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Oxygen radicals alter the cell membrane potential in a renal cell line (LLC-PK1) with differentiated characteristics of proximal tubular cells

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We employed a carbocyanine dye (1,1′,3,3,3′,3′-hexamethylindocarbocyanine iodide) to measure the plasma membrane potential of LLC-PK1 renal epithelial cells exposed to either xanthine oxidase-generated oxygen radicals or to hydrogen peroxide. Measurements were performed using a fluorescent-activated cell sorter to record fluorescence on a cell by cell basis. Initial exposure of cells to low concentrations of either H_2O_2 or xanthine oxidase resulted in a transient increase in membrane potential relative to control cells ($P < 0.001$), followed by an exponential decline in potential ($P < 0.001$). The addition of extracellular catalase diminished the H_2O_2-related decline in potential, consistent with a role for hydrogen peroxide in producing this effect. Pretreatment of cells with inhibitors of intracellular catalase and superoxide dismutase prior to exposure to xanthine oxidase caused an even larger decline in potential ($P < 0.001$). Cells could be partially protected from the radical-mediated loss of potential by incubating them in a hypertonic (400 mosmolal) environment during radical exposure. Similarly, the loss of membrane potential was increased after incubation of cells in a hypotonic (200 mosmolal) environment during radical exposure. These observations are consistent with a reduction in membrane potential effected by exposure to oxygen radicals (including superoxide anion and hydrogen peroxide). This reduction may be prevented, in part, by radical scavenging enzymes and by reducing the degree of cellular swelling in response to oxygen radical exposure.

Introduction

Oxygen radicals are thought to play a role in ischemia-reperfusion [1] and possibly minimal change disease [2] in the kidney, damage observed most commonly in the proximal tubular and glomerular cells. Most work, however, has examined parameters altered only during the later stages of such injury [3]. In this study we examined alterations in cell membrane potential, which may represent an early phase in the evolution of this cellular injury and may precede the formation of frank membrane discontinuities associated with intracellular enzyme leakage. Since the LLC-PK1 cell exhibits multiple differentiated characteristics of the straight segment of the proximal tubule [4], it provides a convenient tissue culture model for the study of this type of injury in vitro.

Various cationic fluorescent probes have been demonstrated to partition between the cell interior

Abbreviation: DiIC1(3), 1,1′,3,3,3′,3′-hexamethylindocarbocyanine iodide.

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and the extracellular medium in a fashion permitting the recording of membrane potentials by quantitation of the level of cellular fluorescence. These dyes have included the cyanine dyes [5–7] for the measurement of membrane potential and rhodamine dyes for the measurement of mitochondrial potential [8–10]. Several authors have adopted these methods for use in the flow cytometer [5,8,9]. In this paper we report on the use of the carbocyanine dye 1,1′,3,3,3′,3′-hexamethylindocarbocyanine iodide (DiIC1(3)) to measure the membrane potential in renal epithelial cells exposed to various concentrations of oxygen radical species. We also examine methods of modifying this response.

Methods

Cell culture

LLC-PK1 renal epithelial cells were harvested between passages 230 and 240 and grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum in a humid environment containing 5% CO_2 at 37°C. Cells were harvested by brief exposure of confluent monolayers to 0.25% trypsin EDTA solution followed by neutralization of the enzyme with fetal calf serum following cellular detachment from substratum. The suspension of cells was centrifuged, the pellet isolated and resuspended in phosphate-buffered saline at pH 7.2. The harvested cells were used in experiments immediately after isolation.

Reagents and incubation techniques

Xanthine oxidase (grade IV), hypoxanthine, catalase, hydrogen peroxide, aminotriazole, cytochrome *c* and diethyldithiocarbamate were all obtained from Sigma. DiIC1(3) was obtained from Molecular Probes (Junction City, OR).

