JPP Journal of Pharmacy and Pharmacology

2003, 55: 669–674

Received October 31, 2002
Accepted January 20, 2003
DOI 10.1211/002235702946
ISSN 0022-3573

Impairment of the renal *p*-aminohippurate transport induced by 6-hydroxydopamine

C. Cojocel and M. S. Thomson

Abstract

In this study, the effects of 6-hydroxydopamine (6-OHDA) on renal *p*-aminohippurate transport were investigated in-vitro using rat renal cortical slices. Cisplatin, a known nephrotoxin, was used as positive control. Renal cortical slices were incubated for 60 min in a cisplatin-containing medium (0.83–5.0 μM) at 37 °C under a 100% O_2 atmosphere. In another series of experiments, renal cortical slices were incubated in a 3.33 μM cisplatin-containing medium for 15–120 min or in a cisplatin-free medium. Subsequently, for each series of experiments, kidney slices were incubated at 25 °C for 90 min in a media containing *p*-aminohippurate. In a further series of experiments, renal cortical slices were incubated for 60 min in a 6-OHDA containing medium (3.125–100 μM) at 37 °C under a 100% O_2 atmosphere. In another series of experiments, renal cortical slices were incubated in a 50 μM 6-OHDA-containing medium for 15–120 min or in 6-OHDA-free medium. Subsequently, for each series of experiments, kidney slices were incubated at 25 °C for 90 min in a media containing *p*-aminohippurate. The results of this study where slices were incubated in 6-OHDA- or cisplatin-containing media indicate that both 6-OHDA and cisplatin induced a time- and concentration-dependent decrease in *p*-aminohippurate accumulation by renal cortical slices. Therefore, similarly to cisplatin, 6-OHDA causes functional injury of renal proximal tubule cells, leading to impairment of transport processes across the cell membrane.

Introduction

Parkinson's disease, known also as striatal dopamine deficiency syndrome, is a degenerative disorder of the central nervous system characterized by muscular rigidity, tremor at rest and postural abnormalities. Studies of the substantia nigra after death in Parkinson's disease have suggested the presence of oxidative stress, depletion of glutathione and a high level of total iron with reduced level of ferritin (Hirsch 1993; Jenner & Olanow 1996). Initially, 2,4,5-trihydroxyphenethylamine (6-hydroxydopamine; 6-OHDA) was prepared by oxidation of dopamine by the ascorbic acid–iron EDTA system (Senoh et al 1959) and has been, since its discovery, a major tool for the investigation of the histochemical localization and function of catecholaminergic systems. 6-OHDA induces generation of free radicals, which can destroy striatal dopaminergic neurons causing parkinsonism in animals and in man (Soto-Otero et al 2000).

Levodopa, which is a very effective drug in the treatment of patients with Parkinson's disease, improves the quality of life and increases life expectancy. However, long-term use of levodopa causes a variety of side effects in parkinsonian patients. The use of 6-OHDA in sympathectomy, its endogenous formation, and its subsequent presence in the urine of parkinsonian patients on levodopa medication (Andrew et al 1993) raises questions concerning 6-OHDA toxicity. The toxic effects of 6-OHDA to brain cells and to the cells of other target organs, such as the kidney, merit close investigation, especially the elucidation of the biochemical mechanism(s) of its potential nephrotoxic effects.

Cisplatin is a very effective antineoplastic agent that has a therapeutic activity against tumours of the bladder, testis, ovary and against other solid tumours (Cojocel & Lock 1999). Administration of the platinum-containing coordinative

Faculty of Medicine, Department of Pharmacology and Toxicology, Kuwait University, Kuwait

C. Cojocel, M. S. Thomson

Correspondence: C. Cojocel, Faculty of Medicine, Kuwait University, P.O. Box 24923, Safat 13110, Kuwait. E-mail: cojocel@hsc.kuniv.edu.kw

Funding: Supported by Kuwait University, Research Grant No. MR 01/00.

complex cisplatin at therapeutic doses produces nephrotoxicity as a major side effect, with an incidence of 15–30% in man (Cojocel & Lock 1999). In rats, renal cortical accumulation of cisplatin leads to necrosis of the proximal tubule cells, late development of intrarenal cysts (Cojocel & Lock 1999) and decreased renal gluconeogenesis. In this study cisplatin was used as positive control. The aim of this in-vitro study was to investigate and compare the effects of cisplatin and 6-OHDA on renal *p*-aminohippurate transport, which is one of the pathways by which many drugs and chemicals enter the renal cells.

