Biochemical Pharmacology, Vol. 51, pp. 1111–1116, 1996.

ELSEVIER

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00144-X

RAPID COMMUNICATION

MAINTENANCE OF HEPATIC GLUTATHIONE HOMEOSTASIS AND PREVENTION OF ACETAMINOPHEN-INDUCED CATARACT IN MICE BY L-CYSTEINE PRODRUGS

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Abstract-Administration of acetaminophen (ACP, 3.0 mmol/kg, i.p.) to β-naphthoflavone-induced C57 BL/6 mice led to the formation of bilateral cataracts within 8 hr with a 71% incidence. The hepatic glutathione (GSH) levels were reduced 99% and lenticular GSH levels reduced 42% in cataractous mice. Cataract formation was completely prevented by the co-administration of the L-cysteine prodrugs 2(*R,S*)-methylthiazolidine-4(*R*)-carboxylic acid (MTCA) and 2(*R,S*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid (PTCA) in two divided i.p. doses totaling 4.5 mmol/kg. 2-Oxo-L-thiazolidine-4-carboxylic acid (OTCA) was nearly equipotent, yielding only one cataract in 16 mice, but D-ribose-L-cysteine (RibCys, 5/16) and N-acetyl-L-cysteine (NAC, 9/14) were much less effective. Hepatic and lenticular GSH were maintained at near normal levels by MTCA, PTCA and OTCA. These results suggest that maintenance of adequate cellular GSH levels in the presence of ACP protects against cataract induction.

Key words. acetaminophen; cataracts; L-cysteine prodrugs; glutathione

ACP§ (4-hydroxyacetanilide, paracetamol), a non-aspirin analgesic/anti-pyretic agent, is used universally by individuals who are unable to tolerate aspirin or aspirin-like drugs and by children for whom aspirin is contraindicated for the control of fever. Whereas ACP is regarded as being devoid of toxicity when used in the recommended dose, chronic and possibly excessive use appears to lead to increased risk of liver and kidney damage over a lifetime [1], damage that is exacerbated by fasting and alcohol use [2,3]. In acute overdose situations, ACP can be lethal unless intervened pharmacologically with NAC within the first 8-10 hr of ingestion [4-6].

The biochemical/pharmacological mechanism of the hepatic and renal toxicity elicited by high doses of ACP is generally accepted as being due to the metabolic transformation of ACP to a reactive species that covalently binds to critical cellular macromolecular targets, triggering a complex series of metabolic events, leading eventually to cell death [7]. In addition to NAC, a number of other substances have been proposed as hepatoprotective and renal protective agents for the palliation of toxicity by high doses of ACP [7].

We are unaware of any epidemiological evidence that chronic, excessive use or accidental overdoses of ACP cause cataracts in humans. However, C57 BL/6 mice, following induction of their hepatic mixed-function oxidase system, are subject to ACP-induced cataracts that morphologically mimic human senile cataracts [8-12].

The ocular lens is characterized by a uniquely high content of GSH, an antioxidant required to maintain lens clarity. A low content of GSH is the first biochemical lesion known to precede opacity formation in every form of

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§Abbreviations: ACP, acetaminophen; MTCA, 2(*R,S*)-methylthiazolidine-4(*R*)-carboxylic acid; PTCA, 2(*R,S*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid; OTCA, 2-oxo-L-thiazolidine-4-carboxylic acid; RibCys, D-ribose-L-cysteine; NAC, N-acetyl-L-cysteine; GSH, glutathione (reduced); and GSSG, glutathione (oxidized).

Accepted 27 February 1996.

irreversible cataract. Both the uptake and incorporation of the amino acid L-cysteine into GSH are rate-limiting steps in its biosynthesis [13,14]. Thus, prodrugs of L-cysteine appear to be suitable compounds for pharmacological intervention to maintain lenticular GSH homeostasis. Auto-oxidation of the sulfhydryl group can be prevented by use of compounds with masked sulfhydryl groups [15,16].

