observed signal intensities with the formation and decay kinetics of the primary radicals and of the spin-adducts themselves. The DMPO–OOH spin adduct, which is continuously produced from DMPO and $O_2^{\cdot}$ (reaction 1), is reported to have a half-life < 1 min and to decay yielding DMPO–OH through a poorly understood mechanism.[21]



(1)

Our data clearly show that under conditions of high and steady $O_2^{\cdot}$ flux generated by the HX/XO system (Figure 2), there is no accumulation of the DMPO–OH spin adduct. The same is true for DMPO–OOH and DMPO–OH produced by $O_2^{\cdot}$ from stimulated-neutrophils at room temperature (Figure 1a, 1e). The failure of the relatively long-lived DMPO–OH to accumulate has been previously observed in several systems[22] and was recently shown[13,20] to result from its rapid destruction by $O_2^{\cdot}$ through reaction 2.



(2)

Since $O_2^{\cdot-}$ contributes to both the formation (reaction 1) and the destruction of DMPO-OH (reaction 2), the DMPO-OH signal is not a valid quantitative marker for assaying superoxide production. For instance, the 1:2:2:1 spectrum observed with neutrophils maintained in HBSS at 37° C (Figure 1b) does not report ·OH generation, but rather a residual formation of $O_2^{\cdot-}$ which is sufficient nor to produce an observable DMPO-OOH signal nor to eliminate the DMPO-OH adduct.[13,20,33] No attempt, therefore, was made in the present work to correlate the DMPO-OH signal intensity with the kinetics of $O_2^{\cdot-}$ or ·OH formation. Conversely, the steady state intensity of the DMPO-OOH signal does correlate with the generation of $O_2^{\cdot-}$ and, therefore, makes DMPO-OOH a valid monitor of superoxide production.

It has previously been reported that the DMPO-OOH spin-adduct decays faster at 37° C than at 25° C.[11] This conclusion is corroborated by our results obtained with cell-free system, as shown in Figure 2. Although $O_2^{\cdot-}$ formation by HX/XO is greater at 37° C than in 25° C, the DMPO-OOH signal observed at 37° C did not increase (Figure 2b and 2a), indicating a faster decay of the spin-adduct at 37° C. The facilitated destruction of DMPO-OOH at 37° C accounts also for the apparent difference between peaks heights seen in Figures 3 and 4. Both full time-courses obtained with EPR and Cyt-c^{III} techniques show that $O_2^{\cdot-}$ production is persistent in RPMI at 25° C but rapidly subsides in HBSS at 37° C. On the other hand, the initial peak of DMPO-OOH signal (Figure 3) was lower at 37° C than in 25° C, indicating a faster destruction of the probe. It has been also suggested that DMPO-OOH decay may be accelerated in the presence of non-stimulated neutrophils by reduction of DMPO-OOH to DMPO-OH;[11,23] however, as we show here, both in cell-free enzymatic (HX/XO) system and also in the presence of stimulated neutrophils, the DMPO-OOH signal is observable and persists at 37° C (Figures 1f and 2b). This rules out the possibility that failure to observe DMPO-OOH produced by neutrophils stimulated in HBSS at 37° C results **solely** from facilitated spin-adduct destruction. As evident from Figures 1 and 4, the temperature-dependent impairment of $O_2^{\cdot-}$ production is particularly significant when neutrophils are maintained in simple buffers like HBSS. In previous studies, indeed, when cells were maintained in simple buffer the DMPO-OOH spin adduct decayed within a few minutes of neutrophil stimulation at 37° C.[11,12] However, even in RPMI, there was a variable decrease with time in $O_2^{\cdot-}$ production rate when the temperature was elevated from 25° C to 37° C. Because the failure to detect the $O_2^{\cdot-}$ spin-adduct of DMPO at 37° C cannot be accounted for solely by a temperature-dependent increase in the decay rate of DMPO-OOH, alternative explanations might be: a) Oxygen depletion; however, although depletion of oxygen results in a cessation of $O_2^{\cdot-}$ production, this is not the explanation for the temperature effect because, as we (Figure 1b) and others[12] have shown, loss of the DMPO-OOH signal occurs at 37° C in simple buffers even in the presence of an adequate supply of oxygen; b) Self-limiting $O_2^{\cdot-}$ production by neutrophils at 37° C; yet the restoration of the DMPO-OOH signal upon re-cooling to 25° C suggests that termination of the respiratory burst is not the whole answer; c) A temperature-sensitive cellular component of the superoxide generating system; one possible candidate may be the

neutrophil's own NADPH oxidase which, when isolated, has been shown[31] to have a V_{max} at 25° C and is susceptible to inactivation at 37° C.[18]

