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AMINO ACIDS' PROTECTIVE EFFECTS ON EXPERIMENTAL ACUTE RENAL FAILURE

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Abstract: This study was carried out to elucidate the nephroprotective effects from a mixture of 8 L-amino acids and the possible mechanism of protection by this amino acid mixture. Acute renal failure model was induced by an intravenous administration of 10 mg/kg cisplatin to male Sprague-Dawley rats. A mixture of 8 L-amino acids or 0.9% saline was infused at a rate of 2 ml/h for 3 h, starting with a 2 ml bolus injection before cisplatin administration. Amino acids showed no acute effect on renal morphology. The infusion of a mixture of 8 L-amino acids increased GFR by 85% in control rats. The abnormalities of urine sodium and potassium excretion caused by cisplatin were markedly attenuated by the administration of the amino acid mixture. With the infusion of this amino acid mixture, cisplatin-induced abnormal state 4 respiration returned to control levels and the depressed state 3 respiration, respiratory control ratio and carbonyl cyanide p-trifluoromethoxyphenyl hydrazone-uncoupled respiration were ameliorated remarkably. A mixture of 8 L-amino acids showed marked nephroprotection against cisplatin-induced acute renal failure in rats and might function through augmentation of the cisplatin-injured renal mitochondrial electron transport-oxidative phosphorylation sequence, probably via stabilizing the membrane (including inner mitochondrial membrane) protein tertiary structure. In addition, this amino acid mixture remarkably increased GFR and decreased urine sodium excretion in rats.

Key words: acute renal failure, cisplatin, nephroprotection, amino acids, kidney tubule, mitochondria
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INTRODUCTION

Acute renal failure (ARF), usually referred to as acute tubular necrosis, is a common syndrome characterized by an abrupt and sustained decline in glomerular filtration with resultant azotemia, caused by acute ischemic and/or toxic injuries. It is not immediately reversible when causes are eliminated.

In order to protect renal function, a variety of pharmacologic interventions have been developed. A series of reports indicate that provision of L-glycine or L-alanine at physiological concentrations strikingly protects kidney tubule cells studied in vitro from multiple types of cell injuries (Baines et al., 1990; Songabe et al., 1996). Several other L-amino acids including serine, proline and α-aminoisobutyric acid also have protective effects in isolated perfused kidneys or in cultured renal tubule cells injured by calcium ionophore (Baines et al., 1990; Weinberg, 1991a). However, the mechanisms of nephroprotection by amino acids are unclear and whether amino acid supplementation *in vivo* will be protective remains to be determined.

The present study was designed to elucidate the nephroprotective effects from a mixture of 8 L-amino acids on cisplatin-induced nephrotoxic ARF model and the possible mechanism of protection by this amino acid mixture.

METHODS

Animal model (Xie et al., 2000)

Male Sprague-Dawley rats, weighing 250-350 g, were used for all experiments. They were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) and then placed on a warmed operating table to maintain body temperature at about 37 ℃ throughout the experiment. The femoral vein was cannulated with a two-

channel vein catheter for infusion of cisplatin (CP) and fluid. Following a 2 ml bolus infusion, 0.9% saline or a mixture of 8 L-amino acids (AA, consisting of alanine and glycine etc.) was then administered into the femoral vein at a rate of 2 ml/h using a constant-infusion syringe pump.

After a midline laparotomy, the abdominal aorta below the renal arteries was catheterized with an intravenous catheter placement set and 0.1ml heparin was added to the connecting tube to avoid blood coagulation. Mean arterial blood pressure (MABP) was measured using a strain-gauge transducer(Gould, Statham) and a magnifier (Hugo Sachs Elektronik, March-Hugstetten) connected to a recorder (ABB Goerz-Metrawatt, Austria). During the experiment, MABP of all animals ranged from 13.3 kPa to 16 kPa.