For many decades the question has persisted: If GSH content could be maintained in the presence of cataractogenic agents, would cataract formation be altered or even prevented? Two thiazolidines yielded promising results in cultured rat lenses and in prevention of naphthalene-induced cataract in the mouse [17,18]. It is the purpose of this work to show the efficacy of certain L-cysteine prodrugs in maintaining normal hepatic GSH levels and in preventing cataract in the acetaminophen-induced murine cataract model.

MATERIALS AND METHODS

Chemicals. The following reagents were obtained commercially: ACP, NAC, OTCA and ß-naphthoflavone (Sigma Chemical Co., St. Louis, MO); Dulbecco's phosphate-buffered saline without calcium or magnesium (Celox Corp., Hopkins, MN); corn oil (Wesson, Hunt-Wesson, Inc., Fullerton, CA). All other L-cysteine prodrugs were synthesized according to published methods [15,16]. Chemicals and other materials required for optical GSH assay by HPLC were obtained as detailed elsewhere [17].

Animals and Pharmacological Protocol. SPF C57 BL/6 mice, 17-21 g males, were received from the Indianapolis, IN, facility of Harlan/Sprague-Dawley. Mice were maintained in approved animal quarters following the NIH requirements and according to the resolution on the Use of Animals in Research by the Association for Research in Vision and Ophthalmology. The mice were acclimated to their new environment for at least 5 days.

Immediately prior to administration, ß-naphthoflavone (0.0375 M solution, 10.2 mg/mL) was dissolved in warm corn oil and administered to mice (0.75 mmol/kg body wt) by a single i.p. injection to induce the cytochrome P450 isozymes. Three days following the administration of ß-naphthoflavone, ACP, dissolved in warm Dulbecco's saline, was administered, i.p. A dose-response study (Table 1) indicated that maximum cataract formation (71% incidence) occurred with 3.0 mmol ACP/kg body wt; hence, this dose was used for all subsequent experiments. Earliest cataract detection and subsequent expiration times for this dose were 4.7±0.9 and 5.0±1.3 hr, respectively, following ACP administration to 12 mice. The standard ACP solution contained 22.7 mg/mL (0.15 mmol/mL) and was administered at a level of 0.02 mL/g body wt.

Table 1. Cataract incidence (at 12 hr) with ACP dose

	ACP (mmol/kg)					
	1.75	2.25	2.50	2.75	3.00	3.25
Cataracts	0/4*	0/4	2/5	4/7	12/17	4/7

*The first numeral is cataract incidence, the second is number of mice; thus, 0/4 indicates 0 cataracts in 4 mice. All mice received β-naphthoflavone pretreatment.

The L-cysteine prodrugs were dissolved in Dulbecco's saline just prior to use. Except for PTCA, stock prodrug solutions were prepared at two concentrations, *viz.* 0.27 and 0.18 mmol/mL, respectively. PTCA concentration was 0.09-0.135 mmol/mL to accommodate its lesser solubility. The prodrugs were administered, i.p., prior to and after ACP, at a level of 0.01 mL/g body wt (or 0.02 mL/g body wt for PTCA) according to the following schedule: 0 time, prodrug (2.7 mmol/kg); 0.75 hr, ACP (3.0 mmol/kg); 1.25 hr, prodrug (1.8 mmol/kg). Mice receiving overnight pretreatment of RibCys were given 2.7 mmol/kg 14 hr before 0 time and 1.8 mmol/kg at 0 time.

Morphology, GSH and GSSG Analysis. Cataract formation was initially detected using a slit lamp microscope or ophthalmic eye loupes for magnification, but thereafter could be easily followed with the unaided eye. Physical type/location of the cataracts was determined with the slit lamp microscope. Cataracts were not graded, as only the distinct presence or absence of opacity was of interest. Mice were observed with increasing frequency during the first 12 hr following administration of ACP. Upon physical and behavioral signs of impending cataract

formation (especially eyelid opening), observations were made continuously. Following the initial 12-hr period, observations were made less frequently but continued for a 3-day period, and thereafter, once a day for an additional 2 weeks. Representative mice of groups that were cataract-free 24 hr post-ACP administration were killed in an atmosphere of 100% CO_2 and used for organ analysis.

