A Correlation between a Proteomic Evaluation and Conventional Measurements in the Assessment of Renal Proximal Tubular Toxicity

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4-Aminophenol (4-AP), D-serine, and cisplatin are established rodent nephrotoxins that damage proximal tubules within the renal cortex. Using high throughput 2D gel proteomics to profile protein changes in the plasma of compound-treated animals, we identified several markers of kidney toxicity. Male F344 and Alpk rats were treated with increasing doses of 4-AP, D-serine, or cisplatin, and plasma samples were collected over time. Control groups received saline or nontoxic isomers, L-serine, and transplatin. Plasma proteins that displayed dose- and temporal-dependently regulated in each study were further characterized by mass spectrometry to elucidate the protein identity. Several isoforms of the rat-specific T-kininogen protein were identified in each study. T-kininogen was elevated in the plasma of 4-AP-, D-serine-, and cisplatin-treated animals at early time points, returning to baseline levels 3 weeks after treatment. The protein was not elevated in the plasma of control animals or those treated with nontoxic compounds. We propose that T-kininogen may be required to counteract apoptosis in proximal tubular cells in order to minimize tissue damage following a toxic insult. In addition, T-kininogen may be required to stimulate localized inflammation to aid tissue repair. We also identified several isoforms of the inter-alpha inhibitor H4P heavy chain in the 4-AP and D-serine studies. In each case, the protein expression levels in the blood samples paralleled the extent of kidney toxicity, highlighting the correlation between protein alterations and clinical chemistry endpoints. A further set of proteins correlating with kidney damage was found to be a component of the complement cascade and other blood clotting factors, indicating a contribution of the immune system to the observed toxicity. These observations underscore the value of proteomics in identifying new biomarkers and in the elucidation of mechanisms of toxicity.

Key Words: kidney; toxicity; proteomics; biomarker; protein profiling; proteases; inflammation.

Proteomic technologies have been highlighted as tools that may aid toxicologists in addressing issues in toxicology. Proteomics might contribute to the field of toxicology by identifying new markers of toxicity or by providing new insights into complex mechanisms of toxicity (Bandara and Kennedy, 2002; Kennedy, 2001). In support of this concept, several reports using 2D gel proteomic technologies have documented novel mechanistic pathways and new biomarkers of toxicity. Specifically, several studies have been performed on rat renal tissue in an attempt to gain new mechanistic insights. The clinical use of the immunosuppressant cyclosporine A (CsA) is limited by adverse effects, of which renal damage is the most prevalent. A proteomic analysis of kidney homogenates from CsA-treated rats defined the calcium-binding protein, calbindin-D (28K), as a novel marker of CsA renal toxicity (Steiner et al., 1996). Subsequent studies in a range of species indicated that rats and humans displayed CsA-mediated renal toxicity, whereas monkeys and dogs did not. Calbindin D was also regulated in a species-specific manner consistent with cellular toxicity (Aicher et al., 1998). Studies using the aminoglycoside antibiotic gentamicin reported changes in a variety of proteins responsible for stress response, glucose metabolism, and lipid biosynthesis (Charlwood et al., 2002; Kennedy, 2002). Several other kidney proteome studies using a variety of nephrotoxins have also been reported. (Cutler et al., 1999; Kanitz et al., 1999; Witzmann et al., 1999). To date, however, surprisingly few proteomic studies have been published analyzing protein changes in blood following a toxic insult. Serum or plasma toxicity markers may be particularly attractive as a clinical tool to monitor patient health as blood samples can be readily taken with a minimal risk to the patient.

