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3,4-Dichlorophenylhydroxylamine cytotoxicity in renal cortical slices from Fischer 344 rats

Monica Valentovic *, John G. Ball, Syam Stoll, G.O. Rankin

Department of Pharmacology, Marshall University School of Medicine, 1542 Spring Valley Drive, Huntington, WV 25704-9388, USA

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Abstract

3,4-Dichlorophenylhydroxylamine (3,4-CPHA) is the *N*-hydroxyl metabolite of 3,4-dichloroaniline. 3,4-Dichloroaniline is a breakdown product of the herbicide Propanil. Previous work has shown that 3,4-dichloroaniline is acutely toxic to the kidney and bladder. The purpose of this study was to examine the in vitro toxicity of 3,4-dichlorophenylhydroxylamine. Renal cortical slices were prepared from male Fischer 344 rats (190–250 g) and were incubated with 0–0.5 mM 3,4-CPHA for 30–120 min under oxygen and constant shaking. 3,4-CPHA produced a concentration and time dependent alteration in lactate dehydrogenase (LDH) leakage, organic ion accumulation and pyruvate stimulated gluconeogenesis. Glutathione levels were diminished within 60 min below control values by 0.1 and 0.5 mM 3,4-CPHA. A 30 min pretreatment with 0.1 mM deferoxamine did not alter 3,4-CPHA toxicity. Alterations in pyruvate stimulated gluconeogenesis and LDH leakage were comparable between vehicle and deferoxamine pretreated tissues. Other studies examined the effect of (1 mM) glutathione, 2 mM ascorbic acid and 1 mM dithiothreitol (DTT) on toxicity. Pretreatment for 30 min with vehicle or 1 mM DTT induced comparable changes in LDH leakage and pyruvate stimulated gluconeogenesis. Pretreatment for 30 min with 1 mM glutathione or 2 mM ascorbic acid reduced 3,4-CPHA toxicity. LDH leakage was not elevated as markedly in renal slices pretreated with glutathione relative to slices pretreated with vehicle. These results indicate that 3,4-CPHA toxicity is through an iron independent mechanism. 3,4-CPHA cytotoxicity was reduced by pretreatment with glutathione or ascorbic acid suggesting formation of a reactive intermediate.

Keywords: Nephrotoxicity; Aromatic amines; Chlorophenylhydroxylamine; F344 rats; Renal cortical slices

1. Introduction

3,4-Dichlorophenylhydroxylamine (3,4-CPHA) is an aromatic ring hydroxylated metabolite of the nephrotoxicant, 3,4-dichloroaniline. 3,4-Dichloroaniline is an essential ingredient in the production of the herbicide Propanil. Propanil is an arylamide, post-emergent herbicide used to control broadleaf grasses in rice, wheat and potato fields (Farm Chemicals Handbook, 1992; Handbook of Pesticide Toxicology, Stevens and Sumner, 1991).

* Corresponding author. Tel.: +1-304-6967332; fax: +1-304-6967391.

E-mail address: valentov@marshall.edu (M. Valentovic).

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The renal effects of 3,4-dichloroaniline have been characterized in Fischer 344 rats (Valentovic et al., 1997). 3,4-Dichloroaniline induced morphological changes to the proximal tubule. Renal functional changes include diminished urine output and hematuria. Proximal tubular function was impaired since renal cortical slice uptake of *p*-aminohippurate, an organic anion, was depressed 24 h after 3,4-dichloroaniline.

Toxicity was also apparent following in vitro exposure of renal cortical slices to 3,4-dichloroaniline (Valentovic et al., 1995, 1997). 3,4-Dichloroaniline addition to renal cortical slices induced a concentration dependent decrease in pyruvate-stimulated gluconeogenesis. Incubation with 2 mM 3,4-dichloroaniline also elevated lactate dehydrogenase (LDH) release above control values.

