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LABORATORY INVESTIGATION

Reactive oxygen molecule-mediated injury in endothelial and renal tubular epithelial cells in vitro

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Reactive oxygen molecule-mediated injury in endothelial and renal tubular epithelial cells in vitro. To investigate renal tubular epithelial cell injury mediated by reactive oxygen molecules and to explore the relative susceptibility of epithelial cells and endothelial cells to oxidant injury, we determined cell injury in human umbilical vein endothelial cells and in four renal tubular epithelial cell lines including LLC-PK1, MDCK, OK and normal human kidney cortical epithelial cells (NHK-C). Cells were exposed to reactive oxygen molecules including superoxide anion, hydrogen peroxide and hydroxyl radical generated by xanthine oxidase and hypoxanthine. We determined early sublethal injury with efflux of ^{3}H-adenine metabolites and a decline in ATP levels, while late lytic injury and cell detachment were determined by release of ^{51} chromium. When the cells were exposed to 25, 50, and 100 mU/ml xanthine oxidase with 5.0 mM hypoxanthine, ATP levels were significantly lower ($P < 0.001$) in LLC-PK1, NHK-C and OK cells compared to MDCK cells while ATP levels were significantly lower ($P < 0.01$) in endothelial cells compared to all tubular cell lines. A similar pattern of injury was seen with efflux of ^{3}H-adenine metabolites. When the cells were exposed to 50 mU/ml xanthine oxidase with 5.0 mM hypoxanthine for five hours, total ^{51}chromium release was significantly ($P < 0.001$) greater in LLC-PK1, NHK-C and OK cells compared to MDCK cells, while total ^{51}chromium release was significantly ($P < 0.001$) greater in endothelial cells compared to all tubular cells. However, lytic injury was the greatest in LLC-PK1 cells and NHK-C cells while cell detachment was the greatest in endothelial cells. MDCK cells were remarkably resistant to oxidant-mediated cell detachment and cell lysis. In addition, we determined ATP levels, ^{3}H-adenine release and ^{51}chromium release in LLC-PK1, NHK-C and endothelial cells in the presence of superoxide dismutase to dismute superoxide anion, catalase to metabolize hydrogen peroxide, DMPO to trap hydroxyl radical and DMTU to scavenge hydrogen peroxide and hydroxyl radical. We found that catalase and DMTU (scavengers of hydrogen peroxide) provided significant protection from ATP depletion, prevented efflux of ^{3}H-adenine metabolites and cell detachment while DMPO (scavenger of hydroxyl radical) prevented lytic injury. In addition, we found that the membrane-permeable iron chelator, phenanthroline, and preincubation with deferoxamine prevented cell detachment and cell lysis, confirming the role of hydroxyl radical in cell injury. We conclude that among tubular epithelial cells, cells with proximal tubular characteristics including LLC-PK1, NHK-C and OK cells were more susceptible to oxidant injury than MDCK cells which originate from distal tubules. Endothelial cells responded to oxidant injury with a greater fall in ATP levels, efflux of ^{3}H-adenine metabolites and cell detachment, while tubular epithelial cells demonstrated greater cell lysis. Finally, it appears that hydrogen peroxide mediates ATP depletion and efflux of ^{3}H-adenine metabolites while hydrogen peroxide and hydroxyl radical mediate cell detachment and cell lysis.

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During the past several years, research has focused on the role of oxidant injury in the pathogenesis of several diseases. Injury mediated by reactive oxygen molecules may be involved in the pathogenesis of aging, carcinogenesis, atherosclerosis, ischemia-reperfusion injury and inflammatory diseases, suggesting that reactive oxygen molecules may be the final common mediator of tissue injury following a number of diverse insults including metabolic, toxic, immunologic, and hypoxic [1, 2]. Reactive oxygen molecules have been shown to play a role in the pathogenesis of several animal models of renal diseases including ischemic injury, acute nephrotoxic nephritis, phorbol myristate acetate and complement activated polymorphonuclear leukocyte glomerular injury, aminonucleoside nephrosis, and aminoglycoside nephrotoxicity [3–11]. Experimental evidence suggests that renal dysfunction in these diseases may be a result of oxidant injury mediated by reactive oxygen molecules including superoxide anion, hydrogen peroxide, hydroxyl radical and hypochlorous acid.

Renal dysfunction as a result of injury mediated by reactive oxygen molecules may occur by at least four mechanisms. First, polymorphonuclear leukocytes and monocytes which infiltrate the glomerulus and interstitium may produce reactive oxygen molecules when activated. Several in vivo studies have demonstrated the critical role of the polymorphonuclear leukocyte in mediating renal injury, while in vitro studies have shown that activated PMN's injure endothelial cell monolayers [7–9, 12–15]. Second, mesangial cells may produce reactive oxygen molecules capable of tissue injury. Renal mesangial cells in culture have been found to generate superoxide anion and hydrogen peroxide when activated with the complement membrane attack complex and opsonized zymosan [16, 17]. Third, reactive oxygen molecules may also be generated during reperfusion following ischemia [18]. Ischemia is associated with rapid depletion of intracellular ATP which is degraded to adenosine, inosine and hypoxanthine [19]. Ischemia is also associated with a calcium-dependent proteolytic conformational change in xanthine dehydrogenase which is converted to xanthine oxidase. Oxygen delivery during reperfusion following ischemia then allows hypoxanthine to be degraded to xanthine

and, subsequently, to uric acid; during this process oxygen-derived free radicals are generated by xanthine oxidase [18]. Because capillary endothelial cells contain xanthine oxidase, endothelial cells have been shown to be at high risk for oxidant injury during reperfusion following ischemia [20, 21]. Fourth, abnormal metabolic processes or metabolism of exogenous substrates may increase the endogenous production of reactive oxygen molecules in the cell. Endogenous production of hydroxyl radical from the metabolism of aminoglycoside antibiotics has been implicated in the pathogenesis of aminoglycoside nephrotoxicity [11]. In addition, a recent study suggests that proteinuria results in iron catalyzed production of hydroxyl radical within the tubular lumen which may initiate progressive interstitial disease [22].

Once generated, reactive oxygen molecules may induce damage to protein through sulfhydryl oxidation, membranes via lipid peroxidation and induce DNA damage by initiating strand breaks [23]. Oxidant injury results in several metabolic alterations including activation of the glutathione redox cycle, elevation of intracellular calcium, DNA damage, ATP and NAD depletion [24–28]. Morphologic alterations including blebbing and detachment due to rearrangement and alterations of cellular filamentous F actin [29]. DNA damage, ATP and NAD depletion, efflux of ^{3}H-adenine and adenosine metabolites occur as an early response to oxidant injury while morphologic alterations, cell detachment, and lytic injury occur considerably later [27–29]. Oxidant-induced ATP depletion is reversible when adenosine is provided in the recovery media but irreversible when oxidant injury is prolonged irregardless of adenosine supplementation [30].