The nephrotoxin 4-aminophenol (4-AP) produces severe necrosis of the pars recta of the proximal tubule in the rat (Davis et al., 1983; Gartland et al., 1989; Green et al., 1969). Although the exact mechanisms by which 4-AP exerts its nephrotoxic action is not completely understood, it is thought to be mediated by the formation of a toxic metabolite 1,4-benzoquinoneimine, which may bind to thiol groups in proteins (Calder et al., 1979; Crowe et al., 1979). Recent studies have shown that the glutathione conjugate of 4-AP is targeted to the kidney, where further processing may occur to produce the proximate metabolite (Fowler et al., 1991, 1994). D-serine is the enantiomer of L-serine, which is also known to selectively damage the pars recta of proximal tubules in the kidney (Ganote et al., 1974; Kaltenbach et al., 1982). The basis for
selective toxicity of D-serine is not fully understood although it has been shown to concentrate in the region of the nephron that is damaged (Imai et al., 1998). D-serine enters cells in the pars recta by re-absorption (Silbernagl et al., 1999) where it is processed by D-amino acid oxidase, leading to the formation of toxic oxidative metabolites. Another well characterized renal toxicant is the antitumor drug cisplatin, which has also been reported to cause renal dysfunction in rats (Goldstein et al., 1981; Safferstein et al., 1981; Wolfgang et al., 1994). Again, damage to the proximal tubules has been observed in the rat mainly confined to the pars recta. Furthermore, in man, cisplatin treatment has been attributed to focal tubular necrosis primary to the distal tubules and collecting ducts, with some dilatation of the convoluted tubules and the presence of protein casts (Dentino et al., 1978; Gonzalez-Vitale et al., 1977). Thus, although all three compounds damage the same part of the nephron, there is little evidence to suggest they act via a similar mechanism or pathway.

We therefore chose to examine the nephrotoxicity of 4-AP, D-serine, and cisplatin in greater detail by monitoring protein changes in the plasma of treated rats using the high-throughput 2D gel proteomics technology developed at Oxford GlycoSciences (OGS; Page et al., 1999; Harris et al., 2002). Here we report the results of several proteins that correlated specifically with the extent of cellular damage to the kidney.

MATERIALS AND METHODS

Compounds. 4-Aminophenol hydrochloride was purchased from Aldrich Chemical Co. (Dorset, U.K.), purified by sublimation as described by Fowler et al. (1991), and then dissolved in isotonic saline for dosing. D-, L-serine, cisplatin, and transplatin were purchased from Sigma-Aldrich Co., Ltd (Dorset, U.K.).

Animals and dosing. Four groups of 20 male Fischer 344 (F344) rats (7–8 weeks old, weight range of 150–200 g on arrival, supplied by Charles River, Ltd, Margate, U.K.) were given a single intraperitoneal injection of either isotonic saline or 4-AP in isotonic saline at 20, 50, or 80 mg/kg at a dose volume of 2.5 ml/kg bodyweight. In addition, two groups of 20 male Alpk rats (Alpk, Wistar-derived) rats (7–8 weeks old, weight range of 201–230 g on arrival, supplied by the Rodent Breeding Unit, Alderley Park, Macclesfield, Cheshire, U.K.) were dosed via the same route with either isotonic saline or 4-AP at 80 mg/kg. Five groups of 20 male Alpk rats were also given a single intraperitoneal injection of either isotonic saline, D-serine in isotonic saline at 75, 250, or 750 mg/kg, or L-serine at 750 mg/kg, at a dose volume of 4 ml/kg bodyweight. A further five groups of 20 male Alpk rats were also given a single intraperitoneal injection of either isotonic saline, cisplatin in isotonic saline at 1, 2, or 6 mg/kg, or transplatin at 6 mg/kg, at a dose volume of 2 ml/kg bodyweight.

Assessment of renal function. For the 4-AP and D-serine studies, animals were sacrificed 4, 8, and 24 h post dosing and following a 3-week treatment-free period by inhalation of an overdose of halothane. For the cisplatin study, animals were sacrificed 24, 48, and 72 h post dosing and following a 3-week treatment-free period. Blood samples were collected via cardiac puncture in heparinized tubes for clinical chemistry assessment and in EDTA tubes for proteomic analysis. The blood was then centrifuged at 1500 g at 4°C for 10 min, and the plasma was removed. For the proteomic samples, protease inhibitors were also added to plasma and all samples were snap frozen in liquid nitrogen prior to analysis. Plasma creatinine and blood urea nitrogen (BUN) were measured using standard procedures, with a Kone automated analyzer. A macroscopic examination was carried out postmortem for signs of toxicity in addition; the left kidney and a sample of liver and heart were removed and fixed in 10% neutral buffered formal saline. Following processing into wax, sections (5 mm) were stained with hematoxylin and eosin and examined by light microscopy.

