Renal Metallothionein and Platinum Levels in Diabetic and Nondiabetic Rats Injected with Cisplatin (43257)

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Abstract. **This study was designed to investigate the relationship between the attenuation of cisplatin-induced nephrotoxicity in experimental diabetes and the increased level of renal metallothionein (MT) reported to occur in this condition. Two groups of male Sprague-Dawley rats were used: 42-day streptozotocin diabetics and age-matched nondiabetics. Half of each group was injected with a nephrotoxic dose of cisplatin (5 mg/kg, ip) and half with vehicle. Four hours after injection, renal MT and platinum (Pt) content were quantified. Mean renal MT concentration in vehicle-injected diabetics was about triple that found in nondiabetics. Comparison of renal MT concentrations in cisplatin-injected diabetics and nondiabetics with their vehicle-injected counterparts suggested an inducing effect of the drug. In contrast to the marked elevations of MT in diabetic kidney, mean renal Pt concentration in the cisplatin-injected diabetic group was only about one-fourth that of the nondiabetic group. No difference was evident in the intracellular distribution Pt between cytosolic and particulate fractions from diabetic and nondiabetic kidneys. It was concluded that: (i) Sequestration of Pt by MT cannot account for the resistance of diabetic kidney to cisplatin toxicity. (ii) Rather, the resistance is due to a significant decrease in renal uptake/retention of cisplatin or derivatives during the critical first few hours after injection.** [P.S.E.B.M. 1991, Vol 197]

Cisplatin (*cis*-diamminedichloroplatinum (II)) is a potent but nephrotoxic anti-neoplastic agent that has become a first-line drug for the treatment of a number of solid tumors. Although cisplatin and/or its aquated derivatives are capable of interacting with a variety of biological nucleophiles (e.g., sulfur and nitrogen) and of promoting lipid peroxidation via free radical generation, the initiating toxic event in renal cells remains undefined. Scott *et al.* (1) have reported that the kidneys of streptozotocin (STZ) diabetic rats are resistant to the toxic action of cisplatin. Although the mechanism of this protective effect of STZ-induced diabetes is unknown, it is clearly separable from the osmotic diuresis (2, 3) that accompanies experimental diabetes. One possible mechanism is suggested by the demonstration of significant elevation of renal metallothionein (MT) in STZ diabetic rats (4). Because of its high cysteine content, this cytosolic protein readily binds many metals and it probably plays a role not only in the normal disposition of essential zinc and copper, but also in the biological transport and intracellular sequestration of toxic heavy metals such as cadmium (5, 6). Given the above observations, it seems reasonable to propose that intracellular binding/sequestration of platinum to renal MT can account for the mitigation of toxicity in the diabetic rats. Indeed, Naganuma *et al.* (7) have shown that pretreatment of mice with MT-inducing metals reduces lethality of high-dose cisplatin. The experiments in the present study were designed to test the hypothesis that the protection from the nephrotoxic action of cisplatin seen in STZ-diabetic rats is related to elevated levels of renal MT.

Materials and Methods

Induction of Diabetes. The experiments were carried out using 30 male Sprague-Dawley rats of about 300 g initial body weight. All were received from the supplier on the same day. On the first day of the experiment, 15 of the rats were rendered diabetic by

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slow injection of STZ (Sigma Chemical Co., St. Louis, MO) into a tail vein at a dose of 65 mg/kg body wt. The STZ for each rat was dissolved immediately prior to injection in citrate buffer (pH 4.5) to yield a final concentration of 32.5 mg/ml. The volume injected was 0.2 ml/100 g body wt. The other 15 rats received an injection of the buffer alone. All rats were maintained in environmentally controlled quarters with a light:dark cycle of 12 hr for 42 days without further intervention other than weekly weighing and testing for urine glucose (Tes-Tape, Eli Lilly, Indianapolis, IN). They were given free access to standard laboratory chow and water throughout the period of the study. One of the STZ-injected rats died on Day 8 for unknown reasons so that the results from 29 animals are presented.

