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MEASUREMENT OF SUPEROXIDE ANION PRODUCTION USING MAXIMAL RATE OF CYTOCHROME (III) C REDUCTION IN PHORBOL ESTER STIMULATED NEUTROPHILS, IMMOBILISED TO MICROTITER PLATES

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Abstract—In the present investigation, a method of studying the maximal rate of superoxide anion ($O_2^{\cdot -}$) production in immobilised human neutrophils using a microtiter plate technique has been developed. The rate of $O_2^{\cdot -}$ production was determined from the rate of reduction of cytochrome (III) C, studied as the increase in absorbance at 550 nm. The protein kinase C activator, phorbol 12-myristate 13-acetate, was used to stimulate $O_2^{\cdot -}$ production. Neutrophils were evenly immobilised as a monolayer to microtiter culture plates to provide a reproducible exposure to the medium. Phorbol ester stimulated $O_2^{\cdot -}$ production was inhibited by staurosporine, a well-known inhibitor of protein kinase C, and by diphenylene iodonium, a potent NADPH-oxidase inhibitor, with IC_{50}-values in this assay of 20 and 220 nm, respectively. The extracellularly produced $O_2^{\cdot -}$ was removed by superoxide dismutase with a half maximal effect of 0.6 μg/mL. The maximal production rate of $O_2^{\cdot -}$ could therefore be estimated by addition of 20 μg/mL superoxide dismutase. Several antioxidants, including butylated hydroxytoluene, nordihydroguairetic acid, probucol and α-tocopherol, were studied and showed neither an effect on $O_2^{\cdot -}$ production nor a scavenging effect. This new method was highly reproducible, and the continuous measurement of $O_2^{\cdot -}$ production was very useful for validating the effect of inhibitors. The developed microtiter technique using immobilised cells has a large capacity and allows different compounds to be tested under comparable conditions, since they are exposed to the cells in a similar way. This is also the first test model which describes $O_2^{\cdot -}$ production as the maximal rate of cytochrome (III) C reduction.

Key words: superoxide anion; neutrophils; microtiter plate; SOD; staurosporine; diphenylene iodonium

Human neutrophils are implicated as mediators of tissue destructive events in several inflammatory diseases, including rheumatoid arthritis [1], myocardial reperfusion injury [2], respiratory disorders [3] and ulcerative colitis [4]. Among the important active substances released by neutrophils are oxygen reactive species such as $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2†). Even though these cells produce radicals and other bactericidal substances in order to defend the host from infection, situations where the host itself is damaged may also occur [5]. *In vivo* neutrophils are activated by substances that bind to receptors located on the cell surface, among which are the bacterial toxins. Most bacteria are phagocytosed and digested, and bactericidal substances are then released into the neutrophil phagocytic vacuole. In some cases, these toxic chemicals are released into the plasma where they might injure the host tissue. It is less probable that $O_2^{\cdot -}$ is a mediator of general tissue injury, since it will not reach far in plasma because it reacts with blood components and has a high self-dismutation rate. Superoxide anions in neutrophils are produced by the respiratory burst enzyme, NADPH-oxidase [6]. It is well known that $O_2^{\cdot -}$ production is linked to the activation of a phospholipid-dependent, calcium-activated PKC, which phosphorylates several intracellular proteins. A 46 kDa protein involved in this reaction has recently been identified [7, 8]. The production of $O_2^{\cdot -}$ in neutrophils occurs via two one-electron transfers to molecular oxygen in the NADPH-oxidase system [9, 10]. Reduction of cytochrome (III) C, determined as the increase in absorbance at 550 nm, where reduced cytochrome C has a maximum, is a common *in vitro* method for detection of $O_2^{\cdot -}$ [11, 12]. In the present study, $O_2^{\cdot -}$ production has been monitored as the maximal rate of cytochrome (III) C reduction.