Materials and Methods

The kidney slice technique was used in a manner similar to that used previously for in-vitro studies of renal cell function, metabolism or toxicity (Cojocel 1990).

Test substances

Cisplatin (*cis*-platinum-II-diammine dichloride), 6-hydroxydopamine (6-OHDA) hydrochloride, lactic acid and other reagents were obtained from Sigma-Aldrich Chemie, GmbH (D-82039 Deisenhofen, Germany).

Experimental protocol

Renal cortical slices were prepared from kidneys of male (8–10 weeks old) naïve 200-g Sprague-Dawley rats. Animal studies were performed in accordance with Kuwait University Guidelines for the Care and Use of Laboratory Animals and rats were maintained on a standard diet with free access to water. Rats were killed and kidneys were removed immediately, decapsulated and placed in ice-cold 0.9% w/v NaCl. Renal cortical slices were prepared from the cortex of both kidneys and pooled together. To achieve a constant slice thickness (0.3 mm), a razor blade and a kidney holder device were used.

Renal cortical slices were pre-incubated for 60 min in a 6-OHDA-free medium or in a 6-OHDA (3.125, 6.25, 12.5, 25, 50 or 100 μM)-containing medium at 37 °C under a 100% O_2 atmosphere. Subsequently, *p*-aminohippurate accumulation by renal cortical slices was determined after a further 90-min incubation in *p*-aminohippurate-containing media.

Renal cortical slices were pre-incubated in a 50 μM 6-OHDA-containing medium for 15, 30, 45, 60, 90 or 120 min or in a 6-OHDA-free medium. Subsequently, *p*-aminohippurate accumulation in renal cortical slices was measured after a second 90-min incubation in *p*-aminohippurate-containing media. For each measured 6-OHDA concentration or incubation time, at least 5 rats were used. A sample of renal cortical slices (80–120 mg) from each rat was used to measure *p*-aminohippurate accumulation.

Renal cortical slices were pre-incubated for 60 min in a cisplatin-free medium or in a cisplatin (0.83, 1.67, 2.5, 3.33, 4.17 or 5.0 μM)-containing medium at 37 °C under a 100% O_2 atmosphere. Subsequently, *p*-aminohippurate accumulation in renal cortical slices was measured after a further 90-min incubation in *p*-aminohippurate-containing media.

Renal cortical slices were pre-incubated in a 3.33 μM cisplatin-containing medium for 15, 30, 45, 60, 90 or 120 min or in a cisplatin-free medium. Subsequently, *p*-aminohippurate accumulation in renal cortical slices was measured after a second 90-min incubation in *p*-aminohippurate-containing media. For each measured 6-OHDA concentration or incubation time, at least 5 rats were used. A sample of renal cortical slices (80–120 mg) from each rat was used to measure *p*-aminohippurate accumulation.

The incubation medium used for control slices was free of cisplatin and 6-OHDA in all series of experiments. The cisplatin- or 6-OHDA-containing medium and the corresponding control medium was composed of (mM): 96.7 NaCl, 7.4 sodium phosphate buffer, 40 KCl and 0.74 $CaCl_2$. Additionally, the incubation medium (pH 7.4) contained 10 mM lactic acid (Cross & Tagart 1950).

Determination of *p*-aminohippurate accumulation

To determine *p*-aminohippurate accumulation in the renal cortical slices, cisplatin- or 6-OHDA-incubated slices or control-incubated slices were transferred to 4 mL of the incubation medium containing 74 μM *p*-aminohippurate.

At the end of first incubation, slices were removed from the incubation media, blotted, transferred to 4 mL 6-OHDA- or cisplatin-free incubation medium containing *p*-aminohippurate and incubated for 90 min at 25 °C under 100% O_2 in a GFL metabolic shaker. After 90 min incubation, slices were removed, blotted, weighed and homogenized in 10 mL of 30% w/v trichloroacetic acid and centrifuged. Incubation medium (2 mL) was treated similarly. The *p*-aminohippurate concentration was measured in the supernatant fluid from slices and incubation medium as previously described (Smith et al 1945) using a Beckman DU 70 spectrophotometer.

The accumulation of *p*-aminohippurate in renal cortical slices was expressed as the slice-to-medium (S/M) concentration ratio. S represents the amount (mg) of *p*-aminohippurate per g of tissue, whereas M represents the amount (mg) of *p*-aminohippurate per mL of medium.

