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Regulation of Cytochrome P4501A Metabolism by Glutathione

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Abstract: Gene expression of cytochrome P4501A (CYP1A) in the rainbow trout *Oncorhynchus mykiss* is dependent on aromatic hydrocarbon receptor signal transduction, and is markedly sensitive to tissue thiol status. Tissue glutathione (GSH) status was manipulated by exogenous GSH, L-buthionine-[S,R]-sulfoximine (BSO), lipoate or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Tissue GSH contents were significantly elevated in GSH- and lipoate-supplemented trout. Hepatic, renal and plasma GSH levels were markedly arrested in BSO-treated trout. Oxidized glutathione (oxidized GSH) levels were significantly elevated in the BCNU-supplemented group. Both BCNU treatment and BSO-induced GSH deficiency increased steady-state levels of hepatic CYP1A mRNA. Additional exposure to 0.1 mg/kg 3,3′,4,4′-tetrachlorobiphenyl marginally suppressed the tetrachlorobiphenyl-dependent CYP1A induction in BSO-treated livers compared with the respective thiol treatment groups. Tetrachlorobiphenyl exposures altered efficiencies of thiol treatments and increased oxidized GSH content in all but the BSO-treated groups. However, exposure to 5 mg/kg tetrachlorobiphenyl altered effects of thiol treatments on CYP1A mRNA to a small extent, but catalytic activity of CYP1A was many times suppressed in BSO-treated and lipoate-supplemented fish. These results suggest that thiol status interferes with CYP1A metabolism in a two-way mode of action and provide further evidence for a cross-talk between cytochrome P4501A and glutathione.

Polychlorinated biphenyls (PCBs) are environmental pollutants with potential carcinogenic properties. The biochemical effects of various PCB congeners, including 3,3′,4,4′-tetrachlorobiphenyl (tetrachlorobiphenyl), are primarily initiated by ligand binding of the biphenyl to the cytosolic aromatic hydrocarbon receptor and subsequent induction of a specific set of cytochrome P450 and conjugation (phase II) enzymes (Safe 1994). Ligand binding of cytosolic aromatic hydrocarbon receptor is followed by the translocation of the complex to the nucleus where it heterodimerizes with the aromatic hydrocarbon receptor nuclear translocator protein (Okey *et al.* 1994). In the form of a heterodimeric complex, aromatic hydrocarbon receptor and aromatic hydrocarbon receptor nuclear translocator bind to enhancer sequences termed aromatic hydrocarbon-responsive elements or xenobiotic-responsive elements that are located in the 5'-flanking region of cytochromes P4501A1 (CYP1A1) and P4501A2 (CYP1A2) and several phase II genes (Nebert 1994). The DNA binding of aromatic hydrocarbon receptor/aromatic hydrocarbon receptor nuclear translocator and subsequent CYP1A1 induction has been well characterized (Bacsi & Hankinson 1996; Dong *et al.* 1996).

CYP1A1 is induced primarily at the transcriptional level. This induction has been described as a model for analyzing mammalian gene transcription (Whitlock *et al.* 1996). Similar to mammals, in fish ligand binding to aromatic hydrocarbon receptor induces CYP1A. Several forms of cloned piscine CYP1A bear closer resemblance to mammalian CYP1A1 than CYP1A2 (Morrison *et al.* 1995; Hahn & Chandran 1996). Induction of CYP1A enhances lipophilic xenobiotic metabolism, however, often the metabolites are more toxic and/or mutagenic than their precursors (Nebert 1994). It has been shown that toxicant-induced CYP1A1 metabolic activity is associated with oxidative DNA damage. Such deleterious effects have been suggested to be triggered by excess reactive oxygen species that are produced during CYP1A1 mediated metabolism (Park *et al.* 1996).

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a central player in a series of critical cell functions including antioxidant defense, detoxication of electrophiles by conjugation, maintenance of thiol-disulfide status and modulation of redox-sensitive signal transduction (Meister 1991; Dröge *et al.* 1994; Sen & Packer 1996). Most of these roles are implicated through the GSH redox cycle. Reactive oxygen species are detoxified either spontaneously and/or by GSH peroxidase catalyzed mechanisms. Oxidized glutathione or glutathione disulfide (GSH disulphide) is formed as the endproduct. In the presence of NADPH, glutathione reductase (GSH reductase) catalyzes the reduction of GSH disulphide to GSH thus ensuring the maintenance of a favourable oxido-reductive (redox) state of glutathione in the cell (Meister 1991). Tissue GSH metabolism can be ma-

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nipulated by the use of L-buthionine-[S,R]-sulfoximine (BSO) and the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). BSO specifically inhibits glutathione biosynthesis (Meister 1991) depleting both intracellular GSH and GSH disulphide contents. BCNU is a cytostatic drug that is commonly used as an inhibitor of GSH reductase activity (Becker & Schirmer 1995). In this way, BCNU can arrest the glutathione redox cycle resulting in the elevation of GSH disulphide within the cell.

Another therapeutic agent that markedly influences GSH metabolism is α-lipoic acid or 6,8-thioctic acid (Packer *et al.* 1995). Following exogenous supplementation lipoate is rapidly taken up by cells and reduced to the corresponding thiol, dihydrolipoate (Handelman *et al.* 1994). Intracellular dihydrolipoate is rapidly released by the cells to the extracellular medium (Handelman *et al.* 1994). Dihydrolipoate is a strong reducing agent that reduces extracellular cystine to cysteine. By doing so, lipoate improves cysteine bioavailability within the cell and boosts GSH synthesis (Han *et al.* 1997). In addition, both lipoate and dihydrolipoate have proven radical scavenging properties. Dihydrolipoate is also known to contribute to the regeneration of other crucial antioxidants (Packer *et al.* 1995). Transcription factors such as nuclear factor B and activator protein-1 are sensitive to reactive oxygen species and the cellular thiol-disulfide redox state. Exogenously supplemented lipoate and the redox state of endogenous glutathione are effective regulators of such redox sensitive gene expression (Dröge *et al.* 1994; Sen & Packer 1996; Sen *et al.* 1996).

