IN VIVO METABOLISM AND DISPOSITION OF THE NEPHROTOXICANT
N-(3,5-DICHLOROPHENYL)SUCCINIMIDE IN FISCHER 344 RATS

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ABSTRACT:

N-(3,5-Dichlorophenyl)succinimide (NDPS) was originally developed as an agricultural fungicide. Previous work indicated that NDPS-induced renal damage in rats is metabolism-dependent and that hydroxylated metabolites might be involved in the nephrotoxic response. In this study, the disposition and nephrotoxicity of [14C]NDPS at two time points (3 and 24 hr) and three doses (0.2, 0.4, and 0.6 mmol/kg) were examined in male Fischer 344 rats. At 3 hr, only approximately 6.0% of the administered dose (0.6 mmol/kg) had been excreted. Elimination was nearly complete by 24 hr, except at the highest dose. Urinary elimination far exceeded fecal elimination at all doses. The urinary metabolites were identified as N-(3,5-dichlorophenyl)succinic acid, N-(3,5-dichlorophenyl)-2-hydroxysuccinamic acid, N-(3,5-dichlorophenyl)-3-hydroxysuccinic acid, and N-(3,5-dichlorophenyl)malonamic acid. N-(3,5-Dichlorophenyl)-3-hydroxysuccinic acid had not been previously detected in vivo. The same metabolites were also detected in the feces, blood, liver, and kidneys of rats. In addition, two novel in vivo NDPS metabolites were detected in liver and kidney homogenates. These metabolites were tentatively identified as N-(3,5-dichlorophenyl)-2-hydroxy succinimide and N-(3,5-dichloro-4-hydroxyphenyl)succinic acid. Dose-dependent increases in blood urea nitrogen levels, diuresis, proteinuria, glucosuria, and covalent protein adducts correlated with increases in oxidative metabolism. Rapid NDPS metabolism could help explain the early onset of nephrotoxicity. These studies provide additional evidence for the importance of oxidative metabolism in NDPS-induced kidney damage.

NDPS1 (fig. 1) was initially developed as an agricultural fungicide (Fujinami et al., 1972). In spite of promising activity, subsequent testing revealed that NDPS was a nephrotoxicant in rats after either acute or chronic administration (Sugihara et al., 1975; Rankin, 1982; Barrett et al., 1983). After acute exposure, NDPS produces renal proximal tubular necrosis that is characterized by increased BUN levels, elevated kidney weights, diuresis, proteinuria, glucosuria, and decreased uptake of organic ions by renal cortical slices (Rankin, 1982). The onset of toxicity is rapid, and renal functional changes can be observed within 3 hr after administration of NDPS (Rankin et al., 1984). Although concerns regarding its toxicity have precluded the use of NDPS as a new agricultural chemical, it was proposed as a useful model nephrotoxicant (Sugihara et al., 1975). Therefore, a careful study of its in vivo disposition could contribute to our understanding of chemically induced nephrotoxicity.

The mechanism of NDPS-mediated nephrotoxicity remains unknown; however, hepatic biotransformation is required to produce kidney damage (Rankin et al., 1986, 1987, 1988; Nyarko and Harvison, 1995; Nyarko et al., 1997). After oral dosing, NDPS (0.4 mmol/kg) was converted to multiple urinary metabolites in rats (Okkawa et al., 1974). Less than 1% of the excreted radioactivity was present in urine as the parent compound. Five metabolites were separated by TLC, but approximately 20% of the total urinary radioactivity remained at the origin of the TLC plates. Using GC/MS, three of the resolved urinary metabolites were identified as NDPSA, DMA, and 2-NDHSA. The remaining two urinary metabolites, comprising approximately 34% of the resolved radioactivity, could not be fully characterized. Blood, tissues, and feces were not evaluated for the presence of NDPS metabolites. Thus, the in vivo metabolic profile of NDPS has not been fully elucidated. Furthermore, nothing is known about NDPS metabolism at early time points or with different doses.

