FEBS Letters 426 (1998) 362–366 FEBS 20141

Retinoids, ω-hydroxyfatty acids and cytotoxic aldehydes as physiological substrates, and H_2-receptor antagonists as pharmacological inhibitors, of human class IV alcohol dehydrogenase

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Received 9 February 1998; revised version received 23 March 1998

Abstract Kinetic constants of human class IV alcohol dehydrogenase (σσ-ADH) support a role of the enzyme in retinoid metabolism, fatty acid ω-oxidation, and elimination of cytotoxic aldehydes produced by lipid peroxidation. Class IV is the human ADH form most efficient in the reduction of 4-hydroxynonenal (k_{cat}/K_m: 39 500 mM^{-1} min^{-1}). Class IV shows high activity with all-*trans*-retinol and 9-*cis*-retinol, while 13-*cis*-retinol is not a substrate but an inhibitor. Both all-*trans*-retinoic and 13-*cis*-retinoic acids are potent competitive inhibitors of retinol oxidation (K_i: 3–10 μM) which can be a basis for the regulation of the retinoic acid generation and of the pharmacological actions of the 13-*cis*-isomer. The inhibition of class IV retinol oxidation by ethanol (K_i: 6–10 mM) may be the origin of toxic and teratogenic effects of ethanol. H_2-receptor antagonists are poor inhibitors of human and rat classes I and IV ($K_i > 0.3$ mM) suggesting a small interference in ethanol metabolism at the pharmacological doses of these common drugs.

Key words: Alcohol dehydrogenase; Retinol; Retinoic acid; Lipid peroxidation; 4-Hydroxynonenal; Alcohol metabolism

1. Introduction

An increasing amount of evidence supports that alcohol dehydrogenase (ADH, EC 1.1.1.1) in vertebrates performs specific metabolic functions in the interconversion of alcohols and aldehydes of particular structures, besides the better known role in alcohol detoxification. The most important pieces of evidence are: the multiplicity of structurally different ADH forms, grouped into classes, that coexist in the same species [1,2]; the distinct kinetic properties of each class [1,3–5]; the characteristic distribution of each class in organs and tissues [6,7]; and the early expression during embryonic development [8].

In the present work we investigate some functional characteristics of human class IV ADH or σσ-ADH. This is a specially interesting ADH because of its distribution in various epithelial tissues such as upper digestive tract mucosa and cornea, but not in liver [6]. Moreover, class IV exhibits high activity with retinoids [4,9] and the expression of mouse class IV in tissues coincides spatiotemporally with the generation of retinoic acid [8,10], which supports a role of the enzyme as a retinol dehydrogenase in the crucial pathway of retinoic acid formation. In the present work we study the kinetics of human class IV ADH with retinoids of physiological relevance, and the inhibition of retinol oxidation by other retinoids (of interest to understand a possible mechanism of regulation) and by ethanol (a possible origin of ethanol toxicity and teratogenesis). In addition we have explored the activity of class IV with other endogenous compounds, putative physiological substrates of class IV, such as ω-hydroxyfatty acids and cytotoxic aldehydes derived from lipid peroxidation. These have been shown to be good substrates for rat class IV ADH [4], but the rat and the human class IV enzymes, although strictly homologous (88% sequence identity), exhibit striking kinetic differences [5], which makes it necessary to separately investigate the human class IV properties. Finally we report the effect of H_2-receptor antagonists, commonly prescribed drugs for peptic ulcer, on the activity of the human and rat alcohol dehydrogenases.

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Abbreviations: ADH, alcohol dehydrogenase; CRBP, cellular retinol binding protein; RXR, retinoid X receptor

2. Materials and methods

2.1. Materials

NAD, NADH, ω-hydroxyfatty acids, retinoids, DEAE-Sepharose and AMP-Sepharose were from Sigma. 2-Hexenol and 2-hexenal were from Aldrich. 4-Hydroxynonenal was provided by Prof. H. Esterbauer, University of Graz (Graz, Austria). Preparation of the alkenal was performed as previously indicated [4]. Other alcohols and aldehydes were from Merck. Cimetidine, ranitidine and famotidine were from Uquifa (Barcelona).

