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INHIBITION OF HUMAN ERYTHROCYTE AND LEUKOCYTE ALDEHYDE DEHYDROGENASE ACTIVITIES BY DIETHYLTHIOCARBAMIC ACID METHYL ESTER

AN *IN VIVO* METABOLITE OF DISULFIRAM

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Abstract—The inhibitory effects of diethylthiocarbamic acid methyl ester (DTC-Me), an *in vivo* metabolite of disulfiram (Antabuse®), on the aldehyde dehydrogenase (ALDH; EC 1.2.1.3) activities in human erythrocytes and leukocytes were studied. ALDH assays were performed by incubating intact isolated blood cells in the presence of different concentrations of DTC-Me, using 3,4-dihydroxyphenylacetaldehyde, the aldehyde derived from dopamine, as the substrate. DTC-Me was more selective as inhibitor of the leukocyte ALDH activity (which resembles the liver "mitochondrial" low K_m ALDH), whereas both disulfiram and diethyldithiocarbamic acid, the reduced monomer of disulfiram, were more selective for the erythrocyte ALDH (which is similar to the "cytosolic" high-K_m ALDH). Diethylthiocarbamic acid, the free acid of DTC-Me, was less potent than DTC-Me, and caused similar inactivation of the erythrocyte and leukocyte ALDH activities. The inhibition of ALDH by DTC-Me could not be completely restored by extensive dilution of intact or sonicated blood cell samples, which indicated that ALDH was irreversibly inhibited. Since the inhibition patterns with DTC-Me agrees with the previously reported patterns of inhibition of the high-K_m and low-K_m isozymes after the administration of disulfiram, the results suggest that DTC-Me might be the active *in vivo* inhibitory metabolite of disulfiram.

Disulfiram (tetraethylthiuram disulfide; Antabuse®) is a potent inhibitor of aldehyde dehydrogenase (ALDH; EC 1.2.1.3), an enzyme which catalyses the oxidation of a great variety of aldehydes to their corresponding acid metabolites. Ethanol ingestion in combination with disulfiram medication results in very unpleasant symptoms (e.g. flushing, tachycardia, hypotension and vomiting; the so-called "disulfiram–ethanol reaction"), which is the rationale for the use of disulfiram in the treatment of alcoholism. These effects are generally believed to be consequences of the elevated concentration of acetaldehyde, the primary metabolite of ethanol oxidation, which accumulates in the body owing to the ALDH inhibition [1].

Upon absorption into the blood, disulfiram is rapidly and completely reduced to its monomer diethyldithiocarbamate (DDC) [2, 3], which is subsequently further biotransformed [4]. DDC was, however, found to be ineffective as inhibitor of rat liver ALDH *in vitro*, opposed to the results observed when it was administered *in vivo* [5]. Consequently, it was suggested that *in vivo* DDC had to be reoxidized to disulfiram [6, 7], or possibly co-oxidized with some endogenous thiol compound to yield a mixed disulfide [8], in order to inactivate ALDH. However, no detectable amount of unchanged drug could be found in blood samples obtained from disulfiram-treated subjects, nor in blood samples spiked with disulfiram [9]. These observations indicate that a metabolite of disulfiram, rather than the parent compound, is the active ALDH inhibitor *in vivo*.

Fig. 1. The structural formula for diethylthiocarbamic acid methyl ester (DTC-Me), an *in vivo* metabolite of disulfiram (Antabuse®).

The present study was undertaken to examine the effects of diethylthiocarbamic acid methyl ester (DTC-Me; the structure is shown in Fig. 1), a recently discovered *in vivo* metabolite of disulfiram [10], on human erythrocyte and leukocyte ALDH activities. These isozymes have been proposed to serve as valuable and easily accessible markers of disulfiram-like side effects of various drug therapies, and, furthermore, as markers of the liver high-K_m and low-K_m ALDH isozyme activities, respectively, which are of special interest during treatment with alcohol-sensitizing drugs (i.e. ALDH inhibitors) [11]. For comparison, assays were also performed with disulfiram, DDC and diethylthiocarbamic acid (DTC), the free acid of DTC-Me.

MATERIALS AND METHODS

Chemicals. DTC-Me and DTC were synthesized as described by Johansson *et al.* [10]. Disulfiram

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(Fluka AG, Buchs, Switzerland) was recrystallized twice from 99.5% ethanol. DDC, dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), sodium bisulphite and NAD^+ were supplied by Sigma Chemical Co. (St Louis, MO). The bisulphite form of 3,4-dihydroxyphenylacetaldehyde (DOPAL) was prepared enzymatically from dopamine by the use of a partially purified rat liver monoamine oxidase (MAO; EC 1.4.3.4) [12]. The concentrations of DOPAL and of the DOPAC standard were determined according to Helander and Tottmar [13].

Blood samples. Fresh human citrated blood samples were obtained from healthy donors at the University Hospital of Uppsala (Sweden). Erythrocytes and leukocytes were isolated by Percoll (Pharmacia AB, Uppsala, Sweden) density gradient centrifugation technique [14]. The cells were counted on a Hycel HC 333 cell counter (Clinicon, Mannheim, F.R.G.), equipped with a multi-channel analyser.

