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Preclinical report

Protective effect of procaine hydrochloride on cisplatin-induced alterations in rat kidney

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Efforts have been made to reduce the undesirable side effects of cisplatin, mainly nephro- and neurotoxicity, but their reduction is usually accompanied by a concomitant inhibition of antitumor activity. The local anesthetic procaine hydrochloride (P.HCl) improves the therapeutic index of cisplatin not only by the reduction of its nephro- and hemotoxicity, but also by an increase of its antitumor activity. We therefore investigated the effects of a combined treatment of cisplatin and P.HCl on rat kidneys and compared this to kidneys from rats treated with a toxic dose of cisplatin or P.HCl alone. Treatment with a saline solution was used as control. Dehydrogenase activities [succinate dehydrogenase (SDH) and NADPH diaphorase reaction demonstrating nitric oxide synthase (NOS/NADPHd)] and phosphatase activities [K^+ *p*-nitrophenyl phosphatase (K^+ pNPPase), alkaline phosphatase (AlPase) and acid phosphatase (AcPase)] were studied on cryostatic sections of kidneys from controls and treated rats. Evidence of heavy morphological damage and altered AlPase and AcPase activities induced by cisplatin were observed in the S3 segment of the proximal tubules. In addition, SDH and K^+ pNPPase activities showed some changes in the distal tubule cells. The NOS/NADPHd activity in macula densa was drastically reduced. Combined treatment of cisplatin and P.HCl greatly attenuated morphological alterations of the rat kidney and reduced the changes in enzyme activities, except for NOS/NADPHd activity, compared to the cisplatin-treated group of animals. The study indicates that, in cisplatin-induced nephrotoxicity, a significant role is played by enzyme activities, in particular K^+ pNPPase and NOS/NADPHd, and that P.HCl can mitigate the nephrotoxicity of cisplatin, possibly by influencing some enzyme activities involved in important renal metabolic pathways. [© 2002 Lippincott Williams & Wilkins.]

Key words: Cisplatin, enzymes, nephrotoxicity, procaine.

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Introduction

Cisplatin is one of the most effective anticancer drugs administered to treat a variety of cancers such as ovarian, testicular, bladder, head and neck, and uterine cervix carcinomas.[1,2] High doses of cisplatin are more effective than low doses in ovarian and colorectal cancer.[2–4] However, high dosage treatment induces nephro- and neurotoxicity. In spite of hydration, hypertonic saline and diuretics to protect against renal complications, a high percentage of treated patients develop from mild to severe renal imbalance characterized by increased serum creatinine levels and uremia, hypomagnesaemia, hypokalemia, and reduced glomerular filtration rate.[5,6]

The mechanisms of nephrotoxicity induced by cisplatin are still a matter of debate. Various data indicate that cisplatin induces oxidative stress,[7–9] lipid peroxidation[10–12] and DNA damage.[13,14] Nevertheless, other factors such as adenosine triphosphatase activity,[15] mitochondrial function,[16] vascular and inflammatory factors,[17–20] and intracellular calcium homeostasis[21,22] are altered by cisplatin as well, and may play a crucial role in its nephrotoxic effect. Finally, all these alterations result in an evident modification of the kidney's histological structure, mainly at the level of the S3 segment of the proximal renal tubule,[8,23,24]

Previous papers have demonstrated that the local anesthetic procaine hydrochloride (P.HCl) increases the therapeutic index of cisplatin, reducing its nephrotoxic and hemotoxic effects, and improving its antitumor activity.[25–27] Although much evidence suggests that the protective and potentiating activity of P.HCl, at least in part, is due to its metabolites *p*-aminobenzoic acid (PABA) and diethylaminoetha-

nol,[28–31] nothing is known about the local action through which P.HCl restores renal function.

This work aims at identifying some of the mechanisms involved in cisplatin-induced alterations of the rat kidney and the effects of P.HCl on cisplatin-treated rats which help to prevent nephrotoxicity. We have therefore studied the morphological aspects of the kidney and some enzyme activities which we suspect may be related to the development of cisplatin-induced toxicity in control rats, and rats treated with cisplatin alone and in association with P.HCl.

Materials and methods

Animal protocols

Male Sprague-Dawley rats (Charles River Laboratories, Calco, Italy) weighing 300–350 g were used. Animals were allowed a 7-day rest before experiments, housed two per cage, maintained at 22°C with a 12 h light/dark cycle, and fed on a standard diet and water *ad libitum*. Four groups of five animals each were then treated: the first with cisplatin alone (Sigma, St Louis, MO; 6 mg/kg in normal saline); the second with cisplatin plus P.HCl (200 mg/kg, the cisplatin/P.HCl solution presented a final Cl^- concentration equivalent to physiological saline); the third group of rats was treated with a single dose of P.HCl diluted in distilled water; the last (control) group of rats was treated with normal saline. In all cases drugs were administered i.p. in a relative volume of 1.9 ml per rat of 400 g body weight.

Five days after treatment, the time of the maximal cisplatin-induced toxicity, animals were killed by ether anesthesia and exsanguination, and kidneys were rapidly excised and processed for morphological observation and cytochemistry. All experiments were performed in accordance with FELASA guidelines and approved by the institutional ethics committee.

Each group was examined by the following procedure: (i) morphological light microscopy with H & E), (ii) ultrastructural observation by conventional electron microscopy, and (iii) histochemical detection of some dehydrogenase and phosphatase activities.

Light microscopy

For morphological observations, sections (8 μm thick) obtained from both paraformaldheyde-fixed and paraplast-embedded pieces and from frozen samples were stained using H & E stain.