We have adapted this method for microtiter plate reading and continuous recording of the rate of $O_2^{\cdot -}$ production, using immobilised neutrophils. On the basis that stimulation of PKC is the major inducer of $O_2^{\cdot -}$ production, the phorbol ester PMA, which is a fast activator of PKC, was used to trigger the production of $O_2^{\cdot -}$ [13]. $O_2^{\cdot -}$ production was defined as the cytochrome (III) C reduction which could be inhibited by SOD [13]. It was therefore expected that staurosporine, a well-known PKC inhibitor [14] and DIPHI, a NADPH-oxidase inhibitor [15], inhibit $O_2^{\cdot -}$ production. Vitamin E may inhibit PKC activity in addition to its

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† Abbreviations: PKC, protein kinase C; $O_2^{\cdot -}$, superoxide anion; H_2O_2, hydrogen peroxide; DMSO, dimethyl sulfoxide; NDGA, nordihydroguairetic acid; DIPHI, diphenylene iodonium; BHT, butylated hydroxy toluene; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate buffer saline; EtOH, ethanol.

antioxidative effect [16]. Thus, it was of interest to investigate the effect of α-tocopherol and some other chain-breaking antioxidants, including probucol, BHT and NDGA.

MATERIALS AND METHODS

Chemicals and materials. Superoxide dismutase (CuZnSOD lot No. 11561220-23, 6100 U/mg) from bovine erythrocytes was purchased from Boehringer Mannheim GmbH (Mannheim, Germany), staurosporine from Fluka Biochemica (Buchs, Switzerland), NaCl and DMSO from Merck (Darmstadt, Germany), Percoll® from Pharmacia LKB Biotechnology (Uppsala, Sweden) and PMA, horse heart cytochrome (III) C, tryphan blue, α-D(+)-glucose, BHT, NDGA and α-tocopherol were from Sigma Chemicals Co. (St Louis, MO, U.S.A.). Probucol and DIPHI were a kind gift from Dr T. Carew, Dept of Medicine, UCSD (San Diego, CA, U.S.A.) and Dr A. Cross, Scripps Clinic (La Jolla, CA, U.S.A.), respectively. Dulbecco's PBS with Mg^{2+} and Ca^{2+} was purchased from Biological Industries (Kibbutz Beth Haemek, Israel) and EtOH, 99.5%, was from Kemetyl (Stockholm, Sweden). All water-soluble reagents were dissolved in water which was deionized and filtered in a Milli-RO®, Millipore Corporation (Bedford, MA, U.S.A.) and further purified on an Elgastat UHP system, Elga Ltd (High Wycombe, U.K.). ICM-Linbro tissue culture multiwell plates for cell culture (cat No. 76-008-04) were from Flow Laboratories (Rickmansworth, U.K.). Vacutainer tubes containing ethylenediamine tetraacetic acid (15%) were from Becton Dickinson (Meyland, France).

Preparation of human neutrophils. Human blood from 1 to 3 healthy donors was collected in Vacutainer tubes. Neutrophils were prepared at room temperature and used within 3 hr and prior to the experiment the cells were slowly shaken to avoid aggregation and sedimentation. Following centrifugation, 1000 *g* for 10 min, the platelet-rich plasma was removed and the buffy coat, including leukocytes, was collected and diluted in 0.5% NaCl. Remaining red cells were lysed by addition of 30 mL distilled water to a 10 mL cell suspension for 1 min, and the lysate was brought back to physiological salt concentration by addition of 10 mL 3.6% NaCl, and then centrifuged at 250 *g* for 10 min. The pellet was dissolved in 1–2 mL 0.9% NaCl and put on top of a Percoll® gradient solution designed for separation of neutrophils. The densities of the Percoll® solutions were 1.0063 g/cm^3 and 1.0075 g/cm^3. 3 mL of each density solution was used to purify neutrophils from approximately 30 mL blood concentrated to 1–2 mL. Following centrifugation at 850 *g* for 10 min, the intermediate neutrophil band was collected and subjected to a second lysis, as described above, and centrifuged at 250 *g* for 10 min. The pellet was resuspended in Dulbecco PBS buffer with 5.6 mM glucose. Cell viability was checked by counting in a Bürker chamber, using 0.25% (w/v) tryphan blue diluted 1:1 with cell suspension.

