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Protective action of glycine in cisplatin nephrotoxicity

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Protective action of glycine in cisplatin nephrotoxicity. Because glycine is cytoprotective for kidney cells in vitro, we investigated its possible action in vivo to protect rats against cisplatin nephrotoxicity, a well-established experimental model of renal tubular injury. Glycine was infused at a dose of 1 mmol per 100 g body weight per hour for 75 minutes, starting 15 minutes before cisplatin, 5 mg per kg, was injected intravenously. Plasma concentration of glycine rose to 3.5 mmol per liter at the time cisplatin was injected. These rats were compared with cisplatin-treated animals treated with L-alanine or with isotonic saline. After five days plasma creatinine of saline-treated rats given cisplatin had risen threefold to 2.6 ± 1.5 mg per 100 ml (mean ± SD), as creatinine clearance fell to 25% of baseline (0.14 ± 0.05 ml/min/100 g). Morphological evaluation disclosed extensive damage involving all S3 segments in the outer medulla as well as the medullary rays of the cortex. In contrast, in rats treated with glycine, plasma creatinine rose only to 1.2 ± 0.2 mg/100 ml and creatinine clearance was maintained at 75% of baseline (0.35 ± 0.05 ml/min/100 g). Glycine also attenuated the weight loss, polyuria, increased fractional excretion of sodium and potassium, decreased urinary osmolality, and renal glycosuria observed in control, saline-treated rats after cisplatin, while substantially decreasing the percentage of S3 tubules with evident morphological injury. Renal platinum content was unaffected by glycine. The administration of L-alanine or the delayed infusion of glycine, starting one hour after cisplatin was given, did not prevent cisplatin toxicity. Thus, high plasma concentrations of glycine achieved during a brief period of time when cisplatin is administered, markedly attenuate cisplatin nephrotoxicity.

Glycine provides remarkable protection against anoxic injury [1, 2] and ouabain toxicity [3] in dispersed rabbit proximal tubules incubated in vitro. Hypoxic injury to medullary thick ascending limbs is prevented by glycine in the isolated perfused rat kidney [4, 5], as is the outer medullary damage from indomethacin and amphotericin in the same experimental model [4]. Systemic infusion of glycine also appears to maintain proximal tubular function following intratubular application of uranyl nitrate [6].

The dosage of cisplatin, a potent, wide spectrum drug utilized in solid tumor chemotherapy, is limited by its renal and neural toxicity. The incidence of deterioration in renal function is high, around 20%, in spite of the use of low divided doses, prolonged infusion periods, saline hydration and diuretics [7]. In the present study we show that the administration of glycine ameliorates tubular injury and renal dysfunction in rats treated with cisplatin.

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Methods

General

Male Sprague-Dawley rats weighing 210 to 330 g were used for all experiments. They were given regular chow and tap water ad libitum. Under pentobarbital anesthesia (65 mg/kg) the right jugular vein was cannulated with two polyethylene tubes (PE-50, Clay-Adams, Parsippany, New Jersey, USA) for the infusion of cisplatin and amino acids. The rats were kept in heated chambers (37°C) throughout the experiment. At the end of the infusion period, the cannulas were removed, the rats were allowed to recover, and were observed over the next five days.

Experimental groups

Four groups of cisplatin-treated rats were examined: 1) glycine-treated rats (GLY, $N = 16$); 2) L-alanine-treated rats (ALA, $N = 6$); 3) rats with late initiation of glycine infusion (D-GLY, $N = 6$); 4) a control group of saline-treated rats (CTR, $N = 20$).

Cisplatin injection

A total of 5 mg/kg cisplatin was injected over 60 seconds through a jugular cannula. A preparation used in clinical practice (Platinol, Bristol-Myers Co., Evansville, Iowa, USA) was dissolved in distilled water, so that each milliliter contained 1.67 mg cisplatin, 16.7 mg of mannitol and 15 mg of sodium chloride.

Amino acid infusion

Glycine was infused at a rate of 75 mg (1 mmol) per 100 g body weight, in 1.06 ml saline per hour. L-alanine was given in an equimolar dose (89 mg/100 grams body wt/1.56 ml saline/hr). (The difference in solute volume resulted from the lower solubility of alanine.) Control rats were given 1.06 ml saline/hr.

