Proximal tubule cell response to radiographic contrast media

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Hardiek, Kathy, Richard E. Katholi, Vickram Ramkumar, and Cynthia Deitrick. Proximal tubule cell response to radiographic contrast media. Am J Physiol Renal Physiol 280: F61–F70, 2001.—Renal dysfunction associated with contrast media (CM) administration is generally attributed to reduced renal blood flow. Studies, however, also suggest direct tubular effects of CM, whose mechanisms remain unclear. This study was conducted to assess the chemotoxic effects of iopamidol, a prototypic CM, on a porcine proximal tubule (PT) cell line, LLC-PK₁ cells. Results indicate that iopamidol did not affect cell viability (determined by trypan blue exclusion and fluorescein staining), but did reduce cell proliferation. Moreover, iopamidol altered mitochondrial function, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and mitochondrial membrane potential. Decreased MTT reduction was evident with all CM tested, and its rapid recovery after CM removal suggests that inhibition of mitochondrial function is reversible. Injury to PT cells by iopamidol is supported by the fact that CM increase extracellular adenosine, an indicator of cellular stress. This study provides greater insight into the mechanism underlying the nephrotoxicity induced by contrast in patients and explains the reversibility of this toxicity.

adenosine; contrast media nephrotoxicity; iopamidol; LLC-PK₁ cells; mitochondria

RENAL DYSFUNCTION AFTER coronary angiography is not uncommon, particularly in patients with preexisting renal failure. Any pathological state that decreases renal function, such as diabetic nephropathy and dehydration, also places an individual at increased risk for contrast media nephrotoxicity (CMN) (29, 30). The nephrotoxicity is characterized by a significant rise in serum creatinine/decrease in creatinine clearance in the absence of other etiology after contrast media (CM) administration (6, 22). Although the full mechanism of impairment is unclear, a combination of reduced renal blood flow and direct interaction of the CM molecule with proximal tubule (PT) cells are thought to be responsible.

The hemodynamic effects of CM consist of a transient efferent arteriole vasodilatation followed by a sustained afferent arteriole vasoconstriction (5, 17). This response is presently attributed to an imbalance of vasomodulators, including endothelin, nitric oxide, and adenosine (4). Studies indicate that endothelin increases after CM exposure but primarily occurs in association with CM volumes greater than 150 ml (8). Nitric oxide, on the other hand, only plays a significant role in CMN, in that decreased concentrations of vasodilators (unrelated to CM) may exacerbate the hemodynamic actions of CM (33). As such, any condition that may increase renal afferent arteriole vasoconstriction or decrease renal arteriole vasodilatation will have a deleterious effect on renal blood flow in the presence of CM. Adenosine, capable of inducing both vasoconstriction and vasodilatation, appears to be the major player in CMN and has been found to elicit a similar biphasic hemodynamic response when administered intrarenally (3). Furthermore, elevated adenosine levels have been found in the urine after CM administration. Although extraneous vasomodulators, such as calcium channel blockers (28) and theophylline (non-selective adenosine receptor antagonist) (18) tend to attenuate CMN, studies also indicate CM directly affect tubular cells.

As CM are primarily eliminated through the kidneys, it is not unreasonable to assume that CM molecules interact with the tubular epithelium. The most commonly reported response to this interaction is PT cell vacuolization (1, 2, 7, 12, 31, 32, and 34). Alteration of vacuoles/lysosomes begins within 2 h of CM administration (32) and suggests cytoplasmic injury as they contain granular electron-dense precipitate (2). Vacuolation, however, did not correlate with loss of function and could not be associated with the osmolality, viscosity, or overall hydrophilicity of the CM (7, 12). For example, iodixanol, a nonionic, isosmolar CM, not only increased vacuolization in PT cells, but also exhibited iodine retention as well (12, 34). Dobrota et al. (12) reported CM retention in PT cells and found that their uptake was comparable to fluid phase endocytosis of dextran. Endocytosis, however, remained fully functional regardless of the extent of PT cell vacuolization.

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and CM retention (12). Of note was the fact that ionic CM generally had a greater effect than nonionics on cells (34), including cytotoxic and cytostatic effects on vascular smooth muscle (35). This confirmed the previous report by Andersen et al (2) of decreased cell viability after metrizoate administration. The combination of these studies indicated that there was a definite interaction between renal cells and CM and that there was a need for further research on the cellular effects of CM.