Electron microscopy

Small fragments (0.5–1 mm) of the kidney from each animal were fixed by immersion for 3 h in ice-cold 1.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) followed by post-fixation in 1% OsO_4 in the same buffer for 1 h at 4°C. Samples were dehydrated with ethanol and embedded in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead cytrate, and examined under a Zeiss EM 900 electron microscope.

Enzyme histochemistry

Kidneys were cut into small pieces and frozen in liquid nitrogen in closed vials. Cryostat sections (8 μm thick) were cut on a cryostat at a cabinet temperature of −21°C. Sections were picked up onto clean glass slides and were stored in the cryostat cabinet until used. Sections were dried for 5 min at room temperature and then incubated in the appropriate incubation medium in order to demonstrate the following enzyme activities:

- Acid phosphatase (AcPase; EC 3.1.3.2) by the method of Burstone.[32]
- Alkaline phosphatase (AlPase; EC 3.1.3.1) by the method of Van Noorden and Frederiks.[33]
- K^+ *p*-nitrophenyl phosphatase (K^+ pNPPase; EC 3.6.1.37) by the method of Halbhuber *et al.*[34]
- Succinic dehydrogenase (SDH; EC 1.3.99.1) by the method of Van Noorden and Frederiks.[33]
- NADPH-diaphorase for NOS visualization (NOS/NADPH-d; EC 1.14.13.39) on fixed sections by the method of Nakos and Gossrau,[35] with our modifications.[36]

We compared the relative intensity of enzyme reactions among treated and untreated rats on the same slide to avoid possible discrepancies resulting from different processing times.

For each enzyme, control sections were incubated in substrate-free media. For K^+ pNPPase, specimens were also incubated in a medium excluding K ions and in a medium containing 10 mM ouabain. For NOS/NADPH-d, fixed sections were alternatively incubated in a medium containing NADH instead of NADPH. Moreover, sections not submitted to pre-fixation were incubated in a complete standard medium to demonstrate both NOS-related and -unrelated NADPHd.

Results

Morphology

Light microscopy analysis revealed that cisplatin treatment alone induced severe alteration of kidney parenchyma when compared to both control and P.HCl-treated rat kidney. Within the cortex, cisplatin induced some tubular dilatation and focal epithelial cell damage in restricted areas, whereas it caused extensive and marked cytolysis of epithelial cells in the outer medulla (Figure 1a and b). Capillaries intermingled with tubules were often dilated. In the inner medulla some tubules appeared dilated. The