In certain human and experimental renal diseases, endothelial cells or tubular epithelial cells may be the prime target of oxidant injury while in other disease processes, the oxidant insult may be generalized with all renal cells exposed to the oxidant stress. To investigate oxidant epithelial cell injury and to explore the relative susceptibility of epithelial and endothelial cells to oxidant injury, we determined cell injury in four renal tubular epithelial cell lines including LLC-PK1, MDCK, OK and normal human kidney cortical epithelial cells (NHK-C), and in human umbilical vein endothelial cells. Cells were exposed to reactive oxygen molecules including superoxide anion, hydrogen peroxide and hydroxyl radical generated by xanthine oxidase and hypoxanthine [31]. We determined early, sublethal injury with efflux of ^{3}H-adenine metabolites and a decline in ATP levels while late, lytic injury and cell detachment were determined by release of ^{51}chromium. In addition, we determined the reactive oxygen molecule(s) responsible for injury in each system by using superoxide dismutase to dismute superoxide anion, catalase to metabolize hydrogen peroxide, DMTU to scavenge hydrogen peroxide and hydroxyl radical, DMPO to trap hydroxyl radical, and iron chelators to prevent iron catalyzed hydroxyl radical generation [32–34].

Methods

Cell culture

Renal tubular epithelial cell lines LLC-PK1 (CRL 139) and MDCK (CCL 34) were obtained from the American Type Culture Collection (Rockville, Maryland, USA) [35, 36]. OK cells were provided by Professor H. Murer, Zurich, Switzerland. NHK-C cells were derived from fragments of human kidney cortex by progressive enzymatic dissociation [37]. All renal cell lines were propagated by routine methods and banked in frozen storage at known passage number. Tubular epithelial cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and nutrient medium F12 (1:1) supplemented with 100 U/ml penicillin, 10 mM HEPES and 10% bovine calf serum (Sterile Systems, Logan, Utah, USA) at 37°C in a humidified atmosphere containing 5% CO_2. For experimental studies, cells were dissociated with trypsin-EDTA, washed free of enzyme and passed to 24-well Costar culture plates. Experiments were performed three to four days later. For each cell line, studies were performed on cultures over a range of no more than five passages. Human endothelial cells were obtained from umbilical veins by collagenase digestion [15, 38]. The endothelial cells were incubated with M199 with 20% pooled human serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 20 mM Hepes buffer at 37°C in a humidified environment containing 5% CO_2 for four to seven days until the cells were a confluent monolayer. Cells from at least three cords were then passed to 24-well Costar plates in the same media as used for primary culture. Twenty-four hours later, after the endothelial cells had attached to the culture dish, the media was removed, the cells were washed and then fed with medium identical to that used for the tubular epithelial cells. Experiments were performed three to four days later when the cells were a confluent monolayer. It was necessary to primary harvest and pass endothelial cells in M199 as DEM/F12 did not support endothelial cell growth until the cells were adherent to the culture plate. Endothelial cells were identified by a cobblestone appearance with phase contrast microscopy and positive direct immunofluorescence for factor VIII related antigen [38].

^{3}H-adenine metabolite release and ATP levels

Early sublethal injury was determined with efflux of ^{3}H-adenine metabolites from preloaded cells and reciprocal decreases in cell ATP levels [15, 28]. Previous studies have shown that exogenous adenine and adenosine are incorporated into adenine nucleotides [39]; when such cells are exposed to oxidant injury, ATP levels decline and ATP degradation products (including ^{3}H-adenine metabolites) are transported from the cell, predominantly in the form of inosine and hypoxanthine [30]. Endothelial and epithelial cells in 24 well plates were radiolabeled for three hours with 1 μCi/ml ^{3}H-adenine (Amersham Corp., Arlington Heights, Illinois, USA) in DEM/F12 (1:1) containing 10% bovine calf serum with 10 mM Hepes and 100 U/ml penicillin. After labeling, the monolayer was washed five times with 1 ml Hanks balanced salt solution containing 0.5% albumin (HBSS+A). Cells were then exposed to 12.5, 25, 50, or 100 mU/ml xanthine oxidase (Boehringer Mannheim, Indianapolis, Indiana, USA) with 5.0 mM hypoxanthine (Sigma Chemical Co., St. Louis, Missouri, USA) in HBSS+A. At the end of one hour, ^{3}H-adenine labeled cells were washed once with HBSS+A. The remaining intracellular label in each well was released by dissolving the cells in 400 μl 2.0% Triton X-100. All fractions were collected and counted for two minutes in a liquid scintillation counter (Packard Instruction Co., Downers Grove, Illinois, USA) after the addition of Optiflour. The percent release was calculated as M+W/M+W+C where M represents the DPM in the media, W represents the DPM in the

wash, and C represents the DPM in the cell fraction. The specific release was determined by substracting the control release from the experimental release [15, 25]; all data is presented as the specific release unless otherwise stated.

ATP levels were measured on cohorts of endothelial and epithelial cells with luciferin-luciferase as previously described [28, 30]. After exposure to the oxygen radical generating system, cells were solubilized with 500 μl 0.5% Triton and acidified with 100 μl 0.6 M perchloric acid and placed on ice until assayed. At the time of assay, the cell suspension was diluted with 10 mM potassium phosphate buffer containing 4 mM $MgSO_4$ (pH 7.4); 500 μl of this was added to 1 ml of 50 mM sodium arsenate buffer containing 20 mM $MgSO_4$ (pH 7.4) to which 25 μl of 40 mg/ml luciferin-luciferase (Sigma Chemical Co.) was added. Light emission was recorded precisely at 20 seconds with a Packard beta counter accepting signals out of coincidence [40]. Protein content was determined on a portion of the cell sample by the method of Lowry et al [41], and ATP was expressed as pmol/μg cell protein.

