Fatty Acid Metabolism in Human Keratinocytes Cultivated at an Air-Medium Interface

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Stratum corneum lipids, which provide the mammalian permeability barrier, display a distinctive fatty acid profile with a predominance of long chain, saturated fatty acids. In addition, linoleic acid (18:2) is present in substantial quantities, implying that it is an important structural component. To investigate selectivity of fatty acid incorporation into epidermal lipids, we examined the metabolism of exogenous fatty acids in cultured human keratinocytes, grown at the air-medium interface to enhance differentiation. Keratinocytes were pulsed with [^{3}H] oleic, [^{14}C] stearic, [^{14}C] palmitic, or [^{14}C] linoleic acids; lipids were extracted and fractionated by thin layer chromatography. All fatty acids were taken up and incorporated into complex lipids in a dose-dependent manner that was linear over the first 60 min. These fatty acids were incorporated predominantly into phospholipids and triacylglycerols; their incorporation could be rank ordered: linoleic > oleic ≥ palmitic > stearic acid. Less than 2% of each fatty acid taken up by keratinocytes was oxidized to CO_2; therefore, these differences in utilization cannot be ascribed to differences in rates of β-oxidation. In pulse-chase studies fatty acids incorporated initially into triacylglycerols, subsequently chased into phospholipids. [^{14}C]Palmitic acid and [^{14}C] acetate were incorporated into sphingolipids more efficiently than the other fatty acids studied. These studies demonstrate that 1) keratinocytes have the ability to incorporate exogenous fatty acids preferentially into complex lipids; 2) triacylglycerols provide a pool of fatty acids for phospholipid synthesis; and 3) palmitate and de novo synthesized fatty acid are preferably utilized for sphingolipid synthesis. *J Invest Dermatol 92:196–202, 1989*

The final product of epidermal differentiation is the formation of the stratum corneum, whose lipid constituents provide the barrier to transcutaneous water loss [reviewed in Ref 1]. Stratum corneum lipids form lamellar bilayers in the intercellular domains [2–4] and are composed predominantly of ceramides and non-polar lipids [5–7]. The fatty acid profile is distinctive: a large proportion are long (C16–C18) and very long chain (C20–C26), saturated species; and the dienoic essential fatty acid, linoleic acid (18:2), accounts for up to 15% of fatty acids in both neutral lipids and ceramides [6,7]. The role of linoleic acid in barrier function is demonstrated by studies in essential fatty acid-deficient animals, where topical linoleic acid corrects barrier function even in the presence of lipooxygenase or cyclooxygenase blockade [8,9]. Certain unusual sphingolipids are also present in mammalian epidermis and stratum corneum, which contain linoleic acid esterified to the terminal ω-hydroxyl group of very long chain (>C35) N-acyl α-hydroxy fatty acids [10–12]. Although linoleate-containing sphingolipids are present in relatively small amounts [6,13], these molecules may be important for stacking of lamellar body discs [14]. Recent in vivo studies have shown that epidermis synthesizes both non-saponifiable lipids and fatty acids [15], and that this lipogenic activity is regulated by barrier requirements [16]. While epidermis must derive linoleic acid from systemic sources, the extent to which non-essential fatty acids derive from the circulation vs. local synthesis is not known.

In order to address this question, we have studied the uptake of exogenous fatty acids by keratinocytes and their incorporation into complex lipids. Keratinocytes were cultivated at an air-medium interface in order to enhance differentiation [17–20]. These studies show that keratinocytes demonstrate different utilization patterns for linoleic, oleic, palmitic, and stearic acids. Moreover, keratinocytes prefer palmitic acid and possibly de novo synthesized fatty acids over the other fatty acids tested for the synthesis of ceramides and glycosphingolipids.

MATERIALS AND METHODS

Lifted Keratinocyte Cultures Our method for keratinocyte cultivation at the air-medium interface has been described recently [19]. Briefly, a suspension of keratinocytes (first passage cells derived from one to three pooled neonatal foreskin populations) and mitomycin C-treated 3T3 mouse fibroblasts were seeded onto Vitrogen 100-collagen gel-coated (Collagen Corp., Palo Alto, CA), Nylaflo membranes (47 mm, 0.2 μm pore size, Gelman Sciences, Ann Arbor, MI), and cultured submerged in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal calf serum (FCS;

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Abbreviations:
BSA: bovine serum albumin
DMEM: Dulbecco's modified Eagle medium
FCS: fetal calf serum
NS: not significant
PBS: phosphate buffered saline
TLC: thin layer chromatography

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Whittaker M.A. Bioproducts, Walkersville, MD), 20 ng/ml epidermal growth factor from mouse submaxillary glands (Collaborative Research, Lexington, MA), 0.5 μg/ml hydrocortisone (Sigma, St. Louis, MO), 1 nM cholera toxin (Sigma), 1.0 μg/ml glutamine (Gibco, North Andover, MA), and antibiotics. When cultures reached confluence, the membranes were lifted onto two stacked 47-mm glass wool pads (Type A/E; Millipore Corp., Bedford, MA) saturated with 10 ml of media without epidermal growth factor in 100-mm dishes. Media were supplemented at the first post-lifting feeding only with 10 μM linoleic acid (Sigma), 2 mM serine (Sigma), 0.6 mM sodium acetate (Sigma), fatty acid-free, bovine serum albumin (BSA; Sigma), and 1 μM α-tocopherol (Sigma) to prevent formation of lipid peroxides [21]. Media without additional supplements were renewed three times weekly, and all studies were performed at 1 week post-lifting. Cultures at this time consist of three or more layers of nucleated cells surmounted by multiple layers of cornified cells; typical lamellar bodies are present ultrastructurally and the lipid composition of these cultures is closer than submerged cultures to that observed for epidermis in vivo [19].

