Stereoselective Characteristics and Mechanisms of Epidermal Carboxylesterase Metabolism Observed in HaCaT Keratinocytes

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There is increasing evidence that epidermal carboxylesterase may be involved in the stereoselective hydrolysis of prodrugs in percutaneous absorption. The present study was designed to evaluate the stereoselective characteristics and mechanisms of ketoprofen ethyl ester hydrolysis by epidermal carboxylesterase expressed in HaCaT keratinocytes. Ketoprofen ethyl ester was mainly hydrolyzed to *R*-ketoprofen by carboxylesterase of human HaCaT keratinocytes. Human carboxylesterase-1 (hCE-1) and human carboxylesterase-2 (hCE-2) were intensively detected in L02 hepatocytes, hCE-2 was also intensively detected in HaCaT keratinocytes, but hCE-1 was not detected in HaCaT keratinocytes. hCE-2 is thus an abundant carboxylesterase in HaCaT keratinocytes which may be responsible for stereoselective hydrolysis of ketoprofen ethyl ester.

Key words HaCaT keratinocyte; human carboxylesterase; ketoprofen ethyl ester; stereoselectivity

The advantages of transdermal drug delivery over other routes of administration have been well documented, including avoidance of variable absorption rates and first-pass hepatic metabolism with oral delivery and improved therapeutic activity.[1] In the past, skin penetration studies usually focused only on the physicochemical factors affecting the transport of drugs across the skin. However, recently, many researchers have also been considering the concurrent epidermal metabolism. Various aspects of the metabolism of xenobiotics in the skin have been reviewed,[2] and it has been demonstrated that skin contains various enzymes including esterase capable of hydrolyzing different esters.[3] Ester prodrugs for carboxylic acid- or hydroxyl-containing drugs are widely used for the enhancement of percutaneous absorption and have been noted to show stereoselective hydrolysis in various tissue preparations[4]; surprisingly, the molecular mechanism of stereoselective hydrolysis in skin has been overlooked.

A great deal of the current knowledge on the metabolic clearance of drugs in the skin is based on studies with homogenates of excised tissues, including excised animal skin or human skin. Inherent drawbacks of these models are the doubtful comparability of animal skin *versus* human skin, the restricted access to human skin, and the potentially high variability of enzyme activity of human skin samples along with the often poorly defined cellular viability of excised tissues.[5] It has been proposed that using cultured HaCaT keratinocytes as a readily available and reproducible *in vitro* metabolism model may circumvent some of these disadvantages.[6] The transformed human HaCaT keratinocyte is a well-established cell model of dermal toxicity and metabolism studies *in vitro*, because it is easily cultured and has the characteristics of basal epidermal keratinocytes.[7]

Carboxylesterase is a group of serine esterases found in numerous animal species and a variety of mammalian tissues. These enzymes hydrolyze many different endogenous and xenobiotic compounds and play a role in the metabolism of numerous drugs.[8] Some researchers have reported the purification and partial characterization of two distinct human carboxylesterases designated human carboxylesterase-1 (hCE-1) and human carboxylesterase-2 (hCE-2).[9] These enzymes are expressed in human liver and are both members of the 60-kDalton serine esterase superfamily, but they differ substantially. Sequence homology between the two enzymes is only 48%. hCE-1 is an 180 kDalton trimer with an isoelectric point of 5.8, whereas hCE-2 is a monomer with an isoelectric point of 4.9. Substantial differences in substrate specificity also exist between the two isoforms.[10]

