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The Role of Biotransformation and Oxidative Stress in 3,5-Dichloroaniline (3,5-DCA) Induced Nephrotoxicity in Isolated Renal Cortical Cells from Male Fischer 344 Rats

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Abstract

Among the mono- and dichloroanilines, 3,5-Dichloroaniline (3,5-DCA) is the most potent nephrotoxicant in vivo and in vitro. However, the role of renal biotransformation in 3,5-DCA induced nephrotoxicity is unknown. The current study was designed to determine the in vitro nephrotoxic potential of 3,5-DCA in isolated renal cortical cells (IRCC) obtained from male Fischer 344 rats, and the role of renal bioactivation and oxidative stress in 3,5-DCA nephrotoxicity. IRCC (~4 million cells/ml) from male rats were exposed to 3,5-DCA (0-1.0 mM) for up to 120 min. In IRCC, 3,5-DCA was cytotoxic at 1.0 mM by 60 min as evidenced by the increased release of lactate dehydrogenase (LDH), but 120 min was required for 3,5-DCA 0.5 mM to increase LDH release. In subsequent studies, IRCC were exposed to a pretreatment (antioxidant or enzyme inhibitor) prior to exposure to 3,5-DCA (1.0 mM) for 90 min. Cytotoxicity induced by 3,5-DCA was attenuated by pretreatment with inhibitors of flavin-containing monooxygenase (FMO; methimazole, N-octylamine), cytochrome P450 (CYP; piperonyl butoxide, metyrapone), or peroxidase (indomethacin, mercaptosuccinate) enzymes. Use of more selective CYP inhibitors suggested that the CYP 2C family contributed to 3,5-DCA bioactivation. Antioxidants (glutathione, N-acetyl-L-cysteine, α -tocopherol, ascorbate, pyruvate) also attenuated 3,5-DCA nephrotoxicity, but oxidized glutathione levels and the oxidized/reduced glutathione ratios were not increased. These results indicate that 3,5-DCA may be activated via several renal enzyme systems to toxic metabolites, and that free radicals, but not oxidative stress, contribute to 3,5-DCA induced nephrotoxicity in vitro.

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Conflict of Interest

The authors have no conflict of interest to declare.

Keywords

Nephrotoxicity; In Vitro; Rat; 3,5-dichloroaniline; oxidative stress

Introduction

Toxicity arising from exposure to anilines, important chemical intermediates used in the production of agricultural, industrial and pharmaceutical products (Lindh et al., 2007; Unger, 1996), has been well established. Exposure to aniline and its chlorinated derivatives has been associated with hematotoxicity (methemoglobinemia, hemolytic anemia; Chhabra et al., 1990; Guilhermino et al., 1998; Pauluhn et al. 2004; Valentovic et al., 1997), splenotoxicity (splenomegaly, elevated erythropoietic activity, hyperpigmentation, fibrosis; Chhabra et al., 1990; Khan et al., 1999; Ma et al., 2008, 2013), hepatotoxicity (hepatomegaly, elevated serum ALT/GPT levels, centralobular necrosis; Valentovic et al., 1995a, 1995b, 1992), and nephrotoxicity (Hong et al., 2000; Lo et al., 1990; Racine et al., 2014; Valentovic et al., 1995a). Chloroaniline induced nephrotoxicity in vivo is characterized by oliguria, decreased kidney weight, proteinuria, hematuria, elevated blood urea nitrogen (BUN) concentration, and decreased organic ion transport in the proximal tubule cells (Rankin et al., 1986; Lo et al., 1990; Valentovic et al., 1995a). Morphological changes occur in both the proximal and distal tubules and collecting ducts, with the greatest abnormalities seen in the proximal tubular cells. These morphological changes include blebbing and vacuolization of proximal tubular cells, occluded lumina with sloughed microvilli and enlarged lumina in the distal tubule (Lo et al., 1990). In vitro exposure of rat renal cortical slices to chloroanilines leads to a significant increase in cytotoxicity as seen by a decrease in organic ion accumulation and an increase in lactate dehydrogenase (LDH) release (Valentovic et al., 1992, 1995a, 1995b). Among the mono- and dichloroanilines, 3,5-dichloroaniline (3,5-DCA) was the most potent nephrotoxicant both in vivo and in vitro (Lo et al., 1990; Valentovic et al., 1995a, 1996).

