Shifts in the Dose-Effect Relationship for the Nephropathy Induced by Cadmium-Metallothionein in Rats after a Reduction in Renal Mass

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ABSTRACT

The aim of the present study was to determine if a reduction of renal mass altered the dose-effect relationship for the nephropathy induced by cadmium-metallothionein. Uninephrectomized (NPX) and sham-operated (SO) rats were given a single i.v. injection of cadmium-metallothionein at a dose of 0.1, 0.2 or 0.3 mg of cadmium per kg, and were sacrificed 24 hr after the injection. Quantitative computerized morphometric analysis of sections of kidney, plasma creatinine and the urinary excretion of some cellular enzymes and plasma solutes were used to evaluate the severity of renal injury at each dose of cadmium-metallothionein. No renal injury was detected in either the NPX or SO rats given the lowest dose of cadmium-metallothionein. At the middle dose of cadmium-metallothionein, renal injury occurred in both the NPX and SO rats, but the severity of injury was substantially greater in the NPX rats. This increased renal injury in the NPX rats correlated with increased renal content of cadmium and increased urinary excretion of cadmium. At the highest dose of cadmium-metallothionein, renal injury was quite severe. Some of the data show that renal injury was more severe in the NPX rats than in the SO rats. In summary, our findings indicate that there is a shift to the left in the dose effect relationship in the nephropathy induced by cadmium-metallothionein. The mechanism for this shift is not known at present, although it appears to be related, in part, to increased renal accumulation and/or retention of cadmium.

After renal mass has been reduced significantly, whether as a result of surgery or renal disease, both structural and functional changes occur in some of the epithelial cells along some segments of the remaining functioning nephrons (Meyer et al., 1991). These changes as a whole constitute the process known as compensatory renal growth, which allows the organism to maintain normal fluid and electrolyte homeostasis, even when renal mass has been reduced by 75% (Zalups et al., 1985; Zalups, 1989; Meyer et al., 1991). Although the adaptive responses that occur in the remaining functional nephrons may enable them to handle the imposed increased physiological demands, these same adaptive responses may change the renal handling and disposition of certain nephrotoxicants. There are data from several studies indicating that there is a shift to the left in the dose effect relationship for the nephropathy induced by analgesics (Molland, 1976; Henry et al., 1983) and inorganic mercury (Ramos-Frendo et al., 1979; Houser and Berndt, 1986; Zalups and Diamond, 1987) in rats that have undergone unilateral nephrectomy. The findings from these studies indicate that NPX rats develop a more severe form of the nephropathy induced by analgesics or inorganic mercury than corresponding control rats.

In the case of the nephropathy induced by inorganic mercury, the shift in the dose-effect relationship for this toxic heavy metal appears to be due to a specific increase in the early accumulation of inorganic mercury that occurs in the outer stripe of the outer medulla (Zalups et al., 1987; Zalups and Diamond, 1987), specifically in the pars recta of the proximal tubule (Zalups, 1991). It should be pointed out that it is the pars recta of the proximal tubule that is affected primarily by the nephrotoxic effects of inorganic mercury (Gritzka and Trump, 1968; McDowell et al., 1976; Zalups and Diamond, 1987; Zalups et al., 1991). The exact mechanism for the increased accumulation of mercury that occurs in the outer stripe of the outer medulla of the kidney after unilateral nephrectomy and compensatory renal growth is not known at present. However, there is some data indicating that it may be related to the

ABBREVIATIONS: NPX, uninephrectomized; Cd, cadmium; MT, metallothionein; SO, sham-operated; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; NAG, N-acetyl-β-D-glucosaminidase.

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content of glutathione (which avidly binds to mercury) in the tubular epithelial cells found in this region of the kidney. The content of glutathione per gram of tissue has been shown to increase significantly in the renal outer stripe of the outer medulla in rats as a result of unilateral nephrectomy and compensatory renal growth (Zalups and Veltman, 1988; Zalups and Lash, 1990).

Inorganic Cd is another nephrotoxic heavy metal. However, it causes injury primarily in the convoluted, rather than in the para recta or straight, portion of the proximal tubule. The nephropathy induced by Cd differs from that induced by inorganic mercury because renal injury occurs generally after more chronic, rather than acute, exposure or treatment (Goyer et al., 1984). Although a number of factors may be involved in the nephropathy induced by Cd, it has been postulated that this nephropathy may be causally related, in part to the reabsorption of complexes of Cd and the metal binding protein MT by the proximal convoluted tubule (Cherian et al., 1976). These complexes are formed in the epithelial cells of the liver and are thought to be released into the blood as a result of hepatocellular necrosis induced by Cd (Tanaka et al., 1981).

Unlike inorganic Cd alone, injected Cd-MT is very nephrotoxic. Extensive necrosis occurs in the renal proximal convoluted tubules of rats within the first 24 hr after exposure (Cherian et al., 1976). The nephrotoxicity of Cd-MT is presumably related to the rate at which MT can deliver inorganic Cd into the intracellular milieu of the epithelial cells along the proximal convoluted tubule. The most likely manner in which Cd-MT is transported inside the epithelial cells of the proximal convoluted tubule is by endocytosis.