This study was conducted to further characterize the cellular response of proximal tubule epithelium to contrast media. Cell responses, including viability and proliferation, were monitored, as well as the cell’s mitochondrial enzyme activity (24). Additionally, as adenosine appears to play an integral part in CMN, its concentrations were monitored to determine whether it might play a role in the direct effects as well as the hemodynamic actions of angiographic CM.

METHODS

Cell culture. LLC-PK1 cells (CRL-1392), a renal proximal tubule cell line of porcine origin, were obtained from the American Type Culture Collection (Rockville, MD) at passage 194, whereas primary human renal proximal tubule epithelial (HRPTE) cells were obtained from Clonetics (San Diego, CA). Cells were cultured in renal epithelial growth media (REGM), media specifically formulated for renal epithelial cells (Clonetics). All cultures were grown on collagen-treated tissue culture plates/flasks in 100% humidity and 5% CO2, with the media being replaced every 2–3 days. Experiments were performed on LLC-PK1 cells of passages 199 to 205 and HRPTE cells of passage 5. Cells were treated with either control media (serum-free REGM) or various doses of contrast media in their nonpyrogenic, pharmacological form diluted in serum free REGM. Contrast agents were donated by their respective companies, including iopamidol (Isovue-370) and iomeprol (Iomeron-400; nonionic, low-osmolar monomers; Bracco Diagnostics, Princeton, NJ), iodixanol (Visipaque) and sodium meglumine diatrizoate (Hypaque-76; nonionic, isosmolar dimer and ionic, high-osmolar monomer, respectively; Nycomed, Princeton, NJ), and ioxaglate sodium meglumine (Hexabrix; ionic, low-osmolar dimer; Mallinckrodt, St. Louis, MO). All other chemicals were obtained from GIBCO (Grand Island, NY) or Sigma (St. Louis, MO), as needed. Gross morphological assessment of the cells was conducted by using an Olympus microscope 2C-35 by using phase contrast or brightfield at 10X. Cells were viewed before and after a 10-min incubation with a 1:2 dilution of culture media with trypan blue (exclusion of the dye indicating viability).

Cell viability. Flow cytometry was used to detect both viable and apoptotic cells. In both assays, cell monolayers were detached with 0.5% trypsin, washed with REGM, and centrifuged at 2,500 rpm in a Fisher Centric centrifuge for 5 min. The pellet was washed and resuspended with Dulbecco’s PBS and diluted to 1 million cells/ml. In viability studies, fluorescein diacetate (0.04 M) was added in a 0.1% vol/vol ratio to the cell suspension. As proximal tubule cells are capable of transporting fluorescein via an anion carrier, those cells staining positive for fluorescein were considered viable (20). Apoptotic cells were assayed by using a kit obtained from Calbiochem (San Diego, CA). Briefly, the cell suspension was combined with 2% media-binding reagent and 0.25% annexin V-FITC and incubated in the dark for 15 min. The suspension was then centrifuged at 1,000 g for 5 min at room temperature, and the supernatant was decanted. The pellet was resuspended in cold 1× binding buffer and propidium iodide (2%). All samples were analyzed on a Becton Dickinson Facs vantage flow cytometer (Krakow, Poland) equipped with a coherent enterprise 488-nm argon laser. Green signals were acquired by logarithmic amplification with an excitation wavelength of 488 and a 530/30-nm filter. Acquisition of 10,000 events was based on linear forward (cell size) and side scatter (cell granularity).

Fig. 1. Morphological examination of LLC-PK1 cells incubated with 100 mg I/ml iopamidol. Cells were grown to confluency and incubated in the presence of serum-free media (SFM) or SFM supplemented with iopamidol for 24 h. A and B: light microscopic representations of control and treated cells, respectively. C and D: control and treated cells stained with trypan blue, respectively. Arrows, nonviable cells stained with trypan blue. Magnification, ×200.
Cell proliferation. Cell proliferation was assessed by using \([^{3}H]\) thymidine incorporation. LLC-PK1 cells were seeded into 96-well plates at a concentration of 200,000 cells/well and allowed to attach and incubate for ~48 h. Twenty-four hours before the results were read, control and experimental media were added followed by 1 \(\mu\)Ci \([^{3}H]\) thymidine per well 4 h later. After 18 h of pulse time, the plates were washed with deionized water and filtered by using a cell harvester. The filter disks were dried and placed in scintillation fluid, and the radioactive counts were determined by using a beta scintillation counter.

