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Effects of 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid on extrahepatic sulfhydryl levels in mice treated with acetaminophen☆

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Abstract

The cysteine (Cys) precursor 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid (PTCA) has been shown to protect against acetaminophen (APAP)-induced hepatic GSH, GSSG, and Cys depletion and hepatic necrosis. The aim of this study was to determine the effects of PTCA on the concentrations of sulfhydryl compounds in extrahepatic tissues, including renal cortex, whole blood, and brain, in C57BL/6 mice treated with hepatotoxic doses of APAP. PTCA (1–5 mmol/kg, i.p.) was administered 30 min after the administration of APAP at a dose (800 mg/kg; 5.29 mmol/kg, i.p.) that depleted hepatic GSH and Cys at 4 hr by 95 and 86%, respectively. Tissue concentrations of GSH and Cys were determined by HPLC. At 4 hr following APAP administration, renal cortical GSH and Cys concentrations were decreased to 64 and 39%, respectively, of vehicle-treated control values, and blood concentrations were decreased to 87 and 30%, respectively, of vehicle controls. Brain GSH and Cys were not depleted by APAP. PTCA at 5 mmol/kg (i) attenuated the APAP-induced depletion of GSH and Cys at 4 hr in renal cortex (78 and 65%, respectively, of vehicle controls), (ii) prevented APAP-induced Cys depletion in blood (670% of vehicle controls) with no effect on GSH concentration (94% of vehicle controls), and (iii) increased GSH and Cys concentrations in brain (119 and 411%, respectively, of vehicle controls). The results demonstrate a high degree of tissue selectivity in the APAP-induced depletion of GSH and Cys, and in the effectiveness of PTCA in maintaining and even elevating sulfhydryl levels in extrahepatic tissues of APAP-treated mice.

Keywords: Glutathione; Acetaminophen (APAP); 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid (PTCA); Kidney; Blood; Brain

1. Introduction

In large doses, the analgesic and antipyretic drug APAP can cause potentially life-threatening hepatotoxicity [1,2]. Liver damage involves the formation of a chemically reactive metabolite, NAPQI [3,4]. The NAPQI formed in small amounts after the usual therapeutic doses of APAP is detoxified by conjugation with GSH, but after large, toxic doses of APAP, GSH is severely depleted and the unconjugated NAPQI binds to vital macromolecules leading to hepatic necrosis [3,5]. Toxic doses of APAP also cause depletion of Cys [6], the rate-limiting substrate of GSH biosynthesis [7]. Protection against GSH depletion and liver damage can be effected by the timely administration of *N*-acetyl-L-cysteine, presumably due to the release of Cys necessary for GSH biosynthesis [8,9]. A class of Cys precursors, the 2-alkylthiazolidine-4-carboxylic acids, which release Cys non-enzymatically, can also protect against APAP-induced hepatotoxicity [10,11]. Our recent studies showed that PTCA protects against hepatotoxicity in mice by attenuating the APAP-induced depletion of hepatic GSH and Cys [6].

Less information is available on the extrahepatic effects of APAP and PTCA. In humans, APAP overdoses occasionally produce acute renal proximal tubular necrosis with or without liver damage [12,13]. In mice, larger doses (600–1200 mg/kg) of APAP are required to produce nephrotoxicity [14,15] compared with doses producing hepatotoxicity (300–500 mg/kg) [5,16]. Also, the mechanism of

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Abbreviations: APAP, acetaminophen; Cys, cysteine; CSSC, cystine; MPA, metaphosphoric acid; NAPQI, *N*-acetyl-*p*-benzoquinone imine; OTC, 2-oxothiazolidine-4-carboxylate; and PTCA, 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid.

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nephrotoxicity in mice may differ from that producing hepatotoxicity. It has been reported that APAP-induced nephrotoxicity involved intrarenal activation of APAP as well as transport of a metabolite derived from the GSH conjugate of APAP (e.g. APAP-Cys) that enters renal tubular cells by a probenecid-sensitive system [14,17].

APAP causes less depletion of renal than hepatic GSH in mice and rats [18,19], but the extent of Cys depletion is not known. Although *N*-acetyl-L-cysteine has been reported to be ineffective in protecting against APAP-induced renal toxicity in humans [13,20], it was of interest to ascertain whether PTCA can attenuate the APAP-induced renal sulfhydryl depletion in a mouse model. APAP has been reported to have no effect on brain GSH concentration in rats [21] and no effect on blood GSH in mice in doses of 300–500 mg/kg, i.p. [22].