Because the epithelial cells along the proximal convoluted tubule undergo significant structural and functional changes after a substantial reduction of renal mass (Meyer et al., 1991), and because Cd-MT exerts its toxic effects along the proximal convoluted tubule, it is possible that as a result of a significant reduction of renal mass and compensatory renal growth there may be an alteration in the handling of Cd-MT along the proximal convoluted tubule. This could potentially affect the nephropathy induced by this metal complex in the remaining renal tissue. Therefore, the present study is designed to test the hypothesis that, as a result of unilateral nephrectomy and subsequent compensatory renal growth in the rat, there is a shift in the dose effect relationship for the nephropathy induced by Cd-MT. The hypothesis will be tested by using several methods for evaluating renal cellular injury, including qualitative histopathological evaluation, quantitative morphometry and measurements of the urinary excretion of some renal cellular enzymes and some plasma solutes. The concentration of Cd in renal tissue and the urinary excretion of Cd will also be measured in order to determine if there is a relationship between these parameters and the pattern of renal injury induced by Cd-MT. Furthermore, the renal content of MT will also be measured to determine if the renal cellular content of MT is altered significantly after the administration of Cd-MT.

**Materials and Methods**

**Animals and operative procedures.** Sprague-Dawley rats weighing 175 to 200 g were purchased from Harlan Sprague-Dawley (Indianapolis, IN) for use in the present study. After several days of acclimation, all the rats were divided into two surgical groups. Unilateral nephrectomy was performed on one group and a sham nephrectomy was performed on the other group.

The operative procedures involved the following steps: 1) all the animals were first anesthetized with 50 mg/kg of sodium pentobarbital (i.p.). 2) After anesthesia was achieved, a small 1- to 1.5-cm flank incision was made through the skin and underlying abdominal muscles on the right side of the body. 3) The right kidney was exteriorized carefully from the body using blunt dissection. Care was taken not to injure the suprarenals during the dissection process. 4) For the animals that underwent the unilateral nephrectomy, a sterile 3.0-silk suture was tied securely around the right renal artery and vein and right ureter. Subsequently, the right renal artery and vein and right ureter were transected distal to the ligature with surgical scissors and then the right kidney was excised. 5) For the animals that underwent the sham-operation, the right kidney was simply placed back into its original retroperitoneal location. 6) After either surgical procedure, the abdominal muscles were sewn back together using sterile 4.0-silk suture. The skin was approximated using 9 mm sterile wound clips, which were applied with a stainless-steel wound clip applicator.

After surgery and during the course of the experiments, the animals were housed individually in plastic metabolic cages. A standard commercial rodent diet and water were provided *ad libitum*. The animals were allowed 12 days to recover from surgery before experimentation. This period was chosen because it allowed sufficient time for the completion of the rapid phase of compensatory renal growth. Usually the rapid phase of compensatory renal growth in the rat is completed by the end of the 7th day after surgery.

**Administration of Cd-MT.** On the morning of the 13th day after surgery, the NPX and SO animals were divided into treatment groups according to the dose of Cd to be administered. The animals were divided up such that a group of NPX (n = 6) and a corresponding group of SO (n = 6) rats received an i.v. dose of Cd-MT that delivered 0.1, 0.2 or 0.3 mg/kg of Cd. Cd-MT was dissolved in 0.9% aqueous sodium chloride. The injection solutions were designed to deliver each dose of Cd in a volume of 2.0 ml/kg. Each dose of Cd-MT was injected into the right femoral vein after the rat was anesthetized lightly with ether.

**Isolation of MT from the liver rats.** MT was isolated from the liver of male Sprague-Dawley rats injected with Cd chloride (1 mg/kg Cd\(^{2+}\)) s.c. once a day for 2 weeks. The purification of the two isoforms of MT 1 and 2 was similar to that described previously (Templeton and Cherian, 1984). Briefly, samples of liver were homogenized (20%). in a 10 mM Tris-HCl buffer, pH 8.6, followed by centrifugation at 27,000 x g for 10 min. The resulting supernatant was heated at 80°C for 2 min and then was centrifuged at 27,000 x g for 10 min. The heated supernatant was fractionated on a Sephadex G-75 column (5 x 95 cm) with 10 mM Tris-HCl buffer (pH 8.6). The 10,000 molecular weight protein fractions containing Cd were pooled and chromatographed on a DEAE-Sephadex ion exchange column using a linear gradient of 10 to 250 mM Tris-HCl buffer (pH 8.6) and then freeze-dried. The amount of Cd and zinc in each sample was measured with an atomic absorption spectrophotometer (Varian Spectra 30, Georgetown, Ontario, Canada) using air-acetylene flame. The amount of MT was estimated by the silver saturation method (Schuhammer and Cherian, 1986). The ratio of Cd to zinc in the MT was about 1:4 in both isoforms and the second isoform of Cd-MT was used in this study.

**Methods used to evaluate renal injury induced by Cd-MT.** The severity of renal injury induced by the three doses of Cd-MT in the NPX and SO rats was evaluated by qualitative and quantitative histopathological evaluations, measurements of the concentration of creatinine in plasma and the measurements of the rates of excretion of some renal cellular enzymes and some renal plasma solutes. The techniques used in these evaluations are described below.

**Urine collection and analysis.** Urine was collected from each of the NPX and SO rats, given one of the three i.v. doses of Cd-MT, for 24 hr immediately before and 24 hr immediately after the administration of Cd-MT. At the completion of either the control or experimental collection period, the volume of urine excreted in 24 hr was determined.
gravimetrically and then a 3.0-ml sample was obtained for the determination of the content of some renal cellular enzymes on that same day. The remainder of urine from each animal was stored in a plastic vessel at −70°C.

The 3.0-ml urine sample obtained from each collection period was divided equally into 3 × 1.0-ml aliquots. Each aliquot was placed in a dialysis bag (Spectropor 1) and dialyzed for 4 hr at 4°C against 1 l of deionized water. The concentration of the cytosolic enzymes lactate dehydrogenase (LDH) (Leathwood et al., 1972) and aspartate aminotransferase (AST) (Sigma Kit No. 58–10, Sigma Chemical Co., St. Louis, MO) in the dialyzed samples of urine was determined spectrophotometrically.

