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Decreased expression of mitochondrial-derived H_2O_2 and hydroxyl radical in cytoresistant proximal tubules

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Decreased expression of mitochondrial-derived H_2O_2 and hydroxyl radical in cytoresistant proximal tubules. Increased production of reactive oxygen metabolites (ROM) can contribute to the initiation phase of nephrotoxic and ischemic acute renal failure (ARF). However, whether altered ROM expression also exists during the maintenance phase of ARF has not been adequately assessed. Since diverse forms of tubular injury can initiate a "cytoresistant state," this study tested whether a down-regulation of ROM expression might develop in the aftermath of acute tubular damage, potentially limiting renal susceptibility to further attack. To test this hypothesis, rats were subjected to either mild myohemoglobinuria (glycerol injection) or bilateral ureteral obstruction and 24 hours later, cytoresistant proximal tubular segments (PTS) were isolated to assess ROM expression. PTS from sham operated rats were used to establish normal values. Both sets of cytoresistant PTS manifested ~75% reductions in H_2O_2 levels, as assessed by the phenol red/horseradish peroxidase technique ($P < 0.01$ to 0.001). A 40% reduction in hydroxyl radical ($^{\bullet}OH$) levels was also observed (salicylate trap method), thereby substantiating decreased oxidant stress in cytoresistant PTS. Catalase, glutathione peroxidase, and free iron levels were comparable in control and cytoresistant PTS, suggesting that decreased H_2O_2 production (such as by mitochondria) was the cause of the decreased oxidant stress. To test this latter hypothesis, H_2O_2 expression by control and cytoresistant PTS was assessed in the presence of respiratory chain inhibitors. Although site 1 and site 3 inhibition markedly suppressed H_2O_2 production in control PTS, they had no impact on H_2O_2 production in cytoresistant PTS, implying that production at these sites was already maximally suppressed. Correlates of the decreased mitochondrial H_2O_2 production were improvements in cell energetics (increased ATP/ADP ratios with Na ionophore treatment) and ~40 to 90% increases in PTS/renal cortical glutathione content. We conclude that: (*1*) proximal tubule $H_2O_2/^{\bullet}OH$ expression can be down-regulated during the maintenance phase of ARF; (*2*) this seemingly reflects a decrease in mitochondrial ROM generation; and (*3*) the associated improvements in glutathione content and/or cellular energetics could conceivably contribute to a post-injury cytoresistant state.

Following the induction of experimental acute ischemic or toxic renal injury, the kidney acquires a modicum of resistance to further attack [1]. If acute renal failure (ARF) already exists, this phenomenon can protect against additional functional and structural deterioration [2]. Conversely, if the initial insult produces only subclinical renal damage, complete protection against *de novo* ARF may result (such as [3]). Previous research has delineated the following manifestations of this "acquired resistance" state: (*a*) it can be triggered by diverse forms of renal damage (such as, ischemia [2, 4], nephrotoxins [1], endotoxemia [5, 6], heat stress [4, 7, 8], urinary tract obstruction [9]); (*b*) an 18 to 24 hour "lag time" following the first insult generally must elapse prior to its emergence [2, 10, 11]; (*c*) once established, the protection is nonspecifically expressed (that is, "cross tolerance" exists, with one form of injury protecting against another [1, 9, 11, 12]); (*d*) it has a direct cellular, rather than a hemodynamic, basis, since decreased proximal tubular necrosis results [1–7, 9–12]; and (*e*) it is probably not a kidney specific state; rather, it may represent a generic consequence of the "stress response" that serves to protect animals, individual organs, and diverse cell types from recurrent damage [13–17].

Although many of the characteristics of acquired cytoresistance have been elucidated, the responsible subcellular determinants of this state have been difficult to define. In part, this is because this phenomenon probably has a multifactorial basis. Since the stress response confers a survival advantage, it is likely that evolutionary redundancy in effector mechanisms has developed [13–17]. Elucidation of protective mechanisms has been further complicated by the fact that differing pathways may be operative depending on the organ, cell type, or form of injury involved. For example, although diverse forms of renal injury can up-regulate the expression of heme oxygenase and ferritin [6], these cytoprotective molecules cannot explain all forms of the cytoresistant state (such as that which follows ischemic renal damage [2, 18]). Similarly, although the heat shock response can protect tubular cells against *in vitro* ATP depletion [4, 7], it does not mitigate *in vivo* ischemic ARF [8]. These observations indicate that no single molecule or mechanism is responsible for all forms of the acquired cytoresistance phenomenon.

Previous work from our laboratory has explored a variety of potential mechanisms of renal tubular cytoresistance by using a combined *in vivo/in vitro* approach. Divergent forms of renal injury have been induced *in vivo* (such as ischemia, myohemoglobinuria, or urinary tract obstruction), and 18 to 24 hours later, proximal tubule segments (PTS) have been isolated for *in vitro* investigation [4, 9, 11, 12]. These studies have demonstrated that irrespective of the nature of the *in vivo* insult, the subsequently isolated proximal tubules manifest resistance to diverse forms of attack (such as hypoxia, mitochondrial respiratory inhibitors, Ca^{2+} overload, oxidant stress, PLA_2). A fundamental feature of this cytoresistance is a primary increase in plasma membrane

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resistance to PLA_2- and oxidant injury [10–12]. However, since no single mechanism likely explains all forms of acquired cytoresistance, it is important to explore additional protective pathways.

A number of recent reports have incriminated H_2O_2 as a critical determinant of several types of renal tubular damage. For example, Guidet and Shah have demonstrated increased H_2O_2 production in response to myohemoglobinuria [19], a result that can be mechanistically linked to the evolution of lethal tubular cell injury [20, 21]. Similarly, Nath et al have demonstrated that H_2O_2 helps mediate $HgCl_2$-induced tubular cell death [22]. Finally, H_2O_2 and/or its downstream product hydroxyl radical, have been implicated as determinants of cisplatin toxicity [23, 24] and aminoglycoside-induced ARF [25]. Since acquired cytoresistance develops against these forms of injury [1, 3, 24, 26, 27], a down-regulation of H_2O_2/•OH expression could potentially be involved.

Given these considerations, the goal of the present study was to assess whether the cytoresistant state might be associated with a down-regulation of H_2O_2 and/or •OH expression. Towards this end, PTS have been isolated from kidneys in which cytoresistance was induced by divergent means (myohemoglobinuria, urinary tract obstruction [9, 12]), and then H_2O_2 and •OH expression were assessed. Since substantial decrements in these reactive oxidants were observed, additional experiments were undertaken to help delineate the basis for this result.

METHODS

In vivo protocols

Male Sprague-Dawley rats (150 to 250 g; Charles River Laboratories, Wilmington, DE, USA), housed under standard vivarium conditions with free food and water access, were used for all experiments. *In vivo* protocols were conducted under pentobarbital anesthesia (30 to 40 mg/kg), administered by intraperitoneal injection.

Myohemoglobinuric renal injury. After induction of anesthesia, rats received an intramuscular injection of 50% glycerol (7 ml/kg; administered in equally divided doses into each upper hind limb). This dose of glycerol was chosen because it induces consistent myohemoglobinuria (rhabdomyolysis/hemolysis) but of insufficient degree to produce renal failure (**Results**). Twenty-four hours following glycerol injection, the rats were re-anesthetized, a midline laparotomy was performed, a 1 ml blood sample was obtained from the abdominal aorta for measurement of BUN and plasma creatinine, and then both kidneys were immediately resected for isolation of proximal tubular segments (PTS), as described below. In each instance, a paired rat subjected to the same experimental procedure, excepting glycerol injection, served as a control.

