BioMetals 1996, 9, 139–142

Evidence for the involvement of nitric oxide in cisplatin-induced toxicity in rats

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Received 1 April 1995; received in revised form 25 September 1995; accepted for publication 26 September 1995

Cisplatin treatment of rats results into a significant increase in the activity of Ca^{2+}-independent nitric oxide synthase (NOS) in kidneys and liver. Significant enhancement of lipid peroxidation in gastric mucosa, kidneys and liver was also observed. The administration of N^G-nitro-L-arginine methyl ester, an inhibitor of NOS, markedly reduced renal and gastrointestinal toxicity, and also decreased the content of blood urea nitrogen, serum creatinine, and incidence of diarrhoea along with a significant inhibition in lipid peroxidation in the target organs. The present report, while demonstrating the beneficial effect of the blockade of NO pathways during cisplatin chemotherapy, may be helpful in developing strategies for combating some of the toxic side-effects of the drug.

Keywords: nitric oxide, cisplatin, NOS, renal toxicity

Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II)), the drug of choice for the treatment of tumors, selectively and persistently inhibits the synthesis of DNA and RNA (Srivastava *et al.* 1978, Prestayko *et al.* 1980, Loehrer *et al.* 1984, Howell 1991, Pil & Lippard 1992, Abrams & Murrer 1993). However the adverse side-effects and toxicity to organs, such as kidneys, gastrointestinal tract, bone marrow, etc., limits the clinical usefulness of this drug (Von Hoff *et al.* 1979, Naganuma *et al.* 1987, Vermeulen *et al.* 1993, Fisher *et al.* 1994, Ravi *et al.* 1995). The mechanism by which cisplatin exerts its cytotoxic response and organ toxicity is not fully understood. A growing body of evidence suggests that nitric oxide ($NO^{\bullet}$), a free radical and a prominent vascular and neuronal messenger molecule responsible for endothelium-derived relaxing factor activity, may play a crucial role towards the development and/or exacerbation of various pathological conditions due to its altered metabolism (Trifiletti 1992, Francis *et al.* 1993, Mascolo *et al.* 1993, Middleton *et al.* 1993, Kam & Govender 1994, Weinberg *et al.* 1994). NO is produced as a result of the metabolism of L-arginine, by the action of the enzyme nitric oxide synthase (NOS) in almost all the cell types (Stuehr 1992). The most striking feature of NO is its reactivity with superoxide anion [$O_2^{\bullet-}$] to form peroxynitrite anion ($ONOO^-$), a powerful oxidant which is freely diffusible and can induce tissue damage as a result of direct action on enzymes and macromolecules (Ignarro 1990, Beckman *et al.* 1990, Wink *et al.* 1991, Lepoivre *et al.* 1992).

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^- + H^+ \rightarrow {}^{\bullet}OH + NO_2 \qquad (1)$$

Under the physiological pH, $ONOO^-$ rapidly dissociates to produce a hydroxyl radical ($^{\bullet}OH$), a molecule with high potency for cell and tissue injury and destruction. This has led to intensive investigations aimed at reducing the extent of oxidative insult involving NO and other reactive oxygen species. Demonstration of the protective effects of *N*-monomethyl-L-arginine (L-NAMA) and *N*-nitro-L-arginine methyl ester (L-NAME) as inhibitors of NOS in a variety of experimental models provided the first hand indication for the involvement of NO in tissue injury and other diseases (Trifiletti 1992, Francis *et al.* 1993, Mascolo *et al.* 1993, Middleton *et al.* 1993, Weinberg *et al.* 1994).

These observations, coupled with the fact that cisplatin exhibits an oxidative response and complications similar to NO, prompted us to investigate the possible involvement of NO in cisplatin-induced toxic responses. The present investigation shows that NO production may be one of the contributing factors in cisplatin-induced toxic responses. These observations may be useful in developing strategies

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for combating some of the toxic side-effects produced by cisplatin during chemoprevention.

Materials and methods

Chemicals

Dithiothreitol, HEPES, L-NAME and cisplatin were obtained from Sigma (St Louis, MO). L-[^{14}C]arginine (300 mCi mmol^{-1}) and L-arginine hydrochloride were purchased from ICN Biomedicals (Aurora, OH), and Dowex 50 W (200–400 mesh, 8% cross-linked, Na^+ form) from BioRad (Richmond, CA). All other chemicals used were of the highest purity grade available commercially.

