Biochemical Pharmacology, Vol. 29, pp. 3026–3029.

0006-2952/80/1101-3026 $02.00/0

Differentiation of normal and inducible rat liver aldehyde dehydrogenases by disulfiram inhibition *in vitro**

(*Received* 18 *February* 1980; *accepted* 10 *June* 1980)

In normal rat liver, at least three isozymes of aldehyde dehydrogenase [aldehyde:NAD(P) oxidoreductase, EC 1.2.1.3 and 1.2.1.5, ALDH] can be identified in mitochondria, microsomes, and/or cytosol [1, 2]. Two additional cytosolic aldehyde dehydrogenase isozymes can be induced in normal rat liver. One is induced by phenobarbital, the other by a variety of xenobiotics including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [3]. Rat hepatomas induced by 2-acetylaminofluorene also have a unique aldehyde dehydrogenase phenotype. This phenotype is characterized by the appearance of a cytosolic isozyme immunochemically identical to the TCDD-inducible isozyme and by the increased activity and shift to the cytosol of a normal liver microsomal ALDH [2]. The properties of these inducible aldehyde dehydrogenases (cytosolic location and mM K_m for acetaldehyde) make it unlikely that they play a major role in ethanol metabolism. Rather, evidence is accumulating that these inducible isozymes are involved in the metabolism of other aldehydes, whether biogenically generated or exogenously administered [4, 5]. We have been interested in determining the functional significance of, and the relationships between, these inducible aldehyde dehydrogenases.

A recent study comparing the subcellular distribution and properties of the hepatoma-specific and normal rat liver aldehyde dehydrogenases indicated both isozyme- and tissue-specific differences in sensitivity to the aldehyde dehydrogenase inhibitor disulfiram [2]. These observations suggested that disulfiram sensitivity *in vitro* may be useful in distinguishing various normal liver and inducible ALDHs in whole tissues and in subcellular fractions.

This paper confirms these initial observations, expands them to include the effects of disulfiram on the TCDD-inducible aldehyde dehydrogenase, and reports several differences in disulfiram sensitivity among the isozymes that are useful in distinguishing them structurally and functionally.

Normal rat liver and rat hepatomas were obtained from adult male Sprague–Dawley rats as described [2]. Male Long–Evans rats treated with TCDD by the method of Deitrich *et al.* [3] were provided by Dr. Richard A. Deitrich of the University of Colorado.

Whole tissues were homogenized in 0.06 M sodium phosphate buffer, pH 8.5, containing 1 mM EDTA, with or without 1 mM 2-mercaptoethanol (hereafter referred to as buffer) as appropriate. Homogenates were made to 1.0% with Triton X-100 and centrifuged as described [2]. Normal liver, hepatoma, and TCDD-treated liver were fractionated as described [2] in 0.25 M buffered sucrose with or without 2-mercaptoethanol as appropriate. The fractions were adjusted to 1.0% with Triton *X*-100 and centrifuged as described [2]. The resulting supernatant fractions were used as the source of aldehyde dehydrogenase.

Aldehyde dehydrogenase activities were determined using a slight modification of the spectrophotometric assay described previously [6]. All assays were performed at pH 8.5 in buffer with or without 2-mercaptoethanol as appropriate. Final substrate concentrations were 8 mM and final coenzyme concentrations, 5 mM. Disulfiram was prepared as a 30 mM stock solution in absolute methanol. Methanol had no effect on aldehyde dehydrogenase activity. We previously established that 100 μM final concentration is the disulfiram concentration producing maximum aldehyde dehydrogenase inhibition in both normal liver and hepatomas [2]. The effect of disulfiram (100 μM) on aldehyde dehydrogenase was determined by mixing 10 μl of the disulfiram stock solution with 2.74 ml of an assay mixture containing a sample in buffer with, or without, added 2-mercaptoethanol, but with no substrate. After a 5-min preincubation, the reaction was initiated by addition of 0.25 ml of substrate solution. Occasionally, the order of addition of buffer, sample and disulfiram was varied to study the effects of 2-mercaptoethanol on disulfiram inhibition.

Activities were expressed in mU/mg protein (1 mU = 1 nmole of NAD(P)H produced/min). Protein concentrations were determined by the method of Lowry *et al.* [7] using bovine serum albumin as standard.

