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Expression and functional characterization of cytochrome P450 26A1, a retinoic acid hydroxylase☆

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ABSTRACT

Retinoic acid (RA) is a critical signaling molecule that performs multiple functions required to maintain cellular viability. It is also used in the treatment of some cancers. Enzymes in the CYP26 family are thought to be responsible for the elimination of RA, and CYP26A1 appears to serve the most critical functions in this family. In spite of its importance, CYP26A1 has neither been heterologously expressed nor characterized kinetically. We expressed the rCYP26A1 in baculovirus-infected insect cells and purified the hexahistidine tagged protein to homogeneity. Heme incorporation was determined by carbon monoxide difference spectrum and a type 1 spectrum was observed with RA binding to CYP26A1. We found that RA is a tight binding ligand of CYP26A1 with low nM binding affinity. CYP26A1 oxidized RA efficiently (depletion K_m 9.4 ± 3.3 nM and V_{max} 11.3 ± 4.3 pmoles min^{-1} pmole $P450^{-1}$) when supplemented with P450 oxidoreductase and NADPH but was independent of cytochrome b5. 4-Hydroxy-RA (4-OH-RA) was the major metabolite produced by rCYP26A1 but two other primary products were also formed. 4-OH-RA was further metabolized by CYP26A1 to more polar metabolites and this sequential metabolism of RA occurred in part without 4-OH-RA leaving the active site of CYP26A1. The high efficiency of CYP26A1 in eliminating both RA and its potentially active metabolites supports the major role of this enzyme in regulating RA clearance in vivo. These results provide a biochemical framework for CYP26A1 function and offer insight into the role of CYP26A1 as a drug target as well as in fetal development and cell cycle regulation.

1. Introduction

Retinoic acid (RA, Fig. 1) is the biologically active form of vitamin A. It is necessary for a multitude of biological functions including reproduction, embryonic development, immune competence, maintenance of healthy epithelia and regulation of apoptosis [1,2]. All of these functions are sensitive to the precise control of RA concentrations, and

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Abbreviations: RA, all-trans-retinoic acid; 4-OH-RA, 4-hydroxyretinoic acid; 4-oxo-RA, 4-oxo-retinoic acid; CYP, cytochrome P450; RAR, retinoic acid receptor; RXR, retinoid x receptor; ER, endoplasmic reticulum; NADPH, nicotinamide adenine dinucleotide phosphate; KPi, potassium phosphate; PBS, phosphate buffered saline; EB, equilibrium buffer; δ-Ala, δ-aminolevulinic acid; NP40, Nonidet P40; CRABP, cellular retinoic acid binding protein.

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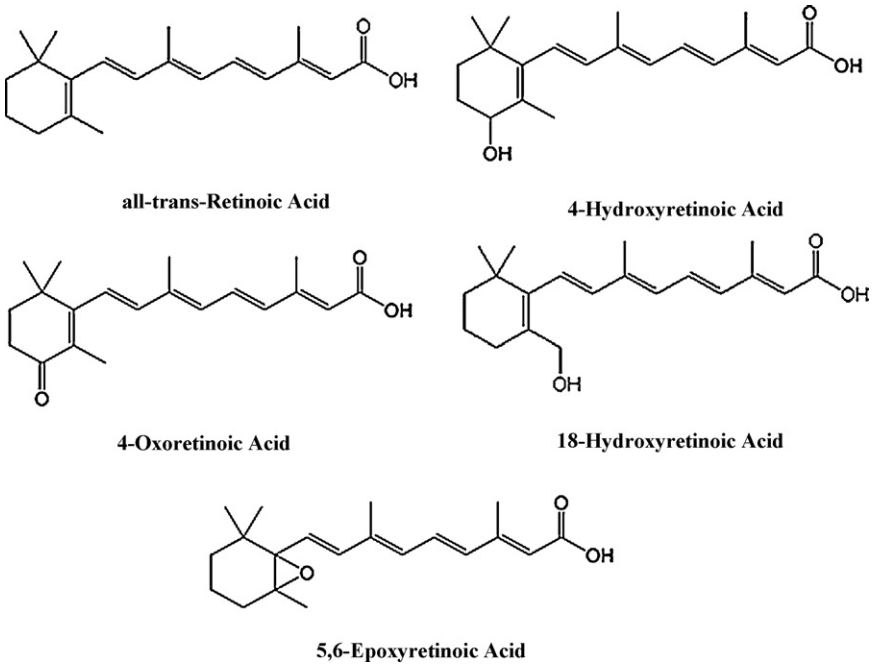