Toxicant-induced aromatic hydrocarbon receptor-mediated gene transcription has been suggested to be redox regulated. In support of this, decreased *in vitro* DNA binding of the aromatic hydrocarbon receptor/aromatic hydrocarbon receptor nuclear translocator heterodimer has been observed under oxidizing conditions (Ireland *et al.* 1995). Previously we have reported that alteration in tissue GSH status sensitively regulates induced hepatic CYP1A gene expression and catalytic activity (Otto *et al.* 1997). In this work we have sought to evaluate the possible role of tissue GSH level and glutathione redox state on basal and toxicant-induced cytochrome P450 metabolism. To address this, BSO-treated GSH deficient and BCNU-treated GSH disulphide-elevated experimental models were used. To elevate tissue GSH levels, animals received GSH- or lipoate-supplementations. We present evidence that manipulations of tissue GSH metabolism, as mentioned above, sensitively modulated the basal cytochrome P4501A system as well as the responses of CYP1A metabolism to tetrachlorobiphenyl.

Materials and Methods

Chemicals. 3,3′,4,4′-Tetrachlorobiphenyl was purchased from Ultra Scientific (Kingstown, RI, USA). 1,3-Bis(2-chloroethyl)-1-nitrosourea was a kind gift from Bristol-Myers Squibb (Montréal, Canada). Ethoxyresorufin, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), bakers yeast glutathione reductase, reduced and oxidized glutathione, L-buthionine-[S,R]-sulfoximine, α-lipoic acid, NADPH, 3-aminobenzoic acid ethyl ester (MS 222) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and 2-vinylpyridine was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were of analytical grade.

Animals. Hatchery reared, immature rainbow trout (70–160 g) were obtained from Lincroft Acres (Campellcroft, ON, Canada). The fish were placed in tanks supplied with dechloraminated City of Ottawa tap water and laboratory acclimated for 1 month before separating the fish randomly into 15 tanks, 7–10 fish per tank, supplied with identical water. Water was saturated with oxygen and maintained at 12±1°. Fish were fed 1.5% of body weight per day, but after daily injections feeding declined. Experiments were undertaken during the spring.

Thiol manipulations and exposure of fish to tetrachlorobiphenyl. Fish received daily intraperitoneally injections of either saline, GSH, BSO, lipoate or BCNU in the evening. Tank water levels were lowered (to approximately 40 l) and fish were mildly anaesthesized by adding 0.05 g/l 3-aminobenzoic acid ethyl ester (MS-222) and 0.1 g/l sodium bicarbonate to the tank. Solutions of each treatment agent were prepared in fish physiological saline (pH 6.8) with the exception of lipoate that was dissolved in 1 part 6 N NaOH and 19 parts saline (pH 7.0) and of BCNU that was dissolved in 1,2 propanediol, cremophore and saline (1:1:3 v/w/v). Saline-treated trout received saline injections (175–400 μl) and GSH-supplemented trout received GSH (0.25 g/kg, 175–400 μl; pH was adjusted to 6.5–6.8 by cautious addition of NaOH just before use). The BSO stock was dissolved in saline with careful heating to 30° and injection volume was 420–960 μl (6 mmol/kg). Lipoate-supplemented fish received volumes of 140–320 μl of lipoate solution (16 mg/kg). BCNU-treated trout received 140–320 μl of BCNU solution (0.15 mmol/kg). Saline, GSH, BSO and lipoate injections commenced 36 hr and BCNU 12 hr prior to tetrachlorobiphenyl exposure. Tetrachlorobiphenyl was dissolved in corn oil heated to 60° to obtain stock concentrations of 0.08 or 3.9 mg/ml. The solution was cooled to approximately 30° and injected intraperitoneally at 0.1 mg/kg and 5 mg/kg (90–210 μl); controls received equivalent volumes of corn oil. Twelve hours after tetrachlorobiphenyl injection, thiol manipulation treatments began (saline, GSH, BSO, lipoate and BCNU) and continued for 3 consecutive days (1 injection/day).

Trout were harvested the morning following a total of 5 days of thiol treatment. Fish were killed by a blow to the head and blood was collected into heparinized syringes from the caudal vein. Plasma was immediately acidified with 5% 5-sulfosalicylic acid and centrifuged to deproteinize. Supernatants were stored at −20°. Organs were quickly excised, freed from blood and snap frozen in liquid nitrogen, and stored at −80° until analyzed.

Preparation of tissue samples. Tissues were homogenized in ice-cold Tris buffer (50 mM, containing 0.25 M sucrose and 1 mM EDTA, pH 7.5) using a Potter-Elvehjem-type glass homogenizer. Homogenates were centrifuged at 10,000×g for 15 min. at 4°. The post-mitochondrial supernatants were further centrifuged at 105,000×g at 4° for 60 min. Microsomes were resuspended by homogenization in 60 mM Tris (containing 0.25 M sucrose, 5 mM EDTA and 20% glycerol, pH 7.4). Microsomes and supernatants were stored at −80° (<2 months) until analyzed for enzyme activities. For glutathione measurements, frozen tissue samples were homogenized in ice-cold 5% 5-sulfosalicylic acid and centrifuged at 4,000×g and 4° for 10 min. Supernatants were collected and stored at −20°.

Biochemical analyses. Total glutathione (=GSH + oxidized GSH; oxidized GSH as GSH equivalents) was determined according to Sen *et al.* (1992) and oxidized GSH according to Griffith (1980). Ethoxyresorufin O-deethylase was assayed at 18°, a temperature which is within the range experienced by this species. Ethoxyresorufin O-deethylase activity was monitored according to Burke & May-

er (1974). Protein content was estimated according to Lowry *et al.* (1951).

