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Radiographic contrast media–induced tubular injury: Evaluation of oxidant stress and plasma membrane integrity

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Radiographic contrast media–induced tubular injury: Evaluation of oxidant stress and plasma membrane integrity.

Background. Experimental and clinical investigations suggest that oxidant stress is a critical determinant of radiocontrast nephropathy (RCN), and that N acetyl cysteine (NAC) can prevent this damage. This study addresses these issues directly at the tubular cell level. Potential alternative mechanisms for RCN have also been sought.

Methods. Isolated mouse proximal tubule segments (PTS), or cultured proximal tubule (HK-2) cells, were subjected to radiocontrast media (RCM) (Ioversol, Optiray 320) exposure, followed by assessments of cellular viability [% lactate dehydrogenase (LDH) release, tetrazolium dye (MTT), uptake] and lipid peroxidation. These experiments were conducted in the absence or presence of a variety of antioxidants [NAC, glutathione (GSH), superoxide dismutase, catalase] or pro-oxidant (GSH depletion, heme oxygenase inhibition) strategies. RCM effects on mitochondrial and plasma membrane integrity were also assessed.

Results. RCM exposure did not induce PTS lipid peroxidation. Neither antioxidant nor pro-oxidant interventions mitigated or exacerbated RCM-induced tubular cell injury, respectively. RCM impaired mitochondrial integrity, as assessed by ouabain-resistant ATP reductions, and by cytochrome c release (before cell death). RCM also induced plasma membrane damage, as indicated by loss of key resident proteins (NaK-ATPase, caveolin) and by increased susceptibility to phospholipase A_2 (PLA_2) attack (increase of $\geq$2 times in free fatty acid and NaK-ATPase release). Hyperosmolality could not account for RCM's toxic effects.

Conclusion. RCM toxicity can be dissociated from tubular cell oxidant stress. Alternative mechanisms may include mitochondrial injury/cytochrome c release and plasma membrane damage. The latter results in critical protein loss, as well as a marked increase in plasma membrane susceptibility to exogenous/endogenous PLA_2 attack.

Radiocontrast nephropathy (RCN) is a leading cause of acute renal failure (ARF) [1]. However, its pathogenesis remains poorly defined. Four dominant injury pathways have been widely discussed. First, radiocontrast medium (RCM) injection induces renal vasoconstriction [2, 3], a process that is thought to arise from an imbalance between endothelium-derived vasoconstrictive and vasodilatory factors (e.g., endothelin, nitric oxide, and adenosine) [4–10]. If severe vasoconstriction is induced, ischemic tubular injury, culminating in cell detachment, apoptosis, or necrosis can each result [11, 12]. Proximal tubules and medullary thick ascending limbs [12] are particularly vulnerable to these events. Second, following tissue ischemia, "reperfusion injury" may occur. This may arise from a number of factors, such as oxidative stress [11] or "re-alkalinization injury," induced by a rapid correction of ischemia-induced reductions in tissue pH [13]. Third, RCM may precipitate in distal tubule lumina along with Tamm Horsfall glycoprotein, forming casts [14–16]. To the extent that sloughed tubular cells are incorporated into these casts, a worsening of intratubular obstruction may result. Fourth, RCM can directly damage tubular cells. This can potentially arise from either RCM-induced elevations in tissue osmolality [17, 18], or from direct contrast-induced cytotoxicity. In this regard, Humes et al [19] reported that RCM (but not the associated hyperosmolality) causes sublethal injury in isolated rabbit proximal tubules, as reflected by decreases in mitochondrial respiration, cellular K^+ leak, and a Ca^{2+} overload state. The pathologic mechanisms by which RCM induced these changes were not defined. However, investigations from a number of laboratories suggest that oxidative tubular stress is involved. This conclusion is supported by findings that RCM injection can induce or exacerbate lipid peroxidation in either normal or dehydrated rats [20, 21]. The fact that administration of N-acetyl cysteine (NAC) to humans may mitigate RCN further supports this view [22, 23].

Progress towards preventing RCN will require additional understanding of involved pathogenic mechanisms. Hence, the present study was undertaken with the following goals in mind. First, define whether RCM does, in fact, induce critical oxidative stress in renal tubular cells. If so, can antioxidants, in general, and NAC, in

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particular, mitigate this damage? Second, since NAC is currently widely used as prophylaxis for RCN, ascertain the spectrum and molecular determinants of its purported cytoprotective effects. Third, given that RCM may induce mitochondrial injury [19] as well as tubular cell apoptosis [12, 17, 18], might these two events be linked (e.g., via mitochondrial cytochrome c release, a potential inducer of apoptosis)? Fourth, since radiocontrast agents are thought to be plasma membrane impermeant, might they alter plasma membrane integrity, inducing either direct membrane damage and/or predisposing the membrane to superimposed attack (e.g., by PLA_2)? Clinical RCN results from a complex interplay of vascular, intraluminal, and tubular cell events. The present investigations were confined to direct tubular cell experiments in order to focus on pathogenic events at this level.

METHODS

Proximal tubule segment experiments

Isolated proximal tubule segment (PTS) preparation. Proximal tubules were isolated from normal CD-1 male mice (25 to 35 g; Charles River, Wilmington, MA, USA) as previously described [24]. They were suspended in an experimentation buffer (NaCl 100 mmol/L; KCl 2.1 mmol/L; $NaHCO_3$ 25 mmol/L; KH_2PO_4 2.4 mmol/L; $MgSO_4$ 1.2 mmol/L; $MgCl_2$ 1.2 mmol/L; $CaCl_2$ 1.2 mmol/L; glucose 5 mmol/L; alanine 1 mmol/L; Na lactate 4 mmol/L; Na butyrate 10 mmol/L; 36 kD dextran, 0.6%; gassed with 95% O_2/5% CO_2; final pH 7.4) to a tubule protein concentration of 2 to 4 mg/mL. Each PTS preparation was warmed to 37°C, divided into 3 to 6 equal aliquots (1.25 or 1.5 mL, depending on the experiment), and placed into 10 mL Erlenmeyer flasks. They were then used in individual experiments, as detailed below.

Effects of NAC on oxidative and hypoxic proximal tubular injury. NAC has been suggested to protect against human RCN [22], a process widely regarded as being mediated by oxidative stress and ischemic tubular injury. Hence, the first set of experiments was undertaken to examine whether NAC can, indeed, block well-characterized forms of these types of tubular damage. Six sets of PTS were prepared and each was divided into 6 equal aliquots as follows: (*1*) control incubation (95% O_2/5% CO_2) for 45 minutes; (*2*) incubation for 45 minutes with 2 mmol/L NAC (Sigma Chemical Co., St. Louis, MO, USA); (*3*) 15 minutes of control incubation followed by a 30 minute oxidative challenge [25 μmol/L ferrous ammonium sulfate, complexed with hydroxyquinoline (HQ), a siderophore, allowing iron (Fe) to gain intracellular access] [25, 26]; (*4*) 15 minutes of incubation with 2 mmol/L NAC, followed by the 30 minute FeHQ challenge; (*5*) 15 minutes of control incubation followed by 15 minutes of hypoxia (95% N_2/5% CO_2)/15 minutes of reoxygenation (95% O_2/5% CO_2); and (*6*) 15 minutes of NAC treatment followed by hypoxia/reoxygenation. Thus, the total incubations were 45 minutes, with a 15-minute preincubation period ± NAC. At the end of the incubations, cell injury was assessed by % lactate dehydrogenase (LDH) release [24].

Glutathione (GSH) effects on oxidative and hypoxic proximal tubular injury. The following experiment ascertained whether NAC confers a greater degree of protection than a comparable amount of GSH. Noteworthy in this regard is that GSH, unlike NAC, is a source of glycine, a potent cytoprotectant against hypoxic cell damage [27]. Hence, it is conceivable that it might be preferable to NAC as a cytoprotectant. To address this issue, the experiment described above was repeated (N = 4 PTS preparations), except that 2 mmol/L of glutathione (GSH) was substituted for 2 mmol/L NAC.