The present studies were undertaken (i) to further elucidate the effects of hepatotoxic doses of APAP on GSH and Cys levels in extrahepatic tissues of mice, including renal cortex, whole blood, and brain, and (ii) to determine the effects of PTCA on extrahepatic sulfhydryl levels in APAP-treated mice.

2. Materials and methods

2.1. Chemicals

APAP, GSH, Cys, CSSC, GSSG, monochloroacetic acid, and heptanesulfonic acid were obtained from the Sigma Chemical Co. MPA was obtained from the Aldrich Chemical Co. PTCA was synthesized [11] using the method of Schubert [23].

2.2. Animals

Male C57BL/6 mice (Charles River Laboratories), weighing 23–28 g, were maintained on a 12-hr light-dark cycle and received food and water *ad lib*. The animals were treated between 8:00 and 9:30 a.m. to minimize the possibility of diurnal effects in tissue GSH concentrations influencing the experimental results. Body weights were recorded before and after treatments.

2.3. Treatments

Mice were killed by cervical dislocation and decapitation at designated times after i.p. injection of 800 mg/kg (5.29 mmol/kg) of APAP in 50% propylene glycol (pH 6–7; total volume injected, 8 mL/kg) to determine the time course of depletion of renal sulfhydryl compounds. In separate experiments, mice were killed either 4 or 24 hr after i.p. injection of various doses of APAP (400, 600, 700, or 800 mg/kg; total volume injected, 8 mL/kg) to determine the optimal dose for further experiments. In experiments on the protective effects of PTCA, mice received different i.p. doses (1.0, 1.75, 2.5, or 5.0 mmol/kg) of the compound in 0.9% NaCl (pH 7; total volume injected, 10 mL/kg) 30 min after the administration of APAP (800 mg/kg). The dose range and time of administration of PTCA after APAP were based on the findings of Nagasawa *et al*. [11] that PTCA (2.45 mmol/kg) administered i.p. 30 min or 2 hr after APAP, provided complete protection against mortality in mice, but was less effective if administered at later times. The PTCA solution was injected immediately after preparation. In control experiments, mice received comparable volumes of the vehicle alone. In some experiments, PTCA (5 mmol/kg) was administered to mice not receiving APAP. Animals were killed 4 hr after APAP administration to determine the extent of sulfhydryl depletion, or at 24 hr to determine the extent of recovery of sulfhydryl levels.

2.4. Tissue preparation

Blood samples (approximately 0.1 mL) were collected from the retro-orbital sinus of the mouse into heparinized tubes. A 20% blood homogenate in 5% MPA (4% final MPA concentration) was prepared. Mice were killed by cervical dislocation and decapitation after blood was drawn. The kidneys and brain were excised, rinsed in ice-cold 0.9% NaCl, trimmed of adherent tissues, and weighed. Samples were kept on ice and processed as rapidly as possible to prevent oxidation and degradation of the sulfhydryl compounds. An 11% tissue homogenate (w/v) of brain or kidney cortex was prepared in 4.4% MPA (final concentration 4%), using an all-glass Tenbroeck homogenizer, and kept on ice. After standing for 20–40 min, the homogenate was centrifuged for 1 min at 10,000 *g*, and the acid-soluble fraction was collected for measurement of sulfhydryl and disulfide compounds.

2.5. Chemical analyses

GSH, GSSG, and Cys were quantified by HPLC using electrochemical detection according to the method of Richie and Lang [24] with slight modifications. The details of the procedure have been described previously [6,22]. Although CSSC was separated from GSH, GSSG, and Cys on the HPLC column, kidney and brain samples contained a small unknown peak having a retention time close to that of CSSC, which precluded accurate measurement of this disulfide. PTCA was eluted from the column later than any of the standards and did not interfere with the analyses.