Obstructive nephropathy. Rats were anesthetized, a midline abdominal incision was made, and both ureters were isolated and ligated at their midpoints with 2.0 silk ligatures. Then, the abdominal wall was sutured in two layers and the rats were allowed to recover from anesthesia. Twenty-four hours later, they were re-anesthetized, a blood sample was obtained for BUN and creatinine, and the kidneys were immediately resected for PTS isolation, as described below. With each experiment, a paired rat, subjected to sham bilateral ureteral ligation, served as a control.

Isolated proximal tubule segment preparation

Proximal tubular segments (PTS) were isolated from the above experimental and control rats using a previously described technique [4]. This method has previously been shown to produce highly comparable (qualitative/quantitative) proximal tubule yields 24 hours following the described *in vivo* protocols [4, 9, 11]. In brief, following kidney resection, the kidneys were cooled on an iced plate, the cortices were dissected, minced, and digested for 30 minutes with a collagenase containing buffer. Following digestion, the tissues were washed twice and then centrifuged through 32% Percoll to isolate viable PTS. The pelleted tubules were washed × 2, and suspended in experimentation buffer (in mmol/liter: NaCl, 100; KCl, 2.1; $NaHCO_3$, 25; KH_2PO_4, 2.4; $MgSO_4$, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 1.2; glucose, 5; alanine, 1; Na lactate, 4; Na butyrate, 10; dextran, 0.6%; oxygenated with 95% O_2/5% CO_2; final pH 7.4). After completing a 15 minutes rewarming period at 36 to 37°C, each PTS preparation was reoxygenated and split into 2 to 6 equal aliquots, depending on the experiment. The aliquots were placed into 25 ml siliconized Erlenmeyer flasks (2.5 ml PTS suspension; ~2 to 4 mg PTS protein/ml). All experiments were performed in a heated shaking water bath maintained at 36 to 37°C. In every instance, one experimental PTS preparation (post-glycerol; urinary obstruction) and one control PTS preparation were studied simultaneously. The following specific protocols were undertaken.

H_2O_2 production in the aftermath of myohemoglobinuric and obstructive injury

PTS were obtained from 5 rats 24 hours post-glycerol injection, from 4 rats 24 hours post-bilateral ureteral obstruction, and from their sham matched controls. After completing the 15-minute rewarming period, an aliquot was removed to assess baseline tubule viability (% lactic dehydrogenase, LDH, release). The preparations were then divided into two or three equal aliquots which were incubated for 30 minutes to assess H_2O_2 production rates [as determined by the phenol red/horseradish peroxidase (HRP) method, as previously described] [21, 28]. In brief, this method is based on the principle that HRP uses H_2O_2 as a substrate to oxidize phenol red, a reaction which results in a linear H_2O_2-dependent increase in phenol red absorbance (monitored at 610 nm). Phenol red (264 μM) and HRP (2.2 μM; from Sigma) were added to each of the PTS aliquots at the start of the 30 minute incubations. Upon their completion, a 150 μl suspension sample was removed to assess cell viability (% LDH release), and then the H_2O_2/HRP/phenol red reaction was quenched by addition of 100 μl 1 N NaOH. The aliquots were centrifuged and the absorbance of each of the supernatants was determined. H_2O_2 concentrations were determined from a standard curve constructed by exogenous H_2O_2 addition (0, 10, 20, 30, 40, 50 μM) to PTS buffer. The specificity of this reaction for H_2O_2 detection in PTS suspensions has been confirmed by quenching with exogenous catalase addition [21].

Assessment of whether cell injury nonspecifically alters H_2O_2 expression

Although no difference in cell viability (LDH release) was observed between control, glycerol, and obstructed tubules under unchallenged conditions (**Results**), a theoretical possibility is that the cytoprotected tubules sustained less sublethal injury during the isolation procedure, and that this theoretical difference might account for differences in reactive oxygen species formation. To help exclude this possibility, H_2O_2 generation by normal and

sublethally damaged PTS were compared. PTS were harvested from 5 normal rats and each preparation was divided into five aliquots: (*1*) control incubation × one minute; (*2*) control incubation × 20 minutes; (*3*) 20 minutes of incubation with 7.5 U/ml porcine pancreatic PLA_2 (Sigma; p 6534); (*4*) 20 minutes of incubation with a sublytic dose of a cytotoxic fatty acid (arachidonic acid; 30 μM, added in 0.5% ethanol); and (5) 20 minutes of incubation with 10 μg/ml of amphotericin B (Pharma-Tek, Huntington, NY, USA). At the end of the incubations, H_2O_2 generation and % LDH release were assessed.

Assessments of catalase, glutathione peroxidase, glutathione, and free iron content

The following experiments sought to determine whether enhanced concentrations of catalase, glutathione peroxidase, glutathione, or reduced free iron content might contribute to decreased H_2O_2 levels in cytoprotected PTS (**Results**). To this end, a total of 31 cytoresistant PTS preparations were prepared (from 14 rats subjected to glycerol treatment and 17 from rats with urinary tract obstruction). Simultaneously prepared PTS from 31 sham treated rats served as controls. Each preparation was used for a single biochemical endpoint (other than LDH release) as follows:

Catalase activity. PTS were obtained from obstructed kidneys, glycerol-treated rats and their corresponding controls (N = 4 each). After completing the 15-minute rewarm period they were assayed for catalase. Samples were lysed by treatment with 1% Triton 100X followed by freeze-thawing. Catalase activity was determined by a previously described enzymatic method (disappearance rate of exogenous 10 mM H_2O_2 following PTS sample addition; H_2O_2 monitored spectrophotometrically at 240 nm) [9, 29]. Values were expressed as U/mg PTS protein, based on the rate of H_2O_2 disappearance using a 500 U/ml standard (# C-40; Sigma Chemical Co., St. Louis, MO, USA), as previously described [29].

Glutathione peroxidase activity. PTS were obtained from rats subjected to glycerol injection, ureteral obstruction, or from their corresponding controls (N = 3 each). After completing the rewarm period, PTS samples were suspended in 0.25 M sucrose, homogenized at 4°C × 10 seconds, and subjected to ultracentrifugation (54,000 rpm, 4°C × 60 min). The supernatants were assayed for glutathione peroxidase by the method of Lawrence and Burk [30]. Results were expressed as μmoles NADPH oxidized per min per mg PTS protein.

Free iron content. PTS were prepared from three sets of post-glycerol treated rats, five rats with urinary tract obstruction, and from their sham treated controls. The samples were incubated for 20 minutes under oxygenated conditions, and then they were subjected to homogenization at 4°C. The samples were diluted 1:1 with deionized filtered water, followed by free iron assay using the Ferrozine technique of Carter [31], as modified by Atamna and Ginsburg [32]. Iron concentrations were calculated from a standard curve constructed with a free iron standard (0, 1, 2, 5, 10, 20, 40, 100 μM; Sigma; # C976-50). All reagents were pre-treated with Chelex-100 to remove contaminating iron.