A plastic tube was inserted in the bladder. Urine was collected in plastic test tube every 30 min interval following cisplatin injection up to 3 h, and used for calculation of urine volume, determination of creatinine and sodium or potassium concentrations. At the termination of the 3h *in vivo* experiment, 2 ml blood sample was taken from the portal vein, placed at 4 ℃ for 30 min and centrifuged at 735(× g) for 10 min to separate serum for creatinine determination. Meanwhile, the superior mesenteric artery and the aorta above and below the renal arteries were ligated and the kidneys were irrigated via the aortal catheter with a perfusion medium (in mmol/L: 138 NaCl, 4.8 KCl, 1 $CaCl_2 \cdot 2H_2O$, 1 $MgCl_2 \cdot 6H_2O$, 8.25 TRIS, 0.8 $Na_2HPO_4 \cdot 2H_2O$, 0.27KH_2PO_4 and 50 mannitol buffered at pH 7.35 with HCL mol/L). Either kidney was taken immediately for isolation of mitochondria. In addition, the pedicles of those kidneys used for morphological studies were tied before perfusion.

Experimental design

The animals were divided into 5 groups at random. Control group(n = 21): under anesthesia, either kidney was removed immediately after a midline laparotomy and renal perfusion, used for mitochondrial isolation. Sham-operated (SHAM-OP) group (n = 16): only 0.9% saline was infused. CP group(n = 12): 10 mg/kg cisplatin was injected over several minutes after a 2 ml bolus infusion of 0.9% saline. CP + AA group(n = 10): 10 mg/kg cisplatin was administered following AA bolus infusion. AA group(n = 16): only AA infusion was given.

Mitochondrial isolation

Mitochondria of the kidney were isolated according to the method of Goldstein (Goldstein, 1975). Mitochondrial pellets were resuspended in incubation medium (in mmol/L: 290 saccharose, 2 HEPES, 2.5 K_2HPO_4, 2.5$MgCl_2 \cdot 6H_2O$, 0.5 EDTA, 5 malate, 5 glutamate and 0.1% bovine serum albumin at pH 7.4 with KOH mol/L) with protein concentration at about 2.14 mg/ml.

Measurement of mitochondrial respiration

Mitochondrial oxygen consumption was measured polarographically at 25 ℃ in a closed 0.45 ml reaction chamber fitted with a Clark O_2 electrode (Eschweiler, Kiel), connected to a recorder. Before starting measurements, the apparatus was calibrated with a calibration medium (2% Na_2SO_3, 0.2% $Na_2B_4O_7$).

0.45 ml mitochondrial suspension containing 5 mmol/L malate and 5 mmol/L glutamate was added to the chamber to initiate the study of mitochondrial respiration. State 3 respiration was induced by the addition of 0.5 mmol/L ADP. After the ADP effect had worn off (phosphorylated to ATP), state 4 respiration was measured. Respiratory control ratio (RCR) is the quotient of state 3/state 4 respiration used as an indicator of the integrity of the mitochondrial preparation, with high values representing a tight coupling between ADP phosphorylation to ATP and electron transport. In addition, the uncoupled rate of O_2 consumption was measured after the addition of 2 nmol/L carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) providing an index of maximal mitochondrial electron transport capacity.

State 3, state 4 and FCCP-uncoupled respiration rates were expressed as nmol $O_2 \cdot min^{-1} \cdot$ mg mitochondrial $protein^{-1}$ · They were measured at least 4 times for each mitochondrial specimen, respectively, and the averages were taken.

Mitochondrial protein measurement

Mitochondrial protein concentration was measured for each specimen by the Lowry method(Markwell, 1978).

Creatinine determinations and calculation of GFR

Serum and urine creatinine were measured

by Jaffe-method. GFR was calculated at 3 h using urine volume and creatinine concentration in the last 30 min interval and accomplished by the standard formula: GFR (ml/min) = urinary creatinine (mg/ml) · urine volume (ml/min)/serum creatinine (mg/ml).

Measurements of urine sodium and potassium concentration

Urine sodium and potassium concentration were measured by flame photometry.

