Structural and Functional Effects of Oleic Acid and Iontophoresis on Hairless Mouse Stratum Corneum

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The aim of this study was to assess the effects of chemical and electrical modes of percutaneous penetration enhancement on the intercellular lipid lamellae of the stratum corneum. Hairless mice were treated with either oleic acid/propylene glycol and iontophoresis separately or together. Permeability barrier function was evaluated by measuring transepidermal water loss and correlated with the structure of stratum corneum intercellular lamellae, as evaluated by electron microscopy, using ruthenium tetroxide postfixation. Transepidermal water loss levels did not change following 1 h iontophoresis alone. In contrast, topical applications of 0.3 M oleic acid in propylene glycol for 1 h increased transepidermal water loss significantly. Moreover, the combined use of iontophoresis plus 0.3 M oleic acid for 1 h further increased transepidermal water loss at equivalent time points. Ultrastructural observations demonstrated both marked disorganization of the intercellular lipid lamellae, as well as the presence of distended lacunae within the stratum corneum in oleic acid/propylene glycol plus or minus iontophoresis-treated stratum corneum. This study provides direct evidence that the oleic acid/propylene glycol system can disrupt the stratum corneum lipid lamellar structures, and that coapplications of oleic acid with iontophoresis further enhance the effects of oleic acid. The synergy between chemical and physical enhancement may afford a new approach to promote transdermal drug delivery. ***Key words: barrier function/intercellular lipid lamellae/transdermal drug delivery. J Invest Dermatol 114:64–70, 2000***

Stratum corneum (SC) lipids, which comprise mainly ceramides, cholesterol, and free fatty acids, are arranged as membrane structures in the SC interstices (Elias, 1983; Elias and Menon, 1991). By virtue of their unique architecture, these membranes subserve a number of functions, the principal one being formation of an essentially watertight barrier, which allows complete control of the internal water balance of the body (Forslind, 1994). Simultaneously, they also represent the principal route for the penetration of small molecules (Naik *et al*, 1995), presenting a significant barrier to the transdermal drug delivery of larger and water-soluble molecules. Hence, a variety of penetration enhancers have been investigated for their ability to enhance the delivery of poorly absorbed drugs across the skin through the SC interstices.

There are a number of chemical enhancers which increase percutaneous absorption by modifying skin barrier function. Oleic acid (OA) increases transepidermal water loss (TEWL) *in vivo* and the synergistic activity of OA and propylene glycol (PG) is well known (Takeuchi *et al*, 1993, 1995). Studies have shown that OA facilitates the transport of drugs through the intercellular domains of the SC (Naik *et al*, 1995; Menon and Elias, 1997). It has been proposed further that the predominant effect of the fatty acid is the formation of phase-separated domains, thereby forming permeability defects at liquid–solid interfaces (Ongpipattanakul *et al*, 1991). Moreover, other amphoteric moieties (e.g., Azone) probably act in a similar fashion (Wiechers and De Zeeuw, 1990).

Physical methods such as sonophoresis and iontophoresis also have been used to enhance transdermal drug delivery. Iontophoresis employs an electric current carried by ions to introduce compounds into the body at the controlled rates. The current is carried by the drug ions as well as by other ions in the iontophoretic field and physiologic milieu, by imposing an electric field across the SC to drive the current (Su *et al*, 1994). The key advantages of iontophoresis are its capability of strongly enhancing the rate of drug delivery compared with passive diffusion, and the fact that it offers the possibility of externally controlled flux modulation (Camel *et al*, 1996; Geest *et al*, 1996; Turner *et al*, 1997).

OA and iontophoresis represent alternate, unrelated methods for the enhanced delivery of molecules through the skin. Recent biophysical studies have demonstrated synergistic effect of iontophoresis and a series of fatty acids on porcine skin *in vitro* (Bhatia *et al*, 1997; Bhatia and Singh, 1998). In order to exploit fully the potential additive and/or synergistic characteristics of chemical *versus* physical enhancers for the delivery of drugs, it is important to understand the mechanism of action of each within the SC. Ruthenium tetroxide (RuO_4), a stronger oxidizing agent than osmium tetroxide, reacts strongly with SC lipids and is more electron dense, permitting visualization of SC lamellar domains by electron microscopy (Swartzendruber *et al*, 1995). In this study, we observed the effects of OA and iontophoresis on permeability barrier function when applied separately or together, and the correlative structural basis for these changes, using RuO_4 postfixation.

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Abbreviations: OA, oleic acid; PG, propylene glycol; RuO_4, ruthenium tetroxide; SC, stratum corneum; TEWL, transepidermal water loss.

