Osmotic diuretics are used successfully to alleviate acute tubular necrosis (ATN) produced by chemotherapeutic agents and aminoglycoside antibiotics. The beneficial action of these agents likely involves rapid elimination of the nephrotoxic agents from the kidney by promoting diuresis. Adenosine A1 receptor (A1AR) subtypes present on renal proximal tubular epithelial and cortical collecting duct cells mediate the antidiuretic and cytoprotective actions of adenosine. These receptors are induced by activation of nuclear factor (NF)-κB, a transcription factor reported to mediate hyperosmotic stress-induced cytoprotection in renal medullary cells. In this study, we tested the hypothesis that induction of the A1AR in renal proximal tubular cells by NF-κB contributes to the cytoprotection afforded by osmotic diuretics. Exposure of porcine renal proximal tubular epithelial (LLC-PK1) cells to mannitol or NaCl produced a significant increase in A1AR. This increase was preceded by adenosine release and NF-κB activation. Expression of an IκB-α mutant, which acts as a superrepressor of NF-κB, abrogated the increase in A1AR. Cells exposed to manniitol demonstrated increased reactive oxygen species (ROS) generation, which was attenuated by inhibiting xanthine oxidase with allopurinol. Allopurinol attenuated both the increase in A1AR expression and NF-κB activation produced by osmotic diuretics, indicating a role of adenosine metabolites in these processes. Treatment of LLC-PK1 cells with cisplatin (8 μM) resulted in apoptosis, which was attenuated by manniitol but exacerbated by selective A1AR blockade. Administration of manniitol to mice increases A1AR. Cells exposed to manniitol demonstrated increased reactive oxygen species (ROS) generation, which was attenuated by inhibiting xanthine oxidase with allopurinol. Allopurinol attenuated both the increase in A1AR expression and NF-κB activation produced by osmotic diuretics, indicating a role of adenosine metabolites in these processes.

This paper is available online at http://www.jbc.org.
Hypertonicity-induced A\(_1\)AR Protects Proximal Tubule Cells

In the proximal tubule and the cortical collecting duct, adenosine stimulates the transport of sodium and phosphate via the apical surface (18), that of sodium and bicarbonate via the basolateral symporters (19) and stimulates chloride channel activity (20). As such, a primary physiological role of renal adenosine is believed to be antidiuresis, mediated through activation of proximal tubule A\(_1\)AR coupled to reabsorptive transport processes (21, 22).

The present study was performed to determine whether exposure to osmotic diuretics could induce A\(_1\)AR in proximal tubular epithelial cells and whether activation of A\(_1\)ARs could contribute to the protective action of mannitol against drug-induced nephrotoxicity.

EXPERIMENTAL PROCEDURES

Cell Culture

LLC-PK\(_1\) cells were cultured in renal epithelial growth media (REGM) supplemented with 0.5% serum, specifically formulated for renal epithelial cells. Cultures were maintained as monolayers on 75-cm\(^2\) tissue culture flasks/well plates at 37 °C, in 5% CO\(_2\), 95% ambient air, with the medium being replaced every 2-3 days.

Animals

Male C57BL/6 mice (6–8-week-old) obtained from Harlan Laboratory (Indianapolis, IN) and were maintained on pulverized food and water. Animals were used according to the guidelines approved by the Laboratory Animal Care and Use Committee of the Southern Illinois University School of Medicine.

Drug Treatment and Sample Collection

Mice were anesthetized by isoflurane inhalation, followed by retro-orbital sinus injection of mannitol (0.8 g/ml/kg body weight) or an equal volume of saline (controls). Animals were sacrificed after 2 h (for NF-\(\kappa\)B activity) or 20 h (for A\(_1\)AR expression by immunohistochemical methods) by cervical dislocation. Kidneys were removed, and the cortices were dissected out, rapidly frozen in liquid nitrogen and stored at −80 °C.

Radioiodin Binding Assays

Cells were treated with mannitol for 24 h and subsequently harvested for radioligand binding assay. Cells were detached in ice-cold phosphate-buffered saline (PBS) containing 5 mM EDTA and resuspended in 50 mM Tris-HCl buffer (pH 7.4), containing 10 mM MgCl\(_2\), and 1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, and 2 μg/ml peptatin (Buffer A). This was followed by homogenization with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 7 for 40 s at 4 °C. Membranes were obtained by centrifugation of the homogenates at 2,000 × g for 10 min, followed by centrifugation of the supernatant at 40,000 × g for 15 min. The final pellet was resuspended in Buffer B to yield a protein concentration of 0.5 mg/ml. The membrane suspensions were then treated with adenosine deaminase (5 units/ml) and incubated at 37 °C for 10 min to eliminate endogenously released adenosine.