Animals

The experimental animals were 8-week-old male albino rats from the Industrial Toxicology Research Centre Breeding Colony, weighing 175 ± 10 g. They were housed in an air-conditioned room and had free access to pellet diet (Lipton India Ltd., Bombay, India) and water. To determine whether the elevated levels of NOS were contributing factors for renal and gastrointestinal toxicity, and oxidative response, male rats were pretreated with a single dose of L-NAME followed by a single injection of cisplatin. Control rats received only cisplatin treatment. The experimental design, dose and treatment schedule of cisplatin and L-NAME are shown in Table 1. After the termination of the experiment, rats were killed by exsanguination. Blood was collected from the abdominal aorta followed by perfusion with cold normal saline until kidneys and liver were free of excess blood. The stomach was removed, washed thoroughly with cold normal saline and cut along the greater curvature as a source for gastric mucosa.

Lipid peroxidation was measured in whole homogenate of liver, kidneys and gastric mucosa in terms of thiobarbituric acid reacting species (TBARS) as described earlier (Srivastava *et al.* 1993). The results are expressed as nmol malondialdehyde formed mg^{-1} protein. The activity of NOS (Ca^{2+}-dependent and Ca^{2+}-independent) in the cytosol fraction of kidneys and liver was quantified by measuring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline in the presence of saturating concentrations of the enzyme's cofactors as essentially described earlier (Salter *et al.* 1991). The activity of the Ca^{2+}-dependent NOS was determined from the difference between L-[^{14}C]citrulline produced from control samples and samples containing 1 mM EGTA. The activity of Ca^{2+}-independent enzyme was determined from the difference between samples containing 1 mM EGTA and samples containing 1 mM EGTA and 1 mM L-NAMA (an inhibitor of NOS). Blood urea nitrogen (BUN) and serum creatinine were determined using standard kits obtained from Sigma. The analysis of significance of difference between the groups was performed by means of Student's *t*-test (Fisher 1954).

Table 1. Experimental design and treatment schedule

Group	Treatments[a]	
	cisplatin (μmol kg^{-1})	L-NAME (μmol kg^{-1})
1	—	—
2	—	50
3	35	
4	35	10
5	35	25
6	35	50
7	35	75

[a] Twelve rats were maintained in each group. L-NAME was administered i.p. 60 min before a single s.c. injection of cisplatin. Animals were observed for 5 days after treatments and subsequently killed for biochemical assay. Preliminary studies have shown that cisplatin treatment alone caused 50% mortality in rats at day 5; therefore this time interval was selected for various studies. The animals were deprived of food 18 h before killing.

Results

The results shown in Table 2 reveal that administration of cisplatin to rats caused significant enhancement of lipid peroxidation in gastric mucosa (262%), kidneys (185%) and liver (134%) compared with saline treatment. Cisplatin also produced severe toxic side-effects, i.e. an increased incidence of diarrohea, and a significant increase in the levels of BUN and serum creatinine (Table 3). The most striking feature of cisplatin administration was the significant production of NO in kidneys and to a lesser extent in liver as evidenced by the enhanced activity of Ca^{2+}-independent NOS (Table 4). The Ca^{2+}-dependent NOS activity was below the detectable level in both the groups and therefore not recorded in the table. The activity of NOS could not be measured in gastric mucosa due to the paucity of material. Administration of L-NAME profoundly reduced renal toxicity as shown by the lowering in BUN and serum creatinine and the gastrointestinal toxicity as shown by a decrease in the incidence of diarrhoea caused by cisplatin. L-NAME also enhanced the average intake of food in cisplatin-treated rats. Treatment with this NOS inhibitor effectively reduced lipid peroxidation in gastric mucosa (49%), kidneys (35%) and liver (22%) in a dose-dependent manner (Table 2). A significant decrease in the production of NO in kidneys and liver after L-NAME treatment (Table 4) clearly demonstrate its role in the cisplatin-induced oxidative response

Discussion

Mounting experimental evidence indicates the implication of metal ions in the generation of reactive oxygen-derived radicals including NO in the development of various pathological conditions culminating in tissue injury destruction and multiorgan failure (Beckman *et al.* 1990, Athar *et al.* 1987, Srivastava *et al.* 1993, Vermeulen *et al.* 1993). NO pathways indicate a general regulatory function, which, if altered, could contribute to the genesis of a wide variety of