Experiments were performed by incubating suspensions of cells (10^4/ml) in phosphate-buffered saline (PBS) at either 20 or 37°C in the presence of hydrogen peroxide (0–100 mM) or xanthine oxidase (0.006–0.08 U/ml). Here, one unit of xanthine oxidase activity is defined as that activity which will convert one micromole of xanthine to uric acid per minute at pH 7.5 at 25°C. In some experiments, preincubation of cells with either 20 mM aminotriazole or 3 mM diethyldithiocarbamate was performed for 40 min at 37°C, after which the cells were isolated by centrifugation and resuspended in the appropriate phosphate-buffered saline solution prior to experimentation. Experiments involving exposure of the cells to xanthine oxidase-generated radicals utilized 0.1 mM hypoxanthine with or without 3 mM glucose in phosphate-buffered saline. In experiments involving incubation under conditions of altered osmolality, phosphate-buffered saline was rendered hypoosmolar (200 mM) by the addition of stoichiometric quantities of distilled water and hyperosmolar (400 mM) by the addition of stoichiometric quantities of sucrose. At the end of the incubation period, the cell suspension was isolated by centrifugation and resuspended in phosphate-buffered saline containing 0.5 μg/ml DiIC1(3) obtained by dilution of a stock solution of 2 mM DiIC1(3) in DMSO stored at 0°C. 15 min were allowed for the dye to equilibrate with the cells in suspension at room temperature prior to measurement. The cells were then analyzed by flow cytometric techniques.

Flow cytometry

Flow cytometric analysis was accomplished on 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 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. Photomultiplier voltages were set at the beginning of the experiment to exclude autofluorescence (unstained cells) from the recorded fluorescent distribution. The pulse height analyzer was then set to record approximately half of the control cell fluorescence distribution in each of two channels, one representing high and the other low fluorescence events. 5000–10000 cells were counted per sample and experiments were performed in triplicate or quadruplicate. Appropriate control samples were run for all agents tested to exclude any effects of catalase, aminotriazole or diethyldithiocarbamate unrelated to oxygen radical scavenging. Dye uptake was quantitated as the percentage of total cells counted with fluorescence registering in the high fluorescence (high mem-

brane potential) channel. Scatter measurements were also performed to determine the relative size of cellular events recorded, again using two channels corresponding to cells of larger than mean scatter size and to those of less than mean scatter size. Relative scatter size was then quantitated as the percentage of total cells counted observed in the upper scatter size channel.

Enzyme and protein assays

Superoxide dismutase activity was determined by cytochrome *c* reduction [11]. Briefly, the rate of reduction of cytochrome *c* by superoxide radicals was monitored at 550 nm utilizing the xanthine-xanthine oxidase system as the source of superoxide radicals. Superoxide dismutase competes for the superoxide anion, decreasing the rate of reduction of cytochrome *c*. The absorbance changes vs. time were determined in a Gilford spectrophotometer model 240. LLC-PK1 cells were incubated in 10 ml Earle's balanced salt solution with or without 3 mM diethyldithiocarbamate. After 40 min incubation at 37°C, the cells were isolated and the suspensions disrupted with a cell disruptor (Ultrasonic model 300) set at maximal output for 3 s. Inhibition of superoxide dismutase activity was expressed as a percentage increase in the slope of absorbance changes vs. time recorded in the first 3 min of reaction in samples with identical protein content.

Catalase activity was determined with the spectrophotometric method described by Cohen et al. [12] by measuring H_2O_2 by titration withn permanganate. LLC-PK1 cells were incubated in Earle's balanced salt solution with or without 20 mM aminotriazole. After 40 min incubation at 37°C, the cells were isolated and the suspensions were disrupted with a cell disrupter (Ultrasonic model 300) set at maximum output for 3 s. The residual $KMnO_4$ was measured by 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 in cell samples was determined with a modified Lowry assay as described by Wang and Smith [13].

Statistical analysis

Statistical analysis was performed using an initial analysis of variance where three or more groups were to be compared. In 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

Membrane potential changes in response to oxygen radicals

Cells were exposed to varying concentrations of hydrogen peroxide for 45 min at 37°C and compared with control cells incubated under identical conditions but without hydrogen peroxide in the incubation medium. The resulting membrane potential was 101 ± 5.0% of control potential (n.s.) at 50 μM hydrogen peroxide, 63.5 ± 2.6% of control at 500 μM ($P < 0.001$) and 66.0 ± 4.4% of control at 5 mM ($P < 0.001$). Depolarization increased with time, however, as exposure to 1 mM hydrogen peroxide resulted in cells with 94.3 ± 6.9% control potential after 15 min exposure, 82.5 ± 9.9% after 45 min exposure and 60.6 ± 9.9% control potential after 95 min exposure.

