Radioimmunoassay measurement of urinary ligandin excretion in nephrotoxin-treated rats

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Summary

1. A radioimmunoassay was used to establish normal urine excretion of ligandin, a renal tubular protein, in rats, and to study the pattern of ligandin excretion after nephrotoxin administration.

2. Validation of radioimmunoassay measurement of ligandin in urine is described. A normal range of 2–46 ng/h was obtained for ligandin excretion in rats (n = 24).

3. Mercuric chloride (HgCl₂; 10 mg/kg) administration resulted in a significant increase in ligandin excretion within 6 h, preceding the onset of significant azotaemia. Serum ligandin concentrations were raised after 12 h, while renal ligandin concentration fell to 30% of control values by 24 h.

4. Rats given HgCl₂ (5 mg/kg) developed massive ligandinuria between 6 and 12 h, which declined to normal values by 72 h. Potassium dichromate (K₂Cr₂O₇; 7 mg/kg) administration produced a comparatively moderate increase in ligandin excretion after 24 h.

5. The time course of ligandin excretion in HgCl₂- and K₂Cr₂O₇-treated rats correlated with the histological sequence of damage to the pars recta and pars convoluta of the proximal tubule respectively.

6. The results of this study confirm that renal ligandin is confined mainly to the pars recta of the proximal tubule, and small quantities of this protein are present in the pars convoluta. Radioimmunoassay provides a sensitive and specific means for measuring ligandin in urine and provides a valuable tool for the detection and study of renal tubular damage.

Key words: glutathione S-transferase, kidney, ligandin, mercuric chloride, necrosis, nephrotoxin, radioimmunoassay, renal tubules, urine.

Introduction

Ligandin (glutathione S-transferase B; EC 2.5.1.18), a protein of molecular weight 46 000 which is thought to play a role in organic anion transport (Litwack, Ketterer & Arias, 1971; Arias, Fleischner, Kirsch, Mishkin & Gatmaitan, 1976; Smith, Ohl & Litwack, 1977), accounts for 2–5% of the soluble protein in liver, kidney and small intestinal mucosa (Fleischner, Robbins & Arias, 1972; Bass, Kirsch, Tuff & Saunders, 1977b). In the kidney, immunofluorescent studies have localized ligandin to the proximal tubule (Fleischner, Robbins & Arias, 1977). Using immunodiffusion and enzymatic techniques, Kirsch, Fleischner, Feinfeld, Goldstein, Kamisaka & Arias (1975) and Feinfeld, Fleischner, Goldstein, Arias & Bourgoine (1975) have reported the presence of ligandin in urine of rats treated with mercuric chloride (HgCl₂). Ligandinuria may therefore afford a sensitive index of tubular necrosis. Indeed, evidence supporting the value of urinary ligandin measurement in the diagnosis and prediction of tubular necrosis in man has been presented (Goldstein, Feinfeld, Fleischner & Elkin, 1976; Feinfeld, Levine, Fleischner & Levine, 1977).
Feinfeld et al. (1975) reported that immunologically detectable ligandinuria occurred in rats after HgCl₂ poisoning, but not after poisoning with potassium dichromate (K₂Cr₂O₇). As HgCl₂ is known to produce selective necrosis of the pars recta of the proximal tubule (Rodin & Crowson, 1962; Biber, Mylle, Baines, Gottschalk, Oliver & MacDowell, 1968), whereas in contrast, K₂Cr₂O₇ affects mainly the convoluted portion of the proximal tubule (Biber et al., 1968), it has been postulated that ligandin is localized in the pars recta of the proximal tubule (Feinfeld et al., 1975). We have recently described a radioimmunoassay technique which provides a sensitive and specific means of quantifying rat ligandin in nanogram concentrations (Bass et al., 1977). The present paper describes the quantitative measurement by radioimmunoassay in ligandin in urine of normal rats and in urine from rats treated with HgCl₂ or K₂Cr₂O₇.