Mitochondrial enzyme activity (24) was measured by using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were incubated for 4 h with 0.5 mg/ml MTT in Dulbecco’s phosphate-buffered saline. The MTT solution was decanted from the wells, and the crystalline byproduct was dissolved in a 1:5 vol/vol solution of 3% sodium dodecyl sulfate and 0.04 N HCl in isopropanol. Samples were read on a MR600 Dynatech microplate reader at test wavelength of 570 and reference wavelength of 630 (calibration 1.99). Mitochondrial activity was reported as a percentage of the control.

MTT is a tetrazolium salt that is actively transported into the cell and reduced to a formazan byproduct via mitochondrial dehydrogenases (23). Conversion of MTT to its colored byproduct can therefore be used to measure various endpoints. Cells must be viable and capable of endocytosis for the cell to reduce MTT, whereas increased cell numbers generate increased byproduct generation, and thus indicate cell proliferation. MTT is converted to its colored byproduct via a group of nonspecific mitochondrial dehydrogenases, including NADH dehydrogenase, malate dehydrogenase, and succinic dehydrogenase. Byproduct formation represents the enzymatic activity of the mitochondria. Alterations in MTT reduction that cannot be explained by changes in viability or proliferation are most likely explained by alterations in the activity of these enzymes. MTT conversion is therefore a good indicator of mitochondrial enzyme activity as well as viability and proliferation.

Adenosine measurement. HPLC was used to quantify extracellular adenosine concentrations in conditioned cell media collected directly from 24-well plates after CM application (21). Duplicate samples were treated with either adenosine deaminase (7 units/ml) or a 1:20 dilution of phosphatase buffer (a combination of 0.15 M KH2PO4 and 0.6 M K2PO4) and incubated for 30 min at room temperature followed by deproteinization with 14% trichloroacetic acid (30-min incubation at 4°C). Samples were then treated with a 2.7% vol/vol mixture of 8 M chloracetaldehyde in 0.62 M potassium phosphate buffer (pH 5.5) or 1 unit/ml xanthine oxidase in phosphate buffer and incubated at 37°C for 30 min, followed by a second incubation at 100°C for 3 min. Samples were filtered with 0.45-\(\mu\)m syringe filters (acrodisc) and placed in the Waters 2690 autosampler.

Hypoxanthine peaks were eluted during a 10-min isocratic run by using 1% (vol/vol) acetonitrile and 0.05 M ammonium dihydrogen phosphate (pH 5.5) on a Keystone (Bellevont, PA) ODS/A, 5-\(\mu\)m (250 \(\times\) 4.6 mm) column that was heated to 45°C. Samples were read at 250 nm on a Waters 490 E ultraviolet detector. Concentrations were determined by net peak height (difference between buffer-treated and xanthine assay was deproteinated by ultra centrifugation at 4,300 g through Centricon-10 microconcentrate filters for 180 min at 10°C. Duplicate aliquots of filtrate were treated with 10% potassium phosphate buffer (a combination of 0.15 M KH2PO4 and 0.6 M K2PO4) or 1 unit/ml xanthine oxidase in phosphate buffer and incubated at 37°C for 30 min, followed by a second incubation at 100°C for 3 min. Samples were filtered with 0.45-\(\mu\)m syringe filters (acrodisc) and placed in the Waters 2690 autosampler.

Hypoxanthine concentrations were eluted with a 15-min gradient of a 2.5% solution of the aforementioned phosphate buffer (buffer A) and a 70:30 vol/vol solution of buffer A and methanol on a Regis (Morton Grove, IL) hi-chrom reverse spherisorb column (5 \(\mu\)m, 2.5 cm \(\times\) 4.66 mm) heated to 35°C. Samples were read at an excitation wavelength of 315 nm and an emission wavelength of 415 nm on a Waters 474 fluorescence detector.

Samples were analyzed by using Millenium software (Waters), and calculations were based on net peak height (difference between buffer and adenosine deaminase-treated samples) and standard curve adenosine peaks.

