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# Lack of response of the rat liver "class 3" cytosolic aldehyde dehydrogenase to toxic chemicals, glutathione depletion, and other forms of stress

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**Abstract**—One of the rat liver "Class 3" cytosolic aldehyde dehydrogenases (EC 1.2.1.3), ALDH3c, is known to be markedly induced by polycyclic aromatic hydrocarbons and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin). In the present study we examined whether hepatic ALDH3c induction is a general response to toxicity. Treatment of Wistar rats for 4 days with known toxic doses of hepatotoxic agents—carbon tetrachloride, dimethylnitrosamine, diethylnitrosamine, aflatoxin $B_1$, and D-ethionine—did not induce ALDH3c enzyme activity. Whereas dimethylaminoazobenzene at 100 mg/kg/day for 4 days did not increase ALDH3c, a 10-fold lower dose of dimethylaminoazobenzene for 4 days produced a 20-fold increase in ALDH3c activity. Treatment with phorone, diethylmaleate or L-buthionine-*S,R*-sulfoximine—which deplete reduced glutathione (GSH) by different mechanisms—did not affect ALDH3c activity. One dose of benzo[*a*]pyrene for 24 hr increased ALDH3c activity by 25-fold. Treatment with both the GSH-depleting chemicals and benzo[*a*]pyrene inhibited ALDH3c induction by 45% to 75%, suggesting a role for GSH during ALDH3c induction. After ALDH3c activity had already been induced by benzo[a]pyrene, however, the GSH-depleting chemicals did not affect ALDH3c activity. No changes in ALDH3c activity were seen 24 or 48 hr after partial hepatectomy, on the fifth day following surgical cholestasis, or after guanethidine-induced sympathectomy. These data indicate that hepatic ALDH3c inducibility in the rat is not a general or direct response to chemical toxicity, or to conditions of GSH depletion or other forms of stress.

*Key words:* Aldehyde dehydrogenase; benzo[*a*]pyrene; toxic stress; oxidative stress; glutathione depletion; rat liver

Rat liver contains at least two cytosolic aldehyde dehydrogenases that can be stimulated by inducers of drug metabolism [1–5]. Phenobarbital-type inducers increase ALDH1 enzyme activity to higher levels in rats having the "responsive" genotype *R/R*, as compared with those having the "nonresponsive" genotype *r/r* [1, 2]. Polycyclic hydrocarbons (such as benzo[*a*]pyrene) and TCDD* are known to bind as ligands to the Ah receptor, resulting in a dose-dependent increase in ALDH3c enzyme activity in rat liver, in human and mouse hepatoma cell lines, and in primary cultures of human and rat hepatocytes [6–10]. During this induction process one sees increased ALDH3c mRNA levels [10–12], and presumably the inducer-receptor complex operates via an AhRE upstream of the *ALDH3c* gene [13], similar to AhREs found upstream of the dioxin-inducible *CYP1A1*, *CYP1A2*, *UGT1*6*, *GSTA1* and *NMO1* genes [reviewed in Ref. 14]. It was recently shown that the ALDH3c induction process is Ah receptor-mediated and that, in the mouse hepatoma CYP1A1 metabolism-deficient mutant *c37* cell line which exhibits strikingly elevated ALDH3c mRNA levels and enzyme activity, introduction of a functional CYP1A1 or CYP1A2 protein is able to repress ALDH3c to wild-type basal levels [10, 12].

ALDH1 and ALDH3c are now known to be the products of different genes. Conventionally, measurement of ALDH1 enzyme activity is carried out with the substrate propionaldehyde (or more precisely, phenylacetaldehyde) plus the coenzyme $NAD^+$ (P/NAD), whereas measurement of ALDH3c enzyme activity is performed with the substrate benzaldehyde plus $NADP^+$ (*B/NADP*) [7]. ALDH3c expression and inducibility has usually been assessed from the ratio of B/NADP to P/NAD. Under physiological conditions, and also after phenobarbital treatment, this ratio is lower than 1.0, whereas treatment of the rat with Ah receptor ligands such as benzo[*a*]pyrene or TCDD produces a ratio of $> 1.0$ [9, 15].

