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APPLICATION OF THE ELECTROCHEMISTRY OF CYTOCHROME *c* TO THE MEASUREMENT OF SUPEROXIDE RADICAL PRODUCTION

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An electrochemical sensor for the measurement of the superoxide anion produced as a result of the respiratory burst of neutrophils is described. The reduction of cytochrome *c* by the superoxide ion was monitored using a surface-modified gold electrode. The current produced was shown to be superoxide-specific and proportional to the number of neutrophils in both purified neutrophil preparations and plasma.

KEY WORDS: Superoxide, amperometric electrode, cytochrome *c*, surface-modified electrode, electrochemistry, neutrophils, respiratory burst, free radicals.

INTRODUCTION

The generation of oxygen-derived, potentially cytotoxic species, namely the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) during the respiratory burst of phagocytic cells such as neutrophils is thought to play an important role in the destruction of invasive microorganisms.[1] These species have also been implicated in the pathogenesis of conditions such as post-ischaemic tissue injury[2] and rheumatoid arthritis.[3] The production of O_2^- is defective in several genetic or acquired conditions that are associated with recurrent bacterial, viral and fungal infections.[4] The diagnosis of these conditions is currently performed by measuring the production of oxygen-derived free radicals by neutrophils which are stimulated by phagocytosable (e.g. bacteria or opsonised zymosan) or soluble (e.g. concanavalin A, or phorbol myristate acetate) agents.[5]

This paper reports an electrochemical method for the measurement of O_2^- that may offer advantages over existing methods such as nitroblue tetrazolium reduction,[6] spectrophotometric measurement of cytochrome *c* reduction,[7] chemiluminescence[8] or spin-trapping[9] in that it is a simple, sensitive, rapid, quantitative assay that does not require highly purified cell preparations and is therefore potentially of use in whole blood samples and in continuous monitoring.

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The production of O_2^- is followed electrochemically by monitoring the reduction of cytochrome *c* at a gold electrode surface-modified with 4,4′-dithiobipyridine. Modification of gold electrode surfaces with 4,4′-dithiobipyridine promotes the stable, reversible electrochemistry of cytochrome *c*.[10] We present evidence demonstrating the specificity of the electrode for the measurement of O_2^- and show that the method correlates well with the standard spectrophotometric assay based on monitoring cytochrome *c* reduction at 550 nm.

In addition, our data shows that the production of O_2^- by stimulated human neutrophils is dependent on the number of cells present when using both purified cell preparations and plasma samples. Unlike other electrochemical O_2^- detection systems based on opsonised electrodes,[11] the biopassive nature of this detection method has potential application for continuous monitoring e.g. in renal dialysis.

MATERIALS AND METHODS

Electrochemistry

A conventional three electrode system was used. The two-compartment glass cell used had a working volume of 0.5 mL, and was surrounded by a water jacket thermostatted at 37°C by a Grant FH15 water circulator. The reference electrode was a saturated calomel electrode (SCE) and the counter electrode a platinum wire. The 4 mm-diameter gold disc working electrode was surface-modified by dipping the electrode into a 1 mM solution of 4,4′-dithiobipyridine (Aldrich) for 1 min, followed by copious rinsing with distilled water. D.c. cyclic voltammograms of cytochrome *c* were obtained using a Hyspec potentiostat and were recorded on a Gould series 60000 xyt chart recorder. Constant potential experiments were achieved using a Rank Brothers electronic stirrer and magnetic follower. The glass cell and teflon-coated follower were silanized with 2% dimethyldichlorosilane solution in trichloroethane (BDH) prior to use with purified cell-suspensions and plasma.

Generation of O_2^- by Xanthine and Xanthine Oxidase

Solutions containing 0.2 mM cytochrome *c* (Type VI, Sigma) and 0.5 mM xanthine (Sigma) in 20 mM phosphate buffer, pH 7.0, containing 100 mM sodium perchlorate as a supporting electrolyte, were incubated at 37°C. The generation of O_2^- was achieved by the addition of xanthine oxidase (XOD, Boehringer) over the concentration range 35–630 nM. In some experiments cytochrome oxidase (final concentration 2 U mL^{-1}) was preincubated with cytochrome *c* and xanthine prior to addition of XOD in order to reoxidise rapidly cytochrome *c* reduced by generation of O_2^-. The effect of cytochrome oxidase was inhibited by the inclusion of CN^- at a final concentration of 0.1 mM prior to addition of XOD. The rate of cytochrome *c* reduction was recorded as a change in current at −25 mV as a function of time in the electrochemical system and as a change in absorbance at 550 mm as a function of time in the spectrophotometric system. Spectrophotometric measurements were carried out on a Cecil CE292 spectrophotometer. The final concentrations and conditions used in both methods were identical.