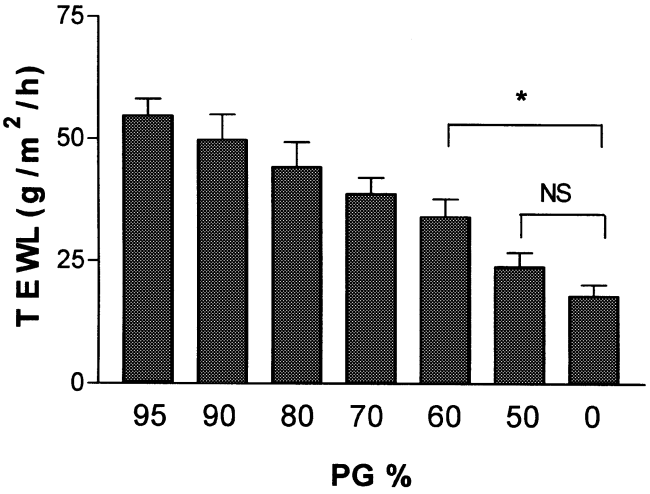

Figure 1. TEWL after application of 0.3 M OA in different concentrations of PG for 2 h. Animals were treated with 0.3 M OA in different concentrations of 95, 90, 80, 70, 60, and 50% of PG in distilled water, and absolute OA, respectively. The elevation of TEWL was dependent on the concentration of PG. Results are represented as mean (± SEM). *$p < 0.05$ *versus* the TEWL after application of absolute OA; NS, nonsignificant.

MATERIALS AND METHODS

Experimental protocols Hairless male mice, 8–12 wk old, were used for these studies. All animal experiments were performed in an ambient environment of 23 ± 1°C, 50 ± 10% relative humidity, and complied with the *Principles of Laboratory Animal Care* (NIH publication # 85–23, revised 1985). Under general anesthesia with 4% chloral hydrate by intraperitoneal injection, groups (n = 6 in each group) of mice received topical absolute OA and PG (extra pure reagents, Sigma, St Louis, MO), as well as 0.3 M OA in different concentrations of PG (graded from 50% to 95%, PG/distilled water, pH 7.4) for 2 h. Other groups were treated with the following concentrations of OA (0.1, 0.15, 0.2, 0.3 M) in a vehicle (PG/distilled water; 9:1) or vehicle alone, each for 1, 1.5, 2, 3, and 4 h. Formulations were applied as follows: 50 μl solutions were applied on one flank; those in solutions were placed on 15 mm diameter filter paper disc, and attached securely to the skin with Tegaderm (3M, Ontario, Canada). After treatment, the patches were removed, wiped with a paper towel, and TEWL measurements were performed (see below), as an indicator of SC permeability barrier function (Ghadially *et al*, 1995, 1996). Another group of animals underwent acute barrier disruption by repeated applications of autoclave tape (3M, Minnesota), four to six times successively. Animals with TEWL rates that exceeded 50 g per m^2 per h (normal < 10 g per m^2 per h) after permeability barrier disruption were included in this study.

Iontophoresis was conducted with an iontophoretic device which delivered 500 mA per cm^2 pulse current (2 kHz ± 10%). An 11 mm diameter electrode patch was positioned on the skin, inserting a piece of filter paper, which containing 50 μl of 0.3 M OA in a vehicle (PG/distilled water; 9:1). Groups then were administered the probe either with or without power supply activation for 1 h; another group received iontophoresis with distilled water for 1 h only. All sites were covered with Tegaderm and secured with paper tape. Finally, another group of animals received 0.3 M OA under Latex occlusion for 1 h. TEWL was measured 10 min after patch removal with a Tewameter TM 210 (Courage + Khazaka, Koln, Germany).

To determine whether application of OA influences the kinetics of barrier recovery after prior induction of a barrier defect, we also observed permeability barrier recovery after tape-stripping alone, in topical OA-treated animals, and in animals treated with iontophoresis with OA. TEWL was measured 2, 4, 6, 12, 24, and 48 h post-treatment.

Electron microscopy At various time points after treatment with the protocols described above, biopsies were taken and minced to ≤ 0.5 mm^3, fixed in modified Karnovsky's fixative overnight, washed in 0.1 M cacodylate buffer, and postfixed in 0.25% RuO_4 in 0.1 M cacodylate buffer for 45 min in the dark at room temperature. After rinsing in buffer, samples were dehydrated in graded ethanol solutions, and embedded in an Epon-epoxy mixture. Ultrathin 60–80 nm sections were examined in an electron microscope (H-500, HITACHI, Japan) after further contrasting with uranyl acetate–lead citrate.