Assessments of glutathione content. PTS were prepared from the following: 5 rats with bilateral ureteral obstruction, 4 rats with prior glycerol injection, and from an equal number of sham operated controls. The PTS were incubated for 20 minutes under control oxygenated conditions, and then the pelleted tubules were extracted in 200 μl of 5% trichloroacetic acid in 2 mM EDTA. Reduced glutathione (GSH) concentrations were estimated with Ellman's reagent [33, 34]. This reagent detects total nonprotein sulfhydryl (NP-SH) groups (GSH and cysteine), with GSH comprising ≥ 90% of the total signal in PTS (R. Zager, unpublished data). A standard curve was constructed with 0, 125, 250, 500, 1000, and 2000 μM reduced GSH standards (from Sigma; #G6529).

To confirm that the GSH alterations observed in the cytoprotected PTS had an *in vivo* correlate, whole renal cortical tissues were obtained from 24-hour post-glycerol rats and from controls (N = 3 each). The tissues were homogenized, extracted, and assayed as noted above.

The following experiment was undertaken to ascertain whether the observed differences in PTS GSH content (**Results**) could explain the observed differences in H_2O_2 expression. Four rats were injected (i.p.) with 4 mmol/kg of L-buthionine-(S,R)-sulfoximine (BSO: an inhibitor of glutathione synthesis). Two hourrs later, PTS were extracted and incubated over 30 minutes to assess H_2O_2 levels. Four sets of control rats studied simultaneously served as controls. One aliquot of each preparation was assayed for GSH to confirm that BSO had reduced PTS GSH levels.

Assessment of mitochondrial contribution to altered H_2O_2 generation rates

The following experiments were undertaken to ascertain the mitochondrial contribution to H_2O_2 production in normal and cytoresistant tubules. PTS were isolated from post-glycerol rats (N = 4), ureteral obstruction rats (N = 4), and from their simultaneous controls. Each preparation was divided into five equal aliquots as follows: (*1*) one-minute control incubation; (*2*) a 20-minute control incubation; (*3*) 20 minutes of incubation with a site 1 mitochondrial respiratory inhibitor (rotenone; 7.5 μM); (*4*) 20 minutes of incubation with a site 2 respiratory chain inhibitor (antimycin A, 7.5 μM); and (*5*) 20 minutes of incubation with a site 3 respiratory chain inhibitor (Na cyanide, CN; 5 mM). Antimycin and rotenone were added from an ethanol stock solution (final ethanol concentration, 0.1%). A comparable amount of ethanol was added to each of the other aliquots to maintain identical incubation conditions (although the ethanol was not shown to exert an independent effect). The incubations were conducted in the presence of phenol red and HRP (see above). After completing the incubations, % LDH release and H_2O_2 production were assessed.

Assessment of PTS/mitochondrial hydroxyl radical production rates

A decrease in H_2O_2 within cytoresistant tubules could result in decreased hydroxyl radical (•OH) production. Thus, if the latter could be documented, it would support the concept of decreased reactive oxidant/H_2O_2 levels in cytoprotected PTS. To this end, PTS were obtained from post-glycerol and ureteral ligation rats and from their corresponding controls (N = 4 each). Each PTS preparation was divided into five equal aliquots, as described above. However, 2 mM Na salicylate (rather than HRP/phenol red) was added to assess •OH production. (Salicylate serves as a hydroxyl radical trap, 2,5 dihydroxybenzoic acid formation, DHBA, being the result [35, 36]). At the completion of the

incubations, % LDH release was ascertained. The remainder of each aliquot was centrifuged and the supernatants were assayed for 2,5-DHBA by HPLC [35, 36]. The specificity of this technique for assessing •OH levels has previously been determined by the suppression of DHBA production with •OH scavengers, but not with catalase or superoxide dismutase [35, 36].

Assessment of cellular energetics

Since the above experiments suggested that cytoprotected tubules had decreased expression of mitochondrial-derived reactive oxygen species (**Results**), experiments were conducted to ascertain whether this might be reflective of a generalized improvement in mitochondrial function and/or in cellular energetics. To this end, PTS were obtained from 5 rats which were either 24-hour post-glycerol treatment or urinary tract obstruction. An equal number of sham treated rats provided controls. Each PTS preparation was divided into 4 aliquots and incubated for 30 minutes under either: (*1*) unchallenged (normal) incubation conditions; or (*2 to 4*) in the presence of different sublytic doses of amphotericin B (2.5, 5.0, or 10 μg/ml of PTS suspension) to drive Na,K-ATPase activity [37]. After completing the 30 minute incubations, a sample of each aliquot was removed to determine % LDH release, and the remainder was used for adenine nucleotide assessments, as previously described [37]. Cellular capacity for ADP → ATP conversion was estimated by determining ATP/ADP ratios [37].

Increased ATP/ADP ratios were observed in the cytoprotected tubules (**Results**). The following experiments were undertaken to assess whether this improvement was due to improved mitochondrial function versus decreased Na,K-ATPase activity or increased glucose utilization/glycolytic ATP production. Four sets of PTS were obtained from control and 24-hour post-glycerol treated rats, each preparation was divided into three aliquots and incubated for 30 minutes under the following conditions: (*1*) control incubation; (*2*) incubation in the presence of 1 mM ouabain (to equalize Na,K-ATPase activity for the two PTS groups); or (*3*) incubation in a glucose free medium that contained 10 mM 2-deoxyglucose (conditions designed to eliminate glycolytic support of ATP production). ATP/ADP ratios were determined at the end of the incubation period.

Oxygen consumption experiments

The following experiment was undertaken to ascertain whether an alteration in tubule oxygen consumption is a correlate of decreased reactive oxygen species formation in cytoprotected tubules. Three sets of PTS were obtained from post-glycerol and control rats and they were used to assess total oxygen consumption (QO_2) and Na,K-ATPase dependent respiration (1 mM ouabain addition). These assessments were performed by transferring tubule aliquots to an oxygen monitoring chamber followed by measurement of O_2 consumption for three minutes both before and after ouabain addition [37]. Values were expressed as nmoles $O_2 \cdot min^{-1} \cdot$ mg PTS protein^{-1}.

Calculations and statistics

All values are presented as means ± 1 SEM. Biochemical values were expressed per mg of PTS protein, unless stated otherwise. Statistical comparisons were performed by either paired or unpaired Student's *t*-test, as appropriate. If multiple comparisons were made, the Bonferroni correction was applied.

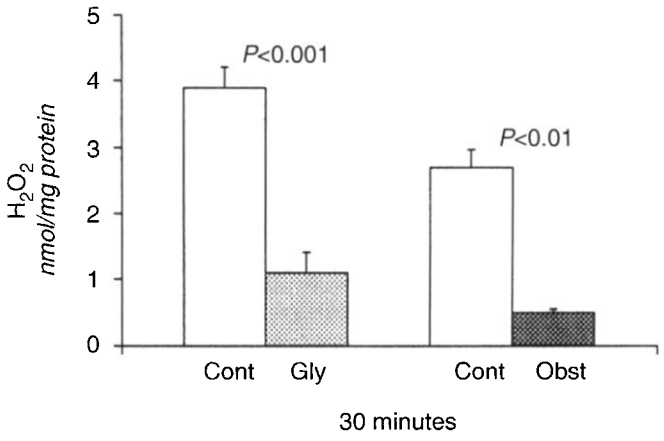

Fig. 1. H_2O_2 expression by control (Cont), post-glycerol (Gly), and obstructed (Obst) tubules during control incubations. An approximate 75% suppression of H_2O_2 levels was noted in both sets of cytoresistant PTS as assessed over the 30 minute incubations.