N acetyl lysine (NAL) and N acetyl methionine (NAM) effects on PTS injury. It is widely assumed that NAC's protective effect is imparted by its free sulfhydryl (−SH) group. However, it remains possible that its acetyl moiety, or possibly its sulfur content, per se, could be responsible. To exclude these possibilities, the impacts of NAL and NAM on Fe-mediated oxidative stress were assessed (NAL contains an acetyl, but not an −SH group; NAM contains sulfur, but not an −SH group). Three sets of PTS were prepared and each divided into 6 1 mL aliquots containing control incubation; incubation with 2 mmol/L NAM; incubation with 2 mmol/L NAL; incubation with 25 μmol/L FeHQ; incubation with 25 μmol/L FeHQ + 2 mmol/L NAM; and incubation with 25 μmol/L FeHQ + 2 mmol/L NAL. A 15-minute pretreatment, followed by a 30-minute Fe challenge, was used, as above. At the end of the 45-minute incubations, % LDH release was determined.

RCM effects on isolated tubule viability and resistance to superimposed injury. The following experiment was undertaken to ascertain whether RCM could exert direct cytotoxicity on freshly isolated PTS, and whether such toxicity might sensitize tubular cells to either superimposed hypoxic or oxidative attack.

Fe-mediated oxidative injury. Each of 4 sets of tubules were equally divided into 4 equal aliquots and treated as follows: (*1*) control incubation for 45 minutes; (*2*) incubation for 45 minutes with 200 μL/mL of the nonionic contrast agent ioversol (Optiray 320, 68%; Mallinckrodt, Inc., St. Louis, MO, USA; stock solution: 702 mmol/L; 320 mg/mL iodine); (*3*) 15-minute control incubation, followed by a 30- minute exposure to 25 μmol/L FeHQ; and (*4*) 15-minute pretreatment with RCM, followed by the 30-minute FeHQ challenge. The final RCM concentration raised buffer osmolality and iodide concentrations by 140 mosm/L and 64 mg/mL, respectively (clinically achievable tubular intraluminal levels). After 45 minutes, injury was assessed by % LDH release. Oxidative damage (lipid peroxidation) was gauged by tubule

malondialdehyde (MDA) content (nmol/mg tubule protein) [28]. Ioversol was chosen for this study as it is representative of currently used nonionic contrast agents. A nonionic agent (rather than an ionic agent) was used because NAC was demonstrated to protect against nonionic contrast agent in previous clinical trials [22, 23].

HYPOXIC CELL INJURY. The above-mentioned experiment was repeated, but hypoxia/reoxygenation (H/R) [26] was substituted for the Fe challenge. To this end, 10 minutes of hypoxia (N_2/CO_2), followed by 20 minutes of reoxygenation, were induced after the 15-minute RCM pretreatment period. Cell injury was again assessed by MDA generation and % LDH release.

Radiocontrast effects on proximal tubular segment ATP/ADP ratios. To ascertain potential RCM effects on mitochondrial adenosine triphosphate (ATP) production, 6 sets of freshly isolated mouse proximal tubules were each divided into 3 aliquots, and incubated for 45 minutes as follows: control incubation; incubation with 100 μL/mL of RCM; or incubation with 200 μL/mL of RCM. At the completion of the incubations, a tubule aliquot was rapidly removed for subsequent determination of % LDH release, and then tubule adenine nucleotides were immediately extracted in 6.67% trichloroacetic acid. After extraction, ATP and adenosine diphosphate (ADP) concentrations were determined by high-pressure liquid chromatography (HPLC) [29, 30]. The state of cellular energetics was gauged by determining ATP/ADP ratios [29, 30].

To determine whether increased plasma membrane Na leak, with increased NaK-ATPase-driven ATP consumption, was responsible for RCM-induced reductions in ATP/ADP ratios, 3 sets of PTS were incubated as follows: control conditions; with 200 μL/mL RCM; with 1 mmol/L ouabain (to block NaK-ATPase) [31]; or with RCM + ouabain. After 45 minutes, ATP/ADP ratios were assessed (see **Results**).

To ascertain whether NAC could attenuate ATP/ADP reductions, 2 sets of tubules were incubated with 200 μL/mL of RCM ± 2 mmol/L NAC. After 45 minutes, ATP/ADP ratios were determined.

Effect of RCM on reoxygenation injury and post hypoxic ATP homeostasis. The experiments above indicate that RCM can both increase hypoxia/reoxygenation-induced cell injury, as reflected by LDH release, and it can lower ATP/ADP ratios in normal tubules. The following experiment was undertaken to ascertain whether RCM must be present during hypoxia to exert this effect, or whether its addition during reoxygenation can suppress recovery of cellular energetics and increase reoxygenation-induced cell death. To this end, 4 sets of tubules were each divided into 4 equal aliquots as follows: control oxygenated incubation for 30 minutes; 10 minutes of hypoxia followed by 20 minutes of reoxygenation; 10 minutes of hypoxia/20 minutes of reoxygenation, the latter undertaken in the presence of 100 μL/mL RCM added at the start of reoxygenation; and 10 minutes of oxygenated incubation followed by 20 minutes of RCM exposure. At the completion of the 30-minute incubations, samples were taken for assessment of ATP/ADP ratios and % LDH release. As a control for the osmotic effect of contrast addition on posthypoxic cell injury, the experiment above was repeated in two additional sets of tubules, substituting equiosmolar mannitol for RCM.

Effect of NaI on proximal tubular cell integrity. RCM can release inorganic iodide, resulting in its urinary excretion [32]. The following experiment was undertaken to ascertain whether free iodide might contribute to RCM cytotoxicity. Three sets of tubules were each divided into 4 aliquots as follows: 45-minute control incubations, and 45-minute incubations with 2.5, 5, or 10 mmol/L NaI. LDH release and ATP/ADP ratios were then determined, as noted above.

Effects of RCM on plasma membrane stability and protein shedding. The following experiments tested whether proximal tubular cell exposure to RCM de-stabilizes the plasma/intracellular membranes, causing critical protein loss and enhanced membrane vulnerability to superimposed (e.g., PLA_2) attack.

Effect of RCM on tubule protein loss. Four sets of tubules were divided into 4 aliquots with control incubation; incubation with 200 μL/mL RCM; incubation with 5 U/mL porcine pancreatic PLA_2 (P6534; Sigma Chemical Co.), which is excluded from traversing the plasma membrane [33]; and RCM + PLA_2. After 45 minutes, % LDH release was determined, tubule aliquots were centrifuged at 4°C, and cell protein loss into the suspending medium supernatant (protein concentrations; bichinchoninic acid method) was assessed.

Effect of RCM on NaK-ATPase and cytochrome c shedding. To ascertain specific sites of protein loss, supernatants of tubule aliquots from the above experiment were centrifuged, saved, and subjected to Western blot analysis for NaK-ATPase as a marker of the plasma (basolateral) membrane and cytochrome c, a mitochondrial inner membrane protein that can initiate apoptosis. Western blots for NaK-ATPase (alpha 1 chain; Santa Cruz Biotechnology; Santa Cruz, CA, USA) and cytochrome c (BD PharMingen, #556433; San Diego, CA, USA) were conducted according to the manufacturer's instructions, and as previously described [34, 35]. Relevant protein bands were visualized by enhanced chemiluminescence and quantified by densitometry [26].

Effect of RCM on caveolin shedding Caveolin is the dominant structural protein of plasma membrane rafts/caveolae (cholesterol/sphingomyelin enriched microdomains associated with a myriad of signaling molecules) [26]. The following experiment assessed whether RCM induces damage to these structures, as denoted by caveolin loss [26]. Four sets of tubules were incubated using control conditions; 200 μL/mL RCM; RCM + 2 mmol/L NAC; or equiosmolar mannitol as an osmotic control.

