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European Journal of Pharmacology 442 (2002) 251–264

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Cisplatin up-regulates the adenosine A_1 receptor in the rat kidney

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Received 27 February 2002; accepted 8 March 2002

Abstract

Cisplatin, a widely used anticancer drug, produces significant oto- and nephrotoxicity. Previous data from our laboratory, using cultured cell lines, indicated that cisplatin increases the expression of the adenosine A_1 receptor subtype through generation of reactive oxygen species and activation of nuclear factor-κB (NF-κB). Since the adenosine A_1 receptor plays an important role in normal renal physiology, this study was performed to determine whether cisplatin modulates adenosine A_1 receptor expression in vivo and whether these receptors play a role in the nephrotoxicity. Male Sprague–Dawley rats, treated with cisplatin (8 mg/kg), developed nephrotoxicity within 3 days, as demonstrated by increased serum creatinine and blood urea nitrogen. Cisplatin also produced a significant increase in malondialdehyde, apoptosis and necrosis in the kidney. The above changes were associated with a time-dependent increase in the expression of adenosine A_1 receptor, as determined by radioligand binding assays, Western blotting and immunocytochemistry, and an increase in adenosine A_1 receptor transcripts. Administration of selective and nonselective antagonists of the adenosine A_1 receptor produced either no change or exacerbated the nephrotoxicity produced by cisplatin. These data indicate that cisplatin can regulate the adenosine A_1 receptor in the kidney and suggest a cytoprotective role of this receptor subtype against cisplatin-induced nephrotoxicity.

Keywords: Nephrotoxicity; Renal failure, acute; Oxidative stress; Apoptosis; Anticancer drug

1. Introduction

Cisplatin is a widely used antineoplastic agent with proven efficacy in the treatment of advanced ovarian cancer (in combination with cyclophosphamide) and testicular cancer (in combination with bleomycin). This drug is also useful against tumors of the head, neck, bladder and endometrium and in the treatment of small cell carcinoma of the lung. The growth inhibitory action of cisplatin is mediated through inter- and intrastrand cross-linking of DNA, with interstrand cross-linking associated with its therapeutic efficacy. Significant side effects of drug use include hearing loss and nephrotoxicity (Loehrer and Einhorn, 1984; Ward and Fauvie, 1977), both of which are dose-limiting factors in therapy (Ozols et al., 1988). The current pharmacological management of nephrotoxicity involves increasing kidney perfusion by osmotic diuretics (such as mannitol and saline) and loop diuretic (such as furosemide) (Anand and Bashey, 1993). The use of diuretics, however, does not necessary preclude some degree of nephrotoxicity observed with cisplatin administration. As such, more research is needed to fully appreciate the area of cisplatin-induced nephrotoxicity.

Recent studies have implicated reactive oxygen species in the development of ototoxicity and nephrotoxicity induced by cisplatin. This agent induces the formation OH^- and other free radicals in cochlear explants (Clerici et al., 1996) and increases peroxide generation in murine L929 and human Jurkat T cells (Sasada et al., 1996). In vivo studies in rats indicated that cisplatin decreases the activities of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase (GSH-Px) (Kruidering et al., 1997; Sadzuka et al., 1994), and decreased the levels of reduced glutathione needed for scavenging reactive oxygen species (Mistry et al., 1991; Husain et al., 1993; Somani et al., 1995; Kuhlmann et al., 1997). This increase in reactive oxygen species could potentially produce DNA damage and stimulate peroxidation of lipid membranes (Salahudeen et al., 1995). In support of this notion, cisplatin has been shown to promote lipid peroxidation in the kidney (Brady et al., 1993;

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0014-2999/02/$ - see front matter
PII: S0014-2999(02)01510-8

Kim et al., 1997). Furthermore, depletion of cellular glutathione potentiates cisplatin-induced lipid peroxidation and cytotoxicity (Anderson et al., 1990).

Our interest in the kidney adenosine A_1 adenosine receptor as a target for cisplatin stems from previous findings that antagonists of this receptor reduced cisplatin-induced nephrotoxicity (Heidemann et al., 1989; Knight et al., 1991; Nagashima et al., 1995). In addition, we have previously shown that cisplatin increased adenosine A_1 receptor expression in ductus deferens smooth muscle cells by generating reactive oxygen species and activating nuclear factor-κB (NF-κB) (Nie et al., 1998). We speculated that the beneficial action of the adenosine A_1 receptor blockade against cisplatin nephrotoxicity is due, in part, to abolition of the function of this increased adenosine A_1 receptor expression. In the renal afferent arterioles, activation of the adenosine A_1 receptor reduce glomerular filtration rate, presumably by constricting these arterioles in the outer regions of the renal cortex (Oswald, 1984). While the afferent arterioles express both adenosine A_1 and A_{2A} receptor subtypes, the efferent arterioles contain exclusively the adenosine A_{2A} receptor subtype (Lang et al., 1985). Other integral functions of adenosine in the kidneys include inhibition of renin release (Arend et al., 1984), stimulation of erythropoietin production (Ueno et al., 1988) and regulation of Na^+, Cl^- and water excretion (McCoy et al., 1993). In addition, adenosine regulates normal cellular metabolic activity and thereby helps to maintain a balance between oxygen consumption and demand (Spielman and Arend, 1991). Since these latter functions are essential the normal functioning of the kidney, it is difficult to reconcile the protective action of adenosine A_1 receptor blockade against cisplatin-induced renal failure.

The goal of this study was to determine the ontogeny of cisplatin-induced nephrotoxicity in the rat and expression of the adenosine A_1 receptor in the kidney. Our results indicate a positive correlation between cisplatin-induced adenosine A_1 receptor expression in the kidney and nephrotoxicity, and suggest that the increase in receptors may serve to negate cisplatin-induced nephrotoxicity.

2. Methods

2.1. Drug treatment and sample collection

Male Sprague–Dawley rats (250–275 g) were obtained from Harlan Laboratory (Indianapolis, IN) and were maintained on pulverized food and water. During the 3-day treatment period, rats were kept in metabolic cages which facilitated the collection of urine. Animals were used according to the protocol approved by the Laboratory Animal Care and Use Committee of the Southern Illinois University School of Medicine.

For the injection of cisplatin, rats were first anesthetized by intramuscular injections of a ketamine/xylazine mixture (5.5 mg/kg xylazine and 17.2 mg/kg ketamine), followed by cisplatin (8 mg/kg body weight) or an equal volume of saline (controls). Injections were performed over a 30-min period using an infusion pump (Harvard Apparatus, South Natick, MA). Urine outputs were determined every 12 h and urine was stored for the assessment of creatinine. Blood samples were collected by heart puncture using tubes containing EGTA and reduced glutathione as anticoagulants. Following blood collection, groups of animals were sacrificed daily (for up to 3 days) using an overdose of the ketamine/xylazine mixture, their kidneys were removed, rapidly frozen in liquid nitrogen and stored at −80 °C. Animals were perfused and fixed with a mixture containing periodate, picric acid, paraformaldehyde or 4% formaldehyde in order to perform immunocytochemistry or apoptosis studies, respectively.

The effects of antagonists blockade of the adenosine A_1 receptor on cisplatin-induced nephrotoxicity were determined by intraperitoneal administration of aminophylline (24 mg/kg), theophylline (24 mg/kg) or DPCPX (0.1 mg/kg) at intervals of 12 h, with the initial dose administered 30 min following cisplatin administration. Caffeine was administered in the drinking water at a concentration of (2 g/l).

2.2. Membrane preparation

Frozen kidneys were first thawed and then homogenized by Polytron (Brinkman, setting 7 for 40 s) in ice-cold 50 mM Tris–HCl buffer containing 10 mM $MgCl_2$, 1 mM EDTA, 2 μg/ml pepstatin A, 10 μg/ml trypsin inhibitor and 10 μg/ml benzamidine (buffer A). Following centrifugation at $1000 \times g$ spin for 10 min, supernatants were centrifuged at $40{,}000 \times g$ for 15 min using an RC5 Sorvall centrifuge. The resulting pellets were resuspended in buffer A to a give a final protein concentration of ~ 1 mg/ml. Prior to performing radioligand binding assays, crude plasma membrane preparations were incubated with adenosine deaminase (5 units/ml) at 37 °C for 10 min in order to degrade adenosine produced during the membrane preparation.