Assay of ALDH activity. The standard assays were performed with intact blood cells according to the method by Helander *et al.* [11]. The reaction mixture contained either 50 million erythrocytes or 5 million leukocytes (giving ALDH activities of 1.12 ± 0.14 and 19.2 ± 8.8 nmol DOPAC formed per hr per 10 million cells, respectively) [11] suspended in 1 ml phosphate-buffered saline (PBS), pH 7.4, containing 50 μM EDTA. The assays were performed under gentle shaking at 37° in a water bath. The reaction mixtures were preincubated for 5 min in the presence of the inhibitors, before the reaction was started by addition of 50 μM DOPAL. The assays were not limited by the endogenous NAD^+-concentration [11]. Disulfiram and DTC-Me were dissolved in methanol (standards were added with equal volumes of methanol) whereas DDC and DTC were dissolved in water. The reaction was terminated after 15 min by addition of 0.2 ml ice-cold 16.8% (w/v) perchloric acid. After centrifugation of the samples at 20,000 *g* for 10 min at 4°, the amount of the acid metabolite (DOPAC) formed was measured in the clear supernatant by high-performance liquid chromatography (HPLC) with electrochemical detection [14].

Assays were also performed to study whether the ALDH activity could be restored after incubation with DTC-Me by removal of the inhibitor through extensive dilution of the samples. Intact erythrocytes or leukocytes were incubated for 10 min in the absence or presence of 5 mM DTC-Me. Thereafter, the ALDH activity was assayed with either intact or sonicated (1 mM NAD^+ was used in the assay mixture) blood cells according to the standard procedure (see above).

RESULTS

The time course of inactivation of the erythrocyte and leukocyte ALDH activities by 1 mM DTC-Me is shown in Fig. 2. The inhibition process was very rapid during the first minute, and then proceeded at a much lower rate. Based on these findings, a preincubation time of 5 min was chosen for the standard assay.

The results from the incubations of intact erythrocytes and leukocytes with disulfiram, DDC, DTC-Me or DTC are shown in Table 1. Disulfiram and DDC were both found to be more selective inhibitors of the erythrocyte ALDH isozyme, although disulfiram was more potent than DDC. DTC showed quite similar inactivation of the erythrocyte and leukocyte activities, whereas DTC-Me was a more selective inhibitor of the leukocyte ALDH activity.

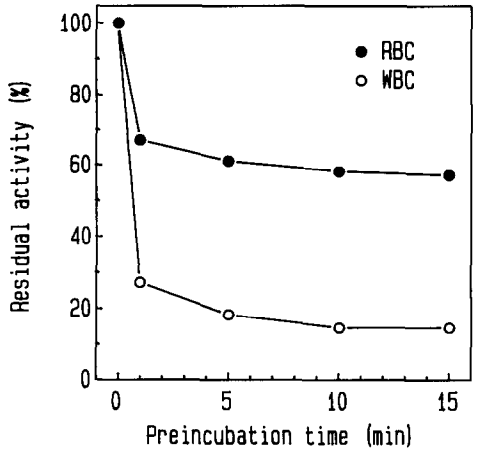

Fig. 2. Time course of inactivation of human erythrocyte (RBC) and leukocyte (WBC) ALDH activities by diethylthiocarbamic acid methyl ester (DTC-Me). Intact blood cells were incubated in phosphate-buffered saline at 37°. After preincubation for different times in the presence of 1 mM DTC-Me, the reaction was started by addition of 50 μM DOPAL. The ALDH activity was assayed by HPLC with electrochemical detection. Data represent mean values of duplicate determinations on blood cell preparations from three different individuals.

In assays studying the reversibility of ALDH inhibition by DTC-Me, only partial restoration of the activity was observed after extensive dilution of the blood cell samples in the assay mixtures. About 30–40% of the inhibition obtained in the standard assay was retained with sonicated and diluted samples.

DISCUSSION

Extensive work has been carried out to elucidate the mechanism behind the ALDH inhibition by disulfiram, and the cause of the "disulfiram–ethanol reaction". The biochemical basis for its action *in vivo* is generally believed to be inactivation of the liver "mitochondrial" ALDH isozyme, with high affinity (i.e. low K_m) for acetaldehyde as the substrate, which primarily is responsible for the oxidation of ethanol-derived acetaldehyde under normal circumstances [15]. This was shown in studies on disulfiram-treated rats where the liver low-K_m ALDH activity was strongly inhibited opposed to the "cytosolic" isozyme, with lower affinity (i.e. higher K_m) for acetaldehyde, which was almost unaffected [16]. In addition, Orientals and South American Indians which manifest disulfiram-like flushing symptoms and elevated acetaldehyde levels in response to moderate alcohol ingestion (e.g. about 50% of the Japanese and Chinese populations) [17] possess a