Hypoxanthine measurement. Extracellular hypoxanthine concentrations were quantified by using a modified method of Wiley (36). Conditioned cell media collected from the MTT

Fig. 2. Viability studies conducted in LLC-PK1 cells incubated with iopamidol. Cells were stained with fluorescein after a 24-h incubation with SFM or SFM supplemented with increasing concentrations of iopamidol. Flow cytometric representations of cells are treated with (B) and without (A) 100 mg I/ml iopamidol. Bottom: viable cells; top: nonviable cells. DCF, 2,7’-dichlorofluorescin diacetate; FL-1, fluorescence of fluorescein; PI, propidium iodide. C: comparison of percent viable cells over the entire range of iopamidol concentrations tested. Data are means &pm; SE of 6 independent experiments and indicate no change in cell viability over the range of iopamidol concentrations tested.
oxidase-treated samples) and standard hypoxanthine curves (see Fig. 3).

Mitochondrial membrane potential. 5′,6′-Tetra chloro-1,1′,3,3′-tetracycl benzimidazolocarbocyanine iodide (JC-1) was used to qualitatively determine alterations in mitochondrial membrane potential (9). Samples were analyzed on a Becton Dickinson Facs caliber-flow cytometer equipped with a coherent enterprise 488-nm argon laser. Green and orange signals were acquired by logarithmic amplification with an excitation wavelength of 490 nm and emission wavelengths of 530 and 590 nm, respectively. Acquisition of 10,000 events was based on linear forward and side scatter. Changes in mitochondrial membrane potential can be qualitatively determined by comparing the amount and intensity of light emitted at 590 and 530 nm. Valinomycin (100 nM in DMSO), a K⁺ ionophore, was used as a positive control for decreased mitochondrial membrane potential, whereas nonylacridine orange (10 μM, Molecular Probes, Eugene, OR) was used as a control for mitochondrial mass. Nonylacridine orange binds to mitochondria regardless of their energized state and indicates the relative number of mitochondria. This control was used to ensure that any alterations in potential were not due to an increase or decrease in mitochondrial numbers. Signals were acquired by logarithmic amplification with an excitation wavelength of 488 and a 530/30-nm filter, whereas acquisition of 10,000 events was based on linear forward and side scatter.

Statistical analysis. Analysis of variance and t-tests were used to analyze the data where appropriate whereas Tukey follow-up tests were performed as needed. Simple main-effect comparisons were conducted by using Bonferroni-adjusted alpha levels. All data are expressed as means ± SE. Percentage of control values were calculated by dividing the raw values of treated cells by the raw values of control cells and multiplying by 100. Significance was determined by \( P < 0.05 \).

RESULTS

Iopamidol (100 mg I/ml) notably altered LLC-PK₁ cell morphology at 24 h (Fig. 1). Treated cells (Fig 1, C and D) appeared shrunken with increased numbers of micronuclei compared with normal, healthy control cells (Fig. 1, A and B). Viability of treated (iopamidol, 100 mg I/ml) cells is also demonstrated in Fig. 1D via trypan blue staining. In Fig. 2, flow cytometry data (fluorescent labeling) confirmed that cells treated with 25, 50, 75, and 100 mg I/ml iopamidol were as viable as control cells.

To rule out apoptosis, cells were assayed for the early marker phosphotidyl serine, with annexin V binding after iopamidol treatment. Control and treated cells did not significantly differ (Fig. 3, A-C), in the percentage of annexin V-positive cells, these values being 15.7 ± 7.9 and 17.0 ± 3.2%, respectively. Dascula and Peer (10), however, had reported significant decreases in kidney cell viability after CM treatment when util...
lizing the MTT assay. Although our results also showed decreased MTT conversion, decreased viability was not consistent with our previous viability studies. To further explore this discrepancy, we measured thymidine incorporation to explore the possibility of decreased proliferation. Iopamidol significantly inhibited incorporation of thymidine into DNA at concentrations of 50, 75, and 100 mg I/ml (Fig. 3D), thymidine incorporation levels being 70.9 ± 13.8, 54.8 ± 3.35, and 44.5 ± 1.76% of control, respectively.

As mentioned above, our assays demonstrated that iopamidol inhibited mitochondrial dehydrogenase activity, as determined by MTT reduction, in a concentration (Fig. 4A)-but not in a time-dependent manner (Fig. 4B). MTT reduction was ~37% of control values after 100 mg I/ml iopamidol treatment and was fully reversible on CM removal (Fig. 4C). Although decreased MTT conversion could be justified through decreased proliferation, the rapid recovery of MTT reduction could not be accounted for. This suggested that MTT activity might be reflecting mitochondrial enzyme function rather than proliferation or viability.

