Use of Electroporation to Accelerate the Skin Permeability Enhancing Action of Oleic Acid

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Rat skin permeability after treatment by electroporation (newly developed frog type electrode, 100 V, 10 pulses), oleic acid/propylene glycol (PG) and a combination of both were investigated using Fourier transformed infrared attenuated total reflectance (FT-IR/ATR) analysis. Electroporation immediately disordered the stratum corneum lipid structure up to a certain threshold level. This action lasted throughout the experiment. This may be attributed to the formation of long lifetime of metastable lipid structures, which may allow molecules to pass to the inside of the stratum corneum due to the electroporation-induced fluidized lipid membranes. Electroporation also altered the protein structure of the stratum corneum. When electroporation was combined with 0.05 M oleic acid/PG, uptake of oleic acid and PG into the stratum corneum was remarkably accelerated compared to the application of only 0.05 M oleic acid/PG to the skin. This indicates that electroporation enables oleic acid and PG to penetrate the stratum corneum easily by disrupting the structure of the latter. PG transfer into the dermis from the epidermis was accelerated, not because of the direct action of electroporation on the dermis, but because electroporation induced the rapidly disordering action of oleic acid on the stratum corneum. Lipid-soluble indomethacin permeated the skin more rapidly when the skin was treated with electroporation plus oleic acid/PG than with 0.05 M oleic acid/PG *in vitro*.

Key words electroporation; oleic acid; co-application; skin structural alteration; Fourier transform/attenuated total reflection spectroscopy

Electroporation enhances drug transport through the skin by reducing the barrier function of the stratum corneum. Drug transport is enhanced by electroporation through the creation of a new aqueous pathway across the lipid bi-layer of the stratum corneum.^{1–5)} Water-soluble and low molecular compounds, peptides, water-soluble macro-molecules, neutral compounds and a lipid-soluble drug have been targeted in studies of enhanced skin penetration using electroporation.^{6–14)} However, the mechanism of action by which electroporation enhances skin permeability has not been fully elucidated, especially in relation to the effects upon skin structure. In this study, we examined the mechanism of action by investigating structural changes of the skin after electroporation using Fourier transformed infrared/attenuated total reflectance (FT-IR/ATR) spectroscopy. We also examined the skin permeability enhancing action of electroporation in the presence of oleic acid (skin penetration enhancer) dissolved in propylene glycol (PG) (electroporation plus 0.05 M oleic acid/PG).

MATERIALS AND METHOD

Materials High purity oleic acid (purity > 99.9%) was supplied by the NOF corporation, Amagasaki, Japan. PG was purchased from Nakalai Tesque, Kyoto, Japan. Indomethacin (IM) was supplied by Sumitomo Pharmaceuticals, Osaka, Japan. All other chemicals were of reagent grade.

FT-IR/ATR Spectroscopic Measurement of Skin Samples Procedures were as described in a previous report¹⁵⁾ with modification. Abdominal skin of male Wistar rats (8–9 weeks old) was mounted between two compartments of diffusion cells (Franz cells) with the dermal side facing the receiver compartment. Three hundred microliters of phosphate

buffer solution (PB) was placed in the donor compartment and the frog type electrode shown in Fig. 1 was attached to the skin surface. Electrical pulses were then delivered to the skin as described below in detail. When either PG or oleic acid/PG was combined with electroporation, PB was displaced by 1 g of PG with or without oleic acid (0.05 M) immediately after electroporation, and the compartment was sealed from the atmosphere using Parafilm®. The receiver compartment was filled with 14.2 ml PBS, pH 7.4. The diffusion cells were immersed in a water bath (37 °C) and the buffered solution was stirred with a magnetic stirrer. The skin samples were removed from the cells at various intervals after incubation. The surface of the stratum corneum was gently wiped with Kimwipes®, and then allowed to dry at ambient temperature (15 min) for FT-IR/ATR assay.¹⁵⁾

Uptake of oleic acid and PG into the skin was determined as described.^{16,17)}

Structural changes of the stratum corneum lipid were determined by measuring the frequency shift of the CH₂ anti-symmetric stretching peak near 2920 cm⁻¹ resulting from the methylene groups of the stratum corneum lipid hydrocarbon chains as reported by Golden *et al.*^{18,19)}

Formation of a β -pleated-sheet in the stratum corneum protein was determined by measuring the increase of absorption at 1519 cm⁻¹ as reported by Ortel *et al.*²⁰⁾

Structural changes in dermal protein were determined by measuring the amide II spectral band near 1550 cm⁻¹ as described.²¹⁾ Absorption intensity was expressed as the ratio of peak intensity at the time of treatment to that at time 0.