2.2. Enzyme preparation

Human class IV ADH was prepared from gastric tissue according to a previously published procedure [11]. From the corresponding DEAE-Sepharose chromatography step of the class IV purification [11], class I γγ-ADH isozymes were isolated and finally purified on an AMP-Sepharose column equilibrated in 200 mM Tris-HCl, 0.5 mM dithiothreitol, pH 8.2, and eluted by a linear gradient of 0–0.4 mM NADH. Recombinant human class IV ADH was purified from *Escherichia coli* cells (Allali-Hassani et al., unpublished results). Rat (Sprague-Dawley) class I was purified from liver as reported [3], but using AMP-Sepharose instead of CapGapp-Sepharose for the last purification step. Rat class IV was prepared from stomach according to [12].

2.3. Enzymatic activity

Activity was determined spectrophotometrically by monitoring the change in absorbance at 340 nm, with a Varian Cary 219 instrument, at 25°C, using an ε_{340} for NADH of 6220 M^{-1} cm^{-1}. Activity was measured in 0.1 M glycine/NaOH, pH 10.0, or in 0.1 M sodium phosphate, pH 7.5. Alcohol oxidation was measured at pH 10.0 or 7.5, with 2.4 mM NAD or 4 mM NAD (rat class IV), while aldehyde reduction was performed at pH 7.5 with 1.33 mM NADH in a 0.2 cm light-path cell. Activity with retinoids was determined as previously indicated [4,13]. 9-*cis*-Retinol was synthesized by reduction of 9-*cis*-retinal with sodium borohydride [14] and the purity confirmed by HPLC. The ε_{326} calculated for 9-*cis*-retinol was 39 733 M^{-1} cm^{-1} in 0.1 M sodium phosphate, pH 7.5, 0.02% Tween 80. Kinetic constants were calculated by using the non-linear regression program Enzfitter

PII S0014-5793(98)00374-3

(Elsevier Biosoft). Inhibition of retinol oxidation by ethanol and other retinoids was analyzed with the COMP program [15].

Inhibition of ethanol oxidation by H_2-receptor antagonists (cimetidine, ranitidine and famotidine) was measured at pH 7.5 or 10.0, using concentrations up to 2 mM inhibitor. Inhibition kinetics by ranitidine was measured at 360 nm, since this drug absorbs at 340 nm, using an ε_{360} for NADH of 4200 M^{-1} cm^{-1}. K_i was determined from the secondary representation of the Lineweaver-Burk plots.

3. Results and discussion

3.1. Physiological substrates

3.1.1. ω-Hydroxyfatty acids. Table 1 shows the kinetic constants of human class IV ADH with different compounds of physiological interest. Medium- and long-chain ω-hydroxyfatty acids are good substrates for the enzyme, with k_{cat}/K_m values increasing as the chain length increases. Kinetics with 16-hydroxy-hexadecanoic acid could not be performed at pH 7.5 because of its low solubility at this pH.

The ω-oxidation of fatty acids has been reported in many tissues, and although it is a minor pathway in normal conditions, it increases in abnormal states such as diabetes or starvation, and by ethanol consumption [16,17]. The oxidation is initiated by the ω-hydroxylase activity of several members of the cytochrome P-450 family and the resulting ω-hydroxyfatty acids are further oxidized to dicarboxylic acids by alcohol and aldehyde dehydrogenases [18]. The kinetic constants with ω-hydroxyfatty acids (Table 1) suggest that human class IV plays a role in this pathway, mostly in the oxidation of long-chain compounds.

3.1.2. Cytotoxic aldehydes. Aldehydes known to be products of lipid peroxidation, and some of the corresponding alcohols were also analyzed (Table 1). All compounds are efficiently transformed by class IV, hexanal being the best substrate because of a very high k_{cat}. The relative k_{cat} values for alcohols and aldehydes of this group of substrates agree with early observations that the 2,3 unsaturation favors the oxidative direction of the ADH-catalyzed reaction [19].