To confirm that the CM molecule mediated these effects, the effects of pharmacological additives contained in the CM preparation, tromethamine and edetate calcium disodium, were tested individually and in combination for their effects on MTT reduction (at concentrations equal to that of 100 mg I/ml iopamidol). As single elements, both edetate and tromethamine had an insignificant (< 1% of control) effect whereas the combination had a small (96.6 ± 0.5% of control) but significant effect. As these additives had little or no effect on MTT conversion, iopamidol was determined to be responsible for the loss of MTT reduction.

Iopamidol was not the only CM to notably affect mitochondrial reductase activity. Iomeprol (nonionic, low-osmolar monomer), iodixanol (nonionic, isosmolar dimer), ioxaglate (ionic, low-osmolar dimer), and diatrizoate (ionic, high-osmolar monomer) had similar effects. A comparison of the effects of these CM did not indicate a single property (e.g., osmolality) to be responsible for this mitochondrial enzyme inhibition, although Fig. 5B demonstrates that there are significant \( P < 0.0001 \) differences among the contrast media and their effects on MTT reduction. Renal damage by CM is often attributed to the high osmolality of CM, but Fig. 5A shows that mannitol, when equiosmolar to iopamidol, did not have the same impact that CM did. All CM showed a significant and concentration-dependent inhibition of MTT reduction and were least “toxic” at concentrations of 25 mg I/ml and the most “toxic” at 100 mg I/ml. As can be seen in Fig. 5B, CM have varying effects over the range of concentrations tested. The differences among agents were most clearly seen at 100 mg I/ml. Iomeprol had the least effect on MTT reduction at 52% of the control, followed by iopamidol and iodixanol, at 39 and 31%, respectively. The two ionic CM, ioxaglate and diatrizoate, had the greatest effect on MTT conversion at 20 and 10% of the control.

Fig. 4. Iopamidol reduces mitochondrial dehydrogenase activity in LLC-PK1 cells. Cells were treated with various concentrations of iopamidol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was determined as described in METHODS. A: cells treated with iopamidol for 24 h showed a concentration-dependent reduction in MTT conversion, a marker of mitochondrial dehydrogenase activity. Data are means ± SE of 36 replicates; *Statistical significance \( P < 0.0001 \). B: the reduction in MTT conversion was evident by 4 h and remained relatively unchanged up to 60 h of iopamidol treatment. C: time course of recovery of mitochondrial function after removal of iopamidol. The zero-hour time point indicates inhibition of MTT reduction in cells treated with iopamidol for 24 h. Data are means ± SE of 8 replications. *Statistically significant difference from the time 0 point.
Loss of conversion in diatrizoate-treated samples may have partially been due to decreased cell attachment to substrate. Results indicate that human proximal tubule cells (HRPTE) respond to CM in a similar manner. Iopamidol (51.9 ± 1.4% of control), iomeprol (51.4 ± 2.2% of control), and iodixanol (52.8 ± 1.8% of control) significantly decreased MTT reduction (100 mg I/ml, 24 h) compared with control. Dose curves of individual CM were also significantly different from one another. These observations suggest that similar effects may occur physiologically.

Adenosine is known to play a substantial role in the hemodynamic actions of CM; however, its function and involvement at a cellular level is unknown. Extracellular concentrations of adenosine increased from 25.2 ± 5.7 (control) to 96.3 ± 3.7, 70.3 ± 5.7, and 74.1 ± 1.81 nm in cells treated with 50, 75, and 100 mg I/ml iopamidol for 24 h, respectively (Fig. 6A). On closer examination, adenosine concentrations were found to peak at 8 h at iopamidol concentrations of 100 mg I/ml (Fig. 6B). In contrast, the levels of hypoxanthine, an adenosine metabolite, were significantly reduced by 50 mg I/ml iopamidol but were nonsignificantly elevated with 75 and 100 mg I/ml (Fig. 6C).