Quantitation of A\(_1\)AR was performed using the tritiated antagonist \(\left[\text{H}\right]\text{DPCPX}\) and the iodinated \(\text{DPCPX}\) for 125I-AB-MECA binding) in

Luciferase Assay

LLC-PK\(_1\) cells were cultured to ~20–40% confluence and transfected with a mixture containing 100–250 ng of plasmid DNA, 500–650 ng of carrier DNA, and ~5 μg DNA of N-1-[2,3-dioleyloxypropyl]-1,3-dimethylimidizolium methylsulfate (LiPofectin) in a volume of 50 μl of Opti-MEM (Invitrogen). The mixtures were incubated for 45–60 min at room temperature and then added to the cultures. After ~6 h, regular renal epithelial growth media (supplemented with 0.5% serum medium) were added to the plate, and it was returned to the incubator for 24 h. For luciferase assays, cells were then lysed using 50 μl of reporter lysis buffer (Promega, Madison, WI) and centrifuged at 4 °C in a microcentrifuge at 12,000 × g. The extract was used immediately or stored at −70 °C. Twenty microliters of cell extract was mixed with 100 μl of luciferase assay reagent at room temperature and the chemiluminescent signal was determined in a luminometer using 1 min counts.

Preparation of Nuclear Extracts

Nuclear extracts were prepared from the cells and renal cortices as described previously (15). Briefly, the samples were suspended in Buffer B (1 ml of 10 mM HEPES, pH 7.9, 10 mM KCl, 0.5 mM MgCl\(_2\), 1 mM EDTA, pH 7.9, 5× Denhardt’s (50× = 0.25 volumes of 4% bovine serum albumin, 0.25 volumes of 4% polyvinylpyrrolidone, 0.25 volumes of 4% Ficoll, 0.25 volumes of dH\(_2\)O, 0.5% sodium pyrophosphate), 0.1% SDS, and 0.1 mg/ml salmon sperm DNA, using 1 × 10\(^6\) cpm/ml of \(\left[{\text{32P}}\right]\)-labeled A\(_1\)AR cDNA probe. Hybridizations were performed by shaking blots in a waterbath at 42 °C for 16–20 h. Following hybridization, blots were washed twice (15 min each) at room temperature in 2 × SSC and 0.1% SDS and twice (20 min each) with 0.1 × SSC and 0.1% SDS at 62 °C. The relative band intensities were determined by densitometric scanning on the GS-250 Molecular Imager (Bio-Rad) after exposing the blots to the imager screen for 1 h.

For Northern blotting experiments, poly(A\(^+\)) RNA samples (5 μg) were electrophoresed on a 1% agarose/MOPS/formamide gel, transferred to nylon membranes, and cross-linked in Stratagene UV cross-linker. Hybridization mixtures contained 5× SSC, 2× Denhardt’s, 0.1% SDS, 0.2 mg/ml salmon sperm DNA, and 50% formamide. Hybridization mixtures (10 ml) were essentially the same, except for the Denhardt’s concentration being 2.5×, and the added 25 labeled cDNA probes encoding the A\(_1\)AR at concentrations of 0.1×–10× cpm/ml. Hybridization and washing conditions were similar as described for Southern blotting. Image was visualized and quantitated using a densitometric scanner (as above). These blots were stripped and reprobed with labeled cDNA probe encoding the human glycosylated dehydrogenase (GAPDH) for normalization.

H\(_2\)DCFDA Fluorescence

Intracellular production of free radicals (reactive oxygen and nitrogen species) was detected in LLC-PK\(_1\), using 2′, 7′-dichlorodihydrofluorescein diacetate (H\(_2\)DCFDA, Calbiochem, San Diego, CA) (25–27). Cells were plated on sterile 12-mm glass coverslips at 400 cells/mm\(^2\) in individual wells of 24-well tissue culture plates. The cells were treated with 100 nm mannitol (Sigma), in the absence and presence of 250 μM allopurinol (Sigma) for 24 h. Coverslips were washed with PBS and the cells loaded with H\(_2\)DCFDA by incubating in 5 μM H\(_2\)DCFDA for 20 min at 37 °C and washed with PBS. The cultures were analyzed for green fluorescence 1 h later using an Olympus fluorescence confocal laser-scanning microscope using argon laser and a ×40 objective.