In the GLY, ALA and CTR groups, infusion was initiated 15 minutes before the cisplatin injection through the second cannula, and was carried out for 75 minutes. In the D-GLY group a 75-minute glycine infusion was started one hour after the injection of cisplatin.

Functional studies

The rats were kept in metabolic cages (Nalge Co., Rochester, New York, USA) for the evaluation of kidney function. Twenty-four-hour urine collections initiated at 10 a.m. were carried out at baseline (day 0), during the first 24 hours following the cisplatin injection (day 1), and on day 5. Corresponding 0.8 ml blood samples were withdrawn from the right femoral vein at the end of each collection period, consisting of a baseline sample taken during the pentobarbital anesthesia, a sample at day 2 under light ether anesthesia, and another at the end of the experiment on day 6. Measurements of sodium and potassium were done with ion selective electrodes (Beckman Lablyte 800, Brea, California, USA). Plasma magnesium was measured by atomic absorption spectrometer (Perkin-Elmer 303, Norwalk, Connecticut, USA). Creatinine and plasma glucose were quantified by colorimetric reactions (Abbot 100 Biochromatic Analyzer, S. Pasadena, California, and COBAS Bio, Roche Diagnostic Systems, Nutley, New Jersey, USA, respectively). Glycosuria was estimated semi-quantitatively using urine Teststrips (Boehringer Mannheim Diagnostics, Indianapolis, Indiana, USA). Urine osmolarity was measured by a freezing point osmometer (uosmette 5004, Precision Systems, Natick, Massachusetts, USA).

Morphologic evaluation

The rats were anesthetized at day 6 with Inactin 100 mg/kg (BYK Gulden Konstanz, Germany). A midline abdominal incision was performed, the right renal pedicle clamped and the kidney removed for cisplatin measurements. Heparin (400 U) was injected intravenously and the left kidney was perfusion-fixed in situ with 1.25% glutaraldehyde (Eastman Kodak Co., Rochester, New York, USA) in 0.1 M phosphate buffer (pH 7.4) through a 19 gauge needle inserted in the aorta. Perfusion pressure was kept at 130 mm Hg, and the superior mesenteric artery and the aorta above and below the renal artery were clamped to provide optimal fixation. The kidneys were sectioned and postfixed in buffered 2% OsO_4, dehydrated and embedded in an Araldit-Epon B12 mixture. Three × 3 mm 1 μm sections containing cortex and outer medulla were cut. The sections were stained with 1% methylene blue and examined by light microscopy in a blinded fashion (interpreted by the examiner without knowing the study groups).

A semiquantitative morphological analysis of proximal straight (S3) tubular damage, the characteristic morphological feature of cisplatin nephrotoxicity [8], was performed, using the following categories: 0 = no damage; 1+, 2+, 3+ = 25%, 50% and 100% outer stripe S3 injury, respectively. Intermediate degrees of injury were also designated. Complete outer stripe destruction, extending beyond this region to involve S3 segments in the medullary rays, was ranked as 4+ and was considered as 125% necrotic proximal tubules for the data analysis. Data were eventually converted to a percentage of examined tubules evincing injury.

Platinum measurement

This was carried out in rats from the CTR and GLY groups, chosen at random, five from each group. Plasma samples from days 2 and 6 and samples of urine collected during the first 24 hours after cisplatin were examined (collection period started 2 h after cisplatin injection). The right kidney, removed at day 6, was weighed and frozen. All specimens were analyzed in duplicate for platinum with a Perkin Elmer model 1100 atomic absorption spectrometer, as described elsewhere [9].

Glycine concentrations

Continuous infusion of glycine, 75 mg/100 g body weight in 1.56 ml per hour was carried out for 90 minutes in two rats. Repeated blood samples with EDTA were taken during the infusion period, quickly centrifuged and the plasma collected and frozen. At the end of the infusion period the kidneys and liver slices were quickly removed, weighed, washed in iced saline and homogenized in 5% trichloroacetic acid. Samples were centrifuged and the supernatant was titrated with NaOH to pH 7. Amino acid assay was performed on the plasma and tissue samples using the Water's Pico-Tag method [10]. Calibration was based on a 200 mM/l standard.

