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Cisplatin-induced Changes in Adenine Nucleotides in Rat Kidney Slices: Amelioration by Tiopronin and Procaine

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Abstract

The adenine nucleotides (ATP, ADP and AMP) in rat renal cortical slices exposed in-vitro to cisplatin, an anticancer drug, were determined by HPLC.

Cisplatin had no effect on total adenine nucleotides in the slices but caused a time- and concentration-dependent decrease in ATP levels with a concomitant increase in ADP and AMP levels. The decrease in ATP and increases in ADP and AMP concentrations became statistically significant after incubation with cisplatin (2 mM) for 90 min or after cisplatin (1 mM) for 120 min. Both tiopronin, a sulphydryl-containing drug, and procaine, an antioxidant, protected against cisplatin-induced changes in the adenine nucleotides.

The results indicate a cisplatin-induced defect in cellular energetics that occurs at a relatively late stage in the process of toxicity to the slices in this in-vitro model. Cisplatin-induced depletion of ATP in the slices might result from an increase in catabolism of ATP to ADP and AMP. Maintenance of the normal concentration of ATP in the slices might be involved in the protection afforded by tiopronin and procaine against cisplatin-induced nephrotoxicity.

Cellular adenine nucleotides play a vital role in the regulation of numerous intracellular processes in normal kidney cells and in the pathogenesis of cell injury such as chemical-induced nephrotoxicity and renal ischaemia (Zager 1991; Henke & Nickel 1992; Rush et al 1992; Henke & Jung 1993; Lock et al 1993).

Cisplatin is currently one of the most important anticancer drugs. It is, however, nephrotoxic, which restricts its clinical use (Von Hoff et al 1979; Choie et al 1981). It has been suggested that mitochondrial dysfunction is a central component of cisplatin nephrotoxicity to proximal tubules (Brady et al 1993) and that cisplatin-induced toxicity to kidney slices in-vitro is critically associated with oxidative damage to mitochondria (Zhang & Lindup 1993) which might lead to failure of energy production or ATP depletion, or both.

Depletion of ATP has been observed in renal cortical slices after incubation with cisplatin (Phelps et al 1987; Zhang & Lindup 1996a) but the mechanisms were not defined. The current work examines whether the depletion of ATP was because of conversion to ADP or AMP, or both. We have also examined the effects of tiopronin (*N*-(2-mercaptopropionyl)glycine) and procaine on cisplatin-induced changes in adenine nucleotides because these two drugs protect against cisplatin-induced nephrotoxicity (Zhang et al 1992; Zhang & Lindup 1994a, 1996a).

Materials and Methods

Materials

Cisplatin, tiopronin, L-cysteine, ATP (disodium salt), ADP (sodium salt) and AMP (sodium salt) were purchased from Sigma (Poole, Dorset, UK), L-glutamic acid (sodium salt) and glycine from BDH (Poole, Dorset, UK), and procaine hydrochloride from Hopkin and Williams (Chadwell Heath, Essex, UK). Other chemicals were of reagent grade.

Preparation of renal cortical slices and incubation conditions

Adult female Wistar albino rats, 210–260 g, were obtained from the Department of Pharmacology and Therapeutics animal house; rat renal cortical slices were prepared as previously described (Zhang & Lindup 1993). The slices (3–5 slices; total weight approximately 80–100 mg) were loaded into glass conical flasks (25-mL Erlenmeyer flasks, Duran, Astell Scientific, Kent, UK) containing 5 mL incubation medium (97 mM NaCl, 40 mM KCl, 0·74 mM $CaCl_2 \cdot H_2O$, 1 mM glycine, 1 mM glutamate, 0·2 mM L-cysteine and 7·5 mM sodium phosphate buffer, pH 7·4, oxygenated with 100% oxygen). Cisplatin, tiopronin and procaine were dissolved in the incubation medium before use. The flasks were oxygenated with pure oxygen for 1 min after addition of the slices or test drugs and stoppered immediately with rubber bungs. The flasks were incubated in a shaking water-bath at 100 cycles min^{-1} at 37°C for various times.