2.6. Statistical analyses

Analyses were performed using the SPSS for Windows computer program (SPSS Inc.). Differences between the means of two groups were evaluated by a two-tailed *t*-test for independent samples. Differences between the means of three or more groups were evaluated by a one-way ANOVA. If the ANOVA indicated a significant difference

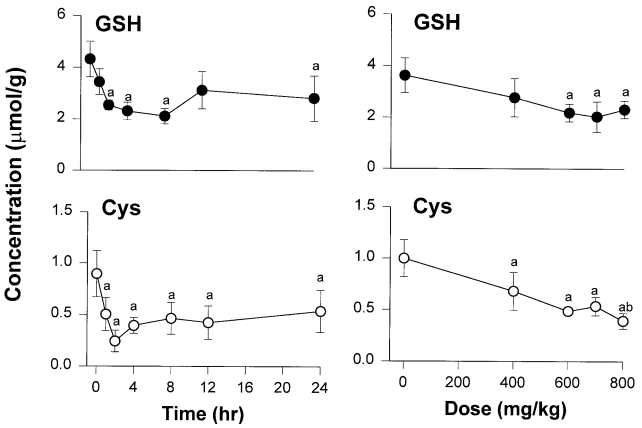

Fig. 1. APAP-induced time- and dose-dependent depletion of renal cortical sulfhydryl compounds. (Left panels) Time dependence: APAP (800 mg/kg) in 50% propylene glycol was injected i.p. at time zero. Symbols represent means ± SD (N = 3–17). Key: (a) significantly different ($P < 0.05$) from values in the untreated group (time-zero sample). (Right panels) Dose dependence: Concentrations of GSH and Cys were determined 4 hr after the administration of APAP (400–800 mg/kg). Symbols represent means ± SD (N = 3–17). Data presented for 4 hr (left panels) and 800 mg/kg (right panels) are the same. Key: (a) significantly different ($P < 0.05$) from values in the vehicle-treated group (dose-zero sample); and (b) significantly different ($P < 0.05$) from the value in the APAP (400 mg/kg) group.

among the groups, the post-hoc test was determined by a test of homogeneity of variances. If the test indicated equal variances, the Tukey test was employed; if the test indicated nonequal variances, the Dunnett T3 test was applied. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Depletion of renal cortical GSH and Cys by APAP

The time course of changes in renal cortical GSH and Cys concentrations after the administration of APAP (800 mg/kg) is shown in Fig. 1 (left panels). GSH and Cys concentrations in untreated control mice (time-zero values) were 4.3 and 0.9 μmol/g, respectively. The concentration of GSH decreased to about 50% of the initial control value over the 4- to 8-hr period following APAP administration. The concentration of Cys decreased to 26% of the initial control value at 2 hr and was about 50% of control over the 4- to 12-hr period. By 24 hr, the concentrations of GSH and Cys had returned to 66 and 53%, respectively, of the initial control values. The relationship between dose and renal cortical GSH and Cys concentrations at 4 hr after APAP administration is also shown in Fig. 1 (right panels). APAP in doses of 600–800 mg/kg produced significant decreases in GSH and Cys concentrations; at the highest dose, the concentrations were 64 and 39%, respectively, of the values in vehicle-treated animals. Renal cortical GSSG concentrations in all samples were <5 nmol/g, and below the limit of sensitivity of the analytical method used.

