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Effects of disulfiram therapy on aldehyde dehydrogenase activity in human leukocytes and erythrocytes

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Aldehyde dehydrogenase (ALDH, EC 1.2.1.3) catalyses the irreversible oxidation of various aldehydes to their corresponding acid metabolites. It is present in most tissues and organs with the highest specific activity in the liver. ALDH is responsible for the oxidation of acetaldehyde, the proximate metabolite of ethanol, to acetic acid. The main part of acetaldehyde oxidation occurs in the liver, and it is known that drugs such as disulfiram (Antabuse), that inhibit ALDH, cause an increased level of acetaldehyde during ethanol elimination. Ethanol ingestion during disulfiram treatment results in very unpleasant symptoms (e.g., flushing, tachycardia and hypotension), which is the rationale of using this "alcohol-sensitizing" drug in the treatment of alcoholism.

ALDH is also present in human blood. Alcoholics have been reported to possess a decreased activity of ALDH, both in blood [1–5] and in liver [6–8]. This indicated that blood ALDH could serve as a marker for alcoholism or, rather, high ethanol intake, since the activity has been reported to return to normal values after abstinence [4, 9]. The good correlation obtained between blood ALDH activity and sensitivity to ethanol in Japanese individuals suggested that blood ALDH may also prove useful as a marker for ethanol sensitivity in orientals [10]. Furthermore, the erythrocyte ALDH activity has been shown to correlate significantly with the hepatic cytosolic ALDH activity in healthy controls, whereas no correlation was obtained for alcoholics [11].

The ALDH activity in human whole blood is practically all due to the activity of the erythrocytes [12]. However, it was recently shown that not only erythrocytes, but leukocytes and platelets as well, possess ALDH activity [13]. Human erythrocyte ALDH, which is similar to the liver cytosolic isozyme (ALDH II), is very sensitive to disulfiram [12, 14]. Since disulfiram is an irreversible inhibitor, and mature erythrocytes lack the ability to synthesize new enzyme and have a life-span of about 120 days, the ALDH activity in blood remains affected for a much longer time than in the liver after the disulfiram therapy has been completed [15, 16]. Thus, the blood ALDH activity (i.e. the erythrocyte activity) is not a suitable marker for the duration of disulfiram inhibition of the liver ALDH.

The present study was performed to compare the effects of disulfiram treatment on human leukocyte and erythrocyte ALDH activities. A method previously used in a study on the biogenic aldehyde metabolism in intact human blood cells [13], and a method for routine analysis of ALDH activity in small samples of whole blood [17], were modified for analysis of the ALDH activity in leukocytes and erythrocytes.

Materials and methods

Chemicals. Dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), sodium bisulphite and NAD were supplied by Sigma (St. Louis, MO). The bisulphite form of 3,4-dihydroxyphenylacetaldehyde (DOPAL) was prepared enzymatically from dopamine by the use of rat liver monoamine oxidase (MAO, EC 1.4.3.4) [18]. The concentrations of DOPAL and of the DOPAC standard were determined as described by Helander and Tottmar [17]. Percoll was obtained from Pharmacia AB (Uppsala, Sweden), and disulfiram (Antabuse, 400 mg tablets) was from Dumex A/S (Copenhagen, Denmark).

Blood samples. Fresh human citrated blood samples were obtained from healthy donors at the University Hospital of Uppsala (Sweden), and, furthermore, from four alcoholic patients (two men and two women) at Beckomberga Hospital (Bromma, Sweden) during disulfiram medication and at different times after the therapy had been completed. The patients had been on medication for at least 2 weeks before taking part in the study. During treatment, disulfiram was given every second day at an oral dose of 400 mg.

Fractionation of blood. Fractionation of blood was performed by Percoll density gradient centrifugation technique as previously described by Helander and Tottmar [13]. Cytocentrifuge preparations of the leukocyte fraction were stained with Giemsa for identification of leukocyte subpopulations. The fraction was found to contain about 60% granulocytes, 30% lymphocytes and 5% monocytes, and less than 5% erythrocytes. The erythrocyte fraction contained less than 0.001% leukocytes.