2.3. Radioligand binding assay

Initial studies suggested that the levels of the adenosine A_1 receptor in the kidney was substantially lower than in the rat brain. In order to obtain a good signal for radioligand binding assays, we opted to use the agonist radioligand ^{125}I-N^6-(4-aminobenzyl)-9-[5-(methylcarbonyl)-ß-D-ribofuranosyl]adenine (AB-MECA) because of its high specific activity. However, since this ligand can bind to both the adenosine A_1 and A_3 receptors, the level of the adenosine A_1 receptor was determined using the selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), to define nonspecific binding. Assays were performed by incubating membranes (100 μg membrane protein) at 37 °C for 1 h with increasing

concentrations of the radioligand in absence (total binding) or presence (nonspecific binding) of 10 μM DPCPX. The incubation volume was 250 μl of buffer A. Following incubations, samples were filtered through GF/B glass fiber filters using a cell harvester (Brandel, Gaithersburg, MD) and quickly washed with 9 ml ice-cold buffer A containing 0.01% CHAPS. Bound radioactivity was determined using a gamma counter (Packard Instrument, Downers Grove, IL). Saturation curve were analyzed by a computer-based curve-fitting program according to a one-state model (Graph Pad PRISM, San Diego, CA).

2.4. Histological examination

Histological examinations of the section were performed under the light microscope after staining the above slides with hematoxylin and eosin stain.

2.5. Western blotting

For quantitation of the adenosine A_1 receptor, membranes were gently homogenized on ice in buffer A containing 3-[(3-cholamidorpropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), with a CHAPS/protein ratio of 2.5:1 (w/w). Samples were then stirred on ice for 1 h and centrifuged at 40,000 × *g* for 15 min. The supernatants were desalted on Sephadex G-25 columns and equilibrated with buffer A containing 0.1% CHAPS. The eluates (100 μg protein each) were dissolved in solubilization buffer and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes, blocked in Blotto buffer (130 mM NaCl, 2.7 mM KCl, 1.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% NaN_3, 0.1% Triton-X 100 and 5% low-fat skim milk) for 2 h and then incubated with the primary antibody for the adenosine A_1 receptor (Alpha Diagnostic International, San Antonio, TX) at 4 °C overnight. After five washes in blocking solution, blots were incubated horseradish peroxidase-labeled goat antirabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at room temperature, washed five times, treated with Enhanced Chemiluminescence Plus reagents (Amersham Pharmacia Biotech) and exposed to Kodak XAR film at room temperature for about 1 min. The relative band intensities were determined by densitometric scanning on GS-250 Molecular Imager (Bio-Rad Laboratories, Hercules, CA) after exposing the blots to the imager screen for 6–12 h.

2.6. Adenosine A_1 receptor immunocytochemistry

For immunochemical localization and quantitation of adenosine A_1 receptor in kidney sections an indirect immunofluorescence method (Yu et al., 1997) was performed, using adenosine A_1 receptor specific antisera (Alpha Diagnostic) and a reagent kit (Molecular Probes, Eugene, OR). Kidney sections (16 μm thick) were mounted on Vectabond coated slides (Vector Laboratories, Burlingame, CA) and dried overnight at 4 °C. The sections were washed with 0.01 M phosphate buffered saline and blocked using 1.5% normal goat serum diluted in 0.05% Triton-X/PBS for 30 min at room temperature. After washing with 0.01 M phosphate buffered saline, the specimens were incubated with primary antibody at a titer of 1:100 for 48 h at 4 °C. This was followed by incubation with rhodamine (TRITC) fluorescent-tagged IgG (1:100 titer) for 1 h. The sections were then incubated with a nuclear stain, SYTOX (titer of 1:300), in dark at room temperature for 5 min. The sections were then rinsed with 10 mM phosphate buffered saline and subjected to fluorescence microscopy using the Olympus fluoview confocal laser-scanning microscope with Argon (488 nm) and Krypton (568 nm) lasers. For quantification of immunoreactivity, 20 fields from six sections each from four different animals were used. Results were analyzed and expressed as in each group as lx/μm^2.

2.7. Terminal deoxy transferase-mediated deoxy uracil triphosphate nick end labeling (TUNEL) assay

Apoptosis was determined using the TUNEL assay kit (Oncogene Research Products, Cambridge, MA), essentially utilizing the manufacturer's protocol. In brief, the kidney sections (16 μ thick) were mounted on slides, permeabilized by incubating with 100 μl of 20 μg/ml proteinase K solution for 20 min and then incubated with 100 μl of 0.3% H_2O_2 for 5 min to inactivate endogenous peroxidase. The sections were then treated at 37 °C with 60 μl of terminal deoxythymidine enzyme mix for 1.5–2 h in a humidified chamber, followed by 100 μl of 0.5 M EDTA, pH 8, for 5 min to stop the reaction. Diaminobenzidine staining was performed by incubating sections with 100 μl of 4% bovine serum albumin in 0.01 M phosphate buffered saline for 10 min, followed by incubation with 100 μl of 1 × peroxidase strepavidin conjugate, in a humidified chamber, for 30 min and subsequent incubation with 100 μl of diaminobenzidine solution for 5 min. After a single rinse with dH_2O, the apoptotic cells with dark brown/gray-black diaminobenzidine stained nuclei and non-apoptotic cells with blue-green to greenish tan nuclei were evaluated using a Leitz Diaplan microscope with digital camera. A positive control generated by 1 μg/μl DNase and a negative control generated by replacing terminal deoxythymidine enzyme reaction mix by dH_2O were included as a marker. Quantification of TUNEL positive cells/nuclei was performed from at least 10 images from four different animals and were expressed as a percent of TUNEL positive cells.

2.8. Annexin V-fluorescein isothiocynate apoptosis detection assay

The cisplatin-induced apoptosis were measured using the Annexin V-fluorescein isothiocynate (FITC) apoptosis detection kit (Oncogene Research Products) and the proto-

col. In brief, the kidney sections mounted on slide were washed with 0.01 M phosphate buffered saline then incubated with 10 μl of media binding agent and 1.25 μl of a fluorescein isothiocynate conjugate of AnnexinV, in the dark for 15 min. The sections were subsequently washed three times with 0.5 ml of cold 1 × binding buffer and 10 μl of propidium iodide was added. Samples were placed on ice away from light and analyzed immediately by fluorescence microscopy using the Olympus fluoview confocal laser-scanning microscope using Argon (488 nm) and Krypton (568 nm) lasers at 20 × magnification. For quantification, about 20 fields from six sections each from four different animals were analyzed and expressed as lx/μm^2.

2.9. RNA preparation and Northern blotting

Isolation of total RNA was performed using Triazol reagent kit (Gibco BRL, Rockville, MD) and selection of poly $(A)^+$ messenger RNA using oligo-dT cellulose was performed as described previously (Davis et al., 1986). For Northern blotting experiments, poly$(A)^+$ RNA samples (10 μg) were electrophoresed on 1% agarose/3-*N*-morpholino] propane-sulfonic acid/formaldehyde gel, transferred to nitrocellulose membrane and cross-linked using a Stratagene UV cross-linker. Prehybridization mixture contained 5 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 2 × Denhardt's (1 × contains 0.2 g/l (w/v) each of polyvinylpyrolidine, bovine serum albumin and Ficoll), 1% SDS, 0.2 mg/ml salmon sperm DNA, and 50% formamide. Hybridization mixture (10 ml) was essentially the same except for the Denhardt's concentration was increased to 2.5 × and the concentrations of ^{32}P-labelled cDNA probes were $1-2 \times 10^6$ cpm/ml. Hybridizations were performed by shaking the blots in a 42 °C water bath for 16–24 h. Following incubations, 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. Blots were then subjected to autoradiography for 1–4 days. These blots were normalized using a labeled cDNA probe encoding the human glyceraldehyde 3-phosphate dehydrogenase. The relative band intensities were determined by densitometric scanning using a GS-250 Molecular Imager, as described above.

2.10. Creatinine and blood urea nitrogen estimation

Urine and plasma creatinine and blood urea nitrogen were measured using Sigma kits (Sigma, St. Louis, MO), according to the manufacturer's instructions. For creatinine estimation, 0.3 ml of plasma or urine samples (diluted 1:10) were incubated for 10 min at room temperature with 3.4 ml of alkaline picrate reagent. The absorbance at 500 nm was determined using a spectrophotometer. Aliquots of acidic reagent (0.1 ml) were then added and the mixtures were incubated for 5 min at room temperature and again read at 500 nm. The differences between two readings were used to calculate creatinine concentration based on a standard curve constructed using creatinine standard. For blood urea nitrogen estimation 5–20 μl of plasma or 1:10 diluted urine samples were incubated for 5 min at room temperature with 3.4 ml of blood urea nitrogen end point reagent supplied with kit and absorbance was measured at 340 nm using a spectrophotometer. The difference between the blank and sample reading was used to calculate blood urea nitrogen concentration from a standard curve.