In tissues prepared in buffer lacking 2-mercaptoethanol, the NAD-dependent aldehyde dehydrogenases were differentially inhibited by disulfiram (Table 1). Disulfiram significantly reduced the NAD-dependent ALDH activities of TCDD-treated and normal liver, with at least 60 per cent of the normal liver aldehyde dehydrogenase inhibited by disulfiram. Hepatoma NAD-dependent ALDH activitiy was only slightly (<20 per cent) inhibited by disulfiram with either aliphatic or aromatic aldehyde substrates.

The NADP-dependent aldehyde dehydrogenases of both hepatoma and TCDD-treated liver were much more sensitive to disulfiram than their NAD-dependent activities, with more than 85 per cent of the TCDD-treated liver NADP-dependent activity inhibited by disulfiram (Table 1). Normal liver NADP-dependent ALDH was slightly less sensitive to disulfiram than its NAD-dependent activity.

Preparing the tissues in buffers containing 2-mercaptoethanol significantly increased the disulfiram inhibition of the NADP-dependent aldehyde dehydrogenase activities in hepatoma and normal liver (Table 1). The inhibition of hepatoma aliphatic NAD-dependent activity was also significantly increased by 2-mercaptoethanol. Mercaptoethanol did not affect the inhibition of any ALDH in TCDD-treated liver.

The final 2-mercaptoethanol concentration reached in the 3 ml assay under these conditions ranged from 1.6 μM when 5 μl of sample was used as an enzyme source to 8 μM with 25 μl of sample. The premixing of disulfiram and buffers containing appropriate concentrations of 2-mercaptoethanol did not affect the disulfiram inhibition of hepatoma or normal liver NADP-dependent ALDH. We confirmed that the increased inhibition is not due to interactions between disulfiram and 2-mercaptoethanol (i.e. mixed disulfide formation) by demonstrating that adding 2-mercaptoethanol to the assay mixture had no effect on disulfiram inhibition of the NADP-dependent ALDH activity unless the reactants were mixed in the order: buffer with 2-mercaptoethanol, sample without 2-mercaptoethanol, then disulfiram. The addition sequence, buffer with 2-mercaptoethanol, disulfiram, then sample, did not show enhanced inhibition.

* Supported, in part, by Grant CA-21103 from the National Institutes of Health.

Table 1. Effect of disulfiram on rat liver aldehyde dehydrogenase

Substrate/Coenzyme	Tissue					
	Normal liver		Hepatoma		TCDD-treated	
	−2-ME*	+2-ME	−2-ME	+2-ME	−2-ME	+2-ME
Propionaldehyde/NAD						
Control activity†	21.9 ± 2.4	24.4 ± 1.6	148.6 ± 20.7	171.0 ± 26.5	487.9 ± 160.7	597.3 ± 105.6
% Inhibition	60.6‡	62.7‡	17.9‡	60.7‡,§	37.2‡	40.7‡
Propionaldehyde/NADP						
Control activity	17.1 ± 2.4	18.1 ± 2.4	197.6 ± 30.5	181.8 ± 34.1	720.2 ± 197.2	681.5 ± 121.2
% Inhibition	44.3‡	83.8‡,§	66.6‡	89.2‡,§	87.5‡	87.9‡
Benzaldehyde/NAD						
Control activity	8.4 ± 1.1	10.7 ± 0.6	205.4 ± 28.6	234.0 ± 57.5	821.7 ± 225.9	927.3 ± 178.2
% Inhibition	66.7‡	71.3‡	12.2	40.4‡	21.3‡	40.0‡
Benzaldehyde/NADP						
Control activity	6.1 ± 0.5	7.0 ± 0.8	549.5 ± 51.4	650.5 ± 168.7	2557.7 ± 848.2	2251.5 ± 393.4
% Inhibition	34.4‡	71.4‡,§	60.9‡	96.9‡,§	86.9‡	94.1‡

* Presence (+) or absence(−) of 2-mercaptoethanol in the tissue preparation buffers.

† Activity (mU/mg protein) is the average ± S.E.M. for at least six determinations in each tissue.

‡ Activity in the presence of disulfiram (100 μM) was significantly different from its corresponding control at at least the $P < 0.05$ level by a paired t-test.

§ Inhibition in the presence of 2-mercaptoethanol was significantly greater than that without 2-mercaptoethanol at at least the $P < 0.05$ level by a paired t-test.

Disulfiram significantly inhibited the NAD-dependent aldehyde dehydrogenase activity of normal liver, hepatoma, and TCDD-treated liver mitochondria and cytosol, as well as the microsomal NAD-dependent activity from TCDD-treated livers (Table 2). Normal liver and hepatoma microsomal NAD-dependent ALDH activity was only slightly reduced by disulfiram. Mercaptoethanol in the fractionation buffer had no significant effect on the disulfiram sensitivity of NAD-dependent aldehyde dehydrogenase in any normal liver, hepatoma, or TCDD-treated liver subcellular fraction.