For routine assays, erythrocytes and leukocytes were counted on a Hycel HC 333 cell counter (Clinicon, Mannheim, F.R.G.), equipped with a multi-channel analyzer.

Assay conditions. Assays were performed by incubating blood cells under physiological conditions in a final volume of 1 ml with DOPAL, the aldehyde derived from dopamine oxidation, as the substrate [13]. The amount of acid metabolite, DOPAC, formed after 15 min of incubation was measured using high-performance liquid chromatography (HPLC) with electrochemical detection [13]. The standard assays were performed with intact cells, since the ALDH activity obtained in incubations with sonicated leukocytes and 0.5 mM NAD only was about 50% of the activity obtained with the intact cells. By contrast, for erythrocytes, the activity obtained with sonicated cells was 3–5 times higher than with the intact cells.

Results and discussion

Assay conditions. The formation of DOPAC during an incubation period of 15 min using 5 million leukocytes/ml, or 50 million erythrocytes/ml, was measured with DOPAL concentrations of 5–100 μM. Both in incubations with leukocytes and erythrocytes, the reaction rate increased sharply at concentrations up to 10–20 μM, and then proceeded more slowly at higher aldehyde concentrations. The formation of DOPAC was linear during at least 20 min of incubation in assays using 50 μM DOPAL and 5 million leukocytes or 50 million erythrocytes. About 7 and 4% of the DOPAL added had been oxidized after 20 min of incubation with leukocytes or erythrocytes, respectively. Furthermore, a good linearity between DOPAC formation and number of cells added in the range of 1–10 million leukocytes/ml, or 5–200 million erythrocytes/ml, was observed in incubations for 15 min with 50 μM DOPAL. The recovery of DOPAC was almost 100%.

These results showed that ALDH was saturated during the assay conditions used, and that the assays were not limited by the endogenous NAD concentration. Thus, a substrate concentration of 50 μM, an incubation time of 15 min, and 5 million leukocytes or 50 million erythrocytes in a final volume of 1 ml was chosen for the standard assay.

HPLC analysis. The HPLC system used gives a good separation between bisulphite-free DOPAL and DOPAC with retention times of about 6 and 8 min, respectively [13].

However, since bisulphite was added to the PCA, only small amounts of free aldehyde were detected. The DOPAL-bisulphite eluted close to the elution front. No interfering peaks were observed in the chromatograms with samples treated with PCA before addition of DOPAL, or in samples incubated in the absence of aldehyde (blanks). No formation of DOPAC was observed in incubations with reaction blanks.

The incubations with intact leukocytes resulted in formation of small amounts of 3,4-dihydroxyphenylethanol (DOPET), the alcohol metabolite formed by reduction of DOPAL, probably by the action of aldehyde reductase (ALR, EC 1.1.1.2). This is consistent with previous results, showing a ratio of 15–20:1 between the acid and alcohol metabolites formed from DOPAL in incubations with human intact leukocytes [13]. The small peak representing DOPET eluted between DOPAL and DOPAC, and did not interfere with the standard assay.

The overall precision was 3% (coefficient of variation) when 10 replicate assays were performed with 5 million leukocytes/ml, or 50 million erythrocytes/ml, from the same pool of blood cells.

Leukocyte and erythrocyte ALDH activities in healthy controls. The ALDH activity obtained in incubations with 5 million intact leukocytes from healthy controls was 2.4 ± 1.1 nmol DOPAC formed/15 min (mean ± SD; range = 1.3–4.4; N = 16, eight men and eight women) or, if related to amount of cells, 19.2 ± 8.8 nmol DOPAC formed per hr per 10 million cells (range = 10–35). The corresponding results obtained with 50 million intact erythrocytes were 1.4 ± 0.2 nmol DOPAC formed/15 min (mean ± SD; range = 1.1–1.8; N = 24, 12 men and 12 women) or, if related to amount of cells, 1.12 ± 0.14 nmol DOPAC formed per hr per 10 million cells (range = 0.9–1.4). The much higher ALDH activity observed in leukocytes than in erythrocytes (about 17 times higher) is in accordance with previous results [13]. However, since the leukocyte:erythrocyte ratio in human blood normally is about 1:800, the ALDH activity in whole blood is practically all due to the activity of the erythrocytes [12].

