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Inhibitory Effects of Calmodulin Antagonists on Urinary Enzyme Excretion in Rats After Nephrotoxic Doses of Mercuric Chloride

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Mercury, in its various inorganic and organic forms, is an important environmental and occupational pollutant and toxicant. Prochlorperazine, a phenothiazine antiemetic, has been reported to protect rats against mercuric chloride ($HgCl_2$)-induced nephrotoxicity (Harrison et al. 1982). Mercuric ion and 12 other divalent metal ions of toxicologic importance inhibit the activity of calmodulin, a ubiquitous intracellular calcium receptor and regulatory protein, at physiologically relevant concentrations (Cox and Harrison 1983). Phenothiazines, including prochlorperazine, are reversible calmodulin antagonists, and as such they interact with divalent calcium at the level of calmodulin (Weiss et al. 1982). It was of interest therefore to evaluate the comparative effects of several phenothiazines on $HgCl_2$-induced nephrotoxicity in rats.

MATERIALS AND METHODS

Rats were obtained from Harlan Industries, Inc., Indianapolis, IN (U.S.A.), were housed individually in polycarbonate cages with hardwood bedding (Ab-sorb-dri, Garfield, NJ, U.S.A.), and were permitted free access to Purina rodent chow (No. 5001, St. Louis, MO, U.S.A.) and tap water. The phenothiazines and thioxanthine used were generous gifts from the following sources: prochlorperazine edisylate, trifluoperazine dihydrochloride, and chlorpromazine hydrochloride, Dr. Barry Berkowitz, Smith Kline & French Laboratories; triflupromazine hydrochloride and fluphenazine hydrochloride, Dr. Robert J. Laffan, E. R. Squibb & Sons, Inc.; perphenazine hydrochloride, Dr. I. I. A. Tabachnick, Schering Corporation; and chlorprothixene, Dr. W. E. Scott, Hoffmann-La Roche Inc. All other chemicals used were of the best available reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Rats were randomly distributed into groups of four each that received either a single i.p. dose of mercuric chloride ($HgCl_2$, 1 mg/kg) or $HgCl_2$ (1 mg/kg) preceded by a phenothiazine or thioxanthine (4.3 μmole/kg i.p. except that the dosage of trifluoperazine was 6.4 μmole/kg). The phenothiazine, thioxanthine, or

diluent was administered as a single dose one hr prior to $HgCl_2$ on the first day of treatment (Day 1) and daily thereafter for an additional four days. All compounds were dissolved separately in aqueous NaCl (0.9 g/100 ml) and diluted to permit injection of 0.3 ml/100 g of body wt. Control rats received no treatment. Animals treated with diluent and/or phenothiazine or chlorprothixene alone did not differ from control animals with regard to enzymuria or histopathologic evaluation.

Rats were weighed on Days 1-7, 15, and 16. On Days 1, 3, 6, and 15, rats were placed in polycarbonate metabolism cages for 24 hr for the collection of urine. The volume of each 24-hr urine sample was recorded, and each sample was centrifuged at 1000 x g to remove sediment. Samples were refrigerated at 5°C until determinations of creatinine, N-acetylglucosaminidase (NAG), and leucine aminopeptidase (LAP) were performed. Creatinine concentration was determined using an alkaline picrate procedure (Sigma No. 555, Sigma Chemical Co.). NAG activity was determined by hydrolysis of p-nitrophenyl-N-acetyl-β-D-glucosaminide according to the method of Lockwood and Bosmann (1979). LAP activity was determined by hydrolysis of L-leucyl-β-naphthylamide (Sigma No. 251). To correct for individual variability in urine production in the rats, urinary NAG and LAP activities were expressed as units/mg of creatinine excreted. Units of enzyme activity were defined as described in the methods indicated (Lockwood and Bosmann 1979; Sigma No. 251). On Day 16, rats were euthanized by exsanguination under chloroform anesthesia. Kidneys were removed, trimmed of fat, weighed as pairs, and fixed in buffered formalin (10 g of formaldehyde/100 ml of phosphate buffer, pH 7). For selected experiments, kidney samples were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Slides were coded, randomized, and read blindly. Lesion severity was scored as described previously (Harrison et al. 1980) using the scores indicated in the footnote to Table 3. Lesion scores from rats that received $HgCl_2$ plus phenothiazine were compared to scores from rats that received only $HgCl_2$ by Wilcoxon's rank sum test for unpaired samples (Snedecor and Cochran 1980). Enzyme activity values were subjected to Student's t test. Lesion severity scores and enzyme activity values were subjected to regression analysis.

