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) subtype present on renal proximal tubular epithelial and cortical collecting duct cells mediates the antiuretic 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 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 mannitol 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 mannitol but exacerbated by selective A₁AR blockade. Administration of mannitol to mice increases A₁AR expression and activation of NF-κB in renal cortical sections. Taken together, these data provide novel mechanisms of nephroprotection by osmotic diuretics, involving both activation and induction of the A₁AR, the latter mediated through activation of a xanthine oxidase pathway leading to ROS generation and promoting activation of NF-κB.

Osmotic diuretic agents, such as mannitol are used in the prophylaxis of acute renal failure induced by antineoplastic agents (1), even though they have been supplemented recently by other agents. The beneficial action of osmotic diuretics derives from a reduction in time the nephrotoxic drugs are in contact with the renal tubules (2, 3) and, in the case of mannitol, through an additional direct antioxidant action (4). Osmotic diuretics also induce a number of genes that aid in cell survival, including those for heat shock proteins, and genes involved in the synthesis of osmolutes, such as aldose reductase, betaine transporter, and the sodium-dependent myo-inositol transporter (5, 6). The induction of these genes is mediated by components of the mitogen-activated protein (MAP) kinase signaling pathways, such as c-Jun N-terminal kinase and p38 MAP kinase (7, 8), and in the case of aldolase reductase, through activation of an osmotic response element, crucial for its regulation by hypertonicity (9). A more recent study also indicates a role for NF-κB-dependent cyclooxygenase-2 (COX-2) expression in protecting interstitial fibroblasts from hypertonic stress (10). In general, the studies described above have all used high osmotic stressors to induce proteins of interest in renal medullary cells, since these cells are subjected to a relatively high osmotic environment in vivo during periods of water deprivation.

The renal proximal tubules represent a primary site of action of osmotic diuretic agents. It is highly permeable to water, and as such, reabsorption of water is essentially isotonic. Chemo-therapeutic agents, such as cisplatin, are actively transported into proximal tubular cells (11) and concentrate in the P3/S3 pars recta segment (12). The concentration of the antineoplastic agent, cisplatin, in the proximal tubular epithelial cells exceeds plasma concentrations by a factor of 5 (13), rendering this segment most susceptible to injury. We have recently shown that cisplatin administration increased the expression of the adenosine A₁ receptor (A₁AR) in different regions of the kidney, including the proximal tube (14), presumably via activation of NF-κB (15).

Adenosine receptor (AR) subtypes show differential localization in the kidney. Specific receptor expression is demonstrated in different nephron segments, such as the glomeruli, the thick ascending limb and collecting duct (16) and the proximal tubules. Most correspondences should be addressed: SIU School of Medicine, Box 19250, Springfield, IL 62974-1222. Tel.: 217-785-2171; Fax: 217-545-0145; E-mail: vramkumar@siumed.edu.

1 The abbreviations used are: COX-2, cyclooxygenase-2; A₁AR, adenosine A₁ receptor; AB-MECA, N²-(4-aminobenzyl)-9-[5-(methylcarbonyl)-6-ribopyranosyl]adenine; DPCPX, 8-cyclopentyl-1,3-dipropyl-xanthine; H₂DCFDA, 2,7-dichlorodihydrofluorescein diacetate; NF-κB, nuclear factor kappa B; R-PIA, R-phenylisopropyladenosine; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; GTPγS, guanosine 5’-O-3-thiotriphosphate; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid.
Hypertonicity-induced A1AR Protects Proximal Tubule Cells

Mice were anesthetized by isoflurane inhalation, followed by retro-orbital sinus injection of mannitol (0.8 g/ml body weight) or an equal volume of saline (controls). Animals were sacrificed after 2 h (for NF-κB activity) or 20 h (for A1AR expression by cDNA microarray infiltration followed by cervical dislocation. Kidneys were removed, and the cortices were dissected out, rapidly frozen in liquid nitrogen and stored at −80 °C.

Radioligand Binding Assays

Cells were cultured with mannnitol 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-Cl buffer (pH 7.4), containing 10 mM MgCl2, and 1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, and 2 μg/ml pepstatin (Buffer A). This was followed by homogenization with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 7 for 40 at a 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 (0.2 U/ml) and incubated at 37 °C for 10 min to eliminate endogenous adenosine.