Two-dimensional gel electrophoresis. Plasma samples were analyzed using the high-throughput 2D gel proteomics technology developed at OGS (Harris et al., 2002; Kennedy, 2001; Page et al., 1999). Plasma samples were initially enriched to remove the high abundance proteins, immunoglobulins (IgG), haptoglobin, transferrin, and albumin, using fast protein liquid chromatography (FPLC). This was achieved by passing the sample through a Hi-trap affinity chromatography column containing immobilized antibodies to each of the plasma proteins highlighted. Approximately 20 µl of each plasma sample was enriched using this method and 55 µg of protein was loaded on to an immobilized pH gradient (IPG) gel (Immobiline DryStrip 3–10 NL, Pharmacia Biotech, U.K.). Enriched samples were then separated by isoelectric focusing (IEF) in the first dimension initially at 300 V for 2 h, followed by a linear ramp from 300 V to 3500 V over 3 h and 3500 V for 19 h. After IEF, the strips were equilibrated for 10 min at 20°C in a solution of 6 M urea: 2% (w/v) DTT; 2% (w/v) SDS; 30% (v/v) glycerol; 0.05 M Tris/HCl, pH 6.8. The strips were then loaded onto supported 9–16% linear polyacrylamide gradient gels for SDS–PAGE. Samples were separated by apparent molecular weight (mw) in the second dimension by electrophoresis for 1 h at 20 mA/gel followed by approximately 5 h at 30 mA/gel at 16°C. Gels were subsequently fixed in 40% vol/vol ethanol:10% vol/vol acetic acid, stained with a fluorescent dye, and scanned using a digital scanner (Harris et al., 2002; Page et al., 1999).

Gel image analysis. One gel image was generated for each plasma sample. Individually resolved protein spots (features) were enumerated and quantified based on fluorescence intensity. Intensity was measured by summing the pixels within the boundary of each feature and represented as a percentage of the total gel image intensity. The protein content (number of features) detected in each gel image has been termed a protein expression map (PEM; Page et al., 1999) Primary images were initially processed using MELANIE III (GeneBio, Geneva, Switzerland) and checked using in-house software and human operators before incorporation into a database (master group) containing all the individual protein data points. In the final stage of processing features were grouped together into linked sets or “clusters,” according to the degree of overlap between them in each gel image. An mci (molecular cluster index) identifies a set of matched features on different images (gels). Thus “mci” represents a protein or proteins at equivalent positions in the 2D separation in different samples.

Mass spectrometry (MS) analysis. Protein features were excised from the gel using an automated robotic cutter and transferred into separate wells of a 96-well plate. Each protein sample was further processed by enzymatic digestion with trypsin to generate peptide fragments (Page et al., 1999). A mass list of peptides for each protein was obtained by using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. Fragmentation spectra from 1-Da mass window were recorded by using a nanospray ionization (Z-spray) source on a Q-TOF instrument (Micromass, Manchester, U.K.). The continuum fragmentation spectra were converted to centered spectra and used to search protein databases using the SEQUEST program. Confirmation of the peptide sequences was accepted when an ion series consistent with y-type fragmentation was observed for the complete peptide sequence (Page et al., 1999).

Proteome data analysis. Proteome analysis was performed using the Rosetta™ software on the 2D gel images of plasma samples taken from control and treated animals (Page et al., 1999). Each study was examined separately and after an initial comparison between control and treated images, further selection of proteins (mcis) was achieved by using RosettaTM to plot the abundance profile (percentage volume) of each protein, as shown in Figure 4. The percentage volume (% Vol) represents the fluorescence of a protein feature as a percentage of the total gel fluorescence for each image. Each protein was...
examined to ascertain if a dose and time response were evident or if the protein was consistently altered in the treatment groups. For the 4-AP study, PEMs containing the 2D gel digital image data for the plasma samples from each control F344 animal (4–24 h) were compiled together to create a group containing 15 images. The PEMs from the 80-mg/kg 4-AP-treated F344 animals (4–24 h) were also grouped together to form a second set of 15 images. The 80 mg/kg group was specifically chosen for analysis because this treatment produced the greatest toxicity at all time points (Table 1). A binary comparison was performed by comparing all the proteins in the control images with tissue repair evident after 3 weeks, consistent with the BUN and plasma creatinine data (Figs. 1a, 2a–c). At the 20 mg/kg dose of 4-AP, mild reversible vacuolation of some proximal tubular epithelial cells was seen at 4 and 8 h but not at later time points (data not shown).