Morphological studies

Two rats for each group were used for this study. One gross section (1mm thick) was made sagittally from each kidney examined and immersed immediately in a vial containing 2 ml buffered 4% glutaraldehyde. After a 2 h fixation at 4 ℃, the specimens were cut to obtain tissue blocks about 1 mm^3, postfixed in 1% OsO_4, and dehydrated in graded ethanol solutions. Following treatment with propylene oxide, the samples were embedded. Semithin sections (1 – 2 μm thick) stained with toluidine blue were used for routine examination. For electron microscopy, ultrathin sections (50 nm thick) were made. After staining with uranyl acetate and lead citrate they were examined and photographed with a transmission electron microscope.

In addition, the remaining tissue samples of each kidney were fixed in formalin and processed for light microscopy.

Statistics

Results were expressed as means ± SEM. The comparisons of GFR and mitochondrial respiration studies were carried out using a one-way analysis of variance with the Student-Neuman-Keuls test. A two-way analysis of variance and the Student-Neuman-Keuls test were utilized for analyses of sodium and potassium concentration in urine. A probability level of $P < 0.05$ was selected as indicating statistical significance.

RESULTS

Morphologic evaluation

After 3h *in vivo* experiments no changes were apparent by both light and electron microscopy in CP, CP + AA and AA groups, as compared to SHAM-OP animals(data not shown).

Protective effect by amino acids on kidney glomerular filtration

At the end of the 3 h *in vivo* experiments, no significant difference in GFR was found between CP (1.50 ± 0.13 ml/min) and SHAM-OP (1.87 ± 0.08 ml/min) groups ($P > 0.05$).

However, in CP + AA (3.30 ± 0.19 ml/min) and AA (3.45 ± 0.21 ml/min) groups, GFR increased by 77% and 85% of SHAM-OP levels, respectively (both $P < 0.01$).

Protective effect by amino acids on kidney tubular excretion

A short time (2.30 min) following cisplatin administration, CP-treated animals showed a 56% continuous increase of sodium concentration in urine throughout the 3h period as compared with SHAM-OP values ($P < 0.01$, Table 1). In contrast to the finding in CP-treated rats, urine sodium concentration in CP + AA and AA groups decreased dramatically (62% and 71% decreases of SHAM-OP values, respectively, both $P < 0.01$, Table 1). Within all groups between various intervals, no significant difference

Table 1 Sodium concentrations in urine throughout the 3 h *in vivo* experiments

Group	Urine Sodium(mmol/L) Concentration					
	0.5	1.0	1.5(h)	2.0	2.5	3.0
SHAM-OP	106.38 ± 6.97	96.80 ± 6.55	96.36 ± 4.66	101.52 ± 7.16	102.76 ± 6.78	106.88 ± 6.87
CP	137.26 ± 16.27*	166.88 ± 16.07*	174.06 ± 16.29*	167.04 ± 13.12*	158.20 ± 2.85*	149.96 ± 11.47*
CP + AA	40.28 ± 4.27*#	41.24 ± 5.06*#	39.76 ± 3.15*#	36.02 ± 3.71*#	38.02 ± 3.95*#	39.74 ± 2.19*#
AA	27.08 ± 2.15*#	25.74 ± 1.79*#	27.48 ± 3.39*#	29.88 ± 3.64	35.24 ± 4.18*#	33.28 ± 3.10*#

All values were means ± SEM. SHAM-OP: Sham-operated; CP: 10 mg/kg cisplatin; CA + AA: 10 mg/kg cisplatin + a mixture of 8 L-amino acids; AA: a mixture of 8 L-amino acids; * $P < 0.01$ compared with sham-operated animals. # $P < 0.01$ versus cisplatin group.

was observed in urine sodium concentration ($P > 0.05$).

Urine potassium concentration in the CP group increased 2.6 times of SHAM-OP levels 30 min after cisplatin injection ($P < 0.01$, Table 2) and remained high thoughout the *in vivo* experiments. However, in the CP + AA group, the increased urine potassium excretion was ameliorated and maintained at SHAM-OP levels throughout the *in vivo* studies and AA had no effect on normal potassium excretion in urine ($P > 0.05$, Table 2). There was also no significantly different urine potassium concentration within all groups between various intervals ($P > 0.05$, Table 2).