RNA slot blot analysis. Total RNA was isolated from 50 mg of frozen liver tissue using a guanidinium isothiocyanate lysis, membrane binding system (RNeasy, Qiagen, Chatsworth, CA, USA). RNA (5 μg/sample) was denatured in 50% formamide, 7% formaldehyde at 65° for 15 min., then transferred to a nylon membrane using a vacuum slot manifold (Gibco/BRL, Burlington, Canada). In addition, an equivalent amount of total RNA from mouse liver was blotted on the same membrane as a negative hybridization control. Blots were probed with a ^{32}P-labelled rainbow trout CYP1A cDNA probe, pfP1–450–3′ (Heilmann *et al.* 1988), ATCC #37657 obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Probes were hybridized for 1 hr at 68° in the presence of 100 mg/ml sonicated calf thymus DNA using a rapid hybridization system (Quickhyb, Stratagene, La Jolla, CA, USA), and washed twice with 2X saline sodium citrate (SSC), 0.25% sodium dodecylsulfate (SDS) at room temperature for 15 min. and once with 0.1 X SSC, 0.25% SDS at 62° for 30 min. Blots were exposed to X-ray film for 8 hr, stripped for 10 min. with boiling 0.1×SSC, 0.1% SDS, and then re-exposed for 24 hr to confirm stripping. Stripped blots were re-hybridized with a ^{32}P labelled oligonucleotide probe (5′-CCG ACA TCG CCG CTG ACC CCT GGC GCC AGT TTA CGT GAG CCG ATC-3′) directed against the D2 region of the trout 28S RNA using the same hybridization conditions as described above. Blots were washed at room temperature as described above with two additional 30 min. washes in 0.1×SSC, 0.25% SDS at 65°, and exposed to X-ray film for 45 min. Relative optical densities of the CYP1A and 28S blots were determined for each sample by densitometry scanning of the X-ray films using a Molecular Dynamics scanning laser densitometer with Imagequant software. Three different blots were made to enable statistical comparison between signal intensities of five different thiol treatments at each tetrachlorobiphenyl concentration and different tetrachlorobiphenyl concentrations within one thiol treatment.

Western blot analysis. Aliquots of equivalent protein content from liver microsomes of each animal were pooled for each treatment group and separated by 12% SDS-PAGE according to Laemmli (1970), then transferred to nitrocellulose membranes. Blots were incubated for 2 hr with a rabbit polyclonal anti-rat CYP1A1/2 antibody (Human Biologics, Phoenix, AZ, USA). Antibody binding was detected by chemiluminescence using a biotinylated anti-rabbit IgG followed by a streptavidin-horseradish peroxidase conjugate (ECL, Amersham, Oakville, Canada). After development, blots were exposed to Hyperfilm ECL (Amersham). Two different Western blots were made to allow qualitative comparison between individual thiol treatments and different tetrachlorobiphenyl exposures within one thiol treatment.

Statistical analyses. Significant differences between the 5 different treatment groups were tested with a one-way analysis of variance, using Statistix 4.1 (Analytical Software, Tallahassee, FL, USA). Significant differences between the various tetrachlorobiphenyl exposures in each treatment group were determined also with a one-way ANOVA. The location of significance was determined using the Least Significant Difference method. A $P<0.05$ or lower was accepted as significant.

Results

GSH status.

Daily saline injections. Tetrachlorobiphenyl exposure increased hepatic GSH and total GSH (i.e., GSH+oxidized GSH) contents in saline-treated fish (table 1). Total GSH levels in 5 mg/kg tetrachlorobiphenyl-exposed saline-treated trout were 1.4-fold higher than in non-tetrachlorobiphenyl controls ($P<0.05$). Hepatic oxidized GSH content increased in both 0.1 mg/kg (2.8-fold) and 5 mg/kg (2.6-fold) tetrachlorobiphenyl-exposed saline-treated trout compared with non-tetrachlorobiphenyl controls ($P<0.01$). The oxidized GSH/GSH ratio, an oxidative stress marker, of the tetrachlorobiphenyl-exposed saline-treated groups approximately doubled compared with that in non-tetrachlorobiphenyl-controls ($P<0.05$). Kidney GSH, total GSH and oxidized GSH levels were not influenced by tetrachlorobiphenyl exposure in the saline-treated groups (table 1).

GSH supplementation. Tissue GSH and total GSH contents in non-tetrachlorobiphenyl and tetrachlorobiphenyl-exposed GSH-supplemented trout significantly increased compared with corresponding treatment groups. In non-tetrachlorobiphenyl controls, the magnitude of tissue GSH and total GSH levels increases in response to GSH supplementation were as follows: liver, 3-fold; kidney, 2-fold; and plasma 14- and 26-fold (for GSH and total GSH, respectively) ($P<0.001$) compared with non-tetrachlorobiphenyl saline-treated controls. Similar increases in tissue GSH and total GSH were also observed in tetrachlorobiphenyl-exposed fish that were GSH-supplemented. Interestingly, the GSH supplementation-dependent increase in hepatic GSH levels was significantly higher in 0.1 mg/kg tetrachlorobiphenyl-exposed fish than in the corresponding non-tetrachlorobiphenyl controls.

That tetrachlorobiphenyl exposure may enhance tissue GSH levels was also evident in the kidney. Renal GSH content in the 0.1 mg/kg tetrachlorobiphenyl group was also significantly higher than that in 5 mg/kg tetrachlorobiphenyl-exposed GSH-supplemented fish. Exposure to lower concentrations (0.1 mg/kg) of tetrachlorobiphenyl was more effective in elevating tissue GSH levels compared to higher (5 mg/kg) concentrations. The elevation of tissue GSH content in 0.1 mg/kg tetrachlorobiphenyl-exposed GSH-supplemented trout was 3.1- (liver) and 2.5-fold (kidney) higher than that in the respective saline-treated group. In comparison, hepatic and renal GSH contents were only 2.4- and 1.9-fold higher in the 5 mg/kg tetrachlorobiphenyl-exposed GSH-supplemented trout compared with the respective saline-treated trout. Tetrachlorobiphenyl exposure did not influence tissue oxidized GSH levels or the oxidized GSH/GSH ratio of GSH-supplemented fish (table 1).

BSO treatment. A marked decrease in tissue GSH content was observed in response to BSO-treatment. In non-tetrachlorobiphenyl controls, BSO treatment induced global tissue glutathione deficiency. Tissue GSH and total GSH contents of BSO-treated fish were reduced in liver (4.9-fold), kidney (3.5-fold) and plasma (3.3-fold) compared with those in saline-treated controls ($P<0.001$). Exposure to higher tetrachlorobiphenyl concentrations potentiated BSO-dependent GSH depletion in the liver. Hepatic GSH and total GSH contents in 0.1 mg/kg tetrachlorobiphenyl-exposed BSO-treated fish were 9- to 10-fold lower than in

Table 1.

Glutathione status in liver, kidney and plasma of tetrachlorobiphenyl (TCB) exposed rainbow trout receiving different thiol treatments.