RESULTS

Degrees of azotemia following *in vivo* protocols

Only slight azotemia was noted by the 24-hour post-glycerol injection (BUN 27 ± 2 mg/dl, creatinine 0.54 ± 0.08 mg/dl vs. controls, 17 ± 1 and 0.30 ± 0.01, respectively; $P < 0.001$; composite values for all experiments). In contrast, severe renal failure resulted from bilateral ureteral obstruction (BUN 133 ± 6, creatinine 3.5 ± 0.2, vs. controls, 19 ± 2 and 0.31 ± 0.3; $P < 0.0001$; composite values).

H_2O_2 production by cytoresistant tubules

Over the course of 30-minute control incubations, normal PTS manifested ~4 nmoles H_2O_2 production/mg PTS protein, compared to just 1 nmole H_2O_2/mg protein for the post-glycerol PTS (Fig. 1, left; $P < 0.001$). A comparable decrement in H_2O_2 expression (~75 to 80%) was also noted in the obstructed PTS (Fig. 1, right). These differences were observed despite highly comparable PTS viability, as denoted by % LDH release at both baseline and at the end of the 30 minute incubations (baseline values: glycerol/controls, 5 ± 0%/5 ± 1%; obstruction/controls, 10 ± 1%/7 ± 0%; at 30 min: glycerol/controls, 6 ± 1%/7 ± 1%; obstruction/controls, 10 ± 1%/8 ± 1%; all NS).

H_2O_2 generation by sublethally damaged PTS

As shown in Figure 2, sublethal cell injury induced by either PLA_2, arachidonic acid (C20:4), or amphotericin B did not increase H_2O_2 production. Rather, each of the challenges caused a slight, albeit nonsignificant, reduction in H_2O_2 generation rates. Each of these challenges induced only sublethal injury (the goal of the experiment; 9 to 11% LDH release for each group).

Catalase, gluthathione peroxidase, and free iron content

Neither catalase nor glutathione peroxidase activity was elevated in either set of cytoprotected PTS, compared to the sham treated controls (Fig. 3). Free iron levels were identical in the control and post-glycerol tubules. The obstructed tubules manifested a slight depression in free iron levels, compared to their

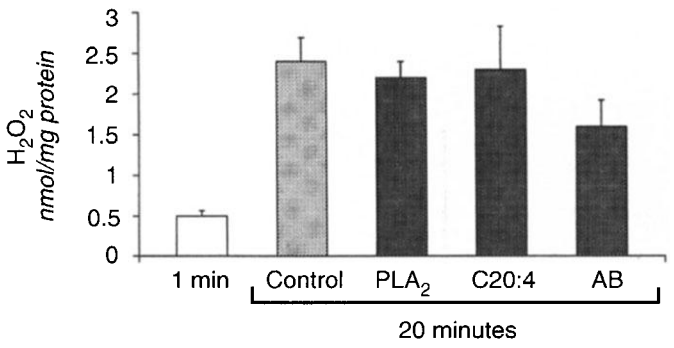

Fig. 2. Cell injury does not non specifically increase H_2O_2 production. Normal tubules maintained under control incubation conditions manifested progressive H_2O_2 generation from 1 to 20 minutes. The amount of H_2O_2 produced was not increased by sublytic doses (no increased LDH release) of PLA_2, arachidonic acid (C20:4), or amphotericin B (AB) addition (all NS vs. 20 min control, cont, values). This indicates that increased H_2O_2 production is not simply a nonspecific consequence of sublethal cell injury.

sham operated controls. However, this may have reflected increased iron expression in the sham operated controls, rather than a decrease in the obstructed tubules, since the iron levels observed in the latter were identical to those observed in the glycerol controls (that is, no sham surgery).

GSH status/BSO effects

In contrast to the above, NP-SH (GSH) levels were significantly elevated in both the post-glycerol and obstructed PTS, compared to their sham treated controls (~90% and 48% increments, respectively; $P < 0.05$; Fig. 4A). This GSH increment was not simply confined to isolated PTS, since a significant increase in GSH content was also observed in whole renal cortical homogenates obtained from post-glycerol rats versus controls (3.5 ± 0.4 vs. 2.6 ± 0.4 μmol/g wet wt; $P < 0.005$).

Pretreating rats with BSO two hours prior to PTS isolation produced a 46 ± 8% reduction in PTS GSH content below control values ($P < 0.025$; not depicted). However, this GSH decrement did not significantly increase PTS H_2O_2 levels (Fig. 4B). This suggests that differences in GSH levels in control versus cytoprotected PTS did not account for the differences in H_2O_2 expression.

Impact of mitochondrial respiratory chain inhibitors on H_2O_2 production

Post-glycerol PTS. Figure 5 presents H_2O_2 production by control and post-glycerol PTS over the course of 20 minute incubations in the absence and presence of site 1 (rotenone), site 2 (antimycin A), or site 3 (Na cyanide) respiratory chain inhibitors. The control PTS (Fig. 5A) demonstrated progressive H_2O_2 generation, with values rising seven- to eightfold over the course of the 1 → 20 minutes incubations. Antimycin A (AA) had no significant impact on this process (H_2O_2 generation not significantly different vs. control incubation). When rotenone (rot) or cyanide (CN) were added to control PTS, ~67% and ~85% reductions in H_2O_2 generation resulted.

The post-glycerol PTS showed virtually no H_2O_2 generation over the course of the 20 minute incubations. When rotenone and cyanide were added to these post-glycerol PTS, no significant decrease in H_2O_2 expression occurred (presumably because mitochondrial H_2O_2 production was already maximally suppressed).

Obstructed PTS. Figure 6 presents H_2O_2 generation by control and obstructed PTS in the presence and absence of mitochondrial respiratory chain inhibitors. In essence, the results of these experiments recapitulated those discussed above: (*1*) the control PTS manifested progressive H_2O_2 production over the 20 minute incubations; (*2*) control PTS H_2O_2 levels were substantially reduced by rotenone and CN, but not by antimycin A addition; (*3*) the obstructed tubules had markedly reduced H_2O_2 expression, compared to the controls; and (*4*) no further decrement could be obtained with rotenone or CN addition.

PTS •OH generation with and without mitochondrial respiratory chain inhibitors

Post-glycerol PTS. Figure 7 depicts •OH production for the control and post-glycerol PTS, as estimated by 2,5-DHBA formation from salicylate. The control PTS manifested brisk DHBA generation, rising to ~50 pmol/mg protein within one one minute and then increasing to ~135 pmol/mg protein by the completion of the 20 minute incubations. PTS obtained from the glycerol treated rats manifested an approximate 40% suppression in DHBA formation compared to the controls, noted at the completion of the 20-minute incubation period ($P < 0.035$). Site 1 (rot), site 2 (AA), and site 3 (CN) respiratory inhibitors caused stepwise reductions in DHBA generation in both the control and post-glycerol tubules. Co-incident with this was a progressive narrowing of the difference in DHBA levels between the control and post glycerol PTS. In the presence of the site 3 inhibitor (CN), no significant difference in DHBA generation was observed for the control and post-glycerol PTS (suggesting that the differences in DHBA production for the two groups likely reflected differences in site 3 •OH formation rates).