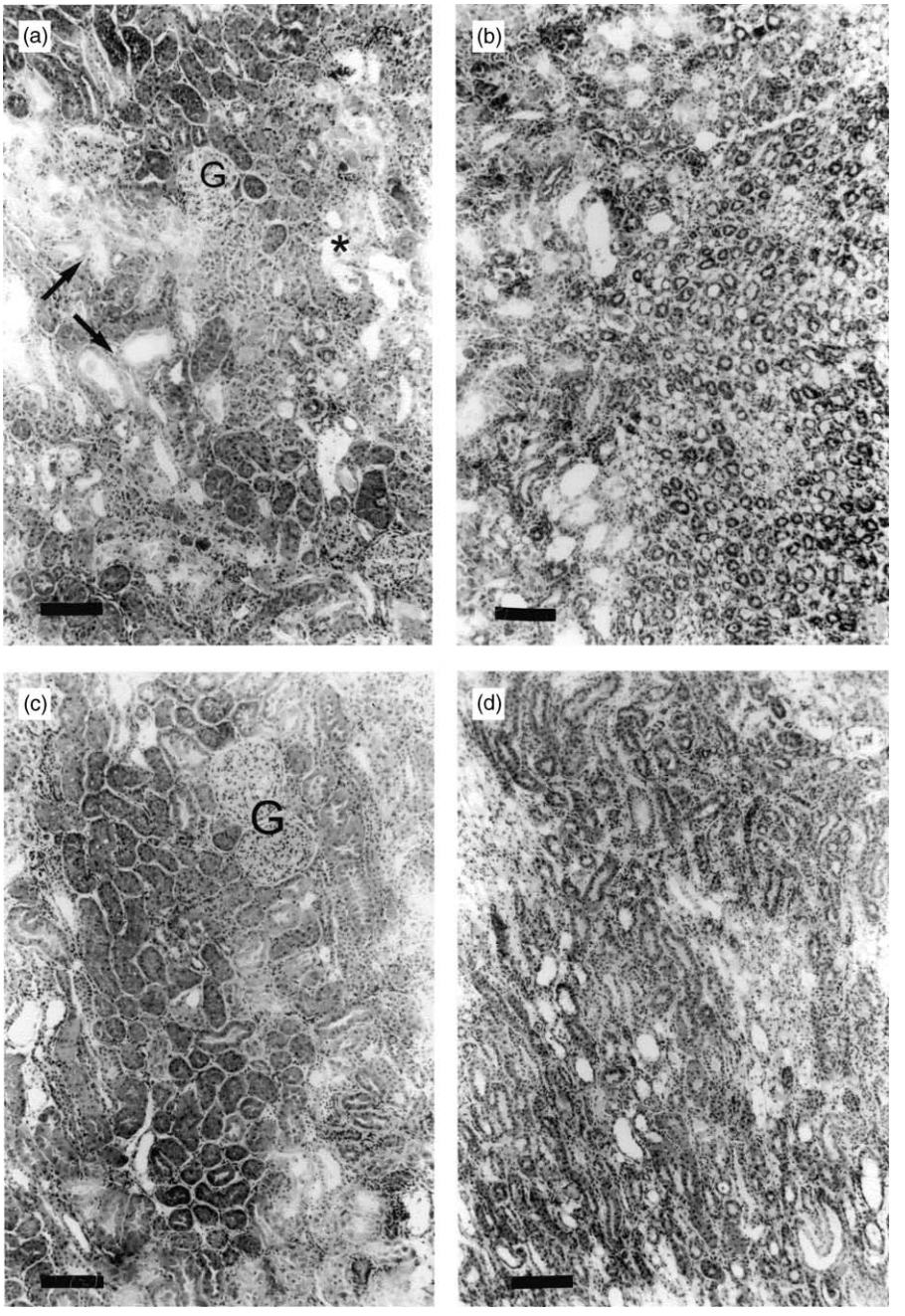

Figure 1. Representative sections from cisplatin-treated rats showing extensive tubular damage (arrows) and marked cytolysis (asterisk) within the outer medulla (a); less severe changes are observed within the inner medulla (b). Comparable sections from P.HCl/cisplatin-treated rats with mild alterations in restricted areas of both outer (c) and inner medulla (d); G, glomerulus (H & E stain). Scale bars: 100 μm.

simultaneous administration of P.HCl plus cisplatin resulted in a much less extensive and severe tubular alteration in every part of the renal parenchyma (Figure 1c and d).

Electron microscopically, epithelia of renal tubules from rats treated with cisplatin alone showed signs of cell alteration in comparison with control and P.HCl-treated rats. In proximal tubule cells, cisplatin induced cytoplasmic vacuolization, alteration of several lysosomes and karyopyknosis. Several cells also revealed loss of the brush border from the apical cell membrane and even rupture of the cell membrane, with cytoplasm pouring into the lumen. In addition, lumen of several tubules was filled with cell debris and flocculent material (Figure 2a and b).

Some cytoplasmic vacuolization and altered mitochondria were also observed in both distal tubules and macula densa (Figure 2c and d); however, the cytoplasmic changes in the cells of both were milder than those seen in proximal tubules. Endothelial cells of the capillaries sometimes appeared damaged, showing vacuolated cytoplasm.

Ultrastructural analysis of kidney samples from rats treated with cisplatin/P.HCl revealed a better preservation of the tubule cells compared to those treated with cisplatin alone. Proximal tubule cells showed mild vacuolization and less frequent disruption of the apical cell membrane; in most samples both basal and apical cell membrane appeared normal (Figure 3a). Distal tubule (Figure 3b) and

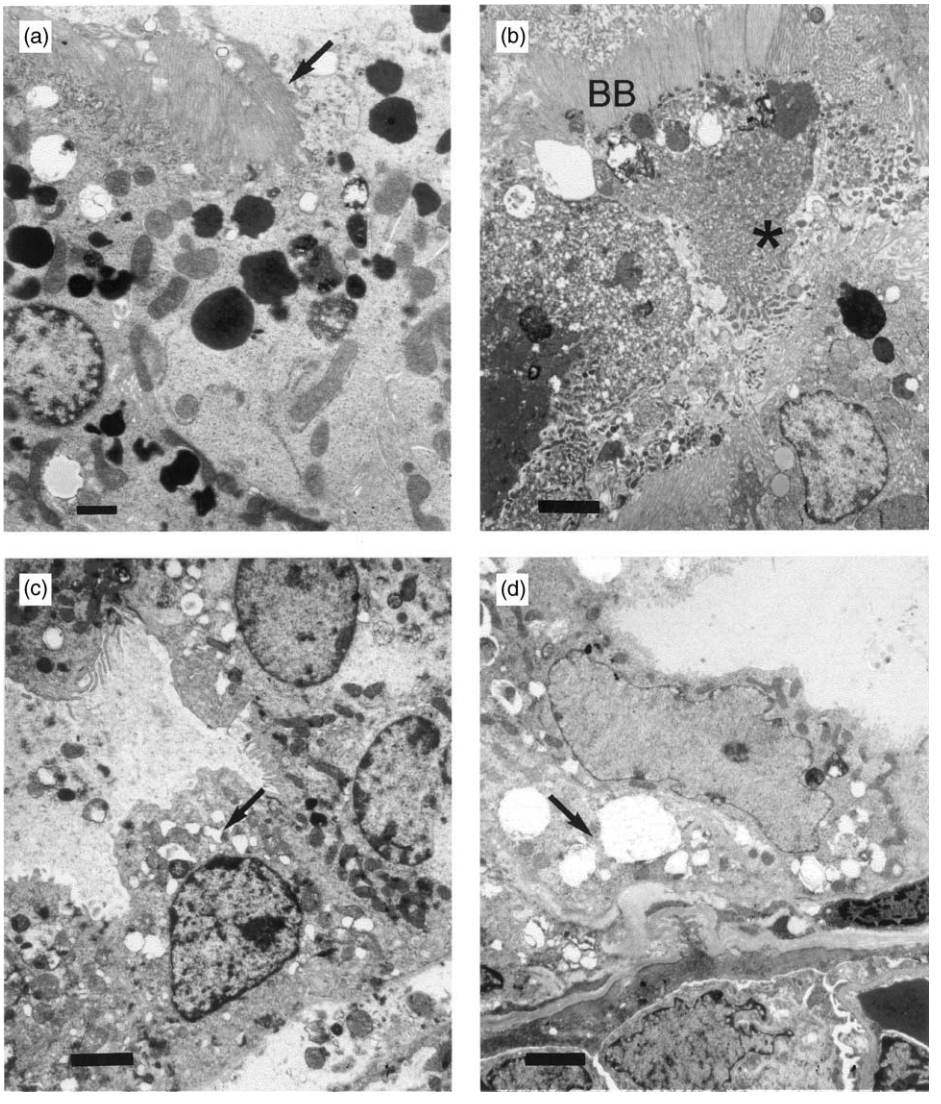

Figure 2. Electron micrographs of different renal tubule segments from cisplatin-treated rats. Portions of severely damaged proximal tubule cells (a): note cell debris within the tubule lumen (b); distal tubule cells (c) and cells of the macula densa (d) show marked vacuolization (arrows) and altered mitochondria. Scale bars: (a) 1.5 μm; (b)–(d) 2 μm.

