Alveolar Permeability Enhancement by Oleic Acid and Related Fatty Acids: Evidence for a Calcium-Dependent Mechanism

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Pulmonary exposure to oleic acid (OA) is associated with permeability alterations and cellular damage; however, the exact relationship between these two effects has not been clearly established. Using cultured alveolar epithelial monolayers, we demonstrated that OA and some other fatty acids (≤ 50 μM) can induce permeability changes without detectable cellular damage. At higher concentrations, however, OA caused severe membrane damage and leakage to solute flux. The permeability enhancing effect of OA was observed with both the paracellular marker ^{3}H-mannitol and the lipophilic transcellular indicator ^{14}C-progesterone. While the effect of OA on transcellular permeability may be attributed to its known effect on membrane fluidity, the paracellular promoting effect of OA and its mechanism are not well established. We postulated that OA may increase paracellular permeability through a Ca^{2+}-dependent tight junction mechanism. Using dual-excitation fluorescence microscopy, we demonstrated that OA can increase intracellular calcium, $[Ca^{2+}]_i$, in a dose-dependent manner. This effect was transient at low OA concentrations (≤ 50 μM) but became more pronounced and sustained at higher concentrations. Free hydroxyl and unsaturated groups were required for this activation since esterified OA (oleic methyl ester) and stearic acid (a saturated fatty acid with equal chain length) had much reduced effects on both the $[Ca^{2+}]_i$ and the permeability alterations. Degree of unsaturation was unimportant since linolenic acid (18:3), linoleic acid (18:2), and OA (18:1) had similar and comparable effects on the two parameters. When the alveolar epithelium was bathed with Ca^{2+}-free medium, OA failed to elevate $[Ca^{2+}]_i$, suggesting that Ca^{2+} influx from the extracellular medium is responsible for the observed $[Ca^{2+}]_i$ rise. This effect of OA was not due to nonspecific membrane damage since the monolayer maintained its integrity and the $[Ca^{2+}]_i$ returned to pretreatment levels after an initial transient rise. Moreover, the permeability alteration was fully reversible upon removal of OA. These results suggest that the alveolar permeability may be reversibly enhanced by sublethal concentrations of oleic acid.

KEY WORDS: oleic acid; alveolar epithelium; permeability; cellular damage; calcium.

INTRODUCTION

Drug delivery through the pulmonary route provides an attractive means for systemic application of therapeutic macromolecules such as peptides and proteins due to its large absorptive surface area and the relatively permeable blood barrier of the alveoli. Previous studies have indicated that while drug administration through this route exhibits improved systemic bioavailability over several other alternate routes, its efficiency is still low as compared to parenteral administration. As a result, attempts have been made to improve pulmonary drug bioavailability, most notably through the use of penetration enhancers. However, problems associated with tissue toxicity and irritation have limited their effective use. Oleic acid (OA) is an unsaturated fatty acid, often employed as a dispersing agent in several aerosol formulations. It also possesses a permeability enhancing and damaging action on lung epithelium, however, the relationship between the two actions as well as their mechanisms is not yet clear. In other epithelia, OA and certain fatty acids have been shown to alter membrane permeability by increasing the motional freedom or fluidity of the membrane phospholipids (1–4). In these studies, unsaturated fatty acids were found to be more effective in inducing permeability changes than their saturated counterparts (2,5). In the lungs, most studies on the effect of fatty acids have focused on OA-induced toxicity. When administered at high doses, either intravenously or by aerosol, OA can cause pulmonary edema and injury (6,7). Because of its potential toxicity as well as its permeability enhancing benefit, the present study investigated the dose–response relationship between these two effects as well as their mechanisms. To facilitate detailed mechanistic studies and to allow accurate quantitation of the effects, an *in vitro* cultured monolayer system of alveolar epithelial cells was utilized. The cells were grown in primary cultures on microporous, tissue culture-treated, polycarbonate substrata. Membrane permeability was evaluated by radioactive flux measurements of two known permeability probes, ^{3}H-mannitol (a hydrophilic paracellular tracer) and ^{14}C-progesterone (a lipophilic transcellular tracer). Membrane damage was quantitated fluorometrically using the membrane integrity indicator propidium iodide. Intracellular calcium, $[Ca^{2+}]_i$, was measured using dual-excitation fluorescence microscopy with the aid of the calcium probe Fura-2 AM (acetoxymethyl ester). Our studies indicated that permeability enhancement by OA can occur in the absence of cellular damage. We also demonstrated that the noncytotoxic effect of OA on paracellular permeability was reversible and mediated through a transient increase in $[Ca^{2+}]_i$ which reversibly altered tight junction structures.