The frozen samples of urine were analyzed later with spectrophotometric techniques to determine the concentration of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) (Lockwood and Bosmann, 1979), total protein (Fesce and First, 1979) and albumin (Doumas et al., 1971; Gustafsson, 1976). The concentration of Cd in the frozen samples was determined by direct current plasma atomic emission spectroscopy.

Collection and handling of tissues. After the final urine samples were collected, the animals were anesthetized with a 50-µg/kg dose of sodium pentobarbital (i.p.). Subsequently, a 1.0-ml sample of blood was obtained from the inferior vena cava. The blood was spun down and a sample of plasma was obtained. The sample of plasma was later analyzed spectrophotometrically to determine the concentration of creatinine (Sigma Creatinine Kit for the colorimetric endpoint determination of creatinine, Sigma Chemical Co.).

After the sample of blood was obtained, the kidney(s) of each of the NPX or SO rats was/were removed and weighed quickly. A sample of the left kidney of each animal was obtained to determine the content of Cd per gram of renal tissue. The determination of Cd in renal tissue was accomplished by atomic absorption spectrophotometry. Another representative sample of the left kidney was obtained to determine the contents of MT. In addition, a 4.0-mm mid-transverse slice of the left kidney was obtained from the left kidney for histopathological and morphometric analysis.

Determination of the content of Cd in urine. Samples of urine were digested in warm trace metal grade nitric acid and then dried. The dried residue of each urine sample was cooled and then redissolved in a solution of 11.6% hydrochloric acid (HCl) and 2.8% nitric acid (HNO3) (v/v). Cd was quantified by direct plasma atomic emission spectroscopy (Beckman SpectraSpan V, Fullerton, CA) at 228.8 emission line. All reagent blanks, standards and samples were buffered with lithium (0.5% w/v) to minimize the effect of matrix variations.

Determination of the content of Cd in renal tissue. The samples of renal tissue used for Cd analysis were first homogenized in distilled water using a polytron. Then samples of the homogenate were diluted 1:1 with concentrated (10 Normal) nitric acid (HNO3) and were left overnight to allow for the digestion of cellular constituents. The content of Cd in diluted aliquots of the digested renal tissue was determined by atomic absorption spectrophotometry using an air-acetylene flame.

Technique for estimating the content of MT in renal tissue. Each sample of renal tissue allotted for MT analysis was weighed and then homogenized in a 0.25 M sucrose buffer (20% w/v) using a polytron (Tecmar, Cincinnati, OH). The homogenates were centrifuged for 15 min at 10,000 × g in a Sorvall refrigerated centrifuge (4°C). Subsequently, the content of MT in the supernatants was determined by the silver saturation method (Scheuhammer and Cherian, 1986). In brief, two aliquots of each sample (0.1–0.2 ml) were diluted with 0.5 M glycine-Tris buffer (pH 8.5). All samples were mixed with 1.0 ml of a silver nitrate solution for 10 min at room temperature. The silver bound to proteins other than MT and free silver were removed by addition of rat hemolysate, heating in a water bath for 2 min and centrifugation (10,000 × g for 10 min). These steps were repeated twice. The heated supernatants contained silver bound exclusively to MT. In order to obtain the level of background silver, a control tube with glycine buffer alone (not containing a sample) was measured by flame atomic absorption spectrophotometry (Varian Spectra-30, Georgetown, Ontario, Canada) using a standard curve for silver. The content of silver in the control tube was subtracted from the content of silver in the samples to calculate the content of silver bound to MT. The amount of MT in each sample was expressed as micrograms of wet weight, assuming a stoichiometry of 17-g atoms of silver bound to 1 mol of MT.

Histopathological and morphometric analysis. The 4.0-mm mid-transverse slice of the left kidney from each of the NPX and SO rats was fixed for 48 hr at 4°C in a fixative containing 4% formaldehyde (v/v) and 1% glutaraldehyde (v/v). The fixative was buffered with 11.6 g/l of sodium dihydrogen phosphate (Na2HPO4) and 2.7 g/l of sodium hydroxide (NaOH). The pH of the fixative was adjusted to 7.35. The total osmolality of the fixative was 1120 mOsmol. After the slices of kidney were fixed, they were dehydrated in an ascending graded series of ethanol, cleared in xylene and embedded in paraffin wax. Sections (3–5 µm) of each embedded slice of left kidney were obtained with a standard microtome and were mounted on glass slides. The sections were later stained with hematoxylin and eosin for histological analysis.

As part of the histopathological analysis, each section of left kidney from each animal was viewed and evaluated under a light microscope using bright field optics. Qualitative assessments of the level of injury to the proximal convoluted tubules in the sections were recorded.