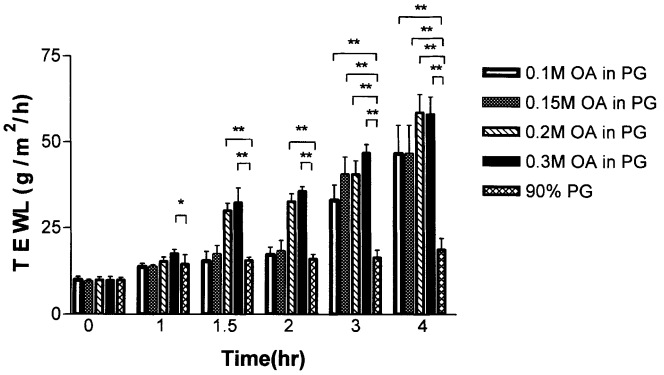

Figure 2. Changes of TEWL after application of different concentrations of OA according to application times. Groups were treated with 0.1, 0.15, 0.2, and 0.3 M OA in 90% PG and 90% PG only, each for 1, 1.5, 2, 3, and 4 h. When 90% PG was used as the vehicle, no significant changes of TEWL occurred. After treatment in different concentrations of OA, TEWL increased in relation to the concentration of OA and application time. Results are represented as mean (± SEM). *$p < 0.05$, **$p < 0.001$ *versus* the TEWL treated with 90% PG by an analysis of variance.

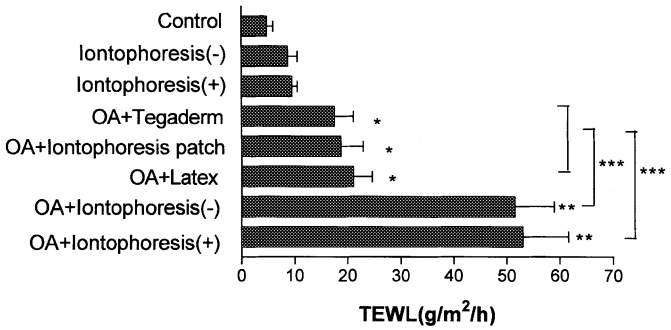

Figure 3. TEWL after application of 0.3 M OA for 1 h. Animals were received iontophoresis alone (+ or – electrode); topical application of OA covered with Tegaderm; iontophoretic patch containing OA; Latex occlusion with OA and iontophoresis with OA, respectively. TEWL was not changed after iontophoresis alone, and increased 2-fold after application of OA with iontophoretic patch without power supply, of levels are similar to topical application of OA which covered both Tegaderm and Latex occlusion. In contrast, the TEWL was increased markedly with a combination of iontophoresis and OA. Results are represented as mean (± SEM). *$p < 0.01$, **$p < 0.001$ *versus* normal TEWL, and ***$p < 0.001$ *versus* TEWL after 1 h application with Tegaderm iontophoresis patch, and Latex by an analysis of variance.

Statistics Statistical significance was determined using a repeated-measures ANOVA test for between group and within group differences, followed by Bonferroni's correction when necessary.

RESULTS

Application of oleic acid change TEWL rates To delineate the effects of OA in PG on epidermal permeability, we first assessed the effects of absolute OA and PG separately on TEWL across intact hairless mouse skin. After 2 h treatment with absolute OA, TEWL levels increased to 17.8 ± 1.6 g per m^2 per h, whereas the change in TEWL after 2 h of treatment with PG alone was 14.5 ± 1.2 g per m^2 per h. To delineate the optimal concentration of PG for OA-induced permeability enhancement, we next assessed the impact of 0.3 M OA in different concentrations of PG on epidermal permeability barrier function. As seen in **Fig 1**, with 2 h application, TEWL increased progressively as the concentration of PG was raised, indicating that PG modulates the effects of OA on epidermal permeability.

Having shown that the extent of changes in TEWL from OA application is influenced by the concentration of PG, we next

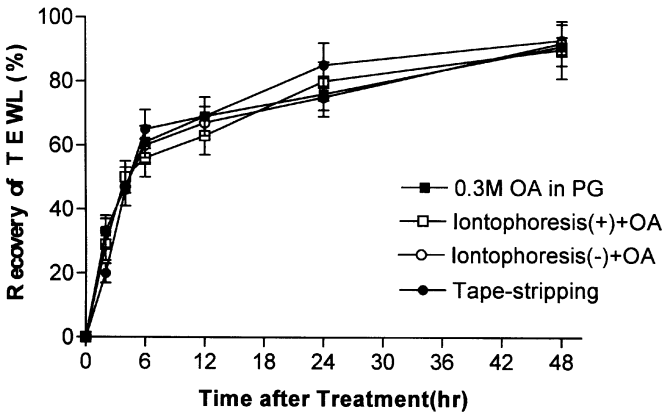

Figure 4. Recovery rates of TEWL after topical application of 0.3 M OA for 2 h and by iontophoresis with OA for 1 h. After 4 h post-treatment, the barrier function recovered by nearly 50%. There were no differences in TEWL recovery in comparison with after tape-stripping. TEWL rates are plotted as the percentage recovery of TEWL. Each point represents the mean (± SEM).