In Vitro Permeation Study of Indomethacin (IM) The same procedure was adopted for the diffusion cell experiment as described above. In brief, skin was electroporated or not under the same conditions used for FT-IR/ATR analysis,

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Fig. 1. Frog Type Needle Point Holder Electrode Used for Skin Electroporation

then one gram of PG containing 1% (w/v) IM with or without 0.05 M oleic acid was placed into the donor compartment. Aliquots of 400 μ l were withdrawn from the receiver compartment at various intervals. Immediately after sampling, 400 μ l of PB was added to the receiver compartment to maintain constant volume. Indomethacin was analyzed by the modified Levison method using HPLC.²²⁾

Electroporation The electroporation device was an ELECTRO SQUARE PORATOR T820 (BTX, San Diego, CA, U.S.A.) delivering square wave pulses with a newly developed frog type electrode (Fig. 1). The voltage, time constant, number of pulses and time interval between the pulses were set at 100 V, 1 ms, 10 pulses and 1 s because preliminary experiments revealed enhancement of transdermal penetration of oleic acid into the stratum corneum under these conditions.

Data Analysis Results are expressed as means $\pm$ standard error (S.E., $n=3-8$). Statistical differences of the data were analyzed using Tukey's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Effect of Electroporation on the Uptake of Oleic Acid and PG into the Stratum Corneum Electroporation plus 0.05 M oleic acid/PG markedly accelerated penetration of both oleic acid and PG into the stratum corneum, compared to only 0.05 M oleic acid/PG (Figs. 2 and 3). With respect to oleic acid, the time required to reach the maximal level of the stratum corneum was significantly shorter for the sample treated with electroporation plus 0.05 M oleic acid/PG than that with only 0.05 M oleic acid/PG. The uptake profiles of PG and oleic acid into the stratum corneum were quite similar. When PG was applied to the skin as either PG without oleic acid after electroporation or PG alone, it hardly entered the stratum corneum.

Fig. 2. Time Profiles of Oleic Acid Penetration into the Stratum Corneum after Treating Rat Skin Surface Either with 0.05 M Oleic Acid/PG or Electroporation Plus 0.05 M Oleic Acid/PG

(○), 0.05 M oleic acid/PG; (●), electroporation plus 0.05 M oleic acid/PG ($n=3$, mean $\pm$ S.E.). The values for electroporation plus oleic acid/PG were significantly different from those for oleic acid/PG at 5, 15, 30, 60, 90 and 120 min ($p<0.05$).

Fig. 3. Time Profiles of PG Penetration into the Stratum Corneum after Treating Rat Skin Surface with 0.05 M Oleic Acid/PG, Electroporation Plus 0.05 M Oleic Acid/PG, PG Alone or Electroporation Plus PG

(○), 0.05 M oleic acid/PG; (●), electroporation plus 0.05 M oleic acid/PG; (△), PG alone; (▲), electroporation plus PG ($n=3-4$, mean $\pm$ S.E.). Values for electroporation plus oleic acid/PG significantly differed from those for oleic acid/PG at 5, 15, 30, 60 and 90 min ($p<0.05$). Values for electroporation plus oleic acid/PG and oleic acid PG significantly differed from both the values for electroporation plus PG and those for PG alone at all times during the experiment ($p<0.05$). Values for electroporation plus PG significantly differed from those for PG alone at 30, 60, 90, 120, 180 and 240 min ($p<0.05$).

These results indicate that electroporation enables oleic acid and PG to penetrate the stratum corneum easily by disordering the stratum corneum. They also indicate that electroporation accelerates the action of oleic acid, which disorders the stratum corneum lipid structure. Elevation of oleic acid uptake into the stratum corneum caused by electroporation suggests that primary pores expand into significantly larger pores by secondary processes which are particular to the condition where oleic acid acts as the enhancer. PG hardly entered into the inside of the electroporated stratum corneum when it was applied alone (without oleic acid). PG is a compound with a molecular weight (M.W.) of 76.10. Compounds of larger molecular size such as metoprolol (M.W.=267)⁷⁾ and fentanyl (M.W.=336),¹¹⁾ compared to PG, can penetrate the electroporated pore of the skin. The size of the membrane channel produced by electroporation is thus presumably large enough to allow PG to pass although the

Fig. 4. Frequency Changes in the CH_2 Antisymmetric Stretching Peak Resulting from Rat Abdominal Stratum Corneum Treated with 0.05 M Oleic Acid/PG or Electroporation or a Combination of Both