	Saline	GSH	BSO	Lipoate	BCNU
LIVER					
[GSH]	1314±181■	3893±222###	269±71###	2263±90***d	1469±191[1]d
[GSSG]	49±12[2]■	159±37*	9±3[1]#	40±7[3]■	117±21*
[TGSH]	1412±180[1]■	4210±214[1]###	287±77[1]###	2343±88***d	1703±222[1]d
Ratio	40±10[1]	42±11	32±5	18±3[3]	81±11##
0.1 mg TCB					
[GSH]	1627±124	5090±523###	177±35###	2077±181	1604±135
[GSSG]	138±15~	205±19*	5±2###	98±24[2]#	131±35
[TGSH]	1903±114	5499±497~###	187±37###	2273±218	1903±182
Ratio	88±14~**ab	43±8[a]	26±6■	46±8.7[3]rb	78±19**
5 mg TCB					
[GSH]	1720±176	4126±441###	114±6###	2163±139	2113±195~
[GSSG]	128±20~	118±34[a]	3±0.4~###	214±24~**a	103±23■
[TGSH]	1975±195~■	4362±478###	119±6~###	2590±162*	2320±170~
Ratio	76±10~**	29±8■[a]	23±3■[b]	100±10~**c	54±16[abc]
KIDNEY					
[GSH]	2021±110	3878±259###	572±34###	2284±58	2088±124
[GSSG]	39±4	120±15###	10±2###	30±5	28±9
[TGSH]	2099±109	4118±259###	591±33###	2343±58	2145±123
Ratio	19±2	32±4**	18±3■	13±2■	14±1■
0.1 mg TCB					
[GSH]	1774±145	4414±277[1]###	502±27###	2046±100	1882±130
[GSSG]	41±6	144±13###	9±1###	31±6	35±4
[TGSH]	1855±152	4701±293[1]###	521±30###	2108±95	1951±134
Ratio	23±3[a]	33±3###a	19±1■	16±3■	19±2■
5 mg TCB					
[GSH]	1783±104	3440±299~##	566±68###	2163±103	2048±228#
[GSSG]	46±5[a]	163±31###	9±0.6###	33±3	29±5[a]
[TGSH]	1874±100	3766±332~##	583±68##	228±102	2107±23#
Ratio	26±4#	47±8***	16±3■	15±2■	15±2■
BLOOD PLASMA					
[GSH]	13±3	184±35###	4±1~##	16±2	31±2
[GSSG]	3±0.4■	157±184###	1±0.1###	5±0.3~*	5±0.6
[TGSH]	19±3	497±104###	6±2~###	26±2	41±6
Ratio	433±190■	893±181#	492±240*	329±42*	145±22■
0.1 mg TCB					
[GSH]	12±2	226±53###	9±2[1]■	18±6*	30±2#
[GSSG]	4±2	128±12###	1±0.2###	4±0.8	3±0.6
[TGSH]	20±2	483±75###	11±2[1]##	26±6	37±2#
Ratio	354±91*	704±129***a	176±57#	299±86[a]	119±26■
5 mg TCB					
[GSH]	16±2	188±20###	6±1###	15±2	29±1##
[GSSG]	4±0.8	163±28###	0.8±0.2###	3±0.4#	4±0.3
[TGSH]	25±2	513±62###	8±0.2###	20±0.4	38±2##
Ratio	286±58	901±188##	197±98	170±21	141±13

Values are means±S.E., n=5. GSH, reduced glutathione; GSSG, oxidized glutathione; TGSH, total glutathione (GSH + GSSG; GSSG as GSH equivalents); ratio, 1000(GSSG/GSH).

Concentrations in nmol/g wet weight (liver and kidney) or μmol/l (whole blood). ###P<0.001, ##P<0.01, #P<0.05, significantly different from all thiol treatment groups exposed to the same TCB concentration. ***P<0.001, **P<0.01, *P<0.05, significantly different from ~-labelled treatment groups exposed to the same TCB concentration; [a,b,c]P<0.05, [d]P<0.01, significantly different from same labelled treatment group exposed to the same TCB concentration; [1]P<0.05, [2]P<0.01, [3]P<0.001, significantly different from ~-labelled TCB exposed groups receiving same treatment. [rrr]P<0.001, [rr]P<0.01, [r]P<0.05, significantly different from other TCB exposed groups receiving the same treatment.

the respective tetrachlorobiphenyl-exposed saline-treated group. In the 5 mg/kg tetrachlorobiphenyl-exposed BSO-treated trout, hepatic GSH and total GSH levels were decreased by 15- to 17-fold compared with the corresponding tetrachlorobiphenyl saline-treated group (P<0.001). Depletion of renal and plasma GSH and total GSH levels, however, were less pronounced in response to tetrachlorobiphenyl exposure and BSO treatment. Kidney and plasma

GSH and total GSH contents in 0.1 and 5 mg/kg tetrachlorobiphenyl-exposed BSO-treated trout were 1.3- to 3.6-fold lower than that in the respective tetrachlorobiphenyl-exposed saline-treated groups.

Hepatic GSH and total GSH contents decreased in 0.1 mg/kg (1.5-fold) and 5 mg/kg (2.4-fold) tetrachlorobiphenyl-exposed BSO-treated groups versus the corresponding non-tetrachlorobiphenyl BSO-treated controls (table 1). In contrast, plasma GSH content was 2.3-fold higher in the 0.1 mg/kg tetrachlorobiphenyl-exposed BSO-treated fish than in non-tetrachlorobiphenyl controls (table 1). Liver oxidized GSH content significantly decreased in 5 mg/kg tetrachlorobiphenyl-exposed BSO-treated trout compared with non-tetrachlorobiphenyl controls, but the tissue oxidized GSH/GSH ratio did not differ significantly between the different BSO-treated groups (table 1).

Lipoate treatment. Lipoate clearly enhanced hepatic GSH and total GSH contents in non-tetrachlorobiphenyl lipoate-treated controls compared with the respective saline-(1.7-fold) or BCNU-(1.5- and 1.4-fold)-treated controls. GSH and total GSH levels of other tissues were not influenced by lipoate treatment. Hepatic total GSH content was 1.3-fold higher in 5 mg/kg tetrachlorobiphenyl-exposed lipoate-treated trout than in the respective saline-treated group (table 1).