A very similar pattern of toxicity was also observed following D-serine treatment, with elevated creatinine and BUN evident in the 250-mg/kg and 750-mg/kg groups between 4 and 24 h, followed by recovery after 3 weeks (Table 2, Fig. 1b). The pattern of tubular necrosis to the pars recta of proximal tubular epithelial cells in the cortex-medullary region. This was visible as an early degenerative change at 4 h, progressing by 8 h and 24 h to widespread coagulative necrosis with tissue repair evident after 3 weeks, consistent with the BUN and plasma creatinine data (Figs. 1a, 2a–c). At the 6 mg/kg cisplatin-treated group at 48 and 72 h post dosing (Table 3, Fig. 1c). Creatinine and BUN levels remained high after 3 weeks, suggesting poor repair of the original lesions, which was confirmed by microscopic examination of kidney tissue sections. At 72 h, necrosis and degeneration of tubular epithelial cells was apparent (Fig. 2e). By 3 weeks, tubular epithelial cells displayed karyomegaly and basophilic staining, with several tubules showing clear tubular dilatation (Fig. 2f).

No damage was observed in the transplatin-treated group at any of the time points examined (data not shown).

**Protocols and Methods**

Using the selection criteria defined in Materials and Methods, a total of 24 protein spots (mcis) were selected from all
three studies for identification by MS. Nine proteins were selected from the 4-AP study, and 11 and 4 from the D-serine and cisplatin studies, respectively. The identities of the proteins from each study, including the biochemical characteristics, are shown for 22 proteins in Table 4. One protein of interest from the 4-AP and D-serine study was identified as the cellular enzyme fumarylacetoacetate hydrolase (FAH) and is described elsewhere (Bandara et al., in press). Several isoforms of the rat-specific T-kininogen protein were identified, one in the 4-AP and cisplatin studies and six from the D-serine study (Table 4). All six T-kininogen proteins were located in the same region of the gel, resolving as a chain of proteins (Figs. 3a–c). Several isoforms of the inter-alpha-inhibitor H4P heavy chain (H4P) protein were also identified in the D-serine and 4-AP studies (Table 4). The H4P proteins were also located to a specific region of the gel and separated into a chain of proteins (Fig. 3a). Of further note was the identification of the retinol binding protein (RBP) in the D-serine study, which has previously been linked to renal toxicity. The regulation of RBP was treatment-specific, as changes were not observed in control animals or the L-serine-treated group (data not shown). In addition, a number of components of the immune system and proteins involved in tissue repair were also detected (Table 4). For mci 24986677 in the cisplatin study, two proteins may have colocalized in one mci position, as both apolipoprotein E and clusterin, were evident from the MS data. Since both proteins were potentially present, it was not possible to determine which one was regulated in response to compound treatment; therefore this mci was not examined further.

The six T-kininogen isoforms increased in the blood of D-serine-treated rats correlating with the extent of cellular damage (Figs. 3b and 3c). An example of the regulation of one isoform (mci 25914552) is shown in Figures 4a and 4b; however, all isoforms responded in a similar fashion (data not shown). T-kininogen levels peaked in the D-serine study at 24 h, correlating with the peak of kidney damage returning to baseline levels after the 3-week treatment-free period (Fig. 4a). T-kininogen was not elevated in control animals or those treated with L-serine (Fig. 4b). Two-factor ANOVA analysis for mci 25914552, comparing 750 mg/kg D-serine and control at all time points, showed significant effects with time and treatment \( (p < 0.01) \) and significant interaction \( (p < 0.01) \). Single-factor ANOVA analysis comparing all doses of D-serine with control at 24 h showed significant effects with
A comparison of D-serine and L-serine at 24 h and 750 mg/kg by the time of the Student’s t-test also showed a statistically significant difference in response ($p < 0.01$). A similar pattern of T-kininogen regulation was also apparent in the 4-AP study in both the F344 and the Alpk rat strains that was consistent with the observed renal damage (Figs. 4c–4e). Again, two-factor ANOVA analysis comparing 80 mg/kg 4-AP and control at all time points in F344 rats showed significant effects with time and treatment ($p < 0.05$). Single-factor ANOVA analysis comparing all doses of 4-AP with control at 24 h in F344 rats showed significant effects with treatment ($p < 0.05$). Two-factor ANOVA analysis comparing 80 mg/kg 4-AP and control at all time points in Alpk rats showed significant effects with treatment ($p < 0.01$) but not with time. However, there was significant interaction ($p < 0.01$). One isoform of T-kininogen was also elevated in cisplatin-treated animals, indicating a specific response to all three renal toxicants (Table 4). Taken together, these findings suggest that T-kininogen may be a general marker of renal proximal tubular toxicity in rodents as at least one isoform is elevated in each study.

