Biochimica et Biophysica Acta 899 (1987) 76–82
Elsevier

BBA 73536

Free radical-mediated membrane depolarization in renal and cardiac cells

James A. Scott [a], Alan J. Fischman [a], Ban-An Khaw [a,b], Charles J. Homcy [b] and Carlos A. Rabito [a]

[a] *Department of Radiology and* [b] *Cardiac Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA (U.S.A.)*

(Received 29 September 1986)

Key words: Membrane potential; Membrane depolarization; Free radical; Superoxide; Hydrogen peroxide; (Rat heart, LLC-PK1 renal cell)

Cell membrane potential was measured with a flow cytometer by quantitating the intracellular accumulation of a fluorescent cationic carbocyanine dye. We used this system to demonstrate depolarization upon the addition of hydrogen peroxide (10–1000 μM) and ferrous chloride (25–100 μM) to cultures of either neonatal rat myocardial or LLC-PK1 renal epithelial cells. Ferrous chloride-induced depolarization was prevented by superoxide dismutase, catalase and dimethyl sulfoxide, suggesting roles for the superoxide anion, hydrogen peroxide and the hydroxyl radical in effecting this depolarization, possibly through a Fenton-type reaction mechanism. Supplementation of either cell type with 2 μM tocopherol acid succinate during growth in tissue culture, prior to exposure to the oxidizing agent, decreased the magnitude of the depolarization in both cell types. The results are consistent with a role for tocopherols in scavenging free radical species responsible for the depolarization of the cell membrane.

Introduction

Oxygen radicals can cause significant cell membrane damage [1–3], resulting in increased membrane permeability and ultimately cell death. The sensitivity of various probes of membrane permeability in detecting membrane injury may be limited, however, because of their relatively high molecular weight and large Stokes radius. For this reason, such probes may detect injury to the plasma membrane at a relatively late stage in its evolution. The membrane potential results from the permeabilities and electrochemical gradients of small ionic species across the plasma membrane, and may provide a more sensitive indicator of cellular injury than probes used to quantitate membrane permeability. Membrane potential can be monitored directly in the intact cell through the use of various cyanine dyes [4]. We [5] and others [6] have previously described this method and used it to examine the role of free radicals in effecting membrane depolarization in cultured cells [7]. In this study we examine the effect of ferrous ions and hydrogen peroxide in producing depolarization of the plasma membrane and the efficacy of various scavenging enzymes in limiting this depolarization.

Abbreviations: DCFDA, 2′,7′-dichlorofluorescein diacetate; DiIC1(3), 1,1′,3,3,3′,3′-hexamethylindocarbocyanine iodide.

Correspondence: J.A. Scott, Division of Nuclear Medicine, Department of Radiology, Massachusetts General Hospital, Boston, MA 02114, U.S.A.

Methods

Tissue culture

LLC-PK1 renal epithelial cells (cell line obtained from the American Type Culture Collection) were harvested between passages 230 and 240 and grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum in a humid environment containing 5% CO_2 at 37°C. Cells were harvested by initial exposure to calcium-free DMEM with 10 mM citrate for 30 min after which the cells were washed and exposed to a 6 U/ml solution of papain in calcium-free DMEM with citrate for 15 min until the cells detached from the culture plate. The suspension of cells was immediately centrifuged, the pellet isolated and re-suspended in Earles balanced salt solution (EBSS) containing 4.5 g/l glucose at pH 7.2. The harvested cells were used in experiments immediately after isolation.

Neonatal rat myocardial cells were cultured according to methods previously described [7]. In brief, the hearts were isolated from 1 day neonatal rats, dissected free of great vessels, divided in half perpendicularly to the long axis of the ventricles and trypsinized in a 0.05% trypsin-EDTA solution. At ten minute intervals, aliquots of trypsin were neutralized with Nu serum (Collaborative Research, Lexington, MA) and centrifuged at 1 000 rpm, for 10 min to obtain a cell pellet, which was then resuspended in culture medium and incubated for seven days prior to experiment. Culture medium was Dulbecco's Minimal Essential Medium (DMEM) containing antibiotics (penicillin, streptomycin, gentamycin), 0.0025 mM thymidine and 20% Nu serum. Cells were harvested by exposure to a 0.05% trypsin-EDTA solution until detachment from the culture plate, at which time the trypsin was neutralized with Nu serum and the cell pellet isolated by centrifugation at 1 000 rpm for 5 min. The isolated cell pellet was re-suspended in EBSS containing 4.5 g/l glucose.