2.11. Superoxide dismutase

Superoxide dismtase activity was determined according to the method of Mishra and Fridovich (1972). Briefly, 100 μl of tissue homogenate (20–30 μg protein) was added to 900 μl of 50 mM carbonate buffer (pH 10.2) containing 0.1 mM of epinephrine and the absorbance measured at 480 nm for 4 min. One unit of superoxide dismtase activity was defined as the amount of enzyme that inhibited the oxidation of epinephrine by 50%.

2.12. Catalase

Catalase activity was determined by a slight modification of the method of Aebi (1984). In these assays, 10 μl of absolute ethanol is added to 100 μl of tissue extract (20–30 μg protein) and placed in an ice bath for 30 min. Triton X-100 (reduced) is added to the mixture. The sample (100 μl) was then mixed with 500 μl of 66 mM of H_2O_2 and 400 μl of 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and the absorbance was monitored at 240 nm for 30 s. The molar extinction coefficient of 43.6 cm^{-1} was used to determine catalase activity. One unit of catalase activity was defined as 1 mmol of H_2O_2 degraded/min/mg protein.

2.13. Glutathione peroxidase

Glutathione peroxidase was determined by a modification of the method of Flohe and Gunzler (1984). In these assays, 100 μl of 100 mM reduced glutathione, 100 μl of 1.5 mM NADPH, 0.34 units of glutathione reductase and 500 μl of phosphate buffer (pH 7.0, containing 1 mM EDTA) were incubated at 37 °C for 10 min. This was followed by the addition of 100 μl of 12 mM *t*-butylhydroperoxide and determining the absorbance of the reaction mixture at 340 nm for 3 min. The millimolar extinction coefficient of 6.22 mM cm^{-1} was used to determine the activity of glutathione peroxidase. One unit of activity is equal to 1 mmol of NADPH oxidized/min/mg protein.

2.14. Lipid peroxidation assay

The assay method used is based on that described by Ohkawa et al. (1979). Tissue homogenates (200 μl) were incubated with 50 μl of 8.1% sodium dodecyl sulfate at

room temperature for 10 min. Following incubation, 375 μl of 20% acetic acid (pH 3.5) and 375 μl of 0.8% thiobarbituric acid (in 0.05 N NaOH) are added and the mixture is boiled for 1 h. The mixture is then cooled and extracted with 1 ml each of *n*-butanol-pyridine mixture (1:3, v/v) to avoid turbidity. The upper layer of each sample was aspirated and the absorbance measured at 532 nm. Concentrations of 2-thiobarbituric acid, mainly malondialdehyde, were determined using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.15. Data analysis

Statistical differences among means were determined using analysis of variance followed by Tukey's post hoc analyses or by the Student's *t*-test.

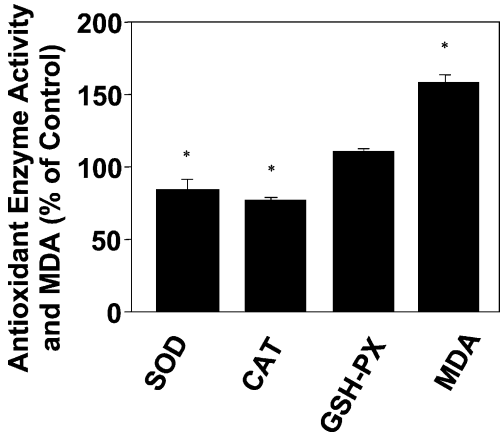

Fig. 2. Cisplatin alters the activities of antioxidant enzymes in the rat kidney. Antioxidant enzyme activities were determined 3 days following cisplatin exposure. Results are presented as the mean ± S.E.M. of four rats. Asterisk denotes statistically significant difference from control.

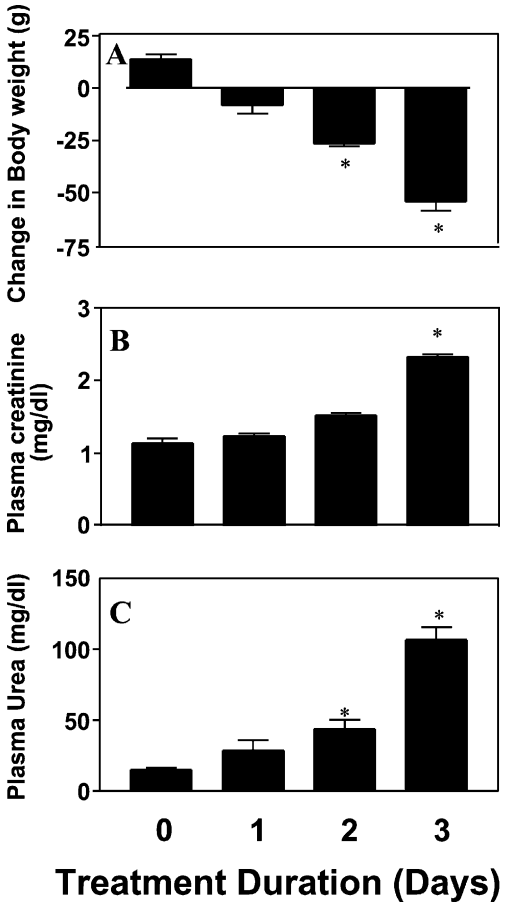

Fig. 1. Cisplatin decreases body weight and induces nephrotoxicity in rats. Rats were administered cisplatin (8 mg/kg) by intraperitoneal injections and tested daily over a 3-day period. (A) Decreases in body weights were 8 ± 4, 27 ± 1 and 55 ± 5 g following cisplatin treatment for 1, 2 and 3 days, compared to the controls which showed a modest weight gain over this period. (B) Increases in plasma creatinine by cisplatin. (C) Increases in blood urea nitrogen induced by cisplatin. Results are presented as the mean ± S.E.M. obtained from four animals per time point. Asterisks indicate statistically significant differences ($P < 0.05$) from the saline-treated control rats.

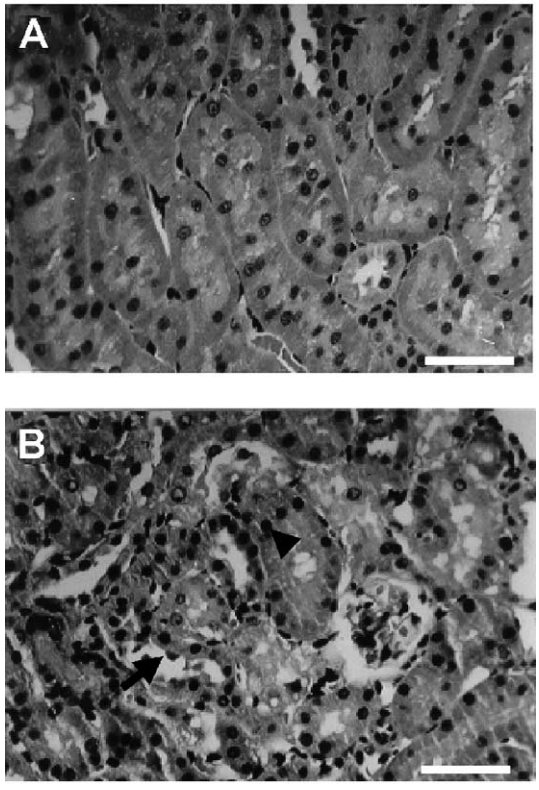

Fig. 3. Cisplatin induces morphological damage to the rat kidney. Kidneys sections were obtained 3 days following administration of cisplatin and examined by light microscopy. Representative sections from control (A) and cisplatin-treated (B) animals stained with hematoxylin and eosin. Injury in the kidney was confined to inner cortex and outer medulla. The epithelial cells of the P_3 segment of proximal tubule showed necrosis (arrow), and pyknotic nuclei (arrow head), a morphologic feature of early cell death in cisplatin treated animals. Control animals had vesicular and prominent nucleoli whereas it shifted to edges of nuclei in cisplatin-treated animals, indicative of apoptotic cells (A,B); bar = 50 μm.

