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Reactive oxygen species and rat renal epithelial cells during hypoxia and reoxygenation

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Reactive oxygen species and rat renal epithelial cells during hypoxia and reoxygenation. To study the importance of oxygen free radical production by and injury to proximal tubule epithelial cells, an *in vitro* model was established. Rat renal proximal tubule epithelial cells in primary culture were subjected to normoxic conditions or 60 minutes of hypoxia and 30 minutes of reoxygenation. Under normoxic conditions, these cells produced superoxide radical, hydrogen peroxide, and hydroxyl radical. During hypoxia and reoxygenation, there was an increase in the production of these reactive oxygen species, detected in the extracellular medium, of 252, 226, and 45 percent, respectively. The production rate of superoxide radical was most markedly increased in the first five minutes of reoxygenation. Studies employing 2,7-dichlorofluorescin which fluoresces when oxidized by peroxides revealed a seven-fold increase in cellular fluorescence in cells studied after hypoxia and reoxygenation compared with control cells. That increased production of reactive oxygen species played a role in cellular injury was demonstrated by an increase in lipid peroxidation during hypoxia and reoxygenation, as well as substantial injury during hypoxia and reoxygenation which could be largely prevented by the addition of superoxide dismutase, catalase, dimethylthiourea, or deferoxamine to the cells. These studies demonstrate that proximal tubule epithelial cells produce reactive oxygen species in increased amounts during hypoxia and reoxygenation, and that these reactive oxygen species are injurious to the cells under these conditions.

Reactive oxygen species (superoxide radical, hydrogen peroxide, and hydroxyl radical) have been implicated in contributing to reperfusion injury of a variety of organs including the central nervous system, myocardium, gut, skeletal muscle, skin, and kidneys [1]. Considerable indirect evidence has been put forth to support the role of oxygen free radicals in mediating postischemic renal injury. Using *in vivo* animal models, many investigators have demonstrated a renal protective role of several free radical scavengers and antioxidants, including superoxide dismutase, allopurinol, and dimethyl sulfoxide, vitamin E, dimethylthiourea, glutathione, deferoxamine, and probucol [2–14], although, some investigators have not demonstrated this effect [15, 16]. Additional indirect, but convincing, evidence for the presence of oxygen free radicals after renal ischemia has been the observation of increased lipid peroxidation in this setting [2, 17–19]. Furthermore, dietary deficiency of selenium (a co-factor for glutathione peroxidase) and/or vitamin E, or inhibition of catalase exacerbated ischemic renal injury [20–22].

The obvious value of these *in vivo* studies is their clinical relevance and the possibility that findings from these studies can be directly applied to clinical studies. The disadvantages of *in vivo* studies are also important. It has not been possible to unequivocally demonstrate the presence of oxygen free radicals following renal ischemia. In addition, it has not been possible to delineate which cell type(s) is responsible for oxygen free radical formation and which cell type(s) is the major target for free radical attack. In ischemic myocardium, oxygen free radical production by infiltrating neutrophils is an important component of injury, and free radical-mediated endothelial cell injury appears to be a major consequence of free radical formation [23]. Studies in the kidney have suggested that proximal tubular cells are the major target for free radical-mediated injury, but again these studies have been indirect. Only free radical scavengers and iron chelators that gained access to the urinary space were found to be protective in an animal model of renal ischemia, whereas scavengers confined to the vascular space had no protective effect [13]. Although we and others have found no definitive role for neutrophils to mediate postischemic renal injury [24, 25], some investigators have found such a role [26–28]. Therefore, we sought to develop an *in vitro* model of renal ischemia that would be useful in defining the role of oxygen free radicals as they relate to one particular cell type, the proximal tubular epithelial cell. We chose to focus on this cell type because of the above cited findings and because histologic studies in rodent models of ischemic acute renal failure show early and severe damage to proximal tubular cells [29]. Many of these changes, such as apical membrane damage with the formation of blebs and loss of brush border, increased membrane permeability, and disruption of cell polarity, might be the consequences of oxygen free radical-mediated injury because oxygen free radicals can peroxidate lipids and thus disrupt membrane function, and can oxidize sulfhydryl groups and enhance proteolysis of essential proteins, such as Na-K ATPase [19, 29–31]. The present studies were undertaken to directly measure reactive oxygen species production by a defined renal cell type in the setting of oxygen deprivation and reoxygenation and to determine the consequences of such oxygen radical formation on cell viability.

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Methods

In vitro studies of hypoxic renal cell injury

Cell culture. Rat renal proximal tubule segments were isolated by the method of Gesek, Wolff and Standhoy [32] which employs collagenase digestion of the renal cortex followed by Percoll density gradient centrifugation. This procedure yielded a preparation primarily consisting of proximal tubule fragments (>95%) with approximately 90% viability by vital dye exclusion. Culture medium was RPMI 1640 which contains amino acids and vitamins, 11 mM glucose, 1 mM Ca $(NO_3)_2$, 5.4 mM KCl, 0.4 mM $MgSO_4$, 103 mM NaCl, 5.6 mM Na_2 HPO_4, 23.8 mM $NaHCO_3$, and 10 mM Hepes to which 10% fetal calf serum, 100 μU/ml penicillin, and 100 μg/ml streptomycin 10 ng/ml epidermal growth factor (EGF), 5 μg/ml transferrin, 5 μg/ml insulin and 10^{-8} M dexamethasone (final concentrations) were added. Tubule fragments were suspended in culture medium and plated onto collagen gel-coated (Type I, Sigma Chemical Co., St. Louis, Missouri, USA) plastic 12-(4.5 cm^2) multiwell plates. Culture medium was change every other day. Primary cultures were used for all studies. The proximal origin of the cultured cells was supported by preliminary studies which showed expression of proximal tubule brush border enzymes and formation of domes, evidence of vectorial transport. Confluent monolayers were assayed in situ for the brush border enzyme alkaline phosphatase using a cytochemical assay as described by Wachsmuth and Torhorst [33]. Virtually 100% of cells showed staining for alkaline phosphatase. Cells were also assayed for gamma-glutamyltranspeptidase activity, another proximal tubule brush border marker, using L-gamma-glutamyl-3-carboxy-4-nitroanilide as the substrate while spectrophotometrically monitoring the reaction at 37°C [34]. Cell homogenates derived from subconfluent monolayers contained 1.038 ± 0.05 μmol/min/mg protein of the enzyme (N = 4), whereas fresh renal cortex had an enzyme activity of 8.88 ± 0.43 μmol/min/mg protein.