In order to quantitate the level of cellular injury and necrosis in proximal convoluted tubules in the sections of the left kidney from all the animals at each dose of Cd-MT, a computerized morphometric system was used. This system consisted of a Nikon Optiphot light microscope with a camera lucida drawing tube attachment, a digitizing tablet (12" × 12"), a computer with an Intel 386 microprocessor and commercially available software that allows for morphometric measurements. In brief, several low power (40×) fields of the renal cortex from each section were projected onto sheets of white paper mounted on the digitizing tablet using the camera lucida drawing tube attachment. Each visual field was first traced on paper. Then, all the tubules that were affected by necrosis in the visual field were traced on paper. Subsequently the total area of the visual field and the total area of the proximal tubules affected by necrosis were determined from the tracings using the digitizing tablet and the computer. The total area of the proximal tubules affected by necrosis in the visual field was divided by the entire area of the visual field and was then multiplied by 100 to arrive at a percentage of the visual field that was affected by necrosis in proximal convoluted tubules. The values obtained from three to four visual fields of sections of kidney from each animal were averaged to obtain a representative value for each animal. After obtaining an average representative value for the percentage of the visual field affected by necrosis for each animal, the value was transformed to a nonparametric rank score using the following method: values of 0% were assigned a score of 0; values of 1 to 10% were assigned a score of 1; values of 11 to 20% were assigned a score of 2; values of 21 to 30% were assigned a rank score of 3; values of 31 to 40% were assigned a score of 4; and a value of 41 to 50% were assigned a score of 5. Scores greater than 5 were not needed in the present study because there was never an instance in which greater than 50% of the visual field was affected by necrosis.

Calculations and statistical analysis. The excretion of cellular enzymes in the urine by the NPX and SO rats during the control and experimental collection periods is expressed as units (or kilounits) of enzyme excreted in 24 hr per gram of wet kidney (1 U = 1 nmol of substrate consumed per min). Urinary excretion of total protein and albumin is each expressed as milligrams of solute excreted in 24 hr per gram of wet kidney. The concentration of Cd in the urine is expressed as micrograms per milliliter. The urinary excretion of Cd is expressed as percentage of the administered dose of Cd excreted in 24 hr per gram of kidney.

The content of Cd in the kidney is expressed as percentage of the administered dose of Cd, per gram of kidney, or in the whole left kidney or total renal mass. The tissue concentration of Cd is also expressed as micrograms per gram. The concentration of MT in the left kidney is expressed as micrograms per gram.

All values are expressed as mean ± S.E.M, except for the data obtained from the histopathological morphometric analysis, which are
expressed as the median and range for assigned rank scores. Differences between means for the renal and urinary data obtained from NPX and SO rats given the same dose of Cd-MT were evaluated statistically using the unpaired Student's *t* test for two independent samples. Differences in the assigned rank scores, for necrosis in cortical proximal tubules, between NPX and SO rats given the same dose of Cd-MT, were evaluated using the nonparametric two-tailed Mann-Whitney rank sum test. All data were evaluated using the 95% confidence interval, which was chosen *a priori*. Therefore, when *P* was less than .05, differences between means or rank scores were regarded statistically significant.

**Results**

**Animal body and kidney weights.** There was no significant difference in animal body weight between corresponding NPX and SO rats, given the same dose of Cd in the form of Cd-MT, 12 days after surgery (table 1). The remnant left kidney in the NPX rats was on average 35% greater in mass than the left kidney in the corresponding SO rats (table 1). This finding confirms the fact that a significant degree of compensatory renal growth occurred in the NPX rats during the 12 days of recovery after surgery. Even though compensatory renal growth occurred in the NPX rats, the total renal mass in the SO rats was significantly greater than that in the NPX rats, which is consistent with observed patterns of compensatory renal growth in the rat (Zalups et al., 1987; Zalups and Diamond, 1987; Zalups and Lash, 1990).

**Histopathology and morphometry.** No evidence of cellular necrosis was found in any segment of the nephron or collecting duct in the renal tissue of either the NPX or SO rats 24 hr after the i.v. administration of the 0.1-mg/kg dose of Cd in the form of Cd-MT (fig. 1).

Cellular necrosis occurred very rapidly in the proximal convoluted tubules in the renal cortex of both NPX and SO rats injected with the 0.2-mg/kg dose of Cd. Necrosis was essentially confined to the proximal convoluted tubules. By the end of the initial 24 hr after injection, the severity of cellular necrosis in proximal convoluted tubules, as determined by qualitative light microscopic observations, was greater in the NPX rats than in the SO rats (fig. 2). On the basis of computerized morphometric analysis of cross-sections of kidney, approximately 30% of the renal cortical area was affected by necrosis in proximal convoluted tubules in the NPX rats, whereas only about 11% of the renal cortical area was affected by necrosis in the same tubular segments in the SO rats. Inasmuch as the percentage of scores do not generally fit a normal or “Gaussian” distribution, it was necessary to convert percentage of scores to rank scores for the purpose of evaluating statistically differences in the severity of cellular necrosis in proximal convoluted tubules. Nonparametric analysis (Mann-Whitney Rank Sum Test) of the rank scores assigned to the NPX and SO rats given the 0.2-mg/kg dose of Cd shows that the level of necrosis in proximal convoluted tubules was significantly greater in the NPX rats than in the SO rats (fig. 1). This confirms the qualitative assessment of renal injury.

Quite extensive necrosis was noted in the renal proximal convoluted tubules of both the NPX and SO rats given the 0.3-mg/kg dose of Cd in the form of Cd-MT. As with the 0.2-mg/kg dose of Cd, cellular injury was localized primarily to the proximal convoluted tubules. On a qualitative basis, the level of proximal tubular necrosis appeared to be more severe in the NPX rats than in the SO rats. Based on morphometric analysis of sections of renal tissue from these animals, approximately 40% of the renal cortex was affected by necrosis in the NPX rats, whereas approximately 33% of the renal cortex was affected by necrosis in the SO rats. Statistical analysis revealed that the level of necrosis in the renal proximal convoluted tubules of the NPX rats was significantly greater than that in the SO rats (fig. 1).