HPLC assay

After incubation the slices were homogenized at 15 000 rev min^{-1} (Polytron PT300; Aggregates PT-DA 3012/2; homogenizing vessel GS 15, Kinematica AG, Switzerland) for 1 min in a mixture of 70% ethanol and 2 mM EDTA, pH 10·9 (5 mL). After homogenization, trichloroacetic acid (20%, w/v; 1 mL) was added and mixed well to precipitate the protein. The mixture was centrifuged at 3000 *g* for 10 min and a sample of the resulting supernatant was frozen at −70°C until analysis.

The cellular adenine nucleotides, ATP, ADP and AMP were analysed by high-performance liquid chromatography (HPLC) (Hull-Ryde et al 1983) with slight modification to the mobile phase. The frozen samples were thawed, neutralized with one

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drop of 70% aqueous ammonia (29·5% NH_3) and diluted with phosphate buffer (0·05 M; pH 7·4).

Liquid chromatography was performed with Spectra-Physics Analytical (California) SCM 400 solvent degasser, P100 isocratic pump, AS1000 autosampler, UV1000 UV detector and HP4600 integrator. A Nucleosil 5-C18 main column (particle size 5 μm; 25 × 0·46 cm i.d.) was obtained from Technicol (Stockport, UK). The mobile phase was 0·1 M ammonium dihydrogen phosphate adjusted to pH 4·5 with 3 M ammonium hydroxide. Before each assay the column was flushed for at least 10 min with 100% methanol then flushed thoroughly with 2% methanol in water. The column was left to equilibrate with the mobile phase before injection of samples.

Isocratic elution at 1·5 mL min^{-1} was performed at room temperature. The loop size was 20 μL; detection was performed at 254 nm. Quantitative calculations were based on peak-height measurement. A series of standards of ATP, ADP and AMP in phosphate buffer (0·05 M; pH 7·4) was analysed each day. For determination of adenine nucleotides a standard mixture of the three nucleotides (2 μM final concentration) was added to each sample and unknown concentrations were expressed as the difference between that of the mixture (standard + sample) and that of the standard.

Statistics

The renal cortical slices for each experiment were obtained from at least three rats and the results are expressed as means ± s.e.m. of five observations. The data were analysed with an unpaired two-tailed Student *t*-test. A probability level of $P <$ 0·05 was considered as indicative of statistical significance.

Results and Discussion

HPLC analysis

Fig. 1 shows the separation of a standard mixture of the adenine nucleotides (2 μM). Retention times were ATP 5·69 min, ADP 6·85 min, and AMP 14·66 min. A large peak of unknown material eluted at 7·97 min but this did not interfere with the assay. Peak-height measurements for standard adenine nucleotides were linear over the range 1–20 μM (data not shown). Peaks identified as ATP, ADP and AMP in extracts of homogenates had retention times similar to those of the corresponding standards (Fig. 2).

Effect of cisplatin

The normal concentrations of ATP, ADP and AMP in the slices were approximately 30–35, 15–20 and 7–10 nmol mg^{-1} protein, respectively (Tables 1–3).

Incubation of slices with cisplatin caused a time- and concentration-dependent decrease in ATP levels, with concomitant increases in levels of ADP and AMP (Fig. 3 and

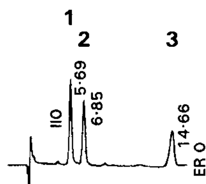

FIG. 1. Typical HPLC elution pattern of a standard mixture of adenine nucleotides (2 μM). 1. ATP (retention time 5·7 min); 2. ADP (6·9 min); 3. AMP (14·7 min).

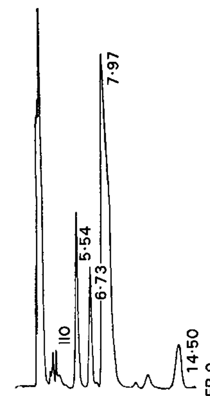

FIG. 2. HPLC determination of adenine nucleotides in an extract of slice homogenate containing 2 μM standard adenine nucleotides. Retention times were 5·5 min (ATP), 6·7 min (ADP) and 14·5 min (AMP).