Statistics

Mean value, standard deviation, analysis of variance, the Student–Newman–Keuls test and the unpaired *t*-test were used for the statistical analysis of the data. The 0.05 level of probability was chosen as the criterion of significance.

Results

Effects of cisplatin on *p*-aminohippurate accumulation in rat renal cortical slices

Incubation of renal cortical slices in media containing various cisplatin concentrations decreased *p*-aminohippurate accumulation in renal cortical slices in a concentration-dependent manner (Figure 1). At a cisplatin concentration

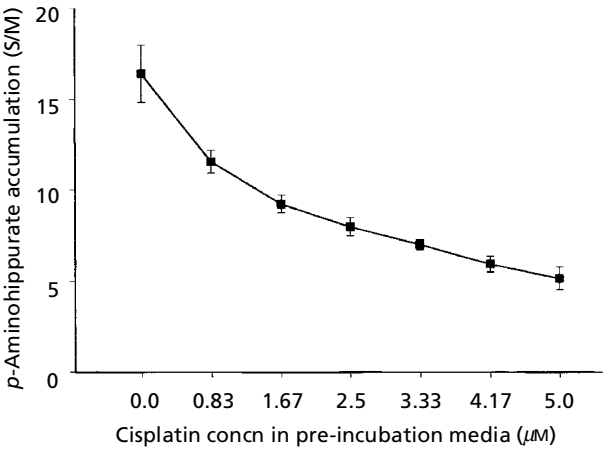

Figure 1 Concentration-dependent decrease of *p*-aminohippurate accumulation after pre-incubation of rat renal cortical slices at 37 °C for 1 h in media containing different cisplatin concentrations or in cisplatin-free medium (controls). *p*-Aminohippurate was measured after a subsequent 90-min incubation in *p*-aminohippurate-containing medium. Symbols represent means ± s.d. of measurements in slices from at least 5 different rats. The *p*-aminohippurate accumulation is expressed as the slice-to-medium (S/M) concentration ratio. When compared with controls, *p*-aminohippurate accumulation decreased significantly ($P < 0.05$) after incubation of renal cortical slices with cisplatin.

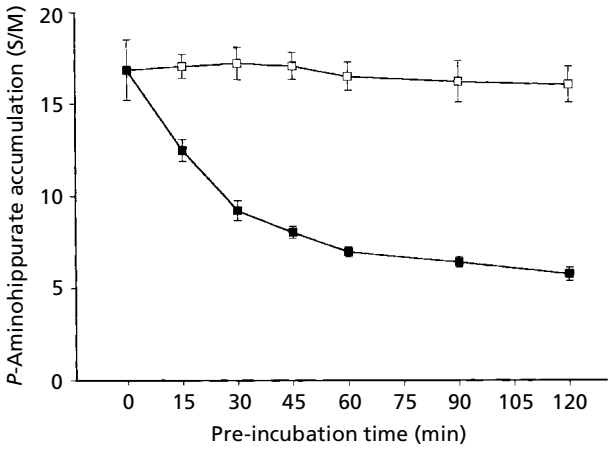

Figure 2 Time-dependent decrease of *p*-aminohippurate accumulation in rat renal cortical slices after pre-incubation of slices at 37 °C in a cisplatin-containing medium (3.33 μM, ■) or in cisplatin-free media (controls, □) for different periods of time. *p*-Aminohippurate accumulation was measured after a subsequent 90-min incubation in a *p*-aminohippurate-containing medium. Symbols represent means ± s.d. of measurements in slices from at least 5 different rats. The *p*-aminohippurate accumulation is expressed as the slice-to-medium (S/M) concentration ratio. When compared with controls, *p*-aminohippurate accumulation decreased significantly ($P < 0.05$) after incubation of renal cortical slices with cisplatin.

of 0.83 μM and higher, *p*-aminohippurate accumulation decreased significantly when compared with controls. At a cisplatin concentration of 3.33 μM, renal cortical *p*-aminohippurate accumulation was decreased by 56.17%, while at 5.0 μM cisplatin, *p*-aminohippurate accumulation was decreased by 68.50%.

Incubation of renal cortical slices for various periods of time in medium containing 3.33 μM cisplatin depressed *p*-aminohippurate accumulation in renal cortical slices in a time-dependent fashion (Figure 2). After incubation of renal cortical slices with cisplatin for 30 min, *p*-aminohippurate accumulation was decreased by 60.27% and after 90 min, *p*-aminohippurate accumulation was depressed by 80.83%.