A great deal of interest has focused on ALDH3c, because it has been described as a tumor-associated aldehyde dehydrogenase detected in many chemically-induced hepatocellular neoplasms [5, 16–18]. The physiological role of this enzyme remains unknown. However, based on kinetic data of the purified enzyme, it has been suggested that ALDH3c may play a role in the oxidation of lipid aldehydes, especially those generated by lipid peroxidation [19, 20]. Furthermore, it has been shown that TCDD and polycyclic aromatic hydrocarbons produce a mild and diffuse hepatotoxic effect, which is associated with an increase in lipid peroxidation of intracellular membranes [21] and oxidative stress. [22].

The purpose of this study was to examine in rat liver the possibility that ALDH3c induction is a non-specific response to various hepatotoxic chemicals and other forms of stress. In addition, we examined the effects of GSH depletion and benzo[*a*]pyrene on ALDH3c activity. GSH depletion was achieved using three chemicals which are known to act [23] by at least two distinct mechanisms: (a) phorone and DEM, both of which react chemically with GSH; and (b) BSO, which inhibits glutamylcysteinyl synthetase.

## *Materials and Methods*

*Chemicals.* All chemicals used were reagent grade. Pyrazole was obtained from Fluka, (Germany); propionaldehyde and benzaldehyde were purchased from Ferak (Germany). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A).

* Abbreviations: ALDH3c, the polycyclic hydrocarbon-inducible cytosolic aldehyde dehydrogenase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; GSH, reduced glutathione; DEM, diethylmaleate; BSO, L-buthionine-*S,R*-sulfoximine; AhRE, *a*romatic *h*ydrocarbon *r*esponse *e*lement; EpRE, *e*lectro*p*hile *r*esponse *e*lement. By convention, italicized *ALDH3c* represents the gene, whereas non-italicized ALDH3c represents the mRNA, protein or enzyme activity.

*Animals.* We used male albino rats (weighing 200–250 g) of the Wistar/Mol/Io/rr substrain, originating from the Animal Center at the University of Kuopio (Finland). The rats were kept in plastic cages (Makrolon) with wood chip bedding (*Populus sp.*), and the animals had free access to tap water and pelleted chow.

*Treatment with hepatotoxic agents.* The following chemicals were administered by intraperitoneal injection, for 4 days at the following daily doses: carbon tetrachloride (5 mg/kg); dimethylnitrosamine (10 mg/kg); diethylnitrosamine (10 mg/kg); dimethylaminoazobenzene (100 mg/kg or 10 mg/kg); D-ethionine (100 mg/kg); and aflatoxin $B_1$, (5 mg/kg).

*Cotreatment with benzo[a]pyrene and GSH-depleting chemicals.* Forty animals were divided into eight groups of five. The control group received no treatment at all. Each of the three experimental groups was treated with a GSH-depleting chemical for 4 days (phorone, 50 mg/kg/day; DEM, 200 mg/kg/day; BSO, 50 mg/kg/day) and killed 24 hr after the last treatment. The next three experimental groups were treated with a single dose of benzo[*a*]pyrene (20 mg/kg) on the first day, plus each of the GSH-depleting chemicals on that day and the next 3 days, as above. The seventh group was given only the single dose of benzo[*a*]-pyrene (20 mg/kg) on the first day and killed on the fifth day. All chemicals, in olive oil, were administered intraperitoneally.

*Treatment with benzo[a]pyrene, then GSH-depleting chemicals.* Twenty-four animals were injected with benzo-[*a*]pyrene (25 mg/kg) for 7 days. Following the seventh day, they were divided into four groups. The first group was not given any further treatment. Each of the remaining groups was treated with a GSH-depleting chemical for 4 days, using the same dosages as described above and killing the rats 24 hr after the last dose. All animals were killed on day 12 of this experiment.