Table 4). The slices incubated with 2 mM cisplatin suffered a dramatic depletion of ATP (65% of control) after 90 min incubation and ADP and AMP were increased to about 150% and 240%, respectively, of control. In addition, ATP concentration was significantly reduced ($P < 0·01$) and AMP was increased ($P < 0·01$), without significant change in ADP, by 1 mM cisplatin after 120 min incubation (Fig. 3 and Table 4). During incubation of slices with cisplatin, the total concentrations of adenine nucleotides (ATP + ADP + AMP) in the slices did not change significantly (Fig. 4 and Table 5).

Moreover, cisplatin reduced the ATP/ADP and ATP/AMP concentration ratios, changes that were attributable to depletion of ATP levels and increases of ADP and AMP levels. These ratios were reduced from control values of 1·7 (ATP/ADP) and 3·7 (ATP/AMP) to 0·8 and 1·0, respectively, after incubation with 2 mM cisplatin for 90 min. Furthermore, cisplatin at 1 mM caused a similar reduction of the ATP/ADP and ATP/AMP concentration ratios at 120 min (Fig 5 and Table 5).

These results therefore showed that cisplatin caused a marked depletion of ATP levels in the slices; this is consistent with our previous observations with a luciferin-luciferase bioluminescence assay of ATP (Zhang & Lindup 1996a; Zhang et al 1996). The mechanisms that underlie cisplatin-induced depletion of ATP are not known but it can in theory be caused either by inhibition of ATP synthesis at the level of mitochondrial respiration or by excessive degradation of ATP to ADP, AMP, possibly with further catabolism to inosine, either with or without alteration of ATP synthesis. Cisplatin in-vivo inhibited state-3 renal mitochondrial respiration (Gordon & Gattone 1986) which led to a decrease in synthesis of ATP. There is no previous report, however, on the effect of cisplatin on ATP synthesis by use of the current model and thus this needs further investigation. ADP and AMP were also assayed to define the metabolic pattern of adenine nucleotides arising from the depletion of ATP. Intracellular ADP and AMP were concomitantly increased with a subsequent decrease in the ATP/ADP or ATP/AMP concentration ratios (Figs 3 and 5 and Tables 4 and 5). Most renal ATP loss was, therefore, probably a result of conversion of ATP to ADP or AMP, or both, by activation of the hydrolysis of ATP.

The exact role of ATP in the mechanism of cisplatin-induced nephrotoxicity is still questionable. Phelps et al (1987)

Table 1. Effects of tiopronin on cisplatin-induced changes in adenine nucleotides in rat renal cortical slices.

	ATP	ADP	AMP	ATP + ADP + AMP	Concentration ratios ATP/ADP	Concentration ratios ATP/AMP
Control	31·5 ± 2·5	20·6 ± 1·2	8·3 ± 0·5	60·5 ± 3.9	1·5 ± 0·1	3·8 ± 0·1
Cisplatin (2 mM)	14·9 ± 0·7*	27·2 ± 0·8*	24·5 ± 1·3*	66·6 ± 2·3	0·5 ± 0·1*	0·6 ± 0·1*
Cisplatin + tiopronin (2 mM)	34·6 ± 2·2†	22·0 ± 0·4†	11·5 ± 0·5†	68·1 ± 2·6	1·57 ± 0·1†	3·0 ± 0·2†

The slices were incubated in the medium with 2 mM cisplatin or cisplatin plus tiopronin (both 2 mM) for 120 min. Each value (nmol (mg protein)$^{-1}$) represents the mean ± s.e.m. of results from five determinations. *$P < 0·01$, significantly different from control; †$P < 0·01$ significantly different from cisplatin alone.

Table 2. Effects of tiopronin concentration on cisplatin-induced changes in adenine nucleotides in rat renal cortical slices.

	ATP	ADP	AMP	ATP + ADP + AMP	Concentration ratios ATP/ADP	Concentration ratios ATP/AMP
Control	35·5 ± 3·1	15·4 ± 1·7	10·4 ± 0·8	61·3 ± 5·0	2·4 ± 0·2	3·5 ± 0·3
Cisplatin (2 mM)	15·0 ± 0·8†	22·5 ± 1·2†	24·3 ± 1·7†	61·8 ± 1·7	0·7 ± 0·1†	0·6 ± 0·1†
Cisplatin + Tiopronin (0·25 mM)	25·2 ± 1·3§	18·5 ± 0·4‡	16·4 ± 1·3§	60·1 ± 3·8	1·4 ± 0·2§	1·6 ± 0·2§
Cisplatin + Tiopronin (0·5 mM)	20·3 ± 1·2§	17·8 ± 0·8‡	10·4 ± 0·5§	48·8 ± 1·2*‡	1·2 ± 0·1§	2·0 ± 0·1§
Cisplatin + Tiopronin (1·0 mM)	29·2 ± 1·7§	16·9 ± 1·2§	11·0 ± 0·6§	57·1 ± 2·3	1·7 ± 0·2§	2·7 ± 0·3§