RNA Preparation, Polymerase Chain Reactions, and Northern Blotting

Isolation of total RNA was performed using TRIzol reagent kit (In-vitrogen) and selection of poly (A\(^+\)) messenger RNA, using oligo(dT)-cellulose as described previously (23). For PCR studies total RNA (1 μg each) was reverse-transcribed using a first strand cDNA synthesis kit (Amersham Biociences) in a total volume of 15 μl. Five microliters of each of the reaction volumes were used for PCR amplification. Primers used included the canine A\(_1\)AR consensus protein sequence ILGNULU (sense) and FALCWLP (antisense) and generated a 770-bp fragment (24). PCR were performed in a total volume of 50 ml using 2.5 mM MgCl\(_2\), using 36 amplification cycles. The amplified products were resolved on 1.2% agarose gels, which were subsequently denatured, neutralized and transferred to nylon filters for Southern blot analysis. Filters were UV cross-linked and prehybridized for 4 h at 42 °C in a mixture containing 50% formamide, 6× SSC (20× SSC = 175 g NaCl, 88 g sodium citrate, pH 7.0), 5× Denhardt’s (50× = 0.25 volumes of 4% bovine serum albumin, 0.25 volumes of 4% polyvinylpyrrolidone, 0.25 volumes of 4% Ficoll, 0.25 volumes of dH\(_2\)O, 0.5% sodium pyrophosphate), 0.1% SDS, and 0.1 mg/ml salmon sperm DNA, using 1 × 10\(^6\) cpm/ml of \(\left[{\text{32P}}\right]\)-labeled cDNA probe. Hybridizations were performed by shaking blots in a waterbath at 42 °C for 16–20 h. Following hybridization, blots were washed twice (15 min each) at room temperature in 2 × SSC and 0.1% SDS and twice (20 min each) with 0.1× SSC and 0.1% SDS at 62 °C. The relative band intensities were determined by densitometric scanning on the GS-250 Molecular Imager (Bio-Rad) after exposing the blots to the imager screen for 1 h.

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Electrophoretic Mobility Shift Assay

Nuclear extracts were incubated with double-strand-specific \(\kappa\)b oligonucleotide (5′-ATGGAGGGGACTTCCAGGC-3′) (28). Similar
Hypertonicity-induced A1AR Protects Proximal Tubule Cells

Characterization of the Adenosine A1 Receptor in LLC-PK1 Cells

The presence of the A1 and A2AAR in LLC-PK1 cells have been determined by functional assays (30), but no studies to date have demonstrated the existence of AR subtypes in these cells by radioligand binding experiments or molecular biology techniques. Therefore, initial studies characterized the A1AR in membrane preparations obtained from LLC-PK1 cells by radioligand binding assays using the agonist radioligand 125I-1\(^-\)N\(^6\)-(4-aminobenzyl)-9-(5-(methylcarbonyl)-\beta\)-ribofuranosyladenine (AB-MECA), along with 10 \(\mu\)M DPCPX to define nonspecific binding. While this radioligand interacts with both the A1 and A2AR (31), albeit with lower affinity for the latter receptor, the use of DPCPX, a selective antagonist to block the A2AR, provides a simple method for distinguishing this receptor subtype from the A1AR. The data were best fitted according to a one-site model by Graph Pad Prism Software (San Diego, CA), which indicates the receptor number \(B_{max}\) of 42.4 \pm 3.1 fmol/mg protein and equilibrium dissociation constant \((K_d)\) of 1.1 \pm 0.3 nM (Fig. 1A). In competition experiments, 125I-AB-MECA binding was inhibited by DPCPX, with an inhibitory constant \((K_i)\) of 1.6 \pm 0.1 ns, characteristic of the interaction of this drug with the A1AR (Fig. 1B). The inability of DPCPX to completely inhibit 125I-AB-MECA binding is indicative of this radioligand also interacting with other ARs (such as the A2AR), which are not targets of DPCPX. Radioligand binding was also inhibited completely by R-phenylisopropyladenosine (R-PIA), a relatively selective A1AR agonist, with a \(K_i\) of 1.2 \pm 0.1 nm (data not shown). This relatively high affinity interaction of R-PIA is also characteristic of its preferential interaction with the A1AR, at lower concentrations, as opposed to the A2AR subtype. Similar to other G-protein-coupled receptors, addition of GTP\(\gamma\S\) to membrane preparations dramatically reduced the population of the A1AR in the high affinity state, and therefore the level of 125I-AB-MECA. The binding of 125I-AB-MECA was reduced by 37.5 \pm 5.3% and 62.2 \pm 7.0%, upon incubation of membranes with 0.1 and 10 \(\mu\)M GTP\(\gamma\S\), respectively (data not shown). The presence of A1AR in LLC-PK1 cells was also confirmed by Northern blotting studies (Fig. 1C, upper), using poly(A)\(^+\) preparations and a bovine A1AR cDNA probe for detection of the transcript. Polymerase chain reactions were performed, using forward and reverse sequences derived from the canine A1AR cDNA. Primers used include amino acid sequence ILGNVLY (sense) and FALCWLP (antisense) common to the A1AR in several species (24). Sequences were identified as the A1AR by Southern blotting using a labeled canine A1AR cDNA probe (Fig. 1C). The figure shows the predicted 770-bp PCR fragments derived from the canine A1AR cDNA (lane 1), water blank (lane 2), LLC-PK1, cells (lane 3), rat kidney (lane 4), and testes (lane 5). Immunocytochemical assays, using a monoclonal antibody (32), were performed to further determine the presence of A1AR on LLC-PK1 cells. As shown in Fig. 1D, the presence of the A1AR was detected as a fluorescent halo, using confocal microscopy.
Mannitol Increases Expression of the Adenosine A1 Receptor in LLC-PK1 Cells—To determine whether the expression of the A1AR is modulated by hypertonicity, LLC-PK1 cells were exposed to 100 mM mannitol for 24 h, and A1AR levels were determined by radioligand binding assay. Saturation curves performed using 

