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Human cytochrome P450 27C1 catalyzes 3,4-desaturation of retinoids

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In humans, a considerable fraction of the retinoid pool in skin is derived from vitamin A_2 (all-*trans* 3,4-dehydroretinal). Vitamin A_2 may be locally generated by keratinocytes, which can convert vitamin A_1 (all-*trans* retinol) into vitamin A_2 in cell culture. We report that human cytochrome P450 (hP450) 27C1, a previously 'orphan' enzyme, can catalyze this reaction. Purified recombinant hP450 27C1 bound and desaturated all-*trans* retinol, retinal, and retinoic acid, as well as 11-*cis*-retinal. Although the physiological role of 3,4-dehydroretinoids in humans is unclear, we have identified hP450 27C1 as an enzyme capable of efficiently mediating their formation.

Keywords: Cytochrome P450; desaturation; mass spectrometry; retinoids; spectroscopy

Retinoids, including derivatives of vitamin A_1 (all-*trans* retinol), vitamin A_2 (all-*trans* 3,4-dehydroretinol), and vitamin A_3 (all-*trans* 3-hydroxyretinol), have numerous important biological functions [1–3] (Fig. 1A). Retinoic acid, an oxidation product of vitamin A_1, interacts with nuclear receptor transcription factors to regulate gene expression during development and in the adult [4,5]. Derivatives of vitamin A_1 also play an important role in vision in humans because 11-*cis*-retinal is the chromophore of most vertebrate visual pigments [6,7]. However, the vitamin A_2 derivative 3,4-dehydroretinal is also used as a visual chromophore in many cold-blooded vertebrates including lampreys, fish, amphibians, and some reptiles [8,9]. The use of this alternate chromophore is associated with freshwater environments, and many animals that migrate between salt and fresh water dynamically switch from vitamin A_1-based to A_2-based chromophores [8]. Because vitamin A_2 has a longer system of conjugated double bonds than A_1, its light absorption spectrum is red-shifted relative to that of A_1. This shift in sensitivity may enhance vision in murky freshwater environments in which longer wavelength light predominates.

Vitamin A_2 derivatives have also been found in other contexts, including in developing embryos in the form of dehydroretinoic acid [10,11]. Reports on the occurrence and function of vitamin A_2-based retinoids in mammals are more limited. Vahlquist and associates reported that vitamin A_2 is found in humans and

Abbreviations
ADR, Adx reductase; Adx, adrenodoxin; APCI, atmospheric pressure chemical ionization; UPLC, ultraperformance liquid chromatography.