The slices were incubated in the medium with 2 mM cisplatin or with cisplatin plus different concentrations of tiopronin for 120 min. Each value (nmol (mg protein)$^{-1}$) represents the mean ± s.e.m. of results from five determinations. *$P < 0·05$, † $P < 0·01$, significantly different from control; ‡$P < 0·05$, §$P < 0·01$ significantly different from cisplatin alone.

Table 3. Effects of cisplatin and cisplatin plus procaine on the concentration of adenine nucleotides in rat renal cortical slices.

	ATP	ADP	AMP	ATP + ADP + AMP	Concentration ratios ATP/ADP	Concentration ratios ATP/AMP
Control	30·3 ± 0·8	168 ± 0·9	7·2 ± 0·5	54·2 ± 1·5	1·8 ± 0·1	4·3 ± 0·3
Cisplatin (1 mM)	22·1 ± 1·8*	24·8 ± 0·9*	13·3 ± 1·0*	60·2 ± 1·4	0·9 ± 0·1*	1·7 ± 0·2*
Cisplatin (1 mM) + procaine (1 mM)	30·2 ± 1·4†	16·7 ± 1·7†	9·8 ± 1·5†	56·2 ± 2·2	1·9 ± 0·1†	3·1 ± 0·2†

The slices were incubated in the medium with 1 mM cisplatin or with cisplatin plus 1 mM procaine for 120 min. Each value (nmol (mg protein)$^{-1}$) represents the mean ± s.e.m. of results from five determinations. *$P < 0·01$, significantly different from control; † $P < 0·01$ significantly different from cisplatin alone.

considered that, because it occurred at an early stage, the depletion of ATP was a sensitive and important indicator of cisplatin-induced toxicity to precision-cut rabbit renal cortical slices. A study with renal proximal tubular cells in-vitro showed, however, that cisplatin did not affect the ATP content of these cells (Courjault-Gautier et al 1994). Our results revealed that depletion of ATP occurred rather later (90 min) during the development of toxicity. With the same experimental model we found that cisplatin-induced damage to mitochondria was an early event and preceded depletion of ATP. These events included depletion of mitochondrial glutathione and protein thiols, inhibition of calcium uptake by mitochondria, lower activity of protein kinase C and induction of mitochondrial lipid peroxidation (Zhang & Lindup 1993, 1994b, 1996b).

Therefore, the cause-and-effect relationship between ATP depletion and cell death could not be clearly established by the current experiments. The depletion of ATP could, however, potentiate cisplatin-induced biochemical events such as inhibition of Na^+,K^+-ATPase and disruption of intracellular calcium homeostasis.

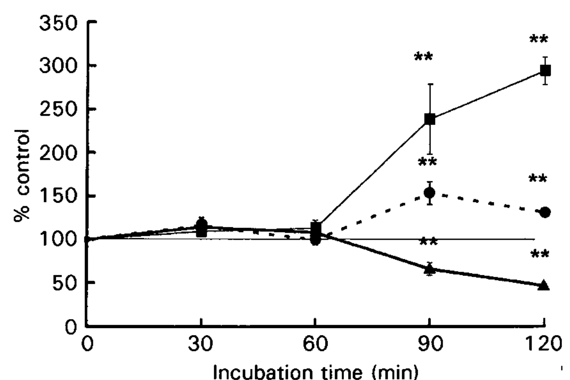

FIG. 3. Time-dependent effects of cisplatin on levels of ATP (▲), ADP (●) and AMP (■) in rat renal cortical slices. The slices were incubated with cisplatin (2 mM) at 37°C for different times up to 120 min. After incubation, adenine nucleotides in the slices were extracted and then determined by HPLC. Each value represents the mean ± s.e.m. of results from five determinations. **$P < 0·01$, significantly different from control.