Eyes and entire livers from representative mice of each group were rapidly excised immediately after death. The lenses were removed, and the livers were divided into three approximate equiportions; the excised tissues were weighed and quick-frozen in liquid nitrogen. The small lenses (average wt: 6 mg) were pooled into groups for analyses; the liver portions were analyzed individually. Following a brief storage time at -80°, frozen tissues were prepared for HPLC analyses of GSH and GSSG content by applying the procedure described for lenses [17].

RESULTS

Approximately 71% of the mice pretreated with β-naphthoflavone, followed by ACP administration, developed bilateral cataracts in less than 8 hr (Fig. 1). All cataracts were initiated at the suture lines, then spread peripherally within minutes, first involving the nucleus, then the cortex and finally resulting in total opacity. The posterior subcapsular region could not be viewed for evaluation at maximum cataract development, and the anterior subcapsular region appeared to be the last involved region. The cataract, when viewed by the unaided eye, was a bright, white shining bead and can be described as being a "total cataract."

Mice that received ACP only exhibited passivity, eyelid closure, and eventual eyelid opening prior to cataract development. When no prodrug or a totally ineffective prodrug was used, these symptoms occurred as early as 1 hr following ACP administration. Mice that never developed cataracts rarely exhibited any toxic effects of ACP.

Three L-cysteine prodrugs, MTCA, PTCA and OTCA [19], were highly effective in cataract prevention during the first 8-hr period. No cataracts formed with these compounds in a total of 40 mice. One OTCA-treated mouse developed bilateral cataracts 23.5 hr post-ACP administration and expired soon thereafter (Fig. 1). Three additional OTCA-treated mice expired (without cataract) within 4 days. MTCA and PTCA were the only L-cysteine prodrugs that maintained all mice in a cataract-free state throughout the observation period with 100% survival.

Neither NAC nor RibCys administered 14 hr before ACP prevented cataract formation as compared to mice given only ACP. RibCys appeared to be more effective when administered 0.75 hr prior to ACP, reducing cataract incidence to about half that of controls. Approximate cataract formation times for mice where NAC, RibCys and RibCys with 14-hr pretreatment proved ineffective were 5.0±2.0, 4.9±1.0 and 5.2±0.7 hr, respectively. Subsequent post-ACP expiration times were 5.6±1.7, 5.7±1.5 and 6.1±0.3 hr, respectively. No additional

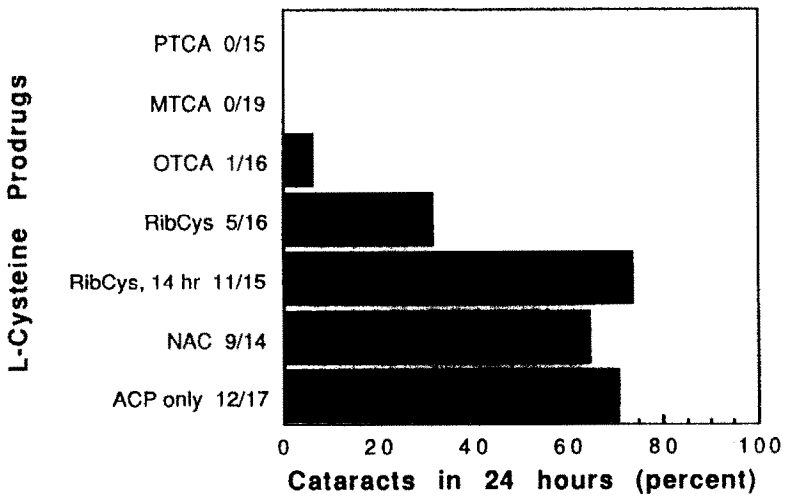

Fig. 1. Attenuation of ACP-induced cataracts in C57 BL/6 mice by prodrugs of L-cysteine. The experimental protocol is given under Materials and Methods and the key to abbreviations is footnoted. RibCys, 14 hr indicates that RibCys was administered 14 hr before ACP.

cataracts developed in any mice beyond the initial 24 hr post-ACP period over the 2-week observation period.