Preparation of Radiolabel [2-^{14}C] Acetic acid, sodium salt (50 mCi/mmole; S.A., ICN-Radiochemicals) was taken up in phosphate buffered saline (PBS) at a final concentration of 1 μCi/μl. Radiolabeled fatty acids, [1-^{14}C] linoleic (S.A.: 59 mCi/mmole; Amersham, Arlington, IL), [9,10 (n)^{3}H] oleic (S.A.: 4.2 Ci/mmole; Amersham) [1-^{14}C] stearic (S.A.: 53 mCi/mmole; ICN-Radiochemicals), [1-^{14}C] palmitic (S.A.: 48 mCi/mmole; ICN-Radiochemicals), [1-^{14}C] oleic acid (S.A.: 56 mCi/mmole; ICN-Radiochemicals) were checked for purity by silica gel thin layer chromatography (TLC) prior to use. Radiolabeled fatty acids (with varying amounts of the corresponding unlabeled fatty acids, as necessary to achieve the desired final concentrations) were solubilized in a small volume of ethanol and transferred to a flask using DMEM without fetal calf serum and containing fatty acid-free BSA (fatty acid:BSA ratio ≤ 3:1), and stirred mechanically at 60 rpm. The ethanol-containing tube was rinsed twice with DMEM and BSA to ensure complete transfer. When necessary, the pH was adjusted to 7.4 with 0.1 N HC1 or 0.1 N NaOH. These preparations were not filtered because preliminary studies showed that up to 40% of the BSA-fatty acid complex was lost onto the Millipore membrane during filtration. The final ethanol concentration in tissue incubations did not exceed 0.2%.

Incorporation of Radiolabel into Lipid To ensure that lifted keratinocyte cultures had sufficient access to the label, in most experiments incubations were performed under submerged conditions by transferring cultures on the Vitrogen-coated membranes to fresh 60-mm dishes and overlaying cultures with media (4 ml) containing the radiolabel. The cellular uptake and incorporation into lipid was reduced by 1/3 or more when cells were incubated with radiolabel under lifted conditions. However, the relative distribution of radiolabel into cellular lipid remained unchanged. At the end of the labeling period cultures were either submerged with gentle agitation in three consecutive 100-mm dishes containing 10 ml ice-cold phosphate buffered saline (PBS) and harvested; or they were rinsed similarly in room temperature PBS, transferred to fresh glass wool pads in 100-ml dishes containing fresh media with 5% FCS, and harvested at various time points. In each experiment studies were performed in triplicate using keratinocytes from a single foreskin preparation. All experiments have been repeated at least twice; data shown are from representative experiments.

Cell Harvesting Cells were harvested by scraping with a razor blade into metered microfuge tubes (Tegal Scientific, Concord, CA). The membranes were rinsed with 500 μl ice-cold PBS, and this rinse was added to the pellet. A cell homogenate was prepared by sonication on ice with 3–4 × 20 sec bursts at the relative output of 35% with a sonic dismembranator (Fisher Scientific Model 300). Prior to the last sonication, the volume was adjusted to 1 ml. Aliquots were taken into scintillation cocktail (Scinti-Verse II, Fischer, Pittsburgh, PA) for scintillation counting (Beckman LS 1800) and DNA content [22]. Lipids were extracted according to the method of Bligh and Dyer [23]; in our studies the extraction efficiency using L-3-Phosphatidyl[N-methyl-3H]1,2 dipalmitoylcholine (S.A. 76 Ci/mmole; Amersham), and [3H]-oleic acid as internal standards were ≥ 89% and ≥ 95%, respectively.

Thin Layer Chromatography Lipid extracts were dried under a stream of nitrogen, redissolved in 2 ml of $CHCl_3$:MeOH (2:1, vols), and a 50 μl aliquot was taken for scintillation counting. Three aliquots were taken for neutral lipid, polar lipid, and sphingolipid fractionation by thin layer chromatography (TLC) on silica-gel plates (Silica gel 60, Merck, Darmstadt, FRG), as previously described [7,24]. Lipid fractions were identified by cochromatography with authentic standards, visualized under black light after spraying with 0.2% aqueous solution of 8-aniline-1-naphthalene sulfonic acid and scanned using a Berthold LB-283 TLC linear analyzer. Appropriate zones were scrapped directly into scintillation cocktail and counted. Data obtained from the linear analyzer correlated well with that obtained by scintillation counting as long as there were 10,000 cpm or more in each of the fractions. Incorporation into each lipid fraction was calculated by multiplying the present radioactivity found in that lipid fraction by the total radioactivity incorporated into lipid. Data are expressed as pmoles exogenous fatty acid incorporated/μg DNA, as calculated from the specific activity of the fatty acid added to the media. Because the specific activity of the intracellular fatty acid pools was not determined, these data may not be the same as the actual quantities of each fatty acid incorporated in complex lipids, but they do permit comparison of utilization between several exogenously supplied fatty acids.