Table I shows the depolarization observed with time upon exposure of cells to 0.08 U/ml xanthine oxidase at 20°C. Data are shown as a percentage of control potential at various times of exposure. The membrane potential shows a linear depolarization ($r = 0.98$, $P < 0.001$) to half its initial value after approximately 35 min. A similar decline in membrane potential was observed after exposure of cells to 500 μM hydrogen peroxide ($P < 0.001$). These changes in potential were observed whether

TABLE I

DEPOLARIZATION UPON EXPOSURE TO XANTHINE OXIDASE

Cells exposed to 0.08 U/ml xanthine oxidase demonstrate a progressive depolarization of the membrane potential relative to control with time of exposure to the enzyme ($r = 0.98$, $P < 0.001$).

Time of exposure (min)	% Control membrane potential
15	77.6 ± 3.9
30	68.0 ± 2.0
45	39.5 ± 1.9
60	19.7 ± 0.8
80	12.1 ± 1.0

TABLE II

RELATIVE MEMBRANE POTENTIAL AFTER EXPOSURE TO 0.006 U/ml XANTHINE OXIDASE

Relative membrane potential is shown for cells exposed to 0.006 U/ml xanthine oxidase after inhibition of intracellular scavenging enzyme by diethyldithiocarbamate or aminotriazole. Measurements are made either 15 or 75 min after the enzyme had been removed from the cells. In all three groups, an initial hyperpolarization is demonstrated, which disappears by the time of the later measurement. Only aminotriazole produces a significant depolarization in treated cells at the time of later measurement. Error bars are ± 1 S.E.

	Control		Xanthine oxidase	
	$t = 15$	$t = 75$	$t = 15$	$t = 75$
Control	46.1 ± 0.8	44.1 ± 0.5	57.3 ± 0.3	49.2 ± 3.9
Diethyldithiocarbamate	48.6 ± 0.7	41.7 ± 2.3	51.5 ± 0.3	40.3 ± 3.1
Aminotriazole	47.6 ± 1.0	39.8 ± 0.1	54.9 ± 0.6	28.2 ± 2.3

or not glucose was included in the incubation medium during radical exposure, although the rate of potential decline was increased in cells incubated without glucose ($P < 0.01$).

Tables II and III show data obtained from cells exposed to concentrations of xanthine oxidase varying from 0.006–0.08 U/ml for 45 min. In cells exposed to the lower (0.006 U/ml) concentration, a transient hyperpolarization of the membrane was observed within the first 15 min after addition of DiIC1(3) (116% of control potential, $P < 0.001$), although this effect had disappeared by 75 min after the addition of DiIC1(3). A similar transient hyperpolarization is seen after exposure of cells to 500 μM (113% control, $P < 0.001$) or 3 mM (110% control, $P < 0.001$) hydrogen peroxide. A significant transient hyperpolarization was not obtained after treatment with the higher (0.08 U/ml) concentration of xanthine oxidase. The magnitude of the transient hyperpolarization was increased when the cells were exposed to hydrogen peroxide in the presence of glucose (113% control potential vs. 105% control potential in the absence of glucose, $P < 0.01$), suggesting that a metabolic effect might be involved in the production of this transient hyperpolarization.

Cells were pretreated with 20 mM aminotriazole or 3 mM diethyldithiocarbamate at 37°C for 40 min (to inhibit intracellular catalase and superoxide dismutase, respectively). This treatment resulted in 96% inhibition of superoxide dismutase activity by diethyldithiocarbamate and 70% inhibition of intracellular catalase. Pretreatment of cells with both aminotriazole and with diethyldithiocarbamate caused a significant decrease in potential compared with cells exposed to xanthine oxidase alone. This decline in potential increased with time, as shown in Tables II and III. At the

TABLE III

RELATIVE MEMBRANE POTENTIAL AFTER EXPOSURE TO 0.08 U/ml XANTHINE OXIDASE

Relative membrane potential is shown for cells exposed to 0.08 U/ml xanthine oxidase after inhibition of intracellular scavenging enzymes by diethyldithiocarbamate or aminotriazole. Measurements are made either 15 or 75 min after the enzyme had been removed from the cells. In the control group, an initial mild hyperpolarization of the memrbane is observed, with depolarization (relative to control) at the later time of measurement. In both aminotriazole and diethyldithiocarbamate-treated cells, there is significant depolarization observed both initially and at the time of later measurement. Error bars are ± 1 S.E.