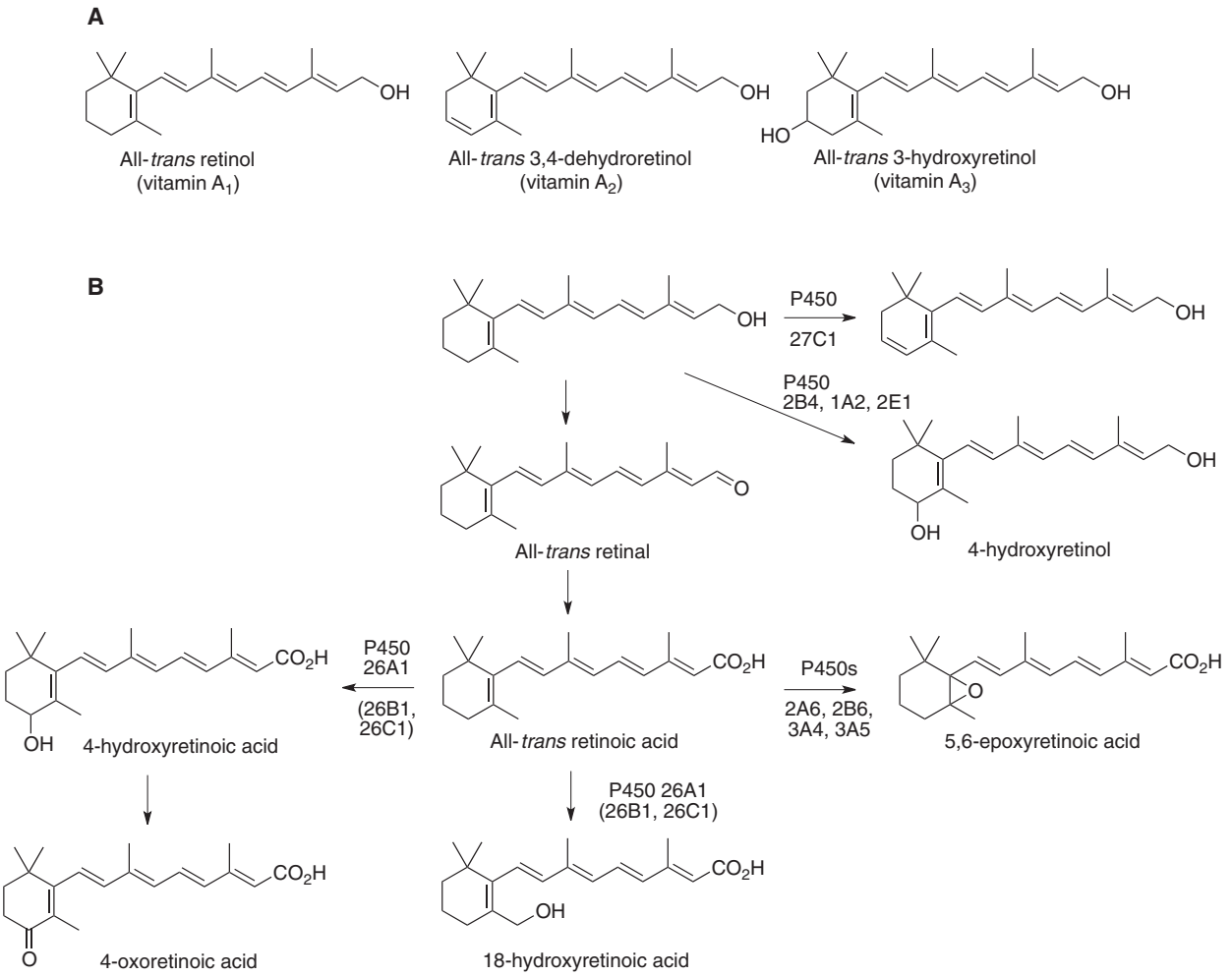

Fig. 1. (A) Forms of vitamin A. (B) Known transformations of vitamin A.

constitutes ~ 25% of the retinoid pool in skin [12–14], with the fraction increasing in psoriasis, atopic dermatitis, and lichen planus [15,16]. However, the function of dehydroretinoids in human skin is currently unknown.

The metabolism of retinoids has been a matter of investigation for some time. Dehydrogenases convert retinol and retinaldehyde to retinoic acid [17]. Most of the studies of oxidation by cytochrome P450 (P450) enzymes have been with retinoic acid (Fig. 1B). Several rabbit P450s can catalyze the 4-hydroxylation of all-*trans* retinol, retinal, and retinoic acid [17]. Human P450s 2B6, 2C8, 3A4, 3A5, and 2A6 can form 4-hydroxy and 4-oxo retinoic acid, P450s 2B6, 2C8, and 2A6 can catalyze 18-hydroxylation, and P450s 2A6, 2B6, 3A4, and 3A5 have been reported to catalyze 5,6-epoxidation [18]. However, the main human liver P450 involved in retinoic acid oxidation is P450 26A1, which forms 4- and 18-hydroxy retinoic acid [19–22]. Human P450s 26B1 and 26C1 appear to carry out similar reactions, but 26B1 has extrahepatic localization and 26C1 is expressed during fetal development [23]. Retinoic acid metabolites also induce P450 26A1 [22]. Thus, a range of human P450s can act upon retinoid substrates.

Desaturation of retinol to 3,4-dehydroretinol (vitamin A_2) (Fig. 1) has been reported in preparations of human breast skin [24] and keratinocytes in culture [25]. More recently UV light exposure was reported to increase the formation of 3,4-dehydroretinol in cultured human keratinocytes [26]. However, the identity of the enzyme that mediates 3,4-desaturation of retinoids in human skin has not been determined. Recently we reported that zebrafish P450 27C1 is an efficient retinol 3,4-desaturase and that this enzyme is expressed in the eye of fish and amphibians where it acts to red-shift the visual chromophore [27]. Although no bird or mammal has been shown to express P450 27C1 in the eye, an intact copy of the gene is present

in the human genome. Here, we report that the human *CYP27C1* gene encodes a retinoid 3,4-desaturase, hP450 27C1, with selectivity for all-*trans* retinol. We hypothesize that this enzyme may catalyze the production of 3,4-dehydroretinoids in human skin.