(○), 0.05 M oleic acid/PG; (●), electroporation plus 0.05 M oleic acid/PG; (■), electroporation alone; (□), control (without treatment) ($n=3-5$, mean $\pm$ S.E.). Values for electroporation plus oleic acid/PG significantly differed from those for oleic acid/PG at 5, 15, 30, 60, 90, 120 and 180 min ($p<0.05$) and for electroporation alone at various times during the experiment. The values for oleic acid/PG significantly differed from those for electroporation alone at 5, 15, 120, 180 and 240 min ($p<0.05$). Values for electroporation alone significantly differed from those for the control at all times during the experiment.

pore size varies depending upon the electrical conditions. The physico-chemical properties of PG such as electric charge, the viscosity and other parameters must also be considered for explaining the poor PG uptake into the stratum corneum *via* the electroporated pore. Weaver *et al.*³⁾ reported that electroporation resulted in increased membrane permeability, particularly for charged ions and molecules. In contrast, Vanbever *et al.*¹⁰⁾ demonstrated that high voltage pulses increased the transdermal permeation of neutral molecules. PG does not have any electric charges (neutral compound). Based on these findings, the observed poor transport of PG into the stratum corneum after electroporation can not be simply explained by the electric charge. Other factors thus have to be considered to explain the possible mechanism. PG is viscous compound. This is considered to be an important property which controls PG transport by skin electroporation, *i.e.*, higher the viscosity, the more the drug may be poorly transported *via* the pores which are large enough for it to pass. However, the exact mechanism underlying this phenomenon is still unclear.

Effects of Electroporation Combined with Use of Oleic Acid/PG on the Lipid Structure of the Stratum Corneum

The results presented in Fig. 4 compare the time profiles of the frequency shift of the IR spectrum in the CH_2 stretching region. Samples were treated by electroporation with or without 0.05 M oleic acid/PG, with 0.05 M oleic acid/PG without electroporation, or untreated control. Compared with the control, treating the stratum corneum with 0.05 M oleic acid/PG shifted the CH_2 antisymmetric stretching peak to a higher frequency near 2920 cm^{-1} and increased the band width (data not shown). Electroporation alone significantly and immediately increased frequency to the threshold level. The frequency shift did not increase any further even when the duration of electroporation was increased. The degree of the shift was similar to that caused by oleic acid/PG 60 min after initiating treatment. When the skin was treated with 0.05 M oleic acid/PG, the frequency shift was significantly

higher compared with the sample treated by electroporation alone. The increase in the frequency shift was greatest when the skin was electroporated with 0.05 M oleic acid/PG, whereas PG alone had no effect. These results indicate that a transdermal high voltage pulsing (100 V, 10 pulses, 1 ms) that creates a local transport region (electroporated pore),¹⁻⁵⁾ may disrupt the stratum corneum lipid structure at the vicinity of the pore. This effect lasted over the duration of the experiment. Powell reported²³⁾ that viable frog skin subsequently recovered its original resistance within 2-3 min after an electric pulse, demonstrating the reversibility of tissue resistance. Our electrical pulse conditions differed from those of Powell. The bio-physicochemical nature of the rat skin also differed from that of the frog skin. The post-pulse membrane behavior can vary significantly depending upon the pulsing voltage and the skin specimen. Pliquett *et al.* indicated that the recovery of the skin resistance after pulsing occurred very rapidly and was almost complete in the experiment with a relatively low voltage single pulse ($<80\text{ V}$). They also pointed out that no recovery of the transdermal resistance was evident when high voltage with multiple pulses such as $<130\text{ V}$ was applied to the skin, *i.e.*, permanent changes in the stratum corneum occurred. Based on these findings, no recovery of the lipid bilayer membrane structures (Fig. 4) may be attributed to the formation of longtime-metastable structures due to high-voltage pulsing (100 V, 10 pulses, 1 ms). A long-lasting elevation of fluidity may allow oleic acid and PG to easily enter into the stratum corneum when they are co-applied to the skin. Under such circumstances, electric pulse-induced pores are considered to remain open. Disappearance of the pores (resealing or shrinking) is also likely to occur as a result of post-pulse behavior of cells. In such a case, the long-lasting membrane disruption is maintained, *i.e.*, the disrupted lipid membrane structures inside are not rearranged over the duration of treatment. The results also show that the stratum corneum structure is more rapidly disordered when electroporated with oleic acid/PG than when treated with oleic acid/PG. PG itself does not appear to disorder the stratum corneum lipid structure. As a result, electroporation accelerates the ability of oleic acid to disrupt the lipid structure of the stratum corneum. Electroporation-induced disruption enables oleic acid and PG to penetrate the stratum corneum easily. These notions are consistent with the above uptake study.