Isolation and Purification of Neutrophils

Polymorphonuclear leukocytes were prepared from freshly collected venous blood using Heparin (12.5 units/mL) as anticoagulant. Blood was added to a 6% Dextran solution in physiological saline (2 mL/20 mL blood) in order to sediment the erythrocytes. After standing for 50 min at 37°C, the supernatant was removed and contaminating erythrocytes lysed with 0.83% NH_4Cl (10 mL/20 mL blood) for 10 min at room temperature. Centrifugation at 100 × g for 10 min yielded a cell pellet, which was washed twice with Hank's balanced salt solution (HBSS) prior to being resuspended in HBSS containing 5 mM glucose and 100 mM sodium perchlorate. The cells isolated were 80% neutrophils and were more than 95% viable as judged by trypan blue exclusion. In other experiments, plasma obtained after erythrocyte sedimentation was used directly in the electrochemical cell. Differing volumes of plasma and resuspended neutrophils were added to cytochrome *c* in HBSS containing 5 mM glucose and 100 mM sodium perchlorate such that the final cell volume was always 0.5 mL and the final concentration of cytochrome *c* 0.2 mM. After a steady background current had been obtained, the respiratory burst was elicited by injection of phorbol 12-myristate 13-acetate (PMA, Sigma) at a final concentration of 2.5 $\mu g\,mL^{-1}$.

RESULTS AND DISCUSSION

Electrochemical Specificity

The specificity of the electrochemical technique for O_2^- was investigated using the xanthine/XOD system to generate O_2^- enzymically. It was found that a change in current at −25 mV at the surface-modified gold electrode occurred only in the presence of cytochrome *c*, xanthine and oxygen upon the addition of XOD. No change in current was observed in the absence of any one of the components. Degassing the system by bubbling argon gas through the cytochrome *c* and xanthine solution in the electrochemical cell completely inhibited the reduction of cytochrome *c*. Further evidence of the oxygen-dependent nature of the system was provided by examining the effect of superoxide dismutase (SOD) on the electrochemical technique. Addition of SOD (40 $\mu g\,mL^{-1}$, specific activity 3300 U mg^{-1}) completely inhibited the production of a current due to the reduction of cytochrome *c* by O_2^- (Figure 1). Figures 1b and 1c show that SOD completely inhibits the current whether it is present before addition of XOD (Figure 1c) or added after the establishment of a current due to the reduction of cytochrome *c* by O_2^- (Figure 1b). In addition it was found that when using SOD at lower concentrations it was possible to demonstrate an SOD activity-dependent inhibition of the current produced — the basis of a great many assays for SOD activity.[4] The purpose of this communication however is not to report another SOD activity assay. The results obtained in the presence of SOD and absence of oxygen strongly indicate the O_2^- specificity of the electrode.

In contrast to the report by Vandewalle and Petersen[12] who suggested that hydrogen peroxide produced as a result of spontaneous dismutation of O_2^- in a competing reaction could oxidise reduced cytochrome *c* and thus interfere with spectrophotometric cytochrome *c* based O_2^- assays we have found that addition of catalase to the electrochemical assay has no effect on the magnitude or rate of production of

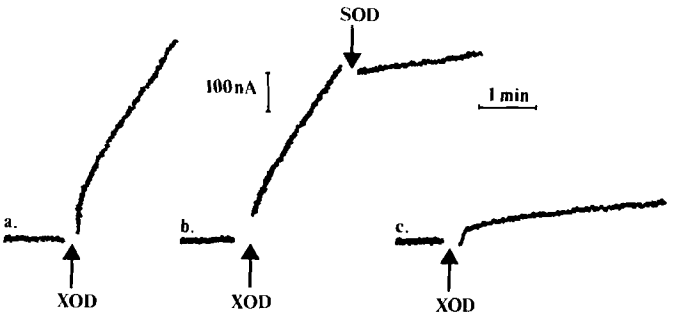

FIGURE 1 Typical current-time profiles of the reduction of cytochrome *c* by O_2^- at a surface-modified gold electrode. (a) 0.28 μM XOD added to a solution of 0.2 mM cytochrome *c* and 0.5 mM xanthine in phosphate buffer, pH 7.0 at 37°C; (b) as for (a) but 40 $\mu g\,mL^{-1}$ CuZn SOD added as indicated; (c) as for (a) but with 40 $\mu g\,mL^{-1}$ CuZn SOD present in solution before addition of XOD.

