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Molecular mechanisms of action of different concentrations of ethanol in water on ordered structures of intercellular lipids and soft keratin in the stratum corneum

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ABSTRACT

Ethanol (EtOH) is one of the bases in topically applied medicines that promote the skin permeation of drugs. Although the effects of EtOH have been attributed to structural modifications in the stratum corneum, the underlying mechanisms, especially the influence of different concentrations of EtOH, have not been examined extensively. Structural modifications in the stratum corneum of hairless mouse due to the application of EtOH/water mixture were herein investigated at the molecular level using synchrotron X-ray diffraction. The results revealed that all EtOH concentrations examined greatly modified the short lamellar structures containing the aqueous layer in intercellular lipids and the structure of keratin fibrils in corneocytes, which can take up hydrophilic compounds. However, the long lamellar and the hydrocarbon-chain packing structures were unaffected by EtOH. Changes to the short lamellar structures were not proportional to the concentration of EtOH. However, the keratin fibril structures changed gradually with increasing EtOH concentration. The X-ray diffraction experiments enabled the effects of different EtOH concentrations on the morphology of the stratum corneum to be assessed by using a number of experimental samples to avoid variations due to individual differences. The results indicated that alterations to the short lamellar structures appeared to be related to the skin permeability of drugs with the application of EtOH/water mixture, and monotonous structural changes in the keratin fibrils with an increase in EtOH concentration may contribute to this permeation as supplement. These results will be useful for the development of new drug formulations containing EtOH.

1. Introduction

Ethanol (EtOH) is widely used as a skin penetration enhancer as well as a skin disinfectant and tonic. In order for drugs to be effective at their target sites, the active ingredients in topical formulations must penetrate the skin barrier, which tightly regulates the entry of external substances. This skin barrier is mainly comprised of the stratum corneum, the outermost layer of skin, which markedly restricts the penetration of drugs. EtOH has been shown to enhance the skin permeation of drugs in topical formulations markedly. Estradiol and fentanyl dermal patches are typical examples of formulations that contain EtOH in order to enhance their absorption into the skin [1–3]. The enhancements reported in the skin permeation of drugs with the application of topical formulations containing EtOH have been attributed to structural modifications in the stratum corneum. The various mechanisms underlying the penetration-enhancing effects of EtOH on the stratum corneum barrier include lipid extraction, increase in lipid fluidity, enhancement of drug solubility in the lipids of the stratum corneum, change in skin hydration, an altered putative pore pathway, alteration in keratinized proteins, and its effects on solvent drugs [4,5]. However, the effects of EtOH on the structures of soft keratin and intercellular lipids in the stratum corneum have not been extensively examined at the molecular level.

In the present study, we thus utilized synchrotron X-ray diffraction to examine the structure of the stratum corneum at the molecular level as well as the effects of EtOH/water mixture on the structures of intercellular lipids and fibrils in the soft keratin. We previously demonstrated that EtOH/water mixture affected the in vitro permeability of drugs through pig skin in an EtOH concentration–dependent manner [6]. The skin permeability of hydrophilic drugs was increased but conversely decreased by low and high concentrations of EtOH, respectively,

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suggesting that the effects of EtOH/water mixture on the structure of the stratum corneum may depend on its volume ratio. In the present study, we measured X-ray diffraction in the stratum corneum of mouse skin as a function of EtOH concentration. Previous studies have already used X-ray diffraction in hairless mouse skin [7,8], hairless rat skin [9], and pig skin [10,11]. Although there are slight differences in the constituents of intercellular lipids among mammalian species, the lamellae have almost the same periodical structure among the mammals. As for the skin permeation of chemical compounds, the skin of rodents, such as rat and mouse, has higher permeability than pig or human skin, whereas the structures of the stratum corneum are similar among these species. Among these mammals, the X-ray diffraction profiles of the stratum corneum in hairless mice showed the most distinct diffraction peaks, and represented the typical characteristics of the stratum corneum in mammals. Therefore, we employed hairless mouse skin in the present study to detect minute changes in the structure of the stratum corneum upon treatment with EtOH/water mixture. The diffraction profile of the stratum corneum has also been investigated in humans [12,13], and has been found to have similar characteristics to that in hairless mice.

In this study, we investigated the molecular mechanisms of action of various volume ratios in EtOH/water mixture on intercellular lipids and soft keratin based on a structural analysis using synchrotron X-ray diffraction experiments in stratum corneum treated with the mixture.

2. Materials and methods

2.1. Materials

EtOH (99.5%, HPLC grade), sodium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Trypsin and trypsin inhibitor were obtained from Sigma-Aldrich Co., Ltd. (St. Louis, MO, U.S.A.). All other reagents and solvents were of reagent grade or HPLC grade, and used without further purification.