Hypoxic cell injury. Cells were studied in a subconfluent stage, usually four days after initial plating. Forty-eight hours before study the culture medium was switched to a glucose-free formulation of the usual culture medium to enhance cell susceptibility to hypoxia/reoxygenation injury. Two hours before study, the medium was changed to glucose-free medium without added growth factors or serum. Cell plates were placed in an airtight glass chamber under a continuous flow of humidified gas and maintained at 37°C: hypoxia (95% N_2, 5% CO_2) for 60 minutes followed by reoxygenation (95% O_2, 5% CO_2) for 30 minutes.

In preliminary studies we found that rat renal proximal tubular epithelial cells in primary culture were quite resistant to injury by hypoxia and reoxygenation if allowed to grow to confluence. Additional preliminary studies showed a clear-cut, inverse relationship between the number of days in culture (or cell density) and the amount of injury produced by 60 minutes of hypoxia and 30 minutes of reoxygenation (measured as LDH release). We speculated that the resistance to hypoxic injury in cells with a slower growth rate (those reaching confluence) may have been due to a transformation from aerobic to anaerobic metabolism after four or more days in culture as has been reported by others [35, 36]. Because of these concerns, we followed the example of Goligorski et al and employed glucose-free media for 48 hours prior to studies [36]. In our preliminary studies we found that specific LDH release during hypoxia and reoxygenation (that amount exceeding the basal release by normoxic cells) was 33.2 ± 4.5% (N = 12) in cells deprived of glucose and only 16.3 ± 4.6% (N = 12) in cells grown in the presence of glucose ($P < 0.002$).

Measurement of reactive oxygen species

Cells were examined for the release into the incubation medium of either superoxide radical, hydrogen peroxide, or hydroxyl radical under normoxic conditions (95% air/5% CO_2) for 90 minutes, or hypoxia for 60 minutes followed by reoxygenation for 30 minutes. Superoxide production was measured spectroscopically as the superoxide dismutase-inhibitable reduction of ferricytochrome C [37]. Ferricytochrome C (horse heart, Type III, Sigma) was added to the cells in a concentration of 80 μM at the beginning of the 90 minute observation period. The incubation media was then removed and the absorbance determined at 550 nm. Samples with superoxide dismutase present served as blanks. All reduction of the cytochrome was inhibited by superoxide dismutase. The incubation medium without any renal cells generated no detectable superoxide radicals when incubated at 37°C for 90 minutes.

Hydrogen peroxide was measured using 4-amnioantipyrine and phenol which in the presence of horseradish peroxidase and hydrogen peroxide yields a chromagen with a maximum absorption at 505 nm and with a molar extinction coefficient of 6.4 × 10^3 M^{-1} cm^{-1} [38].

Hydroxyl radical formation was detected by the degradation of 2-deoxyribose, which when attacked by hydroxyl radical yields malondialdehyde which can be detected by the thiobarbituric acid assay [39]. Deoxyribose was added to the incubation media to yield final concentration of 3 mM. Following a 90 minutes observation, the supernate was removed and assayed for thiobarbituric-reactive material using previously described methods [40]. Exposure of cells to 2-deoxyribose for 90 minutes caused no increase in LDH release over basal levels. Studies were also performed to determine the effects of deferoxamine (150 μM) and of the hydroxyl radical scavenger dimethylthiourea (125 μg/ml) on detectable hydroxyl radical.

Time course of superoxide radical production during hypoxia and reoxygenation

In the preceding studies, the accumulation of superoxide radical, hydrogen peroxide, or hydroxyl radical was determined for a 90 minute period. The mean production rate was determined by dividing net production by 90 minutes to yield the rate per minute. To determine the rate of superoxide radical production at various time points, additional studies were performed in which cells were studied after just five minutes of hypoxia, 60 minutes of hypoxia without reoxygenation, or 60 minutes of hypoxia and just five minutes of reoxygenation. To determine the production rate of superoxide radical in the reoxygenation period, the net amount produced during 60 minutes of hypoxia was subtracted from the amount observed during hypoxia plus reoxygenation and then the production rate (per minute) was calculated.