**Urinary excretion of cellular enzymes.** During the 24 hr before the injection of any of the three doses of Cd-MT, there was no significant difference in the urinary excretion of LDH (fig. 3), AST (fig. 4) or NAG (fig. 5) in terms of kU/24 hr/g of kidney between the NPX and SO rats.

The urinary excretion of all three cellular enzymes measured did not increase significantly from base-line values in NPX and SO rats during the 24 hr after the administration of the 0.1-mg/kg dose of Cd in the form of Cd-MT (figs. 3–5). This is consistent with the fact that no cellular necrosis was observed in the sections of renal tissue from these animals.

During the 24 hr immediately after the administration of the 0.2-mg/kg dose of Cd, the urinary excretion of cellular enzymes increased from control levels in both NPX and SO rats. The urinary excretion of LDH (fig. 3) and AST (fig. 4) increased in both the NPX and SO rats, whereas the urinary excretion of NAG (fig. 5) increased only in the NPX rats. The urinary excretion of all three cellular enzymes was significantly greater in the NPX rats than in the SO rats. These findings are consistent with the histopathological and morphometric data, indicating that the nephropathy induced by the 0.2-mg/kg dose of Cd in the form of Cd-MT was significantly more severe in the NPX rats than in the SO rats.

The urinary excretion of cellular enzymes was also elevated significantly above control levels in both NPX and SO rats during the 24 hr immediately after the administration of the 0.3-mg/kg dose of Cd in the form of Cd-MT (figs. 3–5). Only

**TABLE 1**

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<td>0.1</td>
<td>291 ± 6</td>
<td>27.4 ± 0.7</td>
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<td>1.50 ± 0.04*</td>
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<td>0.2</td>
<td>329 ± 6</td>
<td>31.8 ± 0.8</td>
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<td>2.35 ± 0.04</td>
<td>16.2 ± 1.1</td>
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<td>43.6 ± 0.8</td>
<td>1.48 ± 0.04*</td>
<td>1.48 ± 0.04*</td>
<td>17.6 ± 1.0</td>
<td>19.5 ± 1.5</td>
<td>10.93 ± 0.28*</td>
<td>0.57 ± 0.13</td>
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<td>240 ± 9</td>
<td>43.2 ± 1.8</td>
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<td>2.21 ± 0.14</td>
<td>14.6 ± 1.2</td>
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<td>NPX, n = 6</td>
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<td>278 ± 8</td>
<td>83.5 ± 2.4</td>
<td>1.84 ± 0.06*</td>
<td>1.84 ± 0.06*</td>
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<td>20.6 ± 1.5</td>
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<tr>
<td>SO, n = 6</td>
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* Significantly different (*P* < .05) from the mean for the corresponding group of SO rats.
the urinary excretion of NAG was significantly greater in the NPX rats than in the SO rats after the administration of this dose of Cd-MT. The urinary excretion of LDH and AST was statistically similar in both groups of rats. It is important to note that the excretion of these two enzymes in the NPX rats did not increase greatly above the levels measured in the NPX rats given the 0.2-mg/kg dose of Cd. Contrasting results were obtained with the SO rats in which the greatest increase in enzymuria occurred after the 0.2-mg/kg dose of Cd.

**Urinary excretion of plasma solutes.** The base-line or control level for the urinary excretion of total protein (fig. 6) and albumin (fig. 7), in terms of milligrams of solute excreted per 24 hr per gram of kidney, was significantly greater in the NPX rats than in the SO rats during the 24 hr before the rats were given an i.v. injection of Cd-MT.

The urinary excretion of total protein, albumin and glucose in the NPX and SO rats did not change substantially from base-line values during the 24 hr after the administration of the 0.1-mg/kg dose of Cd in the form of Cd-MT. These data are also consistent with the histopathological data and the enzyme excretion data, which indicate that no significant renal injury occurred in the NPX and SO rats given this dose of Cd-MT.

The urinary excretion of total protein (fig. 6) and albumin (fig. 7) did not change substantially above base-line values in the SO rats treated with the 0.2-mg dose of Cd in the form of Cd-MT. By contrast, the NPX rats injected with the same dose of Cd-MT excreted substantially more protein and albumin above base-line values during the initial 24 hr after being treated. Consequently, the NPX rats excreted significantly more protein and albumin than the SO rats.

The urinary excretion of total protein and albumin in the NPX and SO rats given the 0.3-mg/kg dose of Cd increased above values obtained from the corresponding NPX and SO rats given the 0.2-mg/kg dose. The greatest increase, however, occurred in the SO rats. Nevertheless, the overall rate of excretion of total protein and albumin during the 24 hr after treatment of the 0.3-mg/kg dose of Cd was significantly greater in the NPX rats than in the SO rats.

**Urinary excretion of Cd.** The urinary excretion Cd, in terms of percentage of the administered dose of Cd excreted in 24 hr per gram of kidney, tended to increase in a dose-dependent manner in the NPX and SO rats injected with Cd-MT (fig. 8). However, the increase was minimal between the two highest doses of Cd-MT. During the 24 hr immediately after the administration of each dose of Cd-MT, the urinary excretion of Cd was significantly greater in the NPX rats than in the SO rats.

**Concentration of creatinine in plasma.** At the 0.1-mg/kg dose of Cd, in which there was no apparent renal tubular injury, plasma creatinine was significantly greater in the NPX rats than in the SO rats (fig. 9). The concentration of creatinine increased in the NPX and the SO rats given the 0.2-mg/kg dose of Cd above levels found in the corresponding NPX and SO rats given the 0.1-mg/kg dose of Cd. The concentrations of creatinine in the plasma of the NPX and SO rats given the 0.3-mg/kg dose of Cd were similar to those in the plasma of the NPX and SO rats given the 0.2-mg/kg dose of Cd. At both the 0.2- and 0.3-mg/kg doses of Cd as Cd-MT, the concentration of creatinine in plasma was significantly greater in the NPX rats than in the SO rats.