Quantitation of A1AR was performed using the triitated antagonist 8-cyclopentyl-1, 3-dipropylxanthine ([H]HIPCPX) and the iodinated agonist radioligand N6-(4-aminobenzyl)-9-[5-(methylcarbonyl)-b-p-ribofuranosyl]adenine ([3H]-AB-MECA). For these assays, membrane preparations (∼40 μg of protein) were incubated for 1 h at 37 °C with either radioligand in the absence or presence of theophylline (1 mM) (for [H]HIPCPX binding) or 10 μM DPCPX (for [3H]-AB-MECA binding) in order to define nonspecific binding. The total incubation volume was 250 μl. Samples were then filtered through polyethyleneimine-treated Whatman GF/B glass-fiber filters using a cell harvester (Brandel, Gaithersburg, MD) and washed with 9 ml of ice cold Buffer A (without protease inhibitors), containing 0.01% CHAPS. Bound radioactivity was determined using either a scintillation or gamma counter.

RNA Preparation, Polymerase Chain Reactions, and Northern Blotting

Isolation of total RNA was performed using TRIzol reagent kit (Invitrogen) and selection of poly (A+) messenger RNA, using oligo(dT) 30-mer, as described previously (23). For PCR studies total RNA (1 μg each) was reverse-transcribed using a first strand cDNA synthesis kit (Amersham Biosciences) in a total volume of 15 μl. Five microliters of each of the reaction volumes were used for PCR amplification. Primers used include the canine A1AR consensus sequence ILGNULU (sense) and FALCWLP (antisense) and predictedly generated a 770-bp fragment (24). PCR were performed in a total volume of 50 μl using 2.5 mM MgCl2, 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 dH2O, 0.5% sodium pyrophosphate), 0.1% SDS, and 0.1 mg/ml salmon sperm DNA, using 1 × 106 cpm/ml of 32P-labeled A1AR cDNA probe. Hybrization were performed by blots in a shaking waterbath at 42 °C for 16–20 hr. 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 Image (Bio-Rad) after exposing the blots to the imager screen for 1 h.

Luciferase Assay

LLC-PK1 cells were cultured to ∼20–40% confluency and transfected with a mixture containing 100–250 ng of plasmid DNA, 500–650 ng of carrier DNA and 3 μg DNA of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium 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 culture plate. 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 A (10 mM HEPES, pH 7.9, 30 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 0.5 mM Nonidet P-40, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The mixtures were centrifuged at 5000 × g for 30 s, and the cytosolic extract was separated. The nuclear pellet was washed with excess volume of Buffer B and then resuspended in Buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). After incubating for 5 min at 4 °C with rotation, the extracts were centrifuged (5000 × g, 1 min), and the supernatant was used for DNA binding activity analyses.

Electrophoretic Mobility Shift Assay

Nuclear extracts were incubated with double strand-specific [32P] oligonucleotide (5′-ATGTTAGGGGACTTCCAGGC-3′) (28). Similar
electrophoretic mobility shift assays were performed using a labeled oligonucleotide probe (5'-GGCGCTGTTGACCGCGGAA-3') for the AP-1 transcription factor binding sequence. Incubations were performed at room temperature for 30 min in a total volume of 15 μl of buffer containing 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM dithiorethiol, 1.0 μg of poly(dI-dC), and 10,000 cpm of the labeled probe. The DNA-protein complexes were resolved on nondenaturing 5% polyacrylamide gels, performed with 0.5× Tris borate/EDTA buffer (4.5 mM Tris, 4.5 mM boric acid, 1.0 mM EDTA, pH 8.0). Bands were imaged using the Phosphorimager and analyzed using Optiquant software.