Experimental conditions and analysis. Neutrophils were immobilised to the 96-well microtiter plate by incubation of 250 μL of 1.2×10^6 cells/mL per culture well for 1 hr under oxygen with 5% CO_2, 37°, pH 7.4 in the presence of 120 μM cytochrome (III) C (Fig. 1). After incubation the compounds to be tested were added in 25 μL volumes, and the experiment was started by addition of 25 μL PMA. In the final assay, the volume of the reaction mixture was 300 μL with 0.4 μg/mL PMA, 100 μM cytochrome (III) C and 1×10^6 cells/mL. The production of $O_2^{\cdot -}$ was measured continuously at 37° for 2 hr, as the increase in absorbance at 550 nm on a V_{max} kinetic microplate reader from Molecular Devices (Menlo Park, CA, U.S.A.). The experimental calculations were expressed as the mean of eight determinations. Every microtiter plate contained reference determinations with only PBS, only cytochrome (III) C and both cells and cytochrome (III) C, to which no PMA was added. Compounds which showed a positive effect in the test system were tested with cytochrome (III) C in the absence of cells. All additions were made with an 8-channel pipette except for the addition of PMA where the repetitive Eppendorf Multipette® pipette was used. This was especially tested and found to be the best way to induce a fast PMA response.

Preparation of substances. All compounds except cytochrome (III) C and DIPHI were dissolved in EtOH and further diluted in PBS. The scheme for dilution of α-tocopherol and probucol was 120 mM (99.5% EtOH), 1.2 mM (50% EtOH) and 0.12 mM (10% EtOH). BHT and NDGA were dissolved to 120 mM (99.5% EtOH) and further diluted to 1.2 mM in PBS. Staurosporine 2 mM (99.5% EtOH) was sonicated and diluted to 0.2 mM (99.5% EtOH) and finally to 2 μM in PBS. DIPHI, 1 mM was kept in dry DMSO and further diluted to 120 μM in PBS. Cytochrome (III) C and SOD were dissolved in PBS. PMA (stock solution in EtOH, 1 mg/mL) was diluted to 4.8 μg/mL in PBS giving a final concentration of 0.4 μg/mL. The concentration of each compound used in the assay was as indicated in the figures. Dissolving the compounds as above gives rise to maximal final EtOH and DMSO concentrations of 0.8% (v/v) and 1% (v/v), respectively.

Calculations and statistics. The absorbance, A, was determined at 550 nm, every 22 sec for 2 hr, and the absorbance values from eight wells were averaged. The rate, dA/dt, was calculated from the averaged absorbance readings over time. Through computerised linear regression, a straight line was fitted to each averaged absorbance value and 10 values preceding and 10 values following that value. The slope of the fitted line was used as the rate for that absorbance value. This analysis was repeated for all averaged absorbance values except for the first and last 10 values. All rates were measured at the time of the maximal rate in the experiment with the PMA-activated neutrophils, with no drug added. The determined rates in the presence of various compounds were normalised relative to the rate for PMA versus the rate obtained in the presence of 20 μg/mL SOD. All values were compared within the same microtiter plate. The IC_{50} values were calculated by fitting the equation $(dA/dt)/(dA/dt)_{max} = 1/(1 + [drug]/IC_{50})^n$, with no fixed slope ($n$), to the averaged experiments, and the correlation

Fig. 1. Neutrophils immobilised on the microtiter plate used for activation of superoxide anion production. The cell size was on average 3.2 μm (marker represents 18 μm).