3. Results

3.1. Cisplatin induces nephrotoxicity and changes in body weight

Administration of cisplatin to rats (8 mg/kg) by intraperitoneal injections produced a gradual weight loss over a 3-day period. Reductions in body weight following 1, 2 and 3 days of cisplatin treatment were 8 ± 4, 27 ± 1 and 55 ± 5 g, respectively (Fig. 1A). The development of nephrotoxicity was indicated by increases in plasma creatinine and blood urea nitrogen. Plasma creatinine levels were 1.1 ± 0.1, 1.3 ± 0.1, 1.5 ± 0.1 and 2.3 ± 0.1 mg/100 ml for control rats and those treated with cisplatin for 1, 2 and 3 days, respectively (Fig. 1B). The respective blood urea nitrogen levels were 15.1 ± 1.4, 28.5 ± 7.1, 43.6 ± 6.8 and 106.7 ± 8.8 mg/100 ml for the controls and rats treated with cisplatin for 1, 2 and 3 days, respectively (Fig. 1C). Despite the indications of nephrotoxicity, urine output were increased about two-fold, 1 and 2 days following administration of cisplatin, and remained above control levels by day 3. Urine output, determined in controls and 1, 2, and 3 days after cisplatin administration, were 0.4 ± 0.1, 1.1 ± 0.1, 1.2 ± 0.2 and 0.7 ± 0.1 ml/h, respectively. Furthermore, urine creatinine and urine urea nitrogen were significantly decreased ($P < 0.05$) on day 1 and remained low on days 2 and 3. Urine creatinine levels were 314 ± 27, 171 ± 14, 141 ± 25 and 160 ± 7 mg/100 ml in control, and following cisplatin treatment for 1, 2 and 3 days, respectively. The levels of urea nitrogen in these groups were 10 ± 2, 5 ± 1, 4 ± 1 and 5 ± 1 g/100 ml for control and cisplatin treatment for 1, 2 and 3 days, respectively.

3.2. Cisplatin alters the activities of antioxidant enzymes in rat kidneys

To test whether cisplatin alters the antioxidant status of the kidney, assays for antioxidant enzymes were performed in the kidney samples obtained from control and cisplatin-treated rats. Basal activities of superoxide dismutase, catalase and glutathione peroxidase in the kidney preparations were 28 ± 5, 782 ± 110 and 707 ± 23 units/mg protein, respectively. Results presented in Fig. 2 indicate small but statistically significant reductions in the activities of superoxide dismutase and catalase following cisplatin treatment for 3 days. These changes were associated with a slight increase in the activity of glutathione peroxidase. Importantly, cisplatin treatment increased the levels of malondialdehyde by $58.5 \pm 4.9\%$, indicative of increased oxidative stress. Administration of the selective adenosine A_1 receptor

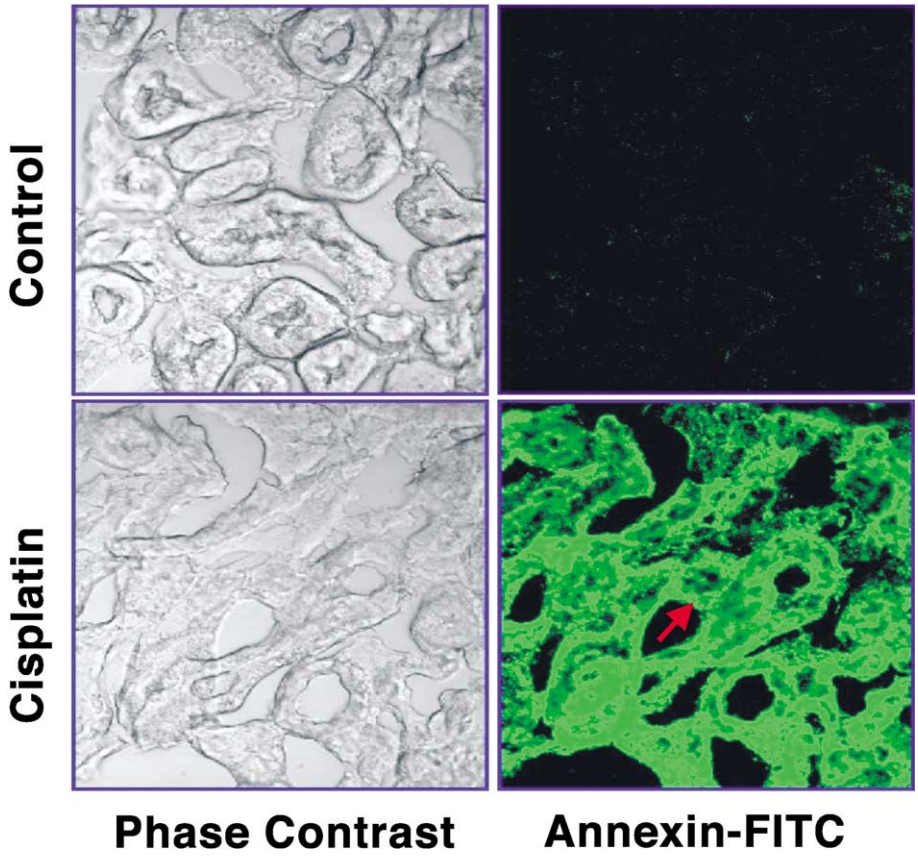

Fig. 4. Cisplatin induces necrosis and apoptosis in the rat kidney. Apoptosis was determined 3 days following cisplatin administration by AnnexinV-FITC on 16 μm thick sections. Early apoptotic cells show bright apple green fluorescence in the cisplatin-treated group.

antagonist, DPCPX, to rats resulted in a increase in malondialdehyde levels by 39.8 ± 16.7% over control. Co-administration of cisplatin and DPCPX increased malondialdehyde levels further to 93.5 ± 22.2% over control, indicative of increased toxicity with concurrent adenosine A_1 receptor blockade.

3.3. Cisplatin produces histological changes in the kidney

Additional evidence of nephrotoxicity were provided by light microscopic examination of kidney sections stained with hematoxylin and eosin. Examination of the kidney 3 days following cisplatin administration indicate injury to the kidney which was confined to inner cortex and outer medulla. The glomeruli, the convoluted portion of the proximal tubule, the distal tubule and the collecting duct were not significantly affected. The epithelial cells of the P_3 segment of proximal tubule showed pyknotic nuclei, a morphologic feature of early cell death, as compared to viable nuclei in control animals. Control animals had vesicular and prominent nucleoli whereas these shifted to edges of nuclei in cisplatin treated animals, indicative of cells undergoing apoptotis (Fig. 3A and B).

3.4. Cisplatin induces renal apoptosis in Sprague–Dawley rats

Apoptosis was determined using both the AnnexinV-FITC and TUNEL assays. Using the AnnexinV-FITC assay, cisplatin treated rats showed bright apple green fluorescent staining characteristic of either early apoptosis or necrosis, in contrast to unstained control cells (Fig. 4A and B). Quantitation of immunofluorescence showed a higher (214 ± 6) lx/μm^2 in kidneys from rats exposed to cisplatin as compared to control (13.5 ± 3) (Fig. 4C). In TUNEL assay cisplatin treated kidneys showed a significant increase in TUNEL positive apoptotic cells with dark brown/gray-black diaminobenzadine stained nuclei (51 ± 2%) while kidneys from control animals appeared nonapoptotic with light brown stained nuclei (3 ± 1%) (Fig. 5A and B). A