MATERIALS AND METHODS

Cell Isolation and Culture

Male, pathogen-free, Sprague–Dawley rats were anesthetized with pentobarbital sodium (150 mg/kg body wt) and the lungs were removed. After removing blood cells and free alveolar macrophages by tracheal lavage, the lungs were excised and filled with phosphate buffer containing elastase (40 U/mL, type I) and DNase (0.006%) and incubated at 37°C for 20 min to free lung cells. After enzymatic digestion, the lungs were finely minced and the digestion was arrested by incubation for 5 min in phosphate buffer containing 25% fetal

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bovine serum and 0.006% DNase. The crude extract was sequentially filtered through 160- and 45-μm screens, then centrifuged, and the resulting cell pellet was spun on a sterile Percoll density gradient. The second cell band from the surface was collected, washed twice, and resuspended in 1:1 F_{12} and Eagle's modified minimum essential medium plus 10% newborn bovine serum and 0.1 μ*M* dexamethasone. The suspended cells were plated onto 0.4-μm pore, 1.2-cm^2 tissue culture-treated Nuclepore filters (Transwell, Costar, Cambridge, MA) at 1.5×10^6 cells/cm^2 in 12-well plates. The cells on filters were maintained in a humidified 5% CO_2 incubator at 37°C and the nutrient medium was changed every 48 hr after plating. Cell confluency was monitored by electrical resistance measurements using the Millicell ERS testing device (Millipore, Bedford, MA). A typical peak resistance value of the resulting monolayers (normally obtained after 5 days) was ≈1.5 kΩ · cm^2.

Permeability Studies

After 5 days in culture, the culture medium was replaced with HEPES-buffered medium (136 m*M* NaCl, 2.2 m*M* Na_2HPO_4, 5.3 m*M* KCl, 10 m*M* HEPES, 5.6 m*M* glucose, 1 m*M* $CaCl_2$, pH 7.4). After an equilibration period of 1 hr, the donor chamber of the filter inset was spiked with ≈1 μCi/mL ^{3}H-mannitol (≈10 ng/mL) or ^{14}C-progesterone (≈5 ng/mL) (NEN Dupont, Boston, MA) in the presence or absence of fatty acids (Sigma Chemicals, St. Louis, MO). Propylene glycol (≤0.1%) was used as a cosolvent for fatty acids. This amount was found to have no significant effects on all observed parameters. The appearance of the radioactivity in the receptor chamber was determined at appropriate time intervals over a period of 3 hr. Membrane permeability was determined from transepithelial flux of the radioactive tracers, which was obtained from the slope of the amount transported vs time plots, according to the equation $P_{app} = J_{ss}/C_d = (dM/dt \cdot A)(1/C_d)$, where P_{app} is the apparent permeability, J_{ss} is the steady-state flux, C_d is the donor radioactive concentration, M is the amount transferred, and A is the exposed surface area. All experiments were conducted at 37°C in multiwell cell culture plates which were continuously agitated on an Adams Nutator 1105 cell rocker (Clay Adams, Parsippany, NJ).

Studies of Cellular Injury

The monolayers were incubated in HEPES-buffered medium containing 1 μg/mL propidium iodide (PI). After the addition of testing agents, the fluorescence signals emitted from the cells were collected through a photomultiplier attached to a fluorescence microscope and counted at excitation and emission wavelengths of 490 and 600 nm, respectively. PI, due to its hydrophilicity, is normally excluded from cells, but if the cell membrane is disrupted, the probe can enter the cell and bind specifically to the cell nucleus. Upon binding, its fluorescence intensity is strongly enhanced; therefore intense nuclear fluorescence indicates membrane damage and cell death. In these experiments, Triton X-100 (1%) was used to permeabilize the cells to establish maximum fluorescence signal. Cell damage was estimated from the maximum and minimum (baseline) fluorescence signals according to the equation

$$\% \text{ Damaged cells} = \frac{\text{Measured signal} - \text{Minimum signal}}{\text{Maximum signal} - \text{Minimum signal}} \times 100\%$$