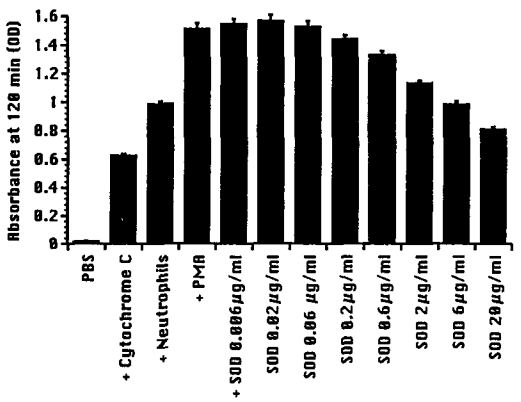

Fig. 2. Absorbance values in the presence of PMA-activated neutrophils and cytochrome (III) C and following addition of SOD. Mean values of eight determinations from one representative experiment.

coefficient, r^2, was determined. Values shown represent means ± SD or ±SEM (indicated).

RESULTS

One hour's preincubation of neutrophils dissolved in PBS buffer at 37° and pH 7.4 showed a confluent and homogeneous layer of cells on the microtiter culture plate (Fig. 1). The viability of the immobilised cells on the culture plate was >95% for at least 3 hr.

The absorbance for the control determinations (see Materials and Methods) was not changed during the length of the experiment (data not shown). The average maximal rate of $O_2^{\cdot -}$ production at the time 7 ± 0.9 min (SEM), $n = 20$, was 15 ± 3.8 O.D./min (SEM), $n = 20$, estimated from different cell preparations in the presence of 0.4 μg/mL PMA. Absorbance values after 120 min determined for a typical experiment including controls with PBS, cytochrome (III) C and neutrophils are shown in Fig. 2. Addition of neutrophils to the microtiter plate showed an absorbance value of 0.2 O.D. Further addition of cytochrome (III) C to the cells increased the absorbance to 1.0 (Fig. 2). This effect was not changed following addition of 20 μg/mL SOD (data not shown). In the presence of PMA, the absorbance values increased from 1.0 O.D. at time zero to approximately 1.5 O.D. after 2 hr (Fig. 2). These values are well within the microtiter reading range. The time course for the absorbance and dA/dt values following two hour's activation with PMA, and in the presence of different concentrations of SOD, are shown in Fig. 3A and B. SOD potently scavenged extra cellular $O_2^{\cdot -}$ with a half-maximal effect of 0.6 μg/mL, $n = 4$ (Fig. 4). The value for the half-maximal effect of SOD was calculated by regression analysis (see Calculations and Statistics). A SOD concentration of 20 μg/mL was therefore used to inhibit $O_2^{\cdot -}$ production to more than 95% (Fig. 4).

High concentrations of added SOD to the PMA-activated cells decreased the absorbance at 500 nm to 0.7 O.D., which is below the absorbance of 0.9 O.D. found initially (Fig. 3A). However, when

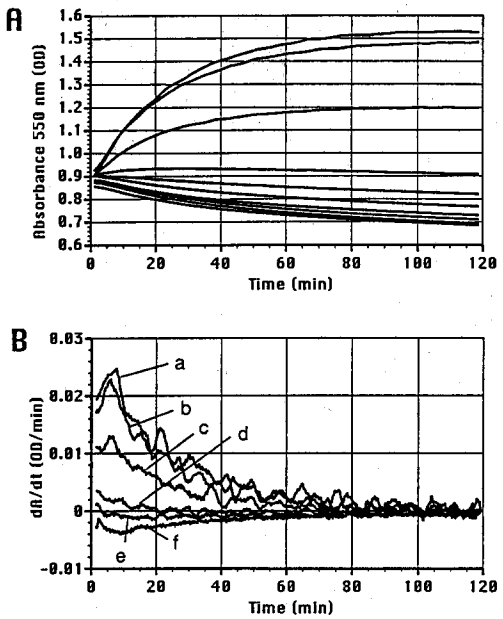

Fig. 3. Effect of SOD on $O_2^{\cdot -}$ production. (A). Representative experiment with 10 different concentrations of SOD expressed in μg/mL; 0, 0.05, 0.5, 2.5, 5.0, 10, 20, 40, 80 and 160 from top to bottom, respectively. (B) Rate of $O_2^{\cdot -}$ production of six traces from the experiment shown in panel A 0.0 (a), 0.05 (b), 0.5 (c), 2.5 (d), 5.0 (e) and 160 (f) μg/mL.