Vanbever *et al.*^{10,11)} reported rapid transdermal penetration by fentanyl, a lipophilic drug, during skin electroporation that overcomes the skin barrier. However, they did not investigate or discuss the mechanism by which skin penetration by this drug was enhanced. According to our present results, the increased fentanyl permeation may be due to increased fluidity of the stratum corneum lipid caused by electroporation.

Effects of Electroporation and Oleic Acid/PG on The Stratum Corneum Protein Structure

Figure 5 shows a time profile of the absorption intensity at 1519 cm^{-1} for a β -pleated-sheet. During electroporation without any other treatment, the absorption intensity was momentarily increased to a threshold level and did not further increase. This effect was more obvious in samples treated with 0.05 M oleic acid/PG, and the increase was maximal after approximately 30 min. During electroporation plus 0.05 M oleic acid/PG, the increase was maximal after approximately 15 min. The results

Fig. 5. Increased Absorption Intensity at the 1519 cm^{-1} Signal Reflecting the Formation of Protein β -Pleated-Sheet in Rat Abdominal Stratum Corneum Treated with 0.05 M Oleic Acid/PG or Electroporation or a Combination of Both

(○), 0.05 M oleic acid/PG; (●), electroporation plus 0.05 M oleic acid/PG; (■), electroporation alone; (△), PG alone ($n=3-5$, mean $\pm$ S.E.). Values for electroporation plus oleic acid/PG significantly differed from those for oleic acid/PG at 5 and 15 min and also from those for electroporation alone and for PG alone at all times throughout the duration of the experiment ($p<0.05$). Values for oleic acid significantly differed from those for electroporation alone at all times over the experiment except at 5 min ($p<0.05$). Values for electroporation alone significantly differed from those for control throughout the experiment ($p<0.05$).

indicate that electroporation itself can momentarily disrupt the protein structure at the time of application to the skin, and maintain its action throughout the duration of the experiment. The results also indicate that electroporation significantly accelerates the protein-disordering actions of oleic acid/PG. Such disruption may reduce the stratum corneum barrier function as well as disordering the lipid structure and contribute to the increased mobility of oleic acid and PG in the stratum corneum.

Effects of Electroporation and Oleic Acid/PG on the Penetration of PG into the Dermis Time profiles of the amount of PG that appeared in the dermis after treating skin with 0.05 M oleic acid/PG, electroporation plus 0.05 M oleic acid/PG, electroporation plus PG and PG alone, respectively are shown in Fig. 6. The permeation rate of PG in the sample treated with 0.05 M oleic acid/PG was significantly enhanced compared to the sample treated with PG alone. The permeation rate of PG to the dermis was much greater when electroporation plus oleic acid/PG rather than oleic acid/PG was applied to the skin. Electroporation itself did not enhance PG permeation. The time required to reach the maximum level of PG in the dermis was significantly shorter for electroporation plus oleic acid/PG than for oleic acid/PG (Table 1). The steady state level was the same between electroporation plus 0.05 M oleic acid/PG and 0.05 M oleic acid/PG samples. Oleic acid is a *cis*-unsaturated fatty acid that can not enter the dermis.²⁴⁾ These findings therefore indicate that electroporation enables PG to enter the dermis easily by enhancing the ability of oleic acid to disrupt the stratum corneum. This then accelerates the PG transfer from the stratum corneum to viable epidermis and the dermis.

Effects of Electroporation and Oleic Acid/PG on the Dermal Protein Structure Figure 7 shows peak height ratios as the duration of treatment increased for the amide II band near 1550 cm^{-1} with respect to the dermis following treatment of the skin with 0.05 M oleic acid/PG, electropora-

Fig. 6. Appearance of PG in the Dermis with Time after Treatment of Rat Skin Surface with Oleic Acid/PG or Electroporation or a Combination of Both

(○), 0.05 M oleic acid/PG; (●), electroporation plus 0.05 M oleic acid/PG; (△), PG alone; (▲), electroporation plus PG ($n=3-5$, mean $\pm$ S.E.).

Table 1. Characteristic Parameter of Oleic Acid/PG and Oleic Acid/PG Plus Electroporation for PG Behavior in the Dermis

	T_{sat} (min)	Slope
Electroporation plus 0.05 M oleic acid/PG	96.0	5.1×10^{-2}
0.05 M oleic acid/PG	186.4	3.1×10^{-2}
PG alone	—	—

T_{sat} is the time at which the level of PG in the dermis peaked. Slope is calculated from the linear portion of the profile of cumulative PG in the dermis.