Statistics

Values in Figures and Tables are presented as the means ± SE or SD, respectively. Non-paired two-tailed Student's *t*-test was applied for the comparisons of platinum content. One or two-way analysis of variance with Newman Keuls test were used for multiple comparisons between and within groups, as detailed below. Baseline values were utilized as covariates for comparisons of repeated measurements within groups. Simple correlations were calculated between functional and morphologic findings in the various experimental groups. Statistical significance was set at $P < 0.05$.

Results

Effect of cisplatin on renal function and structure

All control rats given cisplatin without amino acids developed marked structural damage, usually involving the entire S3 segments in the outer stripe of the outer medullary zone, with lesions extending to the medullary rays (Fig. 1A, Table 1). Renal function deteriorated over five days, with more than a threefold increase in plasma creatinine, from 0.8 ± 0.1 to 2.6 ± 1.5 mg/100 ml (mean ± SD, $P < 0.002$, paired Student's *t*-test), and a drop in creatinine clearance to about one-fourth of baseline values (from 0.48 ± 0.1 to 0.14 ± 0.05 ml/min/100 g weight, $P < 0.005$; Table 1). Glycosuria developed in all rats, averaging 221 ± 105 mg per 100 ml on the fifth day after cisplatin injection. Renal glycosuria was confirmed by normal plasma glucose levels (119 ± 36 mg per 100 ml). Polyuria with reduced urinary osmolality developed, urine volume rising to 2.29 ± 1.62 from 0.56 ± 0.14 ml/hr, and urinary osmolality falling from 1943 ± 275 to 425 ± 137 mOsm (Fig. 2). Fractional sodium and potassium excretions increased ($P < 0.05$) and a substantial decrease in body weight was noted (Table 1, Figs. 2 and 3). The volume of urine excreted by glycine-treated rats during their 75 minute infusion of glycine and cisplatin (1.1 ± 0.1 ml) did not differ from that excreted by control rats receiving cisplatin with saline vehicle (0.9 ± 0.1 ml).

Effect of amino acids on injury induced by cisplatin

In contrast to the findings in the control rats, renal injury was markedly attenuated by simultaneous administration of glycine. Proximal tubular injury was limited to 40% of tubules examined