**Apoptosis Detection**

**Annexin V-FITC Assay**—Cells were washed with PBS and harvested using 1 ml of 0.5% of trypsin/EDTA solution at 37°C. Cells were then centrifuged at 220×g for 5 min and immediately resuspended in the medium. Cells (~5×10^6 cells/ml) were then incubated in the dark for 15 min at room temperature with 10 μl of media binding agent and 1.25 μl of a FITC conjugate of Alexa 568. Cells were next centrifuged at 1,000×g for 5 min and resuspended in 0.5 ml of cold 1× binding buffer (HEPES, NaCl, CaCl₂, MgCl₂, bovine serum albumin). Propidium iodide (10 μl) was then added, and samples were placed on ice away from light and analyzed immediately. Quantification of Annexin V-FITC and propidium iodide signals was performed by a flow cell based bench top FACS Calibur Cytometer (BD Biosciences) with excitation wavelength of 488 nm and transmission wavelengths of 515–545 nm. Early apoptotic cells are reported in the lower right-hand quadrant of the dot plot, while necrotic or late apoptotic cells are reported in the upper right-hand quadrant of the plot. LLC-PK₁ cells were also examined by fluorescence microscopy (using a Olympus fluorview confocal laser-scanning microscope), equipped with Alexa 488 nm and Krypton (568 nm) lasers for green and red fluorescence, respectively. Phase contrast images were observed using transmitted light. Apoptotic cells were detected as bright apple green, while necrotic cells appear with various intensities of yellow-red throughout the cytoplasm. Viable cells remain unstained.

**TUNEL Assay**—Cisplatin-induced apoptosis was confirmed using the TUNEL assays, as detailed by the manufacturer. For this assay, cells were grown on poly-L-lysine-coated coverslips, pretreated with either vehicle or mannitol, and then treated with either vehicle or cisplatin for an additional 24 h. The monolayers were then fixed with 100 μl of 4% formaldehyde in 1× phosphate-buffered saline, cells were treated for 1 h at 37°C with Krypton (568 nm) lasers for green and red fluorescence, respectively. Phase contrast images were observed using transmitted light. Apoptotic cells were detected as bright apple green, while necrotic cells appear with various intensities of yellow-red throughout the cytoplasm. Viable cells remain unstained.

**Protein Determination**

Protein concentrations were determined by the established Bradford protein assay (29) using bovine serum albumin to prepare standard curves.

**Statistical Analyses**

Saturation curves and competition curves were analyzed by a computer-assisted curve fitting program (Graph PAD Prism Software, San Diego, CA). Statistical analyses were performed by the analysis of variance.

**RESULTS**

**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]^-N^-(4-aminobenzyl)-9-[5-(methylcarbonyl)-β-d-ribofuranosyl]adenine (AB-MECA), along with 10 μM DPCPX to define nonspecific binding. While this radioligand interacts with both the A₁ and A₃AR (31), albeit with lower affinity for the latter receptor, the use of DPCPX, a selective antagonist to block the A₂AAR, 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 (Bmax) of 42.4 ± 3.1 fmol/mg protein and equilibrium dissociation constant (Kᵣ) of 1.1 ± 0.3 nM (Fig. 1A). In competition experiments, [125I]^-AB-MECA binding was inhibited by DPCPX, with an inhibitory constant (Kᵣ) of 1.6 ± 0.1 nM, 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 A₃AR), which are not targets of DPCPX. Radioligand binding was also inhibited completely by R-phenylisopropyladenosine (R-PIA), a relatively selective A₁AR agonist, with a Kᵣ of 1.2 ± 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 A₃AR subtype. Similar to other G-protein-coupled receptors, addition of GTP-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 ± 5.3% and 62.2 ± 7.0%, upon incubation of membranes with 0.1 and 10 μM of GTP-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 labeled 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 testis (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 125I-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 observed when the antagonist radioligand ([125I]DPCPX) was used to quantify 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 (data not shown). Additional confirmation of an increase in A₁AR was provided by immunocytochemistry (Fig. 2C). Results obtained showed 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 μM), drugs known to inhibit this transcription factor (34) (Fig. 3A). Furthermore, 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 with an adenoviral vector expressing 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.

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 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

Fig. 4. **Mannitol increases ROS production in LLC-PK1 cells.** A, cells were treated with either vehicle or allopurinol (250 μM) for 2 h, followed by the addition of vehicle or mannitol for 30 min. ROS production was measured by confocal microscopy using the indicator H2DCFDA (5 μM), with wavelength setting of 488 nm. ROS production was detected as an increase in green fluorescence. These experiments were repeated at least three times. B, mannitol-induced increase in NF-κB activity in LLC-PK1 cells was attenuated by pretreatment with allopurinol (250 μM), catalase (200 units/ml), or PDT (50 μM), as detected by decreased band intensity in electrophoretic mobility shift assays. C, pretreatment of LLC-PK1 cells with allopurinol or catalase also attenuated mannitol-induced increase in A1AR expression, as observed by a decrease in the binding of the radioligand [125I]AB-MECA. D, treatment with hydrogen peroxide (200 μM) increased the expression of the A1AR. Pretreatment with catalase (200 units/ml) attenuated the increase in A1AR in response hydrogen peroxide.
Hypertonicity-induced A<sub>1</sub>AR Protects Proximal Tubule Cells