Obstructed PTS. Figure 8 depicts DHBA production by the control and obstructed PTS ± mitochondrial respiratory chain inhibitors. These experiments yielded the following salient results (essentially reproducing those described directly above): (*1*) the obstructed PTS had significantly reduced DHBA production rates versus the control PTS (statistically significant after both 1 and 20 min of control incubation); (*2*) the mitochondrial respiratory inhibitors reduced DHBA generation in the control tubules (indicating that DHBA production in large part reflects mitochondrial •OH generation); and (*3*) each of the mitochondrial inhibitors eliminated the differences in DHBA generation for control versus obstructed PTS.

LDH release for control and experimental PTS

Figures 9 and 10 present composite LDH release results obtained in the above mitochondrial inhibition experiments. Figure 9 demonstrates virtually identical % LDH release values for the glycerol tubules and their sham controls under unchallenged conditions (suggesting the same degree of cell viability under unchallenged conditions). When the mitochondrial inhibitors were applied, significantly less LDH release was observed in the post-glycerol PTS (confirming that the cytoresistance phenomenon was intact in the tubules used to determine H_2O_2/•OH expression). Figure 10 demonstrates these same observations with the obstructed PTS: (*a*) identical % LDH release for obstructed

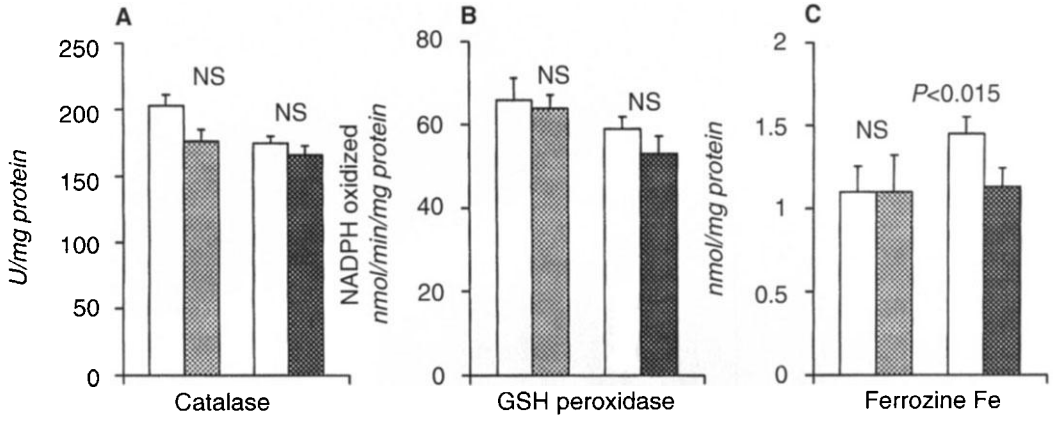

Fig. 3. Catalase (*A*), glutathione (GSH) peroxidase (*B*), and free (Ferrozine) iron (*C*) content in PTS harvested from control (□), post-glycerol (■), and obstructed PTS (▩). Catalase and GSH peroxidase levels did not statistically differ for the different PTS groups. Iron levels were identical for the post-glycerol PTS and their controls. Conversely, iron was slightly lower in the obstructed PTS versus their sham operated controls. However, this appeared to reflect an increase in the sham operated tubules, since the obstructed PTS manifested essentially identical values to the control values established for the post-glycerol PTS.

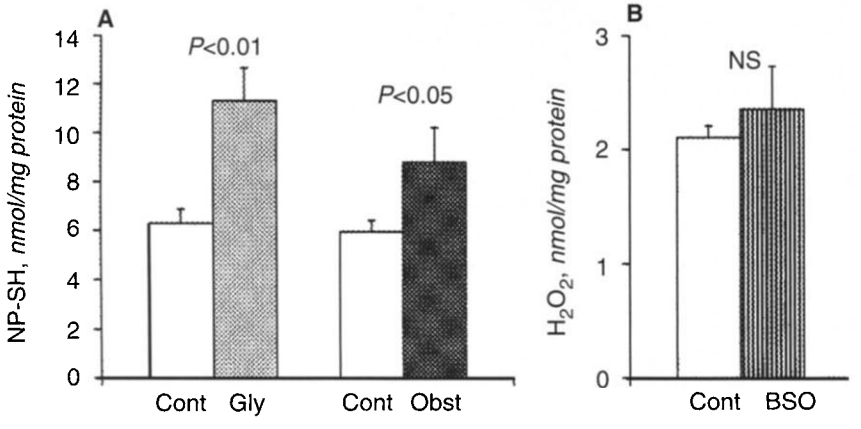

Fig. 4. (*A*) Assessments of glutathione (GSH) status in control and cytoresistant tubules, as determined by total non-protein thiol assay (NP-SH). The post-glycerol (gly) and obstructed (obst) PTS manifested ~90% and ~50% GSH increments, respectively. (*B*) This increase in GSH levels did not appear to be the cause of the reduced H_2O_2 levels in the cytoresistant tubules, since inducing a 50% difference in tubule GSH levels (± BSO treatment) did not significantly impact tubular H_2O_2 expression.

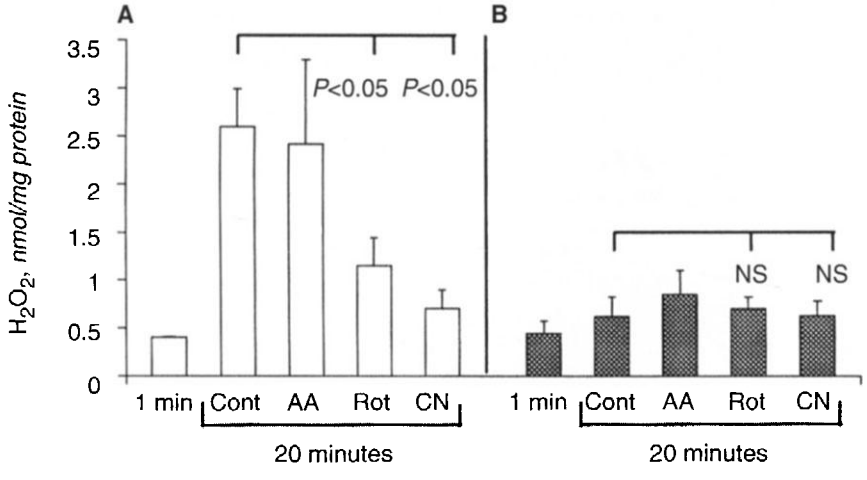

Fig. 5. Impact of mitochondrial respiratory chain inhibitors on H_2O_2 production by control (*A*) and post-glycerol (*B*) PTS. Normal tubules (left panel) showed marked increases in H_2O_2 over time (from 1 min to 20 min of incubation). Site 2 inhibition with antimycin A (AA) did not affect this result. However, site 1 and site 3 inhibition (with rotenone, Rot; and cyanide, CN, respectively) caused marked H_2O_2 decrements. In contrast, neither rotenone nor cyanide reduced H_2O_2 production in the post glycerol PTS, presumably because site 1 and site 3 H_2O_2 generation were already maximally suppressed as a consequence of the cytoresistant state.

and control PTS under unchallenged conditions; and (*b*) significantly less LDH release from the obstructed versus control tubules in the presence of mitochondrial inhibitors (confirming cytoresistance in the obstructed PTS).