Experimental Design and Dosing. The 29 surviving rats were divided into two sets. Set A was made up of six rats and served as a group to test the functional nephrotoxicity of cisplatin under our conditions and its attenuation in the diabetic rats. Set B included the remaining 23 rats and served as the principal group for the testing of the central hypothesis.

For the six rats of Set A, three diabetic and three nondiabetic, an initial blood sample was drawn from a tail vein on Day 42 and each rat was injected with cisplatin as described below for Set B. Ninety-six hours after cisplatin administration, the rats were sacrificed and a blood sample was taken by heart puncture.

On Day 42, Set B rats received, at staggered intervals, an intraperitoneal injection of either 5 mg/kg cisplatin (Alfa Products, Ward Hill, MA) in 0.9% saline (final volume injected = 0.3 ml/100 g body wt) or saline alone, according to the following design: Group 1 Diabetic + saline ($n = 5$), Group 2 Diabetic + cisplatin ($n = 6$), Group 3 Nondiabetic + saline ($n = 6$), Group 4 Nondiabetic + cisplatin ($n = 6$). Exactly 4 hr after each cisplatin injection, the rat was sacrificed with CO_2 in a closed chamber, a blood sample was taken by heart puncture and both kidneys were rapidly removed and placed in ice-cold saline.

Measurement of Plasma Glucose and Blood Urea Nitrogen (BUN). Plasma was assayed for glucose content by the glucose oxidase method with the aid of a Beckman glucose analyzer. BUN concentration was determined colorimetrically by the urease method using a commercially available kit (Sigma).

Renal Cortex Platinum (Pt) and MT Measurements. Both kidneys from each rat in Set B were stripped of their capsules and hemisected longitudinally. A thin (0.3- to 0.5-mm) slice was cut from the cortical surface of each kidney using a Stadie-Riggs microtome and the two slices were pooled. After determining their combined wet weight (about 150 mg), the slices were oven-dried to constant weight at 70°C and reweighed (dry weight). A nitric acid digest of the dried tissue from rats injected with cisplatin (i.e., Groups 2 and 4) was analyzed for platinum content with a Perkin-Elmer model 380 atomic absorption spectrometer fitted with a model HGA 400 programmable graphite furnace and deuterium background correction. Pyrolytically coated graphite tubes were used. Absorbance at an atomization temperature of 2650°C was monitored at 265.9 nm. A standard curve was generated using dilutions of a platinum standard solution (1000 μg/ml) purchased from Sigma. Readings from digests of renal cortex from rats in Groups 1 and 3 (no cisplatin) served as tissue blanks for the diabetic and nondiabetic groups, respectively. These results are reported as μg of Pt/g of cortex in terms of both wet and dry weight. In addition to the slices, approximately 1 g of cortex from each rat, obtained in approximately equal amounts from the two kidneys, was homogenized in 4 vol of ice-cold Tris-HCl buffer (10 m*M*, pH 8.0) using a Polytron homogenizer. The homogenate was spun in a Sorvall Model RC-5B centrifuge at 18,000*g* for 20 min at 4°C (8). The supernatant, representing the cytosolic fraction, was analyzed for MT content using the cadmium saturation assay described by Onosaka and Cherian (8), keeping in mind the precautions noted by Eaton and Toal (9). Values are reported as μg/g wet wt of cortex and reflect the assumption of 7-g atoms of cadmium/mol MT. The cytosolic fractions from the homogenates of rats injected with cisplatin were also analyzed for Pt content by atomic absorption spectroscopy. These results are reported as μg Pt/g wet wt.

Statistical Analysis. Data were analyzed for statistical significance using Student's *t* test and a combination of analysis of variance and the Newman-Keuls test.

Results

Renal Toxicity of Cisplatin. Elevation in blood urea nitrogen concentration within 3–5 days of injection is a commonly used indicator of cisplatin nephrotoxicity (1, 10, 11). Table I presents a comparison of the mean BUN concentrations in the three diabetic and three nondiabetic rats in Set A immediately before and 4 days after cisplatin injection. BUN level before cisplatin injection was significantly higher in the diabetics than in the nondiabetics, indicating decline in glomerular function in long-term uncontrolled STZ diabetes.