**Methods**

**Experimental**

Male Wistar rats (200–250 g) were used throughout this study. Urine was collected into ice-chilled glass tubes from rats individually housed in metabolic cages over urine–faeces separators. Rats were allowed food and water ad libitum. Food was accessible to animals in a small recess away from the collection funnel, and the water bottle spouts contained ball valves. These measures were generally effective in preventing contamination of urine samples with food and drinking water. Fresh solutions of HgCl₂ (37 mmol/l) and K₂Cr₂O₇ (34 mmol/l) were prepared before use. These toxins as well as saline (150 mmol/l) were administered to rats by subcutaneous injection. After each collection period, collection funnels were cleaned with distilled water. Two groups of rats were studied:

**Group 1.** In this group, the effect of HgCl₂ on ligandinuria, ligandinaemia and renal function was assessed. After a basal urine collection period of 24 h, six rats received 10 mg of HgCl₂/kg (37 mmol/kg). Samples of urine were collected 0–6 6–12 and 12–24 h, after administration of the toxin. Tail vein blood samples were obtained before the injection and at the end of each post-injection period. Ligandin and urea were determined in each sample. At 24 h, four of the six rats were killed. Livers and kidneys were rapidly removed, perfused with ice-cold NaCl solution (150 mmol/l: saline), blotted, weighed and homogenized in three volumes to tissue weight of ice-cold sucrose solution (0-25 mol/l) in sodium phosphate buffer (0-01 mol/l), pH 7-4, with a Teflon/glass homogenizer. Homogenates were centrifuged at 100 000 g for 90 min. The supernatant (cytosol) was removed and stored at -20°C for no longer than 1 week before ligandin determination by radioimmunoassay. Ligandin was also estimated in cytosol prepared from the livers and kidneys of four saline-injected control rats, housed under conditions identical with those for the toxin-treated animals.

**Group 2.** The differential effects of HgCl₂ and K₂Cr₂O₇ in producing ligandinuria was assessed in this group. After 24 h of basal urine collection, six rats received HgCl₂ (5 mg/kg; 18 μmol/kg) and six received K₂Cr₂O₇ (7 mg/kg; 24 μmol/kg). These dosages were employed as they reportedly produced renal damage without anuria, thus permitting prolonged urine collection (Biber et al., 1968). Three rats received equivalent volumes of saline and served as controls. After injections, urine was collected from rats over periods 0–6, 6–12, 12–24, 24–36, 36–48 and 48–72 h. Identical doses of HgCl₂, K₂Cr₂O₇ and saline were administered to an additional group of rats from which urine was not collected. Liver and kidney histology were studied on these rats 16 and 48 h after administration of each toxin.

**Analyses**

Ligandin was measured in urine, serum and cytosol by radioimmunoassay. After measurement of volume, urine samples were spun at 2000 g for 30 min at 4°C to remove particulate matter. Blood samples were spun at 12 000 g for 2 min in a Beckman microfuge. A 50 μl sample of serum was diluted to 1 ml with cold assay diluent comprising sodium phosphate buffer (0-05 mol/l), pH 7-4, NaCl (0-15 mol/l), EDTA (0-01 mol/l), bovine serum albumin (0-5%, w/v) and sodium azide (15 mmol/l) before estimation of ligandin. The validation of radioimmunoassay measurement of ligandin in serum has been dealt with in a separate paper (Bass, Kirsch, Tuff & Saunders, 1978). The normal range for serum ligandin in the rat determined in this laboratory is 6–57 ng/ml (Bass, Kirsch, Tuff & Saunders, 1976). The limit of detection for ligandin in serum after dilution as described above was 30 ng/ml. Pure ligandin was prepared as described by Bass, Kirsch, Tuff & Saunders (1977a).

The radioimmunoassay for measuring ligandin
was carried out as detailed by Bass et al. (1977b). In brief, 200 µl samples of samples and serial dilutions thereof were added to tubes containing constant amounts of 125I-labelled ligandin and monospecific rabbit antisem to rat ligandin (no. 982/28/10), at a final dilution of 1:40 000. Separation of antibody-bound from free 125I-labelled ligandin was accomplished by using a double-antibody method. A limit of 1 ng/tube was detectable by the assay. However, in serum and some urine samples, detection of amounts of 0.2 ng/tube was permitted by addition of labelled ligandin after 24 h preincubation of other reagents, followed by a further 24 h first incubation period. The coefficient of variation for 20 determinations of a single sample in a single assay was 7% (mean 12.2 ± SEM 0.83) whereas the coefficient of variation for determination of a single sample in 10 consecutive assays was 12% (mean 27.4 ± SEM 1.07). Urine ligandin excretion was expressed in ng/h. Protein concentration of cytosol was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Glucose in urine was measured qualitatively by means of Ames Labstix. Blood urea was estimated in 10 µl samples of serum using a Beckman blood urea nitrogen Autoanalyzer, and results were expressed as mmol/l of urea. Histological examination was performed on 5 µm sections of formaldehyde-fixed paraffin-embedded tissue samples, stained with Haematoxylin–Eosin.