Assessments of cellular energetics

A correlate of the decreased H_2O_2 expression in cytoprotected tubules was an improvement in cellular energetics (Fig. 11). The

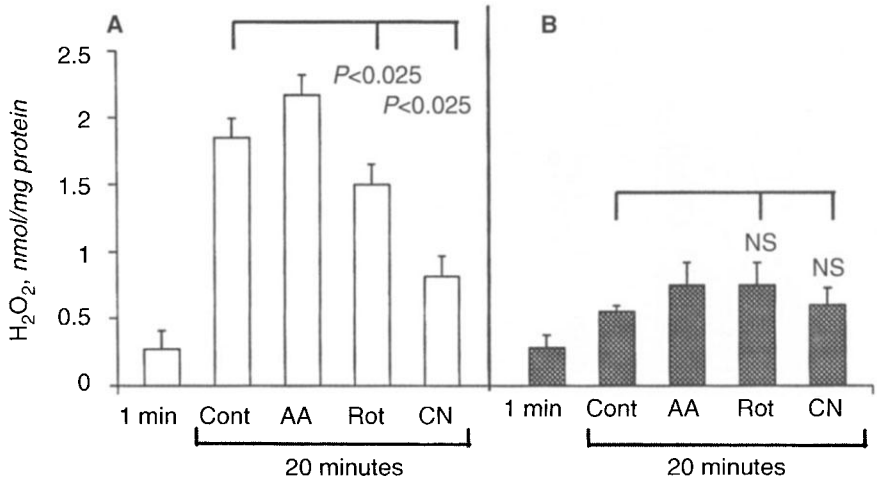

Fig. 6. Impact of mitochondrial respiratory chain inhibitors on H_2O_2 production by control (*A*) and obstructed (*B*) PTS. The results of these experiments fully reproduced those observed with the post-glycerol PTS (see Fig. 5). This suggests that the results were largely a reflection of "cytoresistance," rather than a particular injury model used to induce this state.

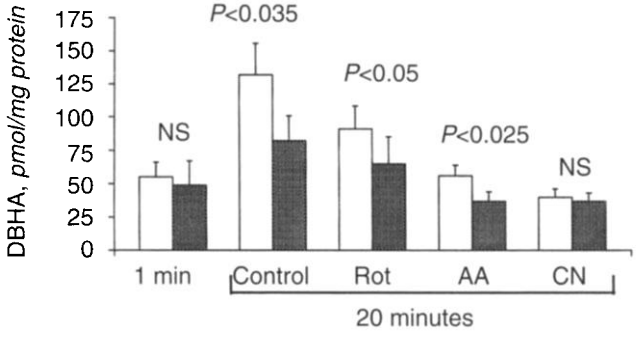

Fig. 7. Hydroxyl radical (OH) production by control (□) and post-glycerol (▨) tubules under normal incubation conditions and in the presence of mitochondrial inhibitors. OH generation by control PTS was denoted by a 2- to 3-fold increase in dihydroxybenzoic acid (DHBA) concentrations over time (from 1 to 20 min of control incubation conditions). The post-glycerol PTS manifested significantly less DHBA production than the controls ($P < 0.035$). Site 1 (rotenone; Rot), site 2 (antimycin A; AA), and site 3 (cyanide; CN) caused a progressive narrowing of the DHBA differences between control (□) and post-glycerol tubules (▨), such that with site 3 inhibition, a significant difference was no longer apparent. These results suggest that the post-glycerol PTS had a significant depression in ·OH generation caused by differences in mitochondrial electron flux, particularly at site 3 of the respiratory chain.

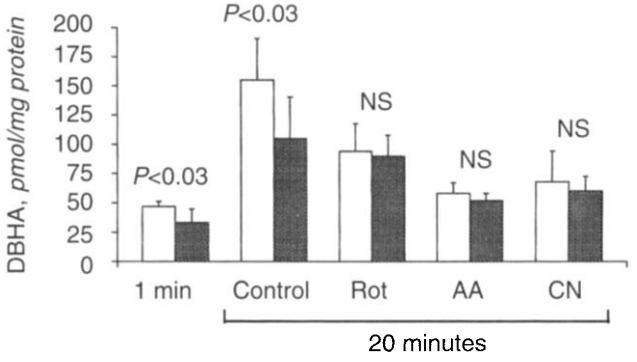

Fig. 8. Hydroxyl radical (·OH) production by control (□) and obstructed (▨) tubules under normal incubation conditions and in the presence of mitochondrial inhibitors. The control tubules manifested a 3-fold rise in DHBA over 1→20 minutes of control incubation conditions. The obstructed tubules had significantly smaller DHBA increments than the controls (noted after both 1 and 20 min). However, each of the mitochondrial inhibitors abrogated this difference between the control (□) and obstructed (▨) PTS. This indicates that the obstructed PTS had depressed OH production, and the site of this differential ·OH production was the mitochondrial respiratory chain.

post-glycerol tubules (Fig. 11A) manifested significantly higher ATP/ADP ratios than the control tubules both under basal conditions (0 AB), and when ATP consumption was driven by increased Na-K exchange (progressive AB addition). Similarly, the obstructed PTS had superior cellular energetics compared to their sham matched controls. However, this was observed only with progressive AB addition and not under basal conditions (Fig. 11B).

When post-glycerol tubules and control tubules were incubated in the presence of ouabain, the former still maintained a higher basal ATP/ADP ratio (9.9 ± 0.4 vs. 8.2 ± 0.4; $P < 0.05$). Similarly, a higher ratio was still observed in the post-glycerol versus control PTS when they were suspended in glucose free/2 deoxyglucose medium (8.2 ± 0.2 vs. 7.5 ± 0.2; $P < 0.015$).

Oxygen consumption experiments

Total QO_2 and QO_2 in the presence of ouabain (Na,K-ATPase independent respiration) did not significantly differ for the control and post-glycerol PTS (Fig. 12). Thus, the reduced H_2O_2/ OH expression in the latter did not correlate with altered oxygen consumption rates.

DISCUSSION

Acute tubular injury triggers a plethora of adaptive and maladaptive responses, the net result of which determines tubular cell fate. Overwhelming insults can produce either tubular necrosis or initiate pathways which culminate in apoptotic cell death [38–40]. Conversely, several fates may befall sublethally damaged tubular cells: (*a*) they may either slough into tubular lumina, increasing tubular obstruction; (*b*) they may be stimulated to mount a proliferative response, thereby reconstituting the tubular epithelium; or (*c*) they may achieve a state of acquired cytoresistance which serves to protect the kidney from further attack. The literature suggests that reactive oxygen metabolites can play critical roles in many of these processes. As examples, increased H_2O_2 and/or OH expression during the initiation phase of injury

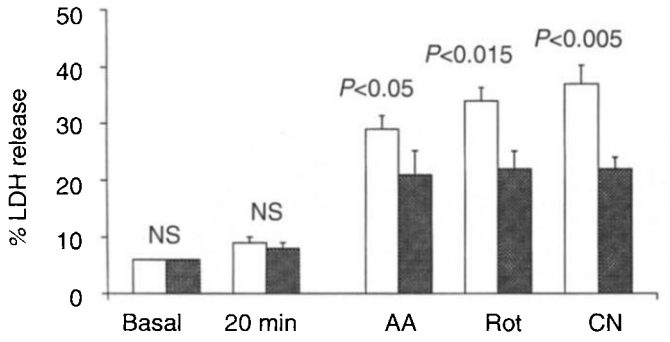

Fig. 9. Percent cell viability for control and post-glycerol tubules, as assessed by LDH release. The control (□) and post-glycerol (▨) tubules manifested no significant difference in cell viability either at baseline (15 min of rewarming) or after completing 20 minutes of control incubation conditions. However, the post-glycerol PTS had significantly less LDH release than the control tubules when challenged with any of the three mitochondrial respiratory chain inhibitors (antimycin A, rotenone, cyanide; AA, Rot, CN), confirming the presence of the cytoresistant state. (The values depicted are the composite results from all of the mitochondrial inhibition experiments presented in Figs. 4 to 8 above).