^{51}Chromium release

Late lytic injury and cell detachment were determined by ^{51}chromium release in cells that were radiolabeled with 1 μCi/ml ^{51}chromium (New England Nuclear, Boston, Massachusetts, USA) in media overnight. Release of ^{51}chromium may be cell associated due to detachment of cells from the culture plate in response to oxidant stress or it is representative of lytic injury with release of intracellular ^{51}chromium into the media. In endothelial cells, both are late responses to oxidant injury occurring three to five hours after exposure to reactive oxygen molecules [13–15, 27, 42]. To differentiate these two responses to oxidant injury, the media and wash portion of ^{51}chromium labeled cells was centrifuged for three minutes, the supernatant was removed and the supernatant and precipitate fractions were counted separately. As with ^{3}H-adenine labeled cells, the remaining intracellular label of cells that remained intact and adherent to the culture plate was released with 2.0% Triton. All fractions were collected and counted in a Beckman gamma counter for one minute. The cell associated ^{51}chromium release (representing detachment of cells from the culture plate) was calculated as CPM in the precipitate fraction/CPM in the precipitate fraction + CPM in the cell fraction that remained adherent to the culture plate, while release of ^{51}chromium representing lytic injury was calculated as CPM in the supernatant fraction/CPM in the supernatant fraction + CPM in the cell fraction that remained adherent to the culture plate. The sum of the release of the precipitate and supernatant fractions was the total ^{51}chromium release. The specific release is the test release minus the control release; all data is presented as the specific release unless otherwise stated.

Scavenger experiments

To determine which oxygen radical(s) mediates injury in each cell line, experiments were performed with 100 μg/ml superoxide dismutase to dismute superoxide anion, 100 μg/ml catalase to metabolized hydrogen peroxide, 10 mM 5,5-dimethylpyrroline N-oxide (DMPO) (Sigma Chemical Co.) to trap hydroxyl radical, and 10 mM dimethylthiourea (DMTU; Alpha Products, Danvers, Massachusetts, USA) as a hydrogen peroxide and hydroxyl radical scavenger [32, 33]. Superoxide dismutase and catalase are each large enzymes that are not membrane permeable while DMTU and DMPO are membrane permeable. DMPO traps hydroxyl radical and decreases formation of products of lipid peroxidation [32]. ^{3}H-adenine release and ATP levels were determined in the presence and absence of the oxygen radical scavengers in cells exposed to 50 mU/ml xanthine oxidase with 5.0 mM hypoxanthine in the tubular epithelial cells and 25 mU/ml xanthine oxidase with 5.0 mM hypoxanthine in endothelial cells for one hour. Cell detachment and lysis were determined with ^{51}chromium release in the presence and absence of scavengers with 50 mU/ml xanthine oxidase for five hours in epithelial cells and 25 mU/ml xanthine oxidase for five hours in endothelial cells.

Iron chelation experiments

To confirm the role of hydroxyl radical in mediating cell injury and to determine the intracellular verses extracellular site of hydroxyl radical generation, we determined cell detachment and cell lysis with the ^{51}chromium release assay. LLC-PK1, and NHK-C cells were exposed to 50 mU/ml xanthine oxidase while endothelial cells were exposed to 25 mU/ml xanthine oxidase with 5.0 mM hypoxanthine and iron chelators of variable permeability including 50 μM deferoxamine (Ciba-Geigy, Basle, Switzerland) which is slowly permeable to cell membranes, 50 μM deferoxamine preincubator for 16 hours to allow for intracellular incorporation, and 50 μM phenanthroline (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) which is rapidly permeable to cell membranes [42–45]. Endothelial cells could not be exposed to oxidant injury after incubation with deferoxamine for 16 hours as the cells became loosely adherent and nonspecific effects were observed.

H_2O_2 assay

The amount of H_2O_2 generated with hypoxanthine-xanthine oxidase in the presence and absence of reactive oxygen molecule scavengers was determined with o-dianioidine-dihydrochloride (o-DD) [46]. Briefly HBSS+A containing 50 mU/ml xanthine oxidase and 5.0 mM hypoxanthine was incubated at 37°C for 15, 30, 60, 90, 120, 180, 240 and 300 minutes with no scavenger, 100 μg/ml SOD, 100 μg/ml catalase, 10 mM DMPO, or with 10 mM DMTU. One hundred microliters of this reaction mixture was incubated for 10 minutes with 5 units horseradish peroxidase and 1.5 ml HBSS with 53 mg/liter o-DD. The absorbance was measured at 470 nm on a Gilford spectrophotometer and compared to absorbance of o-DD alone. The results are expressed as the moles of hydrogen peroxide generated per well (400 μl) and as the concentration (μM) of hydrogen peroxide generated.

Statistical analysis

Data is presented as mean ± 1 SD. Analysis of variance with Student-Newman-Keuls multiple range tests were used to detect differences in the response to oxidant injury between the five cell lines and xanthine oxidase concentrations. To stabilize the variance, analyses were performed on the square root of the measure of the cells response to injury. In the iron chelation studies, analysis of variance was performed on the untransformed data.

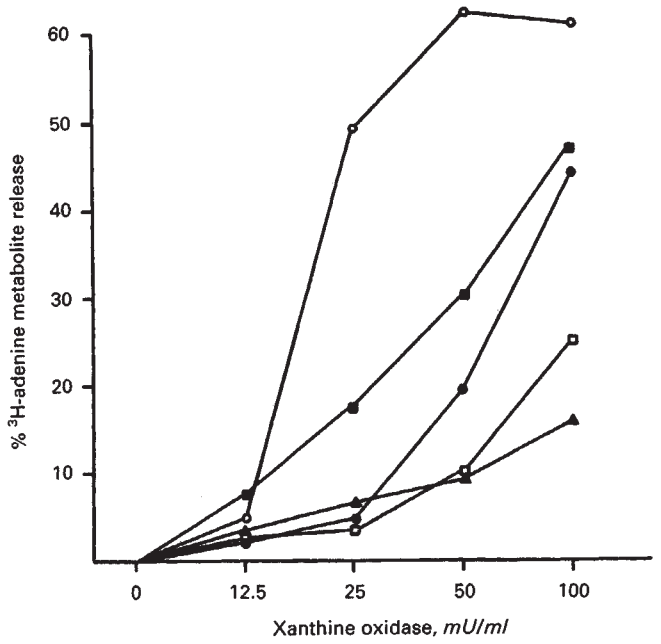

Fig. 1. *^{3}H-adenine metabolite release from HUVEC (○), LLC-PK1 (■), NHK-C (●), OK (□) and MDCK (▲) cells when exposed to oxidant injury generated with 12.5, 25, 50 and 100 mU/ml xanthine oxidase and 5.0 mM hypoxanthine for 1 hour.* Among tubular epithelial cells, release of ^{3}H-adenine was significantly ($P < 0.01$) different in each cell line, while efflux of ^{3}H-adenine metabolites was significantly ($P < 0.01$) greater in endothelial cells compared to all tubular cells. Values represent the mean ± 1 SD of 4 replicates.