Recovery experiments

The estimation of quantitative recovery of pure ligandin added to urine was made as follows. Two pools of urine, each comprising equal volumes of urine obtained from three rats during basal collection periods, were divided into five samples. One sample was assayed for endogenous ligandin concentration, whereas serial dilutions of the remaining samples were assayed after addition of pure ligandin to concentrations between 100 and 20 000 ng/ml.

Gel filtration

2.5 ml of pooled urine samples, obtained during the 6–12 h collection periods from rats in group 1, were chromatographed on a calibrated column (97 cm × 2.5 cm) of Sephadex G-100 in sodium phosphate buffer (0.01 mol/l), pH 7.4, containing NaCl (0.1 mol/l). Ligandin was measured in column fractions (3 ml) by radioimmunoassay.

Statistics

Data from rats in group 1 were analysed statistically (Snedecor & Cochran, 1967). Appropriate statistical comparisons were made on the basis of paired/non-paired observations and the presence of heteroscedascity evidenced by the F-test, and included the paired t-test (urea concentrations), Wilcoxon paired signed-ranks test (ligandinuria) and the unpaired t-test (tissue ligandin concentrations).

Results

Characteristics of radioimmunoassay of ligandin in urine

Immunoreactive ligandin was detected in all urine samples assayed. Twenty-one normal (basal) urine samples yielded a range of concentrations for immunoreactive ligandin between 1.0 and 189 ng/ml, whereas values in some HgCl₂-treated animals were as high as 80 000 ng/ml. Immunoreactive ligandin in normal as well as nephrotoxin-treated rat urine gave immunochemical displacement curves which were similar to the standard curve of the assay (Fig. 1), indicating the presence of protein immunochemically similar to ligandin in these samples. The recovery of pure ligandin added to normal rat urine was between 80 and 99%. A loss of 0–15% of immunoreactivity was associated

![Fig. 1. Comparison between immunochemical displacement curves of normal urine (▲), urine from HgCl₂-treated rats (○) and the ligandin radioimmunoassay standard curve (●). On the ordinate is the percentage ratio of antibody-bound radioactivity (B) to radioactivity bound in the absence of unlabelled ligandin (B₀). On the abscissa (log scale) is shown the amount of unlabelled ligandin and the dilutions of urine.](image-url)
FIG. 2. Elution of immunoreactive ligandin from Sephadex G-100. Chromatography of urine obtained from HgCl₂-treated rats 6–12 h after administration of HgCl₂ (10 mg/kg). V₀ is the void volume; BSA, elution position of bovine serum albumin. Ligandin was measured by radioimmunoassay.

with storage of urine samples for 24 h at either −20°C or 4°C. Similar storage at room temperature resulted in 60% loss of immunoreactivity. Consequently all samples were collected at 0–4°C and stored at this temperature for less than 24 h before assay. Neither HgCl₂ nor K₂Cr₂O₇ added to assay tubes up to concentrations of 40 mmol/l caused interference with radioimmunoassay measurement of ligandin.

Chromatography of urine from HgCl₂-treated rats in Sephadex G-100 yielded a major peak of immunoreactivity (>90%) in the 40 000–50 000 molecular weight region, corresponding to the elution volume of pure ligandin (Fig. 2). Three minor peaks of immunoreactivity were also evident, most likely a result of the presence of aggregated and fragmented forms of ligandin.

Basal excretion of ligandin in urine

The range of values obtained for ligandin excretion in 21 rats calculated from 24 h basal urine collections was 2–46 ng/h (mean 14.86 ± sem: 2.81 ng/h). Thus, for the purpose of this study, an excretion of 46 ng of ligandin/h in the urine was taken as the upper limit of normal.