Assessment of β-Oxidation β-Oxidation was assessed by the method of Rodbell [25], as modified [26]. Briefly, cells were scraped off the membrane, placed in 25-ml Warburg flasks containing a center well, and incubated for 1 h submerged in 4 ml DMEM plus [1-^{14}C]linoleic, [1-^{14}C]oleic, [1-^{14}C]palmitic, or [1-^{14}C]stearic acids. Flasks were flushed with 95% O_2 and 5% CO_2 before the incubation. Hyamine hydroxide (0.4 ml) was added to the central well, and the medium acidified by addition of 1 ml of 2N H_2SO_4. Flasks were incubated for an additional 30 min at 37°C, stored at 4°C for several hours, and 100-μl aliquots were taken from the well from scintillation counting in 8 ml Aquasol (New England Nuclear, Boston, MA). In order to determine the non-enzymatic production of [^{14}C]-CO_2, parallel cultures were denatured by heat (95°C for 15 min) prior to incubation with the radiolabels, and this result was subtracted from experimental samples. Parallel cultures were incubated under the same conditions for determination of both DNA content and incorporation of radiolabel into complex lipids.

Statistics Statistical significances were determined using a two-tailed Student's t test.

RESULTS

Fatty Acid Uptake and Incorporation into Lipid The cellular uptake and incorporation into lipid of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), and linoleic acid (18:2) (10 μM each) were examined over a 24-h time-period (Fig 1*A-D*). [Incorporation into lipid as used here indicates that portion of radiolabel which is retained as lipid (i.e., extracted from the cell homogenate by solvents) and does not include radiolabel that may have been incorporated into complex lipid before further metabolism to CO_2 or water soluble materials.] The uptake and lipid incorporation of all fatty acids studied was linear over the first 2 h. Whereas linoleic acid incorporation did not increase further after 6 h (Fig 1*A*), incorporation of palmitic, oleic, and stearic acids continued to increase up to 24 h (Fig 1*B–D*). During the first hour, the uptake and incorporation of linoleic acid was 12% of radiolabel added, 9.4% palmitic acid, 8% oleic acid and 7.5% stearic acid; but these differences were not statistically significant. Based upon these experiments, we subsequently employed an incubation time of 1 h during which incorporation is linear and no more than 12% of the substrate utilized.