Biotransformation via N-oxidation, N-acetylation, and phenyl ring oxidation are all known pathways of chloroaniline metabolism (Ehlhardt and Howbert, 1991; Hong and Rankin, 1998; Racine et al., 2014). Based on these studies a proposed biotransformation pathway of 3,5-DCA can be seen in Fig. 1. Two of these pathways (N-oxidation and phenyl ring oxidation) have the potential to lead to the formation of toxic metabolites from aniline compounds. For example, N-oxidation of aniline leads to toxic metabolites responsible for hematotoxicity (Harrison and Jollow, 1987, 1986) and splenotoxicity (Khan et al., 2000; Ma et al., 2013, 2008), while formation of the 4-aminophenol metabolite can contribute to nephrotoxicity (Harmon et al., 2005; Rankin et al., 1996; Tarloff et al., 1989). A small number of studies have shown that bioactivation can also contribute to the nephrotoxicity associated with some chloroanilines (Racine et al., 2014; Valentovic et al., 1995b). Putative metabolites of chloroanilines arising from N-oxidation or phenyl ring oxidation are also toxic to the kidney (Hong et al., 1997, 1996; Rankin et al., 2008a). Nonetheless, the enzyme systems responsible for the bioactivation of chloroanilines studied to date and the ultimate nephrotoxic metabolites formed from these compounds are not clearly defined.

Oxidative stress may contribute to the mechanism of cell death with aniline compounds. Harmon et al. (2005) found that oxidative stress played a role in 4-aminophenol-induced nephrotoxicity in vitro, while Hong et al. (1997) showed that in vivo 4-amino-2,6-dichlorophenol (ADCP), a putative metabolite of 3,5-DCA, increased the oxidized to reduced glutathione ratio in kidney, suggesting that ADCP induced renal oxidative stress. In addition, ADCP nephrotoxicity was prevented by pretreatment with antioxidants. However, it is unclear if oxidative stress plays a role in 3,5-DCA nephrotoxicity.

The purpose of this study was to explore, in more detail, the in vitro nephrotoxicity induced by 3,5-DCA. The in vitro cytotoxicity of 3,5-DCA was determined in isolated rat renal cortical cells (IRCC) from male Fischer 344 rats, and the role of renal metabolizing enzyme systems, including CYP isozymes, in the bioactivation of 3,5-DCA to nephrotoxic metabolites was also examined. Lastly, the role of free radicals and oxidative stress in 3,5-DCA-induced nephrotoxicity was explored. The IRCC model was selected for use because this model contains the target cells for 3,5-DCA and maintains metabolic and transport capabilities seen in vivo for several hours, including CYP activity (Cummings et al., 1999; Lash, 1998).

1. Materials and methods

2.1 Experimental Animals

Male Fischer 344 rats (200-250 g) from Hilltop Lab Animals, Inc. (Scottsdale, PA) were used for these experiments. All animals were kept in a controlled environment with a regulated light cycle (12 h on/12 h off), temperature (21-23°C), and humidity (40-55%) with food (Purina Rat Chow) and water available *ad libitum*. Rats were allowed at least one week to acclimate to the animal facilities prior to use in any experiments. Animal use for these experiments was approved by the Marshall University Institutional Animal Care and Use Committee, and animal use was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

2.2 Chemicals

All chemicals used were the highest purity available and were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.3 Isolated Renal Cortical Cell (IRCC) preparation and treatment

Untreated male rats were anesthetized with pentobarbital (75 mg/kg, ip) and isolated renal cortical cells (IRCC) were obtained using the collagenase perfusion method of Jones et al. (1979). Cell viability was initially determined by trypan blue (2% w/v) exclusion and lactate dehydrogenase (LDH) release. IRCC were counted and resuspended in Krebs-Henseleit buffer, pH 7.37, containing 25 mM Hepes and 2% (w/v) bovine serum albumin at a concentration of ~4.2 million cells/ml. IRCC (3 ml) were added to 25 ml polycarbonate Erlenmeyer flasks for a 5 min pre-incubation period in a Dubnoff shaking water bath incubator (37°C, 60 cycles/min) under an atmosphere of 95% oxygen and 5% carbon dioxide. Cells were exposed to various concentrations of 3,5-dichloroaniline (3,5-DCA; 0.5 or 1.0 mM) or vehicle (DMSO) for 60, 90 or 120 min. After the incubation period, flasks