Within the different lipoate treatment groups, hepatic oxidized GSH levels were 2.5-fold increased in 0.1 mg/kg tetrachlorobiphenyl-exposed trout compared with non-tetrachlorobiphenyl controls. Also hepatic oxidized GSH content further increased in 5 mg/kg (2.2-fold) compared with 0.1 mg/kg tetrachlorobiphenyl-exposed group. However, oxidized GSH content and the oxidized GSH/GSH ratio in renal tissue and plasma did not differ significantly between the different lipoate treatment groups (table 1).

BCNU administration. BCNU administration significantly increased hepatic oxidized GSH content and oxidized GSH/GSH ratio in non-tetrachlorobiphenyl controls compared with all other treatments. This increase in oxidized GSH level was accompanied by significantly decreased GSH reductase activities (1.6- to 2.5-fold) compared with respective non-tetrachlorobiphenyl controls (not shown). No further change in the glutathione redox state was observed in BCNU-treated fish in response to tetrachlorobiphenyl exposure. Hepatic, renal and plasma oxidized GSH contents and oxidized GSH/GSH ratios in tetrachlorobiphenyl-exposed groups resembled values of non-tetrachlorobiphenyl BCNU-treated controls.

Tetrachlorobiphenyl exposure also diminished BCNU inhibition of GSH reductase activities (data not shown). Consistent with results from different saline-treated groups, hepatic GSH and total GSH contents increased in 5 mg/kg tetrachlorobiphenyl-exposed BCNU-supplemented trout (1.4-fold) versus non-tetrachlorobiphenyl BCNU controls (table 1).

Cytochrome P450 metabolism and gene expression.

Ethoxyresorufin O-deethylase (EROD) activities. BCNU treatment strongly induced hepatic and renal EROD activities (table 2; $P<0.05$). Basal EROD activities in liver of BSO-treated, and in liver and kidney of lipoate-treated controls were also significantly higher than in GSH-supplemented controls. EROD activities did not differ between GSH-supplemented and saline-treated controls.

Exposure to 0.1 mg/kg tetrachlorobiphenyl significantly increased hepatic EROD activities in saline-, GSH- and BCNU-treated groups. Induction of hepatic EROD activity was markedly suppressed in BSO-treated (6- to 10-fold) and lipoate-supplemented (2.4- to 3.9-fold) trout compared with other treatments. Renal EROD activity was only significantly increased in 0.1 mg/kg tetrachlorobiphenyl-exposed BCNU-supplemented trout compared with other respective treatment groups.

Hepatic EROD activity in 5 mg/kg tetrachlorobiphenyl-exposed GSH-supplemented trout was significantly higher (1.7-fold) than in respective saline-treated controls. Higher concentrations of tetrachlorobiphenyl were required to obtain tissue EROD responses in BSO- and lipoate-treated fish. Exposure to 5 mg/kg tetrachlorobiphenyl significantly elevated hepatic and renal EROD activities in BSO-treated ($P<0.05$) and lipoate-supplemented ($P<0.01$) groups compared with the corresponding 0.1 mg/kg tetrachlorobiphenyl-exposed groups and non-tetrachlorobiphenyl controls. Hepatic and renal EROD activities, however, were still

Table 2.

Hepatic and renal EROD activities in tetrachlorobiphenyl exposed rainbow trout receiving different thiol treatments.

	Saline	GSH	BSO	Lipoate	BCNU
LIVER					
Control	5.6 ± 0.2^{2}	4.8 ± 0.5^{3}■	$10\pm2.5^{\sim *}$	$9.5\pm2.4^{\sim *}$	$84\pm43^{\sim}$■
0.1 mg	$120\pm53^{\tau **}$	$133\pm66^{\tau\tau **}$	$20\pm11^{\sim}$■	$51\pm24^{\sim a}$	$197\pm64^{\sim **a}$
5 mg	$315\pm83^{\sim}$■	$540\pm32^{\sim *}$	$51\pm9^{1\#\#}$	$118\pm4^{2\#\#}$	517 ± 76^{2}
KIDNEY					
Control	$9.3\pm1.0^{\sim}$	$6.6\pm1.2^{\tau\tau\tau}$■	$8\pm0.9^{\sim}$	$12\pm1.1^{\sim *}$	$24\pm2.6^{\sim \#}$
0.1 mg	$15\pm2.8^{\sim}$	$16\pm1.8^{\tau\tau\tau}$	$12\pm1.6^{\sim}$	$22\pm4.5^{\sim \tau}$	$40\pm5.6^{\tau\#\#}$
5 mg	135 ± 23^{3}	145 ± 10	$34\pm4^{3\#\#\#}$	$69\pm3^{3\#\#}$	176 ± 17^{3}

Activities (pmol/min./mg protein) are means±S.E., n=5. For explanation of superscripts, see legend to table 1.