**Content of Cd in renal tissue.** The content of inorganic Cd per gram of kidney decreased in both NPX and SO rats as the dose of Cd-MT was increased (table 1; fig. 10). At all three doses of Cd-MT, the content of Cd per gram of kidney was significantly greater in the NPX rats than in the SO rats 24 hr after treatment. Because the remnant left kidney in the NPX rats was significantly greater in mass than the left kidney in
the SO rats, the percentage of the administered dose of Cd in the entire left kidney was significantly greater in the NPX rats than in the SO rats at each dose of Cd-MT (fig. 11).

**Content of MT in renal tissue.** There was a slight increase in the content of MT per gram of renal tissue when the dose of Cd was increased from 0.1 to 0.2 or 0.3 mg/kg in both NPX and SO rats (table 1). There was no difference in the content of MT per gram of renal tissue 24 hr after the two larger doses of Cd-MT were used in the NPX and SO rats. Only at the 0.1-mg/kg dose of Cd in the form of Cd-MT was there a significant difference in the content of MT per gram of renal tissue between the NPX and SO rats. In this case, the content of MT per gram of renal tissue was greater in the NPX rats than in the SO rats.

**Discussion**

Extracellular Cd-MT is an acute nephrotoxicant that induces injury specifically in proximal convoluted tubules (Nordberg *et al.*, 1975; Cherian *et al.*, 1976; Squibb *et al.*, 1984). Our histo-
Fig. 3. Urinary excretion of the cytosolic enzyme LDH in NPX and SO rats during the 24 hr immediately before and the 24 hr immediately after the animals received a 0.1-, 0.2- or 0.3-mg/kg i.v. dose of inorganic Cd in the form of Cd-MT. Values are mean ± S.E.M. for n = 18 animals for the control urine collection period (0.0 mg/kg of Cd") and n = 6 animals for the experimental urine collection period (0.1, 0.2 or 0.3 mg/kg of Cd"). *Significantly different (P < .05) from the mean for the corresponding group of SO rats treated with the same dose of Cd-MT.

Fig. 5. Urinary excretion of the lysosomal enzyme NAG in NPX and SO rats during the 24 hr immediately before and the 24 hr immediately after the animals received a 0.1-, 0.2- or 0.3-mg/kg i.v. dose of inorganic Cd in the form of Cd-MT. Values are mean ± S.E.M. for n = 18 animals for the control urine collection period (0.0 mg/kg of Cd") and n = 6 animals for the experimental urine collection period (0.1, 0.2 or 0.3 mg/kg of Cd"). *Significantly different (P < .05) from the mean for the corresponding group of SO rats treated with the same dose of Cd-MT.

Fig. 4. Urinary excretion of the cytosolic enzyme AST in NPX and SO rats during the 24 hr immediately before and the 24 hr immediately after the animals received a 0.1-, 0.2- or 0.3-mg/kg i.v. dose of inorganic Cd in the form of Cd-MT. Values are mean ± S.E.M. for n = 18 animals for the control urine collection period (0.0 mg/kg of Cd") and n = 6 animals for the experimental urine collection period (0.1, 0.2 or 0.3 mg/kg of Cd"). *Significantly different (P < .05) from the mean for the corresponding group of SO rats treated with the same dose of Cd-MT.

Fig. 6. Urinary excretion of protein in NPX and SO rats during the 24 hr immediately before and the 24 hr immediately after the animals received a 0.1-, 0.2- or 0.3-mg/kg i.v. dose of inorganic Cd in the form of Cd-MT. Values are mean ± S.E.M. for n = 18 animals for the control urine collection period (0.0 mg/kg of Cd") and n = 6 animals for the experimental urine collection period (0.1, 0.2 or 0.3 mg/kg of Cd"). *Significantly different (P < .05) from the mean for the corresponding group of SO rats treated with the same dose of Cd-MT.
pathological findings confirm this established effect of Cd-MT. The characteristics of renal injury that occur after a single injection of Cd-MT are similar to those that occur after chronic exposure to Cd-salts (Goyer et al., 1984). With Cd-salts, the onset of injury occurs only after long-term chronic exposure, when the concentration of Cd in the renal cortex reaches about 200 μg/g and there is a coinciding increase in the level of Cd-MT in the plasma of blood. It is intriguing that injury can occur in renal proximal convoluted tubules within hours after a single 0.3-mg/kg dose of Cd-MT, when the renal concentration of Cd has only reached a level of about 10 μg/g (Cherian et al., 1976; Suzuki and Cherian, 1987, 1989). In the present study, a similar effect on proximal convoluted tubules was
observed in NPX and SO rats, in which the renal concentration of Cd was somewhat greater than 10 μg/kg. A similar renal burden of Cd after injection of Cd chloride has been shown not to be associated with increased urinary excretion of Cd or renal injury (Dorian et al., 1992). One explanation for the lack of renal injury under the above stated conditions is that Cd in the form of inorganic salts may be accumulated by numerous segments of the nephron, in which Cd in the form of Cd-MT is probably taken up predominantly in the proximal convoluted tubules, where the Cd-MT complex is thought to be abstracted from the luminal fluid by pinocytosis (Cherian et al., 1976). If this is true, then the intracellular concentration of Cd in proximal convoluted tubules would be higher after treatment with Cd-MT. With respect to the renal injury induced by Cd-MT, it is likely that as the filtered load of Cd-MT increases, the binding sites for Cd in the epithelial cells of the proximal convoluted tubules become saturated, resulting in cellular necrosis and release of Cd into the urine. Because extracellular Cd-MT is nephrotoxic, and it increases after chronic exposure to inorganic Cd, it is probable that extracellular Cd-MT plays an etiological role in the development of renal damage after chronic exposure to Cd-salts.