Cells were supplemented with d-alpha-tocopherol acid succinate (Sigma) added to the culture medium to obtain a final concentration of 2 μM. The cells were grown in the presence of tocopherol for five days prior to use in experiments.

Exposure of cells to oxidizing agents

Ferrous chloride (Baker) and hydrogen peroxide 30% (Sigma) were added to aliquots of control and tocopherol-supplemented cells to achieve the stated final concentration. Membrane potential measurements were performed before addition, and at various times up to two hours after addition of the oxidizing agent. Control groups, to which the oxidizing species were not added, were included in all experiments.

Flow cytometry

Flow cytometric analysis was performed with a FACS-III fluorescent-activated cell sorter (Becton-Dickenson) using a 488 nm laser operated at 300 mW. Scatter size was set to bracket the LLC-PK1 or myocyte cell size distribution while excluding both debris and cellular clumps from measurement. A bandpass filter was placed in front of the photomultiplier tube to insure that only fluorescent light was recorded. For DCFDA measurements, photomultiplier voltages were set to record the cellular fluorescence in the lower of two channels at time 0. Fluorescent cells appeared in a second (higher fluorescence) channel as the cells became more fluorescent with time, due to intracellular 2′,7′-dichlorofluorescein diacetate (DCFDA) oxidation [5]. Five to ten thousand cells were counted per sample. For membrane potential measurement, fluorescence channels were set centered at the mean fluorescence of the control cell distribution at time 0, with one channel including cells with less than mean fluorescence and the other channel including those cells with greater than mean fluorescence. Dye uptake was quantitated as the ratio of the number of cells in the upper (high fluorescence) channel to cells in the lower (low fluorescence) channel. Membrane potential recordings were begun at least ten minutes after addition of the fluorescent probe to the cell suspension, at a time when a constant level of dye accumulation had been observed in the control cells, indicating equilibrium.

Relationship between ferrous chloride and intracellular oxidative events

DCFDA (Molecular Probes, Junction City, OR) is a non-fluorescent substance that is taken up, de-acetylated and retained by living cells. The dye remains non-fluorescent within the cell until it reacts with an intracellular active oxygen species

[8], at which time the dye becomes fluorescent. Quantitation of this intracellular fluorescence by flow cytometry [7] thus provides a means of measuring intracellular oxidation events in viable cells. LLC-PK1 cells were incubated in 5 μM DCFDA in EBSS in the presence of ferrous chloride at concentrations ranging from 25 to 100 μM. Cellular fluorescence measurements were performed at 10-min intervals over a period of two hours.

Measurement of membrane potential

The cationic, fluorescent carbocyanine dye 1,1′,3,3,3′,3′-hexamethylindocarbocyanine iodide (DiIC1(3)) (Molecular Probes, Junction City, OR) is a molecule which assumes a potential-dependent distribution between the extracellular environment and the cell interior, permitting quantitation of the plasma membrane potential [4]. This dye may be used in conjunction with flow cytometric technique to establish the plasma membrane potential in single cells [5,6] whose small size may limit the feasibility of microelectrode methods. Since the dye distributes between the cell and its environment in a passive equilibrium governed by the membrane potential, uptake by individual cells may be quantitated with time to document temporal changes in the plasma membrane potential.

Cells were incubated with 1 μM DiIC1(3) in EBSS containing 4.5 g/l glucose for ten minutes or until a constant cellular fluorescence had been achieved, as described above. The suspension of cells was then exposed to the appropriate oxidant and fluorescence was monitored with time.