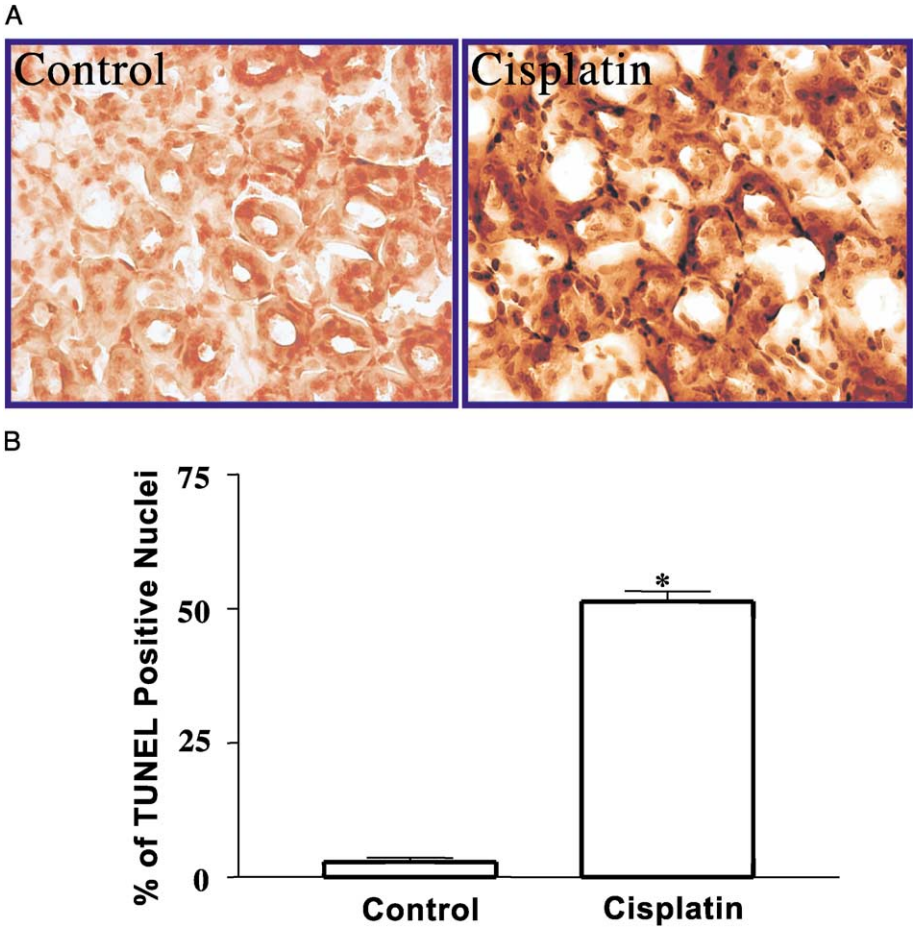

Fig. 5. Cisplatin induces apoptosis and necrosis in the rat kidney. The level of apoptosis was determined by TUNEL assay 3 days following cisplatin treatment. (A) Kidneys obtained from cisplatin-treated rats demonstrate a significant increase in dark-brown to gray-black diaminodenzidine-stained nuclei (arrow), indicative of apoptosis, compared to light brown stained nuclei (nonapoptotic cells). Apoptosis was detected in the columnar epithelial cells lining the cortical proximal convoluted tubules and in the glomerulus, with positively stained nuclei of visceral thin squamous parietal epithelial cells or podocytes. (B) Quantification of TUNEL assay images revealed significant increase (51.4 ± 1.8%) of TUNEL positive nuclei in cisplatin treated kidneys as compared to control (2.8 ± 0.8%). Results are presented as the mean ± S.E.M. of average fluorescence from at least four different animals.

similar finding was reported by Zhou et al. (1999). Maximum apoptosis occurred in the columnar epithelial cells lining the cortical proximal convoluted tubules, while apoptosis was also noted in the glomerulus with positively stained nuclei of visceral thin squamous parietal epithelial cells or podocytes.

3.5. Cisplatin treatment increases the levels of adenosine A_1 receptor in the rat kidney

Due to the low levels of ARs in the kidney, quantitation of this receptor subtype was performed using the agonist radioligand, ^{125}I-AB-MECA, instead of a tritiated radioligand. In addition to the adenosine A_1 receptor, ^{125}I-AB-MECA binds the adenosine A_3 receptor (Olah et al., 1994). The total specific ^{125}I-AB-MECA binding sites (including both the adenosine A_1 and A_3 receptors) could be obtained using *R*-phenylisopropyladenosine (*R*-PIA) (100 μM) to define nonspecific binding. At this concentration, *R*-PIA competes for ^{125}I-AB-MECA binding at both the adenosine A_1 and A_3 receptors. The antagonist, DPCPX, is selective for the adenosine A_1 receptor and competes for ^{125}I-AB-MECA binding at this receptor site. We observed a dose-dependent inhibition of the binding of ^{125}I-AB-MECA by DPCPX, which appeared maximal (about 50% of *R*-PIA sensitive binding sites) at a concentration of 10 μM (Fig. 6A). As such, the level of ^{125}I-AB-MECA binding which was sensitive to 10 μM DPCPX is indicative of the adenosine A_1 receptor. Therefore, adenosine A_1 receptor was quantitated using 10 μM DPCPX to define nonspecific binding.

The specific binding of ^{125}I-AB-MECA was elevated in the cisplatin-treated group. The maximum receptor densities (B_{max}) obtained by Scatchard analyses were significantly higher ($P<0.05$) in the kidneys of animals treated with cisplatin for 3 days (Fig. 6B). The levels of adenosine A_1 receptor averaged 12.4 ± 2.0 and 29.2 ± 3.8 fmol/mg protein (mean ± S.E.M.), respectively. The respective equilibrium dissociation constant (K_d) values for these groups were 1.8 ± 0.7 and 3.8 ± 0.8 nM. Using a single concentration of ^{125}I-AB-MECA (2 nM) to estimate receptor levels, it could be shown that the increase in adenosine A_1 receptor was time-dependent. The relative levels of the adenosine A_1 receptor were 4.1 ± 1.1, 3.1 ± 1.4, 7.1 ± 1.4 and 7.8 ± 1.2 fmol/mg protein for the control kidney and those obtained from rats treated with cisplatin for 1, 2 and 3

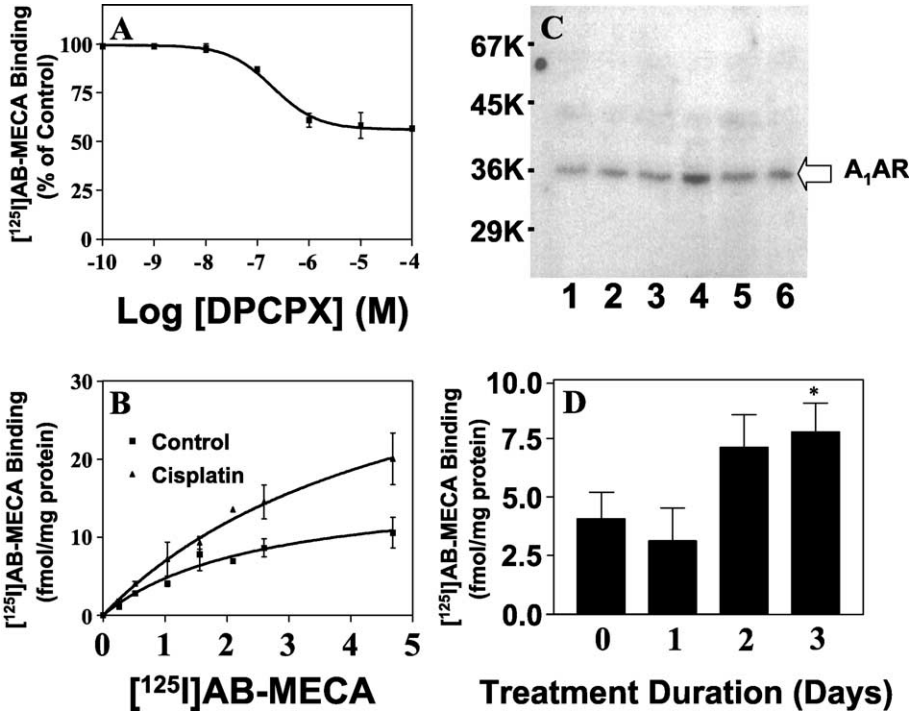

Fig. 6. Cisplatin increases adenosine A_1 receptor expression in the rat kidney. Adenosine A_1 receptor binding studies were detected using the agonist radioligand (^{125}I-AB-MECA). (A) Competition assay by DPCPX, using 100 μM *R*-PIA to define nonspecific binding. Inhibition appeared maximal (~ 45%) at approximately 10 μM DPCPX. (B) Saturation curves performed using ^{125}I-AB-MECA were fitted according to the one-site fit (Graph Pad PRISM), which yielded B_{max} values of 12.4 ± 2.0 and 29.2 ± 3.8 fmol/mg proteins and K_d values of 1.8 ± 0.7 and 3.8 ± 0.8 nM, for the controls and treated rats, respectively. (C) Western blotting for the adenosine A_1 receptor by resolving membrane proteins on a 12% acrylamide gel, transferring the proteins onto nitrocellulose membranes and detecting the adenosine A_1 receptor using specific polyclonal antibody and horse radish peroxidase labeled, goat anti-rabbit IgG, as described in Methods. Lanes 1–3 represent controls, while lanes 4–6 represent cisplatin-treated rats. Adenosine A_1 receptor immunoreactivity was elevated to $210 \pm 37\%$ of control in the cisplatin-treated group. (D) Time course of cisplatin-induced elevation in adenosine A_1 receptor. The levels of adenosine A_1 receptor determined by 2 nM of the radioligand is expressed as fmol receptor/mg protein, and are presented as the mean ± S.E.M. of four animals from each treatment period.