After 45 minutes, % LDH release was determined, the samples were centrifuged, the supernatants concentrated ×10 on a filter, and assayed for caveolin by Western blotting, as previously described [26].

RCM EFFECTS ON PLASMA MEMBRANE SUSCEPTIBILITY TO EXOGENOUS PLA2-INDUCED DEACYLATION. It is well recognized that during ischemic cell injury, PLA_2 becomes activated and induces plasma membrane phospholipid deacylation, culminating in the accumulation of nonesterified (free) fatty acids (NEFA) and lysophospholipids. This experiment tested whether RCM directly causes deacylation and/or sensitizes the plasma membrane to PLA_2 attack. Four sets of isolated tubules were each divided into 4 equal aliquots as follows: control incubation for 30 minutes; incubation with 200 μL/mL of RCM; incubation with 5 U/mL of porcine pancreatic PLA_2; and incubation with RCM + PLA_2. After the 30-minute incubations, % LDH release was assessed, and the remaining samples underwent lipid extraction in chloroform:methanol [33]. The lipid extracts were assayed for NEFA with a commercially available kit (NEFA; Wako, Neuss, Germany) [33]. Results were expressed as nmol/mg tubule protein.

Effect of RCM on hypoxia-induced deacylation. To further test the hypothesis that RCM predisposes to PLA_2-induced membrane injury, intracellular PLA_2-induced deacylation was assessed, rather than porcine PLA_2. Four sets of tubules were each divided into 3 aliquots containing control incubation +2 mmol/L glycine; 15 minutes of hypoxia + 2 mmol/L glycine; and 15 minutes of hypoxia + 200 μL/mL RCM + 2 mmol/L glycine. The glycine was added to prevent, and hence, equalize, cell death. At the end of the experiments, NEFA concentrations and % LDH release were assessed.

Human proximal tubular (HK-2) cell experiments

Cell culture methods. HK-2 cells, an immortalized proximal tubular cell line derived from normal human kidney, were used for all of the following cell culture experiments [36]. The cells were maintained at 37°C with 20% O_2/5% CO_2 in T75 Costar flasks (Cambridge, MA, USA) in keratinocyte serum–free medium (K-SFM) containing 1 mmol/L glutamine, 5 ng/mL epidermal growth factor, 40 μg/mL bovine pituitary extract, 25 U/mL penicillin, and 25 μg/mL streptomycin [36]. At near confluence, the cells were trypsinized and transferred to either additional T75 flasks (for passage) or to 24-well Costar plates (for specific experiments). Cell injury in these experiments was assessed either by determining % LDH release, or by tetrazolium dye (MTT) uptake assay [36].

RCM toxicity in the presence and absence NAC. HK-2 cells were trypsinized and seeded into 24-well cluster plates. Approximately 8 hours later, the cells were maintained under one of the following conditions for 3 days: normal culture conditions; with RCM [100 μL added to 900 μL of K-SFM (i.e., a 1:10 dilution), raising culture medium osmolality by 70 mosm/L]; culture in the presence of 1 mmol/L NAC; or culture with RCM + NAC. After 3 days, cell injury was gauged by % LDH release and by MTT uptake assay ($N = 8$ each).

RCM toxicity in HK-2 cells: influence of superoxide dismutase and catalase. HK-2 cells were seeded into 24-well cluster plates and the plates were divided into the following groups: control incubation; incubation with 250 U/mL of superoxide dismutase (SOD) (from bovine erythrocytes, #S2515; Sigma Chemical Co.); incubation with 5000 U/mL catalase (from bovine liver; C-40, Sigma Chemical Co.); RCM of 100 μL/mL; RCM + SOD; and RCM + catalase. After 3-day incubations, cell injury was assessed by % LDH release and MTT uptake ($N = 8$ determinations each).

RCM toxicity in HK-2 cells: heme oxygenase and endogenous GSH effects. To further gauge the role of oxidant stress in RCM toxicity, two critical intracellular antioxidant defense mechanisms, heme oxygenase and GSH, were inhibited/depleted, respectively, to ascertain whether an exacerbation of RCM toxicity would result. To this end, 24-well plates of HK-2 cells were incubated under one of the following conditions: control conditions; incubation with 50 μmol/L tin (Sn) protoporphyrin, a potent heme oxygenase inhibitor [37]; 1 mmol/L L-buthionine sulfoximine dichloride (BSO) (Sigma Chemical Co.), an agent which induces ~80% glutathione depletion in HK-2 cells via GSH synthesis blockade [38]; RCM, 100 μL/mL; RCM + 50 μmol/L Sn protoporphyrin; and RCM + BSO. Three days later, cell injury was assessed by % LDH release and by MTT assay ($N = 8$ determinations each).

RCM toxicity: effects of xanthine oxidase inhibition. It has previously been hypothesized that adenosine accumulation, possibly the result of ischemia-induced ATP degradation, can both induce renal vasoconstriction [7, 8] and also fuel xanthine oxidase–mediated oxidase stress [39]. The following experiments were undertaken to test this view.

To determine whether RCM causes adenosine accumulation, 18 T 75 flasks of HK-2 cells were divided into 3 groups: control incubation for 3 hours; incubation with RCM (100 μL/mL of culture medium); or incubation with equiosmolar mannitol. After 3 hours, the cells were extracted and assayed for ATP, ADP, adenosine monophosphate (AMP), and adenosine levels [30]. The experimental values were expressed as nmol recovered per flask.

To determine whether xanthine oxidase inhibition mitigates RCM toxicity, 2 24-well cluster plates were each seeded with HK-2 cells and 8 hours later were subdivided as follows: control incubation; incubation with RCM, as noted above; incubation with 50 μmol/L oxypurinol (Sigma Chemical Co.); incubation with 100 μmol/L oxy-

purinol; RCM + 50 μmol/L oxypurinol; and RCM + 100 μmol/L oxypurinol. Three days later, cell injury was assessed by % LDH release.

Hyperosmolality as a mediator of RCM toxicity. It has previously been suggested that RCM toxicity is mediated via inducing osmotic shock, possibly triggering apoptosis [17, 18]. The following experiment addressed whether this is the sole mechanism for RCM-induced cytotoxicity. HK-2 cells, grown in 24-well cluster plates, were divided into 3 groups: control incubation; incubation with 100 μL RCM/mL culture medium (final increase in osmolality of 70 mosm/L); or an equal osmotic dose (70 mosm/L) of mannitol. After 3 days, cell injury was assessed by % LDH release and MTT uptake, as above.

Iodide as a potential mediator of RCM toxicity. Assessment of iodide toxicity. Two 24-well plates of HK-2 cells were divided into 3 treatment groups: control incubation; incubation with 1.25 mmol/L NaI; and incubation with 2.5 mmol/L NaI. After 3 days, toxicity was sought by % LDH release.

Does propylthiouracil (PTU) inhibit RCM cytotoxicity? Propylthiouracil (PTU) can block iodide-mediated apoptosis [40] purportedly by inhibiting iodide incorporation into cellular proteins. Hence, potential PTU-induced protection against RCM toxicity was sought. Two plates of HK-2 cells were incubated under control conditions ± 250 or 500 μmol/L PTU; and with RCM ± 250 or 500 μmol/L PTU. Three days later, % LDH release was assessed.

Calculations and statistics. All values are presented as mean ± SEM. Statistical comparisons were performed by either paired or unpaired Student *t* test. If multiple comparisons were made, the Bonferroni correction was applied. Of note, the two major end points of toxicity used in this study, MTT uptake and LDH release, were first demonstrated not to be artifactually altered by RCM addition (assessment of these end points when the RCM was added, followed immediately by LDH and MTT assay).