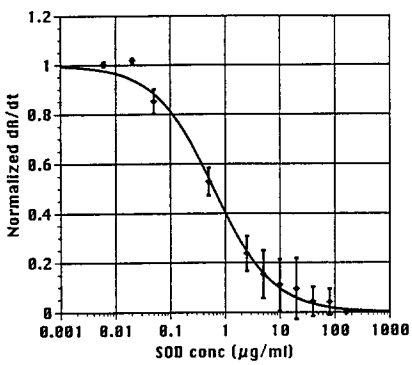

Fig. 4. Effect of SOD on the maximal rate of $O_2^{\cdot -}$ production (dA/dt). Values normalised against rate without SOD minus rate with 20.0 μg/mL SOD. The best fit to the described equation (see Calculations and Statistics) showed an IC_{50} of 0.6 μg/mL and a slope of 0.8. Estimates were from four experiments, where each value represents the mean of eight determinations.

SOD was added together with cytochrome (III) C in the absence of cells, no such effect was found. When H_2O_2 was added, the absorbance decreased even more, an effect also found in the absence of cells. The spectrum of cytochrome C at 550 nm showed that a small amount of its reduced form was present (data not shown). In the presence of high

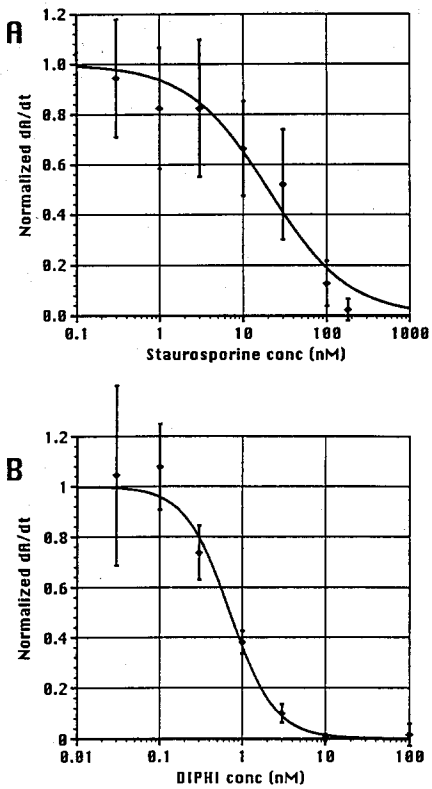

Fig. 5. Effect of staurosporine (A) and DIPHI (B) on the maximal rate of $O_2^{\cdot -}$ production. Values normalised against rate with no drug minus rate with 20.0 μg/mL SOD. The best fit to the described equation (see Calculations and Statistics) showed an IC_{50} of 20 nM and n of 0.90 (r^2 = 0.95) for staurosporine and an IC_{50} of 220 nM and n of 1.6 (r^2 = 0.99) for DIPHI. Estimates were from four experiments, where each value represents the mean of eight determinations.

concentrations of staurosporine and DIPHI, there was no decrease in absorbance (data now shown).

As mentioned in Calculations and Statistics, the effect of added compounds was evaluated as changes in the maximal rate of $O_2^{\cdot -}$ production, which occurred approximately within 10 min after addition of PMA. The PMA-induced $O_2^{\cdot -}$ production was potently inhibited by both staurosporine and DIPHI, with IC_{50}-values of 20 and 220 nM, n = 3, respectively (Fig. 5A and B).

The chain-breaking antioxidants, including BHT (10–100 μM), probucol (10 μM), α-tocopherol (10 μM) and NDGA (100 μM) are all well-known inhibitors of lipid peroxidation, but showed no effect on $O_2^{\cdot -}$ production (data not shown). There was also no direct effect of the tested compounds on cytochrome (III) C. Addition of 100 μM ascorbate increased the absorbance of cytochrome (III) C, both in the absence and the presence of neutrophils and PMA (data not shown).

DISCUSSION

Most of the increased oxygen uptake in neutrophils

is used to form $O_2^{\cdot -}$, which is the major intermediate in the formation of H_2O_2 [10, 11]. The tumour promoter PMA has been described as a strong stimulus for neutrophils, since it activates degranulation, aggregation and superoxide anion formation [12].