The mechanism for 3,4-dichloroaniline renal toxicity is not yet clear. The toxic species that induces 3,4-dichloroaniline nephrotoxicity also still needs to be identified. 3,4-Dichloroaniline is biotransformed in rat hepatic microsomes to two major metabolites, 3,4-CPHA and 2-amino-4,5-dichlorophenol (McMillan et al., 1990a).

Metabolites of another aromatic amine, aniline have been attributed with generation of methemoglobinemia and tissue hypoxia (Kiese, 1966; Gosselin et al., 1984). The phenyl hydroxylamine and nitrosobenzene (Kiese, 1966) metabolites induce oxidation of hemoglobin to methemoglobin. The nitrosobenzene metabolite can undergo reduction to the hydroxylamine and further oxidize another molecule of hemoglobin or nitrosobenzene can form hemoglobin adducts (Kiese and Taeger, 1976).

Studies that have examined 3,4-dichloroaniline toxicity to red blood cells indicate biotransformation is a necessary step for activation. 3,4-Dichloroaniline induced methemoglobinemia is associated with the metabolite 3,4-dichlorophenyl hydroxylamine and its conversion within the red blood cell to 3,4-dichloronitrosobenzene (McMillan et al., 1990b).

The following studies investigated the in vitro cytotoxicity of 3,4-CPHA. An emphasis was placed on: (a) characterization of 3,4-CPHA toxicity in renal cortical slices; (b) alterations in glutathione levels; and (c) examined whether antioxidants or sulfhydryl containing agents could modify 3,4-dichlorophenyl hydroxylamine toxicity.

2. Methods and materials

2.1. Chemicals

3,4-CPHA was prepared by the method of Rondestvedt and Johnson (1977). 3,4-CPHA was prepared via reduction of 3,4-dichloronitrobenzene with hydrazine-palladium on carbon. Recrystallization from benzene-petroleum ether provided pure product in a 60% yield (m.p. 73–75°C). LDH, sulphosalicylic acid, glutathione, deferoxamine and all other compounds were obtained from Sigma-Aldrich (St. Louis, MO) and the products obtained were of the highest quality and purity.

2.2. Animals

Male Fischer 344 rats (weight 190–220 g) were obtained from Hilltop Lab Animals Inc. (Scottsdale, PA). Animals were maintained under a controlled ambient temperature (21–23°C), humidity (40–55%) and light cycle (lights on 06:00–18:00 h). Animals were provided a 7 day acclimation period prior to initiation of experiments.

2.3. Incubation of renal slices

Renal cortical slices were washed in 5 ml of oxygenated Krebs buffer three times for 3 min at 25°C. The tissue was equilibrated for 5 min at 37°C under oxygen. Pyruvate (100 μl, final bath concentration 10 mM) was added to all tissue. A 30 μl aliquot of 3,4-CPHA was added at a final concentration of 0–0.5 mM and tissues were incubated for up to 120 min. Media and tissue were collected to measure LDH leakage, pyruvate stimulated gluconeogenesis and glutathione levels.

Other experiments required a pretreatment of renal slices for 30 min at 37°C with 1 mM reduced glutathione (GSH), 1mM dithiothreitol (DTT), 2 mM ascorbic acid or 50 μM diphenylenediamine

(DPPD). The potential role of an iron mediated radical was examined using 0.1 mM deferoxamine as a pretreatment of the renal slices. Renal slices were pretreated for 30 min with a nonsulfhydryl containing antioxidant, 2 mM ascorbic acid, to examine differences in toxicity between ascorbic acid and sulfhydryl containing antioxidants. Upon completion of the pre-incubation period, 3,4-CPHA was added and tissues were incubated for 90 min. Gluconeogenesis was stimulated by addition of 100 μl of pyruvate (10 mM final concentration, in Krebs) and the tissue was incubated for a final 30 min.