Detection of intracellular hydrogen peroxide

Hydrogen peroxide (and any other lipid hydroperoxides) generated intracellularly during hypoxia and reoxygenation was detected by a fluorescence imaging system and the fluorescent indicator 2,7-dichlorofluorescin diacetate (DCF) [41–43]. DCF (Molecular Probes, Eugene, Oregon, USA) was prepared fresh each day by dissolving in ethanol to yield a 1 mM stock solution which was added to the cells just prior to hypoxia in the amount of 5 μl/ml. After either 90 minutes of exposure to normoxic conditions or 60 minutes of hypoxia followed by 30 minutes of reoxygenation, the cells were washed five times with phosphate buffered saline to remove all extracellular DCF. The culture plate was then transferred to a temperature-controlled stage of an Olympus IMT-2 inverted, phase contrast epifluorescence microscope that is the optical component of an ACAS 570 Interactive Laser Cytometer system (Meridian Instruments, Okemos, Michigan, USA) [44]. The Interactive Laser Cytometer has a 5 watt argon ion laser for exitation in a stationary beam configuration controlled by an accoustically-driven beam splitter. The mechanical stage is computer controlled. The system has software which makes it possible to determine fluorescence in single cells observed undisturbed in tissue culture plates. To quantitate the amount of hydrogen peroxide detected within cells, a standard curve was generated by exposing normal cells to DCF and varied concentrations of hydrogen peroxide. Thus, average cellular fluorescence or fluorescence detected within a single cell could be compared with the standard curve to estimate the amount of hydrogen peroxide within a cell. For these studies the fluorescein-containing cells were excited at 488 nm and emissions above 515 nm were recorded.

Consequences of reactive oxygen species generation

To determine whether enhanced generation of reactive oxygen species by proximal tubular epithelial cells during hypoxia and reoxygenation had an adverse effect on cell viability, cells were studied under control conditions, after hypoxia and reoxygenation, or after hypoxia and reoxygenation while exposed to either superoxide dismutase, catalase, dimethylthiourea or deferoxamine. For most studies, cell viability was determined by measuring the release of lactate dehydrogenase (LDH) into the incubation media. Incubation medium was promptly aspirated with a Pasteur pipette and LDH was determined as the increase in NADH (absorbance at 339 nm at 30°C, extinction coefficient of 6.30 mM^{-1} cm^{-1}) in the presence of 6.5 mM β-NAD and 52 mM L-lactate in 100 mM Tris, pH 9.3 [45]. Cellular LDH was determined after scraping the cells from the tissue culture plate, disrupting the cell membranes by mechanical homogenization, and then measuring LDH in the resulting homogenate. LDH release was expressed as the percent of total cellular LDH (supernate plus cell fraction) recovered in the incubation medium. LDH release correlates with critical cellular injury and loss of other cytoplasmic constituents in cells which have lost cellular integrity [46]. In initial studies, cell viability was also assessed by evaluating nigrosine dye exclusion following hypoxia and reoxygenation. Nigrosine dye (50 mg/ml) in phosphate buffered saline was incubated with cells for five minutes followed by three washes with phosphate buffered saline. Cells were counted under an inverted microscope to determine the percentage of viable cells excluding the dye. Because some viable as well as nonviable cells detached from the plate, the supernatant fluid was centrifuged and the recovered cells were counted and included in the final totals.

To determine if cellular injury was a consequence of hypoxia, reoxygenation, or both, LDH release was measured after 60 minutes of hypoxia as well as after 60 minutes of hypoxia and 30 minutes of reoxygenation. Studies of the effect of reoxygenating cells in 95% air/5% CO_2 rather than 95% O_2/5% CO_2 were also performed. To determine whether superoxide radical generation played a role in post-hypoxic renal cell injury, cells were exposed to superoxide dismutase (40 μg/ml) at the time of hypoxia. As a control study, SOD was inactivated by mixing the scavenger with 0.01 M diethyldithiocarbamate at 56°C for 1.5 hours followed by dialysis against distilled water for 20 hours at 4°C [2].

To determine whether hypoxia and reoxygenation stimulated lipid peroxidation, cell homogenates were examined for malondialdehyde content. Malondialdehyde was measured as thiobarbituric acid-reactive substance by the method of Ohkawa, Ohishi and Yagi [40] and adapted for use in our lab [2, 12, 13].

To evaluate the toxicity of hydrogen peroxide generated during hypoxia and reoxygenation, catalase (200 μg/ml) was added to the media before hypoxia. To determine the specificity of any catalase effect, the ability of heat-inactivated catalase (100°C, 60 min) to protect against lethal cell injury was compared to that of native catalase. Nigrosine dye exclussion, rather than LDH release, was used as an end-point because heat-aggregated catalase tended to interfere with the spectrophotometric assay for LDH. To evaluate the toxicity of hydroxyl radicals generated during hypoxia and reoxygenation, the hydroxyl radical scavenger dimethylthiourea (125 μg/ml) was added to the culture medium before hypoxia. Because the iron-catalyzed Haber-Weiss reaction is believed to be responsible for hydroxyl radical formation within the kidney, the effects of an iron chelator on posthypoxic cell injury were also tested. Deferoxamine was added to the culture medium to attain a final concentration of 150 μM just before hypoxia.

Statistical analysis

All data are shown as mean ± standard error. For all studies concurrent controls were tested. For each experimental condition several different batches of cells were evaluated and at least four wells of cells from each batch were tested. All assay results were corrected for cell protein determined by the Lowry method. Statistical comparison was performed by unpaired *t*-test when the two groups were compared or by the Bonferroni method when multiple groups were compared.

Results

Reactive oxygen species formation during hypoxia and reoxygenation

When cells were observed under control (normoxic) conditions, they produced 28.8 ± 14.0 nmol O_2^-/mg protein in 90 minutes. There was no increase in O_2^- production above base line when cells were exposed to 95% O_2/5% CO_2 for 90 minutes without a preceding period of hypoxia. As shown in Table 1, net production during 60 minutes of hypoxia and 30 minutes of reoxygenation was more than threefold increased ($P < 0.001$).