\[ ^{125}\text{I-AB-MECA} \]

indicate an increase in the number of A1AR in cells exposed to mannitol for 24 h (Fig. 2A). In cells exposed to mannitol, the B_max was increased from 39.7 ± 1.7 to 65.8 ± 6.0 fmol/mg protein, with no significant change in K_D values. Similar increases in A1AR were obtained when the antagonist radioligand \([^{3}H]DPCPX\) was used to quantitate receptor levels (data not shown). LLC-PK1 cells exposed to increasing concentrations of mannitol for 24 h showed dose-dependent increases in receptor number by 90 ± 17, 110 ± 15, and 157 ± 18%, following administration of 50, 100, and 200 mM mannitol, respectively (Fig. 2B). Time course studies indicate significant increases in A1AR at 16 h after addition of mannitol, with further increases observed by 24 h (data not shown). In another series of experiments, addition of NaCl to separate cultures for 24 h resulted in a dose-dependent increase in A1AR expression, which was optimal at 150 mM NaCl (data not shown). Additional confirmation of an increase in A1AR was provided by immunocytochemistry (Fig. 2C). Results obtained show increases in A1AR expression in these cells following exposure of LLC-PK1 cells to mannitol (100 mM). Quantitation of the immunoreactivity by confocal microscopy indicated significant elevations in A1AR by ~80%, following exposure of cells to mannitol.

Additional experiments were performed to test whether the increase in A1AR could be explained by an increase in the steady state level of A1AR-specific mRNA, using Northern blotting assays. Blots probed with the canine A1AR cDNA probe showed a statistically significant increase in the steady-state levels of A1AR-specific mRNA by 68 ± 23% (Fig. 2D). One possible explanation for this observation is that mannitol increases the promoter activity of the A1AR gene. To test this possibility, LLC-PK1 cells were transiently transfected with plasmid pBLPnif/PmtA, which contains the firefly luciferase reporter gene driven by the A1AR promoter (33). Subsequent exposure of these cells to mannitol (100 mM) for 24 h resulted in a 3.4 ± 0.3-fold increase in luciferase activity compared with untreated control cells (data not shown), suggesting that the increase in A1AR mRNA could be caused by the increase in transcription of the A1AR gene.

Induction of A1AR Expression by Mannitol Involves Activation of NF-κB—Because previous studies have shown that hypertonicity increases NF-κB activity in renal medullary cells (10), we tested whether the increase in A1AR expression by these agents was due to activation of NF-κB in LLC-PK1 cells. Cells treated with mannitol (100 mM) demonstrated a significant increase in NF-κB activation, which was reduced substantially following co-incubation of mannitol with, either sodium salicylate (100 μM) or dexamethasone (100 nM), drugs known to inhibit this transcription factor (34) (Fig. 3A). Infection of LLC-PK1 cells with an adenoviral vector expressing a mutant form of IκB-α, which acts as a superrepressor of NF-κB (mIκB-α), abrogated the induction of A1AR by mannitol (Fig. 3A). Infection of LLC-PK1 cells was visualized by fluorescence microscopy, as the IκB-α gene was used was tagged with a green fluorescent protein. Further, the expression of mIκB-α in these cells was confirmed by electrophoretic mobility shift assay to determine nuclear translocation of NF-κB and Western blotting for IκB-α (data not shown). Taken together, these data provide strong support for the involvement of NF-κB in the induction of A1AR expression by mannitol. Additional studies showed that inhibition of the MAPK-ERK kinase (MEK) pathway by PD98059 failed to block the induction of the A1AR by mannitol (data not shown), indicating that this latter pathway does not contribute significantly to this process.