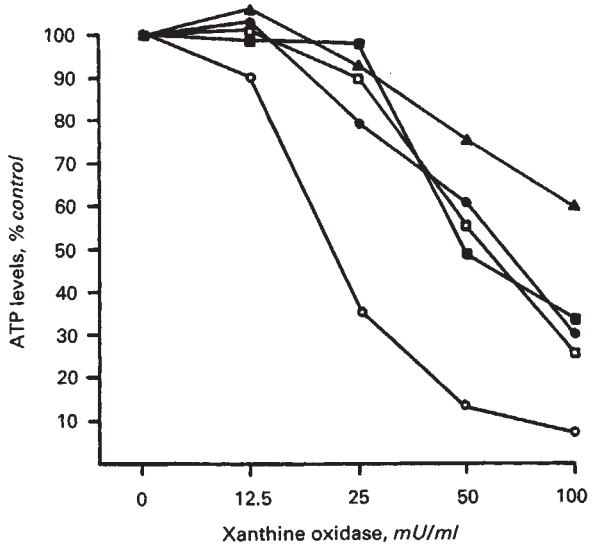

Fig. 2. *ATP levels (% of control) in HUVEC (○), OK (□), NHK-C (●), LLC-PK1 (■), and MDCK (▲) cells, when exposed to 12.5, 25, 50 and 100 mU/ml xanthine oxidase with 5.0 mM hypoxanthine for 1 hour.* Among the tubular cells, ATP levels were significantly ($P < 0.01$) lower in LLC-PK1, NHK-C and OK cells compared to MDCK cells, while at each dose of injury, endothelial cell ATP levels were significantly ($P < 0.01$) lower than tubular epithelial cells. Values represent the mean ± 1 SD of 3 to 4 replicates.

Results

Release of ^{3}H-adenine metabolites and alterations in ATP levels

When epithelial and endothelial cells were exposed to 12.5, 25, 50 and 100 mU/ml xanthine oxidase with 5.0 mM hypoxanthine in HBSS+A for 60 minutes, each cell line demonstrated release of the radioactive label (Fig. 1). Among tubular epithelial cells the ^{3}H-adenine release was significantly ($P < 0.01$) different in each cell line with LLC-PK1 cells, demonstrating the greatest release while MDCK the lowest release. However, the efflux of ^{3}H-adenine metabolites was significantly ($P < .01$) greater in endothelial cells compared to all tubular cells. The spontaneous (control) release was 3.4% for EC, 1.8% for NHK-C, 8.8% for LLC-PK1 cells, 3.7% for MDCK cells and 8.7% for OK cells. Since adenine is primarily incorporated into adenine nucleotides, we measured ATP levels in cohorts of each cell line as used in the ^{3}H-adenine studies. When cells were exposed to 12.5, 25, 50, and 100 mU/ml xanthine oxidase for 60 minutes, ATP levels declined beginning at 25 mU/ml xanthine oxidase (Fig. 2). Among the tubular cells, ATP levels were significantly ($P < 0.01$) lower in LLC-PK1, NHK-C and OK cells compared to MDCK cells. Again, at each dose of injury endothelial cells were significantly ($P < 0.01$) more susceptible to oxidant-induced ATP depletion compared to tubular cells. The ATP content of control cells was 4.95 ± 0.15 pmol/μg protein for EC, 12.3 ± 1.3 pmol/μg protein for NHK-C, 7.4 ± 0.6 pmol/μg protein for LLC-PK1, 6.6 ± 0.6 pmol/μg protein for MDCK and 4.4 ± 0.1 pmol/μg protein for OK cells.

^{51}Chromium release

Cell detachment and cell lysis were determined by ^{51}chromium release. Previous studies in endothelial cells have shown that ^{51}chromium release is a late response to oxidant stress [14, 15, 27]. When each of the four renal tubular epithelial cell lines was exposed to 50 mU/ml xanthine oxidase, ^{51}chromium release first occurred at three hours for LLC-PK1 and OK cells, and at four hours for NHK-C cells, while MDCK cells did not release ^{51}chromium during the five hours after oxidant injury. At three hours the total ^{51}chromium release was 92 ± 1.3% for LLC-PK1 cells, 5.6 ± 1.9% for NHK-C cells, 28.2 ± 2.9% for OK cells and 0.8 ± 0.2% for MDCK cells. At four hours the total ^{51}chromium release was 87 ± 0.1% for LLC-PK1, 39.6 ± 3.4% for NHK-C cells, 28.1 ± 3.3% for OK cells and 0.7 ± 0.1% for MDCK cells. Since cell detachment and lytic injury are also a late manifestation of oxidant injury in tubular epithelial cells, first occurring three to four hours after exposure to oxidant stress, ^{51}chromium release experiments were performed at five hours following oxidant injury.

When endothelial cells and the tubular epithelial cells were exposed to 12.5, 25 and 50 mU/ml xanthine oxidase for five hours, the total ^{51}chromium release was quite variable in the five cell lines (Fig. 3). Among the tubular cells, LLC-PK1 were the most susceptible to injury while MDCK cells were remarkably resistant to cell detachment and lytic injury. Total ^{51}chromium release was significantly ($P < 0.01$) greater in LLC-PK1, NHK-C and OK cells compared to MDCK cells. At each dose of injury, endothelial cells demonstrated significantly ($P < 0.01$) higher total ^{51}chromium release than the tubular cells. When cell detachment and lytic injury were considered separately, differences among the cell lines were evident (Table 1). Endothelial cells demonstrated the greatest degree of cell

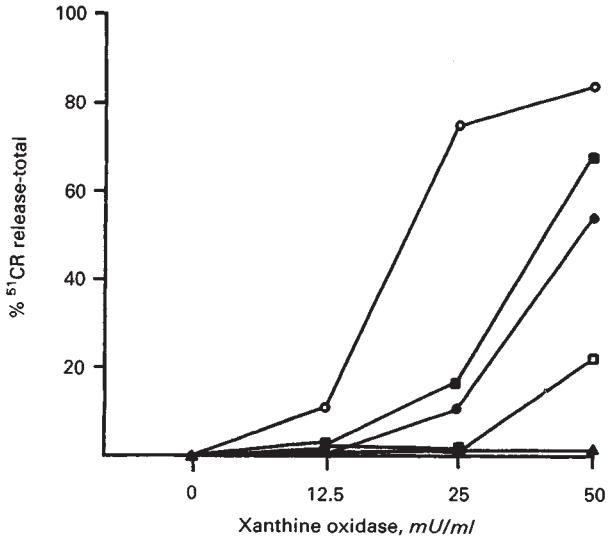

Fig. 3. *Total ^{51}chromium release of HUVEC (○), LLC-PK1 (■), NHK (●), OK (□) and MDCK (▲) cells in response to oxidant injury medicated by 12.5, 25, and 50 mU/ml xanthine oxidase for 5 hours.* Among tubular epithelial cells, release of ^{51}chromium was significantly ($P < 0.01$) greater in LLC-PK1, NHK-C and OK cells compared to MDCK cells while ^{51}chromium release was significantly ($P < 0.01$) higher in endothelial cells compared to all tubular cells. Values represent the mean ± 1 SD of replicates.