Hepatic and lenticular GSH and GSSG levels were determined in randomly selected representative mice of non-treated controls (which received β-naphthoflavone pretreatment), mice receiving ACP alone, and those receiving the L-cysteine prodrugs in conjunction with ACP. Liver GSH concentration dropped 99% in mice treated with ACP alone (Table 2). Neither NAC nor RibCys co-administration was effective in preventing this massive loss of hepatic GSH. In marked contrast, mice treated with PTCA, OTCA and MTCA (which were cataract-free, Fig. 1) maintained hepatic GSH levels that were only 12-19% below control levels (Table 2).

Compared to the liver, GSH loss in all affected lenses was modest. Even the "ACP-only" mice retained 58% of the GSH levels seen in the controls (Table 3), and GSH losses were minimal when treated with NAC, RibCys or PTCA. With MTCA and OTCA, lenticular GSH was increased 9 and 14%, respectively, over control values.

Table 2. Hepatic GSH and GSSG content following various treatments*

Treatment	n	Cataracts†	GSH			GSSG			Deg F
			(nmol/g)	(% of control)	*P*-Value	(nmol/g)	(% of control)	*P*-Value	
Control	3	0	6350±370	100	<0.0001	276±27	100	<0.0001	8
ACP	4	4	51±9	0.8		21±6	7.6		
ACP + NAC	4	4	113±46	1.8	0.0016	25±11	9.1	0.373	11
ACP + RibCys	4	4	147±105	2.3	0.0064	32±19	11.6	0.123	11
ACP + PTCA	4	0	5140±119	80.9	<0.0001	346±107	125	<0.0001	8
ACP + OTCA	3	0	5610±118	88.3	<0.0001	203±55	73.6	<0.0001	11
ACP + MTCA	4	0	5260±440	82.8	<0.0001	366±75	133	<0.0001	11

*All mice received β-naphthoflavone pretreatment. Liver samples were collected immediately following expiration due to ineffective prodrug treatment, or killing of the animals at 24 hr post-ACP for mice lacking toxic symptoms. Three liver samples from each mouse were analyzed individually and averaged. The resulting value was averaged with those from other mice to obtain the indicated values (mean ± SD). *P*-values were calculated with respect to the ACP-treated controls.
†All cataracts were bilateral.

Table 3. Lenticular GSH and GSSG content following various treatments*

Treatment	n	Cataracts	GSH		GSSG	
			(nmol/lens)	(% of control)	(nmol/lens)	(% of control)
Control	22	0	16.2	100	0.246	100
ACP	6	6	9.4	58.0	0.218	89
ACP + NAC	18	18	13.6	84.0	0.262	107
ACP + RibCys, 14 hr	8	8	14.1	87.0	0.279	113
ACP + RibCys	4	4	14.7	90.7	0.476	193
ACP + PTCA	8	0	11.2	69.1	0.259	105
ACP + OTCA	10	0	18.4	114	0.306	124
ACP + MTCA	8	0	17.7	109	0.311	126

*All mice received β-naphthoflavone pretreatment. Lenses were collected immediately after death as noted in Table 2. Lenses from each treatment were pooled for analysis. Pooling of these small lenses is acceptable, as the error in reproducibility of the method is not more than 5% in the experience of this laboratory and of Fariss and Reed [20]. Since the GSH and GSSG concentrations expressed per unit weight of lens are disproportionately affected by increasing hydration associated with cataracts, the values are given as nmol/lens.

GSSG levels likewise decreased dramatically from control levels in livers of ACP-treated mice, even with NAC and RibCys co-administration (Table 2). On the other hand, hepatic GSSG was elevated 25 and 33% above control levels when mice were treated with PTCA and MTCA, respectively, and was reduced only 26% below controls with

OTCA treatment. Table 3 also shows a loss of GSSG in the lenses of ACP-treated mice. Lenticular GSSG increased with every prodrug used, with maximum increase approaching 200% in lenses of RibCys-treated mice. Total GSH plus GSSG increased only in lenses of OTCA- and MTCA-treated mice.