Determination of Intracellular Free Calcium

Intracellular free calcium of the alveolar monolayers was determined using dual-excitation fluorescence microscopy with the aid of a specific calcium indicator, the acetoxymethyl ester of Fura-2. Monolayers were incubated with the probe at a 1 μ*M* concentration in HEPES-buffered medium for 30 min at room temperature to allow ester hydrolysis to take place within the cells, after which they were washed. Changes in Fura-2 fluorescence upon binding with calcium were recorded under the Nikon Diaphot microscope. Excitation light was provided by two monochromators preset at 340 and 380 nm and emitted light was collected through a 510-nm interference filter. The ratio of fluorescence excited at 340 and 380 nm was used to calculate calcium concentrations, according to the equation $[Ca^{2+}]_i = K_d F_o(R - R_o)/F_s(R_s - R)$, where R is the fluorescence ratio, F is the fluorescence measured at excitation of 380 nm, subscripts o and s denote zero and saturated calcium conditions, and K_d is the effective dissociation constant for fura-2. R_o and R_s were determined with the aid of the membrane lysing agent, digitonin (10 μ*M*), and the calcium chelator, EGTA (10 m*M*), respectively.

RESULTS AND DISCUSSION

Effects of Oleic Acid on Alveolar Epithelial Permeability and Cytotoxicity

Figure 1 shows the effect of the concentration of OA (10–100 μ*M*) on transepithelial transport of the paracellular tracer ^{3}H-mannitol and transcellular tracer ^{14}C-progesterone. A concentration-dependent increase in both paracellular and transcellular transport is evident. Fluorescence PI

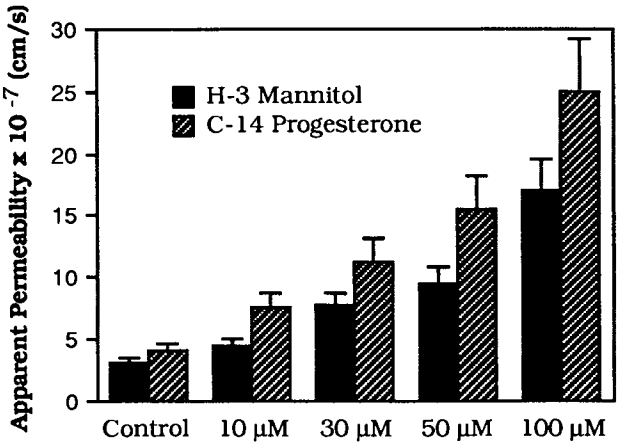

Fig. 1. Effects of concentration of oleic acid on transepithelial transport of ^{3}H-mannitol and ^{14}C-progesterone. The experiments were conducted using alveolar epithelial monolayers grown in primary culture for 6 days. The apparent permeability (mean ± SE; $n = 6$) was calculated from steady-state flux of the radioactive tracers as described under Materials and Methods. All treatment values were significantly greater than controls ($P < 0.05$).