Disulfiram significantly reduced the NADP-dependent activities of all normal liver and TCDD-treated liver subcellular fractions (Table 2), with more than 90 per cent inhibition in all TCDD-treated liver fractions. In hepatoma mitochondria, microsomes, and cytosol prepared in buffers lacking 2-mercaptoethanol, disulfiram significantly inhibited the NADP-dependent aldehyde dehydrogenase (>90 per cent inhibition in all fractions). However, with 2-mercaptoethanol included in the fractionation buffers, the inhibition of hepatoma NADP-dependent aldehyde dehydrogenase was significantly reduced in all fractions (Table 2). This lack of inhibition appears to have been due to an effect of 2-mercaptoethanol on hepatoma NADP-dependent ALDH, because inhibition was duplicated when buffer containing 2-mercaptoethanol and sample without 2-mercaptoethanol were premixed, and then disulfiram was added. As with whole tissue preparations, no significant interactions between disulfiram and 2-mercaptoethanol were observed. Mercaptoethanol did not affect the disulfiram sensitivity of normal liver or TCDD-treated liver NADP-dependent ALDH in any subcellular fraction.

Although several studies have characterized the inhibition of rat liver aldehyde dehydrogenase by disulfiram *in vivo* and *in vitro* [1, 2, 8–11], only one [2] examined the effects of disulfiram on the NADP-dependent isozymes. We previously used disulfiram sensitivity as one variable by which differences in the aldehyde dehydrogenase isozyme composition of normal rat liver and rat hepatomas could be demonstrated [2]. In both tissues, we identified an NAD-dependent μM K_m (isozyme I) and two mM K_m isozymes (II and III) that differ in disulfiram sensitivity. Isozyme I is a mitochondrial isozyme which is very disulfiram sensitive. Isozymes II and III are NAD(P)-dependent and are found in mitochondria and microsomes of both tissues. In the absence of 2-mercaptoethanol, isozyme II is disulfiram insensitive: isozyme III is disulfiram sensitive when NADP is coenzyme. In addition, hepatomas possess a cytosolic NAD(P) isozyme (IV) that is disulfiram sensitive when either NAD or NADP is coenzyme. We propose that isozyme IV is identical to the TCDD-inducible normal liver isozyme [2]. Our observations indicate that the cytosolic TCDD-inducible isozyme should be very disulfiram sensitive with either NAD or NADP and should possess high activity with benzaldehyde and NADP [2]. The results reported here confirm that the TCDD-inducible ALDH possesses these properties. The phenobarbital-inducible aldehyde dehydrogenase may be differentiated from the TCDD-inducible and hepatoma-specific ALDHs because, although it is very disulfiram sensitive [10], it is an NAD-dependent isozyme.

The differential effects of 2-mercaptoethanol on the disulfiram sensitivity of hepatoma and normal rat liver aldehyde dehydrogenase were unexpected. It is generally accepted that disulfiram inhibits aldehyde dehydrogenase by irreversible interactions with enzyme sulfhydral groups [8, 12, 13]. However, only for sheep liver NAD-dependent ALDH has 2-mercaptoethanol been shown to significantly alter disulfiram sensitivity. In sheep liver, the major interactions appear to be between disulfiram and 2-mercaptoethanol, not between 2-mercaptoethanol and aldehyde dehydrogenase [12, 13].

It is unlikely that the effects of 2-mercaptoethanol on rat liver aldehyde dehydrogenase disulfiram inhibition observed here are due to direct 2-mercaptoethanol–disulfiram interactions. Rather, the data indicate that the effects of 2-mercaptoethanol are due largely to interactions between 2-mercaptoethanol and various hepatoma and normal liver aldehyde dehydrogenase isozymes. First, both potentiation and blockade of inhibition may occur when the only source of 2-mercaptoethanol is from the fractionation buffer. Second, both potentiation and blockade of inhibition occur only when 2-mercaptoethanol and aldehyde dehydrogenase interact; premixing of disulfiram and 2-mercaptoethanol did not cause significant potentiation or blockade of inhibition. Third, a clear isozyme- and

Table 2. Effect of disulfiram on aldehyde dehydrogenase from various subcellular fractions