The dose response curves for the uptake and incorporation of the

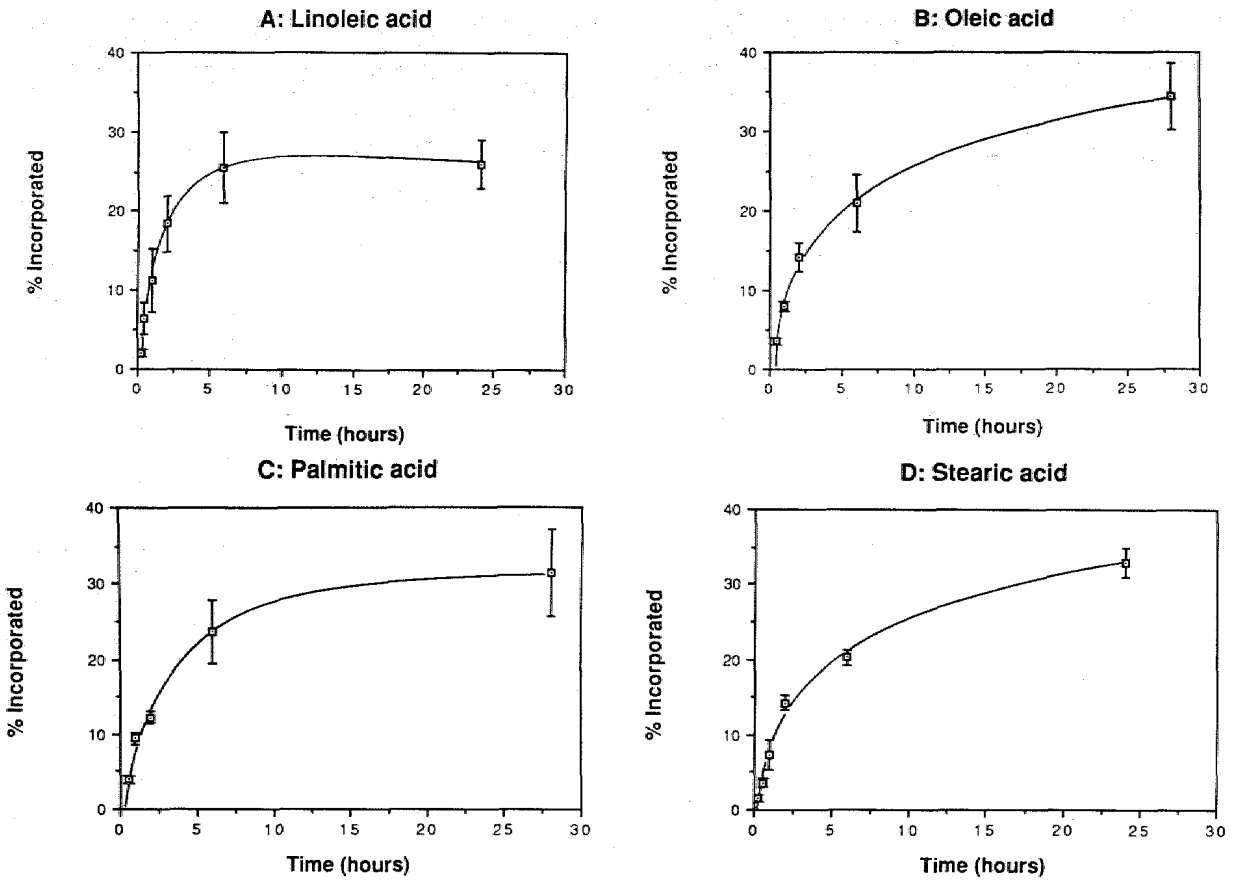

Figure 1. Time course of fatty acid metabolism. The uptake and retention of [^{14}C] linoleic *(panel A)*, [^{3}H]oleic *(panel B)*, [^{14}C]palmitic *(panel C)*, and [^{14}C]stearic acid *(panel D)* were examined over a 24-h time period. Keratinocyte cultures were incubated under submerged conditions with 10 μM of one of the fatty acids (S.A. of radiolabel as added: 59 mCi/mmole; 76 mCi/mmole; 48 mCi/mmole; 53 mCi/mmole, respectively) for 30 min, 1, 2, 6, or 24 h. Cultures were harvested, lipids extracted, and the incorporation into lipid was determined. Data are expressed as percent incorporation into lipid of total added radiolabel and represent the mean ± SD of three dishes each.

four fatty acids are shown in Fig 2*A–D*. The incorporation of linoleic (Fig 2*A*), oleic (Fig 2*B*), palmitic (Fig 2*C*), and stearic (Fig 2*D*) acids was linear over the range of concentrations examined (R values: linoleate, 1.00; oleate, 0.97; palmitate, 0.99; and stearate, 0.96, respectively). At 10 μM concentrations, the uptake of linoleic, oleic, and palmitic acids was comparable; however, at lower concentrations (0.1 μM), the uptake of linoleic acid significantly exceeded the others. In contrast, the uptake and retention of stearic acid were significantly lower than the other three fatty acids at concentrations of 10 μM or more. Based upon these data, a concentration of 10 μM for all fatty acids was employed for subsequent pulse-chase experiments.

Distribution of Radiolabel between Phospholipids and Triglycerides Keratinocytes were incubated for 15 min, 30 min, 1, 2, 6, or 24 h with each of the radiolabeled fatty acids (10 μM), then harvested, and the distribution of radiolabel among complex lipids was assessed by TLC. Data are shown here for the incorporation into phospholipids and triglycerides over the first 6 h of the study (Fig 3*A,B*, respectively). Linoleic acid was rapidly incorporated into phospholipids. Even at the earliest time point (15 min), more than 75% of the radiolabel was incorporated into the phospholipid fraction (Fig 3*A*), and incorporation of linoleic acid into phospholipids continued to increase over the next 6 h. In contrast, the other three fatty acids were incorporated into phospholipids to a much lesser extent during the first 2 h, but labeling of phospholipids continued to increase during the next 22 h (Fig 3*A*). At early time points (<2 h), the extent of incorporation of oleic and palmitic acids into triglycerides (Fig 3*B*) equalled or exceeded that for phospholipids (Fig 3*A*), while with linoleic acid incorporation into phospholipids usually exceeded that of triglycerides in this and other experiments (data not shown). Stearic acid was poorly incorporated into complex lipids at these time points (<6 h) (Fig 3*A,B*), although by 24 h incorporation into phospholipids approached that seen with palmitic acid (data not shown).

Transfer of Fatty Acid Moieties between Lipid Classes In order to compare the pathways of exogenous fatty acid utilization after initial incorporation into cellular lipids, cells were pulse-labeled with 10-μM radiolabeled palmitic, stearic, oleic, or linoleic acids for 1 h, washed, and then maintained in unlabeled medium over a 24-h time period (see *Methods* (Fig 4). After the initial pulse, the amount of radiolabel remaining in the free fatty acid form could be rank ordered: stearic 11.67 > oleic 8.50 > linoleic acids 6.05 ≥ palmitic 5.51, pmoles exogenous fatty acid incorporated/μg DNA, respectively (significances: stearate vs. oleate, $p < 0.01$; stearate vs. palmitate or linoleate, $p < 0.001$; oleate vs. palmitate or linoleate, $p < 0.02$; palmitate vs. linoleate, NS).

Incorporation of linoleic acid into phospholipids at the end of the pulse period was almost twofold higher than that of the other three