Table 1. Effect of hypoxia and reoxygenation on reactive oxygen species formation

	Normoxia	Hypoxia/reoxygenation
Superoxide radical	28.8 ± 14 (16)	101.4 ± 7.3[a] (48)
Hydrogen peroxide	2.55 ± 0.4 (16)	8.31 ± 0.4[a] (16)
Hydroxyl radical	25.9 ± 2.2 (24)	37.6 ± 1.3[a] (36)

Superoxide radical and hydroxyl radical formation are expressed as nmol/mg protein, hydrogen peroxide as μmol/mg protein, for a 90-minute observation period, that is, 90 minutes at basal conditions or 60 minutes hypoxia plus 30 minutes reoxygenation. Numbers in parentheses are the determinations on individual cell wells (*N*). The number of different cell batches studied was 1/4 the above number.

[a] $P < 0.001$

Table 2. Superoxide radical production rates at various times during hypoxia or reoxygenation

Condition	O_2-production rate *nmol/min/mg prot*[a]
Basal	0.16 ± 0.04
Hypoxia	
First 5 minutes	1.52 ± 0.51
Mean (60 minutes)	0.52 ± 0.10
Reoxygenation	
First 5 minutes	6.84 ± 0.06
Mean (30 minutes)	3.00 ± 0.31

[a] These values were determined for the entire observation period indicated, either 5, 30, 60, or 90 minutes (for the basal rate) and then divided by the number of minutes to obtain an *average* production rate to aid comparison. Studies were not performed to ascertain whether production rates *within* each observation period were linear.

A similar pattern was observed for the production of hydrogen peroxide during hypoxia and reoxygenation (Table 1). As can be seen, extracellular accumulation of hydrogen peroxide was approximately threefold greater during hypoxia and reoxygenation ($P < 0.001$). The production of hydroxyl radical was also significantly increased during hypoxia and reoxygenation when compared to control cells ($P < 0.001$, Table 1). Thiobarbituric acid reactive material not derived from hydroxyl attack of 2-deoxyribose (MDA determination on supernatant in the absence of added 2-deoxyribose) was only 2.37% of the total. When either deferoxamine or dimethylthiourea was provided to the cells prior to hypoxia, the hypoxia/reoxygenation-induced increase in hydroxyl radical was not observed: normoxia 88.8 ± 8.7 nmol/mg protein, hypoxia/reoxygenation 112.7 ± 15.0 nmol/mg protein, hypoxia/reoxygenation plus deferoxamine 72.9 ± 4.1 nmol/mg protein ($P = 0.08$ vs. hypoxia/reoxygenation, NS vs. normoxia), hypoxia/reoxygenation plus dimethylthiourea 64.4 ± 3.9 nmol/mg protein ($P = 0.03$ vs. hypoxia/reoxygenation, NS vs. normoxia).

Studies to determine when during hypoxia and reoxygenation superoxide radical production took place are summarized in Table 2. These data were derived by studying four to eight separate wells from two different batches of cells under the various conditions. During hypoxia, there was an increase in the production rate of superoxide radical from the basal rate. A substantial portion of this increased superoxide radical production occurred in the first five minutes of hypoxia. By the design of the study these cells were merely exposed to a continuous flow of nitrogen in a sealed incubation chamber. Therefore, during the first few minutes of the study they were not truly anoxic but merely progressively more hypoxic. Based on the study of Joseph et al, we believe that total anoxia was unlikely to have occurred at any time as a result of gassing the cells with nitrogen [47]. This finding suggests that hypoxia is a stimulus for superoxide radical production. The rate of superoxide radical production was even greater during reoxygenation, particularly in the first five minutes of reoxygenation. Indeed, superoxide radical production was some 40-fold increased in the initial minutes of reoxygenation compared to basal conditions.

These studies demonstrate increased accumulation of reactive oxygen species in the extracellular fluid after hypoxia and, particularly, reoxygenation. The most likely source would be the proximal tubular cells themselves, but studies to directly demonstrate this were also undertaken using DCF to detect intracellular hydrogen peroxide. Fluorescence data for 211 individual control cells from six separate culture plates and for 215 hypoxic cells from six different culture plates were analyzed. Figure 1A shows a phase contrast photomicrograph of a cluster of cells studied under basal conditions. Figure 1B shows a similar cluster of cells studied after 60 minutes of hypoxia and 30 minutes of reoxygenation. Although certain aspects of fine detail may be different between the two groups of cells, at low power both groups of cells look similar. Said another way, after hypoxia and reoxygenation the cells available for fluorescent evaluation appeared to be grossly intact. Nevertheless, there were marked differences in fluorescence between these two groups of cells Figure 1C shows the fluorescent image (in gray scale display) obtained from the same cluster of control cells shown in Figure 1A. Because these cells had a very low level of fluorescence, the entire cluster is outlined. This fluorescent image should be compared to that obtained from the cluster of cells exposed to hypoxia and reoxygenation. The cellular boundaries have been emphasized to aid interpretation of this image. Figure 1D shows that every cell exposed to hypoxia and reoxygenation had a high level of fluorescence. The distribution of cellular fluorescence with DCF for both control and post-hypoxic cells is shown in histogram format in Figure 2. Control cells had a mean fluorescence of 220 ± 26 units. Cells exposed to hypoxia and reoxygenation had a mean cell fluorescence of 1506 ± 64 units, and only 13 percent of the cells had fluorescence less than 250 units. The difference between control and post-hypoxic cells was highly significant ($P < 0.00001$). The fluorescence produced by the addition of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M hydrogen peroxide to normal DCF-treated cells was 668, 821, 962, 2965, and 3756, respectively. Thus, the estimated intracellular hydrogen peroxide concentration of the control cells was less than 1 nM if one extrapolates from the "standard curve," whereas the estimated intracellular hydrogen peroxide concentration of cells following hypoxia and reoxygenation was greater than 1 mM. Since the amount of intracellular hydrogen peroxide is likely to be different from the extracellular concentration of exogenous hydrogen peroxide because of incomplete equilibration and because of scavenging

