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Rabbit 3-hydroxyhexobarbital dehydrogenase is a NADPH-preferring reductase with broad substrate specificity for ketosteroids, prostaglandin D_2, and other endogenous and xenobiotic carbonyl compounds

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ABSTRACT

3-Hydroxyhexobarbital dehydrogenase (3HBD) catalyzes NAD(P)$^+$-linked oxidation of 3-hydroxyhexobarbital into 3-oxohexobarbital. The enzyme has been thought to act as a dehydrogenase for xenobiotic alcohols and some hydroxysteroids, but its physiological function remains unknown. We have purified rabbit 3HBD, isolated its cDNA, and examined its specificity for coenzymes and substrates, reaction directionality and tissue distribution. 3HBD is a member (AKR1C29) of the aldo-keto reductase (AKR) superfamily, and exhibited high preference for NADP(H) over NAD(H) at a physiological pH of 7.4. In the NADPH-linked reduction, 3HBD showed broad substrate specificity for a variety of quinones, ketones and aldehydes, including 3-, 17- and 20-ketosteroids and prostaglandin D_2, which were converted to 3α-, 17β- and 20α-hydroxysteroids and 9α,11β-prostaglandin F_2, respectively. Especially, α-diketones (such as isatin and diacetyl) and lipid peroxidation-derived aldehydes (such as 4-oxo- and 4-hydroxy-2-nonenals) were excellent substrates showing low K_m values (0.1–5.9 μM). In 3HBD-overexpressed cells, 3-oxohexobarbital and 5β-androstan-3α-ol-17-one were metabolized into 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol, respectively, but the reverse reactions did not proceed. The overexpression of the enzyme in the cells decreased the cytotoxicity of 4-oxo-2-nonenal. The mRNA for 3HBD was ubiquitously expressed in rabbit tissues. The results suggest that 3HBD is an NADPH-preferring reductase, and plays roles in the metabolisms of steroids, prostaglandin D_2, carbohydrates and xenobiotics, as well as a defense system, protecting against reactive carbonyl compounds.

1. Introduction

Hexobarbital, a short time-acting hypnotic, is metabolized to 3-hydroxyhexobarbital (3HB) by cytochrome P450, and then to 3-oxohexobarbital (3OB) by 3-hydroxyhexobarbital dehydrogenase (3HBD) (Fig. 1) [1,2]. While 3HB is excreted after conjugation with glucuronic acid, electrophilic 3OB reacts with glutathione to produce 1,5-dimethylbarbituric acid and cyclohexenone-glutathione adduct [3,4]. Because 3HBD is involved in the formation of the reactive 3OB as well as the hexobarbital metabolism, it has been purified and characterized from liver cytosols of rabbits [5–7], guinea-pigs [8,9], mice [10] and golden hamsters [11]. The enzymes are monomeric proteins with molecular weights of 31–42 kDa, and oxidize several other alicyclic alcohols including some 3α- and/or 17β-hydroxysteroids in the presence of either NAD^+ or $NADP^+$ as the coenzyme. However, they differ from one another in their reactivity toward the substrates. In the reactivity toward the α- and β-isomers of 3HB (α-3HB and β-3HB,

Abbreviations: 3HB, 3-hydroxyhexobarbital; 3OB, 3-oxohexobarbital; 3HBD, 3-hydroxyhexobarbital dehydrogenase; AKR, aldo-keto reductase; HSD, hydroxysteroid dehydrogenase; PG, prostaglandin; TBE, 6-*tert*-butyl-2,3-epoxy-5-cyclohexene-1,4-dione; 4*R*-TBEH, 6-*tert*-butyl-2,3-epoxy-4(*R*)-hydroxy-5-cyclohexen-1-one; RT, reverse transcription; BAEC, bovine aortic endothelial cell; LC–ESI-MS, liquid chromatography–electrospray ionization-mass spectrometry.

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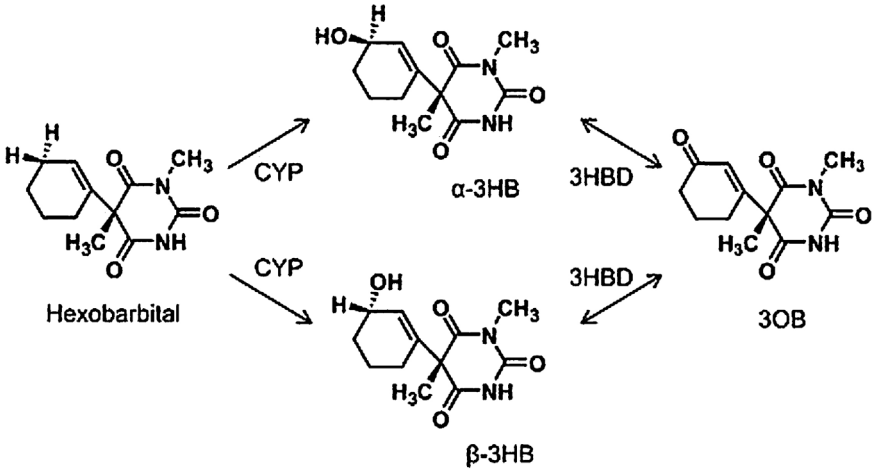

Fig. 1. Metabolism of hexobarbital into 3HB and 3OB. Hexobarbital is oxidized by cytochrome P450 (CYP) into α- and β-isomers of 3HB, which are converted into 3OB by 3HBD. The structures of (+)-hexobaribital and its metabolites are shown.