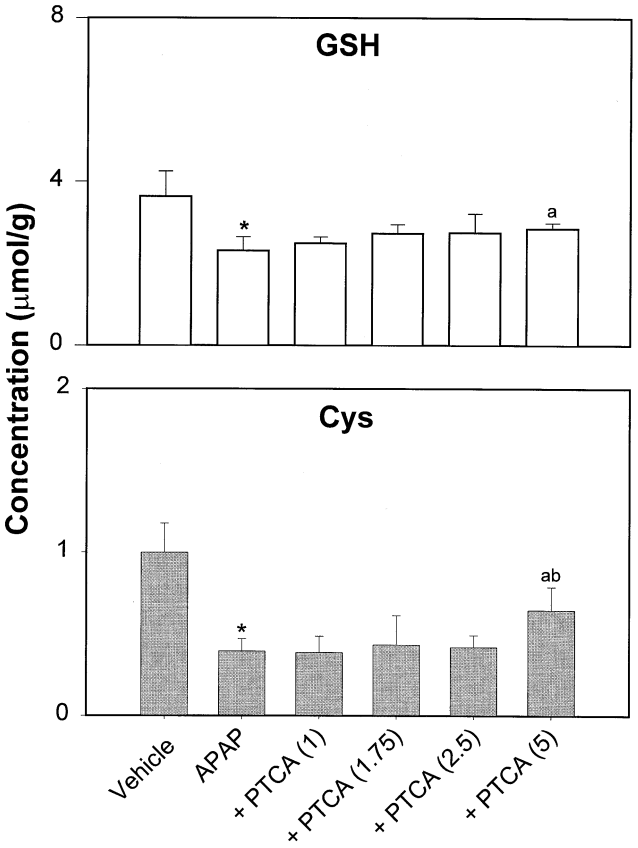

Fig. 2. Effects of PTCA on renal cortical concentrations of sulfhydryl compounds 4 hr after APAP. PTCA was injected i.p. 30 min after APAP (800 mg/kg). + *PTCA* denotes groups receiving APAP *plus* PTCA; the PTCA dose (mmol/kg) is shown in parentheses. Data bars represent means ± SD (N = 3–17). Key: (*) significantly different ($P < 0.05$) from the vehicle-treated group; (a) significantly different ($P < 0.05$) from *APAP alone*; and (b) significantly different ($P < 0.05$) from + *PTCA (1)*.

3.2. Effect of PTCA on APAP-induced depletion of sulfhydryl compounds in the kidney

APAP-induced decreases in GSH and Cys at 4 hr (64 and 39%, respectively, of values in vehicle-treated controls) were attenuated only by the highest dose (5 mmol/kg) of PTCA (78 and 65%, respectively, of vehicle control values) (Fig. 2). In contrast, the APAP-induced decreases in GSH and Cys concentrations at 24 hr (77 and 45%, respectively, of vehicle control values) were prevented completely by PTCA in doses of both 2.5 mmol/kg (123 and 107%, respectively, of vehicle control values) and 5 mmol/kg (127 and 114%, respectively, of vehicle control values), and in the case of Cys, a dose of 1.75 mmol/kg (112% of control value) (Fig. 3). GSSG concentrations were below the limit of sensitivity of the analytical method in all of the samples. The renal cortical concentrations of GSH and Cys at 4 hr in mice receiving PTCA alone at 5 mmol/kg [3.4 ± 1.1 and 0.81 ± 0.20 μmol/g (N = 6), respectively] were not significantly different from the respective values in vehicle-treated controls.

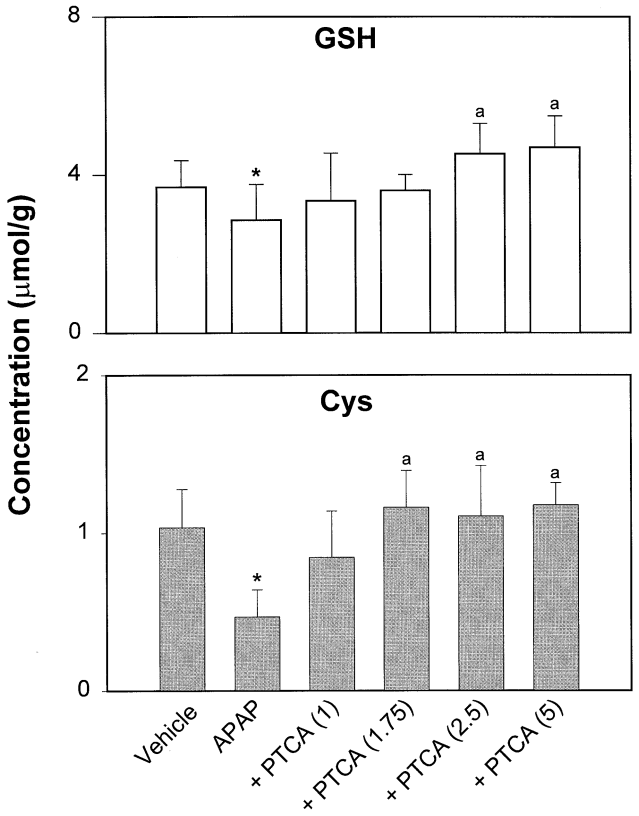

Fig. 3. Effects of PTCA on renal cortical concentrations of sulfhydryl compounds 24 hr after APAP. Data are presented as described in the legend for Fig. 2. Key: (*) significantly different ($P < 0.05$) from the vehicle-treated group; and (a) significantly different ($P < 0.05$) from *APAP alone*.