2.4. Organic ion transport

Renal cortical slices were equilibrated for 15 min in 3 ml of oxygenated Krebs buffer pH 7.4 at 37°C in a Dubnoff metabolic shaker under oxygen and constant shaking (100 cycles/min). Vehicle (DMSO) or 3,4-CPHA were added (30 μl) to yield a final concentration of 0–0.5 mM. The slices were incubated for 30 min prior to the addition of the organic anion *p*-aminohippurate (^{14}C-PAH, sp. activity 60 mC_i/mmol; 0.07 mM, 0.02 μC_i/ml) or the organic cation tetraethylammonium (^{14}C-TEA, sp. activity 4.0 mC_i/mmol; 0.01 mM, 0.02 μC_i/ml) as previously described (Valentovic et al., 1989). Renal slices were incubated 60 min after the addition of PAH or TEA. Upon completion of the incubation period, uptake of organic ions was expressed as the ratio of tissue slices to media (S/M) where S denotes dpm/g tissue and M denotes dpm/ml of incubation media.

2.5. Cytotoxicity assays

LDH release into the media was expressed as a percentage of total LDH using a spectrophotometric kinetic assay (Sigma, Kit # 228). Media glucose was measured using a glucose oxidase enzymatic assay (Sigma, Kit # 510).

2.6. Glutathione determination

Glutathione levels were measured following a 60 min incubation with 0–0.5 mM 3,4-CPHA. Changes in glutathione were monitored as a function of time in renal slices incubated for 15–120 min with 0.1 mM 3,4-CPHA. Renal slices were weighed, homogenized in 0.5 ml of 0.5% sulphosalicylic acid and adjusted to 1 ml. Total glutathione levels were measured using an NADPH coupled reaction described by Andersen (1985). Values were expressed as nmol/g tissue.

2.7. Statistical analysis

Values represent the mean ± S.E.M., with $N = 4$/group. Differences between groups were determined using a one-way analysis of variance followed by a Newman Keuls test. Differences within groups were calculated using a one-way analysis of variance followed by a Dunnett's test. All tests were conducted using a 95% confidence interval.

3. Results

3,4-CPHA was cytotoxic to renal cortical slices. 3,4-CPHA (0.10–0.5 mM) diminished pyruvate stimulated gluconeogenesis (Table 1) in a concentration dependent manner. Glutathione levels were diminished within 60 min as a function of 3,4-CPHA concentration. 3,4-CPHA induced alterations in membrane integrity were confirmed by a rise in LDH leakage (Table 1) relative to vehicle treated tissue. Renal cortical slice accumulation of organic ions was diminished in a concentration manner by 3,4-CPHA (Table 2). Uptake of the organic anion, PAH was decreased ($P < 0.05$) relative to vehicle control at 0.5 mM. Uptake of the organic cation, TEA was inhibited in a concentration dependent manner relative to vehicle controls at 3,4-CPHA concentrations of 0.01, 0.1 and 0.5 mM 3,4-CPHA. 3,4-CPHA (0.5 mM) induced temporal changes in pyruvate stimulated gluconeogenesis (Fig. 1), LDH leakage (Fig. 2) and glutathione levels (Fig. 3).

Pretreatment with the iron chelating agent, deferoxamine, did not reduce 3,4-CPHA toxicity. Alterations in pyruvate stimulated gluconeogenesis and LDH leakage (Table 3) were comparable between vehicle and deferoxamine pretreated tis-

Table 1
The effect of 3,4-CPHA on renal cortical slices[a]

	3,4-CPHA concentration (mM)			
	0	0.01	0.1	0.5
Glucose (mg/g tissue)	2.03 ± 0.11	1.87 ± 0.09	1.40 ± 0.12*	0.53 ± 0.06*
LDH leakage (% of total)	11.6 ± 0.64	11.9 ± 1.2	16.5 ± 1.9	31.7 ± 2.9*
Glutathione (nmol/g tissue)	793 ± 44	713 ± 65*	565 ± 50*	229 ± 43*

[a] Renal slices were incubated with 0–0.5 mM 3,4-CPHA. Cytotoxicity was assessed following 2 h exposure to 3,4-CPHA using LDH leakage and pyruvate stimulated gluconeogenesis. Glutathione levels were measured after a 60 min exposure to the designated concentration of 3,4-CPHA. Values represent mean ± S.E.M. with $N = 4$/group.