	Control		Xanthine oxidase	
	$t = 15$	$t = 75$	$t = 15$	$t = 75$
Control	54.4 ± 1.1	54.5 ± 0.3	57.7 ± 1.1	52.7 ± 1.4
Diethyldithiocarbamate	59.0 ± 0.6	57.6 ± 1.3	44.7 ± 0.8	40.4 ± 2.4
Aminotriazole	55.6 ± 0.6	53.8 ± 1.0	44.7 ± 0.6	37.7 ± 2.0

low concentration of xanthine oxidase, preincubation with aminotriazole produced a transient hyperpolarization (115% control potential, $P < 0.001$) at 15 min after addition of the probe and a decrease in potential (71% control potential, $P < 0.01$) at 75 min. At the higher concentration (0.08 U/ml) of xanthine oxidase a decrease was seen both at 15 min (80% control potential, $P < 0.001$) and 75 min (70% control potential, $P < 0.001$). At the low concentration of xanthine oxidase, preincubation with diethyldithiocarbamate produced a transient hyperpolarization (106% control potential, $P < 0.01$) at 15 min after probe addition and a decrease (97% control potential, n.s.) at 75 min. At the higher concentration of xanthine oxidase (0.08 U/ml), a decrease was present at both 15 min (76% control potential, $P < 0.001$) and 75 min (70% control potential, $P < 0.01$).

Inclusion of 0.1 mg/ml catalase during exposure of cells to 5 mM hydrogen peroxide decreased the magnitude of the depolarization. 5 mM hydrogen peroxide depolarized cells to 41.3 ± 3.4% control potential, while inclusion of catalase resulted in a final potential of 83.5 ± 4.8% of control potential ($P < 0.001$), consistent with involvement of hydrogen peroxide in producing the depolarization.

Influence of osmolality upon membrane potential response to oxygen radicals

Tables IV and V demonstrate the effect of incubating cells in phosphate-buffered saline of varying osmolality during exposure to xanthine oxidase at a 0.05 U/ml concentration. In Table IV, relative scatter size is plotted against osmolality. As expected, cell scatter size is increased to 105% control in the hypoosmolar sample (200 mosmolal) and decreased to 85% control in the hyperosmolar sample (400 mosmolal). At all osmolalities, xanthine oxidase-treated cells were larger than control cells at that osmolality (122% control scatter size at 200 mosmolal, 113% control at 300 mosmolal, 121% control at 400 mosmolal), consistent with a cellular swelling response to xanthine oxidase exposure.

TABLE IV

RELATIVE CELLULAR SIZE AFTER EXPOSURE TO 0.05 U/ml XANTHINE OXIDASE

Relative scatter size is displayed for cells exposed to 0.05 U/ml xanthine oxidase in 200, 300 or 400 mosmolal solutions. While scatter size is inversely proportional to the osmolality of the medium, xanthine oxidase-exposed cells are larger than control cells at all osmolalities tested.

	200 mosmolal	300 mosmolal	400 mosmolal
Control	55.6 ± 1.7	53.0 ± 1.5	44.9 ± 0.9
Xanthine oxidase	67.9 ± 0.2	59.9 ± 1.0	54.5 ± 0.2

TABLE V

RELATIVE MEMBRANE POTENTIAL AFTER EXPOSURE TO 0.05 U/ml XANTHINE OXIDASE

Relative membrane potential is shown for cells exposed to 0.05 U/ml xanthine oxidase in phosphate-buffered saline at either 200, 300 or 400 mosmolal. While membrane potential is decreased after exposure to xanthine oxidase at all three osmolalities, the disparity between control potentials in unexposed and those in xanthine oxidase-treated cells is decreased at the higher osmolality (400 mosmolal) compared to either 200 or 300 mosmolal samples. Similarly, cells incubated in 200 mosmolal media during exposure to xanthine oxidase showed a greater decline in membrane potential than did those cells exposed in a 300 mosmolal environment.