Substrate/Coenzyme	Fraction					
	Mitochondria		Microsomes		Cytosol	
	−2-ME*	+2-ME	−2-ME	+2-ME	−2-ME	+2-ME
			Normal liver			
Propionaldehyde/NAD						
Control activity†	95.8 ± 9.9	79.1 ± 18.6	85.9 ± 11.4	133.3 ± 23.2	2.7 ± 0.5	3.5 ± 0.6
% inhibition	31.7‡	24.0‡	11.5	8.8	85.2‡	60.0‡
Benzaldehyde/NADP						
Control activity	9.3 ± 1.5	10.6 ± 1.1	34.5 ± 3.2	34.1 ± 2.7	2.1 ± 0.4	0.8 ± 0.3
% Inhibition	82.8‡	67.9‡	49.9‡	43.4‡	71.4‡	100.0‡
			Hepatoma			
Propionaldehyde/NAD						
Control activity	220.9 ± 65.4	304.8 ± 64.5	99.2 ± 19.8	212.5 ± 79.4	122.2 ± 23.9	101.4 ± 7.1
% Inhibition	30.0‡	27.1‡	6.8	5.9	56.3‡	53.0‡
Benzaldehyde/NADP						
Control activity	226.6 ± 64.6	380.8 ± 83.9	290.7 ± 98.8	403.4 ± 116.7	581.2 ± 81.2	681.4 ± 62.3
% Inhibition	90.6‡	17.0§	92.7‡	19.9§	97.0‡	27.0§
			TCDD-treated			
Propionaldehyde/NAD						
Control activity	406.2 ± 48.7	408.3 ± 59.8	702.2 ± 44.9	672.5 ± 102.9	691.2 ± 23.6	761.8 ± 23.1
% Inhibition	41.2‡	27.1‡	37.9‡	57.9‡	76.0‡	87.7‡
Benzaldehyde/NADP						
Control actvity	1262.4 ± 156.0	1425.9 ± 287.7	2086.6 ± 164.6	2066.0 ± 318.1	3614.5 ± 161.0	3671.4 ± 310.8
% Inhibition	96.5‡	91.6‡	89.3‡	99.1‡	94.9‡	99.3‡

* Presence (+) or absence (−) of 2-mercaptoethanol in the tissue fractionation buffers.

† Activity (mU/mg protein) is the average ± S.E.M. for at least four determinations in each tissue.

‡ Activity in the presence of disulfiram (100 μM) was significantly different from its corresponding control at at least the $P < 0.05$ level by a paired t-test.

§ Inhibition in the presence of 2-mercaptoethanol was significantly less than that without 2-mercaptoethanol at at least the $P < 0.05$ level by a paired t-test.

tissue-specificity of the 2-mercaptoethanol effects was observed. In whole tissue, the normal liver NAD(P)-dependent and NAD and NAD(P)-dependent hepatoma aldehyde dehydrogenase were affected. No effect was demonstrable on any TCDD-treated liver ALDH nor on normal liver NAD-dependent ALDH. In subcellular fractions, the specificity was even more restricted; only the NAD(P)-dependent hepatoma ALDHs were affected. That TCDD-treated liver disulfiram inhibition was neither potentiated nor blocked by 2-mercaptoethanol indicates that 2-mercaptoethanol was not interacting with hepatoma isozyme IV, but with one or both of the mM K_m NAD(P)-dependent isozymes, II or III.

We are currently unable to explain fully the differing effects of 2-mercaptoethanol on the disulfiram sensitivity of the NAD(P)-dependent hepatoma aldehyde dehydrogenases in whole tissue as opposed to subcellular fractions. It is difficult to directly compare the results obtained in whole tissue preparations and subcellular fractions due to the great differences in specific activity observed [1, 2, 14], and to the possible aggregation and subsequent inactivation of aldehyde dehydrogenase that may occur in whole tissue preparations [3, 14]. TCDD-treated liver ALDHs, however, did not show similar opposing responses to 2-mercaptoethanol and the normal liver potentiation of whole tissue was not apparent in subcellular fractions. These observations suggest that the 2-mercaptoethanol effects were due to variable accessibility of sulfhydral groups in hepatoma isozymes III and perhaps IV to disulfiram caused by 2-mercaptoethanol, perhaps in association with the procedures used to prepare whole tissue homogenates and subcellular fractions. Although this may be further evidence of inherent differences in the ALDHs of the various tissues, confirmation will require comparison of the disulfiram sensitivities of purified preparations of the isozymes involved.