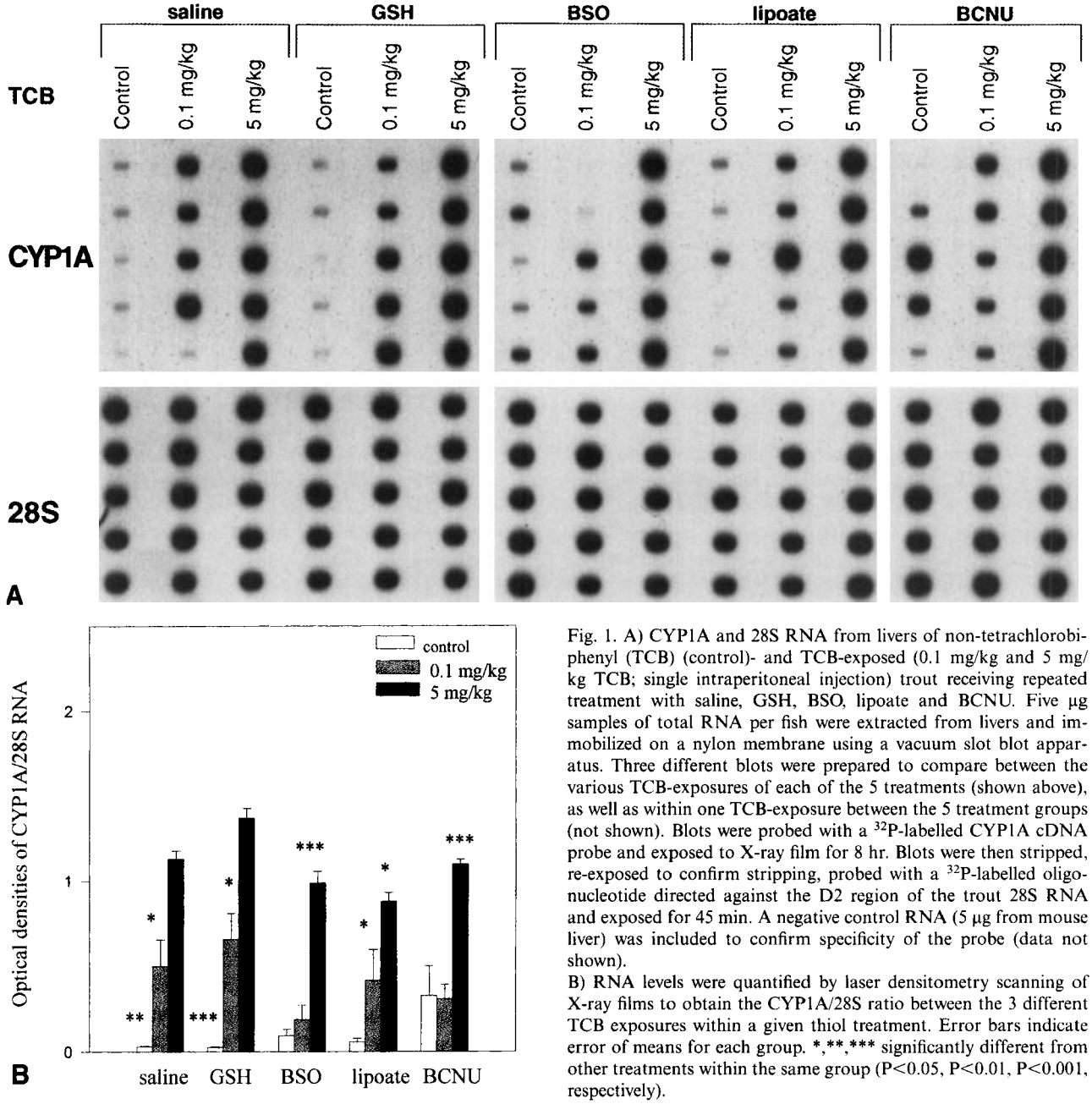

Fig. 1. A) CYP1A and 28S RNA from livers of non-tetrachlorobiphenyl (TCB) (control)- and TCB-exposed (0.1 mg/kg and 5 mg/kg TCB; single intraperitoneal injection) trout receiving repeated treatment with saline, GSH, BSO, lipoate and BCNU. Five μg samples of total RNA per fish were extracted from livers and immobilized on a nylon membrane using a vacuum slot blot apparatus. Three different blots were prepared to compare between the various TCB-exposures of each of the 5 treatments (shown above), as well as within one TCB-exposure between the 5 treatment groups (not shown). Blots were probed with a ^{32}P-labelled CYP1A cDNA probe and exposed to X-ray film for 8 hr. Blots were then stripped, re-exposed to confirm stripping, probed with a ^{32}P-labelled oligonucleotide directed against the D2 region of the trout 28S RNA and exposed for 45 min. A negative control RNA (5 μg from mouse liver) was included to confirm specificity of the probe (data not shown).

B) RNA levels were quantified by laser densitometry scanning of X-ray films to obtain the CYP1A/28S ratio between the 3 different TCB exposures within a given thiol treatment. Error bars indicate error of means for each group. *,**,*** significantly different from other treatments within the same group ($P<0.05$, $P<0.01$, $P<0.001$, respectively).

markedly higher (2- to 11-fold) in 5 mg/kg tetrachlorobiphenyl-exposed saline-treated, GSH- and BCNU-treated groups than in respective BSO-treated and lipoate-treated groups ($P<0.01$). Thus, consistent with the observation in the 0.1 mg/kg tetrachlorobiphenyl group, both BSO and lipoate appeared to suppress tissue EROD response. This suppressive effect was more prominent for BSO than lipoate because hepatic and renal EROD activities were significantly higher in 5 mg/kg tetrachlorobiphenyl-exposed lipoate-treated trout than in the respective BSO-treated group ($P<0.01$).

Hepatic CYP1A gene expression. BCNU treatment markedly increased hepatic CYP1A mRNA levels (fig. 1). Also CYP1A mRNA and the ratio of CYP1A/28S mRNA were significantly increased by BSO treatment alone (fig. 1a). In all treatment groups, induction of CYP1A mRNA expression was directly dependent on the concentration of tetrachlorobiphenyl exposure. CYP1A mRNA and the CYP1A/28S ratio in response to 0.1 mg/kg tetrachlorobiphenyl exposures were marginally lower in livers of BSO-treated than in the respective GSH-adequate tissues. Exposure to higher concentration (5 mg/kg) of tetrachlorobiphenyl strongly in-

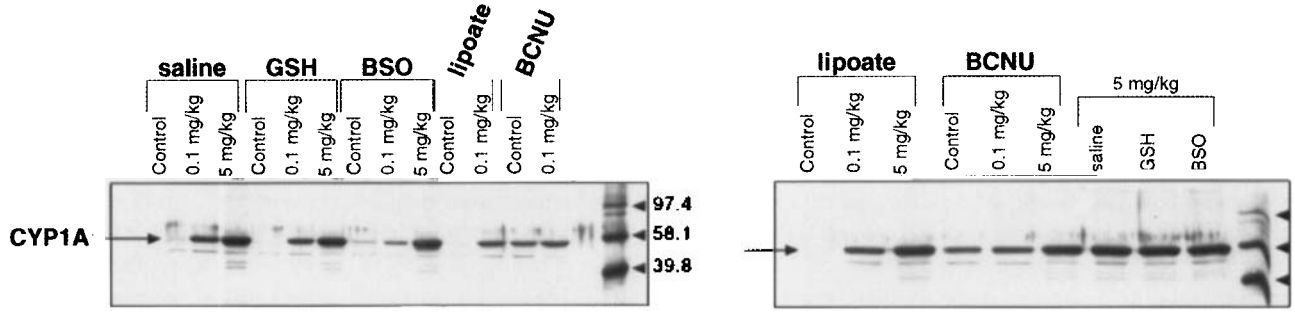

Fig. 2. Western blots of anti-rat CYP1A polyclonal antibody immunoreactive protein from trout liver. Liver homogenates of 5 animals per treatment group were pooled and 20 μg of protein per group were separated by SDS page and electroblotted to nitrocellulose membranes. Blots were probed with polyclonal anti-rat CYP1A from rabbit and detected by chemiluminescent exposure of X-ray film. Two blots were made to allow comparisons between the various tetrachlorobiphenyl-exposures of each of the 5 treatments and within one tetrachlorobiphenyl-exposure between the 5 treatment groups. The arrow indicates the band that co-migrates with rat CYP1A. Arrowheads and numbers at the right indicate molecular weight standards in kD.