Materials and methods

Chemicals

All-*trans* 3,4-dehydroretinol, 3,4-dehydroretinaldehyde, and 3,4-dehydroretinoic acid were synthesized as described elsewhere [27]. All-*trans* retinol, retinal, and retinoic acid were purchased from Sigma-Aldrich. 11-*cis*-Retinal and 11-*cis*-3,4-dehydroretinal were purchased from Toronto Research Laboratories. 4-Hydroxy and 4-oxo (all-*trans*) retinol were prepared by mild alkaline hydrolysis of the acetate esters, which were synthesized as described previously [27].

Enzymes

An N-terminal-modified human (h) P450 27C1 protein was expressed from an *Escherichia coli* codon-optimized cDNA as described previously (M3 construct) [28]. The protein was purified as described using Ni^{2+}-nitrilotriacetic acid (NTA) chromatography [28]. Recombinant bovine adrenodoxin (Adx) [29] and NADPH-Adx reductase (ADR) [30] were expressed in *E. coli* and purified as described previously [27]. Recombinant rat NADPH-P450 reductase was expressed in *E. coli* and purified as described earlier [31,32].

K_d determinations

hP450 27C1 (2.0 μM in 50 mM potassium phosphate buffer, pH 7.4) was included in each of two 1.0-mL glass cuvettes, which were balanced against each other in an OLIS-Aminco DW-2 spectrophotometer (On-Line Instrument Systems, Bogart, GA, USA). A baseline was recorded. Aliquots of retinoids, dissolved in ethanol, were added to the sample cuvette, with an equal volume of ethanol added to the reference cuvette after each addition (the maximum concentration of ethanol added was ≤ 2%, v/v). The contents of each cuvette were mixed (following each addition) using a plumper stick (NSG Precision Cells, Farmingdale, NY, USA, catalog no. P68). A difference spectrum was measured following each addition. The data were fit to plots of $\Delta(A_{390}–A_{418})$ (i.e., A_{max} vs. A_{min}) vs. retinoid concentration, using a quadratic equation of the form

$$\Delta A = \Delta A_{max} \frac{(E_T + L_T + K_d) \pm \sqrt{(E_T + L_T + K_d)^2 - 4E_T L_T}}{2E_T},$$

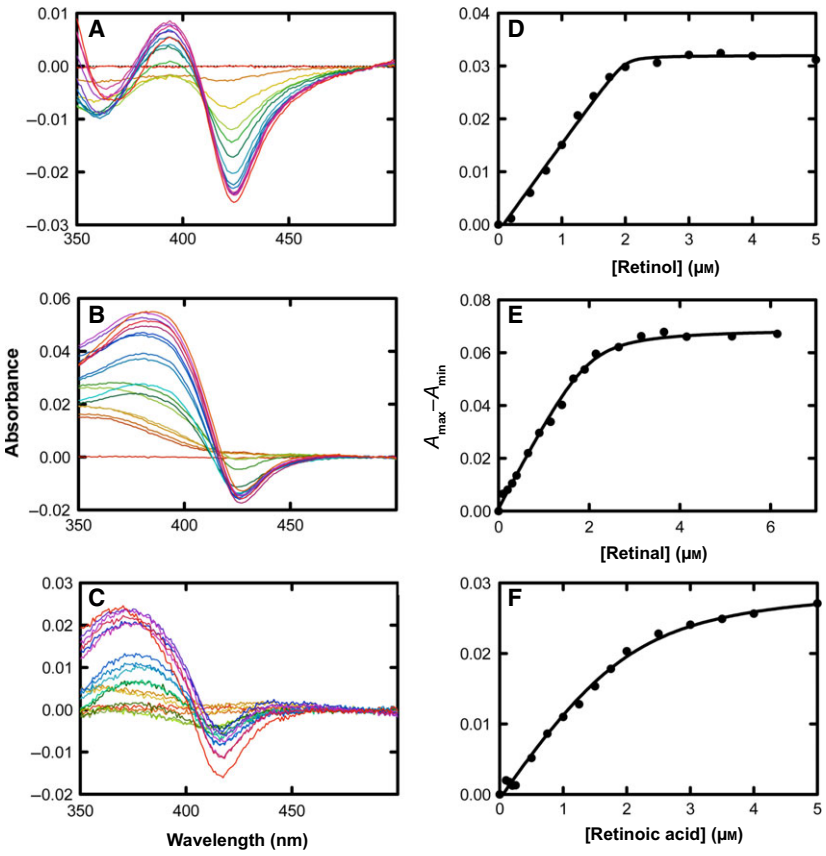

Fig. 2. Binding of all-*trans* retinoids to P450 27C1. Spectral titrations were done as described under Experimental Procedures. Spectral changes are shown parts (A–C). K_d values were estimated using a quadratic expression in GraphPad Prism (see Materials and methods) and are presented ± SE ([P450] = 2.0 μM). No corrections were made for the retinoid absorbance at 390 nm. (A and D) retinol (K_d 0.0056 ± 0.0072 μM); (B and E) retinal (K_d = 0.094 ± 0.026 μM); (C and F) retinoic acid (K_d 0.14 ± 0.08 μM).