* Indicates statistical difference ($P < 0.05$) when compared to control.

Table 2
In vitro effect of 3,4-CPHA on organic ion transport

	3,4-CPHA concentration (mM)					
	0	0.005	0.01	0.05	0.10	0.50
PAH (S/M)	3.15 ± 0.22[a]	2.85 ± 0.25	2.86 ± 0.25	3.06 ± 0.13	2.96 ± 0.29	2.21 ± 0.29*
TEA (S/M)	10.00 ± 1.01	9.22 ± 0.58	8.13 ± 0.24*	9.17 ± 1.02	8.11 ± 0.58*	5.03 ± 1.17*

[a] Values represent mean ± S.D. with $N = 4$ different animals.

* Indicates statistical difference ($P < 0.05$) from control (DMSO) treated tissue.

sues following exposure to 3,4-CPHA. These findings suggest that 3,4-CPHA in vitro toxicity does not require free iron for cytotoxicity to occur. Pre-incubation with the sulfhydryl-containing agent, DTT, did not reduce 3,4-CPHA toxicity. 3,4-CPHA associated effects on pyruvate stimulated (Table 3) and LDH leakage were comparable between vehicle and 1 mM DTT pretreated slices.

Toxicity was reduced by pretreatment with 1 mM glutathione. Glutathione pretreatment prevented 3,4-CPHA associated rise in LDH leakage (Fig. 4). Glutathione did not totally protect renal slices from 3,4-CPHA toxicity since gluconeogenesis (Table 3) was reduced in a similar manner between vehicle and glutathione pretreated tissue in the presence of 0.5 mM 3,4-CPHA. The success of GSH pretreatment to reduce toxicity relative to DTT may be due to differences in intracellular concentration within the renal slices.

Ascorbic acid pretreatment partially reduced 3,4-CPHA cytotoxicity. Pretreatment with ascorbic acid prevented a rise in LDH leakage when slices were exposed to 0.1 mM 3,4-CPHA when compared to vehicle treated slices (Fig. 5). LDH leakage was not reduced by ascorbic acid pretreat-

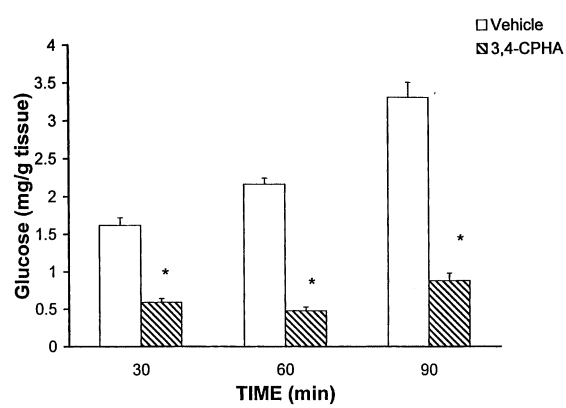

Fig. 1. Time dependent changes in pyruvate stimulated gluconeogenesis. Renal cortical slices were incubated for 30–90 min with vehicle or 0.5 mM 3,4-CPHA. Pyruvate stimulated gluconeogenesis was expressed as mg glucose/g tissue. Values represent mean ± S.E.M. with $N = 4$/group. Pyruvate stimulated gluconeogenesis was decreased ($*P < 0.05$) below vehicle treated tissue within 30 min.