other tubule segments were more similar to controls and P.HCl-treated animals (Figure 3c and d).

Enzyme histochemistry

The results are summarized in Table 1.

AcPase. In both the control and P.HCl-treated rats fine and diffuse granules of reaction product were mainly localized in the proximal tubule cells. Cisplatin treatment induced relevant changes in the distribution pattern of enzyme activity; in the outer medulla AcPase activity was found to be decreased and spottily localized within the cells, mainly in those of proximal tubules (Figure 4a). In rats treated with cisplatin/P.HCl the AcPase activity was found mainly in a granular pattern within the tubule cells; nevertheless, some tracts of proximal tubules in the outer medulla still exhibited variable cytoplasmic activity as coarse deposits or diffuse granules (Figure 4b).

AlPase. The AlPase activity was very intense along the apical membrane of cells constituting proximal tubules in both control and P.HCl-treated rats. Proximal tubules from the kidney of cisplatin-treated animals showed a drastic decrease of reactivity mainly in correspondence with the S3 segment

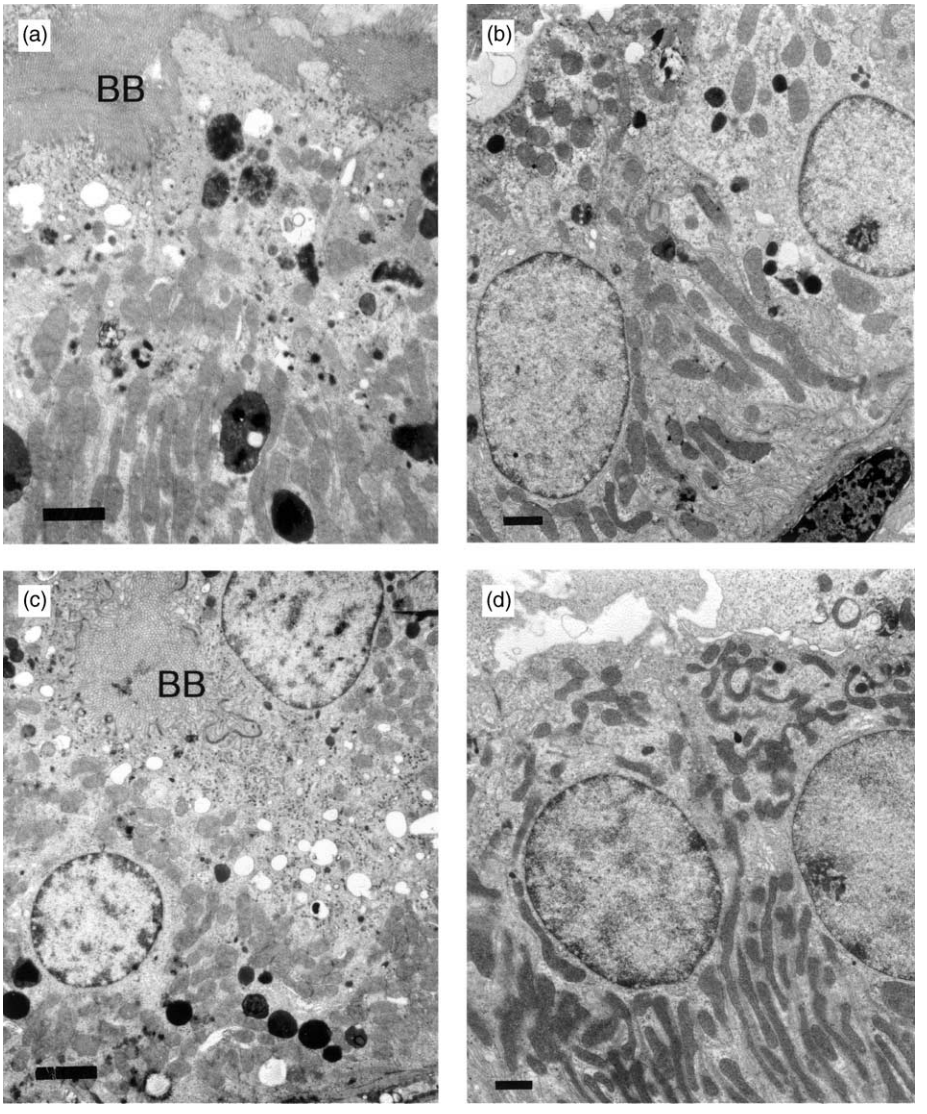

Figure 3. Electron micrographs of proximal (a) and distal (b) tubules in the kidney from cisplatin/P.HCl-treated rats showing little evidence of injury. Comparable sections (c and d) of a kidney from P.HCl-treated rats which appear almost normal; BB, brush border. Scale bars: (a) and (c) 2 μm; (b) and (d) 1 μm.