RESULTS

Isolated proximal tubule experiments

NAC and GSH effects on oxidant and hypoxic injury to isolated tubular segments. As shown in Figure 1, left panel, both FeHQ and hypoxic/reoxygenation (H/R) injury induced ~50% LDH release. NAC treatment exerted significant protective effects. This was most dramatic in the case of Fe-induced oxidant injury, with % LDH release barely rising around baseline values. NAC caused only a minor suppression of H/R injury. NAC by itself did not alter % LDH release.

As shown in Figure 1, right panel, GSH caused an approximate 50% decrease in Fe-mediated LDH release (largely recapitulating NAC's effect). In contrast to NAC, which minimally impacted H/R injury, GSH completely blocked H/R induced cell death.

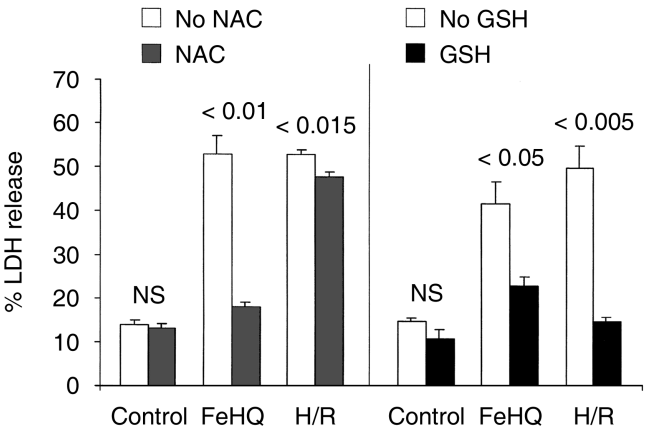

Fig. 1. Relative effects of N acetyl cysteine (NAC) and glutathione (GSH) on iron (Fe) and hypoxia/reoxygenation (H/R)-induced injury to isolated proximal tubular segments (PTS). As shown in the lefthand panel, 2 mmol/L NAC almost completely blocked Fe-hydroxyquinoline (FeHQ)-mediated injury. However, it exerted only a slight, albeit statistically significant, protective effect against H/R injury (15 minutes' hypoxia/15 minutes' reoxygenation). As shown in the righthand panel, 2 mmol/L GSH induced comparable protection against Fe-mediated injury, compared to NAC. However, GSH was far more protective against H/R injury than was NAC (undoubtedly due to GSH's glycine content; see text).

N acetyl lysine (NAL)/N acetyl methionine (NAM) effects on FeHQ-induced injury. Unlike NAC and GSH, neither NAL nor NAM protected against Fe-mediated cell death (% LDH release: control, 12 ± 2%; Fe, 50 ± 2%; Fe + NAM, 53 ± 2%; Fe + NAL, 50 ± 2%; all NS). NAL or NAM did not independently affect LDH release (10 to 12% LDH release for all control, NAL, and NAM exposed tubules).

RCM effects on isolated tubule viability and cell injury responses. The addition of 200 μL/mL of RCM to PTS had no significant effect on tubule viability in the absence of another challenge (Fig. 2). RCM caused a modest, but significant, worsening of hypoxic/reoxygenation injury. However, RCM addition failed to potentiate Fe-mediated injury; rather, a paradoxical trend toward decreased, rather than increased, LDH release resulted (Fig. 2, right). RCM addition did not increase PTS MDA concentrations, either on its own or when present during superimposed H/R or Fe-mediated injury (Fig. 3); rather, a slight, but nonsignificant, trend toward RCM-associated MDA reductions was observed.

RCM effects on proximal tubular segment ATP/ADP ratios. Incubating tubules for 45 minutes with either 100 or 200 μL/mL of RCM caused a dose- dependent decrease in ATP/ADP ratios (Fig. 4, left). This corresponded with only slight, and nonsignificant, changes in % LDH release (Fig. 4, right).

To differentiate between increased ATP consumption

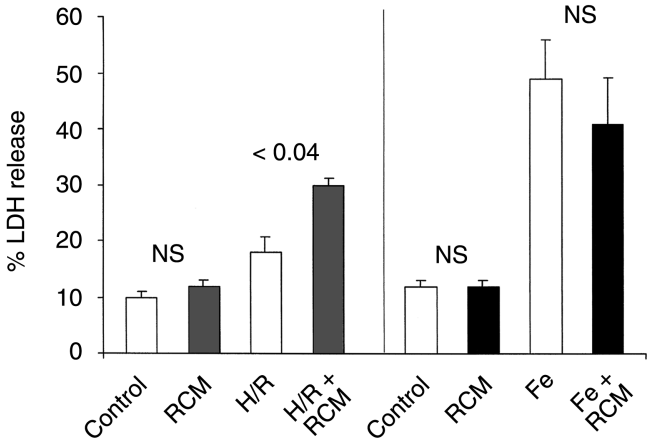

Fig. 2. Radiocontrast medium (RCM) effects on proximal tubule viability in the presence or absence of superimposed hypoxia/reoxygenation (H/R) or iron (Fe)-mediated injury. RCM addition (200 μL/mL) did not, by itself, increase % lactate dehydrogenase (LDH) release (left and right panels). RCM did significantly exacerbate 10 minutes' hypoxic/20 minutes' reoxygenation injury. Conversely, RCM did not worsen Fe-mediated cell death.

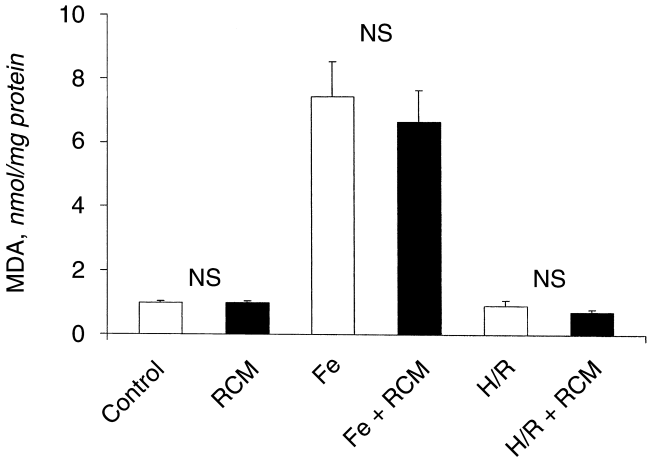

Fig. 3. Radiocontrast medium (RCM) effects on proximal tubular lipid peroxidation, as assessed by malondialdehyde (MDA) concentrations. RCM addition did not cause a spontaneous increase in tubule MDA levels. Whereas iron (Fe) induced a dramatic increase in MDA (as would be expected from an oxidant form of injury), RCM did not augment the Fe-driven MDA increments. Hypoxia/reoxygenation (H/R) injury was not associated with MDA generation, either in the presence or absence of RCM.

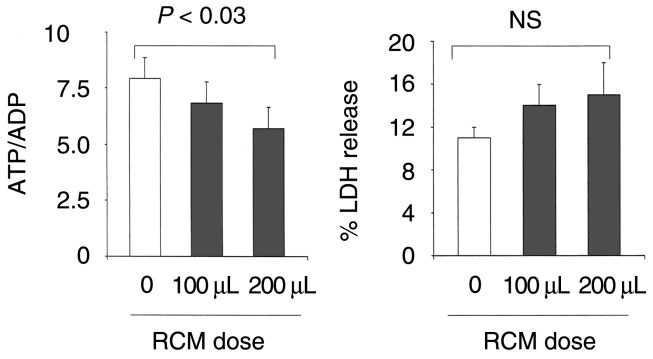

Fig. 4. Radiocontrast medium (RCM) effects on proximal tubule adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratios and cell injury, as assessed by % lactate dehydrogenase (LDH) release. RCM, either 100 or 200 μL/mL, induced a modest, dose-dependent decrease in ATP/ADP ratios (left panel). This was associated with a concomitant slight upward trend in % LDH release, a change that did not achieve statistical significance (right panel).