RESULTS AND DISCUSSION

Urinary excretion of NAG and LAP has proven useful for quantifying the effects of $HgCl_2$ (Harrison et al. 1982) and other nephrotoxicants (Ellis and Price 1975; Stroo and Hook 1977; Jones et al. 1980). In the present studies, enzymuria was maximal within the first 24 hours (Day 1) after administration of $HgCl_2$ to rats (Table 1). By Day 3, NAG excretion had returned to the reference range, and LAP excretion was essentially normal by Day 6. This pattern was similar whether rats were treated with $HgCl_2$ alone (Table 1) or a combination of $HgCl_2$ and a phenothiazine (data not shown). The extent to which phenothia-

Table 1. Urinary excretion of enzyme activity by rats treated with mercuric chloride

Day[a]	N-Acetylglucosaminidase (U/mg creatinine/24 hr)[b]	Leucine aminopeptidase (U/mg creatinine/24 hr)
1	42 ± 5[c]	24 ± 4
3	21 ± 3	5 ± 1
6	17 ± 2	3 ± 0.6
15	15 ± 1	2 ± 0.2

a) On Day 1 rats received a single i.p. dose of $HgCl_2$, 1 mg/kg, n = 31
b) From Harrison et al. 1982
c) Mean ± S.E., n = 31. The reference ranges for urinary NAG and LAP in rats that received no treatment were 10-20 (14.7 ± 0.6) and 0-2 (0.69 ± 0.08) U/mg creatinine, respectively

zines altered $HgCl_2$-induced enzymuria was therefore most clearly revealed by urinary enzyme activity in the first 24 hours post-treatment (Day 1). These data are presented in Table 2. Prochlorperazine, trifluoperazine, perphenazine, and chlorprothixene significantly reduced the extent of $HgCl_2$-induced enzymuria. Fluphenazine lowered NAG and LAP excretion, and chlorpromazine lowered NAG excretion, although these reductions were not statistically significant. Other data (not shown) obtained in our laboratory indicated that a higher dose of chlorpromazine and triflupromazine was capable of reducing $HgCl_2$-induced urinary excretion of both LAP and NAG in rats.

The ability of some of the phenothiazines to prevent enzymuria in rats treated with $HgCl_2$ was interpreted as protection of the renal tubular epithelium against the effects of $HgCl_2$. This interpretation was confirmed histopathologically for prochlorperazine and trifluoperazine (Table 3). These phenothiazines reduced both the frequency and the severity of renal tubular degeneration and necrosis evident microscopically on Day 16. Although the severity of these lesions was maximal about Day 6, it was readily evaluable on Day 16.

To determine the correlation of urinary enzyme values and lesion severity scores, Day 1 NAG and LAP values were assigned a rank of 0 to 4 according to increments of activity chosen arbitrarily for each enzyme. Regression analysis of LAP rank values for rats in the prochlorperazine and trifluoperazine groups and the corresponding lesion severity scores for the same animal revealed a good correlation (data not shown) ($r = 0.84$). Regression of NAG ($r = 0.53$), although not as striking, significantly rejected the lack of a correlation ($P < 0.01$).