sometimes referred to as a Morrison equation, where ΔA_{max} is the extrapolated absorbance difference at infinite ligand concentration, E_T is the total enzyme concentration, L_T is the total ligand concentration, and K_d is the dissociation constant $(Y = B + (A/2)*(1/E)*((K_d + E + X)\sqrt{(K_d + E + X)^2 - (4 * E * X)}))$ in PRISM 5.0 software (GraphPad, La Jolla, CA, USA).

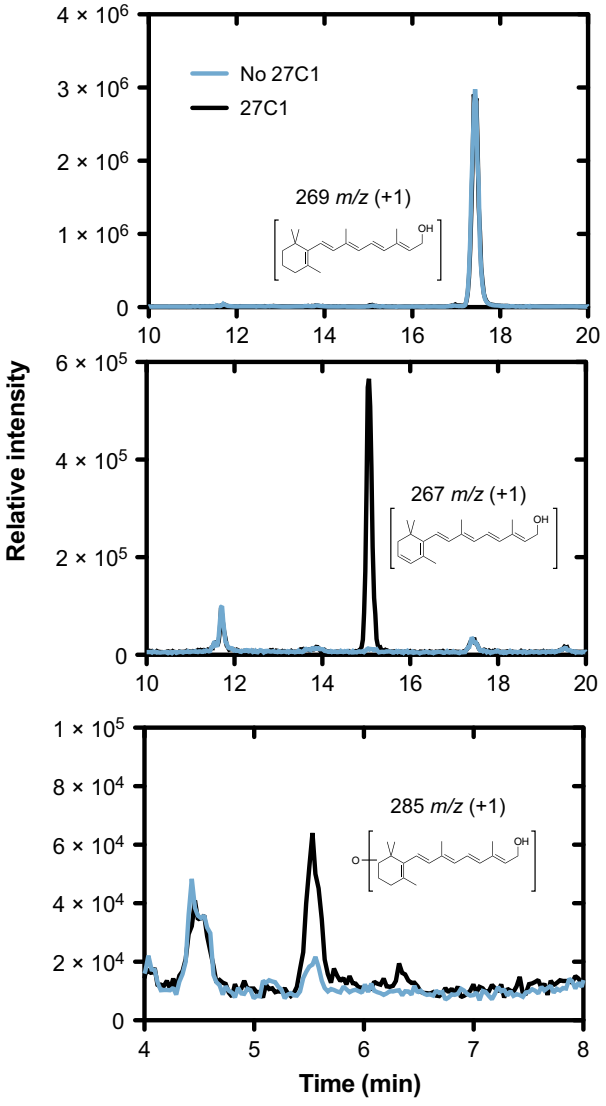

Fig. 3. Desaturation of all-*trans* retinol and 4-hydroxylation by P450 27C1. LC–MS/MS traces of all-*trans* retinol (50 μM) desaturation reactions performed in the presence (black) or absence (blue) of P450 27C1 (0.2 μM). (A) detection of 269 *m/z* (retinol); (B) detection of 267 *m/z* (3,4-dehydroretinol); and (C) detection of 285 *m/z* (oxygenated retinol products). The t_R 5.5-min peak seen in the *m/z* 285 chromatogram (part C) comigrated with standard 4-hydroxyretinol.

Retinoid desaturation assays

Assays for the desaturation of (all-*trans*) retinol, retinaldehyde, and retinoic acid were conducted as described [27]. Unless stated otherwise, typical assay conditions included 0.02–0.05 μM P450 27C1, 5 μM Adx, and 0.2 μM ADR in 50 mM potassium phosphate buffer (pH 7.4). An NADPH-generating system was used, with final concentrations of 10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 1 $IU{\cdot}mL^{-1}$ yeast glucose 6-phosphate dehydrogenase [33]. All reactions and analyses were done in amber glass vials due to the light sensitivity of the retinoids. Extractions were done with ethyl acetate or *tert*-butyl methyl ether, as well as butylated hydroxytoluene (50 $\mu g{\cdot}mL^{-1}$) to prevent degradation of products due to radical damage [17].