*Chemical sympathectomy.* This was achieved with intraperitoneal guanethidine (40 mg/kg/days for 3 weeks). After this treatment, the rat liver noradrenaline levels were found to be decreased to 11.0 ng/g wet weight of tissue, as compared with 82.7 ng/g wet weight of tissue in controls.

*Surgical cholestasis.* The animals were operated under light ether anesthesia. The abdominal cavity was opened, and the bile duct was ligated with a suture. The rats were killed 5 days later.

*Partial hepatectomy.* This procedure was also performed under light ether anesthesia. The anterior two-thirds of the liver was excised after ligation with a suture. The animals were killed 24 hr and 48 hr after the operation, because the most vigorous phase of proliferative liver regeneration is known to take place during this time [24].

*Tissue preparation.* After the rats were killed by decapitation, the livers were homogenized with a teflon pestle in 3 vol (w/v) of ice-cold 0.25 M sucrose solution. The homogenate was first centrifuged at 10,000 *g* for 30 min. An equal volume of 0.024 M $CaCl_2$ in 0.25 M sucrose was added to the supernatant fraction. The diluted supernatant fraction was stirred and left to stand on ice for 10 min. The microsomal fraction was then sedimented by centrifugation at 10,000 *g* for 30 min [25]. The soluble fraction was used for ALDH3c assays.

*ALDH3c enzyme assay.* The enzyme activity was measured according to the method described previously [15]. Values are expressed as nmol of NAD(P)H formed per min per mg of cytosolic protein.

*Protein determinations.* Protein concentrations were determined by the biuret method [26], using bovine serum albumin as the standard.

*Statistical analysis.* Means ± SD of aldehyde dehydrogenase activities are provided for each experimental and control group. Statistical significance was determined by Student's *t*-test.

*Results and Discussion*

*Treatment with hepatotoxic agents.* Acute treatment with classical hepatotoxic agents at known toxic doses did not result in any significant induction of ALDH3c activity (Table 1). In fact, carbon tetrachloride and diethylnitrosamine caused a slight, but statistically significant, 20% decrease in the P/NAD activity ($P < 0.001$). Interestingly, dimethylaminoazobenzene at 100 mg/kg gave no statistically significant increase in ALDH3c activity, whereas a 10-fold lower dose of dimethylaminoazobenzene caused a 15-fold rise in B/NADP activity, indicating ALDH3c induction. This difference in response at the two doses might represent differences in either the toxicity or the pharmacokinetics of the chemical, or some combination of both. The mechanism of ALDH3c induction most likely involves dimethylaminoazobenzene metabolism, during which there is generated a metabolite that activates the *ALDH3c* gene via the EpRE [27, 28]. Another possibility is that the 10-fold differences in dosage of dimethylaminoazobenzene might be due to differential production of a particular inducing metabolite. We found that treatment with higher and lower doses of the hepatotoxicants shown in Table 1 other than dimethylaminoazobenzene did not induce ALDH3c enzyme activity (data not shown).

It has been reported that acute or subacute treatment of rat hepatoma cell lines with diethylnitrosamine or D-ethionine does not induce ALDH3c enzyme activity or mRNA levels [29]. Chronic treatment of Wistar rats with D-ethionine in the drinking water (1 mg/mL, for 2 months), does not cause any change in ALDH3c activity [30]. However, significantly elevated levels of ALDH3c activity have been found in preneoplastic and neoplastic cells *in vivo*, when either diethylnitrosamine or D-ethionine was used as a carcinogenic initiator [5]. These results, including the data in Table 1, thus suggest that increased ALDH3c activity does not represent a general, or direct, response to toxic chemical stress.