The studies described in this report were therefore designed to reevaluate NDPS metabolism in vivo. [14C]NDPS was administered to male Fischer 344 rats at three different doses, i.e. 0.2 mmol/kg (nontoxic dose), 0.4 mmol/kg (threshold toxic dose), and 0.6 mmol/kg (toxic dose). The metabolic profile in urine, feces, blood, and several organ homogenates was determined by HPLC. Nephrotoxicity was assessed by measuring BUN levels, urine volume, and urine contents. Covalent binding of NDPS-derived radiolabel to liver and kidney proteins was also measured. NDPS was rapidly metabolized, which could help explain the early onset of nephrotoxicity. Furthermore, we found that kidney damage was correlated with a shift toward increased oxidative metabolism at higher doses of NDPS.

Materials and Methods

Materials and Animals. NDPS, [14C]NDPS (specific activity, 0.65 mCi/ mmol; radiochemical purity, 99%), and NDPS metabolites were synthesized and purified as previously described (Fujinami et al., 1972; Shih and Rankin,
1989; Griffin et al., 1996). β-Glucuronidase, glucose kit 510-A, urea nitrogen kit 640-A, and Ponceau S were obtained from Sigma Chemical Co. (St. Louis, MO). Sep-Pak cartridges were purchased from Waters (Milford, MA). Male Fischer 344 rats (150–175 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in stainless steel hanging cages, with a 12-hr light/dark cycle, at approximately 22°C and 45–50% relative humidity. Food (laboratory rodent diet 5001; PMI Foods, St. Louis, MO) and water were freely available unless otherwise noted. Rats were allowed a 1-week acclimation period before use in any experiments.

**Equipment.** HPLC analyses were conducted using a Beckman system with a model 421 controller, two model 114M pumps, and a model 165 variable-wavelength detector set at 254 nm. The detector sensitivity was set at 0.1 absorbance units full scale. Chromatograms were printed on a Hewlett Packard model HP 3396A integrator. Fractions were collected with an ISCO Retriever III fraction collector. 1H NMR analyses were conducted with a General Electric (Fremont, CA) GN-500 NMR spectrometer. Chemical shifts are reported in ppm downfield from tetramethylsilane.

**Animal Treatments.** Male Fischer 344 rats were randomly assigned to treatment groups (three or four rats/group). The rats were administered [14 C]NDPS (approximately 10 μCi, ip, in corn oil) at 0.2, 0.4, or 0.6 mmol/kg. Control animals received corn oil only (3 ml/kg). After administration of NDPS, the rats were immediately placed in plastic metabolism cages. The animals were housed in stainless steel hanging cages, with a 12-hr light/dark cycle, at approximately 22°C and 45–50% relative humidity. Food (laboratory rodent diet 5001; PMI Foods, St. Louis, MO) and water were freely available unless otherwise noted. Rats were allowed a 1-week acclimation period before use in any experiments.

**Sample Preparation.** Urine samples from each animal were divided into 0.5-ml aliquots for preparation. One aliquot of urine from each rat was precipitated with 1.5 ml of ice-cold acetonitrile and was then centrifuged at 2000 rpm. The supernatant was removed, passed through a C18 Sep-Pak cartridge, and frozen until HPLC analysis (see below). A second aliquot of urine from each rat was incubated with β-glucuronidase (15,000 units/ml) for 24 hr at 37°C and was then treated as described above. Feces (24 hr time point only) were weighed and homogenized in approximately 30 ml of 25 mM Tris buffer (pH 7.4). A sample was removed for liquid scintillation counting, and the remainder was frozen for later analysis. After thawing, a 2.0-ml aliquot of each fecal sample was precipitated with 6.0 ml of cold acetonitrile and then treated as the urine samples. The fecal pellet from the centrifugation was washed twice with 1.0 ml of acetone and repelleted. Finally, the pellet was digested in 2.0 ml of 1.0 M NaOH and 1.0 ml of dimethylsulfoxide, and the digest was counted to determine nonextractable radioactivity.