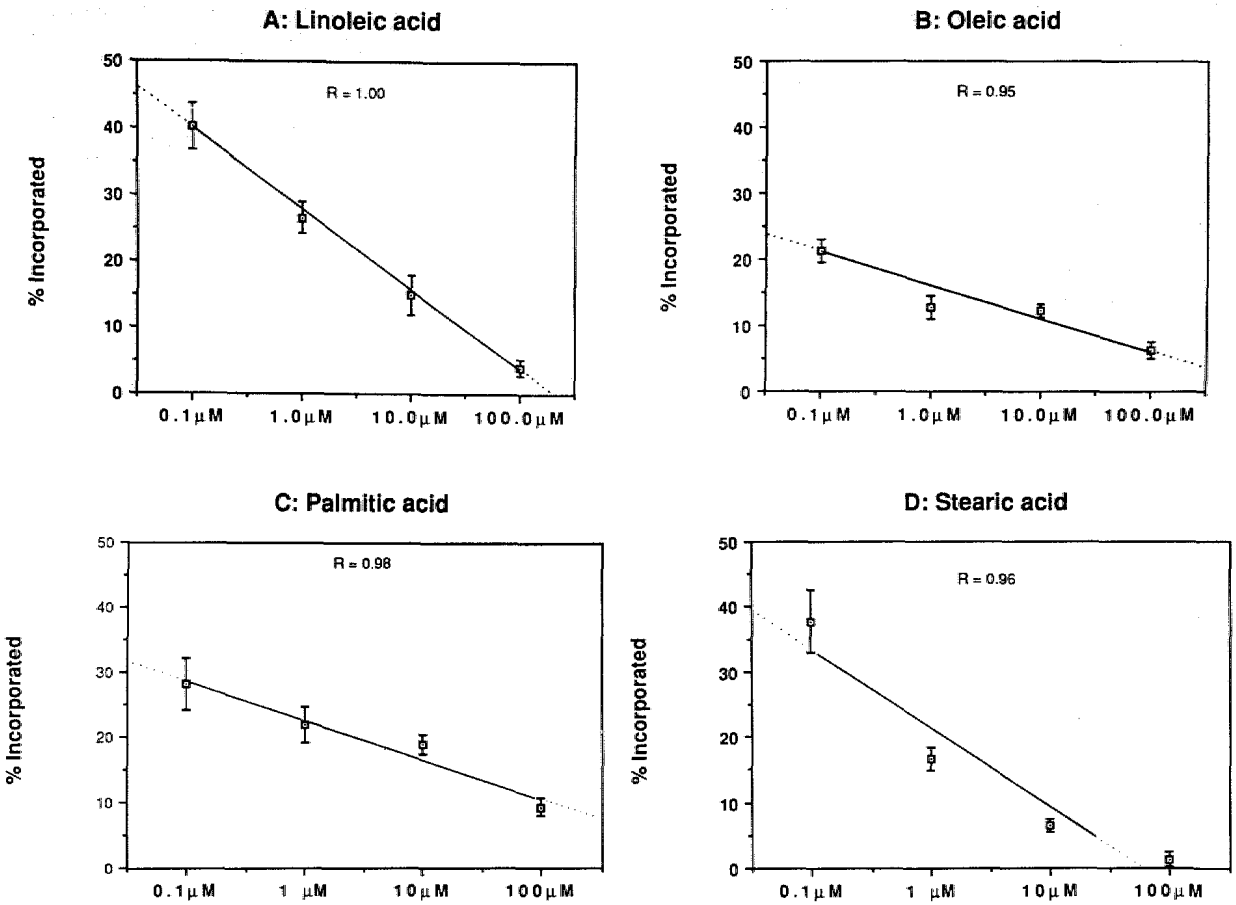

Figure 2. Dose response curves for uptake and retention of fatty acids. [^{14}C]-Linoleic *(panel A)*, [^{3}H]-oleic *(panel B)*, [^{14}C]-palmitic *(panel C)*, and [^{14}C]-stearic acids *(panel D)* in concentrations of 10^{-7}M through 10^{-4}M were incubated with keratinocyte cultures under submerged conditions. After 1 h, cultures were harvested and the incorporation into lipid determined. Data are expressed as percent incorporation into lipid of total added radiolabel and represent the mean ± SD of three dishes each.

fatty acids (pmoles exogenous fatty acid incorporated/ug DNA: linoleate 30.93 > palmitate 18.56 ≥ oleate 11.40 ≥ stearate 9.83; significances: linoleate vs. palmitate, $p < 0.05$; linoleate vs. oleate or stearate, $p < 0.01$; palmitate vs. oleate vs. stearate, NS) Fig 4*A–D*). The incorporation of all radiolabeled fatty acids into phospholipids increased during the 24 h chase (Fig. 4*A–D*). Yet, the incorporation of linoleic acid into phospholipids still significantly exceeded that of the other fatty acids (significances: linoleate vs. palmitate, $p < 0.05$; linoleate vs. oleate or stearate $p < 0.01$). Accumulation of label in phospholipid during the 24-h chase was accompanied by a concomitant decrease in radioactivity in the triacylglycerol fraction, for all fatty acids except stearic acid, where the transfer to phospholipids reflected predominantly a decrease in the radiolabeled free fatty acid fraction. This is consistent with the observation that stearic acid is incorporated into triacylglycerides to a much lesser extent than the other three fatty acids (Fig 3).

The relative and absolute amount of radiolabel incorporated into the sterol ester fraction was comparable for all fatty acids studied and represented a small fraction of the total radiolabel incorporated (Table I). The incorporation of radiolabeled fatty acids into sphingolipids also represented a small fraction of the total; however, at the end of the pulse incorporation of palmitate was greatest (3.11), followed by oleate (1.60) ≥ linoleate (1.43) > stearate (0.84) (pmoles exogenous fatty acid incorporated/ug DNA) (significances: palmitate vs. linoleate, oleate or stearate $p < 0.01$; oleate vs. linoleate NS, linoleate or oleate vs. stearate $p < 0.05$) (Table I). Finally, by the end of the chase period substantially more radiolabeled palmitic acid (12.21 pmoles exogenous fatty acid incorporated/μg DNA) and stearic acid (3.90 pmoles exogenous fatty acid incorporated/μg DNA), accumulated in sphingolipids compared with the other fatty acids. (Significances: palmitate vs. linoleate, oleate, or stearate $p < 0.001$; stearate vs. linoleate or oleate $p < 0.02$; oleate vs. linoleate NS).