Since the loss of $O_2^{\cdot-}$ production is particularly pronounced for cells suspended at 37° C in buffer, experiments performed at room temperature paradoxically better reflect neutrophil-induced $O_2^{\cdot-}$ production than studies carried out at body temperature which are problematical. The choice of buffer over nutrient-supplemented medium by many researchers is dictated by an effort to avoid radical scavenging by media components and to minimize perturbation in optical absorption and chemiluminescence measurements. Similarly, stimulation of neutrophils at 37° C is an attempt to better simulate physiological conditions. In fact, there is no appreciable difference in $O_2^{\cdot-}$ yields (produced either enzymatically or by neutrophils) at 25° C when using either RPMI or HBSS (Figures 1, 3, and 4) and, although $O_2^{\cdot-}$ production is reduced in RPMI at 37° C, it is less affected than in buffer. Therefore, the use of simple buffers instead of nutrient-supplemented medium is unnecessary unless the formation and role of authentic ·OH are being investigated. Warming the cells from 25° C to 37° C only temporarily impaired, but did not irreversibly abolish, the $O_2^{\cdot-}$ forming ability of neutrophils stimulated in simple buffers (with or without fetal calf serum). This finding suggests a temperature-dependent alteration of some cellular function which we are currently attempting to elucidate.

The reversibility of the spin-loss (Figure 1c), the lower temperature-dependent cell vulnerability in medium (Figure 4), and the neutrophil's ability (under controlled conditions) to persistently produce and release $O_2^{\cdot-}$ radicals[13] suggest that the apparent ephemeral nature of the oxidative burst[9-12,24-30] may reflect a non-physiological subsidence in oxy-radical production.

References

1. B.M. Babior, R.S. Kipnes and J.T. Curnutte (1973) Biological Defense Mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *Journal of Clinical Investigation*, **52**, 741–744
2. R.B. Johnston Jr., B.B. Keele Jr., H.P. Misra, J.E. Lehmeyer, L.S. Webb, R.L. Baehner and K.V. Rajagopalan (1975) The role of superoxide anion generation in phagocytic bactericidal acitivity: Studies in normal and chronic degranulomatous disease leukocytes. *Journal of Clinical Investigation*, **55**, 1357–1372.
3. H. Rosen and S.J. Klebanoff (1977) Formation of singlet oxygen by the myeloperoxidase-mediated antimicrobial system. *The Journal of Biological Chemistry*, **252**, 4803–4810.
4. S.J. Weiss, G.W. King and A.F. LoBuglio (1977) Evidence for hydroxyl radical generation by human monocytes. *Journal of Clinical Investigation*, **60**, 370–373.
5. C. Nathan and Z. Cohn (1980) Role of oxygen-dependent mechanisms in antibody-induced lysis of tumor cells by activated macrophages. *Journal of Experimental Medicine*, **152**, 198–208.
6. S.J. Klebanoff (1982) Oxygen-dependent cytotoxic mechanisms of phagocytosis. In *Advances in Host Defense Mechanisms* (eds. J.I. Gallin and A.S. Fauci) Raven Press, New York. 1: 111–162.
7. D.R. Ambruso and R.B. Johnston, Jr. (1981) Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophils, neutrophil particulate fractions, and an enzymatic generating system. *Journal of Clinical Investigation*, **67**, 352–360.
8. J.R. Hatherill, G.O. Till, L.H. Bruner and P.A. Ward (1986) Thermal injury, intravascular hemolysis, and toxic oxygen products. *Journal of Clinical Investigation*, **78**, 629–636.
9. S.B. Shurin, H.J. Cohen, J.C. Whitin and P.E. Newburger (1983) Impaired granulocyte superoxide production and prolongation of the respiratory burst due to a low-affinity NADPH-dependent Oxidase. *Blood*, **62**, 564–571.
10. C.A. Dahinden, J. Fehr and T.E. Hugli (1983) Role of cell surface contact in the kinetics of superoxide production by granulocytes. *Journal of Clinical Investigation*, **72**, 113–121.
11. G.M. Rosen, B.E. Britigan, M.S. Cohen, S.P. Ellington, and M.J. Barber (1988) Detection of