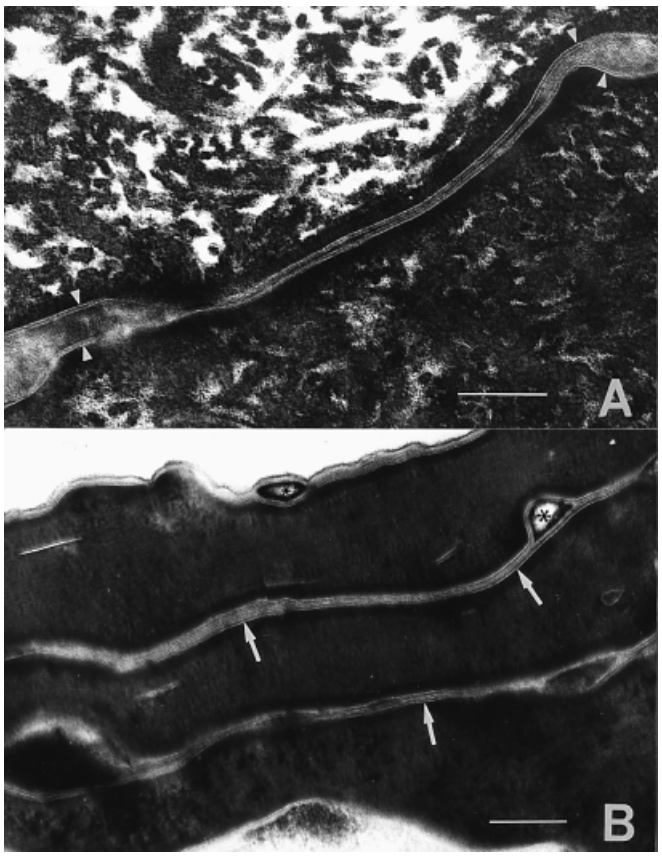

Figure 5. Two hours treatment of absolute PG and OA in hairless mice. (*A*) With PG treatment, foci of individual membranes separated by amorphous materials can be seen (*A*, *arrowheads*). (*B*) Displays after 2 h OA treatment in mouse skin. Note the uniform preservation of the intercellular lipid lamellae (*arrows*) and presence of lacunae (*asterisks*). *Scale bar.* 200 nm.

assessed the ability of different concentrations of OA to influence epidermal permeability at a constant PG concentration (90%). Hence, we measured TEWL after application of 0.1, 0.15, 0.2, and 0.3 M OA for 1, 1.5, 2, 3, and 4 h. A significant increase in TEWL occurred at 0.3 M OA as early as 1 h and with 0.2 M OA by 1.5 h treatment (**Fig 2**). At lower OA concentrations, an increase in TEWL also occurred, but it was further delayed (3 h with 0.1 M and 0.15 M concentrations of OA). Moreover, application of the

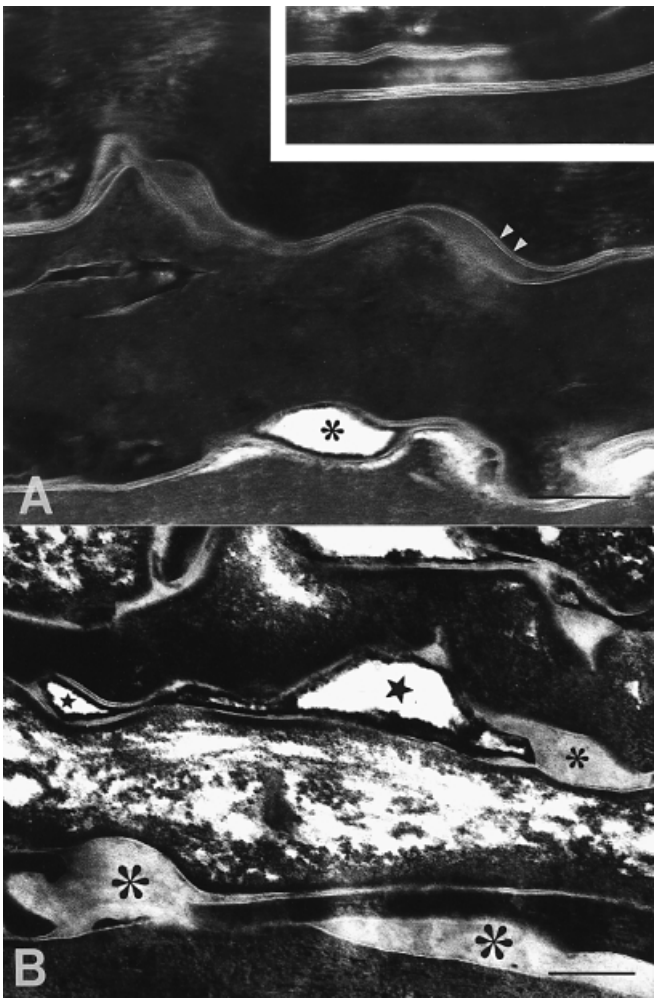

Figure 6. Ultrastructure of topical 0.3 M OA in PG/distilled water (9:1) treated epidermis. After 1 h application, normal-appearing lamellar membrane (*A*, *inset*), and phase-separated lamellar membrane structure (*arrowheads*) as well as lacunae (*asterisk*) coexist within SC (*A*). By 2 h treatment, the formation of lacunae (*stars*), and variable amounts of amorphous material (*asterisks*) filling in the widened SC interstices (*B*). These alterations are considerably more remarkable than those present in after 1 h 0.3 M OA-treated sample (cf. *A*). *Scale bar.* 200 nm.