Table 1. Total, cell detachment and lytic release of ^{51}chromium in EC, NHK-C, LLC-PK1, OK and MDCK cells exposed to 50 mU/ml xanthine oxidase for 5 hours

	% Specific ^{51}chromium release		
Cell line	Cell detachment	Lytic release	Total
EC	63.9 ± 2.2	19.1 ± 1.6	83.0 ± 0.3
NHK-C	24.5 ± 4.2	30.1 ± 3.4	54.6 ± 6.8
LLC-PK1	17.8 ± 1.4	50.0 ± 4.8	67.8 ± 4.7
OK	13.0 ± 1.5	13.0 ± 0.3	26.0 ± 1.5
MDCK	−0.3 ± 0.1	2.3 ± 0.5	2.0 ± 0.4

Values represent the mean + 1 SD of 3 to 4 replicates.

detachment while LLC-PK1 cells were the most susceptible to lytic injury. The majority of ^{51}chromium release in each of the tubular epithelial cell lines was due to lytic injury while the majority of ^{51}chromium release in endothelial cells was due to cell detachment.

Scavenger experiments

To determine the reactive oxygen molecule(s) responsible for injury, we determined ^{3}H-adenine metabolite release, ATP levels, and ^{51}chromium release with reactive oxygen molecule scavengers in endothelial cells and in the LLC-PK1 and NHK-C cells, the three cell lines most susceptible to injury. We used 100 μg/ml SOD to dismute superoxide anion, 10 mM DMPO to trap hydroxyl radical, 10 mM DMTU to scavenge hydroxyl radical and hydrogen peroxide and 100 μg/ml catalase to metabolize hydrogen peroxide. When endothelial cells were exposed to 25 mU/ml xanthine oxidase and epithelial cells to 50 mU/ml xanthine oxidase for one hour, efflux of ^{3}H-adenine metabolites was significantly ($P < 0.01$) reduced by catalase and

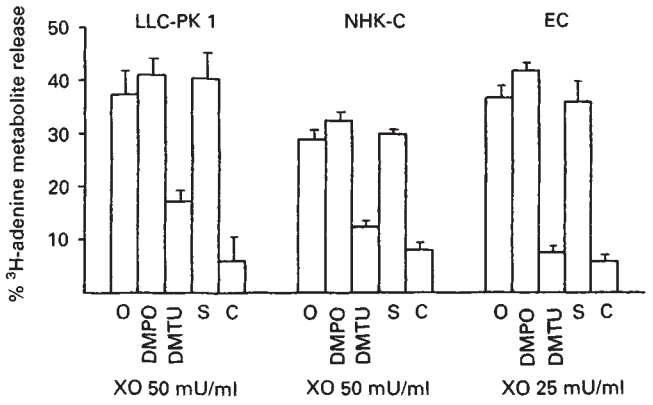

Fig. 4. *Efflux of ^{3}H-adenine metabolites in LLC-PK1, NHK-C, and EC with no scavenger (O), 10 mM DMPO, 10 mM DMTU, 100 μg/ml SOD (S), or 100 μg/ml catalase (C).* LLC-PK1 and NHK-C cells were exposed to 50 mU/ml xanthine oxidase for 1 hour while EC were exposed to 25 mU/ml xanthine oxidase for 1 hour. Hydrogen peroxide radical scavengers, DMTU and catalase significantly ($P < 0.01$) prevented ^{3}H-adenine metabolite efflux, while scavengers of superoxide dismutase and hydroxyl radical did not. Values represent the mean ± 1 SD of 3 replicates.

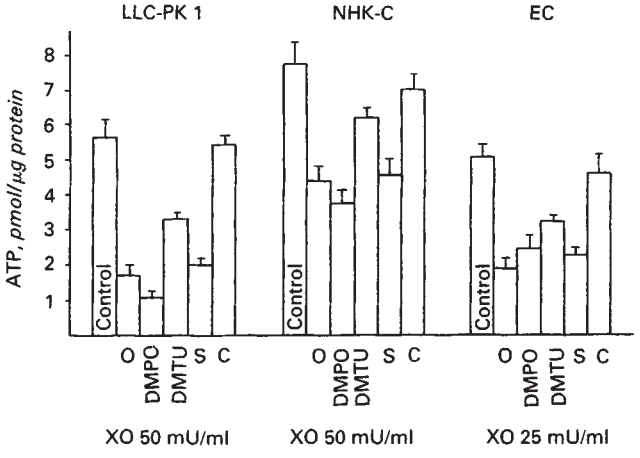

Fig. 5. *ATP levels in LLC-PK1, NHK-C and EC with no scavenger (O) 10 mM DMPO, 10 mM DMTU, 100 μg/ml SOD (S), or 100 μg/ml catalase (C).* LLC-PK1 and NHK-C cells were exposed to 50 mU/ml xanthine oxidase for one hour while EC were exposed to 25 mU/ml xanthine oxidase for 1 hour. Catalase resulted in significant ($P < 0.01$) protection while DMTU resulted in significant ($P < 0.05$) partial protection from ATP depletion. Values represent the mean ± 1 SD of 3 replicates.

DMTU, each scavengers of hydrogen peroxide (Fig. 4). DMPO and SOD did not prevent efflux of ^{3}H-adenine metabolites.