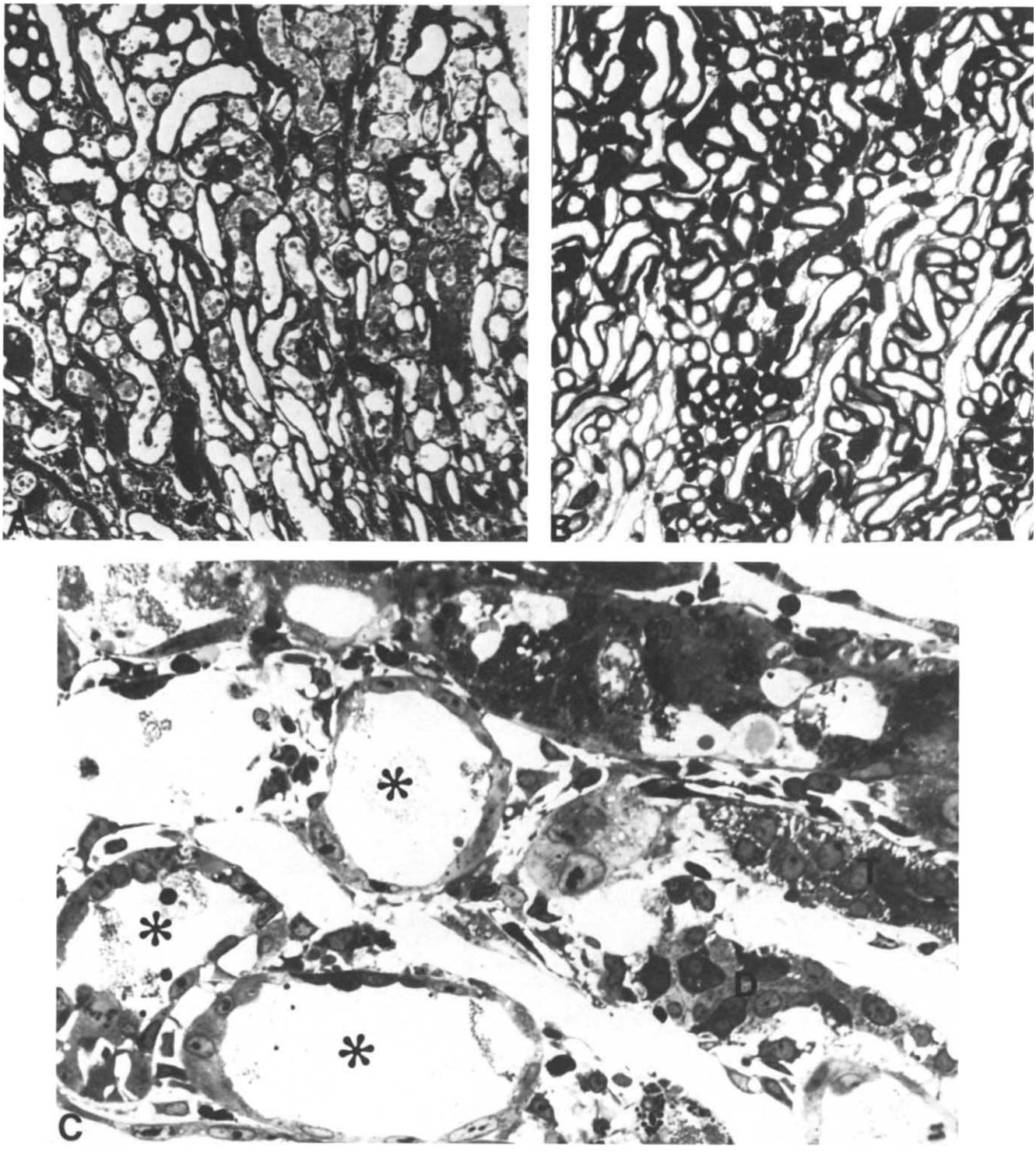

Fig. 1. *Rats given cisplatin (5 mg/kg) and sacrificed five days later.* These photographs are of the outer stripe of the outer medulla, where the cellular mass largely consists of straight (S3) segments of the proximal tubules. In control animals (CTR) typically all S3 segments in this zone show injury (**A**) that is markedly diminished in glycine treated rats (GLY) (**B**). At high power (**C**) injured S3 segments seem to be lined by a thin layer of regenerating epithelium (asterisks). Note the medullary thick ascending limb (T) and collecting duct (D) that are undamaged. (Methylene blue, ×64; ×64; ×500)

Table 1. Effects of glycine and alanine on cisplatin nephrotoxicity

Group (*N*)	Plasma creatinine *mg/100 ml*		Creatinine clearance *ml/min/100 g*		Fractional excretion of Na %		Weight loss *% of initial weight*	S_3 injury %
	Day 1	Day 6	Day 0	Day 5	Day 0	Day 5		Day 6
Control (20)[e]	0.8 ± 0.1	2.6 ± 1.5[a]	0.48 ± 0.01	0.14 ± 0.05[a]	0.54 ± 0.12	1.92 ± 1.36[b]	15 ± 8	104 ± 9
Glycine (16)[e]	0.8 ± 0.1	1.2 ± 0.2[a,c]	0.51 ± 0.11	0.35 ± 0.05[b,d]	0.38 ± 0.22	0.66 ± 0.24	4 ± 7[c]	40 ± 24[d]
Glycine-delayed (6)	0.8 ± 0.1	1.7 ± 0.4[a]	0.39 ± 0.08	0.21 ± 0.06[a]	0.50 ± 0.10	1.80 ± 0.81[b]	8 ± 6	102 ± 5
Alanine (6)	0.8 ± 0.1	2.2 ± 0.7[a]	0.45 ± 0.03	0.16 ± 0.04[a]	0.40 ± 0.16	2.00 ± 0.60[b]	9 ± 7	100 ± 0

Values are mean ± SD. Two-way ANOVA is used for the comparisons of repeated functional measurements within and between the experimental groups. One-way ANOVA is used for the comparisons of tubular injury and weight loss between groups.