phagocyte-derived free radicals with spin trapping techniques: effect of temperature and cellular metabolism. *Biochimica et Biophysica Acta*, **969**, 236–241.
12. F.W. Kleinhans and S.T. Barefoot (1987). Spin trap determination of free radical burst kinetics in stimulated neutrophils. *The Journal of Biological Chemistry*, **262**, 12452–12457.
13. A. Samuni, C.D.V. Black, C.M. Krishna, H.L. Malech, E.F. Bernstein and A. Russo. (1988) Hydroxyl radical production by stimulated neutrophils reappraised. *The Journal of Biological Chemistry*, **263**, 13797–13801.
14. P.E. Newburger, M.E. Chovaniec and H.J. Cohen (1980) Activity and activation of the granulocyte superoxide-generating system. *Blood*, **55**, 85–92.
15. H.J. Cohen, P.E. Newburger, M.E. Chovaniec, J.C. Whitin, and E.R. Simons (1981) Opsonized zymosan-stimulated granulocytes - Activation and activity of the superoxide-generating system and membrane potential changes. *Blood*, **58**, 975–982.
16. A. Boyum, (1968). Isolation of mononuclear cells and granulocytes from human blood. *Scandinavian Journal of Clinical Laboratory Investigations*, **21**, (Suppl 97): 77–89.
17. I. Fridovich (1970) Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *The Journal of Biological Chemistry*, **245**, 4053–4057.
18. M. Markert, P.C. Andrews and B.M. Babior (1984) Measurement of O_2^- production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. *Methods in Enzymology*, **105**, 358–365.
19. B.E. Britigan, G.M. Rosen, Y. Chai and M.S. Cohen (1986) Do human neutrophils make hydroxyl radical? Determination of free radicals generated by human neutrophils activated with a soluble or particulate stimulus using electron paramagnetic resonance spectrometry. *The Journal of Biological Chemistry*, **261**, 4426–4431.
20. A. Samuni, C.M. Krishna, P. Riesz, E. Finkelstein and A. Russo (1989) Superoxide reaction with nitroxide spin-adducts. *Free Radical Biology and Medicine*, **6**, 141–148.
21. E. Finkelstein, G.M. Rosen and E.J. Rauckman (1982) Production of hydroxyl radical by decomosition of superoxide spin-trapped adducts. *Molecular Pharmacology*, **21**, 262–265.
22. C. Mottley, H.D. Connor and R.P. Mason (1986) [^{17}O] Oxygen hyperfine structure for the hydroxyl and superoxide radical adducts of the spin traps DMPO, PBN and 4-POBN. *Biochemical Biophysical Research Communication*, **141**, 622–628.
23. B.E. Britigan, M.S. Cohen and G.M. Rosen (1987) Detection of the production of oxygen-centered free radicals by human neutrophils using spin trapping techniques: A critical perspective. *Journal of Leukocyte Biology*, **41**, 349–362.
24. C. Dahlgren (1987) Polymorphonuclear leukocytes chemiluminescence induced by formylmethionyl-leucyl-phenylalanine and phorbol myristate acetate: Effects of catalase and superoxide dismutase. *Agents and Action*, **21**, 104–112.
25. L. Simchowitz (1985) Intracellular pH modulates the generation of superoxide radicals by human neutrophils. *Journal of Clinical Investigation*, **76**, 1079–1089.
26. H. Gyllenhammar (1989) Mechanisms for luminol-augmented chemiluminescence from neutrophils induced by leukotriene B4 N-formyl-methionyl-leucyl-phenylalanine. *Photochemistry and Photobiology*, **49**, 217–223.
27. K. Cheung, J. Lark, M.F. Robinson, P.J. Pomery and S. Hunter (1986) The production of hydroxyl radical by human neutrophils stimulated by arachidonic acid - measurements by ESR spectroscopy. *Australian Journal of Experimental Biology and Medicine Sciences*, **64**, 157–164.
28. D.A. Hawley, F.W. Kleinhans and J.L. Biesecker (1983) Determination of alternate pathway complement kinetics by electron spin resonance spectroscopy. *American Journal of Clinical Pathology*, **79**, 673–677.
29. R.C. Jandl, J. Andre-Schwartz, L. Borges-Dubois, R.S. Kipnes, B.J. McMurrich and B.M. Babior (1978) Termination of the respiratory burst in human neutrophils. *Journal of Clinical Investigation*, **61**, 1176–1185.
30. J.G. Bender and D.E. Van Epps (1983) Analysis of the bimodal chemiluminescence pattern stimulated in human neutrophils by chemotactic factors. *Infection and Immunity*, **41**, 1062–1070.
31. A.R. Cross, J.F. Parkinson and O.T.G. Jones (1984) The superoxide-generating oxidase of leukocytes: NADPH-dependent reduction of flavin and cytochrome b in solubilized preparations. *Biochemical Journal*, **223**, 337–344.
32. L.S. Kaplow (1965) Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood*, **26**, 215–219.
33. S. Pou, M.S. Cohen, B.E. Britigan and G.M. Rosen (1989) Spin trapping and human neutrophils. Limits of detection of hydroxyl radical. *The Journal of Biological Chemistry*, **264**, 12299–12302.

Accepted by Prof. G. Czapski