were removed, placed on ice, and samples (0.5 ml) taken for lactate dehydrogenase (LDH) release assays. Briefly, samples were centrifuged (3000×g, 3 min), the supernatant was decanted and saved, and the pellet was disrupted with 1 ml of 10% Triton X-100 solution to release cellular LDH. LDH activity was then determined in each fraction as previously described using a kinetic assay based on the amount of NADH produced from NAD (Rankin et al., 2008b). LDH released into the media was expressed as the percent of total (media + pellet).

In separate experiments, IRCC were pretreated with either an antioxidant or an enzyme system inhibitor (Table 1) before exposure to 1.0 mM 3,5-DCA for 90 min, to determine the role of biotransformation of 3,5-DCA on cytotoxicity. Males were chosen for these experiments because our previous studies were performed in male rats (Lo et al., 1994; Valentovic et al., 1995b). All pretreatment times and concentrations were based on previously published studies (Baliga et al., 1998; Harleton et al., 2004.; Harmon et al., 2006, 2005; Katsuda et al., 2010; Lau and Monks, 1987; Lock et al., 1993; O'Brien and Siraki, 2005; Rodriguez and Acosta, 1997; Suzuki and Sudo, 1990; Valentovic et al., 1999).

2.4 Glutathione determination

Following treatment with 3,5-DCA (0.5 or 1.0 mM) or vehicle (DMSO) for 60 or 90 min, IRCC (2.0 ml; 4.2 million cells/ml) were homogenized in 0.5% sulfosalicylic acid (250 µl). Using a glutathione and NADPH coupled reaction; total glutathione (GSH) was determined as previously described (Anderson, 1985; Griffith, 1980; Valentovic et al., 2004). To measure glutathione disulfide (oxidized glutathione; GSSG) 2-vinylpyridine derivation was used as described by Griffith (1980). The ratio of oxidized/reduced GSH was then determined and all results were expressed as percent of control for each set of experiments.

2.5 Protein carbonyl measurement

Protein carbonyl levels were measured after exposure to vehicle (DMSO) or 3,5-DCA (0.5, 1.0 mM) for 60 or 90 min as previously described by Terneus et al. (2007). Briefly, a sample from treated IRCC (0.5 ml) was homogenized in 5× volume Krebs buffer, and total protein was determined using a Coomassie blue spectrophotometric method (Bradford, 1976). Protein OxyBlot kits (Millipore) were utilized to determine protein carbonyl levels following the manufacturers recommended protocol. The assay utilizes a 2,4-dinitrophenylhydrazine (DNP) specific antibody to detect DNP moiety on proteins. DNP moieties arise from the reaction of carbonyl side chains formed under oxidative stress conditions with 2,4-dinitrophenylhydrazine (Terneus et al., 2007).

2.4 Statistics

Data for LDH release is presented as mean ± S.E.M. with an N = 4 separate isolation experiments. Data for oxidized/reduced GSH and Oxyblot experiments are presented as mean percent of control ± S.E.M. with an N = 4 experiments. Data was analyzed by a one-way analysis of variance followed by a Student-Newman-Keuls Test using GraphPad Prism 6.0. Statistical significance was determined at $P < 0.05$.

2. Results

3.1 Time and concentration cytotoxicity studies

To determine the nephrotoxic potential of 3,5-DCA in IRCC, concentration response curves were performed at 60, 90 or 120 min. Cytotoxicity, as determined by increased LDH release, was seen at 90 and 120 min at a 3,5-DCA concentration of 1.0 mM. Cytotoxicity was also seen with 0.5 mM 3,5-DCA, but only at the 120 min exposure time (Fig. 2). Based on these observations, the 3,5-DCA concentration selected for further study was 1.0 mM with an exposure time of 90 min.