Standard curves were constructed for both the LLC-PK1 and neonatal rat heart cells to establish a relationship between relative dye content of the cells and the membrane potential. In brief, cells in the presence of 5 μM valinomycin were exposed to varying external potassium concentrations, at each of which the membrane potential can be calculated using the Nernst equation. In this manner, standard curves are constructed which relate the expression of a given cellular content of the fluorescent probe to the calculated membrane potential.

Catalase assay

Catalase activity was determined using the spectrophotometric method of Cohen et al. [9] by measuring H_2O_2 by titration with permanganate. LLC-PK1 cells or cardiac myocytes were isolated and the cell suspension was homogenized with a cell disruptor (Ultrasonic model 300) set at maximal output for 3 s. The residual $KMnO_4$ was measured using a Gilford spectrophotometer model 240. The results are expressed in terms of the first-order reaction rate constant for samples of identical protein content. Protein concentration of the samples was determined with a modified Lowry assay as described by Wang and Smith [10].

Statistical analysis

Statistical analysis was performed using an initial analysis of variance. In those cases where the *F*-test was significant, the analysis of variance was followed by the unpaired two-tailed *t*-test with Bonferroni's correction for multiple comparisons.

Results

$FeCl_2$-mediated intracellular oxidation of dichlorofluorescein

In order to establish a correlation between extracellular ferrous chloride and intracellular oxidative events, we examined the oxidation of DCFDA in the presence of varying concentrations of ferrous chloride in LLC-PK1 cells. Ferrous chloride at concentrations ranging from 25 to 100 μM increased the intracellular oxidation of 2,7-dichlorofluorescein diacetate, consistent with the increased formation of intracellular oxidizing species. Dichlorofluorescein oxidation was $174 \pm 39\%$ of control at 25 μM ($P < 0.05$) and $202 \pm 47\%$ of control at 100 μM $FeCl_2$ ($P < 0.02$).

Both superoxide dismutase and catalase (0.2 mg/ml) were added to suspensions of LLC-PK1 cells exposed to 100 μM $FeCl_2$ to assay for the involvement of the superoxide anion and hydrogen peroxide in $FeCl_2$-mediated DCFDA oxidation. Superoxide dismutase reduced $FeCl_2$-mediated DCFDA oxidation from $202 \pm 7\%$ to $75 \pm 13\%$ control levels of oxidation ($P < 0.005$) while heat-denatured superoxide dismutase had no significant effect. Similarly, catalase reduced DCFDA oxidation from $202 \pm 7\%$ to $139 \pm 5\%$ control (*P*

< 0.05) while heat denatured catalase did not significantly reduce $FeCl_2$-mediated oxidation.

Effect of ferrous chloride and hydrogen peroxide on the membrane potential of LLC-PK1 and rat myocardial cells

Fig. 1 shows the effect of varying concentrations of ferrous chloride and hydrogen peroxide on the membrane potentials of LLC-PK1 cells during a 90 min exposure. While 5 μM ferrous chloride produced a slight depolarization, a significant depolarization from 35.3 to 27.9 mV was observed at a 50 μM concentration ($P < 0.05$). Similarly, while both 1 and 10 μM hydrogen peroxide had a moderate depolarizing effect on the cells (69% control potential), a significant depolarization was not seen until 100 μM (51% control potential, $P < 0.05$). A further depolarization was obtained at 1 mM hydrogen peroxide (33% control potential, $P < 0.01$).

Fig. 2 shows increasing depolarization observed with increasing time of exposure to hydrogen peroxide. The rate of depolarization, as shown by the steepness of the curve, is greater at the higher concentration of hydrogen peroxide (1 mM) than at the lower 100 μM concentration ($P < 0.05$).