studies also show that at a concentration of 50 μ*M* or below, OA caused no significant cell damage compared to the control, i.e., 8 ± 3 vs 11 ± 4% after a 3-hr incubation period, whereas 100 μ*M* OA severely damaged the cells, i.e., 8 ± 3 vs 74 ± 12% (Fig. 2). The effect of OA at low concentrations on transcellular transport of progesterone is believed to be due to an increase in membrane fluidity since OA is known to promote motional freedom of the membrane phospholipids in various membrane systems (1–4). However, the OA effect on paracellular permeability as well as its promoting mechanism are not clearly understood, although indirect evidence suggests that a calcium-dependent mechanism may participate in such a process. For example, OA has been shown to activate calcium mobilization in a number of cell systems including cardiac myocytes (8), hepatocytes (9), and endocrine cells (10), and an increase in $[Ca^{2+}]_i$ is known to promote tight junction permeability in a variety of epithelia (11–14). To test the hypothesis that OA promotes paracellular permeability via a calcium-dependent mechanism, epithelial monolayers were exposed to varying concentrations of OA (0–100 μ*M*) and their $[Ca^{2+}]_i$ response was monitored. As expected, OA caused a concentration-dependent increase in $[Ca^{2+}]_i$ with the effect being transient at low concentrations (≤50 μ*M*) but becoming more pronounced and sustained at higher concentrations (Fig. 3). The sustained effect of OA is believed to be responsible for the observed cellular damage since an irreversible increase in $[Ca^{2+}]_i$ by a variety of toxicants is known to produce cell lysis and death. In pulmonary epithelium, a high-dose exposure to calcium ionophore was reported to result in elevated phospholipase activities and a parallel increase in cell injury (15). The observation that the effect of OA at low concentrations on cellular calcium is transient also implies that its permeability enhancing effect may be reversible. Indeed, when the monolayers were exposed to OA (50 μ*M* for 1 hr) and then washed with buffer (containing 0.1% ethanol, to aid OA removal), their permeability to ^{3}H-mannitol was completely restored (Fig. 4). Similarly, transepithelial electrical resistance measurements also indicated full recovery of the monolayers' resistance, i.e., from the initial value of 1486 ± 132 to 1069 ± 114 Ω · cm^2 after a 1-hr OA treatment and 1474 ± 137 Ω · cm^2 after OA washout. The above observations suggest an intimate relation between $[Ca^{2+}]_i$ levels and paracellular permeability. A similar reversible effect was also observed in alveolar epithelium treated with a low-concentration calcium ionophore (14).

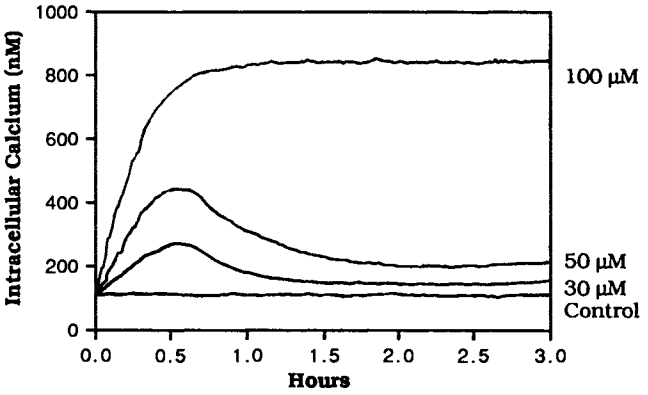

Fig. 3. Effect of concentration of oleic acid on intracellular calcium. The monolayers were loaded with 1 μ*M* Fura-2 AM and then incubated in HEPES-buffered medium containing oleic acid. Intracellular calcium was calculated from the Fura-2 fluorescence ratio excited at 340 and 380 nm. The trace is representative of four experiments obtained from different cell preparations.

Mechanistic Studies of Oleic Acid-Induced Calcium Rise and Its Structural Requirement

To investigate further the mechanisms of OA-induced calcium overloading, we exposed the monolayers to OA in calcium-free medium, and their $[Ca^{2+}]_i$ responses were similarly monitored. Under this condition, OA failed to activate $[Ca^{2+}]_i$ elevation (Fig. 5), suggesting that Ca^{2+} influx from extracellular sources is responsible for the observed $[Ca^{2+}]_i$

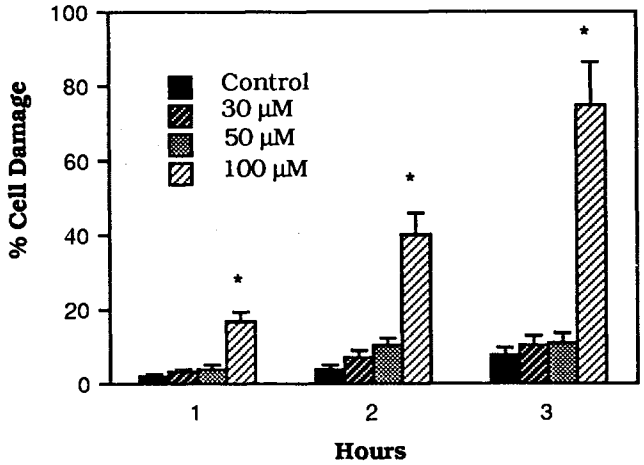

Fig. 2. Effect of incubation time and concentration of oleic acid on epithelial damage. The monolayers were incubated in HEPES-buffered medium containing 1 μ*M* propidium iodide. Cell damage was calculated from nuclear propidium iodide fluorescence. Each data point represents the mean ± SE for ≥6 determinations. (*) Significant increase over controls ($P < 0.01$).