3.2 Effects of flavin-containing monooxygenase (FMO), cyclooxygenase, or peroxidase inhibition

To determine the role of FMOs in 3,5-DCA bioactivation, IRCC were pretreated with the FMO inhibitors methimazole or N-octylamine. The cyclooxygenase activity of prostaglandin H synthase was inhibited with indomethacin, while general peroxidase activity was inhibited with mercaptosuccinate. All pretreatments significantly attenuated 3,5-DCA induced cytotoxicity (Fig. 3).

3.3 Effects of nonselective cytochrome P450 (CYP) inhibition

The role of CYPs in 3,5-DCA induced cytotoxicity was first examined by using the nonselective CYP inhibitors piperonyl butoxide [PiBx], and metyrapone. Results showed PiBx and metyrapone both significantly attenuated 3,5-DCA 1.0 mM induced cytotoxicity (Fig. 4).

3.4 Effects of isozyme selective CYP inhibition

Based on the results from studies using nonselective CYP inhibitors, further studies were designed to explore the role of select renal CYP isozymes on 3,5-DCA induced cytotoxicity. To determine the role of CYP3A, CYP2E1, and CYP2B1/2 isozymes in 3,5-DCA bioactivation, oleandomycin triacetate, isoniazid, and thio-tepa were used to inhibit CYP3A, CYP2E1, and CYP2B1/2 isozymes, respectively. Cytotoxicity induced by 3,5-DCA was not significantly attenuated by any of these pretreatments (data not shown). To determine the role of the CYP2C family in 3,5-DCA bioactivation, the effects of three CYP2C family inhibitors (omeprazole, diethyldithiocarbamate (DEDTCA), and sulfaphenazole) on 3,5-DCA cytotoxicity were examined. Results show that inhibition of the CYP2C family significantly reduced cytotoxicity (Fig. 5).

3.5 Effects of antioxidants on 3,5-DCA cytotoxicity

The effect of pretreating IRCC with antioxidants on 3,5-DCA cytotoxicity was also examined to explore whether oxidative stress or free radicals played a significant role in 3,5-DCA (1.0 mM) induced cytotoxicity. Results showed all five antioxidants (N-acetyl-L-cysteine, α -tocopherol, glutathione, ascorbate and pyruvate) significantly attenuated 3,5-DCA cytotoxicity (Fig. 6).

3.6 Determination Of Oxidative Stress Following Exposure To 3,5-DCA

Since antioxidant pretreatment significantly attenuated cytotoxicity, studies were designed to determine whether oxidative stress following exposure to 3,5-DCA played a role in cytotoxicity. The presence of oxidative stress was determined by examining the ratio of oxidized/reduced glutathione (GSH) after exposure to 3,5-DCA (0.5 and 1.0 mM) at 60 and 90 min. These results were then correlated with the appearance of cytotoxicity. Results showed no significant changes in the ratio of oxidized/reduced GSH at any 3,5-DCA concentration or time point (Fig. 7), even though some cell death was occurring with the 1.0 mM concentration at the 90 min time point (Fig. 2). However, total GSH and reduced GSH were significantly reduced following exposure to 3,5-DCA at both concentrations (0.5 mM and 1.0 mM) and times (60 min, 90 min). Oxidized GSH was unchanged at 60 min and was significantly reduced at 90 min with both concentrations of 3,5-DCA (0.5 mM and 1.0 mM). (Fig. 7)

Protein carbonyl levels were examined as a second marker of oxidative damage to determine if oxidative stress was a causative factor in cell death. Protein carbonyl levels were only significantly increased following exposure to 1.0 mM 3,5-DCA after 90 min (Fig. 8), a time when cell death was evident (Fig. 2). Thus, oxidative damage appears to be secondary to the causative mechanism of cell death.