Kinetic constants suggest that human class IV ADH is effective in the removal of cytotoxic aldehydes produced by lipid peroxidation. It has been previously demonstrated that several human class I ADH isozymes and class II ADH are also efficient in the reduction of 4-hydroxyalkenals [20], class II (ππ-ADH) being the most active, with a k_{cat}/K_m for 4-hydroxynonenal of 28 000 mM^{-1} min^{-1}. The value for class IV is even higher (39 500 mM^{-1} min^{-1}), indicating that class IV ADH is the most effective human ADH in the reductive elimination of 4-hydroxynonenal. The same conclusion was reached for the rat species where class IV was the best ADH in the reduction of 4-hydroxynonenal and of all other cytotoxic aldehydes tested [4]. ADH, aldehyde dehydrogenase and glutathione *S*-transferase are involved in the metabolic elimination of 4-hydroxynonenal. The contribution of class I ADH has been estimated to be about 10% of the total 4-hydroxynonenal metabolism in rat hepatocytes [21,22]. However, the contribution of the ADH reductive pathway in epithelia may be higher than in the hepatic cells because of the presence of class IV, the most active ADH form, in these tissues. Class IV in epithelia may constitute a metabolic protection against cytotoxic aldehydes in areas of active lipid peroxidation.

3.1.3. Retinoids. The kinetic experiments with retinoids were performed with a recombinant enzyme (Table 2), although the constants found are indistinguishable from those obtained with the gastric class IV (results not shown). Except for 13-*cis*-retinol and 13-*cis*-retinal, which are not substrates, the all-*trans* and 9-*cis* isomers of retinol and retinal are effectively transformed by human class IV ADH. The enzyme exhibits the highest catalytic efficiency with 9-*cis*-retinol. Interestingly, in terms of k_{cat}/K_m, alcohols are better substrates than the corresponding aldehydes, supporting a physiological role for class IV in retinol oxidation, in the crucial pathway for retinoic acid generation. Kinetic constants for the all-*trans*-retinoids and 9-*cis*-retinal are similar to those previously reported [9]. For these three substrates, class IV ADH is the most active retinol dehydrogenase form of human ADH [9]. No data are available on the kinetics of 9-*cis*-retinol with the human class I and class II enzymes. Interestingly, several class I isozymes and class II ADH are active with 13-*cis*-retinal [9], but we have not found any activity for class IV with either 13-*cis*-retinol nor 13-*cis*-retinal, in a concentration range of 7–150 μM (Table 2). Thus, while all-*trans*-retinol fits better in the class IV active site than in the class I site [23,24], this seems not to be the case for the 13-*cis*-isomer.

Ethanol inhibits both all-*trans*-retinol and 9-*cis*-retinol oxidation by class IV ADH (Table 3) with K_i values (6–10 mM) in the range of the blood ethanol concentrations after social drinking. This supports the notion that alcohol consumption interferes with the normal metabolism of retinoids and that this could be one of the mechanisms of the toxic and teratogenic effects of ethanol [25].

The relative contribution of ADH (classes I, II and IV) and that of the more specific microsomal retinol dehydrogenases in the retinol-retinal interconversion is controversial [26]. The great amount of ADH in liver (classes I and II) makes the isozymes of these classes suitable for the oxidative elimination of excess of retinol in the organism, which is supported by the

Table 1
Kinetic constants of human class IV ADH with ω-hydroxyfatty acids and products of lipid peroxidation

Substrate	K_m (mM)		k_{cat} (min^{-1})		k_{cat}/K_m (mM^{-1} min^{-1})	
10-OH-Decanoic acid	0.1		59		590	
12-OH-Dodecanoic acid	0.17	(0.07)	660	(2 150)	3 880	(30 750)
16-OH-Hexadecanoic acid		(0.035)		(1 320)		(37 715)
Hexanol	0.14		900		6 430	
Hexanal	0.02		27 230		1 361 700	
2-Hexenol	0.017		3 130		184 100	
2-Hexenal	0.025		2 930		117 200	
4-Hydroxynonenal	0.038		1 510		39 500	

The enzyme was purified from stomach. Activities were determined in 0.1 M sodium phosphate, pH 7.5. In parentheses, activities with 0.1 M glycine/NaOH, pH 10.0.

Table 2
Kinetic constants of human class IV ADH with retinol and retinal isomers

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
all-*trans*-Retinol	15 ± 4	67 ± 10	4 500 ± 1 370
9-*cis*-Retinol	36 ± 4	475 ± 44	13 300 ± 2 020
13-*cis*-Retinol	N.A.		
all-*trans*-Retinal	34 ± 6	110 ± 25	3 300 ± 960
9-*cis*-Retinal	21 ± 5	190 ± 24	8 980 ± 2 350
13-*cis*-Retinal	N.A.		