Similar results were obtained with rat myocardial cells. As shown in Table I, a 30 min exposure to hydrogen peroxide depolarized the cells from a control potential of 80.0 mV to 70.3 mV at 100 μM H_2O_2 ($P < 0.01$) and to 36.1 mV at 1 mM H_2O_2 ($P < 0.001$). Again, depolarization increased with time of exposure to hydrogen peroxide, with further depolarization to 54.7 mV ($P < 0.001$) and 30.2 mV ($P < 0.001$) for the 100 μM and 1 mM concentrations of hydrogen peroxide after a 60 min exposure. Ferrous chloride depolarized cells from a control potential of 80.0 mV to 59.9 mV ($P < 0.01$) and to 53.5 mV ($P < 0.001$) after a 30 min exposure to concentrations of 50 μM and 100 μM, respectively. The myocardial

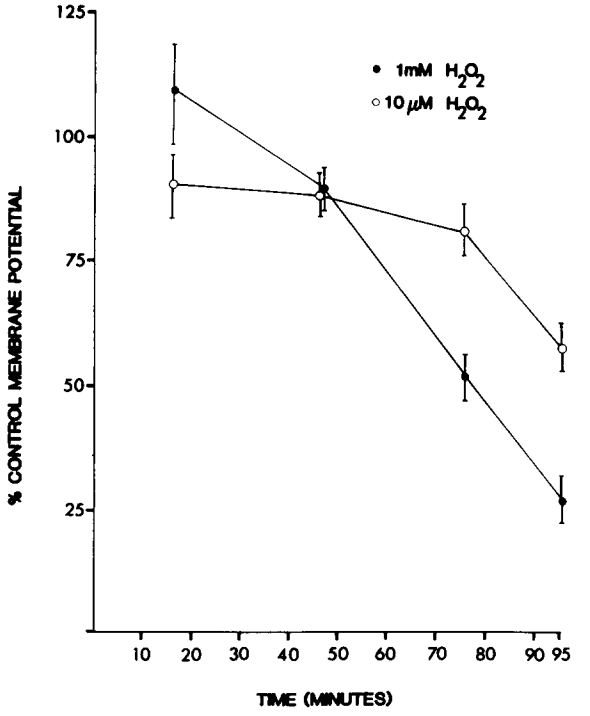

Fig. 1. The effect of a 90-min exposure of LLC-PK1 cells to 1 μM–1 mM hydrogen peroxide or to 5–50 μM ferrous chloride. Note that significant depolarization is observed at concentrations of 100 μM ($P < 0.05$) and 1 mM ($P < 0.01$) hydrogen peroxide and at 50 μM ferric chloride ($P < 0.05$).

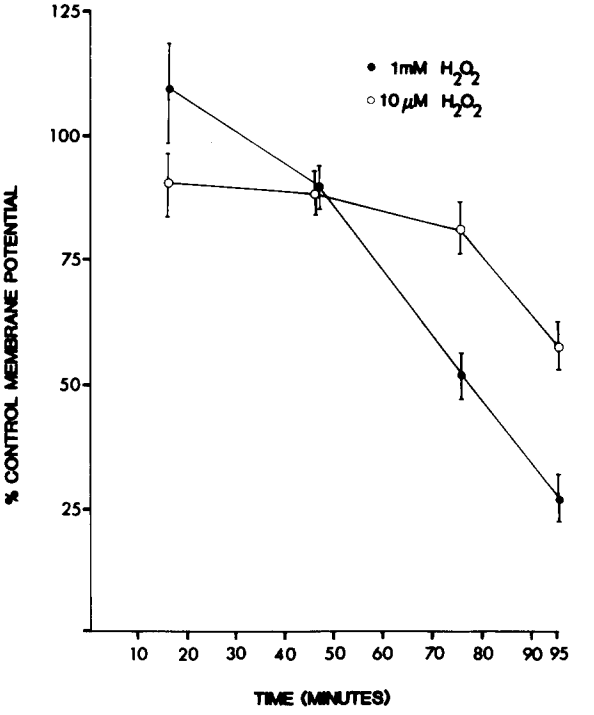

Fig. 2. The H_2O_2-induced depolarization of LLC-PK1 cells is shown with respect to time. Note the more rapid rate of depolarization observed in the presence of 1 mM hydrogen peroxide as compared with the 100 μM concentration.