respectively, Fig. 1), all the enzymes oxidize α-3HB more preferentially than β-3HB, but the activities of the mouse and hamster enzymes toward β-3HB are much lower compared to those of the other animal enzymes. The mouse and hamster enzymes also oxidize *trans*-benzene dihydrodiol that is a representative substrate of dihydrodiol dehydrogenase [10,11]. Guinea-pig 3HBD is distinguishable from the other animal enzymes in its low activity for other xenobiotic alicyclic alcohols (such as 1-indanol and 1-tetralol) and high activity for 17β-hydroxy-C_{19}-steroids, thereby suggesting its identity with testosterone 17β-dehydrogenase (EC. 1.1.1.64) [8,9]. The mouse enzyme also exhibits 17β-hydroxysteroid dehydrogenase (HSD) activity for 17β-hydroxy-C_{19}-steroids and 17β-estradiol [10] that is not the substrate for the guinea-pig enzyme [8]. The rabbit enzyme oxidizes limited 17β-hydroxysteroids such as 5β-androstane-3α,17β-diol [7]. The hamster enzyme exhibits 3α-HSD activity toward bile acids, in addition to the 17β-HSD activity for 5β-androstane-3α,17β-diol [11]. The enzymatic properties of mammalian 3HBDs were studied under optimal pH conditions of 8.0–10.5, where their NAD^+-linked dehydrogenase activities are higher than the $NADP^+$-linked activities. Therefore, 3HBD has been reported to act as a dehydrogenase for the above xenobiotic and steroidal alcohols, but its physiological role has not yet been clarified. Among the mammalian 3HBDs, only hamster 3HBD is so far molecularly cloned and shown to be a member of the aldo-keto reductase (AKR) superfamily [11]. This suggests a possibility that the mammalian enzymes reduce carbonyl compounds, but their substrate specificities in the reduction reaction have not yet been thoroughly characterized.

In rabbits, hepatic 3HBD was reported to be identical to indanol dehydrogenase [6]. In contrast, six multiple forms of indanol dehydrogenase associated with 17β-HSD activity are isolated from the liver cytosol [12]. In addition, cytosolic 17β-HSD [13,14] and dihydrodiol dehydrogenase with 17β-HSD activity [15] exist in multiple forms in rabbit liver. Furthermore, the recent rabbit genomic analysis has predicted several genes that encode HSD-like proteins belonging to the AKR1C subfamily, three of which have recently been identified as NAD^+-preferring 3α/17β-HSDs (AKR1C26-AKR1C28) [16]. In this study, we isolated the cDNA for rabbit 3HBD that has been assigned AKR1C29 in the AKR superfamily. To elucidate the physiological role of AKR1C29 and its functional relationship with the enzymes that metabolize steroids and xenobiotic alcohols, we characterized the properties of the recombinant AKR1C29 at a physiological pH of 7.4, and examined the metabolism of the substrates in the enzyme-overexpressed cells. Our data show that AKR1C29 acts as a NADPH-preferring reductase with a wide range of both aromatic and aliphatic carbonyl compounds including ketosteroids, prostaglandin (PG) D_2 and lipid peroxidation-derived aldehydes.

2. Materials and methods

2.1. Chemicals

Steroids were obtained from Sigma Chemicals (Perth, WA) and Steraloids (Newport, RI), and PGs, 4-oxo-2-nonenal and 4-hydroxy-2-nonenal were from Cayman Chemicals (Ann Arbor, MI). 3OB [5], *trans*-benzene dihydrodiol [17], 4-oxo-2-nonenol [18] geranylgeranial [19], 6α/β-naloxols [20,21], 6-*tert*-butyl-2,3-epoxy-5-cyclohexene-1,4-dione (TBE) [22], 6-*tert*-butyl-2,3-epoxy-4(*R*)-hydroxy-5-cyclohexen-1-one (4*R*-TBEH) and its 4*S*-isomer (4*S*-TBEH) [23] were synthesized as described previously. α- and β-3HBs were kindly denoted by Dr R. Takenoshita (Fukuoka University, Japan). 3-Deoxyglucosone and befunolol were gifts from Nippon Zoki Pharmaceutical Co. (Osaka, Japan) and Kaken Pharmaceutical Co. (Tokyo, Japan), respectively. All other chemicals were of the highest grade that could be obtained commercially.

2.2. cDNA isolation and RT-PCR analysis

A cDNA for AKR1C29 was isolated from a total RNA sample of small intestine of a Japanese white rabbit by reverse transcription (RT)-PCR. The preparation of total RNA, RT, and DNA techniques followed the standard procedures described by Sambrook et al. [24]. The PCR was performed using *Pfu* DNA polymerase (Agilent Technologies, Santa Clara, CA) and a pair of sense and antisense primers, 5′-ttttcatatgatggatcccaagcatcagcgttatgggcatt-3′ and 5′-ttttgtcgactcaatattcatcagaaaatgggtaattt-3′, which contain underlined *Nde*I and *Sal*I sites, respectively. PCR amplification consisted of an initial denaturation step at 94 °C for 5 min, 29 cycles of denaturation (94 °C/30 s), annealing (60 °C/30 s) and extension (72 °C/2 min), and a final incubation step at 72 °C for 5 min. The PCR products were purified, digested with the two restriction enzymes (Life Technologies, Carlsbad, CA), and ligated into the pCold I vectors (Takara Bio Inc., Otsu, Japan) that had been digested with the two restriction enzymes [25]. The sequence of the coding region of the cloned cDNA was analyzed using a Beckman Coulter CEQ8000XL DNA sequencer. The 972-base pair sequence of the