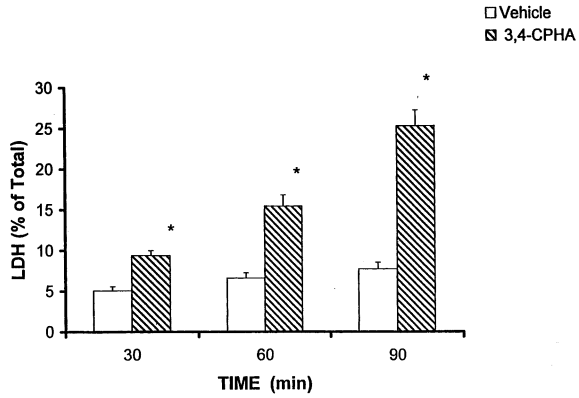

Fig. 2. Time dependent effects of 3,4-CPHA on LDH leakage. Renal cortical slices were incubated for 30–90 min with 0.5 mM 3,4-CPHA. LDH release was expressed as percent of total. Values represent mean ± S.E.M. Groups had $N = 4$/group. 3,4-CPHA induced an increase ($*P < 0.05$) in LDH leakage relative to vehicle treated tissue within 30 min.

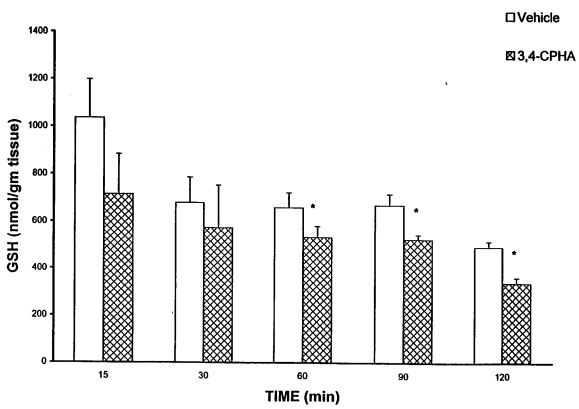

Fig. 3. Alterations in glutathione (GSH) levels as a function of time following exposure to 0.5 mM 3,4-CPHA. Renal cortical slices were incubated for 15–120 min with 3,4-CPHA or vehicle for 60 or 90 min. The amount of glutathione was determined and expressed as nmol/g tissue. Values represent mean ± S.E.M. with five animals/group. An asterisk (*) indicates statistical difference ($P < 0.05$) from respective vehicle treated slices.

ment when slices were exposed to 0.5 mM 3,4-CPHA. Consequently, ascorbic acid was only successful at concentrations below 0.5 mM 3,4-CPHA.

4. Discussion

Previously published studies have shown that the parent compound, 3,4-dichloroaniline, induced cytotoxicity to renal cortical slices (Valentovic et al., 1995, 1997). Pyruvate stimulated gluconeogenesis was inhibited by 0.5 mM 3,4-dichloroaniline and LDH leakage was elevated above control values in the presence of 2 mM 3,4-dichloroaniline (Valentovic et al., 1997). The present study focussed on the in vitro cytotoxicity of 3,4-CPHA, the *N*-hydroxyl metabolite of 3,4-dichloroaniline. Exposure of renal cortical slices to 3,4-CPHA resulted in a depletion of glutathione and decreased organic ion transport. Pyruvate stimulated gluconeogenesis was decreased in the presence of 0.1 mM 3,4-CPHA while LDH leakage was apparent following exposure to 0.5 mM 3,4-CPHA. These findings indicate that the *N*-hydroxyl metabolite is more toxic than its parent compound, 3,4-dichloroaniline since cytotoxicity was detected in slices at a lower level of 3,4-CPHA than had been previously observed for 3,4-dichloroaniline.

Glutathione levels were rapidly reduced by exposure to 3,4-CPHA and addition of glutathione to the incubation media prevented 3,4-CPHA associated rise in LDH leakage from renal slices. The reduction in glutathione may due to glutathione acting as a radical scavenger (Reed, 1990). Total glutathione may be depleted by conversion of the reduced form of glutathione (GSH) to an oxidized GSSG form with donation of an electron to neutralize a reactive intermediate of 3,4-CPHA. Glutathione may also interact with 3,4-CPHA or a metabolite to form a glutathione conjugate. Further studies are necessary to determine the role of glutathione.