Several isoforms of the inter-alpha inhibitor, H4P heavy chain protein, were also altered in the plasma, following either 4-AP or D-serine treatment (Table 4). The properties of one isoform from each study (mci 25918576 from D-serine and mci 24096335 from 4-AP) are shown in Figs. 5a–d. As with T-kininogen, H4P demonstrated time-dependent regulation at the top dose of each compound with peak expression 8 and
and L-serine compared at 24 h and 750 mg/kg by Student’s t-test showed significant effects with time and no interaction. The decrease in a-1-m appears to be specific to cisplatin treatment, particularly as this protein was not identified in the D-serine and 4-AP studies. However, it is possible that a-1-m performs a similar function as the proteins identified in the D-serine and 4-AP studies.

DISCUSSION

In an attempt to identify new markers and new insights into nephrotoxicity, we conducted a proteomic analysis of plasma samples taken from animals treated with a variety of renal toxicants. We selected 4-AP, D-serine, and cisplatin as each has been reported to specifically damage the proximal tubular epithelial cells in the rodent kidney (Davis et al., 1983; Ganote et al., 1974; Goldstein et al., 1981; Green et al., 1969; Kaltstein et al., 1982; Safrstein et al., 1981). Several doses and time points were examined in this study to support the validity of the proteins identified.

A number of protein features were identified from each study that were regulated in a dose-dependent and time-dependent manner, correlating with the extent of renal toxicity. Of particular interest was a series of isoforms of the T-kininogen protein, which were identified in all three studies. Three classes of kininogen have been reported in the literature, the high- and low-molecular-weight kininogens (H- and L-kininogen) are present in rodents and man, whereas T-kininogen is specific to rats (Greenbaum et al., 1992; Takano et al., 1997). Although all three kininogens are predominantly synthesized in the liver, they are alsosecreted into blood. The kininogens are substrates for the kallikrein serine proteases, which cleave the proteins to release bradykinin. Bradykinin is a well-known peptide hor-

| TABLE 2 |
| Plasma Creatinine Data from Serine Rat Treatment Studies |

<table>
<thead>
<tr>
<th>Time post-dose</th>
<th>Control</th>
<th>D-Serine</th>
<th>L-Serine</th>
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<tbody>
<tr>
<td>0</td>
<td>4 h</td>
<td>8 h</td>
<td>24 h</td>
</tr>
<tr>
<td>28 ± 6</td>
<td>24 ± 1</td>
<td>26 ± 5</td>
<td>27 ± 8</td>
</tr>
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<td>25 ± 5</td>
<td>24 ± 2</td>
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<td>24 ± 6</td>
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</tr>
<tr>
<td>750</td>
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</tr>
</tbody>
</table>

Note. D-serine treatments resulted in a dose-dependent increase in creatinine peaking at 24 h post dosing. Each data point represents the mean of 5 animals (n = 5), including the standard deviation.

*A statistically significant change when compared to the concurrent control animal, using Student’s t-test, p < 0.05.

**A significant change of p < 0.01.

24 h post treatment, correlating with the peak of tissue damage (Figs. 5a and 5c). In each case, increased H4P was apparent in the blood at the 24 h time point following treatment with different doses of either D-serine or 4-AP (Figs. 5b and 5d). H4P was not elevated in the plasma of control animals or those treated with L-serine, and the observations were further supported by ANOVA analysis. Two-factor ANOVA analysis comparing 750 mg/kg D-serine and control at all time points showed no significant effects with time or treatment but did show significant interaction (p < 0.05). Single-factor ANOVA analysis comparing all doses of D-serine with control at 24 h showed significant effects with treatment (p < 0.01). D-serine and L-serine compared at 24 h and 750 mg/kg by Student’s t-test showed a statistically significant difference in response (p < 0.05). Two-factor ANOVA analysis comparing 80mg/kg 4-AP and control at all time points in F344 rats showed significant effects with treatment (p < 0.01) but no effect with time and no significant interaction. Single-factor ANOVA analysis comparing all doses of 4-AP with control at 24 h in F344 rats showed significant effects with treatment (p < 0.05).

Thus, like T-kininogen, H4P appears to be a conserved marker of kidney toxicity induced by at least two kidney toxicants.