days, respectively (Fig. 6D). The effect of co-administration of adenosine receptor antagonists and cisplatin on the level of ^{125}I-AB-MECA binding in the rat kidney was also tested. The level of adenosine A_1 receptor in the kidney in the control animals and following administration of theophylline, aminophylline, DPCPX and caffeine were 8.7 ± 0.9, 11.7 ± 2.9, 10.0 ± 1.6, 10.5 ± 1.5 and 11.5 ± 1.5 fmol/mg protein, respectively. None of the values observed with the various antagonists was statistically significant from control. The addition of adenosine A_1 receptor antagonists to cisplatin resulted in no significant increase in adenosine A_1 receptor expression compared to that obtained with cisplatin alone. The values for the cisplatin, cisplatin + aminophylline, cisplatin + DPCPX and cisplatin + caffeine were 14.7 ± 1.3, 12.7 ± 1.7, 14.8 ± 1.6 and 14.9 ± 1.5 fmol/mg protein.

Due to the low expression of the adenosine A_1 receptor in this tissue, Western blotting for this receptor was performed following partial purification of the receptor by size fractionation of the receptor on G50 Sephadex columns. The data indicate a 34–36 kDa protein, whose levels were higher in the cisplatin treated group than controls. Results obtained using six kidneys from each group indicated a statistically significant increase in adenosine A_1 receptor expression ($P < 0.05$) averaging $210 \pm 37\%$ of the control (Fig. 6C).

In order to localize the increase in adenosine A_1 receptor in rat kidney, we performed immunocytochemical studies using an adenosine A_1 receptor specific antibody (Nie et al., 1998). The adenosine A_1 receptor immunoreactivity appeared increased throughout the rat renal cortex, including the Bowman's capsule housing the glomerulus as well as the proximal and distal convoluted tubules (Fig. 7, upper panel). Increased receptor expression was also noted in the transverse sections of medulla. Quantitation of adenosine A_1 receptor immunofluorescence by confocal microscopy showed a significant increase in immunoreactivity in cisplatin treated animals as compared to control animals (Fig. 7, lower panel). Quantitation of the adenosine A_1 receptor immunofluorescence showed a significant increase due to cisplatin, from 47 ± 1 lx/μm^2 average in control animals to 249 ± 1 lx/μm^2 in the cislatin-treated group.

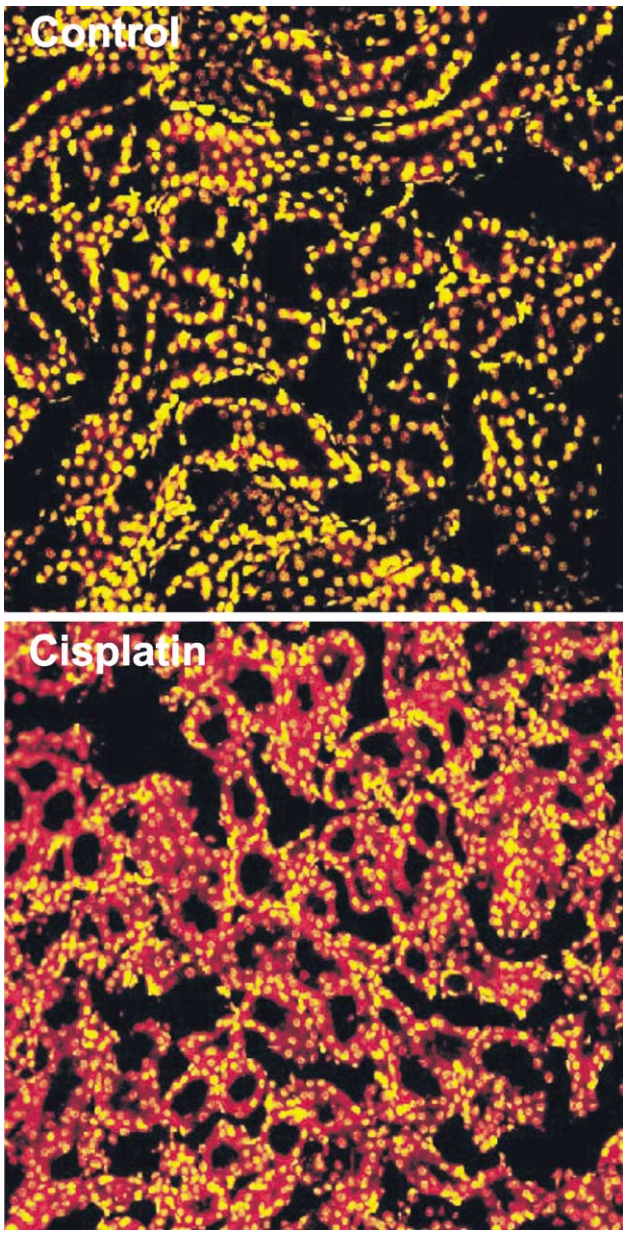

Fig. 7. Immunocytochemical localization of adenosine A_1 receptor in the rat kidney. Kidney sections (16 μm) from animals treated with cisplatin for 3 days were probed for the adenosine A_1 receptor using a monoclonal antibody specific for this receptor. This was followed by incubation with rhodamine (TRITC) fluorescent-tagged IgG (red staining) and a nuclear stain (greenish-yellow) termed SYTOX. Immunoreactivity was detected using an Olympus fluoview confocal laser scanning microscope using Argon (488 nm) and Krypton (568 nm) lasers for green and red fluorescence, respectively. Increases in adenosine A_1 receptor were observed throughout the rat renal cortex including the Bowman's capsule housing the glomerulus as well as the proximal and distal convoluted tubules in cisplatin treated animals. Increased adenosine A_1 receptor expression was also noted in the transverse sections of medulla.

3.6. Cisplatin treatment increased the steady-state levels of adenosine A_1 receptor mRNA in the kidney

Northern blot studies were performed using poly A^+ RNA prepared from rat kidneys to test whether the increase in adenosine A_1 receptor produced by cisplatin was associated with an increase in the steady state level of adenosine A_1 receptor mRNA. The levels of total RNA obtained from the cisplatin-treated groups were progressively reduced with increasing treatment durations, as compared to control animals. Total RNA obtained from the kidneys were 3.7 ± 0.1, 3.6 ± 0.2, 2.9 ± 0.2 and 2.2 ± 0.4 mg/kidney for controls and animals treated with cisplatin for 1, 2 and 3 days, respectively, probably indicative of decreased transcription and/or increased degradation. Two adenosine A_1 receptor transcripts of 5.6 and 3.4 kb were detected by Northern blotting using labeled canine adenosine A_1 recep-