*Possible requirement of GSH during ALDH3c induction by benzo[a]pyrene.* Phorone, DEM or BSO—three chemicals which deplete GSH by at least two different mechanisms—did not alter ALDH3c activity in untreated rats (Table 2, *top*). As has been well documented, benzo-[*a*]pyrene increased the B/NADP activity by 100-fold. Cotreatment with benzo[*a*]pyrene and a GSH-depleting chemical on the first day, plus the GSH-depleting chemical for an additional 3 days, caused a 40–75% inhibition of both P/NAD and B/NADP activities, compared with that of the group receiving benzo[*a*]pyrene alone (Table 2, *top*). These data suggest that adequate pools of GSH might be required during the process of ALDH3c induction by benzo[*a*]pyrene.

*Absence of GSH requirement on ALDH3c already induced by benzo[a]pyrene.* The results were strikingly different when the GSH-depleting agents were administered to animals that had already been treated with benzo[*a*]-pyrene for 7 days (Table 2, *bottom*). In this case, none of the GSH-depleting chemicals affected the already highly induced ALDH3c activity.

Hence, ALDH3c enzyme activity is not inducible under conditions in which oxidative stress is caused by depletion of GSH. It was recently demonstrated that phorone treatment decreases the mitochodrial, as well as the cytosolic, low-$K_m$ aldehyde dehydrogenase activities in rat liver [31]. Table 2 shows that GSH depletion by phorone, DEM or BSO significantly diminishes ALDH3c induction by benzo[a]pyrene; on the other hand, none of the GSH-depleting chemicals, given after ALDH3c induction by benzo[*a*]pyrene, affects the already highly induced ALDH3c activity. It thus appears likely that, although some mechanisms of oxidative stress might be indirectly involved in ALDH3c induction, GSH depletion *per se* is not an effector of the *ALDH3c* gene. On the contrary, physiological levels of GSH seem to be an important factor

Table 1. Effect of hepatotoxic agents on rat liver ALDH3c activity

| Compound | Aldehyde dehydrogenase activity [nmol NAD(P)H/min/mg protein] | | B/NADP:P/NAD ratio |
|---|---|---|---|
| | B/NADP | P/NAD | |
| Control | 1.2 ± 0.2 | 7.8 ± 0.2 | 0.15 |
| Carbon tetrachloride | 1.1 ± 0.4 | 5.8 ± 0.6* | 0.19 |
| Dimethylnitrosamine | 0.8 ± 0.2 | 7.5 ± 0.8 | 0.11 |
| Diethylnitrosamine | 1.3 ± 0.4 | 6.4 ± 0.8* | 0.20 |
| Dimethylaminoazobenzene | | | |
| High dose (100 mg/kg) | 1.6 ± 0.5 | 6.9 ± 0.2 | 0.23 |
| Low dose (10 mg/kg) | 19.0 ± 1.9* | 10.0 ± 0.7 | 1.9 |
| D-Ethionine | 0.8 ± 0.4 | 6.8 ± 1.2 | 0.12 |
| Aflatoxin $B_1$ | 1.5 ± 0.4 | 6.8 ± 2.1 | 0.22 |

Treatment was for 4 days, and each group of animals (N = 5) was killed on the fifth day.
* Statistically different ($P < 0.001$) from the control group.

Table 2. Effect of GSH-depleting chemicals and benzo[*a*]pyrene cotreatment on rat liver ALDH3c activity, and the lack of any effect of GSH-depleting chemicals on ALDH3c activity previously induced by benzo[*a*]pyrene.