TABLE I

THE EFFECT OF A 30-MINUTE EXPOSURE OF RAT MYOCARDIAL CELLS TO FERROUS CHLORIDE OR TO HYDROGEN PEROXIDE

Significant depolarization is observed at concentrations of 50 ($P < 0.01$) or 100 μM ($P < 0.001$) ferrous chloride and at 100 μM ($P < 0.01$)-1 mM ($P < 0.001$) hydrogen peroxide.

	Membrane potential (mV)
Control	80.0 ± 1.6
H_2O_2 100 μM	70.3 ± 2.3 *
H_2O_2 1 mM	36.1 ± 0.9 **
$FeCl_2$ 50 μM	59.9 ± 1.6 *
$FeCl_2$ 100 μM	53.3 ± 0.7 **

* $P < 0.01$ from control.
** $P < 0.001$ from control.

cells were, in general, less sensitive to the effects of hydrogen peroxide than were the LLC-PK1 cells (60-min exposures to 100 μM hydrogen peroxide caused a 29% reduction in the membrane potential of myocardial cells while causing a 72% reduction in LLC-PK1 cells). Both cell types exhibited similar depolarization responses to ferrous chloride.

We examined the effect of various oxygen radical scavengers on ferrous chloride-induced membrane depolarization in LLC-PK1 cells. After a 30 min incubation with 100 μM ferrous chloride, cells were depolarized to 54 ± 3% control potential ($P < 0.001$). Inclusion of 0.1 mg/ml superoxide dismutase increased the potential to 81 ± 3% control ($P < 0.001$ from control, $P < 0.02$ from heat denatured enzyme-treated cells). Inclusion of 0.1 mg/ml catalase increased the potential from 54 ± 3% to 83 ± 3% control in the presence of 100 μM $FeCl_2$ ($P < 0.001$) while heat-denatured catalase-treated cells did not differ from control. Dimethyl sulfoxide (1% v/v) similarly decreased the depolarization in cells exposed to $FeCl_2$ ($P < 0.01$).

Effect of tocopherols upon the depolarization response to $FeCl_2$ and H_2O_2

Table II shows the effect of incubating myocardial cells with tocopherol prior to exposure to 100 μM ferrous chloride or 1 mM hydrogen peroxide. While the membrane potential is depolarized in all groups, the magnitude of the depolarization is

TABLE II

THE EFFECT OF TOCOPHEROL SUPPLEMENTATION OF RAT MYOCARDIAL CELLS DURING EXPOSURE TO FERROUS CHLORIDE OR TO HYDROGEN PEROXIDE FOR 30 AND 60 MINUTES

Significant depolarization is observed after exposure to hydrogen peroxide ($P < 0.005$) and ferrous chloride ($P < 0.005$) in control cells. This depolarization is reduced by tocopherol supplementation after 30- and 60-min exposures in $FeCl_2$ ($P < 0.001$) and after 30-min exposures in H_2O_2-treated ($P < 0.05$) cells.

	Control (mV)		Tocopherol (mV)	
	30 min	60 min	30 min	60 min
Control	80.0 ± 1.7	64.1 ± 1.7	82.3 ± 1.4	70.5 ± 1.4
$FeCl_2$				
100 μM	66.1 ± 1.0	49.2 ± 1.4	79.7 ± 0.6 *	74.1 ± 2.0 *
H_2O_2				
1 mM	68.3 ± 1.3	60.2 ± 1.2	76.1 ± 0.9 **	63.4 ± 2.3

* $P < 0.001$ from similarly-treated control cells.
** $P < 0.05$ from similarly-treated control cells.

significantly less in the tocopherol-treated cells after these exposures. After a 30 min exposure to ferrous chloride, the potential of cells without tocopherol supplementation was 83% of control membrane potential while the potential of tocopherol-supplemented cells was 97% of the control potential ($P < 0.001$). The same effect was seen after a 60 min exposure. After a 30 min exposure to hydrogen peroxide, the potential of cells without tocopherol was 85% of control while tocopherol-supplemented cells were 92% control ($P < 0.05$).