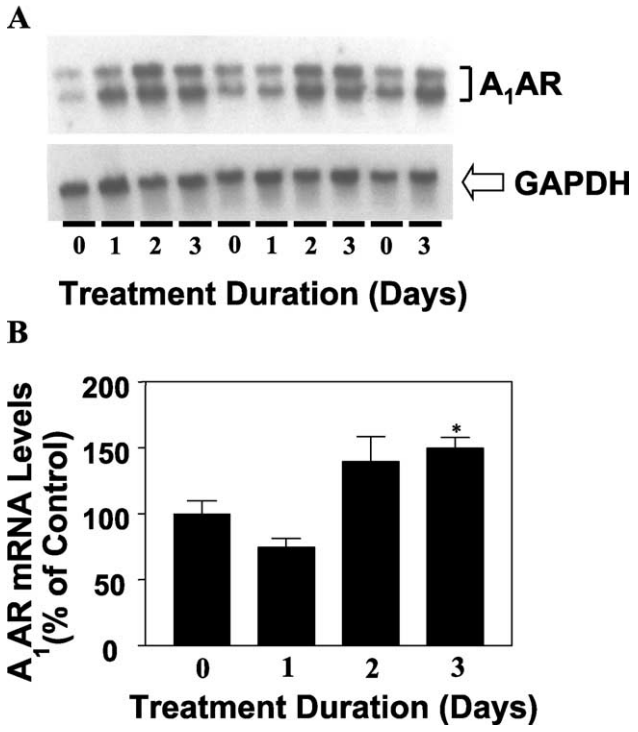

Fig. 8. Cisplatin increased the steady state levels of adenosine A_1 receptor mRNA in rat kidneys. (A) Rats were treated with cisplatin (8 mg/kg) for 3 days, following which kidneys were removed and used for preparing RNA and poly A^+ RNA. Each lane was loaded with 10-μg poly A^+ RNA, which was then resolved on 1% MOPS-formaldehyde-agarose gel. Two adenosine A_1 receptor transcripts (indicated by the arrow) of 5.6 and 3.4 kb were detected. The band intensities were quantitated using a phosphor imager. For normalization, blots were stripped and reprobed with labeled cDNA encoding the human glyceraldehyde 3-phosphate dehydrogenase. (B) Graphical representation of the time-dependent changes in adenosine A_1 receptor mRNA by cisplatin in the rat kidneys. The data are presented as the mean ± S.E.M. of four animals from each time point. Asterisk indicates statistically significant difference ($P<0.05$) from control.

tor cDNA as probe (Fig. 8A). Blots were normalized using a cDNA encoding the human glyceraldehyde 3-phosphate dehydrogenase. The relative amounts of adenosine A_1 receptor mRNA (both 3.4 and 5.6kb transcripts) were 100 ± 10.1, 74.5 ± 6.9, 139.5 ± 19.3 and 149.7 ± 8.2 for controls, for rats treated with cisplatin for 1, 2 and 3 days, respectively (Fig. 8B).

3.7. Effect of AR antagonists on cisplatin-induced nephrotoxicity

To test the role of adenosine A_1 receptor in cisplatin-induced nephrotoxicity, animals were treated with cisplatin followed by AR antagonists. Adenosine receptor antagonists tested include theophylline, aminophylline, DPCPX and caffeine. While theophylline administered alone produced no demonstrable side effects in these animals, the combination of theophylline and cisplatin was lethal to all animals in this group. In addition, one animal treated with the combination of caffeine and cisplatin died between days 2 and 3. Plasma creatinine levels were elevated in the cisplatin treated group, indicative of nephrotoxicity. The administration of cisplatin, followed by aminophylline, DPCPX or caffeine, did not alter the levels of creatinine obtained with cisplatin (Fig. 9A). A similar profile was observed when assays for blood urea nitrogen were performed (Fig. 9B). Administration of adenosine A_1 receptor antagonists alone did not alter the expression of adenosine A_1 receptor in the kidney. A combination of cisplatin and A_1 receptor antagonists also did not elevate receptor expression above that obtained with cisplatin alone (Fig. 9C).

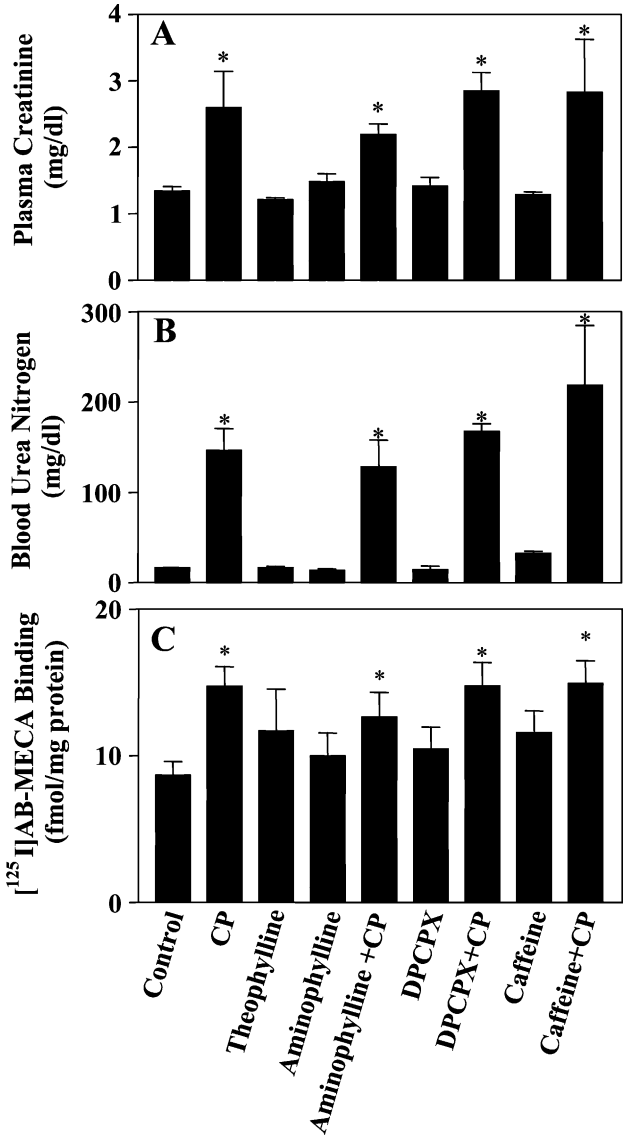

Fig. 9. Effect of AR antagonists on cisplatin-induced changes in kidney function. Rats were administered saline or cisplatin (CP) (8 mg/kg, i.p.) alone or in combination with AR antagonists such as theophylline (24 mg/kg, i.p. twice a day), aminophylline (24 mg/kg, i.p. twice a day), caffeine (2 g/l in the drinking water) or DPCPX (0.1 mg/kg, i.p. twice a day). Assays for plasma creatinine (A), BUN (B) and adenosine A_1 receptor (C) in the kidney were performed as described in Methods. Each point represents the mean ± S.E.M. of four animals.

4. Discussion

This study demonstrates that during cisplatin-induced nephrotoxicity, the level of adenosine A_1 receptor in the rat kidney along with the steady state levels of mRNA encoding the receptor were dynamically regulated. The increase in the levels of adenosine A_1 receptor, we believe, might contribute either to the toxicity of cisplatin or, as we suspect, may be involved in mitigating the renal toxicity of cisplatin. As such, the increase in adenosine A_1 receptor may be viewed as a compensatory mechanism to decrease cisplatin toxicity, since additional data provided by this study indicate that blockade of these receptors exacerbates the nephrotoxicity.

The progressive development of nephrotoxicity following cisplatin treatment, observed in this study, has also been described by other investigators (Chopra et al., 1982; Gordon et al., 1982; Somani et al., 1995) and should be taken into consideration in patients with compromised kidney functions. Evidence of nephrotoxicity include increase in plasma creatinine and blood urea nitrogen, both of which were progressively increased over time. Interestingly, this temporal increase in nephrotoxicity parallels the increase in adenosine A_1 receptor expression, suggesting a cause and effect relationship between these two parameters. The lower levels of both creatinine and urea nitrogen in the urine of cisplatin-treated animals is indicative of decreased glomerular functioning and polyurea. Cisplatin-induced polyurea, demonstrated previously (Clifton et al., 1982; Gordon et al., 1982), likely results from the reduced ability of the kidney to concentrate the urine, probably resulting from disruption of normal transport processes in the cortical tubules and collecting ducts (Lang et al., 1985) and to defects in renal tubular absorption regulated by adenosine (Cornelison and Reed, 1993). Polyurea may also result from cisplatin-mediated decrease in the secretion and release of vasopressin (Clifton et al., 1982; Gordon et al., 1982).

Cisplatin-induced nephrotoxicity was associated with substantial increases in malondialdehyde, indicative of increased oxidative stress. Similar increases in malondialdehyde by cisplatin has been described previously (Sugihara et al., 1987). It has been suggested that cisplatin depletes the cellular levels of reduced glutathione, thereby reduces the ability of the cell to scavenge reactive oxygen metabolites (Kuhlmann et al., 1997). Experimental methods which induce depletion of cellular glutathione exacerbates cisplatin-mediated toxicities (Kim et al., 1997). Cisplatin itself might generate reactive oxygen species and thereby contributes to oxidative stress and damage to the kidney (Sodhi and Gupta, 1986). In contrast, administration of antioxidants and free radical scavengers attenuate cisplatin induced apoptotis in mouse proximal tubular cells (Lieberthal et al., 1996). In support of a protective role of the adenosine A_1 receptor in cisplatin-mediated nephrotoxicity, we observed an increase in lipid peroxidation (determined by malondialdehyde assay) following adenosine A_1 receptor blockade using DPCPX, and further enhancement of lipid peroxidation following co-administration of cisplatin along with DPCPX.