Table 1. Enzyme histochemical staining intensities in control and treated rats

Sample	AcPase	AlPase	K^+ pNPPase	SDH	NOS/NADPHd
Control	+ +	+ + + +	+ + +	+ + +	+ + +
Cisplatin	+	+ +	+ +/+ + + +	+ +/+ + + +	−
P.HCl	+ +	+ + + +	+ + +	+ + +	+ + +
Cisplatin/P.HCl	+ ±	+ + +	+ + ±	+ + ±	−

+ + + + =Very intense reaction; + + + =intense reaction; + + =moderate reaction; + =slight reaction; − =negative.

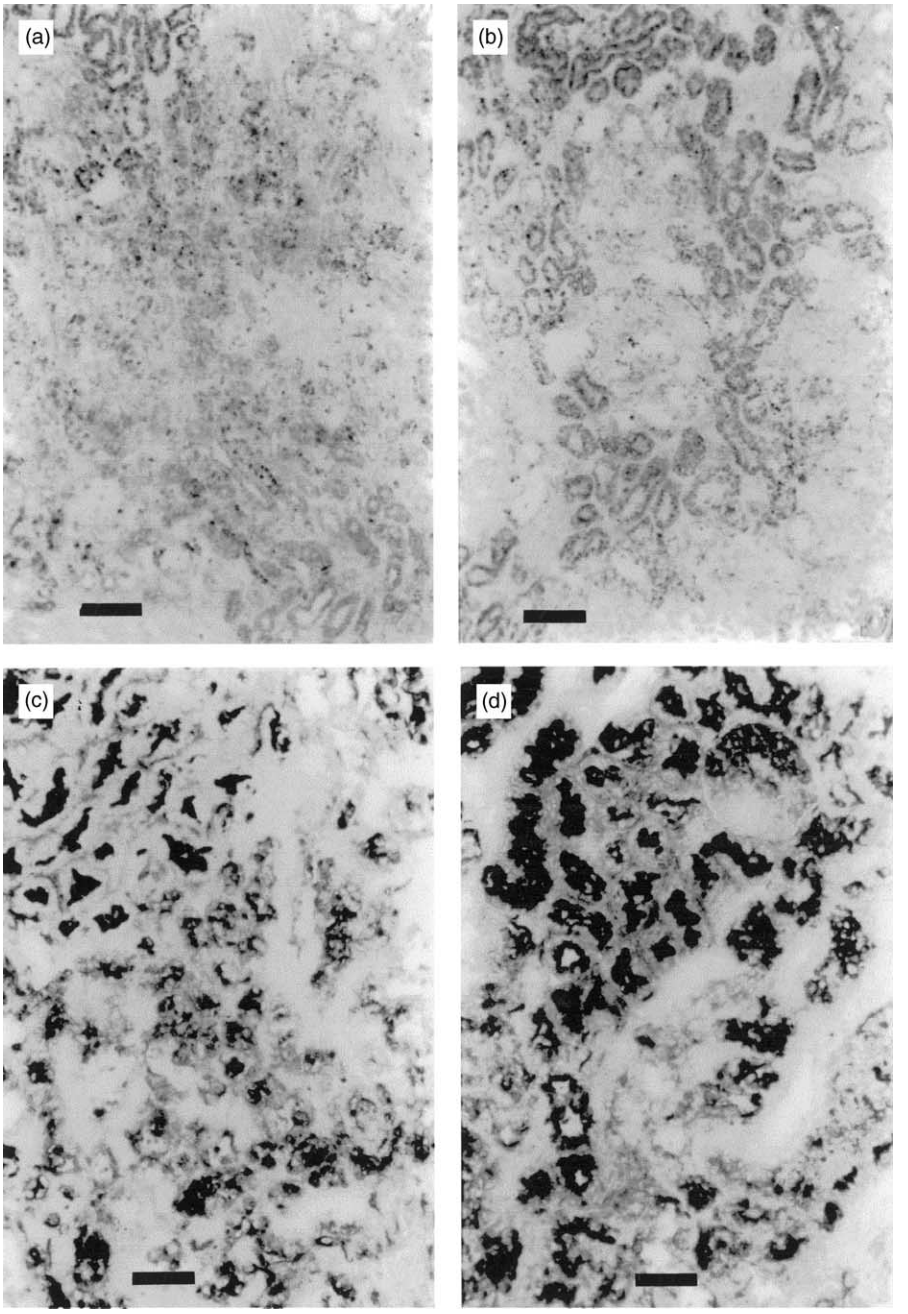

Figure 4. Outer medulla of rat kidney. AcPase activity: a reduction of positivity is observed in most proximal tubules after cisplatin treatment (a) compared to samples treated with P.HCl and cisplatin (b). AlPase activity: light to moderate reaction product is localized on the brush border of the proximal tubule cells from cisplatin-treated samples (c), whereas a strong reactivity is observed in most sections of proximal tubules from cisplatin/P.HCl-treated rats (d). Scale bars: 100 μm.

(Figure 4c). Some proximal tubules in samples treated with cisplatin/P.HCl displayed irregular deposits (Figure 4d).

K^+ pNPPase. In the control and P.HCl-treated animals, the activity of K^+ pNPPase was intense in most distal tubules and in the thick ascending limb of the loop of Henle. In the other tubules, including the proximal tubules, K^+ pNPPase ranged from moderate to weak. The reaction product was always localized in correspondence with the basolateral plasma membrane. After cisplatin administration distal tubules showed activities of different strengths: the intensity of K^+ pNPPase was very strong in some distal tubules in both the cortex and the outer medulla, while other distal tubules showed moderate reactivity and a mild diffusion of the reaction product (Figure 5a). K^+ pNPPase activity after treatment with cisplatin/P.HCl was close to normal in the tubule cells (Figure 5b).