Effects of 6-OHDA on *p*-aminohippurate accumulation in rat renal cortical slices

Incubation of renal cortical slices in incubation media containing various 6-OHDA concentrations decreased *p*-aminohippurate accumulation in renal cortical slices in a concentration-dependent manner (Figure 3). At a 6-OHDA concentration of 3.125 μM or higher, *p*-aminohippurate accumulation decreased significantly when compared with controls. At a 6-OHDA concentration of 3.125 μM, *p*-aminohippurate accumulation was decreased by 30.56% and at 50 μM 6-OHDA, *p*-aminohippurate accumulation was decreased by 75.69%.

Incubation of renal cortical slices for various periods of time in medium containing 50 μM 6-OHDA depressed *p*-aminohippurate accumulation in renal cortical slices in a time-dependent fashion (Figure 4). After incubation of renal cortical slices with 6-OHDA for 30 min, *p*-aminohippurate accumulation was decreased by 46.15% and after 90 min, *p*-aminohippurate accumulation was decreased by 79.49%.

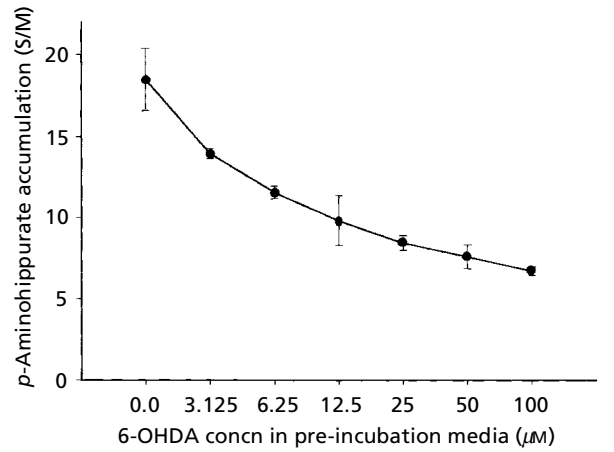

Figure 3 Concentration-dependent decrease of *p*-aminohippurate accumulation after pre-incubation of rat renal cortical slices at 37 °C for 1 h in media containing different 6-OHDA concentrations or in 6-OHDA-free media (controls). *p*-Aminohippurate accumulation was measured after a subsequent 90-min incubation in *p*-aminohippurate-containing media. Symbols represent means ± s.d. of measurements in slices from at least 5 different rats. The *p*-aminohippurate accumulation is expressed as the slice-to-medium (S/M) concentration ratio. When compared with controls, *p*-aminohippurate accumulation decreased significantly ($P < 0.05$) after incubation of renal cortical slices with 6-OHDA.

6-OHDA impaired renal cortical *p*-aminohippurate accumulation to a level comparable with the impairment produced by 10-fold lower concentrations of cisplatin (5.0 μM cisplatin vs 50 μM 6-OHDA).

Discussion

Most drugs and environmental toxicants are excreted into the urine, either in an unchanged form or as biotransformation products. Renal organic anion transporters are involved in the elimination of a large number of drugs (e.g., antibiotics, diuretics, cytostatics), drug metabolites (especially conjugation products with glutathione, glucuronide, glycine, sulfate, acetate) and toxicants and their metabolites (e.g., mycotoxins, herbicides, plasticizers, polyhaloalkenes, hydroquinones, aminophenols), many of which are nephrotoxic. The transport systems involved in organic anion secretion can be functionally subdivided into the well characterized sodium-dependent *p*-aminohippurate system and a recently discovered sodium-independent system (Masereeuw et al 1996). Both systems mediate two membrane translocation steps arranged in series: uptake from blood across basolateral membrane of proximal tubule cells followed by efflux into the urine across the apical cell membrane. As a result of an efficient uptake mechanism against the membrane potential, anionic drugs tend to accumulate within proximal tubule cells, sometimes leading to nephrotoxicity. The organic anion transport system (OAT1) mediates the uptake of organic anions across the basolateral membrane (Sekine et al 1997). *p*-Aminohippurate is an excellent model substrate for the OAT1 and its uptake across the basolateral membrane can be affected by various nephrotoxins (Ullrich 1997). In this study, the effect of cisplatin and 6-OHDA on *p*-aminohippurate accumulation in renal cortical slices was investigated.