Ultraperformance liquid chromatography (UPLC) separations were performed on an Acquity UPLC system (Waters Associates, Milford, MA, USA) coupled with a

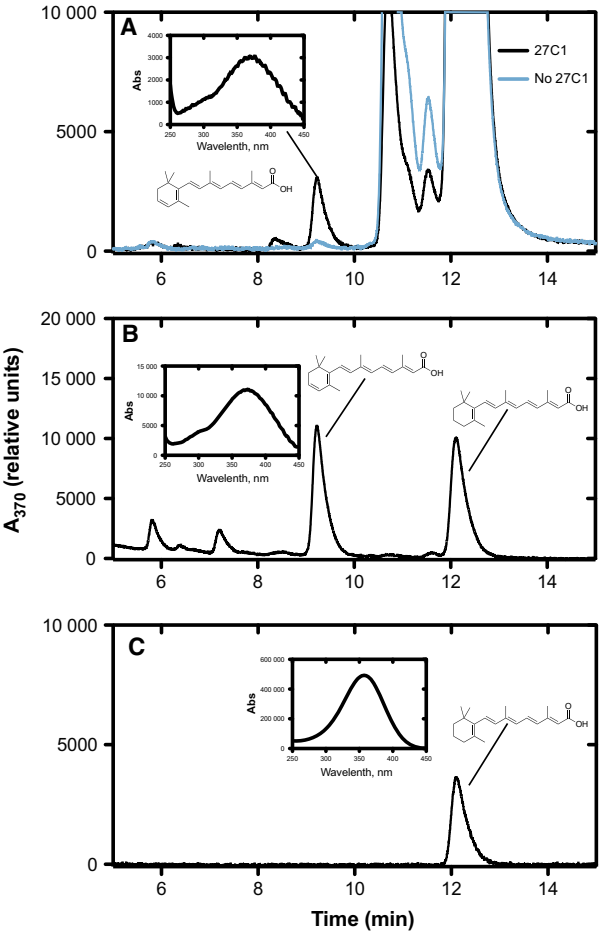

Fig. 4. Desaturation of retinoic acid by P450 27C1. UPLC–UV traces of (A) all-*trans* retinoic acid (50 μM) reaction performed in the presence (black) or absence (blue) of P450 27C1 (0.2 μM); (B) standard mixture of 3,4-dehydroretinoic acid and retinoic acid; (C) standard retinoic acid.

photodiode array (PDA) detector. Samples were separated on an Acquity UPLC BEH octadecylsilane (C_{18}) column (1.7 μm, 2.1 mm × 50 mm) at a flow rate of 0.2 mL·min^{-1} (for retinal) or a Thermo Scientific Hypersil Gold octadecilsilane (C_{18}) column (3 μm, 2.1 mm × 150 mm) at a flow rate of 0.3 mL·min^{-1} (retinoic acid). The column temperature was maintained at 40 °C. Solvent A contained 0.1% HCO_2H in 95% H_2O/4.9% CH_3CN (v/v), and solvent B consisted of 0.1% HCO_2H in 95% CH_3CN/4.9% H_2O (v/v). A gradient program was run from 60% to 70% B (v/v) over 15 min, followed by a column wash at 100% B and re-equilibration back to 60% B (v/v).

The LC–MS/MS analysis was performed on an Acquity UPLC system (Waters Associates) coupled with a Thermo-Finnigan LTQ mass spectrometer (Thermo Scientific, San Jose, CA, USA) with an atmospheric pressure chemical ionization (APCI) source. Samples were separated on an Acquity UPLC BEH octadecylsilane (C18) column (1.7 μm, 2.1 mm × 50 mm) at a flow rate of 0.3 mL·min^{-1}. The column temperature was maintained at 40 °C. Eluent A contained 0.1% HCO_2H in 95% H_2O/4.9% CH_3CN (v/v), and eluent B consisted of 0.1% HCO_2H in 95% CH_3CN/4.9% H_2O (v/v). A gradient program was run as follows: 40–50% B over 10 min, then 60–70% B over 10 min, followed by a column wash at 100% B and re-equilibration back to 40% B (all v/v). MS data were acquired in the positive ion mode and controlled by XCALIBUR 2.1 software (Thermo). Settings were as follows: capillary temperature 275 °C, APCI vaporizer temperature 300 °C, sheath gas flow 50, auxiliary gas flow 5, sweep gas flow 5, source voltage 6 kV, source current 5 μV, capillary voltage 47 V, tube lens voltage 70 V.