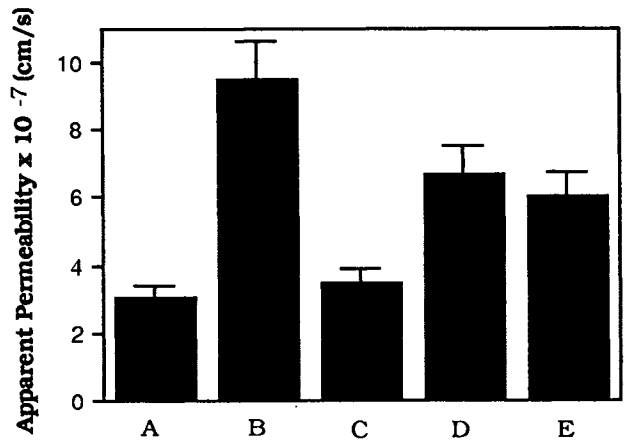

Fig. 4. Effects of oleic acid, oleic acid washout, and nifedipine on paracellular permeability. (A) Control; (B) monolayers treated with 50 μ*M* oleic acid; (C) monolayers after preexposure to 50 μ*M* oleic acid for 1 hr and then washout; (D,E) same as B but in the presence of 1 and 10 μ*M* nifedipine, respectively. Each data point represents the mean ± SE for ≥6 determinations. All values were significantly lower than OA-treated controls ($P < 0.01$).

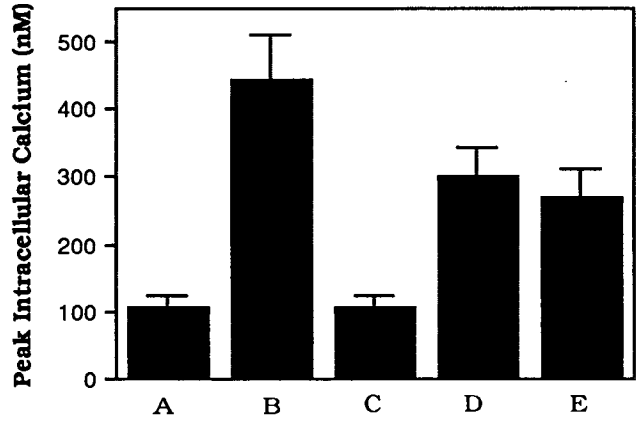

Fig. 5. Effects of extracellular calcium chelation and nifedipine on oleic acid-induced calcium rise. (A) Control; (B) monolayers treated with 50 μM oleic acid in HEPES-buffered medium containing 1 mM Ca^{2+}; (C) same as B but without Ca^{2+} + 100 μM EGTA (to deplete trace Ca^{2+}); (D,E) same as B but in the presence of 1 and 10 μM nifedipine, respectively. Each data point represents the mean ± SE for ≥6 determinations. All values were significantly lower than OA-treated controls ($P < 0.01$).