Qualitative analysis of JC-1 staining indicates an increase in mitochondrial membrane potential after CM treatment (100 mg I/ml iopamidol, 24 h). JC-1 molecules aggregate and emit a specific fluorescence at high membrane potentials, whereas JC-1 aggregates dissociate into monomers that emit a different fluorescence pattern as mitochondrial membrane potential decreases. Figure 7 illustrates the results shown with the treatment of CM. Figure 7A represents JC-1 staining in cells grown in control media whereas positive control, valinomycin (decreased membrane potential, 100 nM), is shown in Fig. 7B. Valinomycin-treated cells have a much more widespread fluorescence pattern with an increase in monomer fluorescence. The JC-1 fluorescent profile for CM-treated cells can be found in Fig. 7C. Staining intensity (yellow < green < red < blue) increased along the aggregate scale whereas the monomer fluorescence decreased. Decreasing JC-1 monomers and increasing JC-1 aggregates suggest that mitochondrial membrane potential is increasing during incubation with CM. Acridine orange (Fig. 8) staining indicated that the rise in mitochondrial membrane potential was not due to an increase in the number of mitochondria, as acridine orange binds to mitochondria independently of its energy state.

**DISCUSSION**

Renal dysfunction is a serious complication that can occur with the administration of CM. Though the incidence of CMN is relatively low (1–2%) in the overall population (34), it poses a serious risk to those individuals with preexisting renal impairment (29, 30). This functional decline has been attributed to a combination of altered renal arteriole hemodynamics, as well as direct cellular effects of CM on renal cells, though past research efforts have primarily focused on the consequences of altered renal blood flow rather than the direct cellular effects. The present study was conducted to explore the potential actions of CM on PT cells. The dose range of CM chosen for these studies was carefully determined to allow for clinical applicability. CM are typically administered at a dose of 1.5 ml/kg, resulting in plasma concentrations of ~10 mg I/ml. Higher doses, however, are not uncommon, leading to plasma concentrations of 15–20 mg I/ml. Proximal tubule concentration, therefore, will be significantly higher as 60–80% of the water and solute content of glomerular filtrate is reabsorbed in this portion of the renal tubule. Beaufils et al. (7) have recorded urinary concentrations of CM as high as 120 mg I/ml in rabbits given clinical doses of CM (7). Our laboratory, therefore, chose a range of contrast concentrations that would potentially include patients with normal and compromised renal dysfunction (25–100 mg I/ml). Of course, the clinical relevance of this study has the limitations of any in vitro study, the lack of shifting concentrations of CM in tubules as well as the absence of a continuous blood supply to tubules. Results, however, indicate that CM injure cells, possibly through inhibition of mitochondrial function.

LLC-PK₁ cells responded to CM incubation with shrinking, a normal response to compounds with an osmolality greater than that of a cell (300 mosmol/kgH₂O). Plasma membranes, however, remained intact as indicated by trypan blue exclusion. Anderson et
al. (15) reported similar findings, as well as increased vacuolization (2), a sign of intracellular damage. Although morphology of these cells was distinctly altered, LLC-PK₁ cells treated with CM for 48–60 h did not show evidence of apoptosis or necrosis. Proliferation, however, was significantly decreased. As such, injury of PT cells may not be reflected in whole organ dysfunction for several h to days, which may explain the clinical course of CMN. The clinical time course of CMN is most likely a reflection of both CM clearance and cell recovery. Patients with normal renal function have a half-life of ~2 h whereas patients with impaired renal function may have the half-life extended to as much as 70 h (13). Although time does not appear to exacerbate the extent of injury inflicted by CM, the cells cannot recover until CM has been eliminated from the kidney. Clinical signs of renal dysfunction generally do not present for 24–48 h after CM administration and often subside within several weeks. This is not an unlikely recovery time if millions of cells must repair themselves and begin to function as a unit once again. Patients with concurrent renal dysfunction may be further compromised, as PT cells have decreased proliferation in the presence of CM.

The mechanism by which cells are injured may be linked to the cell’s diminished ability to reduce MTT to its formazen byproduct in the presence of CM. As mentioned previously, MTT reduction is a reflection of cell viability, proliferation, and mitochondrial enzyme activity. Initially, our MTT assay suggested a significant amount of cell death was occurring, but viability was confirmed by two separate independent viability tests. Decreased proliferation was only partially responsible as indicated by [³H]thymidine incorporation and mitochondrial function recovery experiments. This suggested that CM may be affecting mitochondrial enzyme activity and is supported by the fact that adenosine increases with CM treatment, a reflection of an imbalance between the production and utilization of ATP.