Table 1. Effects of disulfiram and some of its metabolites on aldehyde dehydrogenase activities in human erythrocytes and leukocytes

Compound	Concentration (mM)	Inhibition of ALDH activity (%)[a] Erythrocytes	Leukocytes
Disulfiram	1.0	87.6 ± 6.0[b]	97.3 ± 0.6
	0.1	90.8 ± 4.1	86.8 ± 9.2
	0.01	84.5 ± 9.7	10.5 ± 2.1
Diethyldithiocarbamic acid (DDC)	1.0	93.3 ± 0.2	16.1 ± 3.2
	0.1	77.8 ± 7.7	12.4 ± 7.8
	0.01	25.1 ± 7.9	10.2 ± 3.4
Diethylthiocarbamic acid methyl ester (DTC-Me)	1.0	37.0 ± 6.4	85.8 ± 2.7
	0.1	6.4 ± 2.6	34.2 ± 3.9
	0.01	1.8 ± 3.5	1.6 ± 1.9
Diethylthiocarbamic acid (DTC)	1.0	25.3 ± 7.8	34.8 ± 2.5
	0.1	4.3 ± 6.1	13.0 ± 1.4
	0.01	2.7 ± 2.3	7.2 ± 0.3

[a]Intact erythrocytes or leukocytes were incubated for 5 min in the presence of different concentrations of disulfiram or one of its metabolites, before the ALDH assay was started by addition of the substrate.

[b]Data represent mean ± SD for duplicate determinations on four different blood cell preparations.

defective low-K_m ALDH which due to a structural mutation lacks or have a severely diminished catalytic activity [18]. However, in contrast to the inhibition pattern obtained in *in vivo* experiments, the high-K_m ALDH isozyme is considerably more sensitive to disulfiram than the low-K_m form under *in vitro* conditions [19].

Studies performed *in vitro* revealed that disulfiram, via its reduced monomer DDC, initially forms adducts with cysteine residues of the enzyme followed by the formation of intramolecular disulfide bonds between vicinal SH-groups [6, 19]. Although disulfiram has been suggested not to be an active site-directed reagent [20], these structural changes obviously cause a considerably lowered specific activity of the enzyme. However, the *in vitro* mechanism of inhibition is indicated to be different from the one occurring *in vivo*. For example, *in vivo* ALDH is irreversibly inhibited by disulfiram, whereas the inhibition *in vitro* can be reversed by treatment with various reducing agents [19]. These observations indicate that the inhibition of ALDH *in vitro* and *in vivo* are due to quite different reactions, and that a metabolite of disulfiram, rather than the parent compound, is the active inhibitor *in vivo*.

Diethyldithiocarbamic acid methyl ester (DDC-Me) is a major metabolite of disulfiram, formed by methylation of DDC in the liver and kidney, and it is found in the blood after disulfiram administration [21]. However, like DDC [5], DDC-Me was reported not to be a potent inhibitor of ALDH *in vitro* [22], which indicated the need of biotransformation to give the actual inhibitor. Recently, Johansson *et al.* [10] identified yet another metabolite of disulfiram, DTC-Me, which is formed by subsequent oxidation of DDC-Me. DTC-Me was detected in human blood with a peak concentration about 3 hr after a single oral disulfiram administration [23], and it was found to be a potent and irreversible *in vitro* as well as *in vivo* inactivator of the rat liver low-K_m ALDH [10]. This suggested that DTC-Me might be the active metabolite of disulfiram responsible for the inhibition of ALDH *in vivo*.

The results obtained in the present study show that DTC-Me, in contrast to disulfiram and DDC, is a more selective inhibitor of the leukocyte ALDH activity. With respect to disulfiram sensitivity and induction by Mg^{2+}, the human leukocyte ALDH activity resembles the human liver mitochondrial low-K_m isozyme (ALDH I or E_2) [11], whereas the erythrocyte ALDH is very similar to the liver cytosolic high-K_m isozyme (ALDH II or E_1) [24]. Thus, the inhibition pattern obtained with DTC-Me apparently resembles the pattern observed in the liver during disulfiram treatment. DTC, the free acid of DTC-Me, which has not been identified *in vivo*, caused similar inactivation of the erythrocyte and leukocyte ALDH activities.

The inhibition by DTC-Me showed a very rapid onset, as measured with intact blood cells, but only about 30–40% of this inhibition was retained when the cells were sonicated and extensively diluted in the assay mixtures. This suggests a rapid formation of a saturable complex between enzyme and inhibitor, followed by a slow irreversible covalent reaction which completely inactivates the enzyme.

In conclusion, the present results obtained by DTC-Me on human erythrocyte and leukocyte ALDH activities resemble the inhibition pattern observed in the liver during disulfiram (Antabuse®) treatment. Since DTC-Me is the first metabolite of disulfiram identified that possesses similar inhibitory properties on ALDH *in vitro* as those observed by disulfiram *in vivo*, it is suggested that DTC-Me might be the active *in vivo* inhibitory metabolite of disulfiram.

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