duced CYP1A mRNA expression in all treatment groups studied (fig. 1a,b).

Compared with their respective treatment controls, the magnitude of tetrachlorobiphenyl-induced CYP1A mRNA level in the five different treatment groups (the respective tetrachlorobiphenyl concentrations are indicated in adjacent parentheses) were as follows (fig. 1b): saline-treated, 17-fold (0.1 mg/kg) and 38-fold (5 mg/kg); GSH-supplemented, 27-fold (0.1 mg/kg) and 57-fold (5 mg/kg); BSO-treated, 2-fold (0.1 mg/kg) and 11-fold (5 mg/kg); lipoate-treated, 7.4-fold (0.1 mg/kg) and 16-fold (5 mg/kg); and BCNU-treated, unchanged (0.1 mg/kg) and 3.4-fold (5 mg/kg tetrachlorobiphenyl). BSO and BCNU treatments alone influenced basal expression of CYP1A mRNA, thus the induction of CYP1A is less pronounced in these groups than in the saline-treated or GSH-supplemented groups.

CYP1A protein expression. The response of CYP1A protein expression in the different treatment groups was consistent with CYP1A mRNA expression data (fig. 2). Basal expression of CYP1A protein was low, and CYP1A protein was even less expressed in non-tetrachlorobiphenyl GSH- or lipoate-treated controls. In contrast, BSO-treatment alone induced CYP1A protein expression. Moreover, CYP1A protein was markedly expressed in BCNU-treated controls. Tetrachlorobiphenyl (0.1 mg/kg) markedly induced CYP1A protein in all treatment groups, but protein expression in BSO-treated (0.1 mg/kg) trout was notably weaker than in other treatment groups. CYP1A protein was further induced by 5 mg/kg tetrachlorobiphenyl in all groups and no difference in induction in the different treatment groups was observed.

Discussion

Tissue GSH status.

Various polychlorinated biphenyl congeners enhance the production of reactive oxygen species (Amaro *et al.* 1996; Tithof *et al.* 1996) and thus sensitize cellular antioxidant defenses. Tetrachlorobiphenyl exposure modified both the tissue glutathione pool and the responses of tissue glutathione to the various treatments (table 1). In non-tetrachlorobiphenyl exposed controls, all treatments effectively altered tissue glutathione status. Lipoate treatment enhanced hepatic GSH and total GSH levels. Lipoate has been consistently shown to elevate hepatic GSH content in mammalian species (Packer *et al.* 1995). BCNU inhibits GSH reductase and thus increases intracellular oxidized GSH content (Dröge *et al.* 1994; Becker & Schirmer 1995). However, tetrachlorobiphenyl exposure diminished the lipoate and BCNU effects on GSH status, possibly due to a direct interaction of tetrachlorobiphenyl with lipoate or BCNU.

Lipoate is rapidly reduced in tissues to dihydrolipoic acid, which is a potent reductant (Packer *et al.* 1995; Handelman *et al.* 1994). The elevation of oxidized GSH content with tetrachlorobiphenyl exposure in lipoate-supplemented trout is in contrast to our previous study that showed lipoate diminished increases in hepatic oxidized GSH levels and maintained a low oxidized GSH/GSH ratio in tetrachlorobiphenyl-exposed (1 mg/kg) rainbow trout (Otto *et al.* 1997). However, the increased hepatic GSH content with high tetrachlorobiphenyl exposure, as noted in saline- and BCNU-treated trout, is consistent with previous observations in this species (Otto & Moon 1995).

The significantly enhanced tissue availability of supplemented GSH in 0.1 mg/kg tetrachlorobiphenyl-exposed trout may suggest increased tissue demand for the thiol. GSH serves as a reservoir of cellular cysteine for protein synthesis (Meister 1991). In comparison, GSH-dependent enzymes including GSH peroxidase, GSH reductase and glutathione S-transferase were not significantly influenced by tetrachlorobiphenyl exposure or treatments with saline, GSH, BSO and lipoate (data not shown). However, the loss of BCNU effects on oxidized GSH concentrations resulted from diminished inhibition of GSH reductase in tetrachlorobiphenyl-exposed trout. The present data provide the first evidence that tetrachlorobiphenyl markedly modifies tissue GSH status by the various treatments. In addition, GSH

and BSO treatment effects on GSH status were sensitive to the tetrachlorobiphenyl exposure used (0.1 versus 5 mg/kg). The therapeutic effects of agents such as lipoate and BCNU may thus be altered in pollutant exposed organisms.

CYP1A transcription and catalytic activity.