Ketoprofen is a nonsteroidal antiinflammatory drug (NSAID) and is used through oral or suppository routes for the treatment of pain and inflammation. Given orally, gastrointestinal side effects are most frequently seen. Therefore percutaneous administration of ketoprofen has been studied to minimize gastrointestinal and other possible systemic side effects due to high plasma peak levels after oral administration.[11] Ketoprofen has unsuitable physiochemical properties, and thus various strategies to aid skin penetration have been studied. The prodrug approach represents an alternative method of enhancing the skin permeability of ketoprofen.[12] A successful dermal prodrug of ketoprofen should exhibit optimum lipophilicity and should be stable against chemical degradation prior to hydrolysis within the skin by enzymes.[13] Previously, we reported that ketoprofen ethyl ester (KPE) was hydrolyzed to ketoprofen during *in vitro* penetration and this hydrolysis markedly affected the stereoselective cutaneous penetration.[14] Thus, in the present study, the stereoselective hydrolysis of the prodrug in HaCaT keratinocytes was studied to evaluate the hydrolyzing activity of epidermal carboxylesterase, and RT-PCR was used for studying the expression of hCE-1 and hCE-2 with the expression in L02 hepatocytes as a control.

MATERIALS AND METHODS

Materials RPMI Medium 1640, fetal calf serum (FCS), and Defined Keratinocyte-SFM were obtained from Invitrogen Biotechnology Co. (U.S.A.). The tissue/cell Total RNA Isolation Kit was from Shanghai Watson Biotechnology Corporation. The TaKaRa RNA PCR Kit (AMV) was purchased from TaKaRa Biotechnology Co., Ltd. (Japan). Ketoprofen and *S*-ketoprofen were purchased from Chongqing Southwest Pharmaceutical Company. Ketoprofen ethyl ester was kind-

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ly provided by Professor G. R. Fan (Second Military Medical University, Shanghai). 2-Chloro-3,4-dimethoxybenzil (CDMB) was purchased from Sigma Corporation (India). Isopropyl alcohol (HPLC grade) was from Tedia Company (U.S.A.). Other reagents and solvents were of analytical grade.

Cell Culture HaCaT keratinocytes, a nontumorigenic human keratinocyte-derived cell line, were kindly provided by Professors J. Gu and Q. S. Mi of the Department of Dermatology (Changhai Hospital, Shanghai). Cells were seeded at a density of 2×10^4 cells/cm^2 and grown in Defined Keratinocyte-SFM at 95% relative humidity, 5% CO_2, and 37 °C.[15] The medium was replaced three times a week. For passaging, near-confluent cultures were disintegrated with a 0.25% trypsin solution at 37 °C. The resulting cell suspension was split in a 1 : 10 ratio and plated in culture flasks. Subcultures grew to confluence within *ca.* 8 d. To provide a sufficient stock of cultured cells for subsequent experiments, this propagation step was performed several times. The cells were stored frozen under liquid nitrogen.

L02 human hepatocytes were purchased from the Chinese Cell Biology Institute. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin 100 U/ml, and streptomycin 100 μg/ml at 95% relative humidity, 5% CO_2, and 37 °C.[16] The medium was replaced three times a week. For passaging, near-confluent cultures were disintegrated with a 0.25% trypsin solution at 37 °C. The resulting cell suspension was split in a 1 : 10 ratio and plated in culture flasks. Subcultures grew to confluence within *ca.* 7 d.

Metabolism of HaCaT Keratinocyte Homogenates Near-confluent HaCaT keratinocytes were washed twice with PBS without Ca^{2+} and Mg^{2+} and then collected with a cell scraper in ice-cold PBS (pH 7.4). The cells were homogenized on ice with ultrasound. The resulting homogenate suspension was centrifuged at 4 °C and 2000 ***g***, and the pellet of cell debris was discarded. To obtain a homogenate of uniform protein content, several supernatants were pooled. The homogenates were stored frozen under liquid nitrogen and used within 7 weeks after preparation. For the homogenate metabolism studies, aliquots were thawed at room temperature and kept on ice until use within less than 1 h. The protein content of cell homogenates was determined by the method of Lowry,[17] and bovine serum albumin (BSA) was used as a standard at concentrations of 20 to 100 μg/ml.

Hydrolysis experiments were performed at 12.5—200 μmol/l of ketoprofen ethyl ester in HaCaT keratinocyte homogenates with 34.6 μg/l protein contents at 37 °C. Inhibition studies were performed at 100 μmol/l of ketoprofen ethyl ester and different concentrations (10 nmol/l—100 μmol/l) of CDMB in HaCaT keratinocyte homogenates with 34.6 μg/l of protein contents at 37 °C. PBS (pH 7.4) was used as control. The samples were collected for HPLC assay at different time points after incubation at 37 °C.