Fewer plasma proteins appeared to be regulated in the cisplatin study. Of note were two isoforms of alpha-1-macroglobulin (a-1-m), both of which were decreased following cisplatin treatment (Table 4). Both isoforms were reduced at the top dose of cisplatin (6 mg/kg) at 72 h and 3 weeks after treatment, consistent with the cellular damage (Figs. 6a and 6b). Furthermore, the levels of a-1-m in the transplatin-treated animals at these time points were similar to the saline-treated controls. For both mci’s, two-factor ANOVA comparing the response at 72 h and 3 weeks of control, 1, 2, and 6 mg/kg cisplatin showed a significant response with treatment (p < 0.05) but no significant change with time and no interaction. Similarly, two-factor ANOVA comparing the response at 72 h and 3 weeks of 6 mg/kg transplatin and 6 mg/kg cisplatin showed a significant response with treatment (p < 0.01) but no significance with time and no interaction. The decrease in a-1-m appears to be specific to cisplatin treatment, particularly as this protein was not identified in the D-serine and 4-AP studies. However, it is possible that a-1-m performs a similar function as the proteins identified in the D-serine and 4-AP studies.

TABLE 3
Plasma Creatinine Data from Cisplatin Rat Treatment Studies

<table>
<thead>
<tr>
<th>Time post-dose</th>
<th>Control</th>
<th>Cisplatin</th>
<th>Transplatin</th>
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<tbody>
<tr>
<td>24 h</td>
<td>27 ± 4</td>
<td>29 ± 3</td>
<td>29 ± 3</td>
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<tr>
<td>48 h</td>
<td>27 ± 3</td>
<td>26 ± 2</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>72 h</td>
<td>27 ± 2</td>
<td>31 ± 4</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>3 weeks</td>
<td>39 ± 3</td>
<td>41 ± 5</td>
<td>41 ± 2</td>
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</tbody>
</table>

Note. Although both 4-AP and D-serine treatments resulted in a dose-dependent increase in creatinine peaking at 24 h post dosing, for cisplatin, peak values were at 72 h and 3 weeks post dosing. Each data point represents the mean of 5 animals (n = 5), including the standard deviation.

*A statistically significant change when compared to the concurrent control animal, using Student’s t-test, p < 0.05.

**A significant change of p < 0.01.
monoe capable of a number of biological responses, including the regulation of local blood flow, smooth muscle activity, and the control of vascular permeability. Differential proteolysis of the L- and H-kininogens has also been noted: H-kininogen is specifically cleaved by plasma kallikrein and L-kininogens are preferentially processed by tissue kallikrein highlighting the complex mechanisms that exist to regulate bradykinin levels (Bhoola et al., 1992; Katori and Majima, 1996).

It has been suggested that T-kininogen is cleaved by a specific T-kininogenase to generate T-kinin, which shares many similar pharmacological properties with bradykinin (Barlas et al., 1987; Okamoto et al., 1983). In addition to the liver, T-kininogen is expressed in a wide variety of tissues including the kidney; therefore, it is possible that increased levels in the plasma could result from leakage from any one of these tissues (Hermann et al., 1996; Takano et al., 2000). Since the kidney is the main target of the three toxicants examined in this study, it seems likely that this tissue is the source of kininogen, particularly as we did not observe any cellular damage in heart and liver tissue (data not shown). The elevation of T-kininogen in the plasma by renal toxicants may have dual functions; for example, T-kininogen can inhibit cysteine proteases such as cathepsin B to block cellular damage and aid tissue repair. It is intriguing that cathepsin B is expressed in proximal tubular epithelial cells and has been shown to participate in apoptotic cell death in certain cases (Kugler, 1985; Yokota et al., 1986). Furthermore, we recently identified the cellular enzyme FAA as a marker of kidney damage in these studies and proposed that these toxicants may result in the accumulation of the toxic metabolite fumarylacetoacetate (FAA), which is known to cause apoptosis in tubular epithelial cells (Bandara et al., in press; Sun et al., 2000). If FAA-induced apoptosis is dependent on cathepsin B, it is tempting to suggest that increased levels of T-kininogen may be generated to inhibit apoptosis to protect the local environment from further tissue damage. Second, T-kininogen may also be generated in order to produce more T-kinin, which may in turn be required to initiate localized inflammation around the site of damage. Increased vascular permeability may also serve to reduce further cellular damage and to promote tissue repair.