Pretreatment with deferoxamine prior to exposure to 3,4-CPHA did not reduce cytotoxicity. These results suggest that cytotoxicity mediated by 3,4-CPHA occurs through an iron independent mechanism. A possible alternate explanation is that the deferoxamine did not have sufficient time to chelate excess free iron or that the intracellular concentration of deferoxamine was not sufficient to chelate iron.

Table 3
The effect of deferoxamine, DTT and glutathione pretreatments on 3,4-CPHA toxicity[a]

Pretreatment	3,4-CPHA concentration (mM)			
	0	0.01	0.1	0.5
Glucose (nmol/g tissue)				
Water	1.80 ± 0.35	1.71 ± 0.16	1.28 ± 0.13*	0.56 ± 0.04*
DFX	1.43 ± 0.15	1.61 ± 0.16	1.28 ± 0.11	0.54 ± 0.06*
Water	1.68 ± 0.70	1.75 ± 0.14	1.22 ± 0.09*	0.54 ± 0.13
DTT	1.66 ± 0.06	1.61 ± 0.03	1.14 ± 0.03*	0.43 ± 0.02*
Water	1.25 ± 0.31	1.05 ± 0.30	1.07 ± 0.30*	0.23 ± 0.11*
GSH	1.95 ± 0.49	1.92 ± 0.45	1.70 ± 0.44	0.24 ± 0.06*
LDH leakage (% of total)				
Water	11.4 ± 0.4	10.9 ± 0.2	15.2 ± 1.4	30.4 ± 3.1*
DFX	10.5 ± 1.8	11.8 ± 1.5	14.2 ± 1.0	31.8 ± 2.5*
Water	16.3 ± 0.9	17.1 ± 0.8	20.1 ± 0.5	34.8 ± 2.1*
DTT	18.2 ± 0.2	18.4 ± 0.7	23.5 ± 0.9	44.4 ± 1.1*

[a] Renal cortical slices were preincubated with vehicle or 0.1 mM deferoxamine (DFX), 1 mM DTT or 1 mM glutathione (GSH) for 30 min. Tissues were then incubated with varying concentrations of 3,4-CPHA for 90 min followed by a 30 min incubation with 10 mM pyruvate. Values represent mean ± S.E.M. with $N = 4$/group.

* Indicates different ($P < 0.05$) from respective vehicle treated tissue.

The ability of ascorbic acid to reduce 3,4-CPHA in vitro toxicity would support the hypothesis that toxicity involves formation of a reactive intermediate. Ascorbic acid would be expected to reduce oxidation and lower toxicity of 3,4-CPHA. 3,4-CPHA could undergo oxidation to a reactive intermediate through an enzymatic pathway or via auto-oxidation. Phenylhydroxylamine, the *N*-hydroxyl metabolite of aniline undergoes oxidation to form nitrosobenzene in red blood cells (Kiese, 1966; Eyer, 1988) and in rat liver (Eyer et al., 1980). Ascorbic acid may prevent the oxidation of 3,4-CPHA to 3,4-dichloronitrosobenzene and reduce toxicity mediated by the one electron transfer.

Ascorbic acid has been shown to reduce the toxicity of mono- and dihalo- aminochlorophenol. Pre-treatment with ascorbic acid reduced the in vivo nephrotoxicity of 4-amino-2,6-dichlorophenol (Hong et al., 1997). Pretreatment with ascorbic acid was sufficient to prevent alterations in renal function associated with 4-amino-2,6-dichlorophenol. Ascorbic acid also reduced the in vitro toxicity of 2-amino-5-chlorophenol (Valentovic et al., 1999). Ascorbic acid may reduce cytotoxicity by preventing redox cycling to nitrosobenzene and benzoquinoneimine. Once quinoneimines (Monks et al., 1992) are formed,