Biochemical activity of polychlorinated biphenyl congeners is thought to be initiated primarily through induction of aromatic hydrocarbon receptor-mediated signal transduction (Safe 1994). Enhanced CYP1A metabolism may increase production of reactive oxygen species, which may result in oxidative DNA damage potentially leading to mutations and cancer (Park *et al.* 1996). In the absence of tetrachlorobiphenyl, the various treatments to manipulate tissue GSH status resulted in altered expression of the CYP1A gene in trout (fig. 1). BCNU is chemically relatively unstable and is inactivated by denitrosation in the cytosol by GST. Additional denitrosation occurs in microsomes by cytochrome P450 enzymes that are further induced by phenobarbital and dexamethasone (Weber & Waxman 1993). BCNU alone may activate cytochrome P450s by serving as a substrate, although BCNU effects on GSH redox status may be involved in cytochrome P450 induction. GSH deficiency induced by BSO appears to induce CYP1A mRNA expression and catalytic activity in the absence of tetrachlorobiphenyl. GSH depletion by BSO is known to activate gene expression of conjugation enzymes in an aromatic hydrocarbon receptor independent fashion (Shertzer *et al.* 1995). However, the aromatic hydrocarbon receptor is assumed to play multiple physiological roles in the development of the liver and immune system (Fernandez-Salguero *et al.* 1995). An endogenous substrate for aromatic hydrocarbon receptor has been postulated as being responsible for the constitutive expression of genes such as CYP1A (Nebert 1994). GSH deficiency, i.e. BSO-treatment, may interfere with such expression, providing further evidence for an apparent cross-talk between the GSH and the P450 systems (Otto *et al.* 1997). Such cross-talk is further supported by lower expression of CYP1A protein in GSH- and lipoate-supplemented controls, which both share increased GSH levels, compared with saline-treated controls (fig. 2).

We have previously observed that GSH deficiency by BSO treatment suppressed tetrachlorobiphenyl (1 mg/kg) induced CYP1A mRNA expression (Otto *et al.* 1997). In the present study, low (0.1 mg/kg) tetrachlorobiphenyl exposure marginally suppressed CYP1A mRNA and CYP1A protein expression in BSO-treated trout. However, exposure to 5 mg/kg tetrachlorobiphenyl resulted in a significantly higher hepatic CYP1A/28S ratio in BSO-treated and GSH-supplemented groups than in lipoate- and BCNU-treated trout. Sensitivity of tetrachlorobiphenyl induced CYP1A mRNA expression to depleted tissue GSH content (Otto *et al.* 1997; and 0.1 mg/kg tetrachlorobiphenyl exposure in this study) may be overcome by increased tetrachlorobiphenyl concentrations. The effect of GSH metabolism on CYP1A expression has been described in human cell lines. The silencing of the GSH conjugating enzyme GSTm1 enhances the inducibility of CYP1A1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (Vaury *et al.* 1995).

Interestingly, the increased CYP1A mRNA expression in the 0.1 and 5 mg/kg tetrachlorobiphenyl-exposed groups is accompanied by elevated hepatic oxidized GSH levels in all treatments except BSO-treated trout. We previously observed that lipoate diminished the elevation of hepatic oxidized GSH levels and maintained a low oxidized GSH/GSH ratio in trout exposed to 1 mg/kg tetrachlorobiphenyl (Otto *et al.* 1997). This further coincided with an impaired induction of CYP1A mRNA and protein expression by tetrachlorobiphenyl. Whether oxidized GSH is a factor influencing CYP1A expression can not be resolved with the present results. However, it has been postulated that oxidized GSH may modulate gene transcription, as redox sensitive transcription factors such as nuclear factor B are reported to be not only regulated by cellular GSH, but also oxidized GSH levels (Dröge *et al.* 1994; Mihm *et al.* 1995). A combination of factors including GSH and oxidized GSH levels as well as tetrachlorobiphenyl exposure concentrations may modulate CYP1A transcription and gene expression. Whitlock *et al.* (1996) suggested CYP1A1 is a model for the assessment of inducible gene transcription; the sensitivity of CYP1A to glutathione status may strengthen this model.

High tetrachlorobiphenyl concentrations induced CYP1A mRNA and protein expression in all groups to a similar extent. The observed suppression of tetrachlorobiphenyl-induced EROD activity in lipoate-treated and high tetrachlorobiphenyl-exposed BSO-treated fish was not regulated by CYP1A mRNA or protein levels. With similar expression of CYP1A protein in these treatment groups, a post-translational regulatory mechanism could establish a second stage for thiol regulation of CYP1A. Lipoate shares structural similarity to oltipraz, a dithiolthione, which has been shown to enhance hepatic GSH content and to suppress 3-methylcholanthrene induced EROD activity (Langouët *et al.* 1995; van Lieshout *et al.* 1996). Inhibition of CYP1A enzyme activity has been suggested to involve nitric oxide and haem oxygenase (Kim *et al.* 1995). The protein-bound haem iron of cytochromes is the target for destruction by nitric oxide and increased degradative haem oxygenase activities (Kim *et al.* 1995). The cytochrome P450 apoprotein remains intact, although catalytic activity is lost without the prosthetic haem group. Moreover, GSH supplementation in 5 mg/kg tetrachlorobiphenyl-exposed trout, significantly potentiated hepatic EROD activity compared with the respective saline treated group, in line with previous observations (Otto *et al.* 1997). The current data in combination with our previous observations suggest that regulation of tetrachlorobiphenyl-induced CYP1A by glutathione occurs at two stages, first at the aromatic hydrocarbon receptor-mediated transcription of the CYP1A gene and second at the catalytic activity, most likely by a post-translational mechanism.

Lipoate, N-acetylcysteine and oltipraz are increasingly used in chemoprevention (Packer *et al.* 1995; Smith & Gupta 1996). Inhibition of catalytic CYP1A activity would de-

crease formation of DNA-adducts by P450 metabolites or DNA damage as a result of cytochrome P450 produced reactive oxygen species (Park *et al.* 1996). The precise mechanisms of chemopreventive agents are little known. Lipoate, N-acetylcysteine and oltipraz are pro-GSH agents and their chemopreventive action has not been directly distinguished from their effects on GSH levels. Exogenous GSH is an efficient delivery agent of GSH to tissues in fish, but not in mammals (Otto *et al.* 1997). Fish may serve as models to elucidate whether such inhibition or delayed onset of carcinogenesis is due to synergism of these agents with GSH, inhibition of CYP1A catalytic activity or possibly other mechanisms involved.

In summary, efficient manipulation of tissue thiol status provides a sensitive tool to test the regulation of cytochrome P450 metabolism by tissue glutathione status. Tetrachlorobiphenyl exposure modifies the effectiveness of the various treatments. Treatment with thiols, BSO and BCNU, even in the absence of tetrachlorobiphenyl, influences basal expression of CYP1A. In the presence of tetrachlorobiphenyl, thiols may influence CYP1A metabolism at two stages, at gene expression and/or at a post-translational level. Our present results further support a cross-talk between thiols and cytochrome P4501A and identify a possible signal transduction pathway of antioxidant regulated gene expression.

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