Several isoforms of the inter-alpha inhibitor H4P heavy chain were also identified during our analysis. The inter-alpha inhibitor proteins are also synthesized in the liver and secreted into the blood where they form higher-order complexes with the protease inhibitor bikunin (Fries and Blom, 2000). However, it is currently unclear if H4P is also capable of forming complexes with bikunin, as H4P is sufficiently different from the related H1, H2, and H3 proteins in the region required for

<table>
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<th>Protein Identities from Rat Treatment Studies</th>
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<tbody>
<tr>
<td>mc</td>
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<tr>
<td>----</td>
</tr>
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<td>4-AP study</td>
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</table>

Note. The specific protein numeral identifier (mc) is shown in column 1 and the fold change with respect to the concurrent controls is indicated in column 2. The statistical significance of the fold change, as assessed using the Student’s t-test and associated p-values, is shown in column 3. The pI, molecular weight (mw), and the protein identity (annotation) are shown in columns 4–6, respectively.
binding bikunin (Soury et al., 1998). The inter-alpha inhibitors have also been detected in a wide variety of other organs including the kidney (Businaro et al., 1992). In the rat liver, H4P gene expression can be induced by acute inflammation in vivo, although this is less apparent in man (Daveau et al., 1998; Soury et al., 1998). The bikunin component within the inter-alpha inhibitor complex is thought to be required for the reported protease inhibitory activity (Fries and Blom, 2000). Of particular note is the reported inhibition of plasma kallikrein by bikunin (Morishita et al., 1994). To date it is not known if bikunin can inhibit the tissue kallikrein or the T-kininogenase; however, inhibition of T-kininogenase may be one explanation for the observed increase in plasma T-kininogen. Bikunin is also expressed in the rat kidney and has been shown to be induced in the kidney by calcium oxalate challenge in vivo (Iida et al., 1999). In this study, specific activation of bikunin RNA expression was detected in the kidney but not in the liver (Iida et al., 1999). Therefore, it is possible that 4-AP and D-serine may work in a similar manner to increase bikunin expression to regulate levels of T-kininogen. The H4P protein also appears to be sensitive to the plasma kallikrein degradation, suggesting a complex interplay between the kininogen and inter-alpha inhibitor pathways (Nishimura et al., 1995). The parallels between H4P and T-kininogen induction are striking: both proteins are synthesized in the liver and can be induced by a variety of agents. In addition, both are expressed in the kidney and have been linked with renal dysfunction. We would like to suggest that both pathways are connected and that renal toxins selectively elevate the levels of these proteins in the blood. However, at present we can only speculate on the specific molecular pathways that are involved.

Fewer dose- and time-dependent markers were evident in the cisplatin study. This may be due to the different kinetics observed for cisplatin toxicity and the possibility that the mechanism of action of cisplatin is distinct from D-serine and 4-AP. Of interest was the plasma levels of a-1-m that showed a consistent decrease following treatment with 6 mg/kg cisplatin. The exact function of the alpha macroglobulins in blood has not been clearly defined, although they are known to form tetrameric complexes in vivo (Sottrup-Jensen, 1989). The alpha macroglobulins are also known to inactivate a number of proteinases via binding and sequestering target proteins into inactive higher-order complexes. Although the macroglobulins appear to be broad-spectrum inhibitors, it has been suggested that they may function as backup inhibitors when primary inhibitors have been depleted (Sottrup-Jensen, 1989). As discussed already, we have noted the upregulation of a number of protease inhibitors as markers of renal toxicity; therefore, it would seem likely that a-1-m may be required for a similar function. The decrease in plasma levels of a-1-m can be explained by the removal of the macroglobulin-protease complex, which has been reported to occur via receptor-mediated endocytosis (Kaplan and Nielsen, 1979; Travis and Salvesen, 1983). It is possible therefore, that the molecular pathways exploited by cisplatin-induced toxicity differ subtly from 4-AP and D-serine, but ultimately they result in the same molecular response through inhibiting a group of proteases. Further studies will be required to confirm if these proteins regulate the kallikreins or apoptotic proteases.

We were encouraged by the identification of RBP in the D-serine study, since this protein has been previously associated with renal toxicity. Although RBP is required for the transport of vitamin A in blood, it is also reabsorbed and degraded in the renal proximal tubules (Goodman, 1977). Several reports have focused on the elevated presence of RBP in the urine of patients or animals with renal damage (Brouwer et al., 1988; 1989; Jung et al., 1993). We did not examine urine in our studies; however, we did note a down-regulation of RBP in the plasma of rats treated with D-serine. It is possible that this was not identified in other studies if the protein alteration was not statistically significant or if the protein profiles were not dose- or time-dependent. Nevertheless, the identification of
RBP is important as this confirms that new technologies such as proteomics can uncover established as well as novel markers of toxicity. Several isoforms of fibrinogen and complement C3 were also noted in our studies, highlighting the underlying contribution of the immune system to the toxic insult. It seems likely that these components are expressed to increase local inflammation and promote tissue repair, although the direct pathway from proximal tubular damage leading to alterations of these specific proteins is not clear.