[a] Significantly different from Day 1 ($P < 0.01$)

[b] Significantly different from Day 1 ($P < 0.05$)

[c] Significantly different from Control ($P < 0.01$)

[d] Significantly different from all other groups ($P < 0.01$)

[e] $N = 6$ for creatinine clearance and fractional excretion of Na

(Fig. 1B, Table 1). The increase in plasma creatinine was less marked than in controls (levels rising from 0.8 ± 0.1 to 1.2 ± 0.2 mg/100 ml), and creatinine clearance decreased by only 38% (Table 1). Similarly, alterations in renal electrolyte and water handling were markedly attenuated and weight reduction was minimized (Table 1, Figs. 2 and 3). Glycosuria was minimal or absent, averaging only 17 ± 20 mg/100 ml on day 5. Similar results were obtained whether glycine and cisplatin were infused into the same vein or into different veins.

When glycine infusion was initiated an hour after the injection of cisplatin (D-GLY), the protective effect was almost entirely abolished. Administration of an equimolar dose of L-alanine (ALA) had no effect on the extent of injury and functional derangement (Table 1, Figs. 2 and 3).

Preservation of tubular structure with glycine was also noted in additional studies in which the cisplatin dose was 3.5 mg/kg. In these experiments S3 tubular damage was markedly reduced by glycine (14 ± 26% as compared to 92 ± 17% in controls, $N = 15$ and 23, respectively) but not by L-alanine or glutamine (97 ± 9%, $N = 6$, and 92 ± 15%, $N = 8$, respectively, $P < 0.01$ by one-way ANOVA).

Morphological injury was correlated with plasma creatinine ($r = 0.6$, $P < 0.003$), creatinine clearance ($r = -0.8$, $P < 0.001$) and urinary osmolality ($r = 0.8$, $P < 0.001$) on day 5 in those animals studied.

Concentrations of platinum in plasma, urine and kidneys

Plasma concentration of platinum was slightly lower on day 6 in glycine-treated rats, as compared to controls (Table 2). The concentration of platinum found in kidney tissues on day 6 was similar in both groups, as were plasma concentrations 24 hours after injection and urinary content of platinum on day 1. No correlation was found between renal platinum content and the degree of functional and structural damage.

Plasma and kidney concentrations of glycine

Plasma glycine concentration increased 40-fold from basal values of 159 and 190 μmol/liter in two rats over 60 minutes of infusion. At 15 minutes (when cisplatin was injected in the study groups) plasma concentrations were 3.4 and 3.6 mmol/liter, comparable to the levels (2 mM) found to be protective in earlier studies of isolated perfused kidneys [4, 5]. Kidney glycine concentrations (12.3 and 6.0 μmol/g wet wt) and liver concentrations (8.9 and 6.8 μmol/g wet wt) following 90 minutes of infusion were similar to extrapolated plasma concentrations at that time.

Cisplatin-induced hypokalemia

Prominent hypokalemia was noted in all experimental groups 24 hours after the platinum injection (Fig. 3A). The initial decline in urinary potassium excretion (Fig. 3C) may reflect in part the decrease in intake of food that was documented. Fractional potassium excretion increased in all groups in spite of the hypokalemia, perhaps reflecting impaired renal tubular function (Fig. 3B). For comparison, in a group of four normal rats starved for 24 hours, fractional excretion and total daily urinary potassium were reduced by 62% and 44%, respectively, while plasma potassium declined by only 8%. Plasma magnesium did not fall, but rather increased in the ALA, D-GLY and CTR groups from 1.25 ± 0.16 and 1.24 ± 0.12 mEq/liter on day 1 and 2 to 1.81 ± 0.19 mEq/liter on day 6 ($P < 0.01$, 1 way ANOVA).

Discussion

Recently, glycine has been shown to provide protection against certain hypoxic [1, 2] and metabolic [3] insults in dispersed proximal tubules incubated in vitro. Glycine also improves renal function and reduces hypoxic injury to medullary thick limbs in isolated perfused rat kidneys [4, 5]. In the present experiments carried out in vivo, glycine substantially attenuated morphological and functional damage to the kidneys caused by the administration of cisplatin to intact rats. The dose of glycine administered produced plasma concentrations in the same range as those shown to be protective in earlier experiments in vitro. Equimolar L-alanine, which partially ameliorates damage in isolated perfused kidneys [4, 5] and proximal tubular cells [2, 3, 11], failed to provide protection in our experiments.