Further confirmation of mannitol-induced activation of NF-κB in LLC-PK1 cells was provided using electrophoretic mobility shift assays to determine nuclear translocation of NF-κB in LLC-PK1 cells. As shown in Fig. 3B, exposure of LLC-PK1 cells to mannitol (100 mM) or NaCl (100 mM) for 30 min-induced activation of NF-κB by 2–3-fold, as determined by increased retention of the \(^{32}\text{P}\)-labeled IκB oligonucleotide probe. There was a greater increase in NF-κB activity in cell treated with 100 mM NaCl than with 100 mM mannitol, as would be expected from the higher osmolarity produced by 100 mM NaCl.

To determine the subunit composition of the NF-κB complex, aliquots of the nuclear preparation were each incubated with preimmune serum or with polyclonal antibodies against distinct subunits, p50, p52, RelA, cRel, or RelB. The binding of the antibody to a specific subunit results in a higher molecular weight protein-DNA complex, which appears “supershifted” on a non-denaturing polyacrylamide gel. Our experiments indicate supershifted bands only in the preparations treated with anti-p65 and anti-cRel, suggesting that the NF-κB complex
detected in the nucleus contained predominantly the p65 and c-Rel proteins (Fig. 3).

**Role of Reactive Oxygen Species in Mannitol-induced Activation of NF-κB**—Previous studies from our laboratory indicate that the generation of ROS in response to cisplatin administration is the prime mediator of NF-κB activation by this chemotherapeutic agent, leading to induction of A1AR expression (15). To detect ROS, we utilized the reagent H2DCFDA, which fluoresces on binding with superoxides and peroxynitrite (25–27). In cells treated with mannitol, an increase in ROS generation was observed. The addition of allopurinol alone did not alter the basal ROS production. However, ROS production in response to mannitol was blocked by pretreatment with 250 μM allopurinol (Fig. 4A). Since allopurinol inhibits xanthine oxidase activity, a major source of free radical production in cells, this finding suggests that mannitol-induced ROS generation is mediated primarily by the xanthine oxidase pathway. Moreover, free radical production by this pathway could serve as the trigger for activation of NF-κB. In support of this notion, we showed that mannitol-induced activation of NF-κB was attenuated following exposure of cells to allopurinol (Fig. 4B). Since adenosine serves as a precursor for substrates of the xanthine oxidase pathway, we determined the levels of adenosine released following administration of mannitol. Cells treated with 100 mM mannitol showed a 2-fold increase in extracellular adenosine levels, suggesting that this could serve as a source of free radical production by the xanthine oxidase pathway. Treatment of cells for 24 h with either catalase (200 units/ml) or allopurinol (250 μM) abolished the induction in A1AR...
expression induced by mannitol (Fig. 4C). Additional experiments were performed to determine whether increasing oxidative stress by the addition of H2O2 (200 μM) to the LLC-PK1 cultures for 24 h mimics the response of mannitol. Results shown in Fig. 4D indicate that H2O2 induced A1AR and that this induction was reversed by catalase (200 units/ml) (Fig. 4D).

Mannitol Activates NF-κB and Increases Expression of A1ARs in Renal Cortices of Mice—To determine whether mannitol can induce expression of the A1AR in vivo, mice were administered mannitol by retro-orbital sinus injections (0.8 g/ml/kg mannitol). The diuretic effect of mannitol was evidenced by increased urination of the mice on the bedding in their cages and by increased water consumption. Kidneys were isolated after 20 h and the renal cortices were used to perform radioligand binding assay for the A1AR using [3H]DPCPX. We observed an 45% increase in A1AR expression in kidneys of mannitol-treated mice (5.9 ± 0.61 fmol/mg protein) when compared with control mice (4.1 ± 0.12 fmol/mg protein) injected with normal saline (Fig. 5A). Separate kidneys were used to assess nuclear translocation of NF-κB by mannitol treatment. As shown in Fig. 5B, there was a 3–5-fold increase in NF-κB in cortices obtained from the mannitol-treated when compared with saline-treated animals (Fig. 5B).