Table I. BUN Concentrations in Diabetic and Nondiabetic Rats Injected with Cisplatin

Time	BUN concentration[a] (mg/100 ml)	
	Diabetic	Nondiabetic
Before cisplatin	31.2 ± 0.8	13.5 ± 2.4
96 hr after cisplatin	40.4 ± 2.9	106.6 ± 10.2
P[b]	<0.05	<0.01

[a] Each value is the mean ± SD from three rats.
[b] Statistical significance was assessed by two-tailed *t*-test.

Reyes *et al.* (12) have recently reported very similar BUN values for long-term STZ diabetic Sprague-Dawley rats. Four days after cisplatin administration, mean BUN values were significantly elevated in both the diabetic and the nondiabetic groups. However, a difference of magnitude was evident. While the BUN concentration increased by a factor of 7.9 in the nondiabetics after cisplatin injection, it increased by only 1.3 times in the diabetics.

Assessment of Diabetes. Table II summarizes pertinent physiologic parameters of the two groups of Set B rats injected with cisplatin (diabetic, Group 2 and nondiabetic, Group 4). The values reported are from urine samples, blood samples, and body weight measurements taken on Day 42 immediately before injection of cisplatin. Parameters from the vehicle-injected animals are those expected in normal rats (13). None exhibited glucosuria or elevated plasma glucose concentration and all showed a weight gain. In contrast, all rats in the STZ-injected group were severely diabetic, as indicated by marked glucosuria, plasma glucose concentration in excess of 500 mg/100 ml, and loss of approximately 30% of initial body weight over the 6-week period of the experiment. The BUN concentration in the diabetic animals was about 1.7 times that of the nondiabetic rats, indicating some decline in glomerular function after 6 weeks of uncontrolled diabetes. Because compromised kidneys can swell, tissue water content must be considered if constituent (i.e., Pt and MT) concentrations in control and diabetic rats are to be compared on a tissue wet weight basis. Dry weight to wet weight ratios were calculated for kidney slices taken from diabetic and nondiabetic rats injected with cisplatin (i.e., Groups 2 and 4, as described in Materials and Methods). The slight difference was statistically insignificant.

Renal Cortical Pt Content. Table III presents data comparing the mean concentrations of Pt in the renal cortex of diabetic and nondiabetic rats 4 hr after injection of cisplatin. The mean Pt concentration in a nitric acid digest of cortex slices from diabetic rats was only 25.5% of that measured in slices from the nondiabetic rats. This demonstrates a significantly lower level of uptake and/or retention of cisplatin or derivatives by the diabetic kidney. Also presented are the mean Pt concentrations measured in a cytosolic fraction of cortex homogenates from the two groups. Despite the markedly different renal platinum concentrations in the diabetic and nondiabetic rats, the mean cytosolic concentration of Pt accounted for about 50% of the total in both groups, demonstrating no significant difference in the intracellular distribution of Pt between cytosolic and particulate fractions of the cortex.

Renal MT Content. The mean cortical MT concentrations of the four treatment groups of Set B rats are given in Table IV. Also given are the levels of statistical significance for the three relevant comparisons among the groups. MT levels in the nondiabetic, saline-injected rats (Group 3) agree well with those reported by others for Sprague-Dawley rats (14, 15). The significant stimulatory effect of uncontrolled STZ-induced diabetes on

Table II. Comparison of Physiologic Parameters in Diabetic and Nondiabetic Rats

Parameter[a]	Diabetic[b]	Nondiabetic[c]	P^d
Glucosuria (Tes-Tape)	Positive	Negative	
Plasma glucose (mg/100 ml)	569 ± 41	128 ± 17	<0.01
BUN (mg/100 ml)	40.3 ± 10.5	23.9 ± 3.7	<0.01
Body weights (g)			
Day 1	304 ± 12	294 ± 14	
Day 42	228 ± 25	365 ± 28	
Renal cortex water content (dry wt/wet wt)	0.202 ± 0.017	0.207 ± 0.023	>0.05

[a] All numerical values are the mean ± SD from six rats on Day 42.
[b] Group 2 rats injected intravenously with 65 mg/kg STZ on Day 1.
[c] Group 4 rats injected intravenously with saline vehicle on Day 1.
[d] The statistical significance ($P < 0.05$) between diabetic and nondiabetic rats was assessed with a two-tailed t test.