Leukocyte and erythrocyte ALDH activities during and after disulfiram therapy. The results from the analyses of the ALDH activities in erythrocytes and leukocytes from disulfiram patients, during medication and at different times after the therapy was completed, are shown in Figs 1 and 2. Unfortunately, two of the patients discontinued the study 2 and 3 weeks, respectively, after the disulfiram therapy had been completed, and returned to drinking. The ALDH activity in the erythrocytes returned to control values after about 70 days, which is in accordance with previous observations [15, 16]. However, the leukocyte ALDH activity returned to control values already within 2–4 days after the therapy had been completed. Furthermore, the erythrocyte ALDH activity was inhibited by about 90% during therapy, whereas the leukocyte activity only was decreased by 20–45%. These results are consistent with previous results from a study on isolated human blood fractions, where the leukocyte ALDH activity was much less affected by 50 μM disulfiram than was the erythrocyte ALDH activity [19]. A 95% inhibition of the leukocyte activity could, however, be obtained by performing assays in the presence of 1 mM disulfiram (unpublished observations). In disulfiram medication, the irreversible ALDH inhibition has a slow onset and long duration of action [20]. This might be explained by the need of diethyldithiocarbamate, the reduced metabolite of disulfiram rapidly formed in the blood, to be re-oxidized to disulfiram, or possibly co-oxidized with some natural metabolite to give a mixed disulphide, in order to be a potent inactivator of ALDH [21]. Thus, during medication, leukocytes (probably also immature leukocytes in the bone marrow) are almost continuously exposed to disulfiram, which indicates that the low degree of ALDH inhibition observed in leukocytes is not primarily explained by the *de novo* enzyme synthesis or the much faster turnover of leukocytes than of erythrocytes, but rather reflects the much lower sensitivity of the leukocyte ALDH activity to disulfiram.

The difference in sensitivity to disulfiram between erythrocyte and leukocyte ALDH activities, together with the observation that the activity obtained in incubations with sonicated leukocytes could be increased by about 50% by addition of 0.5 mM $MgCl_2$, whereas the opposite (about 60% reduction) was observed for erythrocytes, indicates that the ALDH activity in leukocytes is mainly due to a different isozyme from that in erythrocytes. The leukocyte ALDH might be similar to the human liver mitochondrial isozyme (ALDH I), which is less sensitive to inhibition by

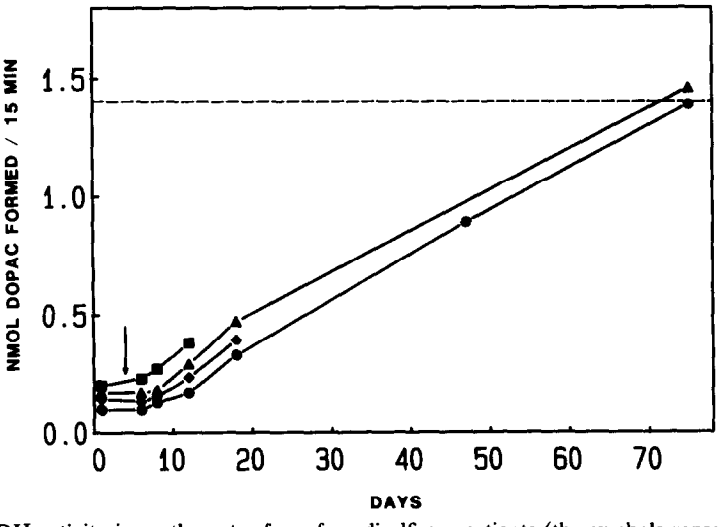

Fig. 1. ALDH activity in erythrocytes from four disulfiram patients (the symbols represent different individuals). The ALDH activity was measured once during treatment and at different times after the disulfiram therapy had been completed. The treatment was completed at day 4 (arrow). The dashed line represents mean erythrocyte ALDH activity from 24 healthy control subjects. The ALDH assays were performed using 50 million erythrocytes according to the standard method (see Materials and methods).