Incorporation of exogenous fatty acids was compared to de novo synthesized fatty acids by examining the incorporation of [^{14}C]-acetate into acyllipids in parallel cultures. Keratinocytes were incubated under submerged conditions with [^{14}C]acetate (5 μCi/ml; 0.75 mM) for 1 h and harvested (pulse) or placed in fresh media without radiolabel and harvested after 24 h (chase). Incorporation of exogenous acetate into sphingolipids did not differ significantly following the pulse or chase (24.48 vs. 17.98 pmoles exogenous acetate incorporated/μg DNA). Sphingolipid synthesis accounted for a significantly greater fraction of acetate incorporation into acyl lipids than observed for any of the exogenous fatty acids (acetate to sphingolipids: 10.7% of total incorporation of radiolabel into lipid vs. palmitate 3.4%; or vs. linoleate, oleate or stearate <2.5%).

β-Oxidation In order to determine whether differences in the utilization of fatty acids reflect differences in their utilization for β-oxidation, we examined CO_2 generation from [^{14}C]-palmitic,

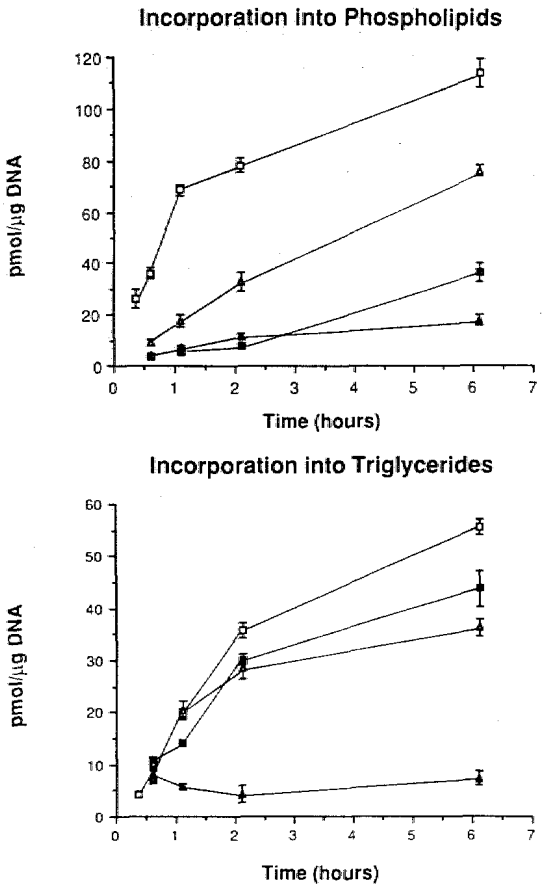

Figure 3. Incorporation of exogenous fatty acids into phospholipids and triacylglycerides. Keratinocytes were incubated for 15 min, 1, 2, 6, and 24 h with [^{14}C]-linoleic *(open squares)*, [^{3}H]-oleic *(closed squares)*, [^{14}C]-palmitic *(open triangles)*, and [^{14}C]-stearic acids *(closed triangles)* (S.A. of radiolabel as added: 59 mCi/mmole; 76 mCi/mmole, 48 mCi/mmole and 53 mCi/mmole, respectively) (see *Methods* and legend to Fig 1). The distribution of radiolabel among phospholipids *(top panel)* and triacylglycerides *(bottom panel)* was assessed by thin layer chromatography. Data are expressed as pmoles of exogenous fatty acid incorporated/μg DNA, and represent the mean ± SD of three dishes each.

[^{14}C]-linoleic, [^{14}C]-oleic and [^{14}C]-stearic acids (see *Methods*). During a 1-h pulse, approximately 2% of each of cell-associated fatty acids were oxidized to CO_2. In contrast, parallel cultures incubated with radiolabeled acetate oxidized 10%–20% of substrate to CO_2, indicating that these cultures were viable and utilizing alternative pathways for energy production. Further evidence that exogenous fatty acids are not utilized extensively for β-oxidation by cultured keratinocytes is given by the observation that total radioactivity in lipid was essentially unchanged at the end of the 24-h chase in the pulse-chase studies described above (Fig 4).

DISCUSSION

In these studies, we have observed distinctive differences in the uptake and incorporation of four exogenous fatty acids by differentiated keratinocyte cultures. Cellular uptake was dependent upon the concentration of fatty acids in the media. All but stearic acid were rapidly incorporated into phospholipids, but at early time points linoleic acid was incorporated more rapidly than were the other fatty acids, and its incorporation into phospholipid was more extensive at all time points (Figs 3 and 4). In pulse-chase studies an

Table I. Incorporation of Radiolabeled Fatty Acids into Ceramides, Glycosylceramides, and Sterol Esters: Pulse-Chase Study[a]