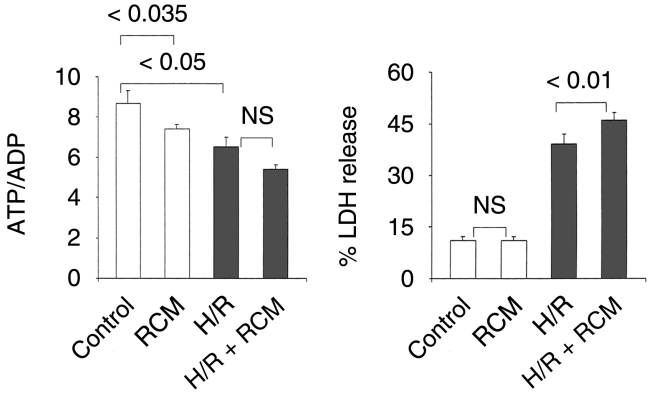

Fig. 5. Effect of radiocontrast medium (RCM) addition on posthypoxic (reoxygenation) adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratios and posthypoxic lactate dehydrogenase (LDH) release. RCM (100 μL/mL) and hypoxia/reoxygenation (H/R) injury each caused significant declines in tubule ATP/ADP ratios (left panel). However, when RCM and H/R were present together, no synergistic decrease in ATP/ADP ratios resulted. Addition of RCM upon reoxygenation caused a modest, but significant, worsening of cell injury, as assessed by % LDH release.

(due to NaK-ATPase activity) and decreased ATP production, the impact of 1 mmol/L ouabain on RCM-induced ATP/ADP ratio suppression was assessed. Ouabain failed to improve the RCM-induced ATP/ADP ratio reductions; rather, a slight additional worsening of the ratios was observed (control, 8.2 ± 0.4; RCM, 7.1 ± 0.3; RCM + ouabain, 5.7 ± 0.3). Thus, RCM likely suppressed mitochondrial ATP generation, rather than increased ATP consumption (i.e., via a NaK-ATPase-dependent mechanism). RCM-induced ATP/ADP ratio reductions, as noted above, were not attenuated by NAC administration (data not shown).

RCM effects on reoxygenation ATP/ADP ratios and reoxygenation injury. ATP/ADP. As shown in Figure 5, left, addition of 100 μL/mL of RCM caused a modest reduction in ATP/ADP ratios in normal tubules ($P < 0.035$). Following hypoxic/reoxygenation (H/R) injury, an approximate 35% reduction in ATP/ADP ratios was apparent, compared to normal tubules ($P < 0.05$). The combination of RCM + H/R injury caused additive, not synergistic, ATP/ADP reductions in posthypoxic tubules.

% LDH RELEASE. Addition of RCM solely during re-

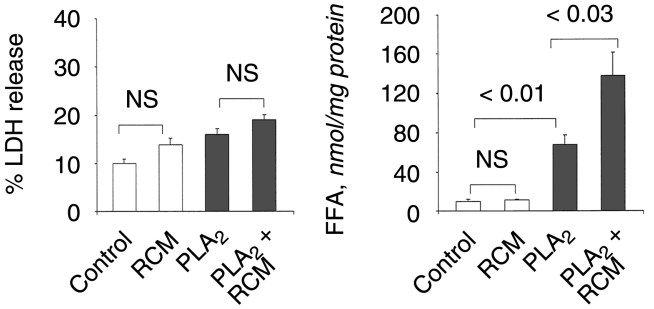

Fig. 6. Effect of radiocontrast medium (RCM) with and without phospholipase A_2 (PLA_2) on % lactate dehydrogenase (LDH) release and free (nonesterified) fatty acid (FFA) levels in isolated tubules. RCM and PLA_2, or the two together, had minimal effects on % LDH release. In contrast, PLA_2 induced an increase of 7 times in free fatty acid (FFA) levels. RCM doubled the amount of PLA_2-induced FFA increases, despite the fact that RCM treatment by itself did not increase FFA content.

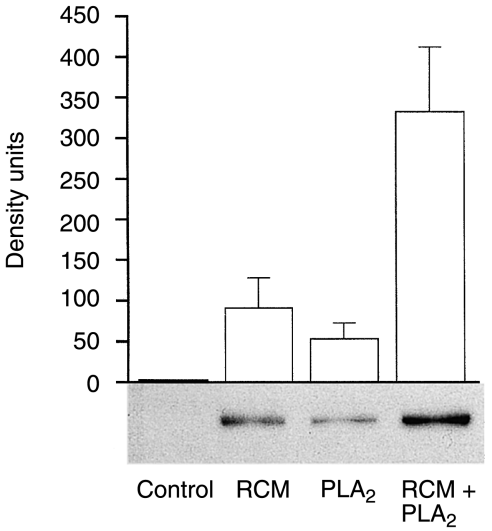

Fig. 7. Western blot analysis of NaK-ATPase released into tubule media. The protein band appears at 112 kD. As can be seen, the control sample demonstrated virtually no NaK-ATPase (alpha 1 chain). Both radiocontrast medium (RCM) and phospholipase A_2 (PLA_2) exposure induced NaK-ATPase release ($P < 0.01$ vs. controls). Combined PLA_2 + RCM treatment yielded significantly more NaK-ATPase than either treatment alone ($P < 0.01$). Statistics were conducted by converting density units into log base 10, followed by unpaired Student *t* test with Bonferroni correction.

oxygenation caused a slight worsening of H/R injury. Thus, when viewed in the context of the Figure 2 data, it appears that RCM can exacerbate H/R injury whether the RCM is present during hypoxia or in the reoxygenation period. Not depicted, equiosmolar mannitol slightly lessened, not worsened, reoxygenation LDH release (43 ± 0.3% vs. 40 ± 0.3%, $P = 0.05$). Thus, RCM's adverse effect on H/R injury was not mediated by an increase in osmolality.

NaI incubation with proximal tubule segments. Incubating tubules for 45 minutes with 2.5, 5, or 10 mmol/L NaI had no demonstrable adverse effect on cell viability, as reflected by completely normal % LDH release (≤11% for all aliquots; control, 11%). ATP/ADP ratios were also unaffected by NaI incubations (8.0 ± 0.1 for all doses of NaI vs. 7.8 ± 0.2 for control tubules, not depicted).

RCM effects on plasma membrane deacylation: susceptibility to exogenous PLA_2. As shown in Figure 6 (right panel), PLA_2 induced a 6- to 7-fold increase in NEFA levels in the absence of a significant increase in cell death (LDH release). The extent of PLA_2-induced deacylation was doubled by the presence of RCM. Again, no significant worsening of cell death resulted (Fig. 6, left panel). The RCM effect on PLA_2-mediated deacylation occurred without RCM exerting an independent effect on NEFA concentrations.

RCM effect on hypoxia (endogenous PLA_2)-induced deacylation. The presence of glycine prevented any hypoxia-induced increase in % LDH release (~10 to 14% for all aliquots, not shown). NEFA concentrations at the end of experiments were as follows: control incubation, 10 ± 1; hypoxia, 31 ± 3; and hypoxia + RCM, 40 ± 3 (nmol/mg protein; $P < 0.05$ vs. hypoxia alone). Hence, the presence of RCM increased the extent of hypoxia-mediated NEFA accumulation under conditions where lethal cell injury was prevented by glycine treatment.

RCM effects on membrane protein loss. TOTAL PROTEIN. RCM caused significant tubule protein loss into the suspending media (protein concentration: RCM, 0.2 ± 0.02 vs. control, 0.12 ± 0.02; $P < 0.0001$). PLA_2 also increased protein loss, but the combination of RCM + PLA_2 caused only a small additional increment (PLA_2 alone, 0.38 ± 0.02 mg/mL vs. PLA_2 + RCM, 0.44 ± 0.02; $P < 0.05$). It had previously been determined that the employed RCM dosage did not affect the employed protein assay method.