A similar effect was seen with PK1 cells exposed for 90 min to 100 μM ferrous chloride, 100 μM hydrogen peroxide or 1 mM hydrogen peroxide. 100 μM ferrous chloride reduced membrane potential in untreated cells to 60 ± 3% of control and in tocopherol-treated cells to 79 ± 6% of control ($P < 0.05$). 100 μM hydrogen peroxide reduced membrane potential in untreated cells to 39 ± 4% and in tocopherol treated cells to 88 ± 4% control potential ($P < 0.001$). 1 mM hydrogen peroxide reduced membrane potential to 33 ± 2% control potential and in tocopherol-treated cells to 51 ± 3% control potential ($P < 0.01$).

Catalase activity, as measured by the rate constant derived from measuring H_2O_2 titrations with

permanganate, did not significantly differ between LLC-PK1 cells and the neonatal myocytes.

Discussion

We studied both intracellular oxidation and plasma membrane potential in response to exposure of LLC-PK1 cells to ferrous salts. Both increased intracellular oxidation, as measured by DCFDA fluorescence [8], and depolarization of the plasma membrane were observed upon exposure of the cells to 50 μM $FeCl_2$. It is likely that the ferrous salts catalyze superoxide and hydrogen peroxide formation through a series of autooxidative single electron reductions of molecular oxygen [2] with regeneration of the ferrous ion by either intra- or extracellular reductants. Intracellular chelation of the iron salt may facilitate this autooxidative process. Both ferrous salt-mediated increases in intracellular oxidation and plasma membrane depolarization were prevented by the inclusion of exogenous catalase and superoxide dismutase. The efficacy of extracellular superoxide dismutase and catalase in limiting intracellular dichlorofluorescein oxidation suggests a trans-membrane distribution of the radical species responsible for the intracellular oxidation of DCFDA. Permeability of the plasma membrane to the superoxide anion has been reported by several authors [12–14]. This permeability would permit scavenging of extracellular radicals to affect intracellular oxidation since the radicals would have access to both the intra- and extracellular compartments. The intercompartmental location of the plasma membrane would permit both intra- and extracellularly-located radicals to participate in effecting plasma membrane depolarization. Inhibition of ferrous ion-mediated membrane depolarization by superoxide dismutase, catalase and dimethyl sulfoxide supports the involvement of the superoxide anion, hydrogen peroxide and the hydroxyl radical, likely in a Fenton-type reaction [1]. In this reaction, the ferrous ion reduces hydrogen peroxide to the hydroxyl radical, which then initiates membrane injury by reaction with cellular constituents. The oxidized ferric ion is re-converted to its ferrous form by reduction effected by either the superoxide anion or other reducing agents within the cell, such as ascorbic acid [1].

Hydrogen peroxide depolarized both PK1 cells and cardiocytes, although the latter cell type appeared more resistant to depolarization. Since intracellular catalase content was comparable between the two cell types, it is possible that this difference in sensitivity to H_2O_2 may be related to differences in other hydroperoxide scavenging enzymes (e.g. glutathione peroxidase) or to differences in membrane lipids between the two cell types. No reproducible differences could be demonstrated between the depolarization responses of the two cell types to the ferrous ion.

The tocopherols are lipid-soluble antioxidants located within the cell membrane, providing the cell with its primary membrane-based defense against radical species, inactivating them before they can initiate potentially destructive peroxidation of membrane lipids [15,16]. It has been estimated that one membrane-based tocopherol molecule protects approx. 1000 polyunsaturated fatty acids from auto-oxidation [16]. Tocopherols have been shown to scavenge a variety of radical species including the hydroxyl and superoxide radical species [17]. In this scavenging process, the less reactive tocopherol radical is produced. Regeneration of the native tocopherol molecule from its radical form is catalyzed by ascorbic acid [18], a mechanism which may be important in vivo in view of the relatively low membrane concentrations of tocopherol in living cells. There is some evidence suggesting that isolated skeletal muscle mitochondria from vitamin E-deficient animals demonstrate an accelerated loss of membrane potential in comparison with non-deficient controls [19]. Also, Chinese Hampster Ovary cells supplemented with 10 μM tocopherol acid succinate for 72 h while in culture showed less oxidant-induced chromatid exchange than did control cells [20].