	Incorporation Into Lipid Fraction (pmol/μg DNA)			
	Linoleic acid	Oleic acid	Palmitic acid	Stearic acid
Glycosphinogolipids				
Pulse	0.78	0.75	0.82	0.41
Chase	0.70	0.81	8.76	2.72
	NS[b]	NS	$p < 0.001$	$p < 0.001$
Ceramides				
Pulse	0.65	0.85	2.29	0.43
Chase	0.29	0.40	3.45	1.18
	$p < 0.01$	$p < 0.001$	NS	NS
Sterolesters				
Pulse	1.10	0.31	0.69	0.19
Chase	0.70	0.91	1.63	0.86
	NS	$p < 0.01$	$p < 0.01$	NS

[a] Keratinocytes were pulsed with [^{14}C]-linoleic, [^{3}H]-oleic, [^{14}C]-palmitic, or [^{14}C]-stearic acids for 1 h and either harvested (pulse) or placed in fresh media for 24 h and harvested (chase) (see the legend to Fig 4 and *Methods*). Data are expressed as pmoles exogenous fatty acid incorporated/μg DNA and represent the mean of three dishes each. Significance was determined by a two-tailed Student's t test.

[b] NS: not significant.

increase in phospholipid labeling during the chase was observed and was accompanied by a decrease in labeling of triacylglycerols (Fig 4). While linoleic, oleic, and palmitic acid were all extensively incorporated into triacylglycerols at early time points (Fig 3), during the 24-h chase, linoleic- and oleic-containing triacylglycerols were used more extensively for phospholipid synthesis than were palmitic-triacylglycerols. On the other hand, palmitic acid was incorporated more extensively than the other fatty acids into sphingolipids, both during a brief (1 h) pulse and during the chase. In general, the relative distribution of stearic acid into major lipid fractions was similar to that of palmitic acid, but the extent of its incorporation into complex lipids, including sphingolipids, was much reduced (Fig 4*D*).

These observed differences in fatty acid utilization may reflect differences either 1) in cellular uptake, 2) in their affinity to fatty acid binding protein(s), 3) in the acyl specificity of enzymes involved in synthesis of complex lipids, or 4) a combination of these factors. Cellular uptake of fatty acids occurs by passive diffusion without specificity for chain length or saturation [27,28]. Recent studies in hepatocytes have demonstrated a second, ATP and sodium-dependent uptake mechanism [29]; however, the acyl specificity of this system has yet to be characterized. The mechanisms[s] for fatty acid uptake by keratinocytes has not yet been addressed. Several fatty acid binding proteins have been described which show restricted tissue distribution and different acyl specificities [30–33]. The function of these proteins is not established, but they may facilitate enzyme-fatty acid interactions [32]. For example, binding to intestinal fatty acid binding protein facilitates formation of the acyl CoA derivatives, which are the preferred substrates for esterification reactions [33]. Fatty acid binding proteins in epidermis and their acyl specificities have yet to be characterized. Finally, there are multiple pathways and enzymes involved in the synthesis of complex acyl lipids. Incorporation of a fatty acid into a specific lipid fraction therefore also reflects the acyl specificity and activity of each of these enzymes. Therefore, complete attribution of the differences in fatty acid utilization patterns that we have demonstrated here will require further study.

In undertaking these studies, we were particularly interested in examining the extent to which differentiated keratinocytes utilize fatty acids for complex lipid synthesis. Recent in vitro [34–36] and in vivo [37] studies have shown that differentiated epidermal cells lack low density lipoprotein receptors and rely instead upon de novo sterologensis. Moreover, Monger et al [38] have shown that the