Blood (0.5 ml) was precipitated with 1.5 ml of cold acetonitrile and centrifuged at 2000 rpm, and the supernatant was removed and frozen for later HPLC analysis (see below). Total blood volumes in the animals were estimated using a formula (13.5 ml of blood/250 g of rat body weight) described by Davies and Morris (1993). The organs were weighed, thoroughly minced, rinsed with ice-cold 25 mM Tris buffer (pH 7.4) to remove blood, and then homogenized in 2–3 volumes of the same buffer. To determine tissue distribution and metabolite content, aliquots of the tissue homogenates were precipitated with 3 volumes of ice-cold acetonitrile and centrifuged at 2000 rpm for 20 min. The supernatant was then removed and stored in the freezer until HPLC analysis. Before and after protein precipitation, 25.0 μl of each sample was counted to determine the recovery of radioactivity (90% in blood, 100% in urine, and 80–100% in the tissues). Samples with low activity were concentrated under a flow of N2 at room temperature before analysis. Aliquots of the liver and kidney homogenates (24 hr time point only) were frozen for subsequent determination of covalent binding to proteins (see below).

**HPLC Analysis.** All samples (see above) were analyzed by the HPLC assay described previously (Griffin et al., 1996). The mobile phase (flow rate, 1.7 ml/min) consisted of a gradient between acetonitrile containing 3% tetrahy-
Results

Three known NDPS metabolites (NDPSA, 2-NDHSA, and DMA) and a previously unreported in vivo metabolite (3-NDHSA) were present in the 3-hr urine sample from a rat that had received 0.6 mmol/kg $^{14}$CNDPS (fig. 2). NDPS was not detected in urine. At this early time point, the total recovered urinary activity was 5.7 ± 1.8% of the administered dose. Collectively, the nephrotoxic metabolites 2-NDHSA and 3-NDHSA (Rankin et al., 1988, 1989) accounted for a significantly greater amount (58.7 ± 0.8%) of the urinary radioactivity than did NDPSA (34.9 ± 1.1%) (fig. 3). A small amount of radioactivity (approximately 4%) (fig. 3, Unk) eluted in the solvent front (2–6 min) and did not correspond to any distinct peaks on the UV chromatograms. To hydrolyze possible glucuronide conjugates, the urine sample was treated overnight with β-glucuronidase. However, there were no significant differences in the HPLC chromatograms (results not shown) or profiles of urinary metabolites before and after β-glucuronidase treatment (fig. 3).

Male Fischer 344 rats were administered $^{14}$CNDPS (approximately 10 μCi, 0.6 mmol/kg, ip in corn oil). Urine was collected for 3 hr and analyzed for the presence of metabolites by HPLC, as described in Materials and Methods. Separation was achieved with a Beckman C18 column, using a gradient of acetonitrile and water. Peaks were detected by UV absorbance at 254 nm. The column effluent was collected at 1-min intervals, and metabolites were quantitated by liquid scintillation counting. The absorbance scale was 0.1 absorbance units full scale.
Radioactivity in aliquots of the homogenized tissues was determined by liquid scintillation counting in corn oil) and were sacrificed 3 hr later. The tissues were removed and homogenized. Radioactivity in aliquots of the homogenized tissues was determined by liquid scintillation counting. Values represent means ± SE (N = 3 or 4 rats).

Male Fischer 344 rats were administered [14C]NDPS (approximately 10 µCi, 0.6 mmol/kg, ip in corn oil) and were sacrificed 3 hr later. The liver and kidneys were removed and homogenized. Aliquots of deproteinized homogenates were analyzed by HPLC. The column effluent was collected at 1-min intervals, and metabolites were quantitated by liquid scintillation counting. Values represent means ± SE (N = 3 rats).

Values that are significantly different (p < 0.05) from the corresponding results at a dose of 0.2 mmol/kg.

Values that are significantly different (p < 0.05) from the corresponding results at a dose of 0.4 mmol/kg.

TABLE 1

<table>
<thead>
<tr>
<th>Tissue Distribution 3 hr after NDPS administration</th>
<th>nmol/kg of tissue</th>
<th>% of administered dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidneys</td>
<td>1124.3 ± 148.2</td>
<td>1.53 ± 0.24</td>
</tr>
<tr>
<td>Liver</td>
<td>565.0 ± 106.6a</td>
<td>2.97 ± 0.58</td>
</tr>
<tr>
<td>Testes</td>
<td>191.3 ± 19.7e</td>
<td>0.45 ± 0.08b</td>
</tr>
<tr>
<td>Adrenal glands</td>
<td>167.5 ± 31.8e</td>
<td>0.009 ± 0.001b</td>
</tr>
<tr>
<td>Lungs</td>
<td>124.5 ± 29.8e</td>
<td>0.12 ± 0.02e</td>
</tr>
<tr>
<td>Heart</td>
<td>113.5 ± 13.8e</td>
<td>0.09 ± 0.02e</td>
</tr>
<tr>
<td>Brain</td>
<td>109.4 ± 14.9e</td>
<td>0.16 ± 0.03e</td>
</tr>
<tr>
<td>Total</td>
<td>1515.9 ± 134.9</td>
<td>5.33 ± 0.87</td>
</tr>
</tbody>
</table>

Values that are significantly different (p < 0.05) from the corresponding results in the kidneys.