90% PG vehicle alone again produced no significant increase in TEWL at all time points, demonstrating that the changes in TEWL cannot be attributed to the effects of PG alone. These results show that the effects of OA on permeability barrier function are affected by the duration of application time, and the concentration of both OA and PG.

Iontophoresis further enhances the effect of oleic acid on epidermal permeability To determine whether iontophoresis influences permeability barrier function in intact skin, we first measured TEWL level after 1 h iontophoresis (see *Materials and Methods*). As shown in **Fig 3**, TEWL levels were similar to normal at both the positive and negative electrode sites. In contrast, after 1 h application of 0.3 M OA in 90% PG under the iontophoretic patch, but without activation of the power supply, TEWL levels increased nearly 2-fold over normal animals, comparable with animals treated with 0.3 M OA alone (cf. **Fig 3** *versus* **Fig 2**). Moreover, in comparison with 1 h topical application of 0.3 M OA alone, there was no significant increase in TEWL after 1 h treatment of 0.3 M OA by Latex occlusion, indicating that the increase in TEWL from OA in these studies could not be attributed to the occlusive effects of the iontophoretic patch (**Fig 3** *versus* **Fig 2**). In contrast, coadministration of iontophoresis for 1 h with

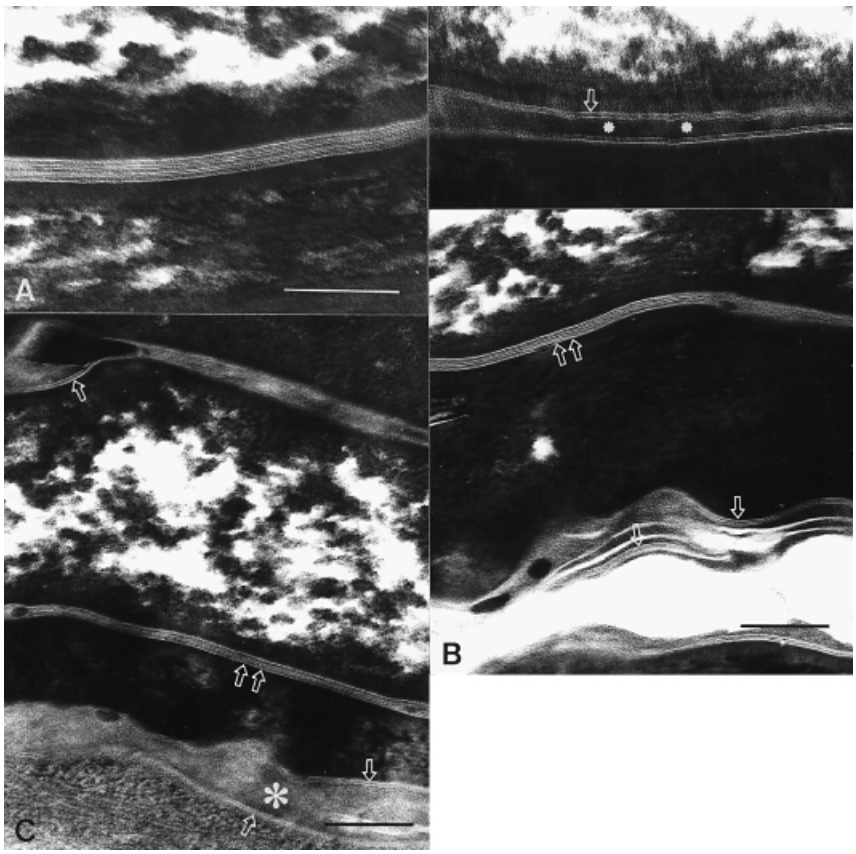

Figure 7. Electron micrographs of iontophoresis for 1 h. (*A*) Shows the normal-appearing lipid lamellae. This sample is taken from the animal treated with the iontophoretic patch without a power supply for 1 h. (*B*) Displays the sample taken from beneath of positive electrode, whereas (*C*) is from the negative electrode. The structures of lipid lamellae appear fragmentary in many sites (*single arrows*) and flocculent materials are within SC interstices (*asterisks*) as well as the appearance of lamellar phase separation (*B*). *Scale bar*: 200 nm.

0.3 M OA significantly elevated TEWL ($p < 0.001$) to levels comparable with those observed after 2 h topical treatment with 0.3 M OA alone (**Fig 3** *versus* **Fig 2**). These results show that iontophoresis enhances the effects of OA on the permeability of intact epidermis.