Cytoprotection Provided by Mannitol Involves Up-regulation of the A1AR—Whereas the protective effects of mannitol on the kidneys have been widely reported (1–4), the mechanism underlying protection remains to be established. Previous studies in our laboratory have demonstrated a protective role of the A1AR in reducing oxidative stress induced by cisplatin in a hamster vas deferens smooth muscle clone (15). We therefore examined whether activation of the A1AR would render the LLC-PK1 cells more tolerant to cisplatin toxicity. The conventional dose of 20 mg/m2/day cisplatin intravenously (35) results in plasma levels of 8–10 μM in a 70 kg man. Since cisplatin is concentrated in proximal tubular cells, it is expected that the levels achieved in these cells would be much higher than the plasma concentration of the drug (13). In order to correlate with a clinically effective plasma concentration of cisplatin, LLC-PK1 cells were pretreated with either vehicle or mannitol (100 mM) for 12 h, followed by administration of either vehicle or cisplatin (8 μM) for an additional 20 h. Flow cytometric analysis (Fig. 6, A and B) indicate that exposure to mannitol alone did not significantly affect apoptosis (upper panel, right). Exposure to cisplatin for 20 h resulted in a significant induction in apoptosis (Fig. 6A, upper panel, middle), with 40.7 ± 2.1% of cells staining positive for Alexa5-FITC. However, pretreatment with mannitol decreased the number of apoptotic...
The protective action of mannitol could also be demonstrated using TUNEL assays to quantify the number of apoptotic cells. In this assay, apoptotic cells are detected as dark brown to gray or black diaminobenzidine-stained nuclei (Fig. 6C). Quantitation of TUNEL assay images showed 3 ± 1 TUNEL-positive cells (8 ± 3%) in the control fields (total cells per field = 37 ± 7) and 2 ± 1 TUNEL-positive cells (6 ± 3%) in the mannitol treatment group (total cells per field = 34 ± 7). Following cisplatin treatment for 20 h, the number of TUNEL positive cells increased to 25 ± 3 (64 ± 8%) (total cells per field = 39 ± 2), while pretreatment with mannitol blocked the induction of apoptosis to 5 ± 1 (16 ± 3%) (total cells per field = 31 ± 5) (Fig. 6D). A similar finding was observed after cells were pretreated with NaCl (100 mM). Pretreatment with NaCl resulted in no significant change in the number of apoptotic cells from control. A total of 2.6 ± 1.0 (10 ± 4%) TUNEL-positive cells were detected per field (total cells per field = 26 ± 1). Following pretreatment with 100 mM NaCl for 12 h, exposure to cisplatin (8 μM) for 20 h did not result in any appreciable change in the number of apoptotic cells, which averaged 5.3 ± 0.5 TUNEL-positive cells per field (16 ± 1%) (total cells per field = 34 ± 1) (data not shown).

Discussion

Osmotic diuresis has commonly been used to alleviate nephrotoxicity due to chemotherapeutic agents (1) and other nephrotoxins. This mode of treatment to prevent nephrotoxicity was first introduced in the clinic by Ozols and Young (36). One factor which contributes to the beneficial action of agents like mannitol is an increase in single-nephron filtration due to an increase in glomerular plasma flow, which reduces the time the drug and renal tubule are in contact (2, 3). Mannitol also inhibits cisplatin-induced lipid peroxidation in rat renal slices, presumably via a direct antioxidant action (4). The administration of mannitol to the ischemic kidney increases renal blood flow, by decreasing the intrarenal vascular resistance through release of vasodilator substances such as prostaglandins or by washing out interstitial sodium, and reducing the sensitivity of the renal vasculature to ischemia-induced stimulation of the renin-angiotensin system (37). Indirect evidence from our laboratory indicates that blockade of the A1AR exacerbated cisplatin nephrotoxicity (14), implying a cytoprotective role of this receptor subtype under normal physiological conditions. However, the possibility that these receptors are also involved in mediating the beneficial actions of mannitol against cisplatin nephrotoxicity has not been studied.

Adenosine, acting via the A1AR, plays an important cytoprotective role against ischemic and chemical stressors, which increase ROS generation. Interestingly, ROS enhance the expression of the A1AR by NF-kB activation, which can then interact with consensus DNA sequences on the A1AR promoter (15). This suggests the presence of a feedback loop involving adenosine, ROS, NF-kB, and the A1AR, whereby the presence of ROS activates an NF-kB-dependent A1AR expression and the activation and induction of the A1AR reduces the toxicity of ROS. Adenosine controls the activity of a number of transport processes and ion channels in the kidney by interacting with renal tubular A1AR on the basolateral and apical membranes (18–22). Thus, up-regulation of the A1AR could serve as an additional mechanism of adaptation to osmotic stress. Recently published data have also shown that mannitol induces the expression of COX-2 in renal medullary interstitial cells through activation of NF-kB, and that this promotes cell survival by stimulating renal medullary blood flow (10). The induction of COX-2 was evident at higher concentrations (>200 mOsm) of mannitol, unlike the induction of the A1AR, which was statistically significant at 50 mOsmol.