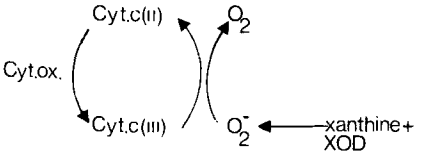

FIGURE 2 The sequence of reactions occurring in the presence of cytochrome oxidase (Cyt.ox.). No nett reduction of cytochrome *c* by O_2^- occurs.

the current observed. Hence we can conclude that the only process being monitored at the electrode is the O_2^--mediated reduction of cytochrome *c* and no reoxidation by H_2O_2 is occurring in this system. Reoxidation of reduced cytochrome *c* would be a major problem to the electrochemical measurement if the rate of reoxidation was much faster than the electrode reaction as would be the case in the presence of cytochrome oxidase (Figure 2). In the presence of O_2^-, generated by xanthine/XOD, and cytochrome oxidase there was no net reduction of cytochrome *c* and therefore no net transfer of electrons to be monitored by the electrode. Preincubation of cytochrome *c*, xanthine and cytochrome oxidase with 0.1 mM CN^- prior to injection of XOD, to initiate the production of O_2^-, prevented the reoxidation of cytochrome *c* reduced by O_2^- by inhibiting cytochrome oxidase. The effect of cytochrome oxidase and its inhibition by CN^- gives further evidence that the electrode specifically measured cytochrome *c* reduction by O_2^-.

Comparison of Electrochemical and Spectrophotometric O_2^- Measurement

Figure 3 shows the response of both methods to incrasing XOD concentrations under identical conditions. As expected there was a linear relationship between the initial rate of change of current and XOD concentration in the electrochemical method and this correlated well with the standard spectrophotometric assay (Figure 4, $y = 0.08x + 0.19$, $r = 0.99$). Since there is no electrochemical equivalent of the molar extinction coefficient this correlation allowed us to equate the rate of change

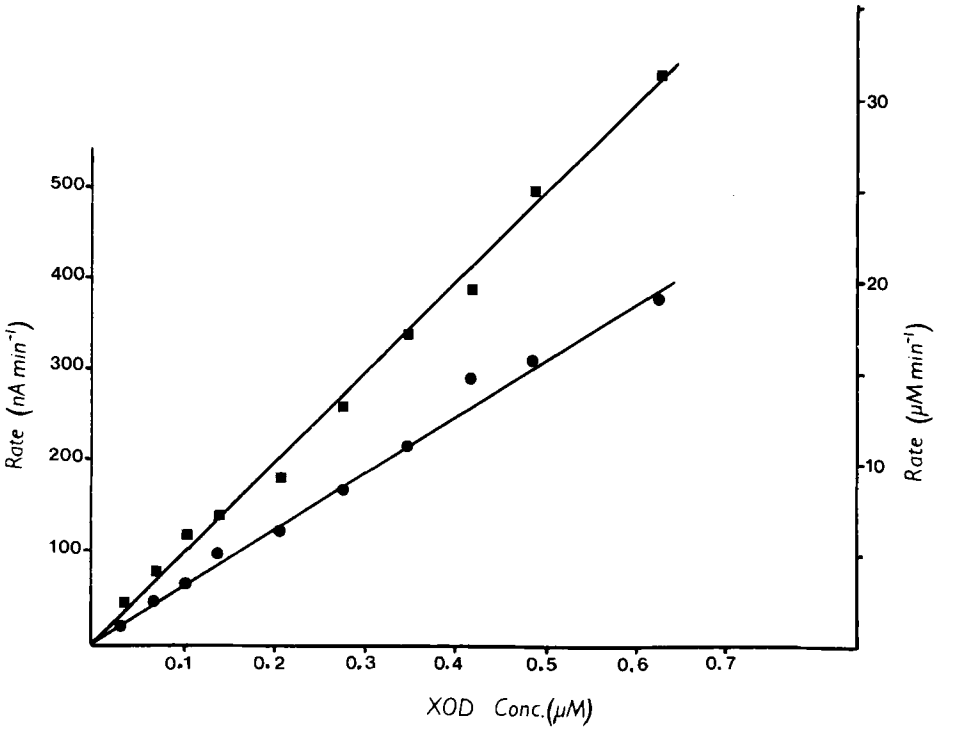

FIGURE 3 The rates of cytochrome *c* reduction at 37°C measured by both the electrochemical (■) and spectrophotometric (●) methods. Each point represents the mean value of six measurements. The conditions were identical for both techniques.

of current to the concentration of cytochrome *c* reduced in subsequent experiments with stimulated neutrophils.

Electrochemical Response to Stimulated Neutrophils

Initial experiments, summarised in Table 1, showed that the calculated rate of cytochrome *c* reduction expected from the electrochemical measurement of O_2^- from stimulated isolated neutrophils (Figure 4) correlated well with the results obtained from the spectrophotometric assay under identical conditions.