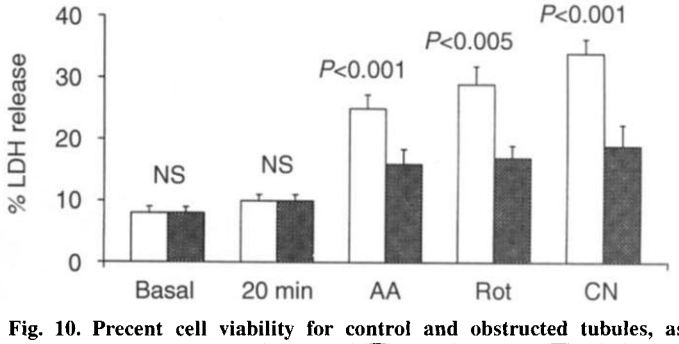

Fig. 10. Precent cell viability for control and obstructed tubules, as assessed by LDH release. The control (□) and obstructed (▨) tubules had identical degrees of LDH release at both baseline (15 min of rewarming) and after completing 20-minute control incubations. However, the obstructed tubules demonstrated significantly less LDH release than the control tubules when challenged with each of the mitochondrial inhibitors (antimycin A, rotenone, cyanide; AA, Rot, CN), confirming the presence of the cytoresistant state. (The values depicted are the composite results from all of the mitochondrial inhibition experiments presented in Figs. 4 to 8 above).

can contribute to necrotic [41, 42] or apoptotic cell death [43], they may alter cell adhesion [44, 45], and they may exert a mitogenic effect [46]. The purpose of the present study was to determine whether alterations in free radical expression might persist beyond the induction phase of acute tubular injury, potentially exerting a delayed effect on at least some of these processes. Of particular interest was the possibility that a down-regulation of H_2O_2/•OH expression might develop within cytoresistant tubules. Since antioxidant drugs have been reported to protect against divergent forms of ARF [20, 22, 23, 25, 41–43], a spontaneous reduction in proximal tubule reactive oxidant metabolite expression ± a concomitant increase in antioxidant defenses (such as GSH) might contribute to the cytoresistant state.

To address this possibility, proximal tubules were harvested from kidneys that had been subjected to one of two highly divergent insults (myohemoglobinuria, urinary tract obstruction), and then H_2O_2 expression was assessed. It was reasoned that if comparable results were obtained while studying highly dissimilar forms of injury, the data would more likely reflect "cytoresistance," rather than peculiarities inherent to a particular form of injury used to induce this state. With this same goal in mind, these two insults were induced in such a way that either minimal azotemia (small glycerol dose) or severe uremia (total bilateral obstruction) was produced. By so doing, the results could be dissociated from changes stemming from marked reductions in single nephron GFR (such as decreased Na reabsorption) or from the uremic state. Reactive oxygen metabolite expression was assessed *in vitro*, rather than *in vivo*, since only the former approach permits direct proximal tubular cell assessments to be made.

The results of these studies indicate that by the 24-hour post-glycerol injection or urinary tract obstruction, an approximate 75% reduction in proximal tubular H_2O_2 expression results. To help validate this finding, •OH levels were assessed. It was reasoned that if decreased H_2O_2 levels exist in cytoresistant tubules, a decrement in •OH, an H_2O_2 by-product, might also result. This appeared to be the case, since a significant lowering of •OH driven-DHBA production was observed in both sets of cytoresistant PTS. It is noteworthy that although both the post-glycerol and obstructed tubules manifested significant reductions in LDH release during ATP depletion injury (that is, confirming the existence of cytoresistance), all sets of tubules had essentially identical degrees of LDH release under unchallenged conditions (baseline and 20 to 30 min oxygenated incubations). This makes it highly unlikely that the differing H_2O_2 and •OH levels in experimental versus control tubules simply reflected a difference in baseline tubule viability. Nevertheless, to further exclude this possibility, control PTS were subjected to sublethal injury (with PLA_2, C20:4, or amphotericin additions) to test whether increased H_2O_2 production would result. If anything, slight decrements, rather than increments, in H_2O_2 levels were noted. Thus, the available data strongly imply that suppressed H_2O_2/•OH expression in the experimental tubules reflected a change which is inherent to the cytoresistant state.

The H_2O_2 decrements in the cytoresistant tubules could reflect either decreased production or increased elimination. Since catalase and glutathione peroxidase are the two principal determinants of intracellular H_2O_2 elimination, their activities were assessed in control and cytoresistant PTS. Highly comparable values were observed, thereby dissociating the H_2O_2 reductions from an up-regulation of these enzymes. Since an increase in GSH peroxidase activity theoretically could result from increased GSH availability, and not just increased enzyme levels, PTS GSH (NP-SH) content was also assessed. Notably, the obstructed and post-glycerol PTS manifested ~50% and 90% increments, respectively. It is noteworthy that increased GSH levels were also observed in whole renal cortical homogenates obtained from post-glycerol versus control rats. This proves that the PTS results had an *in vivo* correlate. To ascertain whether these GSH increments could explain the decreased H_2O_2 levels in cytoresistant PTS, GSH levels were lowered in normal tubules by BSO to determine whether increased H_2O_2 expression would result. However, despite 50% GSH decrements, no significant increase in H_2O_2 levels was observed. This suggests that the increased GSH levels in the cytoresistant tubules did not cause the H_2O_2 decrements; rather, a more tenable hypothesis is that the higher GSH

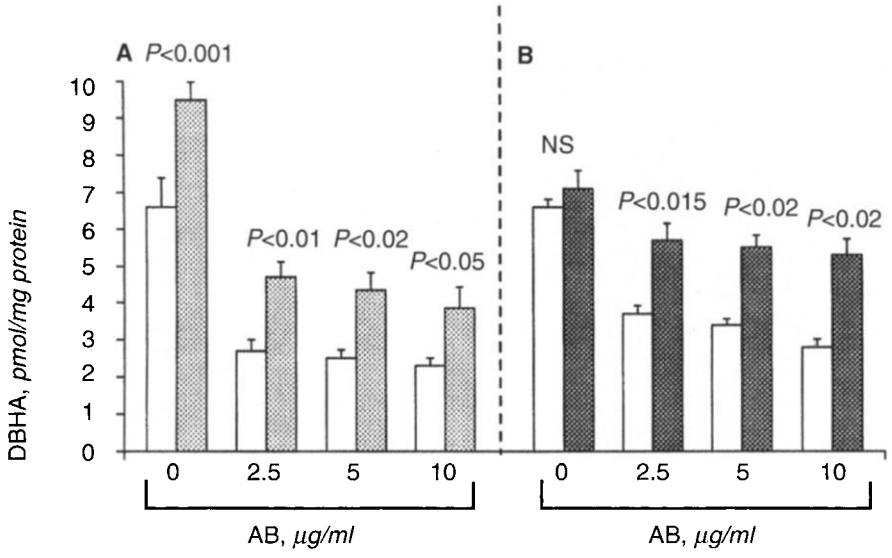

Fig. 11. ATP/ADP ratios for control and cytoresistant tubules under basal conditions and during progressive increments in Na,K-ATPase driven ATP consumption. Both sets of cytoresistant PTS manifested significantly higher ATP/ADP ratios than control tubules at each sublytic dose of amphotericin B (AB) tested (□, control; ▨, glycerol; ■ obstruction). The post-glycerol tubules (*A*) also manifested significantly higher ATP/ADP ratios than control tubules under basal conditions (0 amphotericin B addition). Not depicted, this difference in baseline ATP/ADP ratios for the post-glycerol and control tubules was maintained in the presence of either ouabain or 2-deoxyglucose/glucose free medium, suggesting the improved ATP/ADP ratio did not stem from differences in glycolytic ATP production or a decrease in basal Na,K-ATPase activity (see text).