In rats and mice, cisplatin causes a dose-related, cumulative injury most marked in the straight portion of the proximal tubule (S3) located in the outer medulla and the medullary rays [7, 12]. Injury to the thick ascending limb, while overshadowed by the prominent morphological disruption of S3 cells, can be inferred from the decrease in urinary concentrating ability [13], renal magnesium wasting [14], and marked reduction in urinary excretion of epidermal growth factor, which is contained exclu-

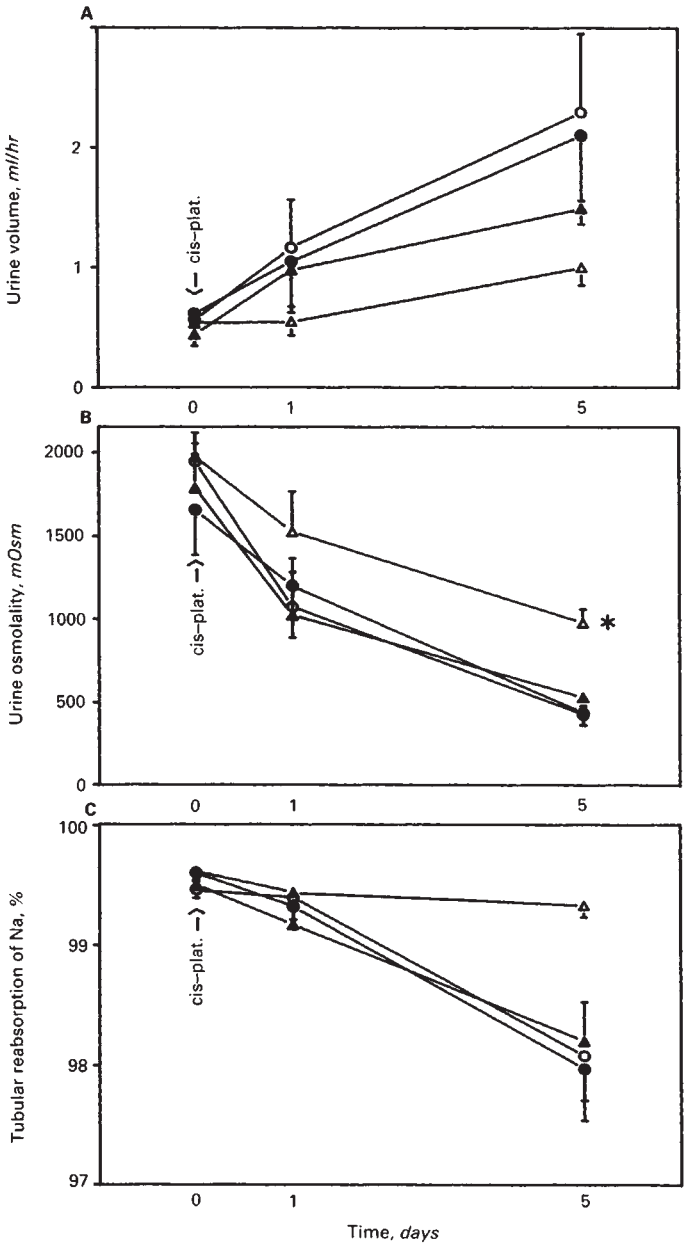

Fig. 2. *Changes in urinary volume, osmolality and fractional tubular reabsorption of sodium after cisplatin, in rats given saline (○—○, CTR), L-alanine (●—●, ALA), glycine (△—△, GLY), or a delayed glycine infusion (▲—▲, D-GLY).* Data are mean ± SE, $N = 6$ for all groups. The changes from day 0 to day 5 in urine volume, urine osmolality and fractional tubular reabsorption of sodium are significant in all groups ($P < 0.05$ by one-way ANOVA) with the exception of an unchanged tubular sodium reabsorption in GLY rats. In this group, as well, changes in urine osmolality and volume were less marked. (* indicates $P < 0.01$ vs. other groups on day 5, by one-way ANOVA).