The enzyme was obtained by expression of its cDNA in *E. coli*. Activities were determined in 0.1 M sodium phosphate, pH 7.5, 0.02% Tween 80. Results are expressed as the mean ± S.E.M. of least three determinations. N.A.: no activity.

inhibition of the process by 4-methylpyrazole [27]. Class IV ADH, which is present in many epithelial tissues but not in liver [6,11] and exhibits high K_m values for short-chain alcohols, seems suitable for a more specific role in cellular retinoid metabolism. There is evidence in favor of microsomal retinol dehydrogenase as a main enzyme in retinol oxidation for retinoic acid generation, such as the recognition of CRBP-retinol as substrate, its specificity towards retinol and its low K_m [28]. However, activity of ADH with CRBP-retinol has not been investigated, while, regarding specificity, retinol dehydrogenases also use hydroxysteroids [29,30]. Finally, although K_m for retinoids is higher for ADH, class IV exhibits a high k_{cat}/K_m ratio (Table 2), which is the best measure of the enzyme contribution at low substrate concentration. This value is not available for retinol dehydrogenase because a pure preparation has not been obtained [31]. Two recent results support the involvement of class IV in retinol metabolism. (1) It has been demonstrated that the initial detection of retinoic acid during mouse embryogenesis (day 6.5) coincides spatiotemporally with co-expression of class IV ADH and class 1 ALDH (an aldehyde dehydrogenase that oxidizes retinal to retinoic acid) [8]. (2) A class IV-like ADH is present in amphibians indicating a general occurrence of the enzyme in vertebrates, that could be related to the general need for retinoid signalling in these animals [32].

The oxidation of retinol to retinal is probably the limiting step in the generation of retinoic acid [26,28]; it is therefore of extreme interest to elucidate the role of the enzymes involved in the process, the mechanism of exposing retinol to the active site of the dehydrogenase from the CRBP-retinol complex and the regulation of the system. The importance of this step may explain the need of a certain degree of redundancy, provided by the existence of several enzymatic systems active with retinol: the cytosolic ADH (classes I, II and IV), the microsomal retinol dehydrogenase (three types) [28], and several types of cytosolic retinol dehydrogenase [33,34]. Contributions of each enzymatic system may differ among tissues. Thus blood vessel endothelium, a tissue with probable requirement for retinoic acid, does not contain microsomal retinol dehydrogenase [35] but it exhibits high ADH activity (class I in humans and class IV in rat) [7].

9-*cis*-Retinoic acid is the only retinoid that interacts with the retinoid X receptor. Its pathway of formation is not clear, although several possibilities have been discussed: dietary origin, cleavage of 9-*cis*-carotenoids, isomerization at the level of all-*trans*-retinoic acid, or isomerization at the level of all-*trans*-retinol and further oxidation to the acid form [36]. The presence of endogenous 9-*cis*-retinol [37], the high activity of class IV ADH with 9-*cis*-retinol (Table 2), the recent report of short-chain dehydrogenases active with 9-*cis*-retinol [30,36] and the existence of aldehyde dehydrogenases active with 9-*cis*-retinal [28,37] support the last possibility.

13-*cis*-Retinol is not a substrate of class IV but it is an effective competitive inhibitor of the oxidation of 9-*cis*-retinol (Table 3). This suggests that it binds strongly to the enzyme, like the all-*trans* and 9-*cis* isomers, but unproductively because the *cis* configuration in the close proximity of the alcohol group may impair the correct positioning of this reactive group in the active site. Interestingly, other retinoid-associated proteins discriminate against 13-*cis*-isomers [28].

All-*trans*-retinoic acid is a powerful inhibitor of the class IV retinol dehydrogenase activity (Table 3). Although the K_i value is high (10 μM) in comparison to the cellular retinoic acid concentrations (0.03–0.2 μM in mouse embryo [38,39]), it may be of physiological significance at the local concentrations in places of active retinoic acid synthesis. The inhibition of the first step by the end product of the pathway may represent a regulatory mechanism of retinoic acid homeostasis.