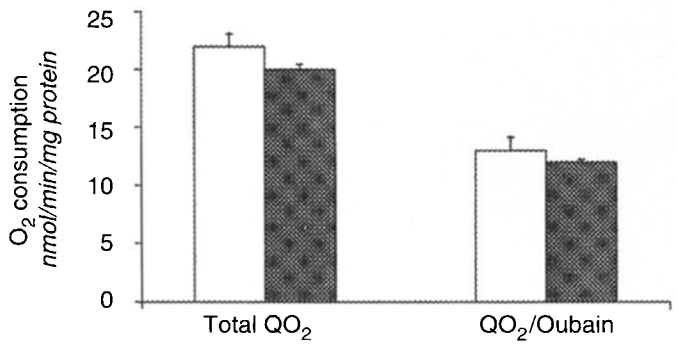

Fig. 12. Oxygen consumption for control (□) and post-glycerol tubules (▨). Total oxygen consumption (total QO_2) did not signficantly differ for control and post-glycerol PTS. Similarly, no difference in QO_2 was observed in the presence of ouabain. These results indicate comparable Na,K-ATPase driven oxygen respiration and Na,K-ATPase independent respiration for control and cytoresistant PTS.

levels were simply secondary reflections of decreased oxidant stress.

Since no evidence was obtained to support increased H_2O_2 disposal in the cytoresistant tubules, we next considered the possibility that the H_2O_2 decrements reflected decreased H_2O_2 production rates. Since tubular injury can up-regulate ferritin expression [6, 22, 26], and since free iron can stimulate tubule H_2O_2 production [21], we tested whether a corrolate of the cytoresistant state is a decrease in free iron content. However, this did not appear to be the case since free iron levels were essentially identical for the normal (non-sham operated), post-glycerol, and obstructed PTS. It is perhaps surprising that a lowering of iron was not observed in the post-glycerol tubules since myohemoglobinuria clearly raises tubule ferritin content [26]. This suggests that although ferritin increments can protect tubules from acute iron loading (such as a heme challenge [26]), they do not depress basal free iron levels. In fact, this could be important since intracellular free iron depletion can cause proximal tubule growth arrest [47]. Were this to occur following acute tubular injury, it could potentially slow recovery from ARF.

Since the mitochondrial electron transport system is the dominant source of reactive oxygen metabolites within proximal tubules [48], experiments were designed to address the possibility that cell injury might alter mitochondrial performance, and that a correlate of this could be reduced H_2O_2/free radical production rates. As an overall index of mitochondrial integrity, cellular ATP/ADP ratios were assessed in control and cytoresistant tubules when they were challenged by progressive increments in Na-K exchange [Na ionophore (amphotericin B) addition, used to simulate state 3 respiration in intact cells] [37]. Irrespective of the amphotericin B dosage, the cytoprotected tubules maintained significantly higher ATP/ADP ratios than the control PTS. These higher ratios were observed despite comparable degrees of LDH release, indicating that they were not simply an indirect reflection of tubule viability. Since an increased ATP/ADP ratio could imply improved glycolysis or decreased Na,K-ATPase driven ATP consumption (and not only improved mitochondrial performance), the former two processes theoretically were equalized in control and post-glycerol tubules by either glucose free medium/2-deoxyglucose treatment or ouabain addition, respectively. That neither treatment negated the heightened unstimulated ATP/ADP ratio in the post-glycerol tubules strongly suggests that the improved cellular energetics did, in fact, have a mitochondrial basis. Finally, we tested whether the postulated improvement in mitochondrial performance might translate into decreased H_2O_2/•OH production rates (presumably because of more "efficient" paired electron transfer to molecular oxygen). To explore this possibility, we hypothesized that if differences in H_2O_2/•OH expression for control and cytoresistant PTS were due to differing degrees of mitochondrial H_2O_2/•OH production, equalizing mitochondrial electron transport at specific sites of the respiratory chain would equalize H_2O_2/•OH expression for control and cytoresistant PTS. Indeed, this was the case, particularly with site 3 inhibition (Na cyanide). Furthermore, it is noteworthy that although site 1 (rotenone) inhibition approximately halved H_2O_2 production in control tubules, it completely failed to inhibit H_2O_2 production in

either post-glycerol or obstructed PTS. This suggests that the cytoresistant tubules already had maximally suppressed mitochondrial H_2O_2 production, thereby explaining why rotenone exerted no additional effect.

It should be recalled that the "stress response" clearly [49–52], and perhaps preferentially [53], impacts the mitochondria, and improved mitochondrial performance can be the result [7, 54]. Thus, the current proximal tubule results, while novel, should not be considered surprising. The subcellular/mitochondrial basis for the presumptive decrements in H_2O_2/•OH production and improved energetics remains unknown. It can, however, be dissociated from an overall alteration in oxygen consumption rates (given the finding of comparable ouabain dependent and independent respiration for control and cytoresistant PTS). This suggests that decrements in single mitochondrial electron transfers to molecular oxygen, rather than an overall decrease in oxygen consumption, *per se*, underlies the suppressed H_2O_2/•OH production rates. Future studies of this issue may require isolated mitochondrial experiments. However, it remains quite likely that the mitochondrial extraction process, and removing them from their normal 'microenvironment' will alter their free radical production rates, potentially complicating this type of data analysis.

The full implications of the present results to the recovery phase of acute renal failure remain to be defined. However, a number of compelling questions arise. For example, since H_2O_2 production can influence cell adhesion, apoptosis, cell regeneration, and cell signaling (such as via Nf-κB, PDGF, stress activated kinses [55–57]), could a decrease in proximal tubular H_2O_2 expression in post-injured tubules affect these processes? Regarding the possible impact of a down-regulation of tubular H_2O_2/•OH production on the cytoresistant state, two points are clear: *First*, this process is not the sole basis for cytoresistance, given that cyanide treatment equalized H_2O_2/•OH production in control and cytoprotected tubules, and still, cytoresistance against ATP depletion was expressed (decreased LDH release). *Second*, even though decreased tubule H_2O_2/•OH production is not essential for the expression of cytoresistance, it might still mechanistically contribute to it. For example, Abul-Ezz, Walker and Shah have demonstrated that when rats are given i.v. GSH to raise renal GSH to the same extent observed in our cytoprotected tubules, marked protection against *in vivo* myohemoglobinuric ARF resulted [20]. Similarly, Paller observed that a comparable increase in renal GSH content protected against post-ischemic ARF [58]. Thus, these results strongly suggest that a decrease in tubule H_2O_2 production, and/or a corresponding 50 to 90% GSH increment, could be a contributing factor to the acquired cytoresistant state, particularly as it is expressed against oxidant forms of attack.

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