rise. Because OA has been shown to activate calcium channels and induce Ca^{2+} influx in cardiac myocytes (8), it is possible that it may do so in alveolar epithelial cells. To test this possibility, the monolayers were exposed to OA (50 μM) in the presence of the calcium channel blocker nifedipine or verapamil (1 and 10 μM) and their $[Ca^{2+}]_i$ levels and paracellular permeability were monitored. Under these conditions, nifedipine significantly but only partially inhibited the two effects of OA (Figs. 4 and 5), whereas verapamil had no significant effects (results not shown). These results suggest a certain degree of specificity of the OA effects and possible involvement of other calcium regulating mechanisms. Because OA is capable of disrupting the membranes, it is possible that the observed $[Ca^{2+}]_i$ rise is due to nonspecific membrane damage. However, the results from PI fluorescence studies indicate that at low OA concentrations (≤50 μM) the cells were be able to exclude the dye after the onset of $[Ca^{2+}]_i$ change and the dye continued to be excluded until 3 hr following OA exposure (Fig. 2). In addition, the reversal of the calcium effect and permeability suggests that the cell membrane maintains its integrity and that these effects are not due to simple membrane leakage. Theoretically, OA may interact with membrane phospholipids and alter their environment, thereby facilitating Ca^{2+} influx. Alternatively, OA may act directly on the channel protein itself. The latter was supported by recent evidence that sulfhydryl modification of membrane-associated proteins reversibly inhibited the OA-induced effect on intestinal permeability (16).

The effects of OA appeared to reside in the free carboxyl group because esterified OA (oleic methyl ester) had no effects on either the calcium induction or the permeability alterations (Table I). The latter observation is consistent with previous studies in perfused rat lungs using oleyl alcohol (7). The dependence of the effects of OA on carboxyl group raises the possibility that local acidity may also contribute to the OA effects. This possibility was tested by conducting experiments using stearic acid, a fatty acid with equal chain

Table I. Structural Effects of Fatty Acids on Paracellular Permeability and Intracellular Calcium in Alveolar Epithelium

Fatty acid[a]	Apparent permeability × 10^{-7} (cm/sec)[b]	$[Ca^{2+}]_i$ (nM)[b]
Control	3.1 ± 0.5	108 ± 17
Oleic methyl ester	3.3 ± 0.6	114 ± 19
Stearic acid (18:0)	4.9 ± 0.7*	232 ± 29*
Oleic acid (18:1 cis Δ^9)	9.5 ± 1.4*	445 ± 46*
Linoleic acid (18:2 cis $\Delta^{9,12}$)	9.7 ± 1.6*	448 ± 38*
Linolenic acid (18:3 cis $\Delta^{9,12,15}$)	9.4 ± 1.5*	439 ± 41*

[a] Concentration, 50 μM.
[b] Mean ± SE; $n \geq 6$.
* Significant increase from the controls ($P < 0.01$).

length and number of free carboxyl groups. The result indicated that, at an equimolar concentration of 50 μM, stearic acid caused much reduced effects on the two parameters (Table I), thereby arguing against the possible contribution of acidity. Because stearic acid is a saturated fatty acid, this result also suggested that the OA effects required the presence of an unsaturated group(s). This observation is in good agreement with previous findings by Aungst (5), which demonstrated that OA was approximately three times more potent than stearic acid in promoting transdermal flux of naloxone. To investigate further the effect of degree of unsaturation of fatty acids, the cell monolayers were treated with various fatty acids differing only in their degree of unsaturation, i.e., linolenic acid (18:3), linoleic acid (18:2), and oleic acid (18:1). In these studies, all three fatty acids exhibited similar and comparable effects on the monolayer permeability and cellular calcium (Table I), suggesting a relatively insignificant role of degree of unsaturation.

CONCLUSION

The present study focuses on the effects and mechanisms of OA- and fatty acid-induced paracellular permeability alteration in cultured alveolar monolayers. Our results indicated a close relation between permeability enhancement and $[Ca^{2+}]_i$ activation caused by OA. At low concentrations (≤50 μM), OA reversibly increased alveolar permeability without causing measurable membrane damage and had only a transient effect on $[Ca^{2+}]_i$ levels. Further enhancement of membrane permeability at high concentrations of OA is probably due to its direct disrupting effect on cellular integrity, which further leads to sustained calcium overload and irreversible cell damage. The effect of OA on $[Ca^{2+}]_i$ appeared to be mediated through Ca^{2+} influx from extracellular source, possibly via specific Ca^{2+} channels and perhaps other Ca^{2+} regulating mechanisms. Carboxyl and unsaturated groups are required for both calcium activation and permeability alterations, however, the degree of unsaturation appeared unimportant. The findings are consistent with the hypothesis that the effect of OA on the paracellular epithelial barrier is mediated by $[Ca^{2+}]_i$ activation and consequent alteration of the tight junction structures.

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