When similar experiments were performed with and without scavengers and ATP levels were measured in response to oxidant injury, a similar pattern was seen (Fig. 5). Catalase significantly ($P < 0.01$) prevented ATP depletion, DMTU resulted in significant ($P < 0.05$) partial protection while no protection was seen with DMPO or SOD. When endothelial cells were exposed to 25 mU/ml xanthine oxidase with 5.0 mM hypoxanthine, ATP levels fell to 1.9 ± 0.3 pmol/μg protein with no scavenger, 2.4 ± 0.7 pmol/μg protein with 10 mM DMPO, 3.1 ± 0.15 pmol/μg protein with 10 mM DMTU, 2.3 ± 0.3 pmol/μg

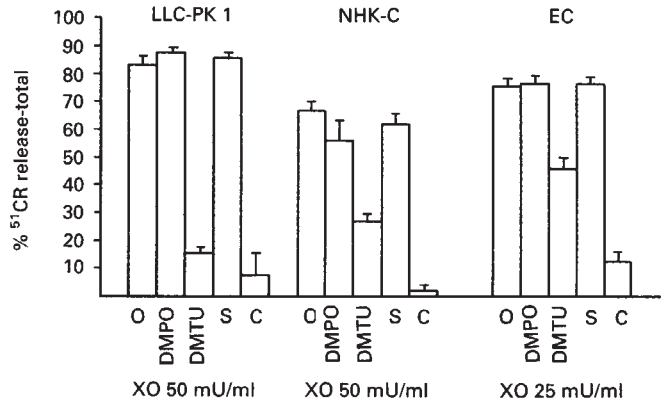

Fig. 6. *Total ^{51}chromium release from LLC-PK1, NHK-C and EC with no scavenger (O), 10 mM DMPO, 10 mM DMTU, 100 μg/ml SOD (S), and 100 μg/ml catalase (C).* LLC-PK1 and NHK-C were exposed to 50 mU/ml xanthine oxidase for five hours while EC were exposed to 25 mU/ml for 5 hours. The hydrogen peroxide scavengers, catalase and DMTU each significantly ($P < 0.01$) prevented total ^{51}chromium release while scavengers of superoxide anion and hydroxyl radical did not alter the ^{51}chromium release. Values represent the mean ± 1 SD of 3 to 4 replicates.

protein with 100 μg/ml SOD and 4.6 ± 0.7 pmol/μg protein with 100 μg/ml catalase while ATP levels were 5.1 ± 0.3 pmol/μg protein in cells not exposed to oxidant stress.

When ^{51}chromium release was determined with and without scavengers, again catalase resulted in significant ($P < 0.01$) protection and DMTU significant ($P < 0.01$) partial protection from total ^{51}chromium release, while scavengers of hydroxyl radical or superoxide anion did not prevent total ^{51}chromium release (Fig. 6). However, when cell detachment and lytic injury were considered separately, a different pattern was observed (Table 2). DMPO did not prevent cell detachment but did significantly ($P < 0.001$) protect LLC-PK1 and NHK-C cells from lytic injury and provided partial protection from lytic injury in EC (Table 2). When LLC-PK1 cells were exposed to 50 mU/ml xanthine oxidase, lytic injury was reduced to 10.2 ± 0.7% in the presence of 10 mM DMPO compared to 50.8 ± 1.7% without a scavenger. When NHK-C were exposed to 50 mU/ml xanthine oxidase, lytic injury was reduced to 11.4 ± 1.1% in the presence of 10 mM DMPO compared to 35.6 ± 1.9% without a scavenger. Since endothelial cells demonstrated less lytic injury than tubular cells, DMPO prevention of cell lysis was less impressive; lytic injury was 8.0 ± 3.0% with 10 mM DMPO compared to 13.2 ± 0.9% without a scavenger. Once a cell has progressed to lytic injury, determination of cell detachment is not possible as the cell is not intact and cannot be precipitated by centrifugation. Thus, the increase in cell detachment observed when cell lysis is prevented with DMPO is not actually due to increased injury due to cell detachment, but rather the ability to detect cell detachment when the cell is intact and precipitable by centrifugation.

Table 2. Total, cell detachment and lytic release of ^{51}chromium in LLC-PK1 cells, NHK-C and EC exposed to oxidant stress with no scavenger and with DMPO to trap hydroxyl radical, DMTU to scavenge hydrogen peroxide and hydroxyl radical, SOD to dismute superoxide anion and catalase to metabolize hydrogen peroxide

	% Specific ^{51}chromium release		
Cell line	Cell detachment	Lytic release	Total
LLC-PK1 *50 mU/ml*			
O	33.3 ± 0.2	50.8 ± 1.7	84.1 ± 1.5
DMPO 10 mM	77.9 ± 0.9	10.2 ± 0.7	88.1 ± 0.2
DMTU 10 mM	6.0 ± 0.1	9.0 ± 0.4	15.0 ± 2.8
SOD 100 μg/ml	33.4 ± 3.0	53.1 ± 3.0	86.7 ± 1.0
CAT 100 μg/ml	6.3 ± 6.1	1.8 ± 0.9	8.1 ± 7.0
NHK-C *50 mU/ml*			
O	31.8 ± 2.8	35.6 ± 1.9	67.6 ± 2.7
DMPO 10 mM	45.6 ± 4.1	11.4 ± 1.1	57.0 ± 5.1
DMTU 10 mM	17.4 ± 2.3	9.7 ± 0.3	27.1 ± 2.1
SOD 100 μg/ml	28.2 ± 2.2	34.2 ± 4.1	62.4 ± 3.0
CAT 100 μg/ml	0.4 ± 0.4	1.4 ± 0.3	1.8 ± 0.4
EC *25 mU/ml*			
O	63.5 ± 4.5	13.2 ± 0.9	76.7 ± 0.1
DMPO 10 mM	69.1 ± 3.6	8.0 ± 3.0	77.1 ± 0.5
DMTU 10 mM	37.7 ± 7.4	8.4 ± 2.7	46.1 ± 5.8
SOD 100 μg/ml	57.0 ± 3.8	18.3 ± 2.0	75.3 ± 0.3
CAT 100 μg/ml	3.8 ± 1.5	8.7 ± 1.9	12.5 ± 3.2

Values represent the mean + 1 SD of 3 to 4 replicates.

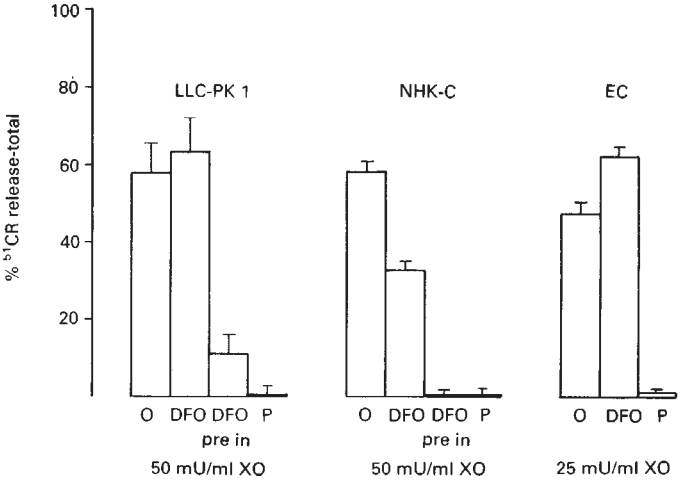

Fig. 7. *Total ^{51}chromium release in LLC-PK1, and NHK-C cells exposed to 50 mU/ml xanthine oxidase with 5.0 mM hypoxanthine for 5 hours with no iron chelator (O), 50 μM deferoxamine without preincubation (DFO), 50 μM deferoxamine preincubated for 16 hours to allow for intracellular incorporation (DFO-pre-in), or 50 μM phenanthroline, a readily membrane permeable iron chelator.* Endothelial cells were exposed to 25 mU/ml xanthine oxidase with 5.0 mM hypoxanthine for 5 hours with no iron chelator, 50 mM DFO and 50 μM phenanthroline. Phenathroline and preincubation with deferoxamine significantly ($P < 0.01$) protection cells from detachment and lysis. Moderate significant protection was also seen in NHK-C cells exposed to DFO without preincubation. Values represent the mean + 1 SD of 3 to 4 replicates.