Table 2. Effect of L-NAME on cisplatin-induced lipid peroxidation

Treatments		Lipid peroxidation (nmole MDA formed mg^{-1} protein)		
cisplatin (μmol kg^{-1})	L-NAME (μmol kg^{-1})	kidneys	liver	Gastric mucosa
—	—	2.01 ± 0.11	3.27 ± 0.14	0.53 ± 0.05
—	50	1.95 ± 0.09	3.19 ± 0.11	0.47 ± 0.04
35	—	3.71 ± 0.13^{a}	4.39 ± 0.12^{a}	1.39 ± 0.09^{a}
35	10	3.47 ± 0.11^{a}	4.11 ± 0.10^{a}	1.11 ± 0.10^{a}
35	25	3.03 ± 0.12^{ab}	3.81 ± 0.12^{ab}	0.94 ± 0.08^{ab}
35	50	2.43 ± 0.08^{ab}	3.49 ± 0.13^{b}	0.77 ± 0.07^{ab}
35	75	2.39 ± 0.10^{b}	3.45 ± 0.09^{b}	0.71 ± 0.05^{ab}

Each value represents the mean ± SE for five to eight animals.
[a] $P < 0.05$ compared with the normal control.
[b] $P < 0.05$ compared with the cisplatin-treated group.

Table 3. Effect of L-NAME on cisplatin-induced toxicity in rats

Treatments		Food intake (g day^{-1})	Diarrohea (%)	BUN (mg dl^{-1})	Creatinine (mg dl^{-1})
cisplatin (μmol kg^{-1})	L-NAME (μmol kg^{-1})				
—	—	35 ± 3	0	17.9 ± 0.6	1.4 ± 0.1
—	50	33 ± 2	0	16.3 ± 0.3	1.3 ± 0.1
35	—	26 ± 3^{a}	100	160.9 ± 7.8^{a}	3.9 ± 0.2^{a}
35	10	26 ± 4^{a}	80	140.1 ± 8.1^{a}	3.9 ± 0.3^{a}
35	25	27.4 ± 4^{a}	50	121.4 ± 7.9^{ab}	3.3 ± 0.2^{a}
35	50	29 ± 0.3	20	87.7 ± 4.5^{ab}	2.6 ± 0.3^{ab}
35	75	30 ± 4^{b}	10	79.7 ± 3.7^{ab}	2.4 ± 0.1^{ab}

Each value represents the mean ± SE of five to eight animals.
[a] $P < 0.05$ compared with the normal control.
[b] $P < 0.05$ compared with the cisplatin-treated group.

Table 4. Effect of L-NAME pretreatment on the activity of cisplatin-induced Ca^{2+}-independent NOS

Treatments	NOS activity (nmol min^{-1} g^{-1} wet tissue)	
	kidneys	liver
No treatment	ND	ND
Cisplatin (35 μmol kg^{-1})	0.73 ± 0.05	0.19 ± 0.03
L-NAME (50 μmol kg^{-1})	ND	ND
L-NAME + cisplatin	0.19 ± 0.02^{a}	0.08 ± 0.02^{a}

Each value represents mean ± SE of five to eight animals. The treatment schedule was the same as described in Table 1.
ND, not detected.
[a] $P < 0.05$ compared with cisplatin treatment.

diseases (Trifiletti *et al.* 1992, Francis *et al.* 1993, Mascolo *et al.* 1993, Middleton *et al.* 1993). Lipid peroxidation is a sensitive marker of injury to cellular membranes involving oxygen-derived free radicals. The present observations on the significant enhancement of lipid peroxidation in the target tissues, i.e. gastric mucosa, kidneys and liver, together with the enhanced production of NO clearly demonstrate the role of NO, and possibly of superoxide radical (O_2^-) as well, in the development of cisplatin-induced toxic responses. The effect of L-NAME pretreatment in reducing the incidence of diarrhoea and renal toxicity in addition provides the evidence for the involvement of NO in the oxidative insult. These findings are consistent with the earlier report where NO has been implicated in the increased incidence of diarrhoea and its prevention by L-NAME pretreatment (Mascolo *et al.* 1993). We have hypothesized that NO production may be responsible for the development of complications associated with exposure of cisplatin to rats. The cisplatin treatment, probably, results in the generation of NO in the gut as evident from the increased lipid peroxidation and the anti-diarrhoeal effect of L-NAME. The interplay of NO in the cisplatin-induced renal toxicity is also shown by the significant protection afforded by L-NAME treatment. These results are in close agreement with earlier reported observations that blocking the production of NO by NOS inhibitors either prevented or reduced the toxic response of a chemical (Trifiletti 1992, Mascolo *et al.* 1993, Weinberg *et al.* 1994). The mechanism by which NO promotes renal toxicity, however, remains to be elucidated. Our findings, nonetheless, signify the beneficial

effect of NO pathway blockade and suggest the usefulness of these observations in developing strategies for combating some of the toxic side-effects of cisplatin during chemotherapy of tumors.

Acknowledgement

The technical assistance of Mr Ramesh Chandra is gratefully acknowledged.

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