Ligandinuria, ligandinaemia and azotaemia after HgCl₂ treatment

Ligandin excretion in six rats before receiving HgCl₂ (10 mg/kg) ranged from 2 to 20.2 ng/h. No urine was obtained from one rat after administration of nephrotoxin, nor from the remaining five animals during 12–24 h collection period. Ligandin excretion in four out of five rats passing urine was greater than the upper limit of normal by 1.7–32 times during the 0–6 h period. For the five animals as a group, ligandin excretion was significantly greater during this period than during the basal period (P < 0.01). During the 6–12 h period, ligandin excretion in all five animals was markedly increased (78–265 times normal; P < 0.001). Glycosuria occurred in each case between 6 and 12 h. Serum ligandin concentrations were within normal limits in all six animals at 0 and at 6 h. By 12 h, serum ligandin concentrations were abnormally elevated in four out of five animals (1.6–23 times normal). By 24 h, all six animals showed abnormal serum ligandin concentrations (1.2–17 times normal).

Ligandin concentration in the kidneys of HgCl₂-treated rats (6.26 ± 1.28 ng/mg of supernatant protein) was significantly decreased to a third of control values (18.24 ± 1.55 ng/mg; P < 0.001). However, there was no difference in hepatic ligandin concentration between HgCl₂-treated rats (44.30 ± 4.69 ng/mg) and control animals (36.76 ± 2.85 ng/mg; P > 0.1).

Patterns of ligandinuria after HgCl₂ and K₂Cr₂O₇ administration

The mean excretion of ligandin in the urine of rats up to 72 h after either HgCl₂ (5 mg/kg) or K₂Cr₂O₇ (7 mg/kg) is shown in Fig. 3. Some rats did not pass sufficient urine for assay during various periods after nephrotoxin administration. The number of specimens suitable for analysis are shown in Fig. 3. Ligandin excretion remained normal during the first 6 h of urine collection after both HgCl₂ and K₂Cr₂O₇ administration.

A dramatic increase in ligandin excretion of 131 times normal was shown by the HgCl₂-treated
Ligandinuria in nephrotoxin-treated rats

FIG. 3. Urine ligandin excretion in rats after administration of either HgCl$_2$ (5 mg/kg) or K$_2$Cr$_2$O$_7$ (7 mg/kg). Each bar shows ligandin excretion as the mean value ± SEM for the number of animals (encircled at the base of each bar) passing sufficient urine for assay during each sampling period.

animals 6–12 h after injection. Over subsequent periods, mean ligandinuria declined in these animals, reaching normal limits at 48–73 h. A different pattern of ligandinuria was evident in K$_2$Cr$_2$O$_7$-treated rats. Mean ligandin excretion in these animals was normal up to 24 h. Thereafter K$_2$Cr$_2$O$_7$-treated rats showed abnormally elevated ligandin excretion. It should be noted, however, that the extent of abnormal ligandinuria developed by K$_2$Cr$_2$O$_7$-treated animals was at least an order of magnitude less than the maximum seen in the HgCl$_2$-treated rats. HgCl$_2$-treated rats developed glycosuria from 6 h, which persisted for the duration of the experiment. Glycosuria was only evident in K$_2$Cr$_2$O$_7$-treated rats after 36 h, lasting up until the final collection period. None of the control animals showed glycosuria or excretion of ligandin above the upper limit of normal at any stage.

On histological examination, the kidneys of animals receiving HgCl$_2$ in the dose employed showed severe tubular damage, particularly in the proximal tubular segments in the outer stripe of the outer medulla (pars recta). Coagulative necrosis in this area was prominent by 16 h, whereas at 48 h the necrotic cytoplasm appeared to have autolysed to some degree and numerous casts were present in Henle's loop and the collecting tubule. No lesion of the distal convoluted tubules or glomeruli was noted, although some necrotic changes were evident in descending and ascending limbs of Henle's loop. Renal tubular damage was also observed in the kidneys of K$_2$Cr$_2$O$_7$-treated animals, which was confined to the pars convoluta of the proximal tubules and did not involve the pars recta, Henle's loop, distal tubule or glomeruli. Changes observed at 16 h included partial loss of the brush border of proximal convoluted tubules with minimal enlargement of nuclei. By 48 h the brush border was lost in most proximal convoluted tubules, and the cytoplasm of this region was granular and vacuolated with hyaline droplets seen in some cells. Furthermore, most proximal tubules showed enlargement of nuclei with a few showing pyknotic degeneration. Liver and kidney sections were normal in saline-treated rats, and no hepatic lesions were evident in HgCl$_2$- or K$_2$Cr$_2$O$_7$-treated animals.