Fig. 7. Time Course of Absorbance Ratio in the Amide II Region Resulting from Rat Dermis Treated with Electroporation Plus 0.05 M Oleic Acid/PG or Electroporation or a Combination of Both

(○), 0.05 M oleic acid/PG; (●), electroporation plus 0.05 M oleic acid/PG; (△), PG alone; (■), electroporation alone ($n=3-5$, mean $\pm$ S.E.). Values for electroporation plus oleic acid/PG significantly differed from those for oleic acid/PG at 30, 60, 90 min and for the control throughout the experiment except at 5 and 15 min ($p<0.05$). The values for oleic acid/PG significantly differed from those for electroporation alone and for PG alone at 120, 180 and 240 min ($p<0.05$).

tion plus 0.05 M oleic acid/PG, electroporation alone and PG alone. The peak height ratio for the samples treated with both electroporation plus 0.05 M oleic acid/PG and 0.05 M oleic acid/PG decreased; however, the lag time differed. The ratio began to decrease soon after starting electroporation plus 0.05 M oleic acid/PG and after 60 min when treated with oleic acid/PG. The values of the peak height ratio for electroporation plus 0.05 M oleic acid/PG at 30, 60 and 90 min after the initiation of treatment were significantly lower than that for

Table 2. Influence of Electroporation on the Action of Oleic Acid/PG upon Skin Permeability of IM in the Rat

	Permeation rate ($\mu\text{g}/\text{cm}^2/\text{h}$)	Lag time (h)
Electroporation plus 0.05 M oleic acid/PG	43.51 ± 4.12	2.71 ± 0.23
0.05 M oleic acid/PG	42.24 ± 2.35	6.02 ± 0.34
PG alone	—	—

Fig. 8. Cumulative Amount of IM that Permeated Rat Skin Detected in the Receptor Compartment as a Function of Time

(○) 0.05 M oleic acid/PG containing 1 w/w% IM; (●), electroporation plus oleic acid/PG containing 1 w/w% IM; (△), PG containing 1 w/w% IM ($n=5-8$, mean $\pm$ S.E.).

0.05 M oleic acid/PG. The degree of the decrease in the peak height ratio was the same between electroporation plus 0.05 M oleic acid/PG and 0.05 M oleic acid/PG at periods of over 120 min. Neither electroporation alone nor PG alone affected the spectral profile near 1550 cm^{-1} over the duration of treatment. These results indicate that the dermal protein structure is altered more rapidly by electroporation plus 0.05 M oleic acid/PG than by 0.05 M oleic acid/PG. They also indicate that the degree of dermal structure disorder at the steady state level where PG flux is maximal was likely to be the same between these two samples. PG that penetrates the dermis may disorder the dermal protein structure.²⁴ Thus, the faster penetration of PG into the dermis induced by electroporation plus 0.05 M oleic acid/PG than by 0.05 M oleic acid/PG causes rapid disruption of the protein structure. These also indicate that electrical pulses induced by a frog type electrode do not affect the dermal protein structure.

Effect of Electroporation Plus Oleic Acid/PG and Oleic Acid/PG on the *in Vitro* Permeation of IM Figure 8 shows the skin permeation profile of IM. Table 2 presents the skin permeability parameters of IM calculated from the permeation profiles. The permeation rate of IM applied to the skin with electroporation plus 0.05 M oleic acid/PG was similar to that when applied with 0.05 M oleic acid/PG. However, the lag time significantly differed. Indomethacin appeared in the receptor compartment much faster *via* electroporation plus 0.05 M oleic acid/PG than in the presence of 0.05 M oleic acid/PG. Indomethacin did not permeate the skin when applied with PG alone. These results indicate that the permeation rate of IM into skin treated by electroporation plus 0.05 M oleic acid/PG is faster than that into skin treated with

0.05 M oleic acid/PG until a steady state level in the permeation rate is reached. This is a result of the more rapid skin disordering action of electroporation plus 0.05 M oleic acid/PG compared to that of oleic acid/PG alone.

CONCLUSION

Electroporation itself immediately disrupted the structure of both the stratum corneum lipid and protein up to a threshold level. This resulted in overcoming the barrier function of the stratum corneum. This may occur in the vicinity of a new aqueous pathway in the stratum corneum. Electroporation effectively accelerated the ability of oleic acid/PG to enhance the skin permeability. Our results indicate that electroporation can be used as a tool to accelerate the action of skin penetration enhancers to improve drug transport through the skin.

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