Also of interest is the potent inhibition of retinol oxidation by 13-*cis*-retinoic acid (K_i 3 μM). The 13-*cis*-retinoic acid is being used in the treatment of dermatological diseases and some forms of cancer, with pharmacological concentrations reaching in blood up to 7 μM [40]. It has been proposed that its actions, both beneficial and highly teratogenic in some conditions, could be related to a higher stability and to a slow isomerization to all-*trans*-retinoic acid, the active compound, since the 13-*cis*-isomer does not bind the nuclear receptors [41]. However, in contradiction with this hypothesis, treatment of experimental animals with high doses of 13-*cis*-retinoic acid does not result in an increase of all-*trans*-retinoic acid concentration [42]. The demonstration that 13-*cis*-retinoic acid is a powerful inhibitor of all-*trans*-retinol oxidation by class IV ADH (Table 3) and, therefore, an inhibitor of the all-*trans*-retinoic acid synthesis, provides an alternative mechanism for the pharmacological actions of this widely used retinoid.

Table 3
Inhibition of human class IV retinol dehydrogenase activity

Substrate	Inhibitor	K_i (mM)
all-*trans*-Retinol	ethanol	6
		9 (37°C)
9-*cis*-Retinol	ethanol	10
9-*cis*-Retinol	13-*cis*-retinol	0.013
all-*trans*-Retinol	13-*cis*-retinoic acid	0.003
all-*trans*-Retinol	all-*trans*-retinoic acid	0.010

Activities were determined at pH 7.5 and 25°C (except where indicated) using the recombinant enzyme. All inhibitions showed a competitive pattern.

Table 4
Inhibition of human and rat ADH classes by H_2-receptor antagonists

Inhibitor	Human		Rat	
	Class I (γγ)	Class IV (σσ)	Class I	Class IV
Cimetidine	No inhib.	Competitive $K_i = 0.51$ mM	No inhib.	Competitive $K_i = 0.32$ mM
Ranitidine	Mixed $K_i = 0.7$ mM	No inhib.	No inhib.	Mixed $K_i = 0.35$ mM
Famotidine	Uncompetitive $K_i = 1.1$ mM	No inhib.	No inhib.	No inhib.

Human classes I and IV and rat class IV enzymes were purified from stomach homogenates. Rat class I was purified from liver. Activities were determined with ethanol and 2.4 mM NAD at pH 7.5 (4 mM NAD and pH 10.0 for rat class IV). No inhib.: no inhibition was found up to 2 mM inhibitor.

The capacity of several retinoids to modulate the retinol oxidation activity of class IV ADH further sustains the physiological implication of the enzyme in retinoid metabolism.

3.2. Inhibition by H_2-receptor antagonists

H_2-receptor antagonists, common drugs for the treatment of peptic ulcer, were used as inhibitors of ethanol oxidation (Table 4). Cimetidine, ranitidine and famotidine affected very differently the human class I (γγ-ADH) and class IV ADH activities. Cimetidine competitively inhibited class IV, while it did not inhibit class I. In contrast, ranitidine and famotidine did not affect class IV ADH activity but inhibited class I. These results resemble only partially those of a previous report [43], although some of the discrepancies could be due to differences in the substrate or assay temperature used.

Regarding the rat enzyme, cimetidine and ranitidine inhibited the class IV enzyme, but none of the studied compounds inhibited class I. In contrast, a previous report [44], using stomach and liver crude homogenates, found inhibition of both class I and class IV by either of the antagonists, although in some cases with high K_i values. From the present and previous works on both species, it can be concluded that cimetidine inhibits ethanol oxidation with a competitive pattern, class IV being more strongly inhibited than class I, while inhibition by other H_2-antagonists shows a more complex pattern, and has more effect on class I than on class IV. These results support significant structural differences between class I and class IV active sites [23,24,45]. In all cases the K_i values are higher than 0.3 mM, well above the pharmacological concentrations of these compounds (0.2–11.2 μM, [44]), suggesting that the usual treatment with the H_2-receptor antagonists studied will have little effect on alcohol metabolism. It has been argued that these drugs can accumulate in gastric cells after an oral dose [44] but, even if this is the case, a partial inhibition of gastric ADH will not practically have any effect because of the small contribution of the stomach to overall ethanol metabolism [11,46,47].

Acknowledgements: We thank Prof. H. Esterbauer for the gift of 4-hydroxynonenal. This work was supported by grants from the Dirección General de Enseñanza Superior (PB95-0612 and PM96-0069), the Comissionat per a Universitats i Recerca (1997SGR 00040) and the Commission of the European Union (BIO4-CT97-2123).

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