NDPS (0.6 mmol/kg) tissue distribution was determined by measuring the amount of radioactivity in homogenates prepared from the major organs at 3 hr. Distribution (in nanomoles per gram of tissue) into the kidneys was significantly greater than that into the liver, testes, adrenal glands, lungs, heart, or brain (table 1). At this time point, we could account for 5.3 ± 0.9% of the total administered dose in the tissues that were examined, and the majority of this amount was present in the liver and kidneys (table 1). In fact, the levels of radioactivity in the hepatic and renal homogenates were high enough that we were able to quantitate the metabolites present (table 2). NDPSA was the major metabolite found in both tissues (approximately 40% of the total); however, a considerable amount of unmetabolized NDPS was also detected. In addition to the compounds found in plasma, we were able to detect two novel in vivo NDPS metabolites in the hepatic homogenates by liquid scintillation counting of the HPLC column effluent (table 2). Based on comparison of their retention times with those of synthetic standards, these metabolites were tentatively identified as NDHS and NDHPSA. Distribution of radioactivity (in nanomoles per gram) in the hepatic and renal homogenates (table 1) was comparable to (i.e. not significantly different from, p > 0.05) the total amount of NDPS metabolites present in each tissue as determined by HPLC (table 2).

Excretion and metabolism of [14C]NDPS (0.2, 0.4, and 0.6 mmol/kg) were also monitored for 24 hr after administration. The total recovery of radioactivity in urine, feces, liver, and kidneys at this time point was <100% at every dose (table 3) and was significantly lower at 0.6 mmol/kg NDPS than at 0.2 or 0.4 mmol/kg NDPS. Fecal excretion increased slightly with increasing dose, whereas total urinary recovery decreased sharply at the highest dose (table 3). Recovery of radioactivity in liver homogenates was significantly greater at 0.6 mmol/kg than at the lower doses. A similar increase in tissue radioactivity at the highest dose was also observed in the kidney homogenates, although the differences were not statistically significant (table 3). Distribution into liver and kidneys (177.7 ± 33.5 and 561.4 ± 176.1 mmol/g, respectively) was significantly greater at the highest dose than at the two lower doses (results not shown). In contrast to the results obtained at 3 hr, the amounts of radioactivity present in hepatic and renal homogenates 24 hr after administration of NDPS (0.6 mmol/kg) were too low for identification and quantitation of individual metabolites by HPLC.

The same four urinary metabolites that were detected in 3-hr urine samples were found in 24-hr urine samples (chromatograms not shown); however, the metabolic profile for NDPS was dose-dependent (fig. 4). For example, 2-NDHSA excretion was significantly increased at 0.6 mmol/kg, whereas the amount of 3-NDHSA remained constant at all three doses. Excretion of NDPSA was significantly less at the highest dose, compared with the lowest dose. Two unknown metabolites (fig. 4, Unk 1 and Unk 2) that eluted in the solvent front (2–6 min) or late in the chromatograms (>20 min) were also found in the 24-hr urine samples. The urinary elimination of these metabolites was unaffected by dose.

Analysis of NDPS metabolites in the feces was difficult. Approximately 25% of the total fecal radioactivity could not be recovered for HPLC analysis. The major fecal metabolite was 2-NDHSA; however, the amount excreted (10–30% of the total) was independent of the dose administered (results not shown). A significant amount of the parent compound (30–35% of the total) was present in the feces at all three doses.

Covalent binding of [14C]NDPS-derived radioactivity to hepatic and renal protein was also assessed at 24 hr. There was no detectable...
difference in binding between liver and kidney at any of the doses (fig. 5). Protein binding was dose-related in both tissues, with binding at the highest dose being significantly elevated, compared with that at the lowest dose.