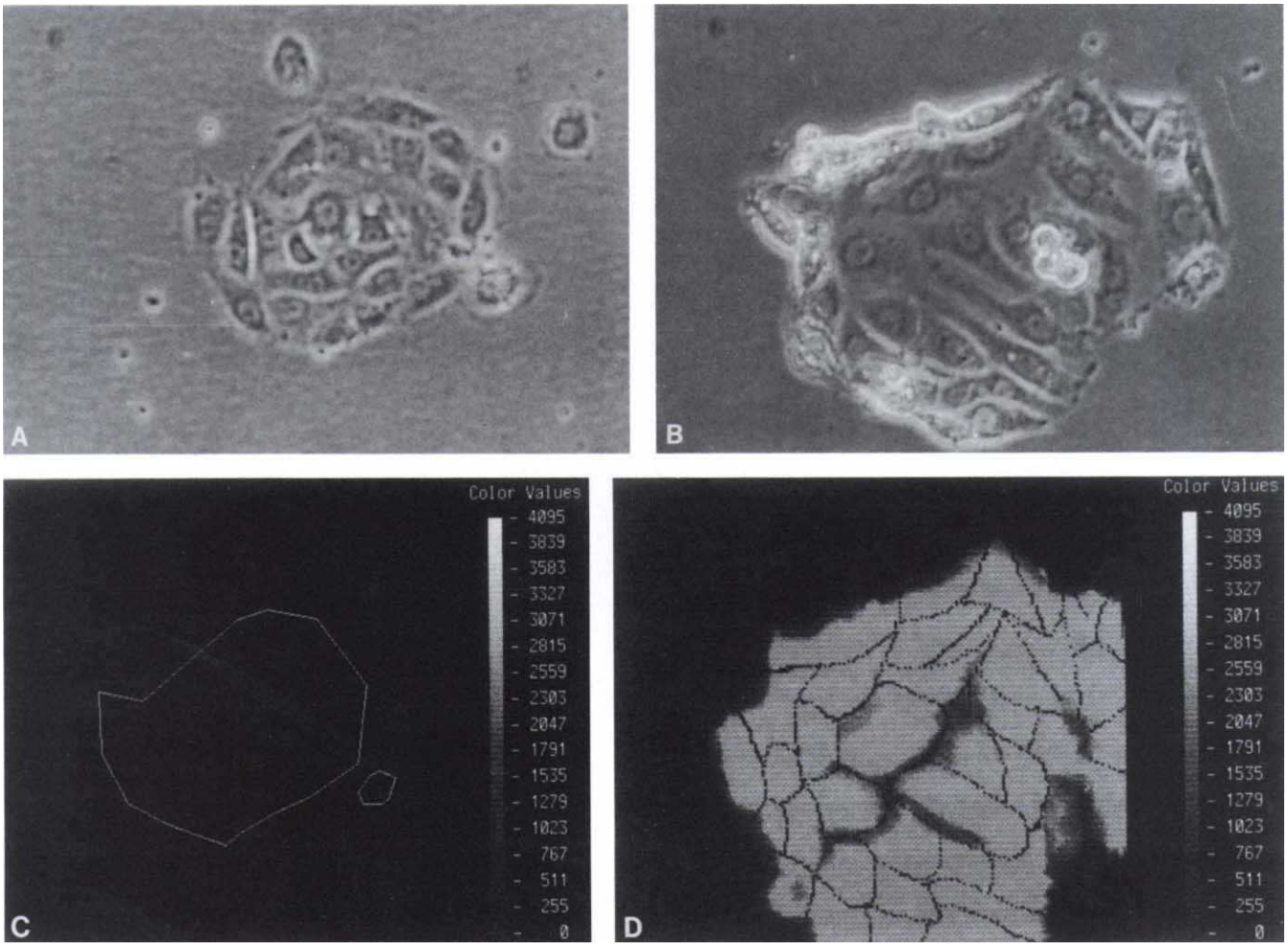

Fig. 1. *Studies of intracellular generation of hydroperoxides during hypoxia and reoxygenation.* **A.** Phase contrast photomicrograph of a cluster of proximal tubule epithelial cells studied under control conditions (100x magnification). **B.** Phase contrast photomicrograph of a cluster of proximal tubule cells after 60 minutes of hypoxia and 30 minutes of reoxygenation (100x magnification). **C.** Fluorescent image (gray scale display) of a cluster of control cells. These are the same cells shown in panel A. The border of the cell cluster is outlined; because this cluster of cells showed little fluorescence above background the image appears blank. **D.** Fluorescent image (gray scale display) of a cluster of cells after hypoxia and reoxygenation. These are the same cells shown in panel B. Note the marked difference in individual cell fluorescence between the cells shown in panels C and D.

by catalase and glutathione peroxidase, this estimate of intracellular hydrogen peroxide concentration is only an approximation.

Cellular injury due to reactive oxygen species

Under control conditions, cells released between 5 and 25 percent of total LDH. There was no increase in LDH release above baseline when cells were exposed to 95% O_2/5% CO_2 for 90 minutes without a preceding period of hypoxia. During hypoxia and reoxygenation, they released 30 to 80 percent of cellular LDH (Fig. 3, Table 3). Nigrosine dye uptake was 4.1 ± 1.2 percent ($N = 8$) for basal conditions and 22.6 ± 1.6 percent ($N = 8$; $P < 0.001$) after hypoxia and reoxygenation. LDH release was 40.4 ± 3.5% ($N = 22$) after 60 minutes of hypoxia versus 54.8 ± 2.1% ($N = 21$) after hypoxia and reoxygenation ($P < 0.01$). Both values were greater than the basal LDH release of 10.7 ± 3.5% ($N = 21$; $P < 0.01$), suggesting than injury occurred during the hypoxic as well as the reoxygenation phase of the experiment.