SDH. SDH activity in control and P.HCl-treated animals appeared as dense deposits, mainly in the cells of the distal tubules. Samples from cisplatin-treated rats showed heterogeneous distribution of the reaction product in the distal tubules. In particular, some distal tubules displayed a very intense reactivity, while others appeared much less reactive (Figure 5c). In the cells of tubules from samples treated with cisplatin/P.HCl (Figure 5d), SDH staining and localization were similar to that of control tissue.

NOS/NADPHd. The strongest activity for NOS/NADPHd in the kidney from control and P.HCl-treated rats was localized in the cells of the macula densa adjacent to glomeruli (Figure 6a). Cisplatin drastically reduced NOS/NADPHd reactivity in correspondence with the macula densa and even after treatment with cisplatin/P.HCl, no staining was present within the macula densa (Figure 6b).

Controls. There were no reactions in any of the samples incubated in substrate-free media. The elimination of K ions or the addition of ouabain in the medium for demonstrating K^+ pNPPase resulted in a drastic reduction of the reaction products. After replacing NADPH with NADH sections incubated for NOS/NADPHd appeared negative. Unfixed sections submitted to NADPHd staining showed intense reaction in macula densa as well as in all the other tubules, thus confirming that, unlike other NADPHd, only the NOS/NADPHd survives fixation.

Discussion

Nephrotoxicity represents one of the major set-backs in the use of the antineoplastic drug cisplatin. The search for different pharmacological strategies to prevent damage to renal function is based on the concept that the therapeutic efficacy of cisplatin is proportional to the delivered dose. Attempts to reduce cisplatin-induced toxicity have been made following different strategies. In particular, decreasing the systemic concentration of the platinum compound (modulation of schedule of administration), modulating its elimination by hydration (clinically used) and administration of diuretics (mannitol and furosemide) which act on renal clearance, or by using antidotes to antagonize the toxic effects of cisplatin or its metabolites (WR2721, sodium thiosulfate, diethyldithiocarbamate) and, finally, using physiological non-protein thiols, such as glutathione (particularly taken up in kidney cells), for the intracellular detoxification of cisplatin (see Anand and Bashey,[37] Treskes and van der Vijgh,[38] and Pinzani *et al.*[39] for reviews).

In previous papers[25–27,31] we demonstrated that P.HCl itself, but also its metabolite PABA,[28,29] could protect rodents against the nephrotoxicity (and hematotoxicity) induced by cisplatin treatment, improving its therapeutic index. Although the complete underlying mechanisms of this protection have not yet been completely elucidated, we know that they correlate with both pharmacokinetic and pharmacodynamic observations. First of all, P.HCl is able to lower the exposure of kidney (and spleen) to platinum in mice treated with cisplatin.[31] This fact causes, of course, the decrease of the general toxic effect of cisplatin or its toxic metabolites on specific organs and molecular targets. Secondly, it has been also demonstrated that P.HCl is able to protect renal tissue against the oxidative stress and lipid peroxidation linked to cisplatin administration.[30,41] The oxidative stress is considered by different authors to be a determinant mechanism for the toxic reaction of renal tissue.

In this paper we analyzed the effect of P.HCl on the morphological changes and alterations of enzyme activities induced by the platinum compound.

A number of studies, with different approaches, have indicated functional and structural alterations in renal tubular segments, mainly in correspondence with the S3 segment of the proximal tubule, in mammals like rats, mice and dogs.[7,23,24] Similar renal damage induced by cisplatin has also been documented in avian kidney.[42] However, despite the large number of reports, the mechanism of toxicity of