Table 2. Effect of phenothiazines on mercuric chloride-induced enzymuria in rats

Treatment[a]	Urinary Enzyme Activity (U/mg Creatinine) on Day 1	
	N-Acetylglucosaminidase	Leucine aminopeptidase
$HgCl_2$	42 ± 5[b]	24 ± 4
$HgCl_2$ plus:		
Prochlorperazine	20 ± 5*	20 ± 8
Trifluoperazine[c]	16 ± 4*	8 ± 5*
Perphenazine	21 ± 5*	8 ± 4*
Fluphenazine	37 ± 6	19 ± 7
Chlorpromazine	28 ± 9	40 ± 23
Triflupromazine	43 ± 9	37 ± 12
Chlorprothixene[d]	25 ± 6*	7 ± 2*

a) Mercuric chloride ($HgCl_2$, 1 mg/kg) was administered on Day 1 as a single i.p. dose alone or in combination with a phenothiazine. Phenothiazines (4.3 μmole/kg/day) were administered i.p. one hr prior to $HgCl_2$ and daily thereafter for 4 days.
b) Mean ± S.E.
c) Dosage was 6.4 μmole/kg/day
d) Thioxanthine antipsychotic
*) Indicated values are significantly lower ($P < 0.05$) than the value for the group that received $HgCl_2$ alone

Phenothiazines are known to produce pharmacologic effects (Baldessarini 1980) that might account for the renal protection observed in the present studies. Some phenothiazines increase renal blood flow and induce varying degrees of diuresis. However, urine volumes were not significantly increased by phenothiazines in the present studies (data not shown). Other investigators have similarly reported that induction of diuresis is not a consistent result of phenothiazine treatment in rats (Siddik et al. 1979). Phenothiazine-induced hypothermia (Baldessarini 1980) might have afforded some protection against cell damage in vivo. This possibility cannot be discounted because body temperatures were not measured in the present study. Pharmacodynamic effects, such as delayed absorption, have not accounted for promethazine cytoprotection against carbon tetrachloride hepatotoxicity in rats (Reddrop et al. 1981). The activity of phenothiazines as radical scavengers was considered, but the

Table 3. Histopathologic evaluation of kidneys from rats treated with mercuric chloride alone or in combination with a phenothiazine

Treatment	Microscopic Evidence of Renal Tubular Degeneration on Day 16	
	Frequency[a]	Severity[b]
$HgCl_2$[c]	12/12	2.0 ± 0.2
$HgCl_2$ plus:		
Prochlorperazine[d]	5/8	0.8 ± 0.3*
Trifluoperazine	1/4	0.3 ± 0.2*

a) The numerator is the number of rats with microscopically evident lesions, and the denominator is the number of rats studied
b) Mean ± S.E. degree of lesion severity using 0 = no lesion, 1 = mild, 2 = moderate, and 3 = severe
c) 1 mg/kg i.p., single dose on Day 1
d) Recalculated from Harrison et al. 1982
*) Significantly lower ($P < 0.05$) than value for group that received $HgCl_2$ alone

present studies indicated that chlorprothixene, a thioxanthine that lacks radical scavenging potential (Mason 1979), was at least as cytoprotective in vivo as the phenothiazines. The design of these studies, permitting one hour for phenothiazine absorption and distribution before $HgCl_2$ administration, minimized the likelihood that a direct chemical reaction might have occurred between Hg^{2+} and a phenothiazine.

Phenothiazines have been shown to bind reversibly to calmodulin in vitro (Weiss et al. 1982). This binding is Ca^{2+} dependent, does not result in the dissociation of bound Ca^{2+}, but does result in functional inhibition of calmodulin activity. Dissociation of phenothiazine restores calmodulin activity in vitro. Hg^{2+} inactivates calmodulin in vitro (Cox and Harrison 1983), presumably by binding to some site on the protein. The present results suggest that Hg^{2+} may bind in vivo to a site or sites on calmodulin that are inaccessible if phenothiazine has been bound first. This possibility warrants further investigation.

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