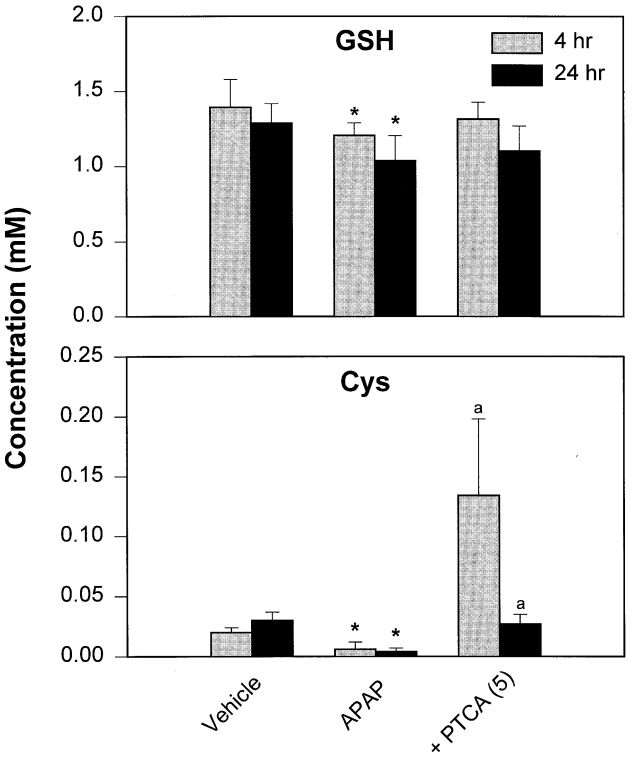

Fig. 4. Effects of PTCA on blood concentrations of sulfhydryl compounds 4 and 24 hr after APAP. PTCA (5 mmol/kg) was injected i.p. 30 min after APAP (800 mg/kg). + *PTCA* denotes groups receiving APAP *plus* PTCA. Data bars represent means ± SD (N = 3–17). Key: (*) significantly different ($P < 0.05$) from the vehicle-treated group; and (a) significantly different ($P < 0.05$) from *APAP alone*.

3.3. Effects of APAP and PTCA on sulfhydryl levels in the blood and brain

Although GSH and Cys levels in the blood and brain were measured at all PTCA doses (1–5 mmol/kg), significant differences from values in APAP-treated animals were observed only at a dose of 5 mmol/kg. Therefore, only data obtained with this dose are reported. The concentrations of GSH and Cys at 4 hr in mice receiving PTCA (5 mmol/kg) alone were 1.3 ± 0.1 and 0.03 ± 0.02 mM (N = 6), respectively, in blood, and 2.0 ± 0.3 and 0.06 ± 0.02 μmol/g (N = 6), respectively, in brain, which were not significantly different from the respective values in vehicle-treated controls.

Whole blood GSH, Cys, CSSC, and GSSG concentrations in untreated control mice were 1.3, 0.02, 0.02, and 0.06 mM, respectively. In vehicle-treated controls, the respective values at 4 hr were 1.4, 0.02, 0.03, and 0.05 mM. APAP caused a small decrease in whole blood GSH concentration (87% of vehicle control value) and a large decrease in Cys concentration (30% of vehicle control value) at 4 hr (Fig. 4). In contrast to the kidney cortex, where partial recovery of sulfhydryl compounds was seen 24 hr after APAP administration, there were further decreases in whole blood GSH and Cys at 24 hr (81 and 13%, respectively, of vehicle control values). There were no significant changes in CSSC or GSSG concentrations at 4 or 24 hr (data not shown). PTCA caused large increases in the whole blood concentrations of Cys (670% of vehicle control values) 4 hr after APAP administration, but did not affect GSH concentrations. At 24 hr, Cys concentrations remained higher than in mice receiving APAP alone.

Brain GSH and Cys concentrations in untreated control mice were 1.9 and 0.05 μmol/g, respectively; in vehicle-treated controls, the respective values at 4 hr were 1.9 and 0.06 μmol/g. APAP had no effect on sulfhydryl concentrations in the brain (Fig. 5). However, PTCA caused large increases in Cys concentrations in the brain (411% of vehicle control values) and augmented GSH concentrations somewhat (119% of vehicle control values) 4 hr after APAP administration.