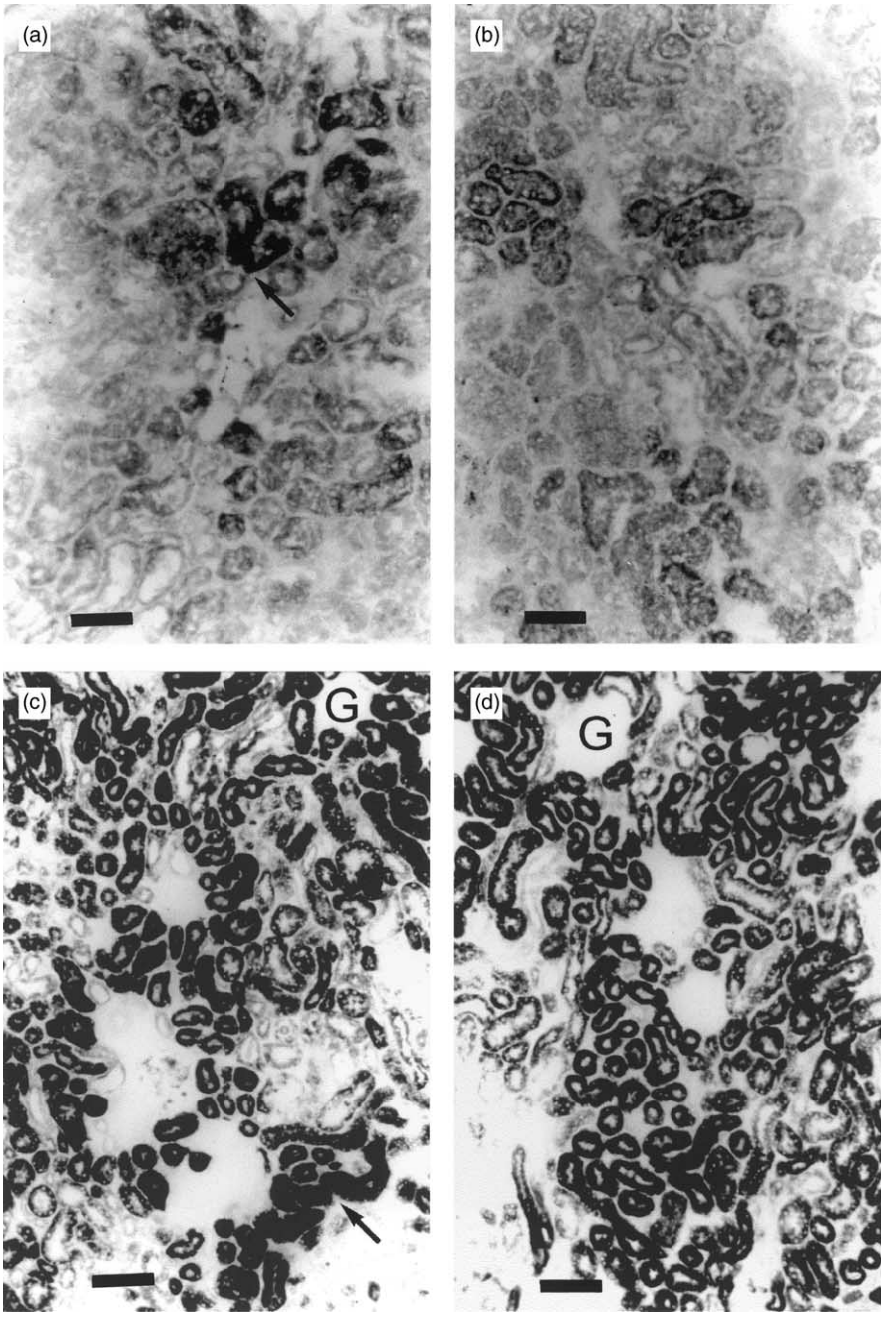

Figure 5. Outer medulla of rat kidney. K^+ pNPPase (a and b) and SDH (c and d) activities. Light to strong K^+ pNPPase activity (arrows) is noticed in distal tubules from cisplatin-treated rats (a), moderate to intense deposits are observed in the same localization in animals treated with P.HCl plus cisplatin. After SDH reaction moderate to strong deposits (arrow) are observed within different tubules in cisplatin-treated rats (c); in the cisplatin/P.HCl-treated sample, tubules show similar reactivity among them (d). Scale bars: 100 μm.

cisplatin is far from clear, probably being multifunctional.[21]

The histochemical analysis of renal tissue at day 5 after treatment followed the indications reported in different papers, and demonstrated that, at toxic doses, cisplatin induces its maximal nephrotoxic activity between day 4 and 7 after treatment, as evidenced in mice and rats by histochemical and chemico-biological analyses.[41,43,44]

In the present study we found that cisplatin treatment induces some alterations in the various segments constituting the renal tubule. By

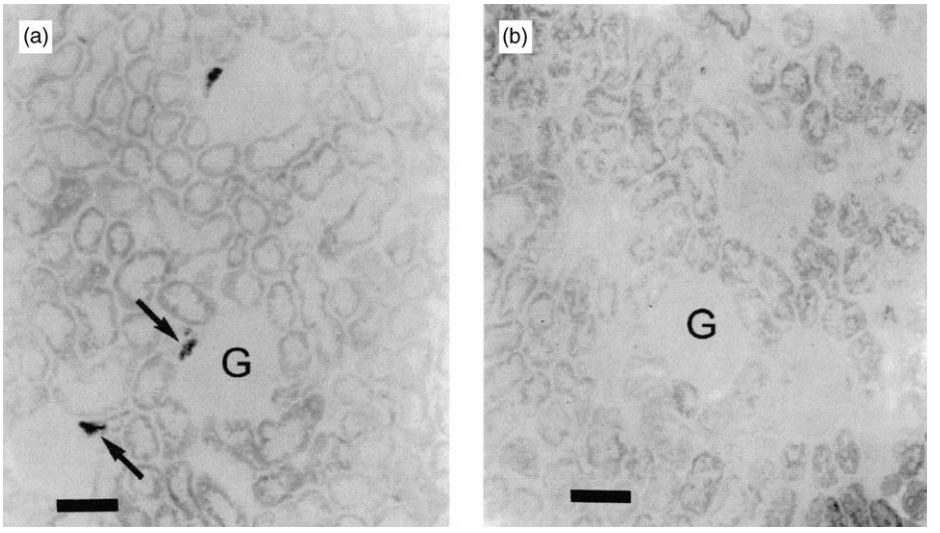

Figure 6. Cortex of rat kidney. NOS/NADPHd activity: strong staining is present in most macula densa of rats treated with P.HCl alone (a), whereas no reaction is observed in samples treated with cisplatin/P.HCl (b). Scale bars: 100 μm.

morphological analysis and histochemical studies of the AlPase and AcPase activities we observed relevant damage to the S3 segment of the proximal tubules.