Table 2 Potassium concentrations in urine throughout the 3 h *in vivo* experiments

Group	Urine Sodium(mmol/L) Concentration					
	0.5	1.0	1.5(h)	2.0	2.5	3.0
SHAM-OP	29.34	31.58	34.76	35.80	38.92	42.36
	±3.03	±3.74	±4.67	±4.14	±4.34	±3.34
CP	126.76	16.42	120.86	127.74	128.44	130.42
	±4.64*	±5.71*	±4.11*	±3.16*	5.71± *	±8.54*
CP + AA	37.98	31.98	38.12	41.84	35.70	36.84
	±4.93	±2.42	±4.11	±4.66	±3.19	±3.52
AA	29.20	29.02	32.12	32.72	30.92	28.40
	±2.97	±3.18	±3.65	±3.89	±3.76	±2.39

All values were means ± SEM. SHAM-OP: Sham-operated; CP: 10 mg/kg cisplatin; CA + AA: 10 mg/kg cisplatin + a mixture of 8 L-amino acids; AA: a mixture of 8 L-amino acids; * $P < 0.01$ compared with sham-operated animals.

Protective effect by amino acids on kidney mitochondrial respiration

The results are presented in Table 3. There were no significant differences in mitochondrial respiration between control, SHAM-OP and AA groups ($P > 0.05$). However, in CP-treated rats, 3 h following cisplatin administration, state 4 respiration doubled that of control values ($P < 0.01$); on the contrary, state 3 respiration, RCR and FCCP-uncoupled respiration decreased by 46% ($P < 0.01$), 74% ($P < 0.01$) 47% ($P < 0.01$), respectively, as compared to that of controls.

Very excitingly, in the CP + AA group, state 4 respiration returned to control levels ($P > 0.05$ versus controls). State 3 respiration, RCR and FCCP-uncoupled respiration were improved greatly compared with CP-treated animals (all $P < 0.01$); but still did not reach control values, the percentages of depression were 16% ($P < 0.05$), 22% ($P < 0.01$) and 21% ($P < 0.01$), respectively, as compared to controls.

Table 3 Renal mitochondrial respiration studies at termination of the 3 h *in vivo* experiments

Group	State 3 respiration (nmol · min^{-1} · mg mito $protein^{-1}$)	State 4 respiration (nmol · min^{-1} · mg mito $protein^{-1}$)	RCR	FCCP-uncoupled respiration (nmol · min^{-1} · mg mito $protein^{-1}$)
Control	1069.82 ± 29.88	145.84 ± 6.04	7.45 ± 0.16	964.76 ± 36.67
SHAM-OP	967.64 ± 37.61	135.36 ± 6.03	7.21 ± 0.17	882.59 ± 22.52
CP	574.55 ± 11.63*	293.64 ± 9.39*	1.97 ± 0.05*	512.23 ± 18.02*
CP + AA	900.35 ± 42.52#$	155.49 ± 8.34	5.85 ± 0.19*#	765.51 ± 34.80*#
AA	995.94 ± 38.75	147.94 ± 6.21	6.78 ± 0.16	926.38 ± 36.54

All values were means ± SEM. PCR: respiratory control ratiol; FCCP: carbonyl cyanide ptrifluoromethoxyphenyl hydrazone; SAHM-OP: Sham-operated; CP: 10 mg/kg cisplatin; CA + AA: 10 mg/kg cisplatin + a mixture of 8 L-amino acids; AA: a mixture of 8 L-amino acids; mito: mitochondrial; * $P < 0.01$ versus control animals. # $P < 0.01$ as compared to cisplatin-treated animals; $ $P < 0.05$ compared with control values.