Discussion

Ligandin is a basic protein of molecular weight 46,000 present in abundance in the cytosol of hepatocytes, renal tubular cells and the non-goblet cells of small intestinal mucosa, which is thought to play a role in the intracellular transport of small molecules (Litwack et al., 1971; Arias et al., 1976). Ligandin has also been shown to possess glutathione S-transferase activity (Habig, Pabst, Fleischner, Gatmaitan, Arias & Jakoby, 1974) and thus contributes to the detoxification process within the cell. Another protective function of this protein, that of sequestering carcinogens, has also been proposed (Smith et al., 1977). Recently, the diagnostic value of serum ligandin, measured by radioimmunoassay, has been explored in relation to hepatocellular necrosis (Bass et al., 1976, 1978). Ligandin excretion in the urine may similarly provide a means of detecting renal tubular damage in experimental animals (Kirsch et al., 1975; Feinfeld et al., 1975) and in man (Goldstein et al., 1976; Feinfeld et al., 1977).

The radioimmunoassay technique provides a highly sensitive and specific means of measuring ligandin (Bass et al., 1977b) and is readily applied to the quantitative assessment of ligandin excretion in the urine. Small quantities of immunoreactive ligandin were detected in normal rat urine and this may be due to leakage of the protein from renal tubular epithelial cells in which ligandin constitutes up to 5% of soluble proteins (Fleischner et al., 1972; Arias et al., 1976). Leakage of ligandin from epithelial cells of the bladder, which contains comparatively minute quantities of the protein (Bass et al., 1977b) may also contribute to normal urinary loss of ligandin. Small amounts of ligandin are normally present in the circulation (Bass et al., 1978), and excretion of ligandin in the urine may provide an important route of elimination of this protein from plasma (N. M. Bass, unpublished...
immunologically detectable ligandinuria. In view of this, ligandin detected in normal urine may arise from glomerular filtration of plasma ligandin.

Feinfeld et al. (1975) reported that after HgCl₂ administration to rats, ligandinuria was detectable in most animals by both immunodiffusion and assay of glutathione S-transferase activity. In contrast, K₂Cr₂O₇-treated rats did not manifest immunologically detectable ligandinuria. In view of the fact that HgCl₂ and K₂Cr₂O₇ in low doses produce selective necrosis of the pars recta (Rodin & Crowson, 1962; Biber et al., 1968) and pars convoluta (Biber et al., 1968) of the proximal tubule respectively, it was concluded that renal ligandin is mainly confined to the pars recta (Feinfeld et al., 1977). Contrary to this hypothesis, however, immunofluorescence studies have demonstrated ligandin throughout all segments of the proximal tubule, and not exclusively in the pars recta (Fleischner et al., 1977). The present study confirms and extends the findings of Feinfeld et al. (1975). Massive ligandinuria was detected by radioimmunoassay in HgCl₂-treated animals with peak excretion occurring 6–12 h after administration of the toxin at both 5 and 10 mg/kg doses. Furthermore, the sensitivity of the radioimmunoassay enabled detection of abnormal ligandinuria occurring within 6 h in rats receiving the higher dose of toxin (group 1). Our studies also reveal a pattern of abnormal ligandin excretion occurring in K₂Cr₂O₇-treated rats. The lesser magnitude of ligandinuria occurring in this group of animals would account for the inability of Feinfeld et al. (1975) to detect ligandinuria by immunodiffusion in the urine of rats after K₂Cr₂O₇ treatment.