4. Discussion

4.1. Effects of APAP

The results of the present study, together with data on hepatic sulfhydryl levels obtained in the same studies [6], extend the available information on the tissue selectivity of

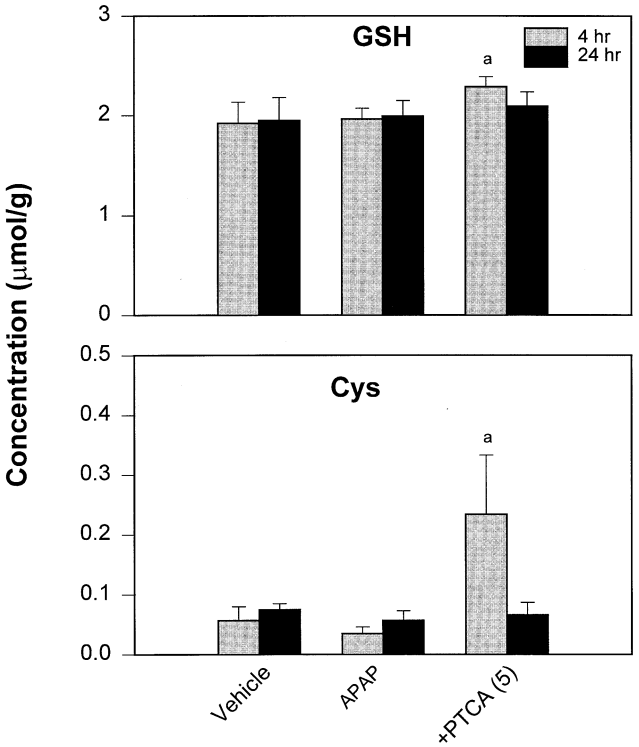

Fig. 5. Effects of PTCA on brain concentrations of sulfhydryl compounds 4 and 24 hr after APAP. Experimental details and data presentation are as described in the legend for Fig. 4. Key: (a) significantly different ($P < 0.05$) from *APAP alone*.

APAP effects. The studies demonstrated tissue differences in APAP-induced sulfhydryl depletion, including differences in the extent, pattern (relative effects on GSH and Cys levels), and time course of depletion. The extent of GSH depletion 4 hr after APAP administration, expressed in terms of the percent decrease in tissue concentration, varied in the order: liver (95% depletion) > kidney (36%) > blood (13%), while the extent of Cys depletion varied in the order: liver (86%) > blood (70%) > kidney (61%). The extent of GSH depletion 24 hr after APAP administration varied in the order: liver (51%) > kidney (23%) ≅ blood (19%), and the extent of Cys depletion varied in the order: blood (87%) > liver (56%) ≅ kidney (55%). Brain concentrations of sulfhydryls were not affected by APAP in mice (Fig. 5), thus corroborating the data of Bien *et al*. [21] who found that APAP had no effect on brain GSH in rats. The above tissue differences could be due to many factors, including differences in the conversion of APAP to the active metabolite NAPQI, differences in GSH turnover rates (synthesis and utilization), and differences in the availability of Cys for GSH synthesis.

The observed differences in the extent of APAP-induced sulfhydryl depletion in the liver and kidney confirm earlier reports that APAP causes greater GSH depletion in the liver in mice [18] and rats [18,19] and demonstrate that it also causes greater Cys depletion in the liver than the kidney (86 vs 61%). Others have shown a higher cytochrome P450 content in rat liver cells than kidney cells [25] and greater covalent binding of radiolabeled APAP metabolites to proteins in the liver than in the kidney in mice and rats [18].

The higher concentration of Cys in the kidney than in the liver could be another contributing factor to the less extensive depletion of renal GSH by APAP. While the renal concentration of GSH (4.3 μmol/g) was only about one-half of the hepatic level (9 μmol/g) in untreated mice, the renal cortical concentration of Cys (0.9 μmol/g) was greater than 4-fold the hepatic level (0.2 μmol/g). Thus, the greater availability of Cys, the rate-limiting substrate in GSH biosynthesis [7], in the kidney could serve to limit GSH depletion in that organ. The proportionately greater depletion of Cys (61%) than GSH (36%) observed in the kidney confirms earlier studies in mice [26] and would also be consistent with proportionately greater conversion of Cys to GSH and, thereby, less extensive depletion of GSH in the kidney.