The data derived from this study clearly indicate up-regulation of the A1AR in proximal tubular cell cultures by osmotic diuretics, which confers protection against cisplatin-induced apoptosis. The A1AR was induced in cells exposed to 50 and 100 mM mannitol, a concentration range that is achieved in the plasma after intravenous infusion (38) and which would be achieved in the lumen of the proximal tubules. Increased A1AR expression was also observed in mice injected with mannitol, indicating that this change might have beneficial application in vivo. The induction of A1AR was much slower than that of p53 (39) and heat shock proteins (40) by osmotic stress in the renal medullary epithelial cells. Thus, the A1AR might contribute to the slower adaptative response induced by osmotic stress.

Activation of NF-kB plays an integral role in the induction of the A1AR in LLC-PK1 cells by osmotic diuretics. This conclusion is supported by the observation that induction of A1AR was abolished following inhibition of NF-kB with sodium salicylate, dexamethasone, or by using a viral vector expressing a super-repressor of NF-kB. Furthermore, when LLC-PK1 cells were transiently transfected with a plasmid...
containing the A1AR promoter coupled to the luciferase reporter gene (15, 33), an increase in luciferase activity was observed upon exposure of cells to mannitol. In addition, exposure to mannitol resulted in an increase in the steady state levels of A1AR-specific mRNA.

The mechanism underlying the activation of NF-κB is presently unclear. In cells treated with mannitol, an increase in ROS generation was observed, which was blocked by co-administration of allopurinol. Since allopurinol inhibits xanthine oxidase activity, this finding directly implicates the generation of superoxides and subsequent H2O2 production in the activation of NF-κB. In support of this contention, we show that mannitol-induced activation of NF-κB was attenuated following exposure of cells to allopurinol. In preliminary studies, we observed a significant rise in the levels of extracellular adenosine (~2-fold increase) following exposure of LLC-PK1 cells to mannitol, suggesting that adenosine released could serve as a source for substrates by the xanthine oxidase pathway. A role of ROS in mediating the hypertonicity-mediated induction in A1AR expression was further supported by the observation that exposure of these cells to H2O2 mimicked the response to mannitol. Furthermore, the addition of catalase (to scavenge H2O2) reversed the induction in A1AR. In addition, our data show that allopurinol also inhibited the increase in A1AR induced by mannitol. Thus, our data suggest that exposure of kidney cells to osmotic diuretics leads to increased adenosine production, which is metabolized by adenosine deaminase to generate oxygen free radicals via the xanthine oxidase pathway. The generation of free radicals can, in turn, mediate NF-κB activation and increase A1AR expression. An important implication of this observation is that the nucleoside adenosine could likely regulate expression of its own receptors through generation of ROS and activation of NF-κB. However, the effect of hypertonic solutions to induce NF-κB activity seems to be cell-type specific, because a previous study from our laboratory demonstrates that, in smooth muscle cells, mannitol inhibits NF-κB activity induced by cytokines and LPS (41). A recent study supports our contention of an NF-κB mediated up-regulation of the A1AR in DDT1MF-2 cells by chronic hypoxia (42).

The relevance of hypertonicity-mediated increase in A1AR is apparent when one examines the effect of mannitol on cisplatin-mediated cytotoxicity. Incubation of LLC-PK1 cells with

**Fig. 6.** Mannitol protects against cisplatin-induced apoptosis. A, flow cytometry: cells were pretreated with 100 mM mannitol (panels c, d, and f) or with the selective A1AR antagonist (DPCPX, 1 μM) (panels e and f), along with mannitol (panels c, d, and f) for 12 h prior to the administration of cisplatin (8 μM) (panels b and d) in the culture medium for an additional 20 h. Apoptosis was determined by measuring the percentage of annexin-positive cells by flow cytometry and plotted in B. B, percentage of apoptotic cells for each treatment was determined in A and plotted as the mean ± S.E. of six independent experiments each. * indicates statistically significant change in apoptosis induced by cisplatin, ** indicates statistically significant potentiation of apoptosis by pretreatment of cells with DPCPX prior to addition of mannitol, *** indicates statistically significant change from cisplatin added alone. C, TUNEL assay: cells were pretreated with either vehicle, mannitol (100 mM), or NaCl (100 mM) for 12 h, followed by the addition of cisplatin (8 μM) for 20 h and then used for TUNEL assays. Apoptotic cells are detected as cells possessing dark brown to gray or black diaminobenzidine-stained nuclei. Cisplatin produced a significant increase in apoptotic cells, which was substantially reduced by pretreatment with mannitol and NaCl. Data are presented as the mean ± S.E. * indicates statistically significant difference from control (p < 0.05). ** indicates statistically significant suppression of cisplatin-induced apoptosis (p < 0.05).
mannitol resulted in significant protection of these cells against cisplatin-induced apoptosis. One explanation for this protective action of mannitol is that this agent serves as a scavenger of hydroxyl radicals (43–44) and thereby reduces oxidant-induced peroxidative damage (45). However, we observed that mannitol increased the generation of ROS in LLC-PK1 cells. Similarly, our data indicate that exposure of cells to cisplatin was associated with increased evidence of lipid peroxidation (as indicated by increased levels of malondialdehyde), whereas mannitol treatment had no effect on malondialdehyde levels (data not shown). So, it is possible that exposure to hypertonicity induces a low level of oxidative stress in these cells, not associated with increased malondialdehyde levels or significant cytotoxicity. Another event that may contribute to the protective action of mannitol is induction of COX-2 expression in LLC-PK1 cells, as observed for the medullary epithelial cells (10), leading to increased cell survival. However, activation of the A1AR, probably by the increased adenosine release (as described above), appears critical for mediating cytoprotection against cisplatin, since blockade of this receptor by DPCPX abolished the protective response elicited by mannitol. The lack of toxic effect of mannitol in these cells is likely due to the low concentrations of mannitol used (50–100 mM) in our studies, observed in the plasma after mannitol infusion (46).