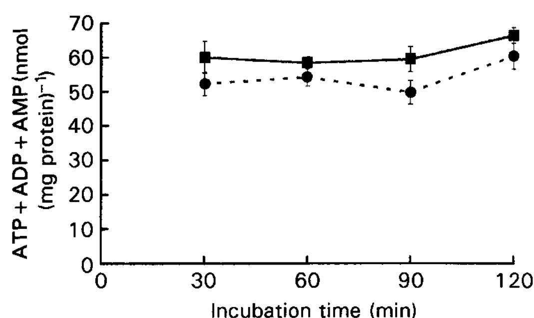

FIG. 4. Effects of cisplatin (2 mM) on total levels of adenine nucleotides in rat renal cortical slices. Control (●), cisplatin (■). Other conditions were as described for Fig. 3.

Table 4. Effect of cisplatin on the concentrations of adenine nucleotides in rat renal cortical slices.

Cisplatin (mM)	ATP	ADP	AMP
0·25	84±9	113±5	113±12
0·5	72±8	117±5	105±9
1·0	65±4*	117±8	151±6*

The slices were incubated in the medium with different concentrations of cisplatin for 120 min. Results are expressed as percentage of control values measured in the absence of cisplatin. Each value (nmol (mg protein)$^{-1}$) represents the mean ±s.e.m. of results from five determinations. *$P<0{\cdot}01$, significantly different from control.

Protective effects of tiopronin and procaine

Our previous work indicated that both tiopronin and procaine protect against cisplatin-induced toxicity to renal slices, including the depletion of ATP (Zhang & Lindup 1996a; Zhang et al 1996), and this was supported by the current results. As shown in Table 1, co-incubation of slices with cisplatin (2 mM) and tiopronin (2 mM) for 120 min significantly protected against cisplatin-induced decreases of ATP levels, decreases of the ATP/ADP and ATP/AMP concentration ratios and increases of ADP and AMP levels in the slices. This protection was also concentration-dependent and tiopronin at 0·25 mM protected against the toxic effects of cisplatin (Table 2). Procaine at 1 mM also provided significant protection against the toxic effects of cisplatin (1 mM) (Table 3). The results suggest that protection by both tiopronin and procaine against the nephrotoxicity of cisplatin might be related to their ability to prevent depletion of ATP in the slices by inhibition of the hydrolysis of ATP to ADP and AMP.

Acknowledgement

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Table 5. Effect of cisplatin on total adenine nucleotide concentrations and concentration ratios in rat renal cortical slices.

		Concentration ratios	
Cisplatin (mM)	ATP+ADP+AMP	ATP/ADP	ATP/AMP
0	51·3±3·7	1·6±0·1	3·0±0·3
0·25	50·6±2·1	1·2±0·2	2·2±0·2
0·5	47·6±2·5	1·0±0·2	2·1±0·2
1·0	50·8±2·4	0·9±0·1*	1·3±0·1*

The slices were incubated in the medium with different concentrations of cisplatin for 120 min. Each value (nmol (mg protein)$^{-1}$) represents the mean ±s.e.m. of results from five determinations. *$P<0{\cdot}01$, significantly different from control.

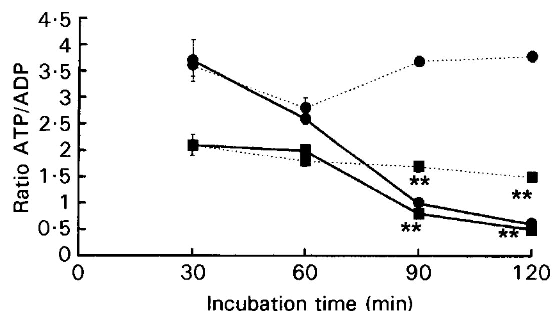

FIG. 5. Time-dependent effects of cisplatin on ATP/ADP and ATP/AMP concentration ratios: ----■----, control ATP/ADP concentration ratio; ----●----, control ATP/AMP concentration ratio; —■—, ATP/ADP concentration ratio for 2 mM cisplatin; —●—, ATP/AMP concentration ratio for 2 mM cisplatin. Other conditions were as described for Fig. 3.

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