The $O_2^{\cdot -}$ produced was efficiently scavenged with 20 μg/mL SOD, which corresponded to inhibition of >95% of the PMA-induced maximal production of $O_2^{\cdot -}$ (Fig. 4). This concentration has been used to define the maximal $O_2^{\cdot -}$ production. SOD had no effect in the absence of PMA. The fast reduction of 100 μM cytochrome (III) C by 100 μM ascorbic acid is caused by differences in the redox potential, 260 mV compared with 58 mV, for the two compounds [17]. Cytochrome (II) C is also rapidly oxidised by H_2O_2 (E_o = 1770 mV) [18] and, since SOD had no direct effect on cytochrome (III) C, it is likely that the detected negative dA/dt-value is caused by the oxidation of H_2O_2. That assumption was supported by the presence of a small amount of cytochrome (II) C, which could be further oxidised. An oxidative effect of H_2O_2 has also been suggested earlier [11]. Neither staurosporine nor DIPHI could reduce or oxidise cytochrome (III) C. The return of the $O_2^{\cdot -}$ production rate to zero after approximately 100 min following addition of PMA (Fig. 3), cannot be due to limitations in the maximal absorbance of the microtiter reader, but to a depletion of PMA or to a complete reduction of cytochrome (III) C.

Using determination of $O_2^{\cdot -}$ production in this system it is not possible to establish on which levels the compounds act. However, using other methods to determine $O_2^{\cdot -}$ production in the absence of cells like the xanthine–xanthine-oxidase method [19] it is possible to perform complementary studies. As expected, both staurosporine and DIPHI inhibited the rate of $O_2^{\cdot -}$ production. The determined IC_{50}-values corresponded well with the reported effects as specific inhibitors of PKC [14] and of NADPH-oxidase [15]. Therefore, one may suspect that staurosporine and DIPHI in this system induced their action on PKC and NADPH-oxidase. Even though ordinary chain-breaking antioxidants may, theoretically, also interact with the production of $O_2^{\cdot -}$ by donation of hydrogen, thereby terminating the radical reaction, they are mainly effective as terminators of lipid radicals [20]. Therefore, it was of interest to examine whether these antioxidants inhibit $O_2^{\cdot -}$ production by other mechanisms, such as interaction with PKC or NADPH-oxidase. Such effects have been suggested for ebselen [21]. However, the antioxidants tested, including α-tocopherol, BHT, NDGA and probucol, showed no detectable effect in our experimental model. Recently, vitamin E has been suggested as a PKC inhibitor [16]. In these studies, detectable effects were found in concentrations around 100 μM, which is 5 to 10 times more than the normal plasma concentration of vitamin E, and also 10 times more than used in the present study. However, the effective concentration of vitamin E in cells and tissues can, due to its lipophilicity, be much higher [22]. Since 100 μM α-tocopherol was difficult to dissolve, further studies using the salt of vitamin E are currently being performed.

The experiments performed in this system using neutrophils immobilised to microtiter plates are reproducible and were acceptable regarding both intra- and inter-batch variations. All situations where a positive result was found were backed up with controls, showing that the compound did not react directly with cytochrome (III) C.

The continuous calculation of dA/dt-values, as described in Calculations and Statistics, has advantages over other methods using the same detector molecule, since this method seems to be more sensitive (compare Figs 2 and 4). It may therefore be concluded that this method is more relevant since changes in $O_2^{\cdot -}$ production can be detected more easily. There are also major advantages with the adaptation of the method to microtiter plates. The cells are immobilised on the microtiter cell culture plate in a monolayer on a defined surface, allowing each interacting substance to be exposed to each individual cell and not to aggregates of cells, which may be the case in a solution. Moreover, the microtiter plate procedure appears to be specially suited to handle multiple samples, since it only requires small amounts of neutrophils and reagents. Another important aspect is that it can be automated and the result evaluation can be efficiently computerised.

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