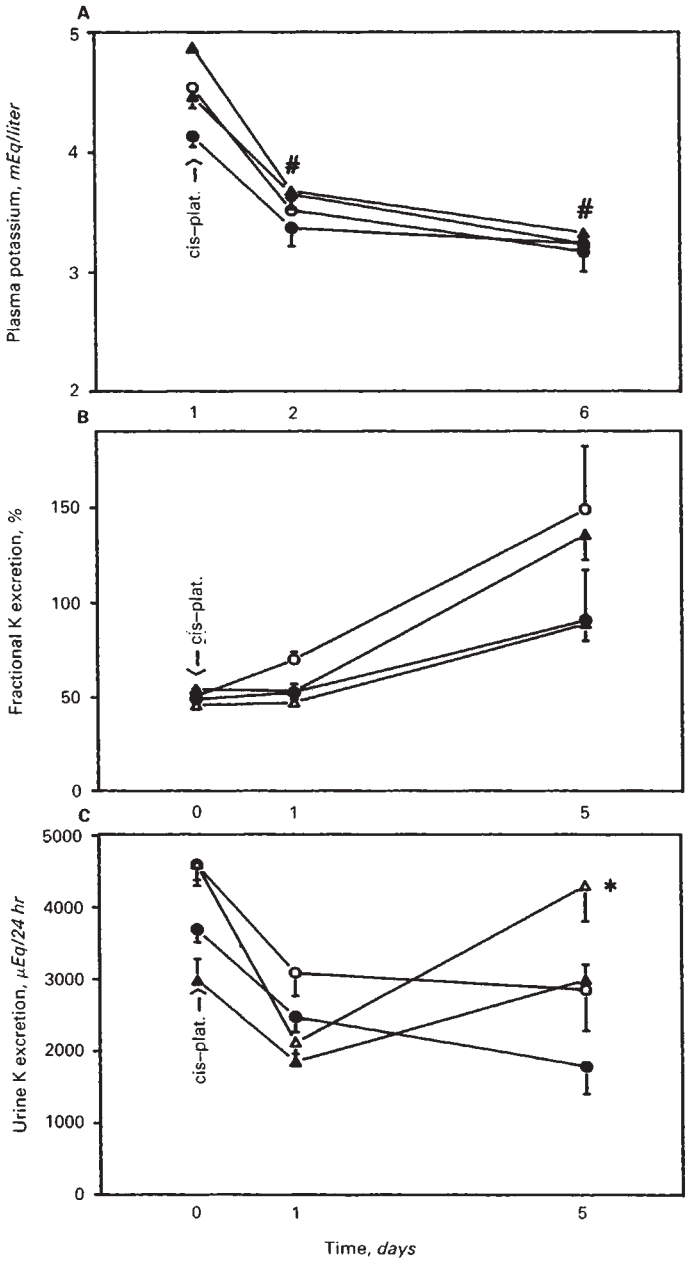

Fig. 3. *Plasma potassium levels* **(A)**, *fractional potassium excretion* **(B)** *and daily potassium excretion* **(C)** *in rats injected with cisplatin 5 mg/kg, with simultaneous infusion of saline (○—○, CTR), glycine (△—△, GLY) or L-alanine (●—●, ALA), or with a delayed glycine infusion (▲—▲, D-GLY).* Remarkable hypokalemia was noted in all groups as early as 24 hours after the injection of cisplatin, and persisted at 5 days. Symbols indicate mean ± SE; $N = 6$ except for plasma potassium in GLY and CTR groups where $N = 8$ and 12, respectively. Plasma potassium fell after cisplatin in all rats (# $P < 0.01$ vs. baseline in all groups by two-way ANOVA). Fractional potassium excretion significantly increased in all groups. The high total daily potassium excretion at day 5 in the GLY group probably reflects increased intake, as indicated by minimal weight loss as seen in Table 2. (*$P < 0.01$ vs. other groups by one-way ANOVA).

sively in this nephron segment [15]. Mitochondrial dysfunction is an early event demonstrated within 20 minutes of exposure of renal tubules to the drug [16]. Ultrastructural analysis reveals early mitochondrial swelling, and thinning and loss of brush borders. The basement membrane becomes electron dense and thickened. Nuclear chromatin is condensed, and lysosomes increase both in size and number. Progressive azotemia evolves over 3 to 10 days following drug administration accompanied by more advanced mitochondrial dysfunction [17]. Altered tubular