Results

Binding of retinoids to hP450 27C1

As reported with zebrafish P450 27C1 [27], we found that the binding of all-*trans* retinol to hP450 27C1 was tight, with a spectrally estimated K_d of 5.6 nM (Fig. 2A, D). All-*trans* retinal and retinoic acid were also tightly bound, with respective K_d values of 0.094 and 0.14 μM (Fig. 2B, C, E, F). Because of the nature of the assays and the enzyme concentrations needed, these low K_d values are considered to contain considerable error, even when quadratic analysis is applied, and may be overestimated. For instance, the titration with retinol shows a sharp inflection point upon saturation of the protein (at 2 μM, Fig. 2D).

Desaturation of retinoids by hP450 27C1

Our initial LC–UV studies demonstrated that all-*trans* retinol was desaturated by hP450 27C1 at the 3,4 position of the β-ionone ring. The results were confirmed by LC–MS analyses (Fig. 3). An oxygenated product was detected in the MH^+ +16

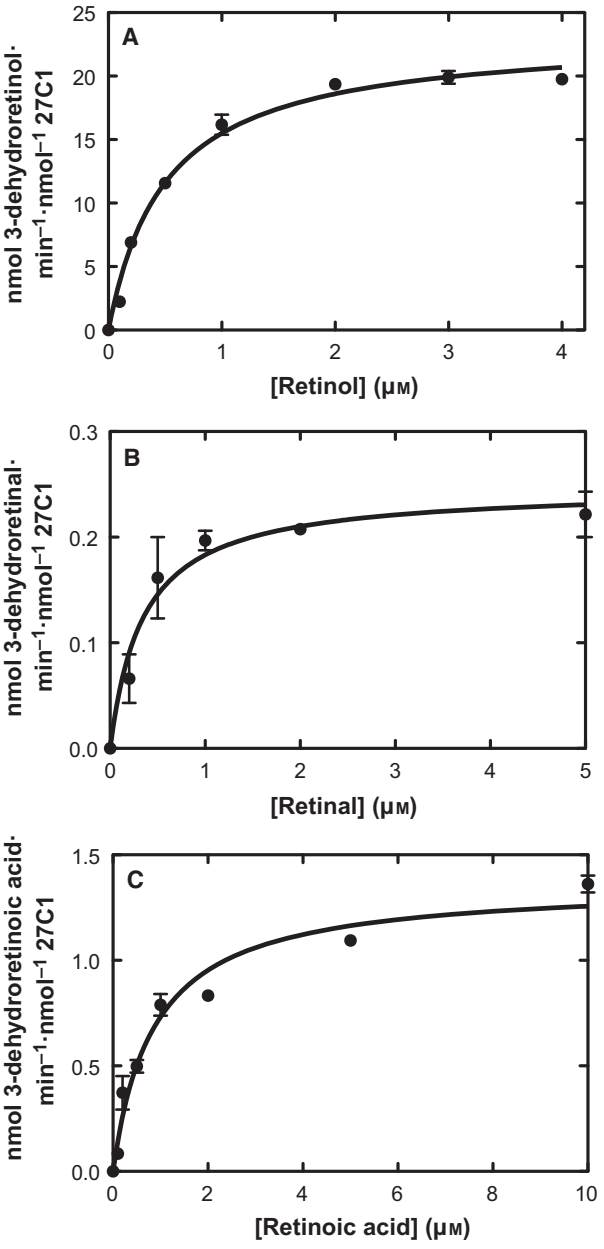

Fig. 5. Steady-state kinetics of (all-*trans*) retinoid desaturation by human P450 27C1. Parameters were estimated using hyperbolic (nonlinear regression) in GraphPad Prism (see Materials and methods). (A) Retinol. k_{cat} 23 ± 1 min^{-1}, K_m 0.50 ± 0.05 μM, k_{cat}/K_m 46 μM·min^{-1} (7.7 (± 0.8) × 10^5 M^{-1}·s^{-1}). (B) Retinaldehyde. k_{cat} 0.25 ± 0.02 min^{-1}, K_m 0.35 ± 0.11 μM, k_{cat}/K_m 0.71 μM^{-1}·min^{-1} (1.1 ± 0.1 × 10^4 M^{-1}·s^{-1}). (C) Retinoic acid. k_{cat} 1.4 ± 0.1 min^{-1}, K_m 0.87 ± 0.15 μM, k_{cat}/K_m 1.6 μM^{-1}·min^{-1} (2.7 ± 0.8 × 10^4 M^{-1}·s^{-1}).

window (*m*/*z* 285, corresponding to addition of one oxygen, that is, hydroxylation). This product comigrated with and had the same UV spectrum as standard 4-hydroxyretinol. Both the desaturation and the 4-hydroxylation activities were dependent upon the addition of the cofactor NADPH and (mitochondrial) ADR and Adx; no desaturation was detected when (microsomal) NADPH-P450 reductase was used instead (data not shown). The yield of the 4-hydroxy product was ~ 10% of the total products, based on the UV spectra and signal responses at wavelength maxima.