	200 mosmolal	300 mosmolal	400 mosmolal
Control	57.0 ± 1.0	53.2 ± 0.7	61.7 ± 1.8
Xanthine oxidase	38.4 ± 0.5	46.1 ± 0.8	59.4 ± 0.6

Table V demonstrates the protective effect of the 400 mosmolal concentration in maintaining membrane potential in the presence of xanthine oxidase. Xanthine oxidase reduced the membrane potential to 67% of the control value at 200 mosmolal ($P < 0.01$), to 87% of control potential at 300 mosmolal ($P < 0.01$) and to 96% control potential at 400 mosmolal (n.s.). This protective effect of 400 mosmolal persisted when cells were returned to 300 mosmolal phosphate-buffered saline prior to potential measurement, suggesting that increased osmolality during exposure to xanthine oxidase is the critical protective step.

Influence of scatter size (cell volume) upon membrane potential

We examined the effect of media osmolality upon membrane potential to ensure that recorded potentials were not affected by cell size indepen-

dently of other parameters. Control LLC-PK1 cells were placed in phosphate-buffered saline ranging in osmolality from 100 to 600 mosmolal and the membrane potential was recorded after 15 min equilibration at 20°C. While the expected differences in cell size were recorded (larger cells being observed in the more hypoosmolar media, see above), there was no significant relationship between scatter size and relative membrane potential. A nonsignificant trend for cells in 300 mosmolal environments to record dye uptakes slightly lower than those cells incubated in 200 or 400 mosmolal solutions was observed.

Discussion

The cyanine dyes are fluorescent lipophilic cations which partion in a potential-dependent manner between the cell and the extracellular space [6,10]. This property permits the recording of membrane potential through the determination of the cellular fluorescence at dye equilibrium, increased membrane potential being reflected by a higher cellular content of the fluorescent probe. These probes can also be used to monitor time-dependent changes occurring in the membrane potential, since depolarization of the cell membrane is accompanied by release of the probe from the cell and reequilibration. Since the rate-limiting step for cellular fluorescence change is the transport of the dye across the membrane, potential changes will not be recorded instantaneously, but will be associated with a time constant (usually in minutes) for re-equilibration of the probe to occur across a membrane of altered potential. Not only do these dyes permit the recording of membrane potential in an accessible and noninvasive manner, but they can be used in conjunction with flow cytometric techniques to quantify the relative membrane potential on a cell by cell basis in a situation where the recording of large populations permits considerable statistical efficiency.

Oxygen-radical-mediated cellular injury is associated with many changes in cellular metabolism, including changes in membrane integrity [14,15], probably resulting from radical-mediated alterations of membrane-associated proteins and lipids. Recently it has been shown that decreases in intracellular ATP content precede changes in membrane permeability to small molecules [16], suggesting that the ultimate (plasma membrane) expression of morphologic injury in response to oxygen radicals may await significant metabolic alterations in the cell. Since membrane potential is maintained by energy-dependent processes within the cell [17], it is likely that early metabolic alterations may be expressed through alterations in the membrane potential (as indicated by the more rapid loss of potential in LLC-PK1 cells incubated without glucose). We thus examined the effect of oxygen radical exposure on the membrane potential of LLC-PK1 cells.

Both xanthine oxidase and hydrogen peroxide caused significant decreases in the membrane potential recorded from LLC-PK1 cells. Hydrogen peroxide reduced plasma membrane potential relative to control at concentrations of 500 μM and above ($P < 0.001$), while lower concentrations (50 μM) had no significant effect after a 45 min exposure. The time-dependence of this depolarization, however (Table I), suggests that both concentration and time of exposure are relevant in determining the magnitude of depolarization. Thus, a lower concentration of hydrogen peroxide might require a longer time of exposure to effect a given depolarization. Also, the ultimate level of depolarization may not be evident immediately, since measurements made 15 and 75 min after cessation of exposure of the cells to xanthine oxidase showed a greater decrease in potential at the later time of measurement. It is likely that continuing cellular damage set in motion at the time of initial oxygen radical exposure leads to this 'delayed' depolarization effect. Similar delayed effects of cellular exposure to active oxygen species have been described by other workers [17,18]. Spragg et al. found no evidence of metabolic injury in endothelial cells at concentrations of hydrogen peroxide below 500 μM [16], although the measurements of cellular energy charge used by these authors may not reflect the same temporal alterations as does the membrane potential in response to hydrogen peroxide.