DISCUSSION

Supplementation of the rabbit tubule incubation medium with glycine at concentrations ranging from 0.25 to 2 mmol/L provided major protection against hypoxic injury (Weinberg et al., 1987). Glycine was protective against lethal cell injury induced by calcium ionophore and was only variably cytoprotective against oxidant lesions in freshly isolated proximal tubules (Sogabe et al., 1996; Weinberg et al., 1991a). Alanine was shown to exert protective effects in perfused kidney (Baines et al., 1990). In the present study, the infusion of a mixture of 8 L-amino acids offered remarkable protection against cisplatin-induced increased excretion of sodium and potassium in urine and renal mitochondrial respiration dysfunction.

The mechanism of nephroprotection by amino acids is obscure. In normal rats, GFR was decreased 32% by the infusion of glycine and alanine at high rate (125 μmol $kg^{-1} \cdot min^{-1}$), suggesting that they both had a nephrotoxic potential when used inappropriately (Zager et al., 1983). However, low and moderate concentrations of amino acids have been clearly shown to produce vasodilation and increase GFR in rats (El Sayed et al., 1991a). The present data confirmed this observation, with GFR increased by 85% of sham-operated values after amino acids infusion, indicating that this amino acid mixture had a remarkable effect on increasing GFR in rats. It was considered that the influence of amino acids on increasing GFR results from their direct action on the kidney, inducing hyperperfusion and hyperfiltration and that prostaglandins, dopamine and nitric oxide might all have a role to play in this process (El Sayed et al., 1991).

Glycine is actively transported by proximal tubule cells via apical and basilateral sodium-dependent uptake mechanism. It has been observed that glycine significantly increases the fractional reabsorption of sodium in isolated perfused rat kidney (El Sayed et al., 1991). The present data showed that amino acids infusion normalizes urine sodium and potassium excretion in cisplatin-treated rats, with urine sodium excretion even lower than that in sham-operated animals. There might be two possibilities to explain this observation. Firstly, cisplatin-induced renal tubule reabsorptive dysfunction is greatly ameliorated by amino acids administration. Secondly, sodium-amino acid co-transports in amino acid-treated rats. Following amino acids infusion, with large quantities of amino acids reabsorbed into the proximal tubules, sodium co-transport occurred, causing an increase in urine sodium reabsorption.

The biochemical and subcellular basis for the nephroprotective effect by amino acids is not fully understood. Although there are many potential metabolic pathways for glycine, most of these are unlikely to contribute to protection because they require ATP and/or synthetic processes, while glycine protective actions occur during profound ATP depletion with preincubation (Weinberg et al., 1987). Consistent with this, the beneficial effects of glycine and alanine do not require their metabolism (Baines et al., 1990). Substitutions on glycine's carboxyl and amino groups or their removal results in loss of potency (Weinberg et al., 1990). 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 been found to protect against renal cell injury in a manner similar to that of glycine, suggesting the stringent requirements of amino acid molecular structure for protection and the involvement of highly specific acceptor-ligand effects on a process critical for functioning (Weiberg et al., 1990). It is assumed that glycine and alanine may play an essential, constitutive role in maintenance of tubule cell structural integrity and prevent tubular disruption through their physicochemical effects, which can stabilize membrane protein tertiary structure (Baines et al., 1990; Weinberg et al., 1991b). The present data showed that cisplatin-induced increased state 4 respiration returned to control levels with amino acid infusion and the depressed state 3 respiration, RCR and FCCP-uncoupled respiration, although they did not reach control values, were ameliorated greatly. Thus it is presumed that this amino acid mixture might exert nephroprotective effects through the functional augmentation of cisplatin-injured renal mitochondrial electron transport-oxidative phosphorylation sequence, possibly by virtue of the stabilizing membrane (including inner mitochondrial membrane) protein tertiary structure. However, further studies are still needed before it could be confirmed this is so.

In summary, the present study suggests that a mixture of 8 L-amino acids offers remarkable protection against cisplatin-induced acute renal failure in rats and might function through augmentation of the cisplatin-injured renal mitochondrial electron transport-oxidative phosphorylation sequence, probably via stabilizing the membrane (including inner mitochondrial membrane) protein tertiary structure. In addition, this amino acid mixture strikingly increases GFR and decreases urine sodium excretion in rats.

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