In summary, disulfiram inhibition *in vitro* is shown to be a useful tool in distinguishing among the various aldehyde dehydrogenase isozymes of normal rat liver, rat hepatomas, and xenobiotic-treated rat liver. The NAD(P)-dependent aldehyde dehydrogenases, as well as the NAD-dependent isozymes, are significantly affected by disulfiram. A significant interaction between sulfhydral reagents and certain aldehyde dehydrogenases, especially in rat hepatomas, is demonstrable.

Development Biology Section,
Department of Biology,
The University of Alabama,
University, AL 35486, U.S.A.

RONALD LINDAHL

REFERENCES

1. S. O. C. Tottmar, H. Pettersson and K.-H. Kiessling, *Biochem. J.* **135**, 579 (1974).
2. R. Lindahl, *Biochem. J.* **183**, 55 (1979).
3. R. A. Deitrich, P. Bludeau, T. Stock and M. Roper, *J. biol. Chem.* **252**, 6169 (1977).
4. R. A. Deitrich, P. Bludeau, M. Roper and J. Schmuck, *Biochem. Pharmac.* **27**, 2343 (1978).
5. D. W. Nebert, *Molec. cell. Biochem.* **27**, 27 (1979).
6. R. Lindahl, *Biochem. J.* **164**, 119 (1977).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
8. R. A. Deitrich and V. G. Erwin, *Molec. Pharmac.* **7**, 301 (1971).
9. R. A. Deitrich, A. C. Collins and V. G. Erwin, *J. biol. Chem.* **247**,7232 (1972).
10. T. Koivula and M. Koivusalo, *Biochim. biophys. Acta* **410**, 1 (1975).
11. O. Tottmar and H. Marchner, *Acta pharmac. tox.* **38**, 366 (1976).
12. T. M. Kitson, *Biochem. J.* **151**, 407 (1975).
13. T. M. Kitson, *Biochem. J.* **175**, 83 (1978).
14. F. Simpson and R. Lindahl, *J. exp. Zool.* **207**, 383 (1979).

Biochemical Pharmacology, Vol. 29, pp. 3029–3031.

0006-2952/80/1101–3029 $02.00/0

Calcium and frequency-dependent release of norepinephrine

(*Received* 1 *April* 1980; *accepted* 3 *June* 1980)

Brown and Gillespie [1] showed that the release of norepinephrine (NE) from the cat spleen increased with increasing stimulation frequency, up to 30 Hz. NE output was about 5-fold greater at 30 than at 10 Hz; above 30 Hz the output progressively declined. Although the exact mechanism responsible for the increase in NE output with stimulation frequency is not clear, Kirpekar *et al.* [2] proposed that facilitation of transmitter release with increase in stimulation frequency may be a reflection of a greater accumulation of calcium in the nerve terminals during a train of pulses at a high frequency as compared to a low frequency. If calcium plays such a role in the frequency–output relationship, then changing the extracellular concentration of calcium ions should modify the frequency-dependent NE release, i.e. at low extracellular calcium concentrations the output at the lower frequency of stimulation should be much more reduced than the output at the higher frequency. Conversely, at high extracellular calcium concentrations the output should be preferentially enhanced at low as compared to high frequencies. We report here the effect of calcium on the frequency–NE output relationship in the perfused spleen of the cat.

Cats were anesthesized with ether, followed by chloralose (60 mg/kg, i.v.). The cats were then given iproniazid (20 mg/kg, i.v.) to inhibit monoamine oxidase. After 30 min the spleens were isolated and perfused *in situ* with oxygenated Krebs-bicarbonate (Krebs) solution at a rate of about 7 ml/min at 35°, as described previously [3]. Control venous perfusate samples were collected for 2 min before nerve stimulation, for 2 min during stimulation at 5 and 30 Hz, and for 4 min during stimulation at 1 Hz. To label the endogenous stores of NE with [^{3}H]-NE, a single injection of 200 μCi of [^{3}H]-NE (10.43 Ci/mole) was made into the femoral vein; the spleen perfusion was started 30 min after the injection. The splenic nerves were stimulated with supramaximal monophasic rectangular pulses of 1–2 msec duration at 1, 5 and 30 Hz for a total of 200 stimuli.

The spleens were perfused with Krebs solution for 30 min before nerve stimulation. The nerves were stimulated at 1, 5 and 30 Hz at intervals of about 15 min in normal Krebs solution, and then in low- or high-calcium solution. The order of perfusion with different calcium solutions was reversed, so that in some experiments the first perfusion was with low calcium. Perfusion with high-calcium solution was usually done during the last period. To prepare high-calcium (10 mM) solution, KH_2PO_4 and $NaHCO_3$ were