Table III. Concentration and Distribution of Pt in Renal Cortex of Diabetic and Nondiabetic Rats Injected with Cisplatin

	Pt[a] (μg/g wet wt)		
	Whole slice	Cytosol fraction	Cytosol/ whole
Diabetic[b]	2.47 ± 1.53	1.38 ± 0.94	0.51 ± 0.18
Nondiabetic[c]	9.67 ± 1.15	4.65 ± 0.79	0.48 ± 0.09
P^d	<0.01	<0.01	>0.05

[a] Each value is the mean ± SD from six rats determined on Day 42 4 hr after administration of cisplatin.
[b] Group 2 rats injected intravenously with 65 mg/kg STZ on Day 1.
[c] Group 4 rats injected intravenously with saline vehicle on Day 1.
[d] Statistical significance was assessed with a two-tailed t test.

Table IV. Renal Cortical Metallothionein Concentrations in Diabetic and Nondiabetic Rats

Treatment	MT[a] (μg/g wet wt)		P^b
	Diabetic	Nondiabetic	
	Group 1	Group 3	
Saline	59.4 ± 11.1	21.4 ± 2.5	<0.01 (1 vs 3)
	Group 2	Group 4	
Cisplatin	84.8 ± 26.4	34.8 ± 9.7	
P^b	0.15 (1 vs 2)	0.01 (3 vs 4)	

[a] Each value is the mean ± SD from either five (Group 1) or six (Groups 2, 3, and 4) rats.
[b] Statistical significance ($P < 0.05$) was assessed by two-way analysis of variance.

renal MT content previously reported by Chen and Failla (4) was clearly evident in this study. Among the saline-injected animals, the renal MT concentration in the diabetic rats (Group 1) exceeded that observed in the nondiabetics (Group 3) by a significant factor of about three. Cisplatin injection caused an increase in the mean renal MT concentrations in both diabetics (Group 3) and nondiabetics (Group 2). However, the increase over that seen in saline-injected rats was statistically significant for the nondiabetic rats (Group 3 versus Group 4 comparison), but not so for the diabetics (Group 1 versus Group 2 comparison). Variability in renal MT concentrations among individual rats was greater in the cisplatin-injected groups, as indicated by wider ranges (data not shown) and greater standard deviations (as percentage of mean).

Discussion

Although maximal functional impairment of the rat kidney occurs within 3–5 days of cisplatin dosing (16), studies of the kinetics of cisplatin biodistribution (17, 18) and of the effects of metal-binding agents (19) indicate that the initial toxic event occurs within 4 hr. Accordingly, we have quantified and compared the renal concentrations of MT and Pt in diabetic and nondiabetic rats 4 hr after injection of a nephrotoxic dose of cisplatin. The results of this study lead to the following two major conclusions: (i) sequestration of Pt by MT cannot account for the resistance of the diabetic rat kidney to cisplatin toxicity; and (ii) the protection seems rather to be associated with reduced uptake and/or retention of Pt complexes by the kidneys of diabetic rats in the critical first few hours after cisplatin injection.

The data from Set A rats presented in Table I serves to demonstrate the differences in the nephrotoxic action of cisplatin between the diabetic and nondiabetic groups of rats. The attenuation of the rise in BUN level 4 days after cisplatin injection is clear, indicating that the kidneys of the uncontrolled STZ-induced diabetic rats were resistant to cisplatin toxicity. Besides confirming the results of Scott *et al.* (1), these data suggest that the significant difference in renal platinum concentrations in the diabetic and nondiabetic rats is associated with reduced nephrotoxicity.