Iron chelation studies

In each cell line, cell detachment and cell lysis was significantly ($P < 0.001$) prevented by phenanthroline and preincubation with deferoxamine (Fig. 7). In LLC-PK1 cells, ^{51}chromium release was 57.6 + 7.8% without an iron chelator, 63.8 + 8.4% with 50 μM deferoxamine, 11.3 + 9.8% in cells preincubation with 50 μM deferoxamine for 16 hours and −2.7 + 2.6% in cells exposed to 50 μM phenanthroline. In NHK-C cells, ^{51}chromium release was 58.7 + 1.6% without an iron chelator, 33.5 + 0.9%

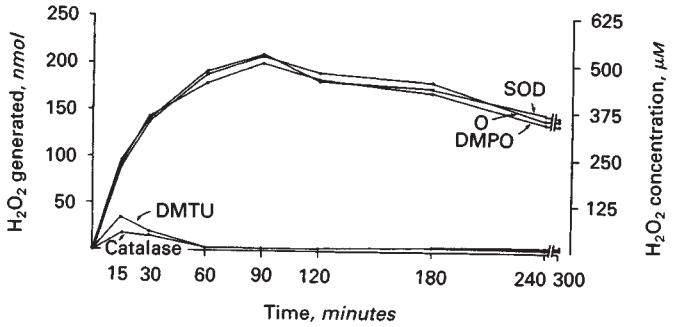

Fig. 8. *Nanomoles of hydrogen peroxide generated per well (400 μl) and μM concentration of hydrogen peroxide generated with 50 mU/ml xanthine oxidase and 5.0 mM hypoxanthine from 15 to 300 minutes, without (O) and with 100 μg/ml SOD, 10 mM DMPO, 10 mM DMTU, or 100 μg/ml catalase.* Both catalse and DMTU were efficient scavengers of hydrogen peroxide with catalase being slightly more efficient at 15 minutes. Values represent the mean of 2 replicates.

with 50 μM deferoxamine, 1.6 + 0.7% in cells preincubated with 50 μM deferoxamine for 16 hours, and 0.2 + 0.5% with 50 μM phenanthroline. In endothelial cells, ^{51}chromium release was 47.8 + 3.7% without an iron chelator, 63.8 + 3.1% with 50 μM deferoxamine, and 0.3 + 0.7% in cells exposed to oxidant injury with 50 μM phenanthroline. Thus, phenanthroline which is readily membrane permeable and preincubation with deferoxamine which is slowly membrane permeable prevented cell detachment and cell lysis. Deferoxamine without preincubation did not prevent ^{51}chromium release in LLC-PK1 cells and endothelial cell and resulted in significant ($P < 0.01$) partial protection from cell injury in NHK-C cells.

H_2O_2 assay

Since hydrogen peroxide and hydroxyl radical derived from hydrogen peroxide was found to be responsible for injury, and since hydroxyl radical is derived from hydrogen peroxide, we determined the amount of hydrogen peroxide generated with 50 mU/ml xanthine oxidase and 5 mM hypoxanthine with and without the scavengers. As shown in Figure 8, the amount of hydrogen peroxide generated was not altered in the presence of DMPO or SOD. Catalase and DMTU were each effective scavengers of hydrogen peroxide with catalase being more effective at the 15 minute time point.

Discussion

Several previous studies have investigated the mechanisms and response of endothelial cell monolayers to oxidant injury while very few studies have investigated the response of tubular epithelial cells to oxidant injury. Because tubular epithelial cells are likely to be exposed to oxidant stress in several disease processes [3–11], we studied oxidant-mediated renal tubular epithelial cell injury and, determined the relative response of epithelial cells including LLC-PK1, NHK-C, MDCK, OK and endothelial cells to oxidant injury generated with hypoxanthine and xanthine oxidase. We found that ATP levels declined and ^{3}H-adenine metabolites were released as an early response to oxidant injury, while cell detachment and lytic injury occurred several hours later. Among the epithelial cell lines, LLC-PK1 cells were the most susceptible to injury while MDCK cells demonstrated the lowest degree of ATP depletion, and efflux of ^{3}H-adenine metabolites and were resistant to cell detachment and lytic injury. Normal human kidney epithelial cells responded to oxidant stress with a fall in ATP levels, efflux of ^{3}H-adenine metabolites and late lytic injury to a slightly lower degree than LLC-PK1 cells. OK cells showed a similar pattern of response to oxidant stress but the magnitude of injury was less than NHK-C and LLC-PK1 cells. Thus, the tubular epithelial cell lines with proximal tubular characteristics were more sensitive to oxidant injury than MDCK cells. While the relative resistance of MDCK cells to oxidant injury may be related to its distal tubular characteristics, other possibilities including the species of origin and the adhesive qualities of MDCK cells are also likely to play a role [36]. Future studies of oxidant injury in an in vitro system will need to consider these marked differences and variable susceptibility to oxidant injury.

In a previous study of MDCK cells, transepithelial electrical conductance and permeability of the paracellular pathway increased when cells were exposed to reagent hydrogen peroxide directly or hydrogen peroxide generated with glucose-glucose oxidase [47]. Reagent hydrogen peroxide also caused some morphologic alterations including focal detachment and disruption of the normal pattern of the actin-cytoskeleton of MDCK cells. However, the concentrations of hydrogen peroxide used in that study ranged from 800 μM to 4000 μM compared to 50 to 550 μM concentrations used in this study [47]. Similarly, a previous study in LLC-PK1 cells found that reagent hydrogen peroxide and hydrogen peroxide generated with hypoxanthine-xanthine oxidase altered the cell membrane potential in LLC-PK1 cells [48]. Hydrogen peroxide concentrations of 500 μM resulted in a decline in membrane potential to 63.5% of control, while exposure to 80 mU/ml xanthine oxidase for 30 minutes resulted in a decline of membrane potential to 68.0% of control.