Fig. 1 – Chemical structures of all-trans-retinoic acid and its previously identified metabolites, 4-OH-RA, 4-oxo-RA, 18-OH-RA, and 5,6-epoxy-RA.

both an excess and a deficiency of RA are detrimental for life [3]. Complex systems have evolved to regulate RA concentrations via biosynthesis and metabolism as well as sequestration and targeting by specific binding proteins [3–5]. Our research is focused on characterizing the mechanisms that control the biodegradation and elimination of RA in humans.

RA can exist as three geometric isomers, all-trans-RA, 13-cis-RA and 9-cis-RA. Of these, all-trans-RA is believed to be the primary biologically active isomer [6]. The biological effects of RA are mainly mediated by all-trans-RA binding to nuclear RA receptors (RAR) that regulate the transcription of multiple target genes [7,8]. The elimination of RA in mammals is mediated metabolically *via* multiple oxidations [9], but the identity of enzymes responsible for the elimination of RA isomers in various human tissues is not well established. To date, the major identified metabolites are 4-hydroxy-RA (4-OH-RA), 4-oxo-RA, 18-hydroxy-RA, and 5,6-epoxy-RA (Fig. 1), which appear to be further oxidized to more polar metabolites [10,11]. Although there is evidence that 4-OH-RA, 18-OH-RA and 4-oxo-RA can bind to RAR [12] they do not appear to contribute to RA signaling during mouse development [13]. Previous studies have shown that common drug metabolizing P450 enzymes, namely CYP2C8, CYP2C9 and CYP3A catalyze the conversion of RA to 4-OH-RA, 4-oxo-RA and 5,6-epoxy-RA [11,14–16]. However, the K_m values were much higher than the circulating concentrations of RA, the actual K_m of RA hydroxylation in liver microsomes, and the K_d values of RA with the cellular retinoic acid binding proteins (CRABPs) [17,18], suggesting that these enzymes do not contribute significantly to the clearance of RA *in vivo*.

Recently, the CYP26 family was discovered as a group of RA metabolizing enzymes that may be responsible for RA catabolism [19]. In mammals, the CYP26 family consists of three highly conserved enzymes, CYP26A1, CYP26B1 and CYP26C1 [20–22]. In transiently transfected cell lines, both CYP26A1 and CYP26B1 metabolize RA to a series of oxidized metabolites [20,21] but CYP26A1 has been suggested as the most important enzyme of the CYP26 family [23]. Although use of transfected cells has been instrumental in producing initial information on the catalytic activity and substrate specificity of CYP26 enzymes, the low expression levels of CYPs in these systems have prevented detailed mechanistic and kinetic characterization of CYP26A1 and other members of this family [23].

Both CYP26A1 and CYP26B1 are essential for development. Cyp26a1 null mouse embryos die during mid-gestation and Cyp26b1 null pups die soon after birth with both knock-outs showing multiple developmental defects [24–26]. Despite the apparent critical role of the CYP26 enzymes in regulating RA concentrations, their activity and expression pattern in human tissues and their cellular localization are unknown, although transcripts of CYP26A1 and CYP26B1 have been detected in most human tissues [27]. Since no recombinant enzyme system has been available, the exact metabolic pathway and kinetics of RA metabolism by CYP26A1 are unknown. The goal of this study was to conduct a basic biochemical characterization of CYP26A1, and to establish the main metabolic determinants of RA oxidation by CYP26A1.

2. Materials and methods

2.1. Reagents

RA isomers and Acitretin (I.S.) were purchased from Sigma–Aldrich (St. Louis, MO) and 4-oxo-RA from Toronto Research