3. Discussion

Human exposure to 3,5-DCA can occur in a variety of settings, including during the manufacture of 3,5-DCA-based pesticides (e.g. iprodione, vinclozolin), dyes, etc. and through environmental exposure (e.g. application of pesticides, accumulation in waste water). Human exposure data to 3,5-DCA is extremely limited and blood levels of 3,5-DCA following pesticide or 3,5-DCA exposure are not reported for humans. Exposure to vinclozolin during manufacture has been monitored by measuring 3,5-DCA levels in the urine, which can be as high as ~1.4 mg/g creatinine (Zober et al., 1995). However, these levels have not resulted in harmful renal effects. Thus, nephrotoxicity in humans would most likely occur only through accidental poisoning, as is seen for aniline (Gosselin et al., 1984). A single nephrotoxic dose of 3,5-DCA in Fischer 344 rats (0.8 mmol/kg, ip) would result in an estimated blood level of 1.25 mM (Lo et al., 1990), a concentration 2.5 times higher than the minimal nephrotoxic concentration used in this study. However, whether the human kidney would be exposed to these minimal in vitro nephrotoxic concentrations of 3,5-DCA is unclear at this time.

This study was the first to show the importance of renal biotransformation in 3,5-DCA induced cytotoxicity. Inhibition of FMO, CYP and peroxidase activity all attenuated cytotoxicity suggesting that a toxic metabolite is formed following exposure to 3,5-DCA and that multiple enzyme systems are capable of bioactivating 3,5-DCA and/or its metabolites (Fig. 1). Antioxidants also offered protection from 3,5-DCA induced cytotoxicity, suggesting that free radicals are involved in the mechanisms of 3,5-DCA bioactivation and/or cytotoxicity. If the parent compound alone was responsible for cytotoxicity, inhibition of biotransformation systems would lead to either no changes in cytotoxicity or an increase in toxicity if one of the pathways inhibited was a major detoxifying mechanism.

Thus, renal bioactivation of 3,5-DCA contributes to 3,5-DCA induced cytotoxicity in an IRCC model.

N-Oxidation, *N*-acetylation, and phenyl ring oxidation are three primary routes of chloroaniline biotransformation (Ehlhardt and Howbert, 1991; Hong and Rankin, 1998). Based on these observations and the biotransformation of other chloroanilines in rats, a proposed biotransformation pathway for 3,5-DCA is shown in Fig. 1. Of these potential biotransformation pathways, it is known that chloroacetanilides, which arise from *N*-acetylation catalyzed via *N*-acetyltransferase enzymes, possess much reduced nephrotoxic potential compared to the parent chloroanilines (Rankin et al., 1993, 1995). Therefore, it is unlikely that *N*-acetylation of 3,5-DCA to 3,5-dichloroacetanilide (3,5-DCAA) would contribute to the renal cytotoxicity of 3,5-DCA. *N*-Acetyltransferase inhibitors were not evaluated for their ability to reduce 3,5-DCA nephrotoxicity for this reason. However, studies with 3,5-DCAA are required to confirm its renal effects and role in 3,5-DCA nephrotoxicity.

Another purported metabolic pathway that could contribute to 3,5-DCA induced nephrotoxicity is *N*-oxidation. *N*-Oxidation of aromatic amines is known to be catalyzed by multiple enzymes including CYPs, FMOs, prostaglandin H synthase, and peroxidases (Bakkenist et al., 1981; Corbett et al., 1980; Golly and Hlavica, 1985; McMillan et al., 1990; Ochiai et al., 2006; Sun et al., 2007; Yanni et al., 2010). *N*-Oxidation of 3,5-DCA would lead to 3,5-dichlorophenylhydroxylamine (3,5-DCPHA) (Fig. 1), whose nephrotoxic potential is yet to be determined. However, it would not be surprising if 3,5-DCPHA contributed to 3,5-DCA induced nephrotoxicity because 3,4-dichlorophenylhydroxylamine is a known nephrotoxicant in vitro using a rat renal cortical slice model (Valentovic et al., 2001). The results of the current study indicate that multiple enzymatic systems that catalyze *N*-oxidation can contribute to 3,5-DCA bioactivation, and that *N*-oxidation is a potential pathway leading to one or more ultimate, cytotoxic 3,5-DCA metabolites.