1H NMR (500 MHz) spectra for the synthetic standards and known urinary metabolites were very similar (results not shown). Peak assignments for synthetic 3-NDHSA were as follows (values represent chemical shifts, in ppm downfield from tetramethylsilane): 2.8 (dd, 2H, CH₂), 4.58 (m, 1H, CH), 7.1 (s, 1H, Ar-H₄), and 7.9 (s, 2H, Ar-H₂₋₆). The following peaks were identified in the NMR spectrum of 3-NDHSA isolated from rat urine: 4.55 (m, 1H, CH), 7.1 (s, 1H, Ar-H₄), and 7.7 (s, 2H, Ar-H₂₋₆). The methylene resonance in the isolated 3-NDHSA sample was obscured by a residual water peak.

Compared with controls and the two lower doses of NDPS, BUN levels were significantly elevated 24 hr after administration of 0.6 mM/kg NDPS (table 4). Urine protein contents were also increased in a dose-dependent manner. NDPS (0.6 mM/kg) produced marked diuresis in the rats. Kidney weights were significantly elevated at the highest dose of NDPS (table 4). Liver weights, body weights, and urine glucose contents were not altered by NDPS at any of the doses used in these experiments (results not shown).

Discussion

NDPS was previously shown to be extensively metabolized when administered orally to rats; however, a substantial proportion (approximately 34%) of the urinary metabolites could not be identified (Ohkawa et al., 1974). Because NDPS must undergo initial cytochrome P450-mediated biotransformation in the succinimide ring to exhibit nephrotoxicity (Rankin et al., 1986, 1987, 1988; Nyarko et al., 1997), it is important to more fully characterize the metabolic fate of this compound in vivo. Furthermore, kidney damage can be observed within 3 hr after dosing (Rankin et al., 1984), although nothing is known about the metabolism of NDPS at early time points. In addition, NDPS nephrotoxicity exhibits a steep dose-response relationship (Rankin et al., 1985), whereas in vivo biotransformation of this compound has been evaluated only at a single dose (0.4 mM/kg) (Ohkawa et al., 1974). Therefore, the purpose of the experiments described in this report was to evaluate NDPS metabolism at an early time point and at several different doses.

Although only a small fraction (approximately 6%) of the dose was excreted 3 hr after administration of a nephrotoxic dose of [14C]NDPS (0.6 mM/kg), we were able to detect several metabolites in rat urine (fig. 2). In addition to the known in vivo metabolites NDPSA, 2-NDHSA, and DMA (Ohkawa et al., 1974), we found 3-NDHSA (a structural isomer of 2-NDHSA) (fig. 1) in the urine samples. Together, these four metabolites accounted for approximately 96% of the urinary radioactivity. 3-NDHSA was previously detected only in vitro (Nyarko and Harvison, 1995; Griffin et al., 1996; Nyarko et al., 1997), but it was probably one of the metabolites that was not fully characterized by Ohkawa et al. (1974). Assignment of this metabolite as 3-NDHSA was confirmed by NMR. The remaining radioactivity (approximately 4%) eluted from the HPLC column in the solvent front and was not clearly visible as a peak in the UV chromatograms. We are uncertain regarding the composition of this peak, although it may contain polar metabolites such as succinic acid or malic acid (Ohkawa et al., 1974; Nyarko and Harvison, 1995; Griffin et al., 1996). Overall, these results are important because they confirm that nephrotoxic metabolites, such as 2-NDHSA and 3-NDHSA (Rankin et al., 1988, 1989), can be generated from NDPS at an early time point, when toxicity initially occurs (Rankin et al., 1984). Thus, rapid NDPS biotransformation correlates with the early onset of renal damage.

Treatment of urine samples with β-glucuronidase had no effect on the urinary metabolic profile of NDPS (fig. 3), which suggests that glucuronide conjugates are not produced from this compound. These results are consistent with those of Ohkawa et al. (1974) and our previous in vitro experiments (Nyarko and Harvison, 1995). However, we cannot exclude the possibility that labile glucuronide conjugates may be produced from NDPS or one of its metabolites. For example, acyl glucuronides, which could be formed from any of the acidic NDPS metabolites, are prone to degradation and isomerization (Faed, 1984; Spahn-Langguth and Benet, 1992). Furthermore, Rankin et al. (1995, 1996, 1997) obtained indirect evidence that glucuronide or sulfate conjugates of NDHS or 2-NDHSA may contribute to the
nephrotoxic effects of NDPS. The formation of putative conjugative metabolites from NDPS and their potential roles in the toxicity of this compound require further study.