Like other organs, the kidney requires iron for its metabolic processes. Ferritin is a ubiquitous protein that is responsible for sequestration, storage and detoxification of intracellular non-functioning iron (Richardson & Ponka 1997). 6-OHDA reacts with Fe^{3+} and, more rapidly, with $FeOH^{2+}$. This is of particular importance because tissue iron is stored in the protein ferritin. The ability of 6-OHDA to remove iron from ferritin and plasma transferrin (Borisenko et al 2000) may explain, in part, its brain and kidney cytotoxicity. In the kidney the filtered iron enters the tubular lysosomes across the brush-border membrane by endocytosis and may produce tubular damage by the generation of reactive free radicals (Nankivell et al 1994).

There is a growing body of evidence arising from animal studies and studies using cell cultures that 6-OHDA acts as a cytotoxic via two independent biochemical mechanisms of action: by forming reactive oxygen species and by being a potent inhibitor of mitochondrial respiratory chain complexes I and IV (Woodgate et al 1999). Melatonin, a highly effective endogenous free-radical scavenger (Reiter 1998), prevents 6-OHDA-induced apoptosis in naïve and neuronal PC12 cells (Mayo et al 1998). Further, 6-OHDA has been shown to induce a dose-dependent reduction in the total cardiac choline acetyltransferase and to cause cardiac malformations in an animal study (Ho et al 1999).

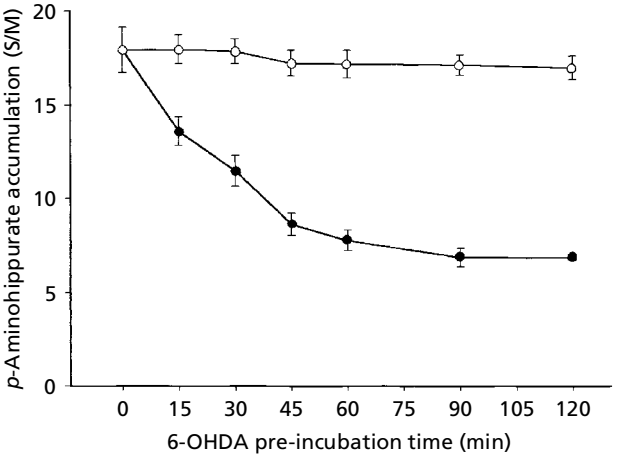

Figure 4 Time-dependent decrease of *p*-aminohippurate accumulation in rat renal cortical slices after pre-incubation of slices at 37 °C in a 6-OHDA-containing medium (50 μM, ●) or in 6-OHDA-free medium (controls, ○) for different periods of time. *p*-Aminohippurate accumulation was measured after a subsequent 90-min incubation in *p*-aminohippurate-containing medium. Symbols represent means ± s.d. s.d. of measurements in slices from at least 5 different rats. The *p*-aminohippurate accumulation is expressed as the slice-to-medium (S/M) concentration ratio. When compared with controls, *p*-aminohippurate accumulation decreased significantly ($P < 0.05$) after incubation of renal cortical slices with 6-OHDA.

The toxicity of endogenously formed 6-OHDA is a matter of concern in parkinsonian patients. The results of more recent studies indicate that 6-OHDA may cause apoptosis or apoptosis and necrosis after exposure of the PC12 neuronal cell line to 25 μM or 50 μM 6-OHDA, respectively (Ochu et al 1998). Similarly, in our study, incubation of renal cortical slices for 60 min in the presence of 25 μM and 50 μM 6-OHDA hydrochloride caused a significant decrease in *p*-aminohippurate accumulation in renal cortical slices (64.45% and 75.69%, respectively). Therefore, 6-OHDA-induced subcellular structural injuries such as cytoplasmic hydrolyzation and swelling of mitochondria in the proximal convoluted tubule and pars recta (Walther & Romen 1981) resulted, in our study, in impairment of *p*-aminohippurate transport into proximal tubule cells.

Levodopa (L-DOPA) is the cornerstone treatment of Parkinson's disease (Miyasaki et al 2002). It crosses the blood–brain barrier, and is then converted to dopamine, thus re-establishing dopaminergic effects. After dopamine biotransformation, the metabolically inert dopamine 3-*O*-sulfate is the predominant sulfate conjugate that is excreted in the urine of parkinsonian patients (Jenner & Rose 1974). Analysis by GC-MS showed that the excretion of 5- and 6-hydroxydopamine (5- and 6-OHDA) was significantly higher in the urine of L-DOPA treated parkinsonian patients than of those not treated with L-DOPA (Andrew et al 1993).