Reoxygenation in 95% air resulted in less cellular injury than reoxygenation in 95% O_2. LDH release was 25.0 ± 5.1% ($N = 5$) for basal conditions, 45.2 ± 1.5% ($N = 5$) after hypoxia and reoxygenation in 95% O_2/5% CO_2 ($P < 0.005$), and 27.4 ± 28.% ($N = 5$) after hypoxia and reoxygenation in 95% air/5% CO_2. Reoxygenation in air rather than O_2 also resulted in lesser amounts of O_2^- formed. Cells subjected to hypoxia and reoxygenation in 95% O_2/5% CO_2 produced 62.4 ± 6.3 nmol O_2^-/mg prot/90 min, whereas those subjected to hypoxia and reoxygenation in 95% air/5% CO_2 produced 23.7 ± 13.7 nmol O_2^-/mg prot/90 min ($P < 0.01$). The reduced cytotoxicity and O_2^- production rate resulting from reoxygenation by air can probably be explained by a delayed return of medium pO_2 to normoxic values when reoxygenated in room air. At five minutes of reoxygenation, the period of maximal rates of O_2^-

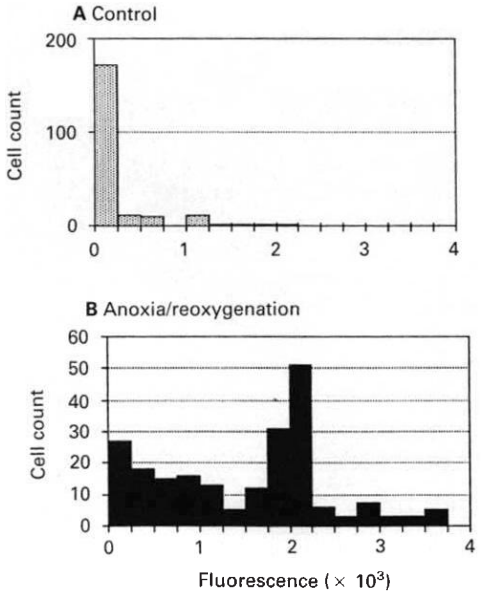

Fig. 2. *Distribution of individual cell fluorescence in proximal tubule epithelial cells treated with 2,7-dichlorofluorescin and either control (normoxic) incubation or 60 minutes hypoxia plus 30 minutes reoxygenation.*

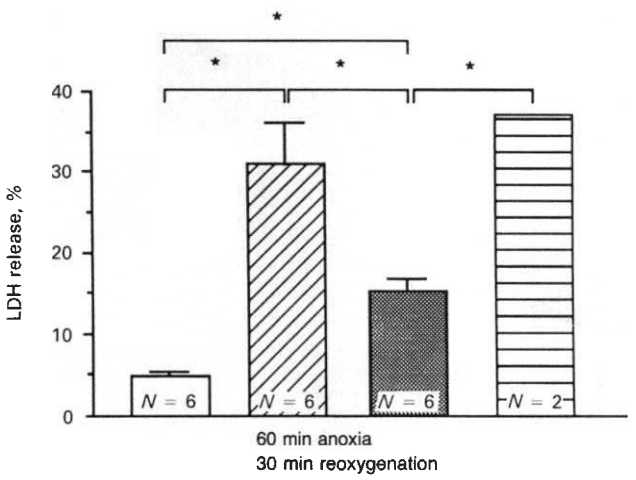

Fig. 3. *Effect of superoxide dismutase (SOD) on cellular injury, measured as lactate dehydrogenase (LDH) release, due to 60 minutes hypoxia and 30 minutes reoxygenation.* The n value is the number of different *batches* of cells tested. Because the number of individual cell wells tested was 4 per batch, the number of cell wells tested was 24, 24, 24, and 8, respectively. Symbols are: (□) control; (▨) anoxia; (■) anoxia + SOD; (▤) inactivated SOD; $^*P < 0.05$.

formation, the pO_2 had reached 167 mm Hg when the reoxygenation gas was 95% O_2/5% CO_2 (basal or normoxic pO_2 in the medium overlying cells taken from the CO_2 incubator which contains 95% air/5% CO_2 was 160 mm Hg). Therefore, in the first critical minutes of reoxygenation cells reoxygenated with air would have remained hypoxic, whereas those reoxygenated with 95% O_2 were "normoxic."

Figure 3 shows that injury due to hypoxia and reoxygenation could be significantly attenuated by including superoxide dismutase in the incubation medium. This was demonstrated to be due to the specific ability of superoxide dismutase to detoxify superoxide radical because enzymatically inactive SOD provided no protection against hypoxia/reoxygenation injury. Hypoxia and reoxygenation was also associated with an increase in lipid peroxidation measured as a cellular malondialdehyde content. Cells studies under control conditions had a malondialdehyde content of 0.42 ± 0.04 nmol/mg protein ($N = 12$). After hypoxia and reoxygenation, this increased to 0.89 ± 0.13 nmol/mg protein ($N = 22$; $P < 0.05$). Exposure of cells to superoxide dismutase during hypoxia and reoxygenation lead to a non-significant decrease in cellular malondialdehyde content (0.68 ± 0.06 nM/mg protein; $N = 12$).