Several sets of data from the present study indicate that nephropathy induced by low doses of Cd-MT is more severe in NPX rats, in which compensatory renal growth has occurred, than in SO or normal rats. Our findings show that plasma creatinine, the rates of urinary excretion of the cellular enzymes LDH, AST and NAG and the rates of excretion of plasma proteins (including albumin) are significantly greater in NPX rats than in SO rats when treated with a 0.2 mg/kg dose of Cd in the form of Cd-MT. These findings are consistent with qualitative histopathological and quantitative morphometric analyses, which indicate that the degree of necrosis in the proximal convoluted tubules is significantly greater in the NPX rats than the SO rats at this dose of Cd-MT. The morphological analyses also indicate that the level of cellular necrosis in the proximal convoluted tubules of the NPX rats given a 0.3-mg/kg dose of Cd in the form of Cd-MT is significantly greater than that in corresponding SO rats treated similarly. However, the differences in the severity of renal cellular necrosis between the NPX and SO are not nearly as great at the 0.3-mg/kg dose of Cd as they are at the 0.2-mg/kg dose of Cd. In fact, in the present study, the differences in renal injury between the NPX and SO treated with the 0.3-mg/kg dose of Cd could only be detected by morphometric analysis and the urinary excretion of NAG.

It should be pointed out that cellular necrosis in the proximal convoluted tubules was very severe in the kidney(s) of both NPX and SO rats treated with the highest dose of Cd-MT, whereas cellular necrosis was apparently absent in the kidney(s) of both the NPX and SO rats treated with the 0.1-mg/kg dose of Cd. This narrow range of no effect to severe effect confirms the findings of others, which indicate that the dose-effect curve for Cd-MT has a very steep slope (Suzuki and Cherian, 1987). Our findings indicate that the dose-effect curve is not only steep for NPX rats, but it is shifted to the left when compared with the dose-effect curve for SO rats. One would predict from our data that NPX rats begin to develop the nephropathy induced by Cd-MT at some dose that does not induce any renal injury in SO or normal rats. In general, one would predict that NPX rats are more susceptible to the nephrotoxic effects Cd-MT than SO or normal rats.

As a whole, the urinary excretion of LDH, AST, NAG and proteins (including albumin) increased in proportion to the level of necrosis in proximal convoluted tubules that could be detected histologically and quantitated by morphometric analysis. Previous reports (Squibb et al., 1984; Suzuki and Cherian, 1987) have shown that enzymuria can be used as an indicator of renal damage induced by Cd-MT. The findings in the present study confirm this and also show that enzymuria can be used as a good indicator to differentiate levels of renal injury, induced by low doses of Cd-MT, between NPX and SO rats.

In the absence of treatment with Cd-MT, the urinary excretion of total protein and albumin was significantly greater in NPX rats than in SO rats used in the present study. This confirms the findings of a previous study, in which it was shown that NPX rats excrete more protein and albumin than SO rats in the absence any treatment (Zalups and Diamond, 1987). Because it is well established that single nephron glomerular filtration rate is elevated in the remaining functioning nephrons after a significant reduction of renal mass (Meyer et al., 1991), there may be increased filtration of larger molecular weight proteins as a result in changes in the filtration coefficient, which occurs to promote an increase in the rate of filtration. Although the base-line levels for the urinary excretion of total protein and albumin were greater in the NPX rats than in the SO rats, the urinary excretion of protein and albumin remains a reasonably good indicator of renal injury induced by low doses of Cd-MT in both NPX and SO rats, because it correlates well with the urinary excretion of cellular enzymes and histopathology.

A shift to the left has also been observed in the dose-effect relationship for the nephropathy induced by low i.v. doses of inorganic mercury in NPX rats using similar markers for renal injury as those used in the present study (Zalups and Diamond, 1987). In brief, it was demonstrated that cellular necrosis in
the pars recta of proximal tubules, as defined by qualitative and quantitative morphological analysis and the urinary excretion of a number of cellular enzymes (including LDH, AST and NAG) and plasma solutes (including protein and albumin), was significantly more severe in NPX rats than in SO rats. There are a couple of other studies that have shown that a reduction of renal mass in rats leads to the animals developing a more severe form of the nephropathy induced by inorganic mercury than normal rats (Houser and Berndt, 1986; Ramos-Frendo et al., 1979). In addition, some investigators have shown that the nephropathy induced by analgesics is made more severe in rats as a result of a reduction of renal mass (Molland, 1976; Henry et al., 1983).

The exact mechanisms involved in the shift in the dose-effect relationship for the nephropathies induced by renal tubular toxicants after renal mass is reduced are not fully known at present. The changes in the expression of renal cellular injury are probably due to some factors associated with compensatory renal growth. Shortly after the renal mass in mammals has been reduced significantly, the remaining functional renal tissue undergoes marked structural, biochemical and physiological changes. Hypertrophic changes occur in the epithelial cells of a number of segments of the nephron, which are accompanied by increases in various aspects of intracellular metabolism and electrolyte and solute transport (Meyer et al., 1991). Renal blood flow and single nephron glomerular filtration rate also increase in association with the compensatory changes associated with a significant reduction of renal mass.