2.2. Animals

Male hairless mice (HR-1, 8–9 weeks old) were obtained from Saitama Experimental Animals Supply Co., Ltd. (Sugito, Saitama, Japan). All animal studies were conducted according to the recommendations of the Institutional Board for Animal Studies, Josai University (Sakado, Saitama, Japan).

2.3. Sample preparation process

Hairless mouse skin was separated from the abdominal and dorsal region. After the removal of excess fat, the skin was soaked with 0.1% (w/v) trypsin in pH 7.4 phosphate-buffered saline (PBS) at 4 °C for 16 h. After being incubated for another 4 h at 37 °C, the stratum corneum was separated from the skin. It was then treated with 0.1% (w/v) trypsin inhibitor and rinsed in distilled water three times. Following this, EtOH/water mixture of 0/100, 20/80, 40/60, 60/40, 80/20 or 100/0 v/v% was applied to the dried stratum corneum for 2 h. By reducing the content of EtOH/water mixture from stratum corneum treated by the mixture the EtOH/water mixture content in the stratum corneum was adjusted to be 20 wt%. An approximately 5-mg piece of the sample was placed into a capillary glass tube and immediately sealed for the X-ray diffraction experiment. We used 25 mice in the experiment and obtained four samples per mouse. In addition, 13–14 samples were used for each experiment; the samples treated by EtOH/water mixture were randomly selected in order to avoid excessive use of a specific region or specific individual.

2.4. X-ray diffraction study

The X-ray diffraction study was performed at the beamline BL40B2 (Structural Biology II Beamline) of SPring-8 (Harima, Hyogo, Japan). X-ray diffraction profiles were recorded using an imaging plate system (R-AXIS IV; Rigaku Corporation, Tokyo, Japan) with a 30 cm × 30 cm area. The X-ray wavelength was 0.0709 nm and sample-to-detector distance was approximately 500 mm. Reciprocal spacing [$S = (2 / \lambda) \times \sin\theta$] was calibrated from the lattice spacing ($d = 5.838$ nm, where d is the lamellar repeat distance) of silver behenate at room temperature, where 2θ is the scattering angle. The exposure time was 15 s. The diffraction pattern was circularly averaged in order to obtain the radial intensity profile. The profile was obtained from samples with almost the same weight of stratum corneum. Photon counting for the profile was performed at 1152 pixels in the range of $S =$ 0.05–3.0 nm^{-1}, and we counted the photon number at each pixel.

3. Results

3.1. Effects of various volume ratios in EtOH/water mixture on the ordered structure of intercellular lipids

Fig. 1 shows the X-ray diffraction profiles ($S = 0.05$–3.0 nm^{-1}) of the stratum corneum of hairless mice following treatments with various volume ratios in EtOH/water mixture. We determined the X-ray diffraction pattern from approximately one dozen stratum corneum samples with almost the same volume after the application of EtOH/water mixture at each volume ratio. Diffraction peaks were due to the repeat distances of the lamellar structures in the small-angle region as well as the lattice constants of the lateral hydrocarbon-chain packing structures in the wide-angle region. Fig. 2a shows the X-ray diffraction profiles in the small-angle region within the range of $S = 0.10$–0.40 nm^{-1}. Three partially overlapping peaks derived from the short and long lamellar structures were detected within the range of $S = 0.12$–0.25 nm^{-1}, and these profiles changed according to the volume ratio. In addition, the single diffraction peaks derived from the long and short lamellar structures were observed near $S = 0.30$ and $S = 0.37$, respectively. Fig. 2b shows the X-ray diffraction profiles in the wide-angle region within the range of $S = 2.0$–3.0 nm^{-1}. Two diffraction peaks near $S = 2.4$ nm^{-1} and $S = 2.7$ nm^{-1} corresponded to the hexagonal and orthorhombic hydrocarbon-chain packing structures and only the orthorhombic hydrocarbon-chain packing structures, respectively. In this experiment, the stratum corneum sample from hairless mice showed split peaks for the orthorhombic hydrocarbon-chain packing structures near $S = 2.7$ nm^{-1}.

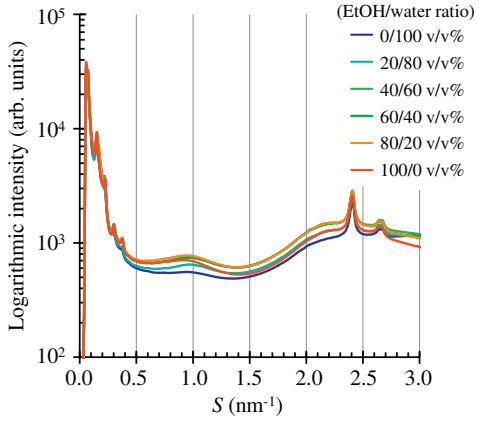

Fig. 1. X-ray diffraction profiles in the hairless mouse stratum corneum as a function of the volume ratios in EtOH/water mixture, where n is the number of stratum corneum samples.