Application of OA does not prolong the kinetics of barrier recovery Having shown that OA treatment induces a barrier defect in intact epidermis, we next determined whether such treatment also could alter the kinetics of barrier recovery. After 0.3 M OA treatment alone for 2 h, or coadministered with iontophoresis for 1 h, TEWL rapidly recovered towards normal, such that by 4 h TEWL levels had normalized by nearly 50% in all groups (**Fig 4**). Moreover, there was no significant difference in rates of barrier recovery following OA treatment alone *versus* OA coadministered with iontophoresis. These results demonstrate first, that there is no toxic effect of coadministration of OA with iontophoresis; and second, that these treatments alone and together do not prolong the kinetics of permeability barrier recovery.

Electron microscopy demonstrates striking alterations on SC membrane domains To gain further insights into the mechanisms by which OA and iontophoresis affect epidermal permeability, we next examined RuO4 postfixed SC. We first compared the ultrastructural changes that results from sites treated with either absolute PG or OA alone or 0.3 M OA in 90% PG for 2 h. With absolute PG treatment alone, intercellular lamellar membrane structure appeared normal, but foci of amorphous material occurred, which enlarged forming lateral clefts, leading to lamellar phase separation (**Fig 5*A***). Likewise, following absolute OA treatment, normal lamellar membrane structures persisted within the SC intercellular spaces, but microlacunae appeared occasionally (**Fig 5*B***). After application of 0.3 M OA for 1 h, both normal-appearing and phase-separated lipid lamellae as well as lacunae, coexist within the SC interstices, but lamellar organization was not altered (**Fig 6*A***). These results are accordance with the functional observations, which demonstrate that application of 0.3 M OA for 1 h did not induce notable alterations in SC lipid lamellar structure (cf. **Fig 2**). By 2 h after application of 0.3 M OA, membrane structure and their lamellar organization were drastically altered (**Fig 6*B***). Two types of nonlamellar domains were present, including enlarged lacunar domains which contained electron-dense material, and large quantities of less electron-dense, flocculent material displacing and disrupting the intercellular lamellae. These results show that application of OA causes distinct abnormalities in the SC interstices.

We next assessed both the structural basis for the effects of iontophoresis alone and for iontophoresis plus OA on epidermal permeability barrier function, and to delineate how combinations of the two further influence barrier properties, Ultrastructural changes after iontophoresis with or without additional OA applications for 1 h were compared. There were no changes in intercellular membrane structural organization in samples taken beneath the iontophoretic patch with the inactivated power supply (**Fig 7*A***). With iontophoresis alone, minimal changes in the intercellular lamellar structures could be seen in samples taken from beneath either the positive or negative electrodes (**Fig 7*B***, ***C***). Both sites showed focal lamellar phase separation (**Fig 7*B***), and fragmentation of lamellar membrane as well as amorphous nonlamellar material (lacanae) within the SC interstices (**Fig 7*C***). In contrast, after 1 h application of iontophoresis in combination with 0.3 M OA, further structural abnormalities were seen within the SC; i.e., both enlargement of lacunae and flocculent, electron-dense materials displacing/replacing the intercellular lamellar membranes (**Fig 8**). These observations resembled those following topical 0.3 M OA administration alone for 2 h (cf. **Fig 6**), demonstrating that the combination of OA with iontophoresis causes variable amounts of phase separation, lacuna formation, and lamellar disruption within the SC interstices. Finally, the normalization of permeability barrier function rates by 48 h following