The absence of a large effect of APAP on blood GSH theoretically could be due to a lack of metabolic activation in red blood cells. The small magnitude of the APAP-induced decrease in blood GSH concentration (13%) at a time of larger decreases in the liver (95%) and kidney (36%) suggests that blood GSH, greater than 99% of which is located in red blood cells [27], is not readily available to replenish depleted stores in the liver and kidney. Supporting this possibility is evidence that red blood cells, unlike many other tissues, export GSSG but not GSH [28]. Also, APAP did not affect the blood GSSG concentration. Alternatively, the small magnitude of the decrease in blood GSH could theoretically be due to enhanced synthesis from Cys, which would result in maintenance of GSH levels and would be consistent with the observed large decrease in blood Cys concentration.

The lack of effect of APAP on brain sulfhydryl concentrations cannot be attributed to exclusion of the drug from the central nervous system, since Fischer *et al*. [29] showed that peak APAP concentrations in the brain were close to those in plasma, liver, and kidney after a toxic dose of APAP in mice. However, Emeigh Hart *et al*. [30] demonstrated binding of anti-APAP antibodies to paraffin sections of liver and kidney but not brain from mice receiving toxic doses of APAP. Also, the observation that brain cytochrome P450 levels are only about 1–3% of hepatic levels [31] suggests that the lack of effect of APAP on brain sulfhydryl concentrations may be due to the lack of cytochrome P450-mediated conversion to NAPQI.

4.2. Effects of PTCA

In addition to protecting against APAP-induced hepatic GSH depletion and toxicity [6,11], PTCA increased sulfhydryl levels in kidney, blood, and brain in mice receiving large doses of APAP. As with APAP, there were tissue differences in the magnitude and pattern of PTCA effects on sulfhydryl levels. It attenuated APAP-induced depletion of

GSH and Cys in the kidney, as in the liver [6], prevented APAP-induced Cys depletion with no effect on GSH in the blood, and increased Cys (large effect) and GSH (small effect) concentrations in the brain.

PTCA was less effective in attenuating APAP-induced GSH and Cys depletion in the kidney than in the liver; for example, a dose of 2.5 mmol/kg is effective in the liver [6], whereas a dose of 5 mmol/kg was required in the kidney. Further, a dose of 5 mmol/kg increases GSH and Cys levels by 1,244% and 700%, respectively, in the liver [6] but by only 23 and 64%, respectively, in the kidney of APAP-treated mice. Some possible explanations of the tissue selectivity include organ differences in uptake of the drug, the relative rates of synthesis and utilization of sulfhydryl compounds, and the time courses of sulfhydryl depletion by APAP and repletion by PTCA. Another theoretically possible explanation for the greater hepatic effect involves the lower concentration of Cys in the liver (0.09 μmol/g in vehicle-treated controls and 0.01 μmol/g after APAP) than the kidney (1.0 μmol/g in controls and 0.39 μmol/g after APAP). Nagasawa *et al*. [11] have provided evidence that PTCA is non-enzymatically converted to Cys and suggested that the rate of conversion is greater at lower Cys concentrations. Thus, greater release of Cys in the liver could account for a greater effect on Cys and GSH levels in this tissue.

The pattern of PTCA effects on blood sulfhydryl levels resembled its effects on the brain (i.e. a predominant increase in Cys). Since Cys is taken up by red blood cells [32], the failure of a high blood Cys level (670% of vehicle control) after PTCA to replenish the depleted GSH presumably is not due to an inability of Cys to enter the cells. The lack of effect of PTCA on blood GSH could be due to a low GSH synthetic capacity and turnover of red blood cells [33,34].

The selectivity of the effects of PTCA on brain sulfhydryls in mice treated with APAP was similar to that reported for Cys and the Cys precursor OTC in untreated mice and rats. As with PTCA, which increased brain Cys and GSH to 411 and 118%, respectively, of vehicle-treated control levels, Anderson and Meister [35] found that Cys and OTC each caused a large increase in brain Cys with little or no increase in GSH in fasted mice and rats. Pileblad and Magnusson [36] demonstrated regional differences in the effects of OTC and Cys in the brain. OTC increased Cys in the striatum and substantia nigra but did not increase GSH in either region. Similarly, Cys caused a large increase in Cys in some regions and a small increase in GSH in only one of the regions studied [36].

In conclusion, the present results demonstrate a high degree of tissue selectivity in APAP-induced depletion of GSH and Cys, as well as in the effectiveness of PTCA in increasing sulfhydryl levels in APAP-treated mice.

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