NDPS-derived radioactivity levels were highest in the kidneys and liver, which suggests that these organs may be important sites for distribution and metabolism at 3 hr (table 1). Furthermore, distribution into the kidneys (in nanomoles per gram) was greater than that into the other organs. This implies that the kidneys are able to accumulate NDPS or its metabolites, which could be an important factor in the selective nephrotoxicity of this compound. The results also suggest that the nephrotoxic species are very potent, because only a small percentage (approximately 1.5%) of the total NDPS dose could be accounted for in the kidneys at this time point.

We were able to detect metabolites in both liver and kidney homogenates 3 hr after NDPS dosing (table 2). Because metabolites were found in plasma and there was no evidence for NDPS biotransformation in vitro by renal preparations (Henessy and Harvison, 1995; Griffin et al., 1996), we believe that the metabolites were generated in the liver and were transported to the kidneys through the circulation. In addition to NDPSA, 2-NDHSA, 3-NDHSA, and DMA, we were able to detect small amounts of NDHS and NDHPSA in the hepatic homogenates. These two metabolites were previously detected only in plasma and urine (table 3). The induction of nephrotoxicity in the rats was confirmed by marked diuresis, as well as elevations in BUN levels, urine protein contents, and kidney weights (table 4). Similar changes in kidney function were previously reported for NDPS (Rankin, 1982; Rankin et al., 1984, 1985; Kellner-Weibel et al., 1995; Nyarko et al., 1997). The recovery of administered radioactivity in the urine was significantly lower at 0.6 mmol/kg NDPS than at the two lower doses (table 3). Comparable results were reported for acetaminophen (Newton et al., 1983b; Tarloff et al., 1989) and are probably indicative of renal failure. The 24-hr urinary metabolic profile was similar to that obtained in the 3-hr study (figs. 3 and 4), and we were able to account for 85–90% of the urinary radioactivity as NDPSA, 2-NDHSA, 3-NDHSA, and DMA at all three doses. However, at a dose of 0.6 mmol/kg there appeared to be more oxidative metabolism (i.e. 2-NDHSA production) than hydrolytic metabolism (i.e. NDPSA production). These findings are important because 2-NDHSA is more toxic than NDPS (Rankin et al., 1988), whereas NDPSA is less toxic (Yang et al., 1985). Therefore, a shift toward increased formation of a nephrotoxic metabolite such as 2-NDHSA with increasing dose could account for the dose-response relationship reported by Rankin et al. (1985) and may indicate that route A (fig. 1) predominates over route B at higher doses. We could account for approximately 70% of the radioactivity at 0.6 mmol/kg NDPS (table 3). The remainder may be sequestered in tissues (e.g. fat) that were not examined.

Covalent binding to proteins can indicate that a reactive, potentially toxic metabolite was generated in tissues (Pumford and Halms, 1997). In fact, selective covalent binding to renal proteins was previously reported for the potent nephrotoxicant para-aminophenol (Crowe et al., 1979). We found that NDPS-derived covalent binding increased with dose in both the liver and kidney; however, binding levels were not significantly different between the two tissues at any dose. In addition, binding paralleled the total tissue burden of radioactivity. This lack of organ selectivity may indicate that covalent binding does not contribute to NDPS-induced cytotoxicity in the kidney. However, it is possible that the critical molecular targets for covalent binding in the two organs are different (Cohen et al., 1997). A reassessment of covalent binding of NDPS to specific subcellular targets (e.g. mitochondria), rather than total protein binding, may be worthwhile.

In conclusion, we have demonstrated that NDPS metabolism occurs rapidly and may contribute to the early onset of nephrotoxicity that is observed with this compound. In addition, the extent of oxidative metabolism appears to be correlated with dose and increasing nephrotoxicity. These experiments have provided additional information on the correlation between NDPS-induced kidney damage and the in vivo biotransformation of this compound.