When the response of tubular cells to oxidant injury was compared to that of endothelial cells, we found that human endothelial cells suffered the greatest degree of injury as determined by efflux of ^{3}H-adenine metabolites, depletion of ATP, and cell detachment but were less susceptible to lytic injury. In general, endothelial cells were more susceptible to oxidant injury than the tubular epithelial cells. This increased vulnerability of endothelial cells to oxidant stress might explain their relative susceptibility to injury in some renal diseases thought to be mediated by oxidant injury [7, 49–51]. Because endothelial cells produce and release vasodilators such as endothelial-derived relaxing factor and vasoconstrictors such as endothelin, alterations in vascular tone and microvascular blood flow may result from oxidant-induced endothelial cell injury [52–54].

To determine the reactive oxygen molecules responsible for injury, we utilized superoxide dismutase to dismute superoxide anion, catalase to metabolize hydrogen peroxide, DMPO to trap hydroxyl radical, and DMTU to scavenge hydrogen peroxide and hydroxyl radical. DMTU was originally thought to be a specific hydroxyl radical scavenger without effects on other reactive oxygen molecules including hydrogen peroxide [55]. However, recent studies have questioned the specificity of DMTU as a hydroxyl radical scavenger by demonstrating that DMTU is an efficient scavenger of hypochlorous acid and hydrogen peroxide [33, 56]. In this study, we also found that DMTU is an efficient scavenger of hydrogen peroxide. DMTU concentrations of 10 mM was as effective as catalase in scav-

enging hydrogen peroxide at later time points and only slightly less effective than catalase at earlier time points. In this study with EC, NHK-C and LLC-PK1 cells, we found that scavengers of hydrogen peroxide (catalase and DMTU) protected the cells from ATP depletion, prevented release of ^{3}H-adenine metabolites and prevented cell detachment while scavengers of superoxide anion or hydroxyl radical had no effect. However, scavengers of hydroxyl radical nearly totally prevented lytic injury in LLC-PK1 cells and NHK-C cells and provided partial protection in endothelial cells.

To confirm the role of hydroxyl radical in cell injury, we used the iron chelators deferoxamine and phenanthroline to prevent iron catalyzed production of hydroxyl radical. We found that the membrane permeable iron chelator phenanthroline and preincubation with deferoxamine, which is slowly membrane permeable, but not deferoxamine in the absence of preincubation prevented cell detachment and cell lysis. Deferoxamine without preincubation confered partial protection from injury to NHK-C cells. The mechanisms for this response is unknown but may be related to more rapid penetration of DFO in NHK-C cells. Since hydrogen peroxide is freely membrane permeable, these findings suggest that extracellularly-generated hydrogen peroxide enters the cell and reacts with intracellular iron to form hydroxyl radical at an intracellular site. The intracellular generation of hydroxyl radical from extracellular hydrogen peroxide has recently been described in endothelial cells and P388D1 cells [43, 57]. In addition, these and other studies have demonstrated that the iron used to catalyze generation of hydroxyl radical is derived from an intracellular site in the target cell [43, 57, 58]. Interestingly, we found that cell lysis was prevented by DMPO (which traps hydroxyl radical once formed) and iron chelators (which prevent hydroxyl radical generation) while cell lysis but not cell detachment was prevented by DMPO. The mechanism for this differential response is unknown but may be related to their different mechanism of preventing hydroxyl radical injury. DMPO traps hydroxyl radical but does not totally eliminate lipid peroxidation [33] such that DMPO may be less efficient at preventing hydroxyl radical injury than iron chelation. In addition, deferoxamine also prevents the generation of perferryl and possibly ferryl ions [45], adding to the mechanisms of preventing cell injury by iron chelation. Thus, both hydrogen peroxide and hydroxyl radical derived from hydrogen peroxide resulted in cell injury; hydroxyl radical was responsible for lytic injury and contributed to cell detachment while hydrogen peroxide was responsible for ATP depletion, efflux of ^{3}H-adenine metabolites and contributed to cell detachment.

Previous in vivo and in vitro studies to determine specific reactive oxygen molecule(s) responsible for injury have yielded variable results [5–8, 11–14, 25]. Several in vivo studies of ischemic and nephrotoxic renal injuries suggest that hydroxyl radical derived from hydrogen peroxide mediates renal injury [6, 11, 59]. Some in vivo studies utilizing PMN's to generate reactive oxygen molecules suggest that hydrogen peroxide and hypochlorous acid generated from hydrogen peroxide mediate renal injury, while in vitro studies utilizing activated PMN's have suggested that hydrogen peroxide mediates endothelial cell injury [7–9, 12–14, 25]. Surprisingly, superoxide dismutase usually does not prevent injury [7, 8, 13–15]. The variable results obtained in these in vivo and in vitro studies may be related to the relative susceptibility of different cells to oxidant injury, to the different end points used to determine injury, and to the mechanism of generation of reactive oxygen molecules (that is, reperfusion, activated PMN's, toxic, etc.). It is likely that cell injury results from the interaction of all the reactive oxygen molecules generated.

The precise pathophysiologic mechanisms responsible for the differential response of the five cell lines to oxidant injury is unknown. While the species of origin may account for some of the differences, the cell type within a species also appears to be important, as demonstrated by the different responses seen in human endothelial and human renal tubular epithelial cells. Previous studies have shown the importance of the glutathione redox cycle and catalase as defense mechanisms against oxidant stress in endothelial cells and proximal tubular preparations [24, 25, 60]. Other intrinsic differences in the cells such as the composition of the cell membrane, and the distribution and number of enzymes that contain critical sulfhydryl groups are likely to play a role in the susceptibility of cells to oxidant stress.

In conclusion, we demonstrate that endothelial cells and renal tubular epithelial cells respond to injury mediated by reactive oxygen molecules with an early decline in ATP levels and efflux of ^{3}H-adenine metabolites, while cell detachment and lytic injury are late responses to oxidant stress. Among the tubular epithelial cells, cells with proximal tubular characteristics, including LLC-PK1, NHK-C and OK cells, were more susceptible to oxidant injury than MDCK cells. Endothelial cells were more susceptible to ATP depletion and cell detachment than tubular cells, while LLC-PK1, and NHK-C cells were more susceptible to lytic injury. Finally, scavengers of hydrogen peroxide prevented efflux of ^{3}H-adenine metabolites, depletion of ATP levels and cell detachment, while scavengers of hydroxyl radical and iron chelators prevented lytic injury and cell detachment.

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