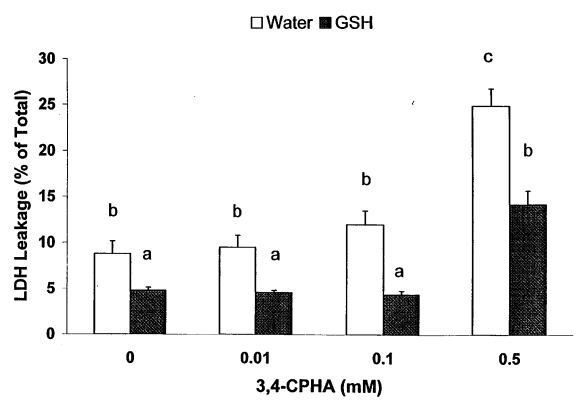

Fig. 4. The effect of glutathione pretreatment on 3,4-CPHA toxicity. Renal cortical slices were pre-incubated for 30 min in 5 ml of oxygenated Krebs buffer with 0 or 1 mM reduced glutathione (GSH). Renal slices were then incubated for 90 min with 3,4-CPHA at varying concentrations. The slices were then incubated for 30 min with 10 mM pyruvate to stimulate gluconeogenesis. Values represent mean ± S.E.M. with $N = 4$/group. Groups that are different from each other ($P < 0.05$) are denoted by different superscripts.

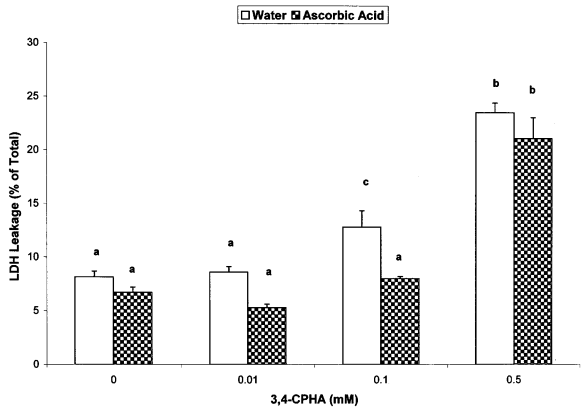

Fig. 5. The effect of ascorbic acid pretreatment on 3,4-CPHA toxicity. Renal cortical slices were pre-incubated for 30 min in 5 ml of oxygenated Krebs buffer with 0 or 1 mM reduced glutathione (GSH). Renal slices were then incubated with 3,4-CPHA at varying concentrations for 90 min followed by an additional 30 min incubation with 10 mM pyruvate to stimulate gluconeogenesis. Values represent mean ± S.E.M. with $N = 4$/group. Groups that are different from each other ($P < 0.05$) are denoted by different superscripts.

these intermediates are highly reactive with intracellular protein. Quinoneimines (Monks and Lau, 1992) and nitrosobenzene are also capable of undergoing redox cycling that would induce oxidative stress within the target tissue.

Ascorbic acid pretreatment reduces the renal toxicity of 4-aminophenol or para-hydroxyaniline. Fowler et al. (1993) reported ascorbic acid reduced in vivo 4-aminophenol toxicity in Fischer 344 rats. Pretreatment with ascorbic acid of rabbit proximal tubules, greatly reduced the cytotoxicity of 4-aminophenol (Lock et al., 1993). Ascorbic acid pretreatment of rabbit proximal tubule was able to maintain LDH leakage at levels comparable to control values following exposure to 4-aminophenol and prevented a depletion in intracellular glutathione levels.

Based on the present studies, 3,4-CPHA is directly toxic to renal cortical slices at a concentration that is lower than its parent compound, 3,4-dichloroaniline. The toxicity is associated with a depletion of glutathione and diminished pyruvate stimulated gluconeogenesis. 3,4-CPHA toxicity may be mediated by oxidation to form a reactive intermediate that is prevented by the presence of ascorbic acid.

Acknowledgements

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