DISCUSSION

This work was based on the concept that if the depletion of GSH due to bioactivated ACP could be counteracted with continuous biosynthesis of GSH by supplying the rate-limiting substrate, L-cysteine, toxic effects of ACP metabolites, including cataract formation, might be prevented. Due to the toxicity at high levels and effervescent nature of L-cysteine, use of prodrugs, especially those having oxidatively stable masked sulfhydryl groups, provide preferred sources of L-cysteine available "on demand" through chemical equilibria. NAC (having a free sulfhydryl group) and RibCys were shown by others to protect against ACP hepatotoxicity in mice [16,21]. Efficacy of eleven L-cysteine prodrugs was compared in rat lenses incubated in the presence of [^{14}C(U)]-glycine. The L-cysteine released from the effective prodrugs was incorporated *de novo* into [^{14}C]-GSH. MTCA, PTCA, NAC and RibCys were the most effective stimulators of lenticular GSH biosynthesis in these 48-hr *in vitro* studies [17].

Hepatic GSH was maintained at 80% or more and toxic symptoms were eliminated by the thiazolidine prodrugs MTCA and PTCA. Only 0.8% GSH was retained in their absence (Table 2). This is consistent with the concept that the depletion of the hepatic GSH pool by ACP was counteracted by newly synthesized GSH containing the L-cysteine moiety derived from these thiazolidines [17].

Four other prodrug treatments were either ineffective or were less effective than those with MTCA and PTCA. Evaluation of the effectiveness of OTCA must be tempered by the appearance of one cataract and four (25%) deaths in a 96-hr period. NAC was not effective in these acute studies, although it was effective in stimulation of GSH biosynthesis during a 48-hr period *in vitro* [17]. Two major differences between the structure of NAC and the effective thiazolidines may be involved. NAC has a free sulfhydryl group subject to oxidation, and the requirement for deacetylase catalysis to form L-cysteine may lead to an organelle and/or time limitation. This is in contrast to rapid non-enzymatic solvolytic dissociation of MTCA and PTCA, which is initiated even prior to absorption. Elevation of murine hepatic and kidney GSH by RibCys was maximal at 16 hr [22], but RibCys administered 14 hr before ACP was ineffective in these *in vivo* trials. A larger dose for long time periods may be required, since administration just prior to ACP was partially effective (Fig. 1).

The lack of toxic symptoms and retention of high levels of hepatic GSH with co-administration of MTCA and PTCA were associated with complete prevention of cataract induction by ACP (Fig. 1). The mechanism of cataract induction by ACP is unknown. Cataractogenesis was dissociated from hepatotoxicity in DBA/2 mice, which are refractory to cataractogenesis by ACP [10,11]. Immunological evidence suggests that cytochrome P450 (1A1/1A2) can be induced in the non-pigmented epithelium of the ciliary body of C57 BL/6 but not DBA/2 mice [12], suggesting possible ACP bioactivation independent of the liver.

The degree of hepatic GSH depletion (99%, Table 2) was not mirrored by the lens, as shown by the loss of only 42% of the lenticular GSH content by ACP treatment (Table 3). Lenticular GSH losses were less or nonexistent with prodrug administration (Table 3). Several possible explanations exist. Since the lens is not capable of induction of the required cytochrome P450 isozyme, the activated form of ACP must be formed elsewhere and dissolved in the aqueous humor, which is secreted by the ciliary body to bathe the lens. The ciliary body might be less efficient than the liver in bioactivation, and the metabolized ACP formed there or derived systemically is likely to react locally prior to secretion. Within the lens GSH loss by conjugation with the reactive forms of ACP may well be greatest in the initial sites of absorption, i.e. the single cell layer which forms the epithelium where GSH levels are highest. Due to the small lens size, GSH content of the entire lens was determined, meaning that high local concentrations would be masked by dilution with regions of lower concentration.

This work probed the question of whether GSH homeostasis might be maintained and cataracts prevented by L-cysteine prodrugs in the presence of ACP. That they do is an observation without precedent.

Acknowledgements - This work was supported, in part, by NIH research grant EY-01197-21. W.B.R. is a Research to Prevent Blindness Senior Scientific Investigator; H.T.N. is a Department of Veterans Affairs Research Career Scientist. The authors appreciate the helpful counsel on cataract morphology by Jay H. Krachmer, M.D.

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