While the level of adenosine A_1 receptor is quite low, compared to the expression of this adenosine receptor subtype in the brain, testes and adipocytes (Stiles, 1992), these receptors are readily detected by [^{125}I]AB-MECA, an agonist radioligand which interacts with both the adenosine A_1 and A_3 receptors (Olah et al., 1994). This radioligand has a much higher specific activity than the tritiated antagonist radioligand DPCPX. Since the rat adenosine A_3 receptor is relatively insensitive to methylxanthines (Olah et al., 1994), the use of the unlabeled DPCPX (10 μM) to define non-specific binding allowed for an adequate distinction of the adenosine A_1 versus A_3 receptor binding sites. The estimated receptor number determined using this radioligand is comparable to that obtained for the adenosine A_1 receptor subtype in the rat renal microvessels and glomeruli using the agonist [^{125}I]HPIA (Freissmuth et al., 1987). A portion of the sites which were insensitive to DPCPX could possibly represent the adenosine A_3 receptor, since the use of the agonist *R*-PIA (which interacts with both the adenosine A_1 and A_3 receptors) define yielded a higher level of specific binding sites with [^{125}I]AB-MECA. The presence of this latter receptor subtype in the kidney has been confirmed by polymerase chain reactions (Zhou et al., 1992). Alteration of the putative adenosine A_3 receptor subtype by cisplatin was not addressed in this study.

Since cisplatin is known to inhibit the overall DNA transcription through inter- and intrachain cross-linking and initiating DNA strand breaks, it was surprising that cisplatin up-regulates the adenosine A_1 receptor, purportedly at the level of the adenosine A_1 receptor gene (Nie et al., 1998). In an in vitro cell culture system, we have shown that up-regulation of the adenosine A_1 receptor is mediated by activation of NF-κB through the action of reactive oxygen species (Nie et al., 1998). Two potential sites for this transcription factor have been identified on the human adenosine A_1 receptor gene (Nie et al., 1998). Whether similar NF-κB sites are present in the rat adenosine A_1 receptor promoter is yet to be determined. As discussed above, one source of reactive oxygen species is cisplatin itself (Powis, 1991; Sodhi and Gupta, 1986). Another potential source of reactive oxygen species is adenosine released in response to cisplatin exposure. The adenosine metabolite, inosine, is metabolized via the xanthine oxidase pathway to yield uric acid. Activation of this metabolic pathway generates reactive oxygen species. Thus, adenosine may mediate two opposing functions in the cell. On one hand, this agent can mediate protection via its interaction with the adenosine A_1 receptor. On the other hand, adenosine, via metabolism by xanthine oxidase, can generate reactive oxygen species. This latter mechanism of reactive oxygen species generation may play a major role in cisplatin-induced nephrotoxicity. Another potential source of reactive oxygen species is nitric oxide synthase. Cisplatin

has been shown to increase the inducible form of nitric oxide synthase which can contribute to ototoxicity (Watanabe et al., 2000). Even though some increase in nitric oxide (NO) may be beneficial to the kidney by promoting glomerular filteration, tubular absorption and intrarenal renin secretion (Noris and Remuzzi, 1999), NO may also induce nitrosylation of cellular proteins (Stamler et al., 1992), thereby exacerbating cisplatin-induced toxicity. As such, inhibition of nitric oxide synthase by N^G-nitro-L-arginine methyl ester resulted in a marked reduction in blood urea nitrogen, serum creatinine and lipid peroxidation (Ueno et al., 1988).

It was expected that augmentation of the signal transduction pathway would be detrimental to the kidney since adenosine mediates constriction of the renal afferent arterioles and decreases glomerular filtration rate (Oswald, 1984). Administration of cisplatin has previously been shown to decrease glomerular filtration rate within 48–72 h (Winston and Safirstein, 1985). We speculated that this decrease results from vasoconstriction as a result of increased release of adenosine and/or increased expression of in the preafferent arteriole by cisplatin. Interestingly, the time point at which the decrease in glomerular filtration rate was attained matched the time at which up-regulation of the adenosine A_1 receptor was obtained (2–3 days). As such, it was reasoned that administration of adenosine A_1 receptor antagonists would ameliorate the renal failure produced by these agents. Accordingly, Knight et al. (1991) demonstrated that inhibition of the by a low dose of DPCPX reduced cisplatin-induced tubular necrosis, while higher doses had added beneficial actions to reduce plasma creatinine and urea levels. Other studies have also demonstrated amelioration of cisplatin-induced acute nephrotoxicity by antagonists of the adenosine A_1 receptor (Heidemann et al., 1989; Nagashima et al., 1995). In addition, adenosine A_1 receptor antagonists have proven useful in the treatment of acute nephrotoxicity induced by contrast media (Katholi et al., 1995) and glycerol (Bidani and Chrchill, 1982; Bowmer et al., 1986). The reason for the contradiction between our study and these is not clear. Our data clearly indicates no change or an exacerbation of cisplatin-induced nephrotoxicity by AR antagonists. We believe that exacerbation of cisplatin toxicity by these antagonists is due to a reduction of the normal functional roles of adenosine interacting with the adenosine A_1 receptor. In keeping with this line of reasoning, we suggest that up-regulation of adenosine A_1 receptor by cisplatin in the kidney may serve as an attempt of this organ to circumvent the toxic action of this drug. In this respect, sites other than the pre-afferent arteriole may be involved. An increase in adenosine A_1 receptor, along with an increase in the levels of adenosine may further inhibit the release of renin (Arend et al., 1984), enhance erythropoietin production (Srivastava et al., 1996) and enhance Na^+ and water excretion, in addition to the transport of chloride transport (McCoy et al., 1993). Since adenosine is also involved in regulating the normal cellular metabolic activity, the increase in adenosine A_1 receptor may help to maintain a balance between oxygen consumption and demand (Spielman and Arend, 1991). However, the damage inflicted by cisplatin is likely too great and overwhelms any compensatory measure.

Other explanations can also account for the difference observed in this study compared to similar other studies. The dose of cisplatin used was higher that those used in the other studies (Heidemann et al., 1989; Nagashima et al., 1995; Knight et al., 1991). In these studies, the dose of cisplatin were 5–6 mg/kg body weight. The higher dose used in this study could overwhelm the ability of adenosine A_1 receptor antagonists to attenuate the nephrotoxicity. Furthermore, the high dose may also lead to increased drug binding to plasma proteins and competes with other drugs for these binding sites. This could lead to more of the free drugs in the plasma, probably resulting in enhanced toxicity. Furthermore, the route of administration used for drug delivery was intravenous, while in our study, an intraperitoneal route was used. It is possible that the drug is more rapidly cleared following intravenous administration than following intraperitoneal administration. In human, the elimination half-life of cisplatin in plasma following intravenous administration is 25–50 min (Chabner et al., 2001). As such, more of the drug would be present in vivo for a longer period.

The development of nephrotoxicity by cisplatin was also associated with significant increase of apoptosis and necrosis, indicative of damages to renal cells leading to cell death. Significant apoptosis occurred in the cortical proximal convoluted tubules and in the glomerular cells. Similar necrosis has been presented by other investigators (Lieberthal et al., 1996; Lau, 1999). In primary cultures of mouse proximal tubular cells, antioxidants delayed cisplatin-induced apoptosis but not necrosis, indicating a role of reactive oxygen species in mediating apoptosis but not necrosis (Lieberthal et al., 1996). This suggests that oxidative stress may contribute to cisplatin-induced nephrotoxicity.

In summary, the results presented indicate that cisplatin-induced nephrotoxicity is associated with increased oxidative stress, up-regulation of the adenosine A_1 receptor and apoptosis. While the role of this increase in adenosine A_1 receptor in unclear at present, given the functional importance of adenosine in the kidney, it is possible that the increase in adenosine A_1 receptor likely confers a cytoprotective role.

Acknowledgements

We would like to acknowledge the technical assistance of Drs. Nat Modi, Robert Helfert and Robert Struble. We also would like to thank Terry Sommers, Shari Randall and Sandeep Pingle for their excellent technical support.

This study was funded by grants from the National Institutes of Health (HL56316-01) and by funds from the Central Research Committee (Southern Illinois University School of Medicine).

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