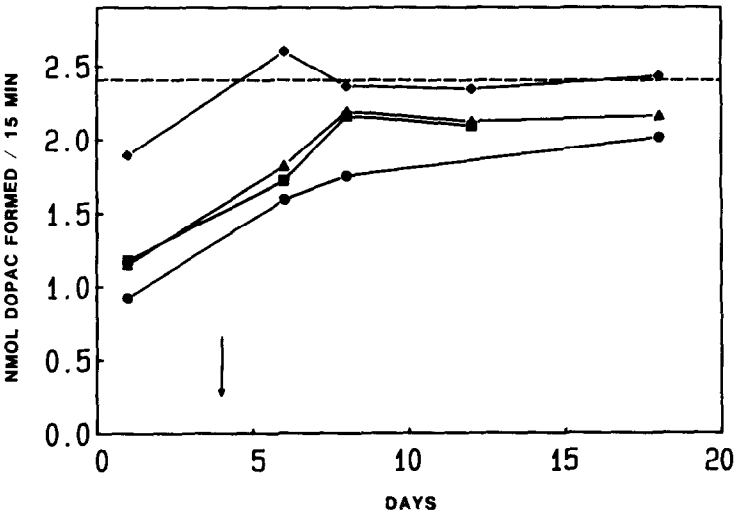

Fig. 2. ALDH activity in leukocytes from four disulfiram patients (the symbols represent different individuals; see Fig. 1). The ALDH activity was measured once during treatment and at different times after the disulfiram therapy had been completed. The treatment was completed at day 4 (arrow). The dashed line represents mean leukocyte ALDH activity from 16 healthy control subjects. The ALDH assays were performed using 5 million leukocytes according to the standard method (see Materials and methods).

disulfiram [22] and, furthermore, is stimulated by Mg^{2+} ions [23].

In conclusion, our results show that the erythrocyte ALDH was almost completely inhibited during disulfiram treatment, whereas the leukocyte activity was much less affected. Since mature erythrocytes, in contrast to leukocytes, lack the ability to synthesize new enzyme, the erythrocyte ALDH is not a suitable *in vivo* marker for the degree and duration of ALDH inhibition by irreversible inhibitors (like disulfiram). However, if the leukocyte ALDH is similar to the liver mitochondrial isozyme, which is primarily responsible for the oxidation of ethanol-derived acetaldehyde under normal circumstances, it could serve as an easily accessible marker for the conditions in the liver during disulfiram therapy.

In summary, the aldehyde dehydrogenase (ALDH, EC 1.2.1.3) activity was determined in leukocytes and erythrocytes from healthy control subjects, as well as from patients during disulfiram treatment and at different times after the therapy had been completed. In healthy controls, the specific ALDH activity of leukocytes was about 17 times higher as compared to the erythrocyte activity. In patients during disulfiram treatment, the erythrocyte ALDH was inhibited by about 90% and did not return to control values until after about 70 days after the therapy had been completed. By contrast, the leukocyte activity was only inhibited by 20–45% during treatment, and returned to control values within 2–4 days after the therapy was completed.

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Studies on the interaction of minoxidil with prostacyclin synthase *in vitro*

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Minoxidil (6-(1-piperidinyl)-2,4-pyrimidinediamine-3-oxide, Lonolox®) is a potent antihypertensive agent in man [1] and has been implicated to act as a direct smooth muscle vasodilator by a yet undefined biochemical mechanism [2]. Interestingly, as a side effect the drug promotes hair growth in man for which the underlying mechanism is also obscure (cf. [3] and literature cited herein). Recently, Kvedar *et al.* reported that minoxidil selectively inhibits prostacyclin (PGI_2)* synthesis of cultured endothelial and smooth muscle cells from bovine aorta [3]. Prostacyclin synthase had been previously characterized as a cytochrome P-450 protein [4] and therefore spectral changes could be expected with minoxidil. In the present study we have investigated the interaction of minoxidil with solubilized prostacyclin synthase in order to demonstrate that the target for the inhibitory action of minoxidil is the heme active site of prostacyclin synthase.