The LC–UV analyses were used to obtain steady-state kinetic parameters for the desaturation of all-*trans* retinol, retinal, and retinoic acid (Fig. 4). Retinol was the most efficiently desaturated substrate (Fig. 5A–C), with an estimated catalytic efficiency of 7.7×10^5 $M^{-1} \cdot s^{-1}$ under our conditions.

11-*cis*-retinal was also a substrate for hP450 27C1 and is compared to all-*trans* retinaldehyde in Fig. 6). Two peaks were repeatedly seen in the analyses of the 11-*cis* products (Fig. 6D), with the second eluting component migrating at the position of an authentic commercial standard of 11-*cis* dehydroretinal. The earlier eluting component had very similar UV and mass spectra. On the basis of the literature [3], we believe this to be an isomer of 3,4-dehydroretinal, different from 3,4-dehydro 11-*cis*-retinal.

Discussion

We provide evidence that the 'orphan' hP450 27C1 protein [28] is a retinoid 3,4-desaturase. The catalytic activities are similar to the zebrafish P450 27C1 enzyme, although somewhat less efficient [27]. The low K_m and K_d values with retinoids, along with the overall catalytic efficiency, argue that retinoids are the endogenous substrates. The catalytic efficiency of hP450 27C1 in all-*trans* retinol desaturation (7.7×10^5 $M^{-1} \cdot s^{-1}$ under our conditions, Fig. 5) is 200-fold higher than the best