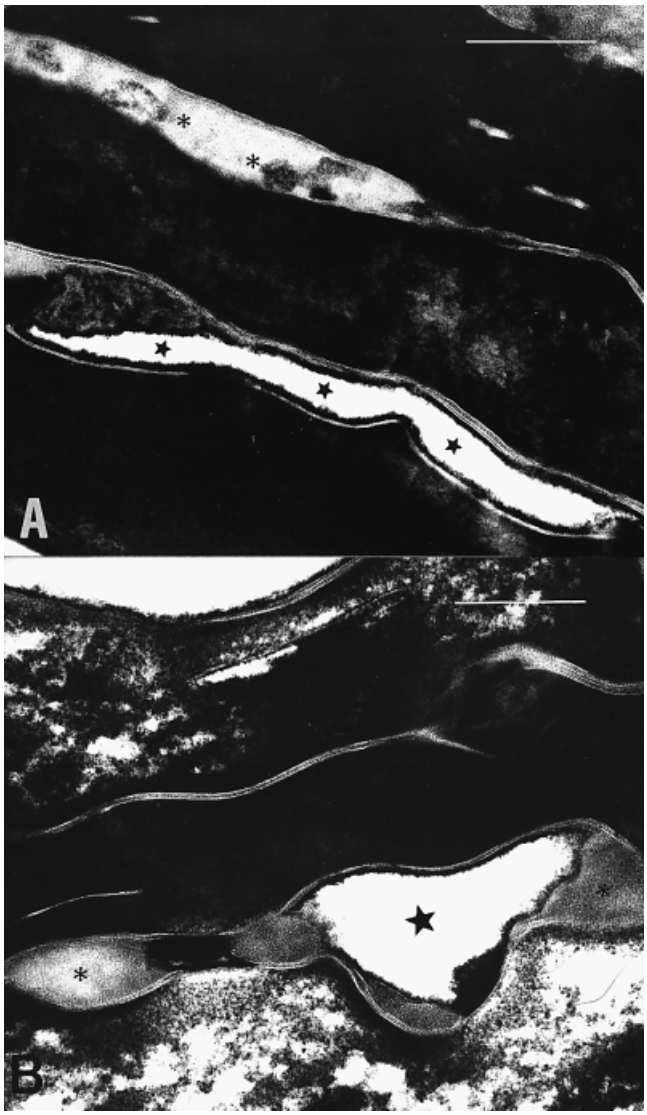

Figure 8. Ultrastructural observation of SC after iontophoresis with 0.3 M OA for 1 h, which demonstrates the alterations in lipid membrane structure leading to barrier abnormality. The dilated lacunae (*stars*), electron-dense, and amorphous relatively electron-lucent material are observed in the widened intercellular spaces of SC (*asterisks*). (*A*) Positive electrode; (*B*) negative electrode. *Scale bar*: 200 nm.

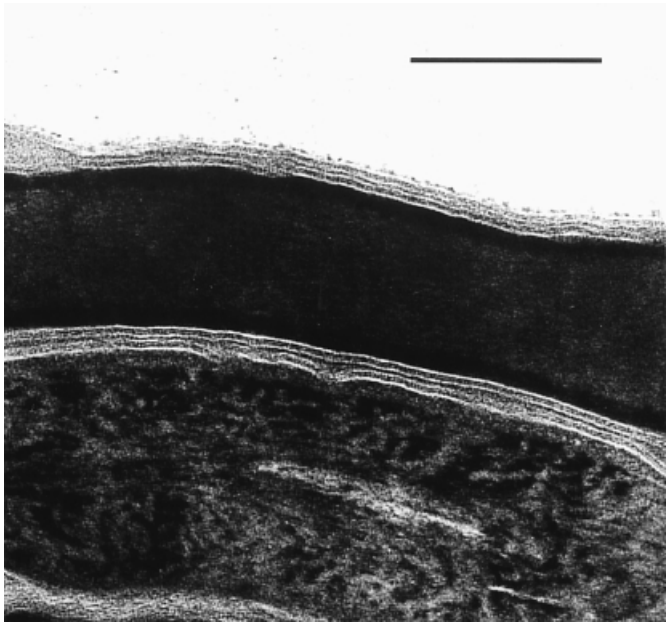

Figure 9. Forty-eight hours post-OA treatment. At this stage of near-complete barrier repair, accompanied the presence of normal lipid lamellae within the intercellular space of SC. *Scale bar*: 200 nm.

either OA treatment alone or OA coadministered with iontophoresis was accompanied by the reappearance of normal-appearing intercellular lamellar membranes (**Fig 9**, OA application alone; the result of OA coadministration with iontophoresis is similar; not shown).

DISCUSSION

SC, the outermost epidermal layer is required for terrestrial life, is the principal regulatory barrier to the transcutaneous traffic of water and exogenous substances. Normal SC allows only a limited number of drugs to be delivered in therapeutic amounts through the skin. As a consequence, methods that compromise skin barrier function would be predicted to increase the therapeutic efficacy of dermatologic formulations and transdermal drug delivery.

Penetration enhancers, such as OA have been widely employed in attempts to deliver poorly absorbed drugs through the SC. OA is believed to reduce skin resistance by disorganizing the tightly packed SC lipid architecture(Gay *et al*, 1989; Francoeur *et al*, 1990). The penetration enhancement effects of OA may result in increased solid–fluid phase separation of SC lipid lamellar domains (Francoeur *et al*, 1990; Ongpipattanakul *et al*, 1991; Pechtold *et al*, 1996). In fact, recent results obtained by attenuated total reflectance infrared spectroscopy suggest that the action of OA could be due to both mechanisms; i.e., increased lipid fluidity and lamellar phase separation (Naik *et al*, 1995). OA in combination with ethanol not only increase the average lipid acyl chain disorder of treated SC, but also leads to extensive lipid extraction (Clancy *et al*, 1994). Any changes in the lipid matrix, in particular increases in the fluidity of hydrocarbon chains or lipid phase separation would result in higher permeability. It has been suggested that PG may work by a direct action on epidermal structural proteins which increases the solubility of proteins and also denatures proteins causing them to lose their secondary, tertiary, and quaternary structures, thereby increasing the partitioning of compounds between the vehicle and the SC whereas OA acts by decreasing barrier permeability function itself (Goldsmith, 1978). Finally, there is a well-established synergy between the enhancer activity of OA and PG vehicles (Goldsmith, 1978; Barry, 1993; Takeuchi *et al*, 1993, 1998; Lin *et al*, 1996; Tanojo *et al*, 1998).