Miniature swine chronically treated with 6-OHDA showed effective renal sympathectomy and induced alterations in renal function when compared with controls

(Thomas & Zambrawski 1991). Abnormalities of renal function in rats was also observed after neonatal sympathectomy with 6-OHDA (Appenroth & Braunlich 1981). Further, treatment of rats for one week with 6-OHDA resulted in a distinct increase in urinary volume and sodium and potassium excretion, when compared with controls (Sato et al 1987). However, little is yet known concerning the effects of 6-OHDA on renal cell function. The results of this study, showing a concentration- and time-dependent decrease of *p*-aminohippurate accumulation in renal cortical slices by 6-OHDA, represent first-time evidence of 6-OHDA-induced cytotoxic effects in renal cortical cells. When the effects of 6-OHDA on *p*-aminohippurate transport are compared on a micromolar basis with those of cisplatin, it appears that 6-OHDA has comparable effects on renal *p*-aminohippurate accumulation, but at higher concentrations.

Free radical toxicity may be the underlying cause of deterioration of nigra cell and impairment of renal cortical cell function in patients with Parkinson's disease. Membrane lipids in nigra cells typically undergo free-radical-induced oxidative damage resulting in lipid peroxidation (Hirsch 1993; Jenner & Olanow 1996) or DNA base alterations (Ferger et al 2001). Nigral cells from parkinsonian patients also seem to lack adequate amounts of antioxidant substances such as glutathione (Hirsch 1993). The available data indicate that 6-OHDA-quinone(s) and generation of reactive oxygen and nitrogen species play a direct role in the pathophysiology of Parkinson's disease (Plumbo et al 1999; Jameson & Linert 2000).

The cytotoxic 6-OHDA is formed from the oxidation of dopamine, resulting in parkinsonian patients from the conversion of levodopa, in the presence of free iron(II) and hydrogen peroxide. Iron(II) is known to interact with hydrogen peroxide, via Fenton's reaction, producing hydroxyl radicals or ferryl species that readily hydroxylate dopamine to form the nontoxic 5-OHDA and the cytotoxic 6-OHDA (Kinzel et al 1999). The 6-OHDA thus formed is, in turn, able to reduce and possibly release iron, as iron(II), from the iron-storage protein ferritin (Linert & Jameson 2000). This continuous cycle forms strongly oxidizing radicals that damage renal cells and impair the function of components such as the renal cell membrane and DNA. Plasma transferrin appears to be the major source of iron in the kidney (Apodaca et al 1994). After internalization of the diferric transferrin via receptor-mediated endocytosis into an endosome, iron is released from transferrin and is then transported through the membrane by the Nramp2 transporter (Canonne-Hergaux et al 1999). Subsequently, iron enters the intracellular iron pool and apotransferrin remains bound to the transferrin receptor and is then released by exocytosis (Richardson & Ponka 1997). Iron that entered the renal cells can be stored in ferritin or can be used for various metabolic functions such as hydroxylation of dopamine to form the cytotoxic 6-OHDA.

Drugs and chemicals such cefaloridine, 2-amino-5-chlorophenol, meropenem and imipenem induce formation of reactive oxygen species and reduce *p*-aminohippurate accumulation in the renal cortical cells (Cojocel 1990; Valentovic et al 1999; Yousif et al 1999). However, the biochemical mechanism of action by which 6-OHDA decreases renal cortical *p*-aminohippurate accumulation is not yet known.

Conclusion

It is conceivable that 6-OHDA, which is formed following treatment of parkinsonian patients with levodopa, is filtered by the glomerulus, reabsorbed or secreted in the proximal tubule cells and a certain amount is excreted in the urine (Andrew et al 1993). After entering the renal tubule cells, 6-OHDA could release iron(II) from ferritin and undergo a continuous autocatalytic cycle, similar to that occurring in the brain, resulting in production of reactive oxygen and nitrogen species causing cytotoxic effects resulting in decreased *p*-aminohippurate accumulation. Formation of intracellular free radicals, such as the hydroxyl radical, analysed by ESR spin trapping technique (Oldfield 1991), could cause dysfunction of renal cell-membrane lipids, nucleic acids and certain enzymes, thus leading to cytotoxicity and possible cell death. The identification by mass spectrometry of 5-OHDA and 6-OHDA in the urine of parkinsonian patients undergoing L-DOPA therapy (Andrew et al 1993) may indicate the presence of such oxidative stress reactions in the kidney leading to cell membrane functional injuries. Further in-vitro and in-vivo studies may provide deeper insight into the biochemical or molecular mechanism(s) of 6-OHDA cytotoxicity.

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