Catalase also provided marked protection against hypoxic renal cell injury, as shown in Table 3. Heat-inactivated catalase had no protective effect. Hypoxia/reoxygenation caused 34.9 ± 0.8% of cells to take up nigrosine ($N = 9$). Catalase treatment decreased the fraction of cells dying during hypoxia and reoxygenation to 0 ± 1.3% ($N = 12$; $P < 0.01$). Heat-inactivated catalase treatment of cells exposed to hypoxia and reoxygenation resulted in 36.1 ± 1.0% of the cells dying ($N = 12$; not significantly different from hypoxia/reoxygenation, $P < 0.01$ vs. native catalase). In addition, the hydroxyl radical scavenger dimethylthiourea and the iron chelator deferoxamine were protective against posthypoxic renal cell injury (Table 3).

Table 3. Effects of several antioxidant agents on renal cell injury following hypoxia and reoxygenation

	LDH release % *total cellular LDH*		
Antioxidant	Normoxia	Hypoxia/ reoxygenation	Hypoxia/ reoxygenation plus antioxidant
Catalase	25.4 ± 4.5 (8)	50.6 ± 4.9 (16)	13.2 ± 0.8[a] (16)
Dimethylthiourea	22.1 ± 1.3 (8)	80.1 ± 3.4 (8)	45.6 ± 5.8[a] (4)
Deferoxamine	9.7 ± 1.3 (12)	43.0 ± 5.2 (12)	9.0 ± 2.0[a] (14)

Antioxidants were added to the incubation media before hypoxia in the following concentration: catalase 200 μg/ml, dimethylthiourea 125 μg/ml, or deferoxamine 150 μM. Numbers in parentheses are the number of determinations performed.

[a] $P < 0.001$ vs. hypoxia/reoxygenation

Discussion

These studies clearly demonstrate that primary cultures of rat renal proximal tubular epithelial cells produce all three major reactive oxygen species under ambient conditions. During hypoxia and reoxygenation, the production of these reactive oxygen species was increased 3- to 10-fold. When exposed to hypoxia and reoxygenation, these cells also suffered considerable damage which was markedly reduced by superoxide dismutase, catalase, dimethylthiourea, or deferoxamine.

Although these findings might have been predicted from *in vivo* studies, such as those from our laboratory, they provide important new data to support a role for reactive oxygen

species in producing ischemic injury. *In vivo* studies have yielded only indirect evidence for the existence of oxygen free radicals during ischemia and reprefusion. We have now directly demonstrated that renal epithelial cells produce increased amounts of superoxide radical, hydrogen peroxide and hydroxyl radical during hypoxia and reoxygenation, and that under these same conditions reactive oxygen species are responsible for epithelial cell injury. These studies also demonstrate, at least in the case of superoxide radical, that the bulk of oxygen free radical formation occurred during reoxygenation following hypoxia. This is consistent with *in vivo* findings that renal cortical malondialdehyde content insignificantly increased during ischemia, but significantly increased following reperfusion [2]. In another study employing excretion of ethane as a marker for lipid peroxidation, increased lipid peroxidation was detected only when kidneys were reperfused following ischemia [17].

This model system can, therefore, be used to further study the cellular events consequent to oxidant generation after hypoxia when an *in vitro* system is required. Another frequently employed *in vitro* system, freshly isolated proximal tubule segments, has not yielded evidence to support a role for reactive oxygen species in postischemic renal injury [48].

The ability of superoxide dismutase to protect epithelial cells against hypoxic injury confirms previous *in vivo* findings by us and many others [2–7]. However, we were previously unable to demonstrate a protective effect of catalase when administered to rats prior to renal ischemia [2]. We hypothesized that the inability of catalase to protect against renal ischemia was due to its large size (molecular weight approximately 250,000) which precluded significant glomerular filtration and thus limited access to proximal tubules via the urinary space. As noted above, in the *in vivo* model of renal ischemia, only those scavengers which underwent glomerular filtration provided protection [13]. The present *in vitro* studies demonstrate that if oxygen free radical scavengers can be delivered to the cell's extracellular compartment, then significant protection can accrue. The present studies still do not define whether an intracellular location is necessary for these scavengers to provide protection. Hence, it might be necessary for these epithelial cells to endocytose these protective substances in order to provide optimal protection against free radical-mediated injury. Studies employing radiolabeled scavengers or scavengers immobilized to the extracellular space will be required to further investigate this question. On the other hand, it is quite possible that these scavengers are protective from an extracellular location if they are in close proximity to the cell membrane. Previous *in vivo* studies demonstrated the appearance of iron capable of catalyzing the Haber-Weiss reaction in the urinary space after ischemia and reprefusion [13]. The present studies have demonstrated the appearance of superoxide radical, hydrogen peroxide, and hydroxyl radical in the extracellular space. Thus, it is possible that significant protection against free radical injury can result from scavengers confined to the extracellular space. This does not preclude the possibility that those scavengers which enter the cell might provide an even greater degree of protection.

Hypoxia and reoxygenation caused a significant increase in reactive oxygen species production by proximal tubular epithelial cells.[1] However, even under basal conditions these cells generated reactive oxygen species. In a recent report, Rovin and colleagues reported that proximal tubule cells, as well as papillary collecting duct and cortical collecting tubule cells, produced superoxide radical and hydrogen peroxide [48]. Basal production of superoxide radical by proximal tubule cells was similar to that observed in the present study. These investigators also demonstrated that production of oxygen free radicals could be enhanced by stimulation with phorbol myristate acetate, zymogen, or heat-aggregated immunoglobulin. We believe ours is the first report to document an increase in reactive oxygen species formation by proximal tubule epithelial cells during hypoxia and reoxygenation.