Table 2. Platinum levels in plasma, urine and kidney

Group	Control ($N = 5$)	Glycine ($N = 5$)
Plasma platinum day 2 *μg/ml*	0.44 ± 0.04	0.39 ± 0.07
Plasma platinum day 6 *μg/ml*	0.11 ± 0.02	0.08 ± 0.01[a]
Kidney platinum day 6 *μg/g wet wt*	6.80 ± 1.11	6.40 ± 0.76
Urine platinum day 1 *μg/24 hrs*	105 ± 156	90 ± 126

Values are mean ± SD.
[a] $P < 0.02$ vs. control, by non-paired Student's *t*-test.

transport leads to polyuria and losses of sodium chloride, potassium and magnesium [7, 12, 18–20].

Cisplatin is predominantly excreted by the kidney. Some 50% of injected cisplatin is excreted in the urine over the first 24 hours in the free, unbound form [21]. Urinary excretion of platinum rapidly diminishes subsequently, owing to its binding by plasma proteins and deposition in tissues. The kidney retains platinum to a greater extent than any other organ. Renal platinum accumulation in the rat is completed over the first six hours, and at 24 hours some 1% of an injected dose of 5 mg/kg dose is found in the renal parenchyma.

The biochemical and subcellular basis for renal toxicity from cisplatin is not fully understood. It is presumed that the high platinum concentration at the corticomedullary junction of the kidney results from active tubular transport. Low intracellular chloride ion concentration is thought to lead to a conversion of cisplatin to a toxic nucleophilic complex [7]. Because it is rapidly bound to plasma and tissue proteins, it is likely that the process of cell injury is initiated during the first minutes after the injection of cisplatin into the bloodstream.

In this perspective, the protective effect of glycine, given early over a brief period of time, is instructive. High plasma and parenchymal glycine levels are apparently required at the same time that platinum is injected in order to prevent kidney damage, as delayed glycine administration was found to be far less effective. It is noteworthy that glycine did not affect the content of platinum in the kidneys of rats given cisplatin, nor did it alter the concentration of platinum in plasma and urine, measured 24 hours after the cisplatin administration. Glycine might conceivably change the binding of free cytosolic platinum, inactivate toxic platinum species [22], or augment their lysosomal accumulation [23]. An interaction between cisplatin and glycine before mixing occurred in the circulation seems unlikely, since the administration of both agents through different veins (jugular and femoral) did not alter the results.

The mechanism of glycine-induced protection is obscure. Glycine and L-alanine do not reduce the utilization of oxygen by renal tubular cells [4, 5] and do not affect ATP stores [2, 11, 24]. Weinberg et al suggested that glycine, serving as a substrate for glycine N-acyltransferase, might prevent secondary cell destruction by neutralizing free fatty acids elaborated from damaged membranes [1], but there is as yet no direct evidence for this hypothesis. Glycine protection apparently occurs without substantial metabolism of the amino acid [11, 25].

It seems possible that the protective effect of glycine may be related to its three-dimensional structure, as certain glycine analogs that bind to glycine receptors in the central nervous system have recently been found to protect against renal cell injury in a manner similar to that of glycine [25, 26].

Hypokalemia has been reported as an uncommon complication of cisplatin treatment, and is usually attributed to hypomagnesemia [14, 27, 29, 30]. In the present experiments, serum potassium fell by about 1 mEq/liter, to 3.8 ± 0.2 during the first 24 hours after cisplatin administration, and further decreased to 3.1 ± 0.2 over the next four days. A similar degree of hypokalemia was seen in animals treated with glycine. The hypokalemia cannot be attributed to hypomagnesemia, as magnesium levels actually increased in the ALA, D-GLY and CTR groups, probably as a result of advanced renal failure. Though decreased intake of food probably contributed to a negative potassium balance, renal potassium losses presumably also played a role in inducing and perpetuating hypokalemia, as indicated by the failure of fractional excretion of potassium to fall and, indeed, its progressive increase from day 2 to day 5.

In summary, glycine infusions given to rats during the administration of cisplatin remarkably attenuates nephrotoxicity, as assessed by kidney morphology and function. L-alanine, on the other hand, was not effective in blunting cisplatin toxicity. Further studies are required to evaluate the clinical importance of this observation and to elucidate the mechanisms involved in the protective effect of glycine.

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