We also noted significant alterations in the distal tubule, which showed an unhomogeneous distribution of both K^+ pNPPase and SDH, and in the macula densa, where NOS activity, as revealed by NAPDHd histochemistry, was absent after cisplatin treatment. In both tubule segments some morphological alterations were also ultrastructurally evidenced. As a consequence, the entire tubule seems to be involved in changes induced by the employment of cisplatin, although in a different way. These observations confirm previous studies conducted *in vitro* which had confirmed that cells from different tracts of the nephron display different sensitivities to the cytotoxic effects of cisplatin.[45]

The present data also demonstrate that most of the histochemical modifications induced by cisplatin treatment can be attenuated by the combined treatment with P.HCl. In fact, morphological changes were markedly less than those observed in rats treated with cisplatin alone. Moreover, a better preservation of membrane enzyme activities, i.e. AlPase and K^+ pNPPase, was evidenced.

The Na^+/K^+ ATPase enzyme, the activity of which is considered to be implicated differently in cisplatin nephrotoxicity, deserves a special comment. Some data suggest that this pump is responsible for cisplatin transport within the renal cells across basolateral cell membranes of renal tubules thus causing nephrotoxicity.[46,47] On the other hand, cisplatin seems to drastically affect the ATPase activity both in *in vivo* and *in vitro* models.[15,48]

Our study presents evidence regarding K^+ pNPPase reactivity in cisplatin-treated samples: we observed some distal tubules with very strong reactivity intermingled to less reactive tubules; P.HCl attenuated the inhomogeneity of this reactivity.

Na^+/K^+ ATPase, acting as a sodium pump, consumes ATP as an energy source. Therefore, it can be reasonably supposed that tubules with strong Na^+/K^+ ATPase consume more ATP than in normal conditions. In fact, decreased ATP levels with a concomitant increase in ADP and AMP levels have been demonstrated *in vitro* on renal slices treated with cisplatin.[49,50] In addition, it has been demonstrated that P.HCl can provide significant protection against the toxic effects of cisplatin, including the depletion of ATP.[50]

Cisplatin has been found to affect mitochondrial functions[51,52] and our findings concerning SDH activity seem to confirm these studies. In our samples some distal tubules displayed weak SDH activity and altered mitochondria. On the other hand, the concomitant increase of SDH activity observed in other tubules possibly supporting the Na^+/K^+-dependent pump activity might eventually result in mitochondrial impairment.

Once again the simultaneous administration of cisplatin/P.HCl seems to provide a good protection as clearly evidenced by the more comparable SDH and K^+ pNPPase activities found in cisplatin/P.HCl-treated and control rats.

Cisplatin has been found to bind irreversibly to SH groups.[53,54] This binding inhibits a number of SH-containing enzymes, including ATPases.[55,56] In addition, it induces a rapid loss in the concentration of mitochondrial protein-SH.[30,52,53]

Some experimental data[31] suggest that P.HCl may alter the otherwise irreversible binding of platinum-containing species to proteins and/or other reactive nucleophiles (e.g. SH-containing molecules) and on this basis we can hypothesize that this molecule plays a protective role against cisplatin-induced injury on SH-containing proteins.

Concerning NOS activity, different studies have demonstrated that a constitutive basal NOS activity is localized at the macula densa and it is considered to be mainly involved in renal blood flow regulation (tubuloglomerular feedback response), as well as in renin synthesis and release.[57,58] In particular, it has been demonstrated that NOS in the macula densa is of a neuronal type and Ca^{2+}/calmodulin dependent. In this study we observed a strong staining for NOS/NADPHd in control rats and in rats treated with P.HCl in correspondence with the macula densa. This reactivity was drastically reduced after cisplatin treatment and it was not restored after cisplatin/P.HCl treatment. To interpret these findings we would like to mention a previous report[59] which has demonstrated a direct interaction between hydrolyzed forms of cisplatin and the calcium-binding sites of the calmodulin molecule in the stomach of the rat: this interaction, indirectly, inhibited nNOS activity in the pyloric sphincter. We may deduce that a similar mechanism may also compromise the nNOS activity of the macula densa of the cisplatin-treated rats.

In the kidney, the inhibition of nNOS can alter renal blood flow thus contributing to the nephrotoxicity of cisplatin.[20,60,61] In this regard, it has been demonstrated that N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS activity, prevents glycine from protecting against cisplatin-mediated nephrotoxicity in the rat, suggesting the importance of hemodynamic mechanisms in cisplatin nephrotoxicity.[62]

The fact that NOS/NADPHd alterations induced by cisplatin, in contrast to the other altered enzyme activities we considered, cannot be significantly restored by P.HCl might depend on the inability of this drug to protect the calmodulin molecule from interaction with hydrolyzed forms of cisplatin. Alternatively, another possible explanation is the decrease of the coenzyme substrate for the reaction induced by cisplatin. Indeed, in renal slices incubated with the drug, the latter induces a decrease in the NADPH-dependent lucigenin-enhanced chemiluminescence and a decrease in the formation of NADPH-dependent superoxide anions.[63]

In synthesis, the effects of P.HCl on kidney from cisplatin-treated rats could be interpreted on the basis of different mechanisms affecting the enzyme activities resulting in the loss of essential components (such as coenzymes) for the catalytic function or modified cellular composition or degradation of the enzyme itself. This could explain the different susceptibility of the enzymes we studied towards cisplatin and procaine.

In conclusion, the results of the previous[25,30] and present studies point to a significant attenuation of renal alterations induced by cisplatin after cisplatin/P.HCl treatment, thus suggesting that P.HCl plays a significant role in the control of the nephrotoxic effects of cisplatin, without reducing its antitumor activity.

Acknowledgments

It was approximately 15 years ago that Dr Mauro Esposito began his studies on the protective effects of procaine and its metabolites against the nephrotoxicity of cisplatin. We would like to dedicate this paper to his memory.

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