Iontophoresis refers to the enhancement of percutaneous absorption from the transfer of ions or charged drugs through the skin in the presence of an electric current. Previous studies have shown that the *in vitro* permeability of hydrocortisone increases following application of an iontophoretic current, suggesting a current-induced effect (Wang *et al*, 1993). A combination of reduced SC barrier properties with induced solvent convective flow, during an applied electric current makes the SC lipid lamellae interior more accessible to water and ions (Pechtold *et al*, 1996). This would imply that during iontophoresis, enhanced ion and water transport through the skin is associated, at least in part, with the SC lipid matrix. In this study, the observation that TEWL increases significantly whereas iontophoresis is coapplied with OA, implies that iontophoresis might: (i) produce discrete sites located in the SC (Cullander and Guy, 1991), or (ii) reduce resistance of the SC that facilitates OA penetration (Burnette and Bagniefsky, 1988), allowing OA easily to act more readily on the lipid lamellae of the SC.

The iontophoretic modulation of chemical enhancement provides a potential means of optimizing iontophoretic

transdermal drug delivery. It is interesting to note the increased TEWL caused by iontophoresis with OA, which is anticipated to have an effect on the intercellular lipid domains. As the SC function is based upon the molecular architecture of these lipids (Elias, 1991; Menon and Elias, 1997), compromising this membrane structure, by increasing lipid disorder, should result in increased drug transport. Alternatively, the influence of an applied electric field could drive OA into the SC directly, or enhance a process termed electro-osmosis (Vanbever *et al*, 1996). Another possible scenario is that the concentration of current flow through appendageal "shunts" or other skin imperfections, which results in significant local heating owing to the resulting high-current densities. This heating may disorder the adjacent intercellular lipids with a concomitant increase in overall skin permeability (Bhatia and Singh, 1998). It is possible that fatty acids change the conformation of SC proteins and as a result open up aqueous channels, which are utilized by iontophoresis to provide further enhancement (Bhatia and Singh, 1998). In each case, the presence of OA may compromise local structure, and thereby facilitate drug transport.

The present ultrastructural studies also provide insights into the potential mechanism of barrier alterations performed by OA +/− iontophoresis. RuO_4 stains SC lipids and delineates the intercellular domains and lipid lamellar structures, giving the novel information about previous unrecognized microdomains in the SC (Hou *et al*, 1991; Menon *et al*, 1992; Ghadially *et al*, 1995, 1996; Swartzendruber *et al*, 1995). The ultrastructural studies suggest that absolute PG does not appear to alter SC lipid lamellae significantly, consistent with results of Takeuchi and coworkers' studies, who showed that PG penetration through the epidermis does not substantially disorder the skin barrier (Menon *et al*, 1992; Takeuchi *et al*, 1993, 1995). In contrast, we showed that application of absolute OA induces the lacuna formation, whereas lipid membrane unit structures are preserved (cf. **Fig 5*B***). This may reflect the inability of absolute OA to be taken up by the SC, thereby yielding minimal impact on permeability barrier function. Finally, when OA and PG are coapplied, distinct disorganization of the SC lipid lamellar structure is observed. Thus, our results indicate that PG is an important factor influencing the transport of solutes.

Previous *in vitro* studies in hairless mouse skin assessing ions and current fluxes using a vibrating probe have implicated the appendageal pathway as the predominant route of electrically assisted drug delivery (Cullander and Guy, 1991). And cathodic iontophoretic transport of a fluorescein dye in human skin showed that transport can occur through a "pore" such as a hair follicle, a sweat duct, or imperfection in the skin (Burnette and Ongpipattanakul, 1988). Because there is no sweat gland in hairless mouse, for the clinical practice of electrically enhanced administration of drugs, it must be account the differences between mouse and human skin. Also, Bond and Barry (1988) compared the effects of pretreatment with a range of penetration enhancers on the permeability of human abdominal and hairless mouse skins to a model permeant, 5-flurouracil. Suggesting that there was no consistent relationship between enhancer effects on the two skin types, and concluded that the hairless mouse model should not be used to predict the effects of penetration enhancers in human skin. Therefore, further studies are required to determine the differences between mouse and human skin.

In summary, these results demonstrate first, that the OA/PG system can disrupt the SC lipid lamellar structure, and second, that the uptake of OA into the SC depends on the concentration and the duration of application time. Finally, coapplications of OA and iontophoresis enhance the action of OA on the SC lamellar lipids. The combination of chemical methods and iontophoresis may provide a way of reducing the application time of the accelerant necessary to produce an effect, thereby promoting transdermal drug delivery with increased therapeutic efficacy. The relevance of these observations for human skin, however, remains to be determined.

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