The increase in superoxide radical and hydrogen peroxide production by hypoxia and reoxygenation was approximately 300%, whereas the detectable increase in hydroxyl radical was only half that amount. This is not surprising since hydroxyl radical has much greater reactivity with many cell components compared with superoxide radical and hydrogen peroxide. Superoxide radical has been demonstrated to egress erythrocytes and pulmonary endothelial cells via anion channels [49–51]. Hydrogen peroxide also readily exits cells across the cell membrane. However, hydroxyl radical is so highly reactive that it has an effective distance of less than 100 nm [52]. Thus, even if hydroxyl radical were produced on an equimolar basis with hydrogen peroxide, the amount which reached the extracellular detector molecule, 2-deoxyribose, would be much less since a substantial proportion of the hydroxyl radical produced would react with molecules other than 2-deoxyribose. If one assumes that no hydroxyl radical would reach the extracellular space, then these studies provide indirect evidence for the extracellular generation of hydroxyl radical. Such a possibility fits nicely with previous *in vivo* findings that iron that has gained access to the extracellular space could generate hydroxyl radicals in a site-specific manner there [13].

Because we were able to detect increased amounts of reactive oxygen species in the extracellular space, the question may be raised as to the initial site of their production. Do these reactive oxygen species truly derive from intracellular sites of production? To answer this question, we made use of 2,7-dichlorofluorescin (DCF) diacetate which fluoresces when oxidized by hydrogen peroxide or other peroxides. DCF is highly permeable for cells. Once within the cell, esterases cleave the acetate moieties and the resulting polar compound, 2,7-dichlorofluorescin, is trapped within the cell. The polar compound is more readily oxidized by peroxide [41, 42]. This technique has been used to study hydrogen peroxide in neutrophils and in renal cells exposed to free radical-generating systems [41–43]. Using this technique, we detected a dramatic increase in intracellular peroxide after hypoxia and reoxygenation. In some studies, all cells exposed to hypoxia showed intense fluorescence, such as the cells shown in Figure 1D. Occasionally, the fluorescence was more heterogeneous. However, in all cases, the fluorescence was randomly located and there was no

[1] In this study the appearance or accumulation of reactive oxygen species has been measured. Theoretically this could be due to either increased production or to decreased degradation (scavenging), or both. Although we believe the former mechanism to be more likely, the latter possibility should also be considered.

difference in intensity between cells in the center of a cluster compared with cells located on the periphery. An additional striking difference between control and post-hypoxic cells was the degree of leakage of the fluorescent indicator from the cells. After the 90-minute experimental period, the cells were washed five times to remove extracellular DCF. In control cells, this maneuver was quite effective and the background (extracellular) fluorescence was low and did not increase substantially for at least of 20 minutes. On the other hand, after hypoxia and reoxygenation, leakage was a greater problem. By 20 minutes background fluorescence was 4 to 10 times that observed in control cells. For this reason, only fluorescent images obtained within five minutes of the cell washing procedure were analyzed. Even so, background fluorescence was approximately twice that seen in control cells. Since this level of background fluorescence is first subtracted from the cell values before individual cell fluorescence was calculated, cell leakiness might have contributed to an underestimation of intracellular peroxide concentration. This leakiness of hypoxic cells also demonstrates a well known consequence of hypoxia and reoxygenation, increased permeability of cell membranes to cytoplasmic constituents.

The studies reported here were not designed to identify the source of oxygen free radicals, although the experiments employing DCF show that an intracellular source can be implicated. Based on the work of McCord and others, xanthine oxidase-mediated superoxide radical production has received considerable attention [1]. Mitochondria also deserve attention as a source of superoxide radical and hydrogen peroxide. Even under basal conditions, a small fraction (usually less than 10%) of mitochondrial oxygen consumption proceeds by univalent or divalent reduction to yield superoxide radical and hydrogen peroxide [53]. When the terminal electron transport proteins are reduced, this fraction increases [54]. Indirect evidence in support of this mechanism for superoxide radical production was the finding of an increase in superoxide production during the initial period of hypoxia. Other sources of free radicals such as prostaglandin synthetase, catecholamine auto-oxidation, and peroxisomes cannot be excluded [54]. However, in our model a role for infiltrating neutrophils or macrophages can be unequivocally excluded. This model also suggests that oxygen free radicals need not be produced by renal artery endothelial cells or glomerular mesangial cells in ischemic acute renal failure for oxygen free radical-mediated injury to occur. This model appears to be well suited for future studies into the precise intracellular source of reactive oxygen species.

An additional comment regarding this model is apropos. The use of cells within four days of culture is recommended by the studies of Larsson, Aperia and Lechene, who found that rat renal proximal tubular cells in culture dedifferentiate after four or more days in primary culture [54]. Even with these attempts to standardize our protocol, some variability in the susceptibility to hypoxic injury persisted. Thus, in some batches of cells LDH release after hypoxia and reoxygenation was as low as 25%, whereas in others (for example, Fig. 3 and Table 3) LDH release was as high as 50%. Because each experiment employed control cells from the same batch of cultured cells, this did not present a problem for interpretation of the results.

In summary, rat renal proximal tubule cells in primary culture generate superoxide radical hydrogen peroxide, and hydroxyl radical under basal conditions and at greatly increased rates during hypoxia and reoxygenation. The free radicals so generated during hypoxia and regeneration are responsible for cell injury (increased lipid peroxidation and cellular permeability) that can be greatly attenuated by a variety of antioxidants, including exogenous superoxide dismutase or catalase, dimethylthiourea, and deferoxamine.

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