Increasing the number of neutrophils exposed to the stimulus in both purified neutrophil preparations and plasma resulted in a linear increase in electrochemical response as expected (Figure 5). It is not entirely clear why many fewer cells in plasma should give rates of cytochrome *c* reduction which are much larger than were obtained using the same number of purified neutrophils (Figure 5). Although it is possible to reduce cytochrome *c* with other species which may be present in plasma, such as ascorbic acid, the electrode was allowed to achieve a steady background current prior to the addition of PMA to stimulate the neutrophils. The current generated by addition of PMA after the plasma background had been established was inhibitable by SOD at the concentration used in the xanthine/XOD system, and therefore it

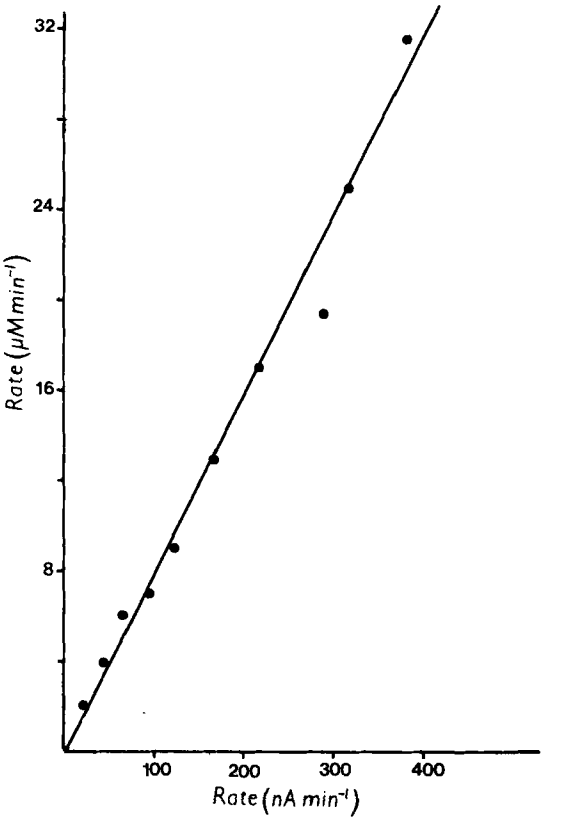

FIGURE 4 The correlation between the electrochemical and spectrophotometric measurement of O_2^- generated by the xanthine/XOD system at 37°C. All rates in both assays have been corrected for the rate observed in the presence of CuZn SOD (40 $\mu g\,mL^{-1}$).

TABLE 1
Production of O_2^- by stimulated human neutrophils measured both electrochemically and spectrophotometrically. The expected rates were extrapolated from Figure 4 and the actual rates measured spectrophotometrically.

	No of neutrophils used	Electrochemical rate ($nA\,min^{-1}$)	Calculated rate of cyt. *c* reduction ($\mu M\,min^{-1}$)	Measured rate of cyt. *c* reduction ($\mu M\,min^{-1}$)
Donor I	1.5×10^6	140	11.0	12.8
Donor II	1.0×10^6	115	9.0	8.0

would appear that the current was produced specifically by the reduction of cytochrome *c* by O_2^-. One possible explanation for the observed plasma results is that cells in plasma are far better preserved and thus more responsive than isolated neutrophils which have undergone the purification procedure and are in a less physiological medium.

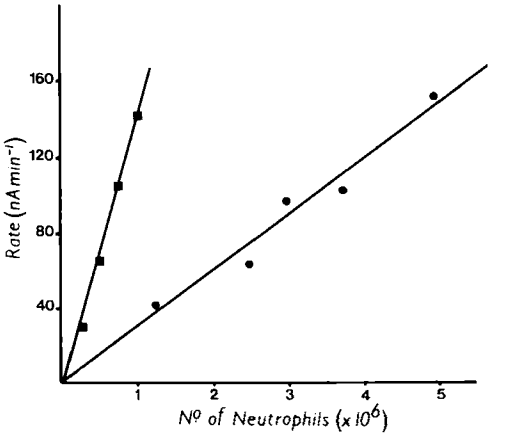

FIGURE 5 Electrochemical measurement of O_2^- generated by stimulated human neutrophils in both purified neutrophil preparations (●) and in plasma obtained after erythrocyte sedimentation (■). The cells were stimulated by the addition of 2.5 $\mu g\,mL^{-1}$ phorbol 12-myristate 13-acetate. Each point represents the mean of three measurements for each sample. All results have been corrected for the rate observed in the presence of CuZn SOD (40 $\mu g\,mL^{-1}$).

CONCLUSION

The electrochemical sensor has been shown to be specific for measurement of the superoxide anion and the reduction current $i\alpha[XOD]\ \alpha[O_2^-]$. The method correlated well with the spectrophotometric O_2^- assay when used both for chemical generation and biological generation of O_2^-. The electrochemical technique can be applied to measurement of O_2^- in biological samples without the need for extensive cell purification and in the presence of spectral interferences. Further work using this method to monitor O_2^- production in post-ischaemic tissue injury is currently in progress.

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