Further oxidation of 3,5-DCPHA would result in 3,5-dichloronitrosobenzene, which can be oxidized to 3,5-dichloronitrobenzene (3,5-DCNB). 3,5-DCNB has been shown to reduce renal gluconeogenesis at 1.0 mM and increase LDH release at 2.0 mM or higher concentrations after a 90 min exposure in renal cortical slices from Fischer 344 rats (Hong et al., 2002). It is unlikely that 3,5-DCNB is the ultimate toxic species following 3,5-DCA 1.0 mM, since a sufficient concentration of 3,5-DCNB to cause LDH release could not be reached with the 3,5-DCA concentration used in the current study. However, *N*-hydroxyl and nitroso compounds have been shown to alter cellular function by covalently binding to cellular nucleophiles (e.g. GSH, thiols of proteins, etc.) or redox cycling to produce reactive oxygen species, leading to increased cell death (Eyer and Ascherl, 1987; Kiese and Taeger, 1976; Stiborová et al., 1992; Umbreit, 2007; Valentovic et al., 1997). Therefore, any 3,5-dichloronitrosobenzene formed from 3,5-DCPHA could be contributing to 3,5-DCA cytotoxicity.

Aromatic ring oxidation is another potential biotransformation pathway that could contribute to 3,5-DCA bioactivation. CYPs can catalyze aromatic ring oxidation, and since CYP inhibition reduces 3,5-DCA cytotoxicity (Fig. 4), production of phenolic metabolites of 3,5-

DCA could be an important contributing factor in 3,5-DCA nephrotoxicity. Oxidation of the aromatic ring can lead to two possible metabolites, 2-amino-4,6-dichlorophenol and 4-amino-2,6-dichlorophenol (Fig. 1). While not much is known about 2-amino-4,6-dichlorophenol nephrotoxicity, 4-amino-2,6-dichlorophenol has been shown to be a potent nephrotoxicant both *in vivo* and *in vitro* (Hong et al., 1997; Rankin et al., 1994, 2008a; Valentovic et al., 1997). Interestingly, addition of a chloro group to the 4-position of 3,5-DCA to form 3,4,5-trichloroaniline produces a 3,5-DCA derivative without the ability to form significant amounts of 4-amino-2,6-dichlorophenol. However, 3,5-DCA and 3,4,5-trichloroaniline have equal nephrotoxic potential at 90 min, and 3,4,5-trichloroaniline is more potent as a nephrotoxicant than 3,5-DCA at 120min in IRCC (Racine et al., 2014). Thus, although 4-amino-2,6-dichlorophenol is a nephrotoxicant, it does not appear to be the ultimate nephrotoxic metabolite arising from 3,5-DCA *in vitro*. Studies with 2-amino-4,6-dichlorophenol are ongoing to determine its nephrotoxic potential. Thus, the role of aminophenol metabolites in 3,5-DCA cytotoxicity remains to be fully determined, but oxidation at the 4-position of 3,5-DCA does not appear to be a critical bioactivation pathway.

Since the general CYP inhibitors (piperonyl butoxide and metyrapone) were able to significantly attenuate cytotoxicity, further studies were conducted looking at the role of selective CYP isozymes which are found in the kidney. Cummings et al. (1999) found CYP2E1, CYP2C11, CYP2B1/2, and CYP4A2/3 in freshly isolated rat proximal and distal tubular cells. CYP2E1 expression was higher in distal tubular cells than proximal tubular cells, while CYP2C11 was higher in proximal tubular cells than distal tubular cells. CYP3A1/2 was not detected in the proximal tubular cells but was found in total kidney homogenate, which may indicate why oleandomycin, a CYP3A inhibitor, was not effective in attenuating 3,5-DCA cytotoxicity. The inability of thio-tepa (CYP2B inhibitor) and isoniazid (CYP2E inhibitor) to attenuate 3,5-DCA cytotoxicity, suggests that these CYPs are not critical for 3,5-DCA bioactivation. Of the selective CYP inhibitors we used, only sulfaphenazole, omeprazole, and diethyldithiocarbamate (DEDTCa) were able to attenuate 3,5-DCA cytotoxicity. These three inhibitors all show a preference to inhibit the 2C family of rat isozymes (Eagling et al., 1998; Kobayashi et al., 2003), suggesting that the 2C family may play a role in the bioactivation of 3,5-DCA. The CYP2C family in rats facilitates *N*-hydroxylation, as well as aromatic ring oxidation (Cribb et al., 1995), which supports one or both of these pathways as contributing to 3,5-DCA bioactivation.