It is interesting that, as a result of unilateral nephrectomy and compensatory renal growth, shifts can occur in the dose-effect relationship for both a nephropathy induced by a nephrotoxicant that primarily affects the proximal straight tubule (inorganic mercury) and one that primarily affects the proximal convoluted tubule (Cd-MT). The shifts in the dose-effect relationship for these nephropathies may be related directly to alterations in the accumulation and/or retention of each type of nephrotoxicant in the segment of the proximal tubule that it affects adversely.

There is some experimental evidence linking the shift in the dose-effect relationship for the nephropathy induced by inorganic mercury to increased accumulation of inorganic mercury in the outer medulla. Renal accumulation of inorganic mercury in the rats treated with a nontoxic dose of mercuric chloride increases significantly after unilateral nephrectomy and compensatory renal growth. The increase is due to a specific increase in the accumulation of inorganic mercury in the outer medulla (Zalups et al., 1987), specifically in the outer stripe of the outer medulla (Zalups and Lash, 1990; Zalups, 1991). Recent histochemical evidence indicates that the increased accumulation of inorganic mercury in the renal outer stripe of the outer medulla is due to a specific increase in the accumulation of the metal in the pars recta of proximal tubules. The increased accumulation of inorganic mercury may be linked in part to increases in the intracellular concentration of glutathione in the outer medulla. Glutathione avidly binds to inorganic mercury and has been shown to increase within cells present in the renal outer medulla of rats as a result of compensatory renal growth (Zalups and Veltman, 1988). More recently, the increases in intracellular glutathione in the renal outer medulla of NPX rats have been shown to occur specifically in the outer stripe of the outer medulla (Zalups and Lash, 1990).

Once the cellular necrosis induced by inorganic mercury begins to occur in the pars recta of proximal tubules, the urinary excretion of inorganic mercury increases in proportion to the level of necrosis that is present in this segment of the nephron (Zalups et al., 1988). At low toxic doses of inorganic mercury, NPX rats excrete more inorganic mercury per gram of kidney than SO rats, which correlates with the level of renal injury as determined by histopathological analysis and the urinary excretion of cellular enzymes and plasma solutes (Zalups and Diamond, 1987; Zalups et al., 1988).

The shift in the dose-effect relationship for the nephropathy induced by Cd-MT in NPX rats may also be related to altered renal accumulation or retention of the nephrotoxicant. The findings in the present study show that, at each dose of Cd-MT that was used in the present study, the content of inorganic Cd per gram of renal tissue was significantly greater in the NPX rats than in the SO rats. This increase does not appear to be related directly to a specific increase in the renal synthesis of MT in the NPX rats, because there was no significant difference in the content of MT per gram of renal tissue between the NPX and SO rats at the two highest doses of Cd-MT. There was a significant difference in the content of MT per gram of kidney between the NPX and SO rats given the apparently nontoxic dose of Cd-MT (0.1 mg/kg of Cd). At the two highest doses of Cd-MT used in the present study, where necrosis in the proximal convoluted tubules was evident, the content of MT per gram of renal tissue increased slightly in both NPX and SO rats. However, the final result was that the content of MT per gram of renal tissue was similar in the NPX and SO rats. It is possible that at the two higher doses of Cd-MT, the content of MT in the renal tissue of the NPX rats was significantly greater than that in the renal tissue of the SO rats during the early stage of the nephropathy, before cellular necrosis occurred. However, once the proximal convoluted tubules became intoxicated, the level of necrosis was greater in the NPX rats and increased amounts of MT could have been released from the tubular epithelial cells into the urine; thus, bringing the content of MT per gram of kidney down to a level similar to that in the SO rats.

It should be pointed out that recent data indicate that the renal capacity to synthesize MT after induction of synthesis by inorganic mercury or zinc is increased significantly in rats as a result of unilateral nephrectomy and compensatory renal growth (Zalups and Cherian, 1992a,b). Moreover, the data show that renal cortical accumulation of inorganic mercury in NPX rats is increased significantly above that in SO rats as a result of increased renal cortical capacity for zinc to induce MT synthesis. In the present study, the concentration of MT in the kidney increased in both NPX and SO rats after the administration of Cd-MT, but it is unclear whether this increase was due to new synthesis of MT or uptake of filtered Cd-MT by renal tubular epithelial cells.

In addition to increased accumulation or retention of Cd and increased renal cellular injury in the remnant kidney of NPX rats after treatment with Cd-MT, there was increased urinary excretion of Cd, on a per gram of kidney basis in the NPX rats. The urinary excretion of Cd increased in both the NPX and SO rats as the dose was increased, but the amount Cd excreted, per gram of kidney, was always significantly greater in the NPX rats. Although the pattern for the urinary excretion of Cd did not completely parallel the pattern for renal injury that occurred in the NPX and SO rats, the elevated rate of excretion of Cd in the NPX rats given the two highest doses of Cd-MT
is consistent with the fact that there was an increased level of renal injury in the NPX rats at those doses. Thus, the urinary excretion of Cd may reflect differences in the severity of the nephropathy induced by low doses of Cd-MT between NPX and SO rats.

In summary, our findings show that NPX rats that have been given sufficient time for compensatory renal growth to occur develop a more severe form of the nephropathy induced by low doses of Cd-MT than SO or normal rats. Our findings also show that the remnant kidney of NPX rats accumulates or retains more Cd and excretes more Cd, on a per gram of kidney basis, than the normal kidneys in SO rats, which may correlate with the increased level of renal injury that occurs in the NPX rats.

References