HPLC Assay of Metabolites Metabolites in the collected samples were acidized (pH 4) with HCl 0.1 mol/l and extracted with diethyl ether. The quantitative determination of metabolites was performed using HPLC (liquid chromatograph with two LC-10AT$_{VP}$ pumps and 20-μl Rheodyne injector, SPD10A detector, and computer integrating system, Shimadzu, Kyoto, Japan). A chiral α-acid glycoprotein column (150 mm×4 mm i.d., 5 μm, Chrom Tech Ltd., Sweden) was used to separate enantiomers of ketoprofen. The mobile phase was composed of isopropyl alcohol : phosphate buffer (400 : 1, v/v).[14] The phosphate buffer was prepared with 0.01 mol/l KH_2PO_4 1000 ml and triethylamine 2 ml, pH 7.0, adjusted with phosphoric acid. A constant flow rate of 0.9 ml/min was maintained. The metabolites were detected by UV absorbance at 258 nm. The chromatographic separations were carried out at ambient temperature. Samples of 20 μl were injected onto the chromatograph.

Table 1. Primer Sequences Used for RT-PCR

Gene	Genebank no.	Code	Primer sequence (5′ to 3′)	Product length/bp
hCE-1	NM_001266	Sense	CAG CCT GCA GAA CCA TGG A	656
		Antisense	CGC AGG CAG TGA ACC ATG A	
hCE-2	NM_003869	Sense	TCA CCG CAG TGG AGT CAG A	527
		Antisense	CAC CAC CGT GGA GAT GAC A	

bp, base pair.

RT-PCR Total RNA was extracted from HaCaT keratinocytes and L02 hepatocytes with the tissue/cell total RNA isolation kit according to the manufacturer's instructions. Crude RNA was purified with isopropanol and repeated ethanol precipitation, and contaminating DNA was destroyed by digestion with RNase-free DNase I (20 min, 25 °C; Invitrogen Biotechnology Co.). Total RNA (1 μg) was used for the synthesis of the first-strand cDNA. The reverse-transcription (RT) reaction was incubated at 42 °C for 2 h, and 99 °C for 5 min, followed by 5 °C for 5 min. The resulting cDNA was used as the template for PCR amplification.

PCR amplification was performed with 2 μl of first-strand cDNA added to a reaction mixture containing 5 μl of 10×Ex Tag buffer (Mg^{2+} free), 3 μl of $MgCl_2$ (2.5 mM), 1 μl of each of two primers, 4 μl of dNTPs (2.5 mM each of dATP, dGTP, dCTP, and dTTP), 0.25 μl of Ex Tag DNA polymerase (5 U/μl, TaKaRa), and nuclease-free water to 50 μl. The specific primers of hCE-1 and hCE-2 were obtained from Invitrogen Biotechnology Co. (see Table 1). RNA was used instead of the cDNA sample for PCR amplifications performed as a negative control. RNA from L02 cells known to express hCE-1 and hCE-2 mRNA abundantly served as the positive control. For each type of cell, PCR was done with GAPDH as the internal control. The primers used were 5′-TGA AGG TCG GTG AAC GGA TTT GGC-3′ (sense) and 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′ (antisense).[18] The reaction mixture was amplified in a thermocycler (Biometra, Germany) for a 2 min denaturation at 94 °C, 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 10 min.

The PCR products were analyzed by electrophoresis in 1% agarose gels and viewed under UV light after ethidium bromide staining. A 2000-bp DNA ladder was run in parallel as a molecular weight marker. Product specification was confirmed by direct DNA sequence analysis.

Statistical Analysis Results are presented as mean± S.D. All analyses were performed with SPSS10.0 software.