Comparison of five different CM did not reveal any specific property to which these results could be attributed. The ionicity of CM appeared to have the greatest influence on MTT activity followed by the molecular structure (monomer vs. dimer). Experiments evaluating the effect of hyperosmolar mannitol indicated a small effect of osmolality; however, comparisons with CM showed a wide range of responses. For example “low”-osmolar CM (i.e., 796 mosmol) had the least effect followed by isosomolar (i.e., 290 mosmol) CM, and finally “high”-osmolar (i.e., 2,016 mosmol). In reality, a combination of all CM properties is responsible for the effects on PT cells. To evaluate the application of these results to human tissue, MTT-reduction experiments were repeated in primary HRPTE cells and indicated that a similar phenomenon occurred. Although cells demonstrated a similar inhibition, the response patterns to various CM were different, implying that human cells may interact with CM somewhat differently.

As mentioned previously, adenosine has been implicated as a mediator of renal dysfunction induced by CM and is well known to reflect cellular stress (14). For these reasons, we chose to monitor the extracellular adenosine concentrations of cells treated with CM. Our results of increased adenosine may be an indirect repercussion of mitochondrial dysfunction. Nonfunctioning mitochondria fail to produce adequate amounts of
ATP to support normal cellular activity whereas high levels of adenosine may ensue as a consequence of increased ADP and AMP dephosphorylation. This may explain adenosine’s lack of a dose response to CM. Higher concentrations of CM may stress the cell to such an extent that it cannot regenerate ATP, whereas lower concentrations of CM may allow for such an occurrence. Adenosine is freely diffusible and may cross the membrane and exert its effect through various membrane receptors, explaining the actions of CM on the renal arteriole. Additionally, increased adenosine concentrations may offer an explanation for the generation of free radicals suggested to be involved in CMN (25, 37). Adenosine is metabolized to hypoxanthine (explaining their inverse relationship in Fig. 6, A and C) that can produce free radicals through xanthine-oxidase activity.

Mitochondrial dysfunction was confirmed through the JC-1 aggregate assay. This assay indicated an increase in mitochondrial membrane potential. Typically, dysfunction is exhibited by a loss of potential; however in states of high ATP utilization and low oxygen consumption, membrane potential can increase via a proton leak (11, 19). By creating a mild uncoupled state, the cell prevents large bursts of superoxide production, thereby protecting itself from oxidative injury and possible death (16). Furthermore, this increase in mitochondrial membrane potential decreases dehydrogenase activity, particularly succinate dehydrogenase. This could potentially explain the loss of MTT conversion in the presence of CM, as the agent requires succinate dehydrogenase and various other dehydrogenases to be converted to its formazen byproduct.

Fig. 7. Qualitative measure of mitochondrial membrane potential after CM treatment. Mitochondrial membrane potential was qualitatively observed by using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolecarbocyanine iodide (JC-1) staining after 24-h CM treatment. A: JC-1 staining for cells grown in renal epithelial growth SFM (control). B: cells treated with 100 nM valinomycin, a positive control for decreased mitochondrial membrane potential. C: the JC-1 staining in cells treated with 100 mg I/ml iopamidol (24 h). Increased JC-1-monomer fluorescence indicates a decrease in potential. JC-1 monomers decrease and aggregates increase in CM-treated cells, suggesting a rise in mitochondrial membrane potential. Staining intensity: yellow < green < red < blue.

Fig. 8. Qualitative assessment of mitochondrial number via acridine orange (AO) staining. Acridine orange staining was employed as a control for the JC-1 assay. Acridine orange binds to mitochondria regardless of their energetic state. A: control cells. FS, fluorescence. B: cells treated with 100 mg I/ml iopamidol. Comparison of the 2 profiles suggests that mitochondria number remain unaffected by CM treatment.
In summary, this study extended the knowledge of the cellular effects of CM, which apparently injures PT cells without altering their viability and is characterized by inhibition of MTT reduction. Ionicty appeared to have the greatest effect on inhibition; however, molecular structure and osmolality also have an effect. The theory of cellular injury/stress is supported by increased adenosine and may be a consequence of mitochondrial dysfunction (as indicated by reduced JC-1 aggregation) and an explanation for renal vasoconstriction.

CMN poses a serious risk to patients with chronic renal insufficiency undergoing procedures that utilize these agents, such as coronary angiography (29). This study provides further insight into the mechanism(s) by which CMN occurs and may open new avenues of prevention and/or treatment for CMN. Future studies are needed to confirm that CM do alter mitochondrial activity and that as a result ATP levels decrease generating high concentrations of adenosine. Additional studies are needed to explore the cellular actions of adenosine in acute renal failure.

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