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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 A<sub>1</sub>AR 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 A<sub>1</sub>AR, plays an important cytoprotective role against ischemic and chemical stressors, which increase ROS generation. Interestingly, ROS enhance the expression of the A<sub>1</sub>AR by NF-κB activation, which can then interact with consensus DNA sequences on the A<sub>1</sub>AR promoter (15). This suggests the presence of a feedback loop involving adenosine, ROS, NF-κB, and the A<sub>1</sub>AR, whereby the presence of ROS activates an NF-κB-dependent A<sub>1</sub>AR expression and the activation and induction of the A<sub>1</sub>AR 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 A<sub>1</sub>AR on the basolateral and apical membranes (18–22). Thus, up-regulation of the A<sub>1</sub>AR 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-κB, and that this promotes cell survival by stimulating renal medullary blood flow (10). The induction of COX-2 was evident at higher concentrations (>200 mOsmol) of mannitol, unlike the induction of the A<sub>1</sub>AR, which was statistically significant at 50 mOsmol.

The data derived from this study clearly indicate up-regulation of the A<sub>1</sub>AR in proximal tubular cell cultures by osmotic diuretics, which confers protection against cisplatin-induced apoptosis. The A<sub>1</sub>AR 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 A<sub>1</sub>AR expression was also observed in mice injected with mannitol, indicating that this change might have beneficial application in vivo. The induction of A<sub>1</sub>AR was much slower than that of p53 (39) and heat shock proteins (40) by osmotic stress in the renal medullary epithelial cells. Thus, the A<sub>1</sub>AR might contribute to the slower adaptative response induced by osmotic stress.

Activation of NF-κB plays an integral role in the induction of the A<sub>1</sub>AR in LLC-PK<sub>1</sub> cells by osmotic diuretics. This conclusion is supported by the observation that induction of A<sub>1</sub>AR was abolished following inhibition of NF-κB with sodium salicylate, dexamethasone, or by using a viral vector expressing a super-repressor of NF-κB. Furthermore, when LLC-PK<sub>1</sub> 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-H9260B 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-H9260B. In support of this contention, we show that mannitol-induced activation of NF-H9260B 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-H9260B 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-H9260B. However, the effect of hypertonic solutions to induce NF-H9260B activity seems to be cell-type specific, because a previous study from our laboratory demonstrates that, in smooth muscle cells, mannitol inhibits NF-H9260B activity induced by cytokines and LPS (41). A recent study support our contention of an NF-H9260B 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
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 arteriole.

Previous studies have demonstrated induction of apoptosis in LLC-PK1 cells by cisplatin (50). The mechanism(s) underlying this event is only recently 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 thereby prolongs cell survival (56). The exposure of renal inner medullary cells to hyperosmotic stress induces the expression of heat shock proteins (57), p53 and MDM-2 (58), suggesting that these proteins might contribute to the protection afforded by the mannitol-induced hypotonicity. The induction of other antiapoptotic proteins by hypertonic stress needs to be addressed in the future.

A number of early studies have indicated a beneficial action of mannitol therapy against cisplatin nephrotoxicity (for review see Ref 1). In addition, the use of mannitol is indicated in ischemic injury to the kidney (59). However, recent trends have moved more toward hydration as a treatment alternative to cisplatin-mediated nephrotoxicity (60). While the use of mannitol in this study might not be in line with current therapy for cisplatin-mediated nephrotoxicity, we believe that the present data provide good evidence that activation of NF-κB and the A1AR by different agents could provide a useful prophylaxis against nephrotoxic drugs. One point to consider is that the induction of the A1AR required longer term exposure of cells to hypertonicity, generally between 16–24 h. We also observed a significant increase in A1AR in vivo 20 h following administration of a single dose of mannitol. This suggests that a treatment strategy worth considering is the prophylactic use of mannitol 24 h before chemotherapy to induce A1AR expression, in addition to its current use (or hydration) just prior to chemotherapy to enhance drug efficacy.

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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Hypertonicity-induced A1AR Protects Proximal Tubule Cells