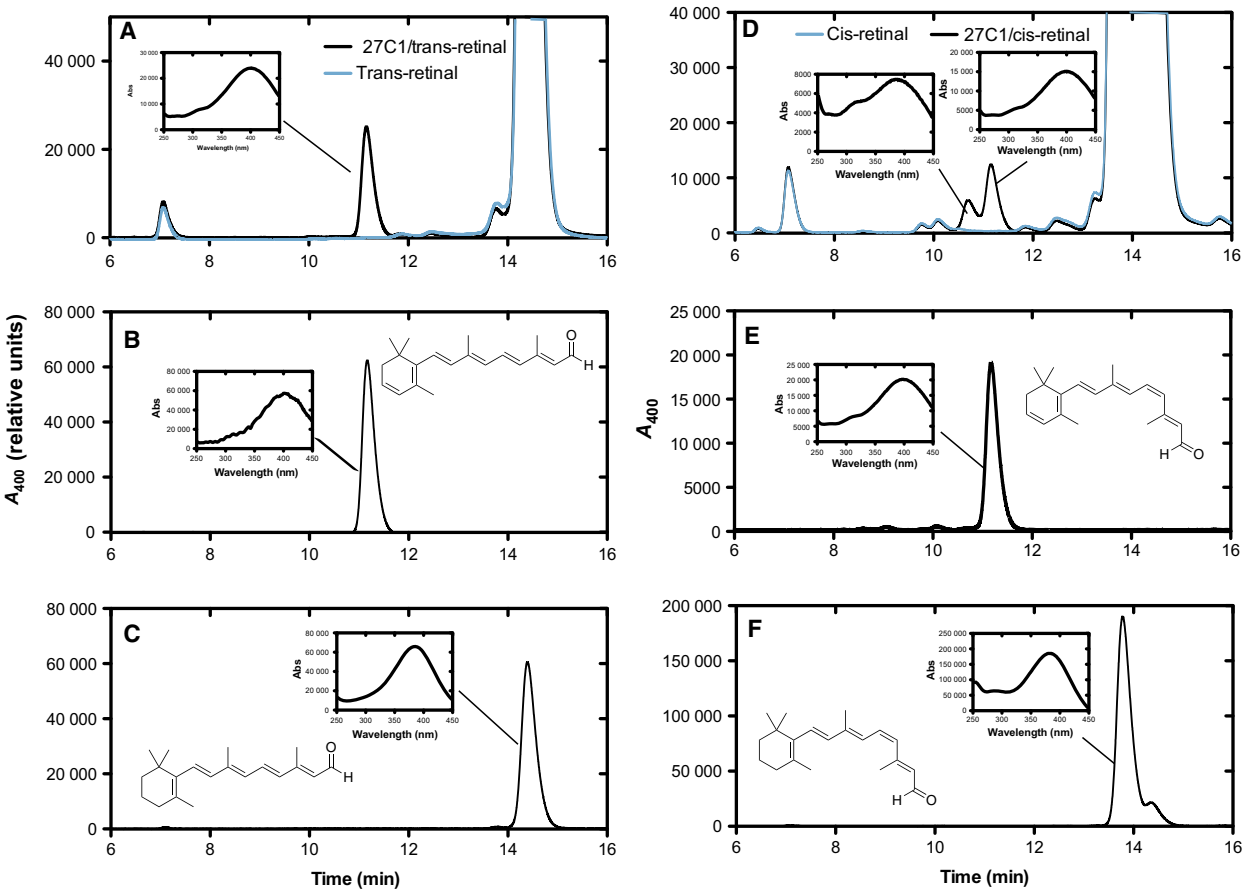

Fig. 6. (A) HPLC of reactions of all-*trans* retinal (50 μM) done with P450 27C1 (black line); (B) standard all-*trans* 3,4-dehydroretinal; (C) standard all-*trans* retinal; (D) HPLC of reactions of 11-*cis*-retinal (50 μM) done with P450 27C1 (black line) and without; (E) standard 11-*cis* 3,4-dehydroretinal; (F) standard 11-*cis*-retinal.

efficiency of a rabbit P450 (2B4) for any retinoid oxidation ($\sim 3 \times 10^3$ $M^{-1} \cdot s^{-1}$) [17]. The activity of hP450 27C1 is surprising in that P450s 27A1 and 27B1, the most closely related proteins, are recognized for their abilities to oxidize unrelated vitamin D compounds (as well as cholesterol in the case of P450 27A1) [23]. In other experiments not presented here, we prepared hP450 27A1 (38% sequence identity) and were able to detect a low rate of desaturation of all-*trans* retinol. The rate was 0.23 nmol dehydroretinol formed $min^{-1} \cdot nmol^{-1}$ P450 27A1 at a substrate concentration of 10 μM, compared with a k_{cat} of 23 nmol dehydroretinol formed $min^{-1} \cdot nmol^{-1}$ for P450 27C1, that is, 100-fold higher, Fig. 5. Thus, some degree of retinoid desaturase activity may be a common feature of this P450 subfamily.

We had reported that zebrafish P450 27C1 was only a desaturase, devoid of other activities [27]. In our preliminary studies with hP450 27C1, we only observed the desaturated products. However, with a shallower HPLC gradient, we could detect all-*trans* retinol 4-hydroxylation activity with the human enzyme, although the extent was only ~ 1/10 of the desaturation. No 4-oxo retinol was detected (based on use of a commercial standard), but we have not extended the search (longer incubation times, etc.). Some P450 desaturases are known in plants [34,35] and yeast [36,37], but in mammals [23,38–40] P450-mediated desaturation reactions have always been minor in comparison to the associated oxygenations (alcohol products) [23,38–40]. The biochemical basis for why P450 27C1 strongly favors desaturation is currently unknown. However, studies with nonheme iron oxygenases have revealed that a few key amino acid residues can control a delicate balance between desaturation and hydroxylation pathways [41,42].

Our previous work using mRNA blots indicated that h*CYP27C1* transcripts are detectable in human liver, kidney, and several other tissues [28]. In contrast, LC–MS assays of liver have failed to demonstrate the presence of the protein (data not shown) and we have been unable to detect retinol desaturation activity in human liver homogenates (data not shown). Further studies on the cellular localization of hP450 27C1 are in progress.

The exact physiological function of retinoid desaturation in mammals is currently unclear. Desaturated retinoids are not thought to be involved in human vision, as the characteristic red-shift associated with the use of the vitamin A_2-derived chromophore [27] has never been reported in man. In contrast, vitamin A_2 (3,4-dehydroretinol) has been reported to constitute about 25% of retinoids in human skin [12–14] and skin cells had been shown to catalyze this reaction [24,25]. It has been reported that ultraviolet light exposure increases the biosynthesis of dehydroretinoids in human keratinocytes in culture [26]. The latter finding raises the intriguing possibility that 3,4-dehydroretinoids might play a role in UV light protection in human skin.

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Author contributions

FPG and JCC conceived the study. FPG and VMK coordinated the study. VMK, LDN, RF and KMJ conducted enzyme incubations and HPLC assays and analyzed the resultant data. RF and LDN measured binding affinities. YX and FPG expressed and purified Adx and ADR. JME and MBT performed preliminary retinoid analyses. FPG analyzed data. FPG and VMK wrote and all authors edited and approved the manuscript.

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