Both *N*-hydroxylation and aromatic ring oxidation can lead to an increase in free radicals: either as metabolites undergoing redox cycling or directly from oxidation during metabolism (Harmon et al., 2006; Michail et al., 2013), and *N*-hydroxyl, *N*-nitroso and aminophenol metabolites can induce cell death via oxidative stress mechanisms (Harmon et al., 2005; Lock et al., 1993; Umbreit, 2007; Valentovic et al., 1997). Antioxidant pretreatment proved to be highly effective in attenuating 3,5-DCA cytotoxicity, with all antioxidants offering protection, suggesting that free radicals may play a role in cytotoxicity. Oxidative stress was measured by looking at the ratio of GSSG/GSH and increases in protein carbonyl levels. If oxidative stress played a significant role in the mechanism of cellular death, an increase in the GSSG/GSH ratio should occur prior to cytotoxicity, as seen with compounds such as

para-aminophenol (Harmon et al., 2005). However, in the case of 3,5-DCA, there was no significant increase in the GSSG/GSH ratio, and the significant increase in protein carbonyl levels only occurred after there was an increase in cytotoxicity. These data suggest that oxidative stress is not responsible for cell death in 3,5-DCA induced nephrotoxicity in vitro, and that the antioxidants may be offering protection, at least in part, by scavenging one or more radical metabolites produced during the metabolism of the amino group or an aminophenol metabolite (e.g. 2-amino-4,6-dichlorophenol) (Fowler et al., 1991, 1993; Job et al., 1978).

Total glutathione levels decreased following 3,5-DCA exposure, even though the GSSG/GSH ratio didn't significantly change (Fig. 7). In the absence of oxidative stress, it is likely that the reduction in total glutathione levels was due to the formation of a reactive 3,5-DCA metabolite(s) that was(were) detoxified by reaction with reduced glutathione. Both addition of GSH and N-acetyl-L-cysteine, which protect cells by being converted to GSH (Lauterburg et al. 1983), to IRCC attenuated 3,5-DCA cytotoxicity. Thus, at least part of the mechanism of protection from GSH could come from reacting with a reactive 3,5-DCA metabolite. It is also possible that the reduction of the GSSG levels seen at 90 min was an effort to restore GSH concentrations to a level offering cellular protection. However, further studies are needed to more clearly determine the role(s) of glutathione in attenuating 3,5-DCA cytotoxicity.

4. Conclusions

The results of this study determined that, as in rat renal cortical slices, 3,5-DCA is toxic to IRCC in a time- and concentration-dependent manner. Results also suggest that 3,5-DCA is potentially bioactivated in the kidney via several biotransformation pathways and that the CYP2C family is the major contributing renal CYP family for bioactivation. In addition, oxidative stress does not appear to play a significant role in the causative mechanism of 3,5-DCA cytotoxicity. While these studies provide insight into the role of biotransformation enzymes in bioactivating 3,5-DCA to nephrotoxic metabolites, studies are underway to determine the ultimate toxicant(s) in 3,5-DCA induced nephrotoxicity.

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Abbreviations

3,5-DCA	3,5-dichloroaniline
IRCC	isolated renal cortical cells
LDH	lactate dehydrogenase
ALT	alanine aminotransferase
GPT	glutamic-pyruvic transaminase

GSH	glutathione
GSSG	oxidized glutathione
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenylhydrazine
FMO	flavin-containing monooxygenase
CYP	cytochrome P450
PiBx	piperonyl butoxide
DEDTCa	diethyldithiocarbamate
ASC	ascorbate
NAC	N-acetyl-L-cysteine
3,5-DCAA	3,5-dichloroacetanilide
3,5-DCPHA	3,5-dichlorophenolhydroxylamine
3,5-DCNB	3,5-dichloronitrobenzene

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Figure 1.

Proposed Renal Biotransformation Pathway of 3,5-DCA. Abbreviations: CYP=Cytochrome P450, FMO=Flavin-containing Monooxygenase, NAT= N-acetyltransferase.

Figure 2. 3,5-DCA induced cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats following exposure for 60, 90 and 120 min. Each bar represents the mean $\pm$ S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P < 0.05$.

Figure 3.