After administration of HgCl₂ in low doses similar to that given to group 2 rats in the present study, electron microscopy has revealed subtle changes throughout the proximal tubule from as early as 15 min, while severe changes are seen in the proximal pars recta by 6 h (McDowell, Nagle, Zalme, McNeil, Flamenbaum & Trump, 1976). After 12 h, plasma membranes of the pars recta show distortion and rupture (Gritzka & Trump, 1968) and complete necrosis of this region is evident by 24 h (McDowell et al., 1976; Gritzka & Trump, 1968). Thus the onset and progression of ligandinuria shown by HgCl₂-treated rats in group 2 coincides with the histological pattern of tubular disruption after HgCl₂ poisoning. The earlier onset of abnormal ligandinuria in group 1 rats could have resulted from the higher dose of HgCl₂ employed, precipitating earlier necrosis of the pars recta (Rodin & Crowson, 1962). It is interesting to note that a significant increase in ligandin excretion occurred before significant azotaemia in group 1 rats, suggesting that necrotic changes in the pars recta may precede the onset of renal failure in this animal model. However, an apparent causal relationship between necrosis of the pars recta and the early pathogenesis of renal failure after HgCl₂ poisoning must be considered in the light of the recent observations of McDowell et al. (1976) and Zalme, McDowell, Nagle, McNeil, Flamenbaum & Trump (1976). These workers have postulated on the basis of elaborate sequential histochemical, histological and functional studies, that in low doses, HgCl₂ interacts with the entire brush border of the proximal tubule, leading to diminished sodium reabsorption, increased renin release and haemodynamic changes resulting in decreased glomerular filtration rate and accompanying disturbances of renal function. Necrosis of the pars recta appeared to be a relatively late event occurring as a result of further Hg²⁺ accumulation in this region. In the present study, group 1 rats received a relatively high dose of HgCl₂ and the development of significant ligandinuria before significant azotaemia in these animals may indicate that, under these conditions, necrosis of the pars recta may occur sooner after the administration of the toxin, and thus play a more important role in the initial stages of acute renal failure.

Glycosuria is seen in rats after administration of several nephrotoxins (Wright & Plummer, 1974) and is thought to result from diminished glucose reabsorption in the proximal tubule (Biber et al., 1968). In the present study, glycosuria was associated with peak excretion of ligandin in the urine of both HgCl₂ and K₂Cr₂O₇-treated animals. An interesting finding was the later onset of abnormal ligandinuria and glycosuria in K₂Cr₂O₇-treated animals. Histological examination revealed necrosis of the pars convoluta at 16 h after K₂Cr₂O₇ administration, which increased in severity by 48 h. Others have also shown the presence of necrosis of the pars convoluta from 48 to 72 h after K₂Cr₂O₇ administration to rats (Tapp, Carroll & Kovacs, 1965; Biber et al., 1968), although data on earlier changes are lacking. Thus it appears from the available evidence that ligandin is present in relatively small quantities in the pars convoluta and that necrosis of the pars convoluta of sufficient severity to produce ligandinuria after a small dose (7 mg/kg) of K₂Cr₂O₇ occurs only 24 h after administration of the toxin.
Elevated serum ligandin was also demonstrated after HgCl₂ administration. It is likely that increased serum ligandin in this situation obtains from the necrotic pars recta. Indeed many tubular enzymes excreted in increased quantities after tubular necrosis are simultaneously raised in the serum (Wolf & Williams, 1973). It is unlikely that ligandinaemia observed in HgCl₂-treated rats might have resulted as a manifestation of liver damage, as this is not a documented feature of HgCl₂ poisoning (Clarkson, 1972). Furthermore, in the present study, the livers of HgCl₂-treated rats were normal on histological examination and contained normal concentrations of ligandin. However, the possibility exists that ligandinaemia after HgCl₂-induced renal failure may also result from diminished excretion of ligandin from the plasma, especially as urinary excretion is important as regards elimination of this protein from the circulation (N. M. Bass, unpublished work).

In conclusion, the results of the present study establish the value of ligandin measurement by radioimmunoassay as a sensitive means of detecting tubular necrosis, especially when there is involvement of the pars recta. The radioimmunoassay technique for measuring ligandin should thus provide a valuable tool for the experimental study of renal failure, and may be of use in screening tests for potentially nephrotoxic compounds administered to rats. The value of ligandinaemia as a means of detecting toxic tubular damage in man (Goldstein et al., 1976) and predicting acute renal failure after renal transplantation (Feinfeld et al., 1977) have recently been established, and serve as an impetus towards the development of a radioimmunoassay for ligandin in man.

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