The exact role of A1AR in the kidneys has been controversial, and there is no consensus as to whether these receptors protect the kidneys or whether they mediate cytotoxicity. However, a recent study by Lee et al. (47) demonstrates convincingly that mice lacking the A1AR exhibit increased apoptosis and necrosis, secondary to renal ischemia and reperfusion. Another recent study (48) demonstrates a cytoprotective role of the A1AR in the kidneys. Adenosine has also been shown to protect human proximal tubular cells from severe ATP depletion injury (49). Furthermore, data from our laboratory (14) provide indirect evidence for a protective effect of A1AR activation, because AR antagonists potentiated the toxicity of cisplatin.

The beneficial effect of agonists which have been described above could be due to a direct action on renal tubular epithelial cells, such as those in the proximal tubules. However, nephrotoxicity might result from A1AR-dependent constriction of the
renal afferent arterioles, thereby reducing blood flow to the kidney. It is likely that the net beneficial effect of A1AR activation results when the direct tubular beneficial effects outweigh the direct vasoconstrictor action. We propose that mannitol produces selective induction of A1AR in the renal proximal tubules and other nephron segments, but not the afferent arteriole. Such a scenario is possible since we have shown that mannitol inhibits NF-κB in cultured vascular smooth muscle cells (41), which would confer a reduction in A1AR expression in the afferent arterioles.

Previous studies have demonstrated induction of apoptosis in LLC-PK1 cells by cisplatin (50). The mechanism(s) underlying this event is currently being elucidated. Cisplatin-induced apoptosis is likely initiated by a number of proteins, which can “sense” DNA damage, such as nuclear excision repair proteins, mismatch repair proteins, DNA-dependent protein kinase, and high-mobility group proteins (51). DNA damage may then be communicated to other proteins involved in cell cycle arrest such as p53 (52), proapoptotic proteins such as Bax and Bak (53) and antiapoptotic protein Bcl-2 (54). Cisplatin-induced apoptosis also involves mitochondrial release of cytochrome c and sequential activation of caspase-8, caspase-3 and caspase-6 (55). Cells resistant to cisplatin toxicity demonstrate high levels of Bcl-2 and a reduction in the accumulation of p53. Bcl-2 suppresses the induction of Bax and therefore prolongs cell survival (56). The exposure of renal inner medullary cells to hyperosmotic stress induces the expression of heat shock protein 70 and confers additional protection to proximal tubular epithelial cells (41). In addition, increased adenosine released following exposure to osmotic diuretics could further stimulate A1AR, which would confer a reduction in vasoconstrictor action. We propose that osmotic diuretics, our data suggest that osmotic diuretics, which can mediate the direct vasoconstrictor action. We propose that osmotic diuretics, which can induce cell cycle arrest such as p53 (52), proapoptotic proteins such as Bax and Bak (53) and antiapoptotic protein Bcl-2 (54). Cisplatin, Chemistry, and Biochemistry of a Leading Anticancer Drug, pp. 111–134, Wiley-VCH, Basel, Switzerland.

In summary, the present study demonstrates that the A1AR gene could serve as an important target for modulation by osmotic diuretics. Our data suggest that osmotic diuretics, through activation of NF-κB, could induce expression of the A1AR. In addition, increased adenosine released following exposure to osmotic diuretics could further stimulate A1AR, thereby conferring additional protection to proximal tubular cells. We believe that the combined effect of A1AR activation and induction provides a novel mechanism by which osmotic diuretics protect renal proximal tubular cells against cisplatin-mediated nephrotoxicity.

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