RESULTS

HPLC Method Development and Validation For

HPLC analysis, 20 μl of the racemate of ketoprofen solution was injected into the Shimadzu LC-10A system at ambient temperature. The enantiomers of (*R*, *S*)-ketoprofen were baseline chiral separated within 20 min (Fig. 1).

Linearity was evaluated based on the correlations between the peak areas and the concentrations of the analytes. The linearity was acceptable in the range of 2—40 μmol/l for ketoprofen enantiomers. The equation of calibration curves for *S*-ketoprofen was $C=0.119+1.145\times10^{-4}A$ ($r=0.9999$), and that for *R*-ketoprofen was $C=-0.151+1.136\times10^{-4}A$ ($r=0.9999$). Solutions of known concentration (typically 20 μmol/l) of *R*-ketoprofen and *S*-ketoprofen were injected five times, indicating the repeatability of the proposed method. The mean values for precision were 0.50% (RSD) for *R*-ketoprofen and 0.54% (RSD) for *S*-ketoprofen. The accuracy of the method was determined by investigating the recoveries of each enantiomer at three levels, *i.e.*, 60, 100, and 140% of the known concentration, by spiking the sample solution (20 μmol/l) with the standard solutions. The average recoveries of (*R*)- and (*S*)-ketoprofen were found to be 100.07% and 99.94%, respectively.

Metabolism Studies with HaCaT Keratinocyte Homogenates Ketoprofen ethyl ester was stable in PBS (pH 7.4) at 37 °C, but it was mainly hydrolyzed to *R*-ketoprofen in HaCaT keratinocyte homogenates at 37 °C. The concentration of *R*-ketoprofen in the homogenates was linear with incubation time. The effect of ketoprofen ethyl ester concentrations on the metabolite formation rate was measured at substrate concentrations ranging from 12.5 to 200 μmol/l (Table 2). The carboxylesterase metabolism in HaCaT keratinocyte homogenates fit the Michaelis–Menten equation (Fig. 2). The values of K_m and V_{max} of the carboxylesterase in HaCaT keratinocyte homogenates were 1.003×10^{-3} mol/l and 1.025 $\mu mol\cdot h^{-1}\cdot mg^{-1}$ protein, respectively.

2-Chloro-3,4-dimethoxybenzil (CDMB), a specific inhibitor of hCE-2,[19] showed potent inhibition of the metabolism of ketoprofen ethyl ester in HaCaT keratinocyte homogenates. The inhibitory effect of CDMB depended on its concentration in homogenates (Fig. 3), and the amounts of *R*-ketoprofen formed at 100 μmol/l of ketoprofen ethyl ester in HaCaT keratinocyte homogenates decreased with the increase in CDMB concentration. The hydrolysis of ketoprofen ethyl ester was very significantly inhibited if the concentration of CDMB in HaCaT keratinocyte homogenates exceeded 100 μmol/l ($p<0.01$, 100 μmol/l CDMB *vs.* 0 μmol/l CDMB), and thus hydrolysis enzyme of ketoprofen ethyl ester in HaCaT keratinocyte homogenates was hCE-2.

RT-PCR Analysis of hCE-1 and hCE-2 mRNA Expression The presence of hCE-1 and hCE-2 mRNA in HaCaT keratinocytes and L02 hepatocytes was identified with RT-PCR using specific hCE-1 and hCE-2 primer pairs. As a result, a DNA band corresponding to 527 bp was confirmed in HaCaT keratinocytes. In the L02 human hepatocytes, as the positive control, two DNA bands corresponding to 656 bp and 527 bp were confirmed (Fig. 4). For the mRNA of GAPDH, the internal control, a DNA band corresponding to 938 bp was indicated in HaCaT keratinocytes as well as in L02 hepatocytes.