Tocopherol supplementation of growth media significantly limited the depolarization observed in PK1 cells in response to both ferrous ion and hydrogen peroxide. Supplementation of myocytes with tocopherol had a lesser, but significant, protective effect (Table II). These differences between the cell types may be related to differences in peroxidizable lipid content or intrinsic antioxidant concentrations in the two cell types.

We conclude that ferrous ion-generated oxygen

radicals and hydrogen peroxide cause early and sustained depolarizations of the plasma membrane in both LLC-PK1 renal epithelial cells and in rat neonatal myocytes. This depolarization is increased with increasing concentrations of oxidant and with increasing time of exposure to the oxidizing species. The depolarization response is limited by supplementation of the cells with exogenous antioxidants prior to exposure to the oxidizing species.

Acknowledgements

This work is supported in part by NIH Ischemia SCOR HL-56212, NIH Grant AM 27401 and by a grant from R.J.R. Nabisco. Dr. Scott is the recipient of an NIH New Investigator Research Award CA33570.

References

1 Halliwell, B. and Gutteridge, J.M.C. (1985) Mol. Aspects Med. 8, 89–193
2 Aust, S.D., Morehouse, L.A. and Thomas, C.E. (1985) J. Free Radicals Biol. Med. 1, 3–25
3 Grankvist, K. and Marklund, S.L. (1986) Int. J. Biochem. 18, 109–113
4 Shapiro, H.M. (1981) Cytometry 1, 301
5 Cantiello, H.F., Scott, J.A. and Rabito, C.A. (1987) Am. J. Physiol., in the press
6 Jenssen, H.-L., Redmann, K. and Mix, E. (1986) Cytometry 7, 339–346
7 Scott, J.A., Khaw, B.A., Homcy, C.J. and Rabito, C.A. (1987) Biochim. Biophys. Acta 897, 25–32
8 Bass, D.A., Parce, J.W., Dechatelet, L.R., Szejda, P., Seeds, M.C. and Thomas, M. (1983) J. Immunol. 130, 1910–1918
9 Cogen, G., Dembiec, D. and Marcus, J. (1970) Anal. Biochem. 34, 30
10 Wang, C.S. and Smith, P.L. (1975) Anal. Biochem. 63, 414–417
11 Mak, I.T. and Weglicki, W.B. (1985) J. Clin. Invest. 75, 58–63
12 Lynch, R.E. and Fridovich, I. (1978) J. Biol. Chem. 13, 4697–4699
13 Rumyantseva, G.V., Weiner, L.M., Molin, Y.N. and Budker, V.G. (1979) FEBS Lett. 108, 477–480
14 Gus'kova, R.A., Ivanov, I.I., Kol'tover, V.K., Akhobadze, V.V. and Rubin, A.B. (1984) Biochim. Biophys. Acta 778, 579–585
15 Fukuzawa, K., Takase, S. and Tsukatani, H. (1985) Arch. Biochem. Biophys. 240, 117–120
16 Ivanov, I.I. (1985) J. Free Radicals Biol. Med. 1, 247–253
17 Fukuzawa, K. and Gebicki, J.M. (1983) Arch. Biochem. Biophys. 226, 242–251
18 Scarpa, M., Rigo, A., Maiorino, M., Ursini, F. and Gregolin, C. (1984) Biochim. Biophys. Acta 801, 215–219
19 Quintanilha, A.T. and Davies, K.J.A. (1982) FEBS Lett. 139, 241
20 Weitberg, A.B., Weitzman, S.A., Clark, E.P. and Stossel, T.P. (1985) J. Clin. Invest. 75, 1835–1841