NAK-ATPASE. Western blotting demonstrated that RCM caused significant shedding of NaK-ATPase (alpha chain; 112 kD) into the tubule medium (control, nondetected; RCM, 85 ± 40 density units) (Fig. 7). PLA_2 also caused NaK-ATPase shedding, a result which was dramatically and synergistically increased by the presence of RCM. As noted above (Fig. 6, left), there was a lack of a significiant increase in % LDH release, indicating that this NaK-ATPase loss was not simply a nonspecific result of RCM- or PLA_2-induced cell death.

CYTOCHROME C. As shown in Figure 8, RCM caused dramatic cytochrome c loss into tubule buffer (seen at 15 kD). This was also found with PLA_2. However, RCM + PLA_2 did not exert a synergistic effect on cytochrome c shedding. Again, there was a lack of increase in % LDH release, indicating that the cytochrome c loss was not simply a nonspecific response to cell death.

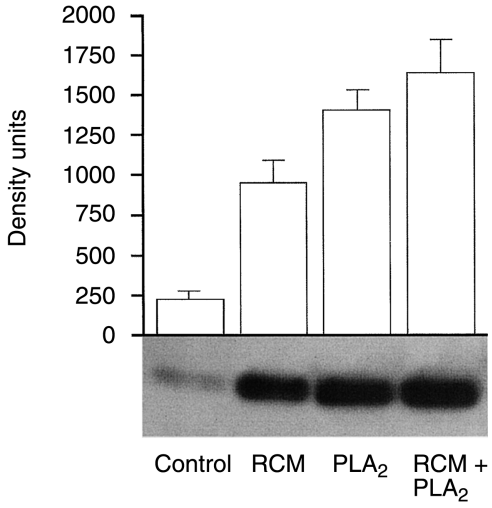

Fig. 8. Western blot analysis of cytochrome c release into tubule media. The protein band appears at 15 kD. Both radiocontrast medium (RCM) and phospholipase A_2 (PLA_2) induced extremely large increases in cytochrome c release, compared to control tubules ($P < 0.01$ and <0.001, respectively). Combined RCM + PLA_2 caused more cytochrome c loss than did RCM alone ($P < 0.035$).

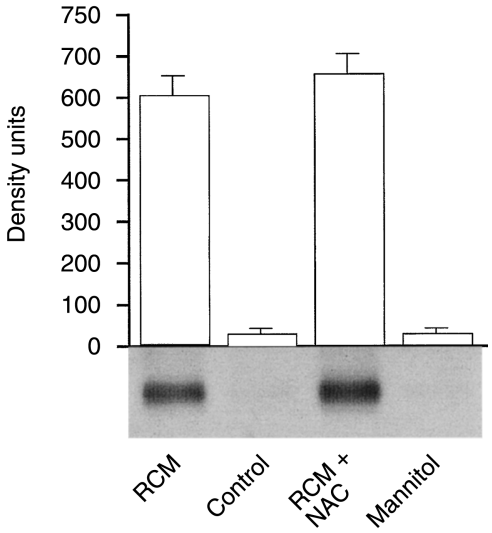

Fig. 9. Western blot analysis of caveolin release into tubule media. The protein band was observed at 22 kD. Only trivial amounts of caveolin were observed in samples obtained from tubules maintained under control conditions or treated with mannitol. However, radiocontrast medium (RCM) treatment caused marked caveolin release ($P <$ 0.01 vs. controls), a result which was not diminished by N acetyl cysteine (NAC) treatment.

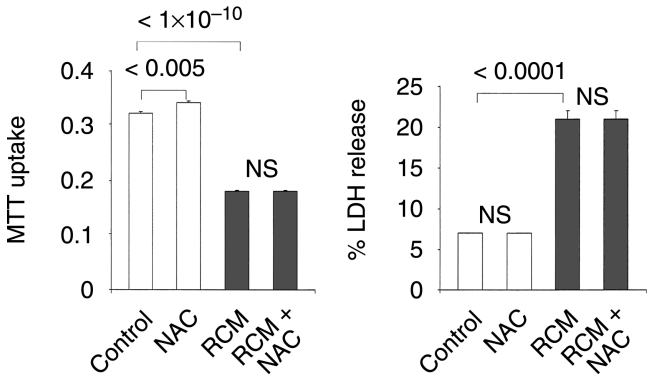

Fig. 10. Effect of radiocontrast medium (RCM) ± N acetyl cysteine (NAC) on HK-2 cell viability, as assessed by tetrazolium dye (MTT) uptake or lactate dehydrogenase (LDH) release assay. Incubating HK-2 cells with RCM for 3 days caused significant toxicity, as reflected by reduced MTT uptake and increased LDH release. NAC did not attenuate this toxicity, as assessed by either assay. NAC, by itself, slightly increased MTT uptake.

Caveolin. Media obtained from tubules incubated under control conditions showed a barely discernible caveolin band at 22 kD (Fig. 9). Conversely, RCM treatment caused a marked increase in caveolin expression ($P <$ 0.01 vs. control). Noteworthy was that mannitol did not cause increased caveolin release, indicating that RCM's effect was not simply due to an increase in media osmolality. NAC treatment did not diminish the RCM-induced caveolin increases, consistent with a lack of a protective effect (as described below). In none of the incubations did a significant increase in % LDH release result (≤14%, all samples), indicating that the caveolin loss was not simply due to cell death.

HK-2 cell experiments

RCM toxicity in the presence or absence of NAC, catalase, and SOD. RCM induced significant HK-2 cell injury, as indicated by both a 50% reduction in MTT dye uptake (which directly correlates with viable cell numbers) [34], and by a significant increase in LDH release (Fig. 10; black bars, white bars: RCM, no RCM, respectively). NAC, by itself, slightly increased MTT uptake in the absence of RCM. However, NAC completely failed to protect against RCM toxicity, as assessed by either MTT assay or LDH release. Furthermore (not depicted), neither catalase (Cat) nor SOD exerted any protective effect against RCM, as assessed by either MTT uptake (~50% reductions with RCM ± Cat or ± SOD) or by % LDH release (control, 8 to 10%; RCM, 30 ± 1%, RCM + Cat, 32 ± 21%; RCM + SOD, 30 ± 1%; all NS).

HK-2 cell RCM toxicity: effects of Sn protoporphyrin and BSO. Heme oxygenase inhibition with Sn protoporphyrin had no discernible independent effect on HK-2 viability, as assessed by MTT uptake or % LDH release (Fig. 11). It also failed to increase RCM toxicity, as assessed by either assay method. Rather, Sn protoporphyrin seemed to reduce RCM toxicity, as assessed by % LDH release.

BSO caused significant toxicity in HK-2 cells, as indicated by a 50% reduction in MTT uptake (Fig. 11). Despite this toxicity, it did not predispose to RCM toxicity, since comparable reductions in MTT uptake oc-

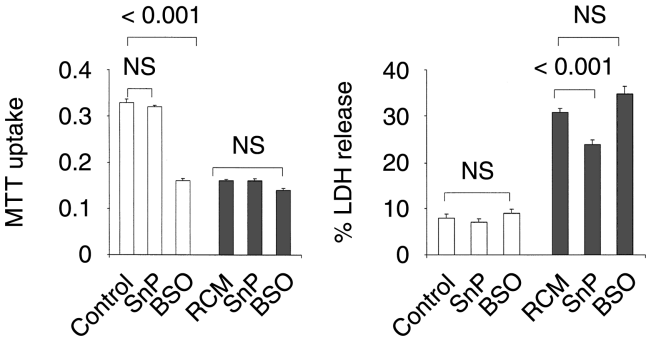

Fig. 11. Tin protoporphyrin (SnP) and L-buthionine sulfoximine dichloride (BSO) effects on radiocontrast medium (RCM) HK-2 cell toxicity. SnP had no significant effect on HK-2 cell viability in the absence of RCM. It also failed to worsen RCM toxicity. Rather, a paradoxical decrease in RCM-induced lactate dehydrogenase (LDH) release was observed. BSO caused significant cytotoxicity in the absence of RCM. However, in the presence of RCM, it failed to exacerbate cell death. Thus, these experiments indicated that depleting/inhibiting two important antioxidants [heme oxygenase and glutathione (GSH), respectively] had no effect on the severity of RCM toxicity. Symbols are: (□) without RCM; (■) with RCM. Assessments were made after 3 days' RCM exposure.