The direct DNA sequencing of the hCE-1 mRNA RT-PCR product from L02 hepatocyte mRNA showed 99% (641/646) homology with the published human hCE-1 mRNA sequence, and the hCE-2 mRNA RT-PCR product showed 100% (527/527) homology with the published human hCE-2 mRNA sequence. The direct DNA sequencing of RT-PCR product from HaCaT keratinocyte mRNA showed 100%

Table 2. Metabolite Formation Rates of Ketoprofen Ethyl Ester in Homogenates

Initial concentration of ketoprofen ethyl ester (μmol/l)	Formation rate of *R*-ketoprofen ($\mu mol\cdot h^{-1}\cdot mg^{-1}$)
12.5	0.013
25.0	0.024
50.0	0.056
100.0	0.095
200.0	0.133

HaCaT keratinocyte homogenates containing 12.5—200 μmol/l ketoprofen ethyl ester were incubated at 37 °C for 4.0 h. Concentrations of metabolite ketoprofen at different time were analyzed by HPLC with an AGP chiral column. The formation rate of *R*-ketoprofen was calculated by linear regression analysis between the *R*-ketoprofen concentrations and the sampling time.

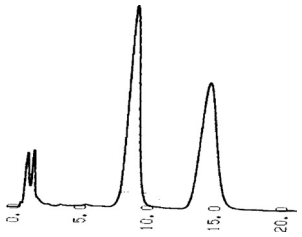

Fig. 1. HPLC Chromatogram of Ketoprofen Enantiomers

The Shimadzu LC-10AD system was used for assaying ketoprofen enantiomers on the chiral AGP column (150 mm×4 mm i.d., 5 μm). The mobile phase was composed of isopropyl alcohol : phosphate buffer (400 : 1, v/v). The UV detection wavelength was 258 nm. The chromatographic separations were carried out at ambient temperature. Peaks: *R*-ketoprofen (t_R=9.11 min), *S*-ketoprofen (t_R=14.75 min).

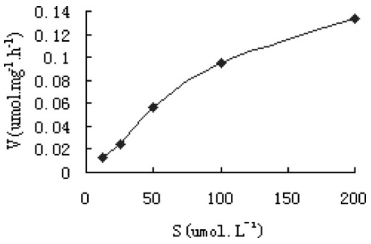

Fig. 2. Michaelis–Menten Plot of Ketoprofen Ethyl Ester Metabolism in HaCaT Keratinocyte Homogenates

V, metabolite formation rate ($\mu mol\cdot h^{-1}\cdot mg^{-1}$ protein); S, initial concentration of ketoprofen ethyl ester ($\mu mol\cdot l^{-1}$).

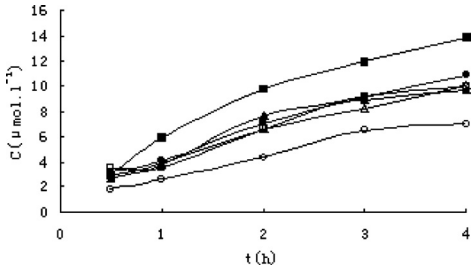

Fig. 3. Concentration of *R*-Ketoprofen in HaCaT Keratinocyte Homo genates at Different Concentrations of CDMB (n=3)

HaCaT keratinocyte homogenates containing ketoprofen ethyl ester 100 μmol/l and CDMB 0.01—100 μmol/l were incubated at 37 °C for 4.0 h. Concentrations of metabolite ketoprofen at different times were analyzed by HPLC with an AGP chiral column. ■, 0 μmol/l; □, 0.01 μmol/l; ▲, 0.1 μmol/l; △, 1 μmol/l; ●, 10 μmol/l; ○, 100 μmol/l.

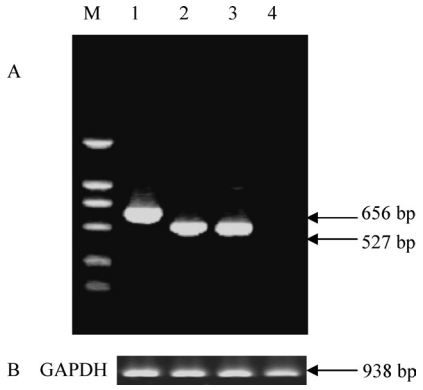

Fig. 4. mRNA Expression of hCE-1, hCE-2, and GAPDH in RT-PCR Analysis

(A) Lane M, DNA marker 2000; lane 1, RT-PCR sample of hCE-1 from L02 hepatocytes as positive control; lane 2, RT-PCR sample of hCE-2 from L02 hepatocytes as positive control; lane 3, RT-PCR sample of hCE-2 from HaCaT keratinocytes; lane 4, RT-PCR sample of hCE-1 from HaCaT keratinocytes; (B) mRNA of GAPDH as the internal control was expressed in L02 hepatocytes and HaCaT keratinocytes.