| Compound (mg/kg) | Aldehyde dehydrogenase activity [nmol NAD(P)H/min/mg protein] | | B/NADP:P/NAD ratio |
|---|---|---|---|
| | B/NADP | P/NAD | |
| Control | 2.1 ± 1.0 | 10.2 ± 0.8 | 0.21 |
| Phorone | 2.5 ± 0.8 | 10.1 ± 0.1 | 0.25 |
| DEM (100) | 3.6 ± 1.4 | 12.0 ± 2.1 | 0.30 |
| DEM (200) | 2.8 ± 1.1 | 13.4 ± 3.6 | 0.21 |
| BSO | 3.9 ± 1.3 | 12.2 ± 2.2 | 0.31 |
| Benzo[*a*]pyrene (BaP) | 326 ± 6.1* | 85.5 ± 4.1*† | 3.8 |
| Phorone + BaP | 81.5 ± 12.5*† | 32.5 ± 1.3*† | 2.5 |
| DEM (100) + BaP | 182 ± 8.1*† | 45.1 ± 1.7*† | 4.0 |
| DEM (200) + BaP | 147 ± 19.4*† | 43.1 ± 5.0*† | 3.4 |
| BSO + BaP | 130 ± 14.3*† | 46.3 ± 8.0*† | 2.8 |
| *Compound (days of treatment)* | | | |
| Control | 3.8 ± 0.7 | 10.1 ± 0.8 | 0.38 |
| Benzo[*a*]pyrene (7) | 1120 ± 170 | 169 ± 23.9 | 6.6 |
| Benzo[*a*]pyrene (7) then phorone (4) | 1290 ± 370 | 149 ± 32.3 | 8.7 |
| Benzo[*a*]pyrene (7) then DEM (4) | 1130 ± 290 | 154 ± 19.5 | 7.3 |
| Benzo[*a*]pyrene (7) then BSO (4) | 1060 ± 332 | 139 ± 28.3 | 7.6 |

Treatment for the first 10 groups (N = 6) was for 4 days and rats were killed on the fifth day.
Treatment for the last 5 groups (N = 6) was for 7 + 4 days, and the animals were killed on the twelfth day.
* Statistically different ($P < 0.001$) from the control group.
† Statistically different ($P < 0.001$) from the benzo[*a*]pyrene-treated group.

in the process of ALDH3c induction. Using similar experimental conditions, we have also found that neither phorone nor DEM has any effect on the induction of ALDH1 by phenobarbital (data not shown).

*Partial hepatectomy, surgical cholestasis and chemical sympathectomy.* No increases in ALDH3c enzyme activity were found 24 and 48 hr after partial hepatectomy, on the fifth day following surgical cholestasis, or after chemical sympathectomy (data not illustrated). Treatment of rat and mouse hepatoma cell lines with clofibrate, a potent hypolipidemic agent that causes peroxisome proliferation, also fails to induce ALDH3c enzyme activity and mRNA levels (V. Vasiliou and D. W. Nebert, unpublished data). Because of the negative results presented in the present study with sympathectomized rats, the possibility that ALDH3c might be involved in catecholamine metabolism also appears unlikely.

*Concluding remarks.* In summary, using a variety of toxic chemicals, we have shown the ALDH3c induction is not a general response to toxic chemical stress. We have also found that ALDH3c expression remains unchanged during the proliferating phase of hepatic regeneration, or following cholestatic liver injury or chemical sympathectomy. Although it has been suggested that ALDH3c might play a role in the oxidation of lipid aldehydes and especially those generated by lipid peroxidation [19, 20],

we have found that carbon tetrachloride, a well-known inducer of lipid peroxidation [9, 32], fails to induce ALDH3c activity in rats.

Finally, we have shown that ALDH3c is not inducible under conditions in which oxidative stress is caused by the depletion of GSH. We found that phorone-, DEM- or BSO-induced GSH depletion does not lead to increases in rat liver ALDH3c activity. It has recently been shown in murine *14CoS/14CoS* cell cultures, which express elevated levels of ALDH3c activity [10], that GSH levels are about 3 times normal [33]. Therefore, neither toxic stress nor physiologic stress nor GSH-depletion-mediated oxidative stress appears to be necessary or essential for ALDH3c induction to occur.

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