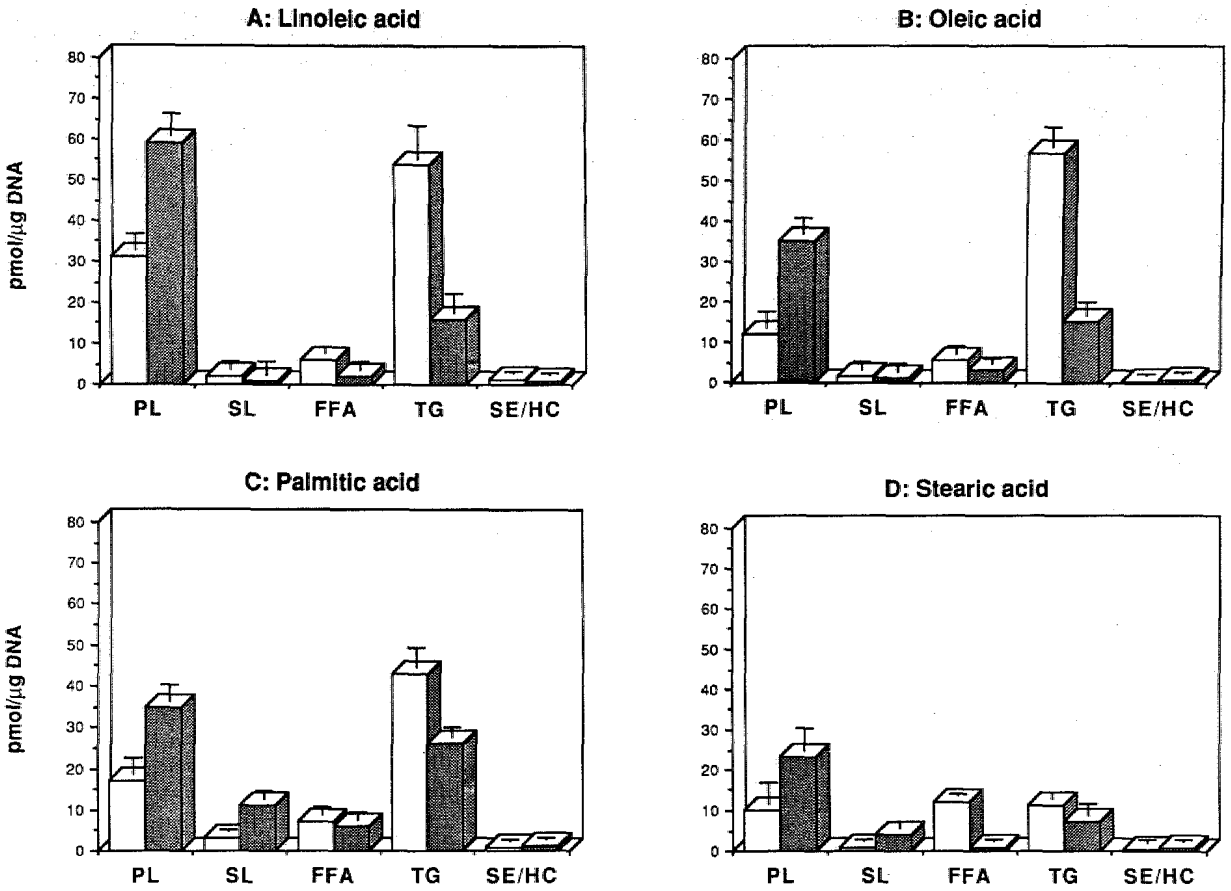

Figure 4. Lipid distribution of exogenous fatty acids: Pulse-chase study. Keratinocytes were pulsed with [^{14}C]-linoleic *(panel A)*, [^{3}H]-oleic *(panel B)*, [^{14}C]-palmitic *(panel C)*, or [^{14}C]-stearic *(panel D)* acid for 1 h under submerged conditions (S.A. of radiolabel as added: 59 mCi/mmole, 76 mCi/mmole, 48 mCi/mmole and 53 mCi/mmole, respectively). One set was harvested at the end of the pulse period and the incorporation into lipid fractions determined *(open bars)* (see *Methods* and legend to Fig 1). Another set was placed in fresh media without radiolabel and harvested after 24 h *(closed bars)*. Data are expressed as pmoles exogenous fatty acid incorporated/μg DNA, and represent the mean $\pm$ SD of three dishes each.

stratum granulosum is an extremely active site of de novo fatty acid and sterol synthesis. Furthermore, Grubauer et al [14] have shown that epidermis synthesizes fatty acids in response to perturbation of barrier function. However, epidermis must utilize exogenously supplied fatty acids as well, because essential fatty acids are important constituents of normal epidermal cell membranes [7]. And, in Refsum disease, where plant-derived phytanic acid accumulates due to deficiency of a catabolic enzyme [39], complex lipids of epidermis also accumulate this exogenous fatty acid [40]. These studies also clearly demonstrate the capacity of keratinocytes to take up both essential and non-essential fatty acids and to incorporate them into complex lipids. These results would suggest that the high rates of epidermal fatty acid synthesis observed in vivo reflect a requirement for fatty acid which exceeds available supply from the circulation, rather than an inability of epidermis to incorporate and utilize exogenous fatty acids.

In undertaking these studies, we were particularly interested in using differentiated keratinocyte cultures to investigate fatty acid utilization for sphingolipid synthesis. Ceramides and glucosylceramides are generated in significant quantities only by differentiated cultures [19,20] and are of acknowledged importance for barrier function [reviewed in Ref 1]. We observed that the relative incorporation of radiolabeled acetate into ceramides and glycolipids was greater than for any of the exogenously supplied fatty acids studied, including palmitate, the major long chain fatty acid synthesized in most cells. This may suggest that de novo synthesized fatty acids are the preferred substrates for sphingolipid biosynthesis; however, to determine this it would be necessary to know the specific activity of intracellular substrate pools. Moreover, although epidermal sphingolipids are a repository for very long-chain saturated acyl species, stearic acid, the longest fatty acid studied, was poorly incorporated into sphingolipid, again suggesting that de novo synthesized fatty acids may be the preferred substrate. Whether the preference for palmitic acid reflects utilization for formation of the sphingosine base as well as for N-acyl fatty acids was not determined in this study. However, it has been shown that exogenously supplied [^{3}H]-palmitate was incorporated exclusively into the N-acyl moiety of ceramides in organ culture guinea pig skin [42]. Linoleic acid, which is an important constituent of some epidermal sphingolipid species [6,11], was not preferentially incorporated into ceramides and glycosphingolipids. Although our cultures were supplemented with additional linoleic acid to avoid essential fatty acid deficiency [43], omission of linoleic acid supplementation did not influence incorporation of radiolabeled linoleate into sphingolipids (Schürer and Williams, unpublished observations). Failure of these cultures to utilize linoleic acid extensively for sphingolipid synthesis may reflect the observation that even this lifted culture system does not completely approximate epidermal differentiation, with lesser

quantities of lamellar bodies and sphingolipids than occur in vivo [19]. However, using a similar air-exposed human keratinocyte culture system, Ponec et al [44] have shown the presence of small quantities of acylglucosylceramides and acylceramides, the linoleate-containing species [11,12]. Further studies will be necessary to determine if linoleate is preferentially incorporated into these sphingolipid species in this culture system.

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