(524/524) homology with the published human hCE-2 mRNA sequence. From these data, we inferred that the carboxylesterase expressed in HaCaT keratinocytes mainly belonged to hCE-2.

DISCUSSION

Ketoprofen and *S*-ketoprofen were stable in PBS (pH 7.4) and HaCaT keratinocyte homogenates, and had no inversion between the *R*-enantiomer and *S*-enantiomer similar to the published report on human hepatoma cells.[20] Ketoprofen ethyl ester was also stable in PBS, but it was mainly hydrolyzed to *R*-ketoprofen in HaCaT keratinocyte PBS homogenates, and CDMB potently inhibited this hydrolysis process. These data show that carboxylesterase in HaCaT keratinocytes had substrate selectivity, and easily hydrolyzed *R*-ketoprofen ethyl ester (the concentrations of *S*-ketoprofen ethyl ester in HaCaT keratinocytes were constant), and thus HaCaT keratinocyte line was a better model for studying the prodrug metabolism of percutaneous absorption *in vitro*.

The utilization of lipophilic prodrugs is a useful method to enhance the transdermal absorption of therapeutic agents.[21] Dermal enzymes can convert prodrugs into pharmacologically active forms and also inactivate or detoxify xenobiotics. To evaluate the effectiveness of prodrugs on the skin, skin metabolism must be studied.[4] Therefore such study using the HaCaT keratinocyte cell line may be useful for observing the metabolism of prodrugs in percutaneous absorption.

Ketoprofen, a well-known 2-arylpropionic acid NSAID, is a racemic mixture of two enantiomeric forms, *R* and *S* isomers. Although the antiinflammatory role of the two enantiomers is not fully characterized, it is known that *R*-ketoprofen is a weak cyclooxygenase inhibitor, being *ca.* 100 to 1000 times less potent than the *S*-enantiomer *in vitro*, and it is therefore assumed to contribute only marginally to antiinflammatory protection. *R*-ketoprofen accounts for most of the analgesic activity of ketoprofen and is a promising analgesic without the NSAID side effects.[22,23] Ketoprofen ethyl ester was enantioselectively hydrolyzed to *R*-ketoprofen in HaCaT keratinocytes by hCE-2, and thus HaCaT keratinocyte are better carriers for synthesizing chiral drugs by biological methods.

Carboxylesterase is a group of serine esterases found in numerous animal species and a variety of mammalian tissues. These enzymes hydrolyze many different endogenous and xenobiotic compounds and play a role in the metabolism of numerous drugs.[24] Some researchers have reported the molecular characterization and function of two distinct human carboxylesterases designated hCE-1 and hCE-2 in human liver tissue.[25] In L02 human hepatocytes, hCE-1 mRNA and hCE-2 mRNA were significantly expressed, and thus the RT-PCR method reported in this paper is reliable and may be used in studying the expression of carboxyesterase in other tissues. In HaCaT keratinocytes, hCE-1 mRNA was not expressed or only very weakly, and the expression level of hCE-2 mRNA was significantly, similar to that of hCE-2 mRNA in L02 hepatocytes, and therefore the enantioselective hydrolysis of the chiral ester prodrug in the HaCaT keratinocyte line was mainly due to hCE-2.

In summary, hCE-2 is an abundant carboxylesterase in HaCaT keratinocytes which may be responsible for stereoselective hydrolysis of ketoprofen ethyl ester. This pilot study reinforces the methods of improving percutaneous absorption by prodrugs.

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