The inclusion of 0.1 mg/ml catalase largely prevented the decline in membrane potential effected by exposure to 5 mM hydrogen peroxide, consistent with the postulated role of hydrogen peroxide in producing the depolarization. Xanthine

oxidase also produced a significant decline in membrane potential at a 0.05 U/ml concentration of the enzyme ($P < 0.01$).

A transient hyperpolarization of the membrane potential occurs soon after initial exposure of the cells to low concentrations of xanthine oxidase or hydrogen peroxide. This hyperpolarization disappears within approx. 15 min is not seen at higher concentrations of the oxidizing agents and is greater in the presence of glucose. One possible explanation for this phenomenon is increased intracellular delivery of oxygen resulting from the successful intracellular scavenging of low levels of oxygen radical species and resulting stimulation of cellular metabolism. Supporting this hypothesis is the observation that the effect is much less evident in the presence of inhibitors of the intracellular scavenging enzymes superoxide dismutase and catalase (Tables II and III).

Increased radical-mediated depolarization is observed in cells in which the intracellular scavenging enzymes have been previously inhibited. This suggests that intracellular radical species are responsible for the production of a significant component of the observed depolarization. The production of intracellular oxidizing events in response to the extracellular generation of oxygen radicals has been previously documented in this system (Scott, J.A. and Rabito, C.A., unpublished data) and transmembrane passage of the superoxide anion has been described by several other authors [19,20]. Thus, internal cellular damage may be caused via the intracellular action of externally generated active oxygen species. It is possible that the hydroxyl radical, generated by the iron-catalyzed Fenton reaction [21] from the superoxide-mediated reduction of hydrogen peroxide, may be in part responsible for the observed depolarization. In this case, one could account for the depolarization by postulating an increase in hydroxyl radical formation effected by increases in the concentration of one or both reactants in the Fenton reaction. Catalase (by decreasing the concentration of hydrogen peroxide) would thus decrease the hydroxyl radical concentration, and thus diminish the depolarization effect. Aminotriazole and diethyldithiocarbamate (by increasing concentrations of hydrogen peroxide and superoxide anion, respectively) would increase the concentration of these reactants, and thus of the hydroxyl radical product, increasing the depolarization. The extreme reactivity of the hydroxyl radical frequently complicates assessment of its role in cellular processes. Scavenging agents may not have sufficient access to or concentration in compartments where the radical is formed, and thus may not be able to inactivate the hydroxyl radical before it reacts with cellular constituents.

Owing to the close interrelationships between cellular metabolic systems for the superoxide anion and catalase, it is difficult to alter the concentration of one of these species in isolation. The superoxide anion is known to inhibit catalase activity and hydrogen peroxide to inhibit superoxide dismutase [22,23]. Thus, an inhibitor of one of these scavenging enzymes may secondarily inactivate the other radical species as well. Also, decreasing the activity of superoxide dismutase via diethyldithiocarbamate inhibition will increase the concentration of superoxide but would theoretically decrease that of hydrogen peroxide (the latter being the end-product of superoxide dismutation).

An early response of the LLC-PK1 cell to oxygen radical exposure is an increase in cellular volume, as shown in Table IV. Previous workers have suggested that cellular injury may be ameliorated by limiting the degree of cellular swelling occurring in response to various injurious agents [24]. Thus we examined the ability of hyperosmolar agents in reducing the membrane potential changes effected by exposure to active oxygen species. Membrane depolarization is most pronounced in cells exposed to xanthine oxidase in hypoosmolar solutions and depolarization is markedly reduced when the cells are exposed to xanthine oxidase while in a 400 mosmolal phosphate-buffered saline solution. These data suggest that artificially maintaining cell volume during oxygen radical exposure exerts a protective effect upon the membrane potential. The mechanism of this effect is not clear, but may involve the prevention of focal membrane ruptures which have been described in metabolically depleted cells exposed to conditions predisposing to cellular swelling [25]. Consideration must also be given to the possibility of radical scavenging by the sucrose molecule which, being a disaccharide of glucose (a hydroxyl

radical scavenger) and fructose, might act as a radical scavenger itself.

In conclusion, we find that active oxygen species including hydrogen peroxide and superoxide produce a transient hyperpolarization followed by a massive depolarization of the membrane in LLC-PK1 cells. This depolarization may be ameliorated by either oxygen-radical scavenging enzymes or by preventing the uncontrolled increase in cell volume occurring in response to the radical load.

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