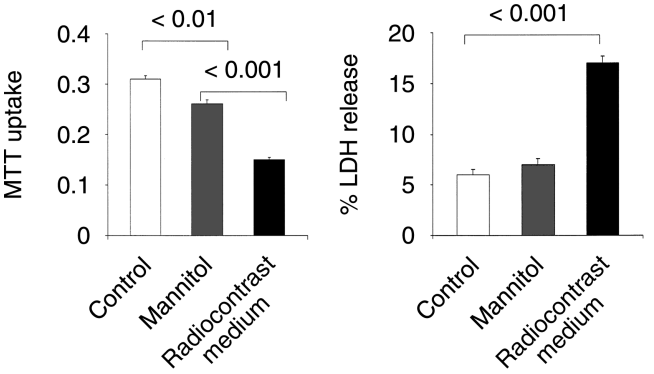

Fig. 12. Equiosmolar radiocontrast medium (RCM) versus mannitol on HK-2 cell viability. Mannitol caused a modest decrease in tetrazolium dye (MTT) uptake (consistent with either an antiproliferative effect or sublethal toxicity). However, it did not cause an increase in lactate dehydrogenase (LDH) release. Conversely, RCM caused a marked suppression of MTT uptake, as well as a significant increase in % LDH release. Thus, RCM cytotoxicity cannot be fully attributed to a hyperosmolar state. Assessments were made after 3 days' RCM exposure.

curred whether or not RCM was present. BSO also failed to increase RCM-mediated LDH release. Thus, in sum, the above experiments indicated that neither heme oxygenase inhibition nor GSH depletion predisposed HK-2 cells to RCM-mediated cell death.

RCM toxicity: effects of xanthine oxidase inhibition. Oxypurinol, the xanthine oxidase inhibitor, did not confer protection against RCM toxicity, as assessed by % LDH release. After 3-day RCM exposures, % LDH release for control, RCM, RCM + 50 μmol/L oxypurinol, and RCM + 100 μmol/L oxypurinol were 6 ± 0.5%, 26 ± 0.4%, 27 ± 0.4%, and 25 ± 0.5%, respectively. Oxypurinol did not exert independent toxicity, as assessed by LDH release (5 to 7% for all control and oxypurinol samples). The failure of oxypurinol to confer protection against RCM toxicity was observed, despite the fact that RCM addition to HK-2 cells caused an increase of three times in adenosine concentrations (Table 1). The increase in adenosine was associated with a decrement in total adenine nucleotide (TAN) (ATP +ADP + AMP) content.

Mannitol vs. RCM effects on HK-2 viability. As shown in the lefthand panel of Figure 12, both mannitol and RCM caused significant decreases in MTT uptake. However, the degree of suppression was approximately twice as great with RCM, indicating that its cytotoxicity was not simply the result of hyperosmolality. This point was underscored by the fact that RCM, but not mannitol, caused a significant increase in LDH release (Fig. 12, right). Lastly, as shown in Table 1, while mannitol caused a trend toward decrements in adenine nucleotides, significant reductions were only observed with RCM treatment.

Table 1. HK-2 cell adenine nucleotide profiles under control conditions and following 3-hour exposures to either equiosmolar mannitol or radiographic contrast medium (RCM)

Group	ATP	ADP	AMP	TAN	Adenosine
Control	72 ± 3	5.6 ± 1	1.5 ± 0.2	80 ± 3	3.2 ± 0.6
Mannitol	66 ± 1[d]	4.5 ± 0.5[d]	1.2 ± 0.1[d]	71 ± 1[d]	4.2 ± 0.05[d]
RCM	59 ± 2[a]	4.1 ± 0.2	0.6 ± 0.1[a]	64 ± 2[b]	9.6 ± 0.04[c]

Abbreviations are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; TAN, total adenine nucleotide. ATP, ADP, AMP, and TAN profiles are shown compared to the control. Radiographic contrast medium (RCM) treatment for 3 hours caused significant reductions in ATP and ADP, resulting in a decrease in TAN, and an increase in adenosine content. Equiosmolar mannitol showed a slight, nonsignificant effect on adenine nucleotides compared to the control. Values are given as nmol/flask.

[a] $P < 0.01$ vs. control

[b] $P < 0.01$ and <0.05 vs. control and mannitol, respectively

[c] $P < 0.001$ vs. control and mannitol

[d] NS vs. control

Iodide as a potential cytotoxic agent. Inorganic iodide (1.25 or 2.5 mmol/L) caused no cytotoxicity, as assessed by LDH release (all control and NaI values ≤5%). Furthermore, PTU did not block RCM's cytotoxic effect: [control, 5 ± 1%; PTU, 5 ± 1%; RCM, 17 ± 1% ($P <$ 0.001 vs. control); RCM + PTU, 17 ± 1%].

DISCUSSION

In 2000, Tepel et al [22] reported that NAC given prior to and the day of RCM administration significantly reduced the risk of ARF. Given the lack of other specific agents for preventing RCN (excepting NaCl loading), this positive finding has led to NAC's frequent use in the clinical setting. Because NAC can function as an antioxidant, it has been assumed that this action is responsible for its protective influence. However, NAC may also attenuate RCM-induced renal vasoconstriction

[41]. Furthermore, it remains to be proven that NAC's protective action stems solely from its free sulfhydryl, versus its acetyl, content [41]. Given these considerations, NAC-mediated protection cannot necessarily be equated with an antioxidant effect expressed at the tubular cell level.

To gain further insight into this issue, we sought to demonstrate that NAC can mitigate proximal tubular oxidant injury, and that this effect is, indeed, dependent on the compound's sulfhydryl content. NAC almost completely blocked Fe-mediated cell death in isolated tubules; conversely, neither NAL nor NAM exerted any protective effect. This supports the notion that NAC's protective action is, indeed, a function of its −SH, rather than its acetyl (or sulfur), content. It is notable that both renal ischemia and oxidative stress are thought to be critical pathogenic factors in RCM nephropathy. Because GSH can potentially block both injury pathways by providing cytoprotective glycine [27] plus functioning as an antioxidant, relative NAC versus GSH-mediated protection was assessed. Whereas each induced comparable protection against oxidant tubular stress, only GSH completely blocked hypoxic cell death. This raises the possibility that GSH (or the combination of NAC + glycine) might be a more potent cytoprotective strategy than NAC monotherapy.

NAC's ability to protect tubular cells against RCM toxicity is predicated on the assumption that RCM, in fact, mediates oxidative stress. To evaluate this issue, isolated tubules were incubated with RCM, followed by assessments of lethal cell injury (LDH release), and MDA generation. Furthermore, the ability of RCM to exacerbate superimposed oxidative and hypoxic/reoxygenation injury was assessed. Surprisingly, no evidence in support of RCM-induced oxidative stress could be gleaned from these experiments: RCM did not increase tubular MDA content; RCM did not predispose cells to superimposed Fe-mediated oxidative stress; and while RCM potentiated hypoxic cell injury [19], this change was dissociated from any increase in MDA content. Thus, in sum, no evidence for RCM-induced oxidative stress could be gleaned from any of these isolated tubule experiments.