Materials and methods

Minoxidil was a gift from the Upjohn Co. (Kalamazoo, MI). Piperidine and pyrimidine were obtained from Aldrich-Chemie (Steinheim, F.R.G.) and imidazole, 6-keto-$PGF_{1\alpha}$ and tranylcypromine from Sigma-Chemie (Deisenhofen, F.R.G.). [1-^{14}C]PGH_2 was prepared as described [5]. Bovine aortic microsomes were isolated according to published procedures, resuspended in 10 mM KP_i buffer (0.1 mM BHT, 0.1 mM DTT, 0.1 mM EDTA, 20% glycerol, pH 7.5) and solubilized with 0.5% cholate/0.2% lubrol PX (v/v) for 30 min at 0–4° followed by centrifugation for 60 min at 100,000 *g*. The supernatant, referred to as solubilized prostacyclin synthase, contained 1.61 nmol/ml cytochrome P450 (cf. [6]). Optical difference spectra for determining the binding affinity of minoxidil were recorded with a Cary 118 spectrophotometer (Varian, Darmstadt, F.R.G.) between 350 and 500 nm [7]. The inhibitory effect of minoxidil on prostacyclin synthase activity was assayed upon incubation of [1-^{14}C]PGH_2 with the enzyme followed by HPLC radiochromatographic analysis of [1-^{14}C]-labelled 6-keto-$PFG_{1\alpha}$, the stable hydrolysis product of PGI_2.

Results and discussion

The addition of minoxidil to solubilized prostacyclin synthase (Fig. 1) caused a concentration-dependent increasing difference spectrum typical for a nitrogen ligand binding to cytochrome P450 [7]. The observed spectral change with a peak at 438 nm and a trough at 417 nm is similar to the difference spectrum produced by *trans*-2-phenylcyclo-propylamine (tranylcypromine, 435 vs 415 nm with a K_s-value of 120 μM [6]), the most well-known inhibitor of prostacyclin synthase. From the corresponding titration curve (Fig. 1) a spectral dissociation constant of 2.4 μM can be calculated which, with respect to the enzyme concentration employed (1.6 μM), reflects an almost stoichiometric binding of minoxidil to the heme active site of prostacyclin synthase (therefore the term "K_s" and not K_s should be used).

In order to clarify which nitrogen atom of the minoxidil molecule is involved in the binding process, difference spectra were recorded also with piperidine and pyrimidine. None of these compounds caused a spectral change similar to that of minoxidil in concentrations of up to 1 mM, suggesting that the primary amino group attached to position 2 of the pyrimidine ring interacts with the heme iron (data not shown). Neither minoxidil nor tranylcypromine produced difference spectra with solubilized thromboxane synthase from human platelets in concentrations of up to 1 mM (data not shown). Since the small imidazole molecule selectively interacts with thromboxane synthase [7] but not with prostacyclin synthase (data no shown), this also indicates that there exists a characteristic difference in the topology of the active site of both enzymes.

Thus, only the primary amino groups of minoxidil or tranylcypromine seem to have access to the heme iron of prostacyclin synthase whereas aromatic nitrogen derivatives seem to be sterically hindered. This is just opposite for thromboxane synthase.

* Abbreviations used: BHT, butylated hydroxytoluene (2,6-di-t-butyl-*p*-cresol); DTT, DL-dithiothreitol; PG, prostaglandin; PGI_2, prostacyclin. *Enzymes:* Guanylate cyclase (EC 4.6.1.2); prostacyclin synthase (EC 5.3.99.4); thromboxane synthase (EC 5.3.99.5).