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Expression of Carboxylesterase Isozymes and Their Role in the Behavior of a Fexofenadine Prodrug in Rat Skin

Teruko Imai [1,*], Satomi Ariyoshi [1], Kayoko Ohura [1], Takashi Sawada [1], Yuichiro Nakada [2]

[1] Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan
[2] Santen Pharmaceutical Company, Ltd., Osaka, Japan

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ABSTRACT

The expression of carboxylesterase (CES) and the transdermal movement of an ester prodrug were studied in rat skin. Ethyl-fexofenadine (ethyl-FXD) was used as a model lipophilic prodrug that is slowly hydrolyzed to its parent drug, FXD (MW 502). Among the CES1 and CES2 isozymes, Hydrolase A is predominant in rat skin and this enzyme was involved in 65% of the cutaneous hydrolysis of ethyl-FXD. The similarity of the permeation behavior of ethyl-FXD in full thickness and stripped skin indicated that the stratum corneum was not a barrier to penetration. However, only FXD was observed in receptor fluid, not ethyl-FXD, presumably because of the high degree of binding of ethyl-FXD in viable skin. The rate of hydrolysis of ethyl-FXD was much faster than steady-state flux, such that the influx rate was the rate-limiting process for transdermal permeation. Although Hydrolase A levels gradually increased in skin taken from rats aged from 8 to 90 weeks, variations in the expression levels of the esterase hardly affected the conversion of prodrug. The present data suggest that the slow hydrolysis of the prodrug of an active ingredient in viable skin followed by slow diffusion of active drug may provide a useful approach to topical application.

Introduction

Skin plays an important role in the body as a permeability barrier to the surrounding environment, particularly the uppermost layer of the epidermis, the stratum corneum. In the development of a topical pharmaceutical preparation, the partition of drug into the intercellular lipid phase in the stratum corneum is the first critical step in skin permeation.[1] Ester formation is a useful approach to achieving sufficient penetration of a drug through the skin, as esters have good membrane permeability because of their lipophilicity. Ester compounds are used in two different ways, as prodrugs or antedrugs. Ester prodrugs are modified active ingredients with less pharmacological effect.[2] In contrast, antedrugs are pharmacologically active and are converted to inactive or less active metabolites in the systemic circulation, thus avoiding systemic adverse effects.[3] Most steroidal drugs used clinically as topical preparations are antesteroids, which are rapidly inactivated by enzymes in plasma and liver.[4,5] There are fewer commercially available ester-type prodrugs for topical application, due primarily to the lack of information on dermal metabolism and difficulties in controlling both the permeation rate and the rate of conversion of the prodrug.

In most studies on esterase-mediated xenobiotic metabolism in human skin,[6] the molecular nature of the responsible esterase has not been ascertained. Human esterases are highly active and able to hydrolyze substances extensively during permeation through *in vitro* human skin, for example, 3-alkyl esters of naltrexone[7] and ester derivatives of fluroxypyr.[8] Previously, we have shown that rat skin also shows high esterase activity, and that the major esterase is carboxylesterase (CES: EC.3.1.1.1).[9] In that study, caproyl propranolol was extensively hydrolyzed by CES during its permeation through *in vitro* rat skin.

Carboxylesterase is the most abundant esterase in mammals, present in a variety of organs including skin,[10] and is involved in the hydrolysis of most clinically used prodrugs. Mammalian CESs are grouped into five families on the basis of homology with human CES1 enzyme, hCE1 (CES1A1).[11] Enzymes in the CES1 and CES2 families are the main esterases involved in the hydrolysis of prodrugs and xenobiotics, and they show different substrate specificities.[12,13]

Abbreviations used: BChE, butyrylcholinesterase; BNPP, bis-*p*-nitrophenyl phosphate; CES, carboxylesterase; FXD, fexofenadine; hCE1, human carboxylesterase 1; hCE2, human carboxylesterase 2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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* *Correspondence to:* Teruko Imai (Telephone: +81-96-371-4626; Fax: +81-96-371-4639).
E-mail address: iteruko@gpo.kumamoto-u.ac.jp (T. Imai).

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It has been reported that human skin expresses hCE1 as its major hydrolase and to a lesser extent the CES2 isozyme, hCE2.[14] We have shown that rat skin possesses several CES1 isozymes including Hydrolase A, which is 77% homologous to hCE1, as its major esterase.[9] However, details of the expression of CES1 and CES2 isozymes in rat skin have not been clarified and variations of expression with aging also remain unclear, in spite of their importance in the approach to prodrugs.

In general, the activity of cutaneous CES isozymes is much lower than their hepatic counterparts. However, lipophilic prodrugs are extensively metabolized in the skin due to their retention in viable epidermis and dermis.[8] The conversion of lipophilic esters into their hydrophilic metabolites allows rapid permeation through the viable skin under the stratum corneum. Caproyl propranolol is such a prodrug, with a high penetration into the skin, where extensive hydrolysis allows rapid permeation of the parent drug into viable skin. However, there are difficulties in using prodrugs for topical application as, to be suitable, a prodrug must offer not only good skin penetration, but also sustained release of active drug by slow hydrolysis, followed by slow diffusion of active drug through viable skin. As diffusivity in skin strongly correlates with the size of the permeant,[15] compounds with relatively large molecular weight may be suitable for topical application by means of a prodrug.