A major limitation underlying these isolated tubule studies is that this model permits only limited incubation periods (generally ≤1 hour), potentially too short for lethal RCM cytotoxicity to develop. To circumvent this problem, additional studies were conducted using a more prolonged model of RCM toxicity: incubation of HK-2 cells with RCM for 3 days, followed by assessments of toxicity by MTT dye uptake and/or by % LDH release. In an effort to prove a mechanistic role for oxidative stress in RCM toxicity, protection against it was sought using a wide variety of antioxidants, including NAC, SOD, catalase, or oxypurinol. In no case was protection observed. The negative result with oxypurinol was particularly noteworthy, given that RCM addition to HK-2 cells induced an increase of three times in adenosine, an 'upstream' substrate for xanthine oxidase. Pursuing the reverse strategy, we attempted to sensitize HK-2 cells to potential RCM-induced oxidant stress by blocking endogenous antioxidant pathways (inhibition of heme oxygenase with Sn protoporphryin, glutathione depletion with BSO). However, neither of these approaches exacerbated RCM-induced damage. The latter results were particularly noteworthy, given that BSO on its own was cytotoxic (indicating the susceptibility of HK-2 cells to oxidant stress), and Sn protoporphyrin paradoxically mitigated RCM cytotoxicity. Hence, these HK-2 results are compatible with the isolated tubule data, indicating a lack of RCM- induced oxidant stress. It should be noted that these in vitro results are not necessarily in conflict with in vivo clinical data. For example, while Tepel et al [22] found positive results with NAC against RCN, Briguori et al [23] failed to show protection, except in a small subset of retrospectively analyzed patients. Furthermore, two additional recent studies each reported that NAC exerted no protective effect [42, 43]. Thus, our findings that NAC did not blunt in vitro RCM toxicity are consistent with these latter two reports.

A hypertonic milieu (e.g., resulting from high dose mannitol or sucrose injection) has frequently been linked to the induction of tubular injury and clinical ARF. This raises the issue of the relative importance of hyperosmolality versus compound-specific toxicity in RCN-induced tubular damage. This issue has previously been debated. For example, Humes et al [19] suggested that hyperosmolality was not a major factor, based on his studies in isolated tubule segments. However, studies by Hizoh and Haller [17] suggested that osmolar changes may be primary, given that both hypertonic NaCl and RCM caused DNA fragmentation in cultured MDCK tubular cells. Because NaCl, unlike contrast agents, undergoes active transport, conclusions based on it are not necessarily germane. Therefore, in the present study, HK-2 cells were incubated with RCM or with equiosmolar mannitol (as a nontransported solute matched control) and resultant toxicities were compared. While each factor may play a role, compound specific toxicity appears to be dominant, as indicated by the following: although both mannitol and RCM suppressed MTT uptake, the reductions were approximately twice as great with RCM treatment; only RCM induced membrane disruption, as assessed by LDH release; RCM, but not mannitol, caused significant declines in tubule cell ATP; and RCM, but not mannitol, caused plasma membrane caveolin release (see below).

While the molecular determinant of RCM cytotoxicity remains unknown, it is intriguing to speculate upon the possibility that it stems from its iodine content. Following

RCM administration, small amounts of iodide (ranging from 0.5 to 5% of the total administered dose) are released and excreted in urine [32]. Iodide is a preferential substrate for peroxidase-mediated free radical (hypoiodous acid) generation, and protein iodination/denaturation can result [40]. Hence, we considered the possibility that iodide might mediate some of RCM's cytotoxic effects. However, NaI addition did not adversely impact isolated tubule or HK-2 cell viability, as assessed by ATP/ADP ratios and/or LDH release. Furthermore, PTU, which blocks protein iodination, exerted no protective effect. Thus, the concept that free iodide is linked to RCM toxicity appears unlikely.

As previously noted by Humes et al [19], RCM can impair mitochondrial energetics, resulting in reductions in tubular ATP. Our results are consistent with this conclusion, given that RCM addition caused ouabain-resistant tubular ATP declines, as well as a depression in HK-2 cell ATP content. However, only 15 to 20% ATP reductions were observed. Given that ~50% and ~90% ATP depletion are thought to be required to induce tubular cell apoptosis and necrosis, respectively [44], it is highly unlikely that the presently observed ATP declines were sufficient, by themselves, to induce lethal cell damage. Hence, we sought a second potential mechanism by which mitochondrial injury might give rise to RCM toxicity.

Under normal circumstances, cytochrome c is associated with the inner mitochondrial membrane. However, with mitochondrial injury, it reaches the cytosol, where it can initiate caspase activation and ultimately cell death. Given its low molecular weight (15 kD), we hypothesized that released cytochrome c should readily traverse the plasma membrane, thereby being detectable in isolated tubule buffer if prior mitochondrial release occurs. Indeed, this appeared to be the case. With RCM addition, dramatic cytochrome c loss into the cell supernatant solution was observed in the absence of increased LDH release. It is notable that sublethal doses of pancreatic PLA_2, which primarily attacks the plasma membrane and secondarily depresses mitochondrial function [31], also caused cytochrome c release. This suggests that RCM-induced mitochondrial damage, as reflected by cytochrome c leak, is a downstream result of plasma membrane damage. That neither PLA_2 nor RCM caused lethal cell injury (increased LDH release) in these experiments indicates that the cytochrome c loss into the tubule media reflected a prelethal tubular cell event. The link between plasma membrane injury and secondary mitochondrial damage, and the ultimate role of cytochrome c release in the expression of RCM toxicity (e.g., potential induction of apoptosis), will each require substantial additional investigations.

The final goal of this study was to prove that RCM impacts cell injury by directly altering plasma membrane integrity, and ultimately, membrane susceptibility to attack. Several pieces of experimental evidence have been gathered to support this "membrane injury" hypothesis. First, RCM addition to proximal tubules caused a rapid loss of cell protein into the suspending media. That this was paralleled by the loss of two critically important plasma membrane proteins (NaK-ATPase and caveolin) supports the concept of RCM-induced "membrane destabilization," and indicates potential mechanisms by which RCM might disrupt cell integrity. Second, RCM dramatically sensitized the plasma membrane to exogenous PLA_2 attack. This was evidenced by synergistic NaK-ATPase loss with RCM + PLA_2; an RCM-induced doubling of exogenous PLA_2-induced deacylation; and increased hypoxia-induced deacylation in the presence of RCM, despite "clamping" lethal cell injury with glycine. It is notable that PLA_2-induced deacylation is a critical determinant of ischemic and hypoxic cell damage. That this process was greatly enhanced in the presence of RCM provides an important new insight into mechanisms by which RCM predisposes to ischemic and hypoxic cell death (e.g., Figs. 3 and 6).

CONCLUSION

The present study provides the following new insights: RCM-induced cytotoxicity can be dissociated from tubular cell oxidant stress (at least in vitro). This is consistent with recent findings [23, 42, 43] that NAC may not prevent clinical RCM-induced ARF; RCM toxicity cannot solely be explained by the induction of a hypertonic milieu, as previously suggested; rather, direct compound-related toxicity appears to play the dominant pathogenic role; the molecular basis for RCM-mediated cytotoxicity remains elusive at this time. However, release of inorganic iodide, with subsequent iodide toxicity, appears not to be involved; and RCM can de-stabilize the plasma, and potentially, the mitochondrial membranes. This can result in the loss of critical plasma membrane (e.g., NaK-ATPase, caveolin), and mitochondrial (cytochrome c) proteins, and an increase in plasma membrane susceptibility to PLA_2 attack. Given the importance of each of the above processes to cellular integrity, the current findings provide new potential insights into mechanisms of RCM tubular toxicity and its associated ARF. Whether the above observations, gathered using tubular cells, also have relevance to endothelial cells remains unknown at this time. However, given that vasoconstriction is a consequence of RCM adminstration, this represents an interesting question that potentially deserves future investigation.

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