In this initial report, we chose to analyze the blood compartment of rats treated with established nephrotoxins. Using traditional endpoints such as kidney histopathology and blood clinical chemistry analysis, we confirmed the extent of cellular damage at each dose and time point. We identified several plasma proteins that correlated with the severity of kidney damage, although it is difficult to conclusively establish if these proteins are released from the kidney or if they are produced in another organ in response to the toxic insult. Since our studies were designed to include multiple doses and time points using well-characterized toxicants that specifically damage the renal proximal tubules, we believe that these protein changes most likely reflect distinct damage to the kidney cortex. The specific combined alteration of these particular plasma proteins may distinguish renal toxicity from other forms of toxicity. Furthermore, it is also possible that this combination of proteins may specifically distinguish proximal tubular toxicity from other types of renal toxicity. However, further renal toxicants would need to be evaluated to support this concept. In future experiments it will be necessary to confirm that the changes reported here are specific to renal toxicity by monitoring the proteins following treatment with other organ-specific toxins. This may require, for example, the

**FIG. 4.** T-kininogen expression levels in the D-serine and 4-AP studies. The relative abundance of T-kininogen (mci 25914552) in plasma samples is displayed as a percentage of the total gel fluorescence (% Vol.). The histogram represents the average spot volume (n = 5) and error bars indicate the standard error of the mean (SEM). T-kininogen levels increase in plasma with the most prominent expression at 8 and 24 h, decreasing to control levels by 3 weeks (a). A treatment-related increase in expression is apparent with increasing doses of D-serine at the 24-h time point (b). T-kininogen (mci 24096396) levels show a similar temporal regulation in 4-AP-treated group with maximal expression at 24 h, disappearing by the 3-week time point in F344 rats (c). A dose-dependent increase is apparent in F344 rats with increasing doses of 4-AP at the 24-h time point (d). T-kininogen levels are also shown for the Alpk rat treated with 4-AP (e).
generation of isoform-specific reagents to T-kininogen or H4P for screening larger numbers of samples. This would also support the proteome data by confirming that these changes are demonstrable using alternative techniques such as Western blotting or enzyme-linked immunosorbent assays (ELISA). Antibody reagents of this type may be necessary for developing diagnostic assays for routine screening in the future. Alternatively, it may also be plausible to screen further samples by 2D PAGE. By monitoring the changes in all of the proteins identified and using pattern recognition software, it may be possible to classify samples as toxic or normal (Harris et al., 2002).

Our preliminary data points to an interesting and complex interconnecting pathway involving protease inhibitors and components of the immune system. Although it is possible to

FIG. 5. H4P expression in D-serine and 4-AP studies. The relative expression (abundance) of H4P in plasma samples is displayed as % Vol. The histogram represents the average spot volume ($n = 5$) and error bars indicate the standard error of the mean (SEM). H4P (mci 25918576) levels increased in plasma with the most prominent expression at 8 and 24 h, decreasing to control levels by 3 weeks (a). A clear treatment related increase in expression is apparent with increasing doses of D-serine at the 24-h time point (b). H4P (mci 24096335) levels show a similar temporal regulation in 4-AP treated group with maximal expression at 24 hr disappearing by the 3-week time point in F344 rats (c) with a dose response at 24 h (d).

FIG. 6. Alpha-1-macroglobulin expression in cisplatin study. The relative expression of a-1-mac in plasma samples is displayed as a % Vol. The histogram represents the average spot volume ($n = 5$) unless indicated otherwise, and error bars indicate the standard error of the mean (SEM). The decrease in a-1-m levels for mci24986627 is shown in (a) and for mci 24986618 in (b).
speculate on the potential mechanisms that may occur, further studies are required to confirm our hypothesis. Nevertheless, we conclude that proteomics is a powerful tool for identifying new molecular pathways and biomarkers of disease.

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system in the development of hypertension and approaches to new drugs based on this relationship. *Jpn. J. Pharmacol.* 70, 95–128.