Effects of FMO, cyclooxygenase, and peroxidase inhibitor pretreatment (PreTX) on 3,5-DCA cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats after 90 min. Each bar represents the mean $\pm$ S.E.M. for N=4-5 separate isolation experiments.

*Significantly different from DMSO control, $P < 0.05$. ▽Significantly different from the 1.0 mM 3,5-DCA value, $P < 0.05$.

Figure 4.

Effects of nonselective cytochrome P450 inhibitor pretreatment (PreTX) on 3,5-DCA cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats after 90 min. Each bar represents the mean $\pm$ S.E.M. for N=4-5 separate isolation experiments.

*Significantly different from DMSO control, $P < 0.05$. ∇ Significantly different from the 1.0 mM 3,5-DCA value, $P < 0.05$.

Figure 5.

Effects of 2C isozyme selective cytochrome P450 inhibitor pretreatment (PreTX) on 3,5-DCA cytotoxicity in isolated renal cortical cells obtained from male Fisher 344 rats after 90 min. Each bar represents the mean $\pm$ S.E.M. for N=4-5 separate isolation experiments.

*Significantly different from DMSO control, $P < 0.05$. ∇Significantly different from the 1.0 mM 3,5-DCA value, $P < 0.05$.

Figure 6.

Effects of antioxidant pretreatment (PreTX) on 3,5-DCA cytotoxicity in isolated renal cortical cells obtained from male Fischer 344 rats after 90 min. Each bar represents the mean $\pm$ S.E.M. for N=4-5 separate isolation experiments. *Significantly different from DMSO control, $P < 0.05$. ▽Significantly different from the 1.0 mM 3,5-DCA value, $P < 0.05$.

Figure 7.

The oxidized/reduced glutathione (GSSG/GSH) ratio following exposure to 3,5-DCA for 60 (A) or 90 (B) min. Total, oxidized, and reduced GSH following exposure to 3,5-DCA for 60 (C) and 90 (D) min. Each bar represents the percent control $\pm$ S.E.M. for N=4-6 separate isolation experiments. *Significantly different from DMSO control, $P < 0.05$.

Figure 8.

Figure shows Oxyblot data following exposure to 3,5-DCA for 60 (A) and 90 (B) minutes.

Each bar represents the percent control $\pm$ S.E.M. for N=4-6 separate isolation experiments.

*Significantly different from DMSO control, $P < 0.05$.

Table 1

List of antioxidants and enzyme inhibitors with primary targeted enzyme systems. NS = nonselective, PiBx = Piperonyl butoxide, DEDTCA = diethyldithiocarbamate

Pretreatment (PreTx) Compound	PreTx Conc.	PreTx Time (min)	Mechanism or Enzyme Inhibited	Reference
N-Acetyl-L-cysteine	2.0mM	30	Antioxidant	Katsuda et al. 2010
α -Tocopherol	1.0mM	5	Antioxidant	Suzuki et al. 1990
Glutathione	1.0mM	30	Antioxidant	Lau et al. 1987
Ascorbate	2.0mM	5	Antioxidant	Lock et al. 1993
Pyruvate	1.0mM	15	Antioxidant	Harmon et al. 2006, 2005
Methimazole	1.0mM	30	FMO	Rodriquez et al. 1997
N-Octylamine	2.0mM	5	FMO	Rodriquez et al. 1997
Indomethacin	1.0mM	15	Cyclooxygenase	Lau et al. 1987
Mercaptosuccinate	0.1mM	15	Peroxidase	Racine et al. 2014
PiBx	1.0mM	15	NS CYP	Baliga et al. 1998
Metirapone	1.0mM	5	NS CYP	Lock et al. 1993
Oleandomycin triacetate	0.5mM	30	CYP3A1/2	Racine et al. 2014
Thio-tepa	0.1 mM	5	CYP2B1/2	Harleton et al. 2004
Isoniazid	1.0mM	5	CYP2E1	Racine et al. 2014
DEDTCA	0.1mM	30	CYP2C > CYP2E1	Eagling et al. 1998
Omeprazole	0.01mM	30	CYP2C	Racine et al. 2014
Sulfaphenazole	0.1mM	30	CYP2C	Eagling et al. 1998, Kobayashi et al. 2003