In this study, firstly we confirmed the expression levels of CES1 and CES2 isozymes in rat skin and evaluated the effect of aging on these expression levels. Then, we studied a possible example of the prodrug approach for topical application using a lipophilic prodrug. Fexofenadine (FXD), a non-sedating antihistamine, was selected as a candidate drug. It has molecular weight of 502, indicating a likelihood of slow diffusion in skin. To improve the partition of FXD into the stratum corneum, ethyl-FXD, in which the carboxyl group of FXD is substituted by an ethyl group, was selected as a prodrug. We have previously reported that ethyl-FXD has only 0.12 of the hepatic extraction ratio because of slow hydrolysis in rat liver where Hydrolase A is abundant.[16] Gradual hydrolysis was therefore also expected in rat skin. The permeation and hydrolysis behavior of ethyl-FXD in rat skin was studied using *in vitro* full-thickness and tape-stripped skin. Finally, we discuss the validity of the prodrug approach for topical applications.

Materials and Methods

Materials

FXD and ethyl-FXD were synthesized according to the procedure reported by Giacomo et al.,[17] and their identity and purity were confirmed by infrared spectroscopy, nuclear magnetic resonance spectroscopy, atomic analysis, and HPLC. Bis-(*p*-nitrophenyl) phosphate (BNPP) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Ethopropazine and bovine serum albumin (BSA; fraction V) were purchased from Sigma-Aldrich Company (St. Louis, MO). All other chemical reagents were of analytical grade.

Animals and Tissue

Male Wistar rats (8 weeks) were purchased from Kyudo Company, Ltd. (Saga, Japan). They were housed in an air-conditioned room with free access to commercial chow and tap water. Rats were anaesthetized with ether, and their abdominal hair was carefully shaved. Twelve hours later, rats were fasted for 12 h before being sacrificed by exsanguination from the external jugular vein under ether anaesthesia. Abdominal skin was removed using a scalpel and dissection scissors. All animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University.

RNA Extraction and Quantification of mRNA Expression

Total RNA was isolated from rat skin using TRIzol reagent (Life Technologies Japan Ltd., Tokyo, Japan). First-strand cDNA was synthesized using Oligo(dT) primer with ReverTra Ace (Toyobo, Osaka, Japan). Real-time quantitative polymerase chain reaction (PCR) was performed using Bio-Rad iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) with SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) and the specific primers reported previously.[18] Thermal cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s/55°C for 30 s. Reactions were carried out in triplicate. Relative mRNA levels were calculated using the $2^{-\Delta Ct}$ method. β-Actin was used as a reference gene.

Preparation of Skin Homogenates and 9000g Supernatant (S9) Fractions

The abdominal skin was minced, mixed with seven volumes of ice-cold 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.15 M KCl, and homogenized using Polytron (Kinematica, Lucerne, Switzerland) on ice. Whole homogenate was filtered with a funnel through buffer-soaked cotton. After centrifugation of the skin homogenate at 9000*g* for 20 min at 4°C, the supernatant (S9) was obtained. Protein content was determined by the method described by Bradford with BSA used as standard.[19] These preparations were stored at −80°C until use.

HPLC Analysis

FXD and ethyl-FXD were analyzed by Jasco HPLC system (Jasco International Company, Ltd., Tokyo, Japan) consisting of a pump (PU-980), UV detector (875-UV, 218 nm), autosampler (AS-2055Plus), column oven (860-C0), and a data processor (Shimadzu Chromatopac CR-7A, Shimadzu Company, Kyoto, Japan). An aliquot of the sample was injected onto a Inertsil ODS column (2.5 μm, 4.6 × 150 mm; GL Sciences, Tokyo, Japan) and eluted at a flow rate of 1.0 mL/min with a mobile phase of acetonitrile/methanol/12 mM ammonium acetate buffer (pH 4.0) 30:10:60. The temperature of the column was maintained at 40°C and the injection volume was 100 μL.

Hydrolysis Experiments

Skin S9 (200 μL) diluted with 50 mM HEPES (pH 7.4) buffer at 200 μg/mL was preincubated for 5 min at 37°C. The hydrolysis reaction was initiated by the addition of 5-40 μM of ethyl-FXD dissolved in dimethyl sulfoxide (DMSO). Reaction (1 h) was terminated by addition of 400 μL of ice-cold acetonitrile/methanol (3:1). After centrifugation, supernatant was analyzed by HPLC. In the inhibition experiments, BNPP, a specific inhibitor of CES, and ethopropazine, a specific inhibitor of butyrylcholinesterase (BChE), were preincubated for 5 min before addition of ethyl-FXD. The final concentration of DMSO was less than 0.5%, which has no effect on hydrolytic activity.

Protein Binding of Ethyl-FXD and FXD in Skin Homogenate

The homogenates of stripped skin were preincubated with diisopropyl fluorophosphate (final concentration 1 mM) at 37°C for 10 min, to inhibit hydrolysis of ethyl-FXD. Then FXD or ethyl-FXD dissolved in DMSO was added at a final concentration of 50-500 μM. After incubation for 30 min at 37°C, the homogenates were filtered through cheesecloth and centrifuged at 9000*g* for 20