The approximate tripling of renal MT concentration in the 42-day diabetic rats who received no cisplatin over their nondiabetic counterparts (Table IV, Groups 1 and 3) is notable and extends the results of the shorter-term study of Chen and Failla (4), which documented a doubling after 14 days of STZ. The mechanism of the induction of MT in experimental diabetes is undefined, but may be related to changes in trace metal distribution (4). Clearly, a number of metals such as bismuth and zinc can induce synthesis of renal MT, but the ability of Pt to do so is uncertain. In an examination of the cellular binding of Pt after cisplatin administration in rats, Mason *et al.* (20) found no evidence for the existence of an inducible Pt-binding protein in the kidney. Zelazowski *et al.* (21) administered multiple injections of cisplatin and its inactive isomer transplatin to rats and found that while Pt-MT complexes were present in liver and kidney cytosol, neither Pt derivative induced MT content in these tissues. In contrast, Farnworth *et al.* (22) and Reeves and Saari (14) found (in mice and zinc-fed rats, respectively) significant elevations in renal MT content following cisplatin administration. In the current study, the mean renal MT concentration in the nondiabetic rats injected with cisplatin was a statistically significant 63% higher than that in the corresponding saline-injected animals, indicating that renal MT can be induced within 4 hr of administration by cisplatin or by an aquated derivative, as suggested by Farnworth *et al.* (22).

A similarly rapid induction of renal MT by cadmium has been reported by Shaikh and Smith (23). A trend toward induction was also evident in the diabetic rats. In these animals, mean renal MT concentration was about 40% higher than that measured in the kidneys of saline-injected diabetics, but the difference was not statistically significant. Two factors which probably, at least in part, account for the lesser induction in the diabetics are the level of renal MT in these rats prior to cisplatin injection and the significantly lower Pt concentration (Table IV).

Indeed, the observation that the mean renal cortical slice Pt concentration in the diabetic rats was only 25% of that measured in the nondiabetic rats, despite a tripling of renal metallothionein concentration, strongly argues against a Pt-sequestration mechanism in the resistance of diabetic kidney to cisplatin toxicity. This conclusion parallels that of Suzuki and Cherian (15), who have recently examined the role of intracellular sulfhydryl compounds in the interaction of cisplatin with the kidney. Their results demonstrate little binding of Pt to renal MT 24 hr after dosing, even in rats whose MT levels were induced by prior $ZnSO_4$ injection. In contrast to our results, however, these workers saw no induction of MT after cisplatin administration. It should be noted that our results do not necessarily preclude a contribution of MT to the protection by, for example, a free radical-scavenging mechanism (24). But they do clearly suggest that reduced levels of Pt complexes in the kidney are primarily responsible for the protection phenomenon. The fact that the distribution of Pt between the cytosolic and particulate cell fractions did not differ in diabetic versus nondiabetic rats suggests a general, rather than selective, depression of platinum uptake or retention.

While the results of the current study do not allow a conclusion about the mechanism of the reduction of

the Pt concentration in the diabetic kidney, the fact that the cytosol to whole slice concentration ratio was virtually the same in the diabetic and nondiabetic animals suggests that the mechanism involves either accelerated excretion or reduced uptake of cisplatin or derivatives by the diabetic kidney, rather than selective alteration of intracellular particulate or cytosolic Pt pools. This could conceivably have been due to alterations within the kidneys secondary to the diabetic state. One obvious effect of diabetes is osmotic diuresis which might accelerate Pt excretion. However, Scott *et al.* (2) have demonstrated that glucose-induced diuresis offers no protection from cisplatin nephrotoxicity. A more likely possibility is decreased uptake by the tubular cells, a mechanism which, though little studied in the kidney, seems clearly to play a role in the acquired resistance of some tumor cells to cisplatin toxicity (25). Alternatively, the mechanism may be primarily extrarenal, involving, perhaps, changes in the disposition of Pt derivatives in STZ-induced experimental diabetes.

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