# Serine-palmitoyl transferase activity in cultured human keratinocytes

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**Abstract** Sphingolipids comprise approximately 25% of the stratum corneum lipids and are considered critical constituents of the epidermal permeability barrier. Whether sphingoid base structures are synthesized in the epidermis or whether they are derived from circulating or dermal sources is not known. We report here the initial characterization of serine-palmitoyl transferase (EC 2.3.1.50; SPT), the rate-limiting enzyme in the synthesis of sphingolipids, from cultured human neonatal keratinocytes. Subcellular fractionation studies demonstrated that 79% of the total cellular SPT activity was associated with the microsomes. The specific activity of keratinocyte SPT was 270 ± 20 pmol/min per mg of microsomal protein, a level significantly higher than activities reported in other tissues. Keratinocyte SPT showed an apparent $K_m$ for L-serine of 0.40 (± 0.04) mM, with an alkaline pH optimum (8.2 ± 0.4). Keratinocyte SPT utilizes palmitoyl-CoA preferentially over other saturated or unsaturated acyl-CoA substrates; increasing acyl-CoA chain lengths above C16 by one or two carbons was less detrimental to activity than similar decrements in chain length. Finally, the mechanism-based inhibitors L-cycloserine and β- chloro-L-alanine, demonstrated potent inhibition of keratinocyte SPT activity, with 50% inhibitory concentrations of approximately 3.0 and 25 μM, respectively. In summary, we have found that cultured human neonatal keratinocytes contain unusually high levels of serine-palmitoyl transferase activity, and that the substrate specificity of keratinocyte SPT may determine the base composition of epidermal sphingolipids.— **Holleran, W. M., M. L. Williams, W. N. Gao, and P. M. Elias.** Serine-palmitoyl transferase activity in cultured human keratinocytes. *J. Lipid Res.* 1990. **31:** 1655–1661.

**Supplementary key words** sphingosine base

Stratum corneum is enriched in sphingolipids, particularly ceramides, which form the intracellular membrane bilayers, that are thought to mediate cutaneous barrier function (rev. in 1, 2). Recent work has shown that the epidermis is an active site of cholesterol and fatty acid synthesis, and that the synthesis of these lipids is regulated in response to perturbations in barrier function (3, 4). Although Yardley and Summerly (5) first suggested that the epidermis must possess the capacity to synthesize sphingolipids, to date, sphingolipid synthesis has been demonstrated only in cultured keratinocytes (6–8). Whether the epidermis can synthesize these compounds is unknown, and this capacity would be dependent upon the content and activity of the requisite synthetic enzymes.

The initial committed step in the synthesis of all sphingolipids is the transfer of a fatty acyl group from a coenzyme A derivative to L-serine, forming the long-chain base precursor, 3-ketosphinganine (3KDS) (9, 10). This reaction is catalyzed by the enzyme, serine-palmitoyl transferase (SPT) (EC 2.3.1.50), which appears to be the rate-limiting step for the formation of sphingolipids in brain and liver tissues (11, 12). SPT has been extensively studied in microsomes from rat liver (12–15), rat and mouse brain (11, 16), and several other tissues (17–21). In addition, the enzyme has been characterized both in *Hansenula ciferri* (10, 21, 22) and in *Bacteriodes melaninogenicus* (23), where dissociation into a soluble enzyme form allowed the only partial purification reported to date (24).

We present here the first characterization of SPT from passaged cultured human keratinocytes (CHK) grown in a serum-free medium. Keratinocyte SPT was shown to be a membrane-associated, pyridoxal phosphate-requiring enzyme. Moreover, CHK-derived microsomal preparations contain the highest levels of SPT reported to date, and the enzyme demonstrates a substrate specificity for saturated, fatty acyl-CoA substrates that correlates with the base composition of epidermal sphingolipids.

## MATERIALS AND METHODS

### Materials

Reagent-grade organic solvents were obtained from Sigma Chemical Co. (St. Louis, MO). Pyridoxal phosphate, dithiothreitol, fatty acyl CoA derivatives, sphingo-

Abbreviations: SPT, serine palmitoyl transferase; 3KDS, 3-keto-sphinganine or 3-keto-dihdrosphingosine; PCoA, palmitoyl coenzyme A; PyrP, pyridoxal phosphate; SPL, sphingolipid; DTT, dithiothreitol; KGM, keratinocyte growth medium; CHK, cultured human keratinocytes.

sine base, and β-chloro-L-alanine also were purchased from Sigma. L-cycloserine was purchased from Fluka Chemical Co. (Pittsburgh, PA), while [G-$^3$H]L-serine was obtained from ICN Radiochemicals (Irvine, CA), HEPES buffer was purchased from Fisher Scientific (Santa Clara, CA). Keratinocyte growth medium (KGM) was purchased from Clonetics Corp. (San Diego, CA).

### Cell culture

Keratinocytes were isolated from human neonatal foreskins by a modification of the method of Pittelkow and Scott (25, 26). Cells were added to 100-mm plastic Petri dishes at a density of 1–2 × $10^4$ cells/$cm^2$, and grown initially in 10 ml of KGM with 0.07 mM calcium. The cultures were maintained at 35°C under 5% $CO_2$ in air, with medium changes performed three times weekly. After the cells reached 70–80% confluence, they were switched to KGM medium containing 1.2 mM calcium, and harvested at 1 week post-confluence.

### Microsomal isolation

Keratinocytes were harvested as follows. The medium was aspirated, the cells were rinsed twice with cold PBS (calcium/magnesium-free, 4°C), and then scraped from the Petri dish. The cells were sedimented, the homogenization buffer was added (HEPES 50 mM, pH 7.4, containing 10 mM EDTA, 5 mM DTT, and 0.25 M sucrose), and cells were disrupted by sonication using a Fisher Sonic Dismembranator (Model 300, Artec Systems Corporation, Farmingdale, NY) at 35% power, three times for 10 sec with pauses of 1 min while kept on ice. Differential centrifugation (4°C) was performed first at 800 *g* (15 min), then 10,000 *g* (15 min), followed by microsomal sedimentation at 100,000 *g* (60 min). The microsomal pellet was resuspended in storage buffer containing 50 mM HEPES (pH 7.4), 5 mM DTT, and 20% glycerol (v/v), and stored at −70°C until used. No loss of enzyme activity was observed upon freezing or subsequent thawing, with activity stable for over 6 months at −70°C. The protein content of various subcellular fractions was determined by the Bradford procedure (27) using bovine serum albumin (BSA) as standard. Assay reagent and BSA were purchased from Bio-Rad (Richmond, CA).

Microsomes were also prepared from rat liver as a known source of SPT activity. Adult rats (Sprague-Dawley from Bantin-Kingman), maintained on a normal diet, were anesthetized with ether, killed, and the liver, was quickly excised and rapidly frozen by freeze clamping in liquid nitrogen. The tissue was suspended in three volumes of cold homogenization buffer and subjected to two separate bursts of a Polytron PCU2 tissue homogenizer (Kinematica GmbH, Lucerne, Switzerland) on ice for 15 sec at 80% intensity, and allowed to cool for 30 sec between bursts. Cells were then disrupted by sonication and differential centrifugation was performed as above.

### Serine palmitoyl transferase (SPT) assay

The assay for SPT activity follows the method of Williams, Wang, and Merrill (12). Briefly summarized, the assay buffer contained 100 mM HEPES, pH 8.3 (20°C), 5.0 mM DTT, and 2.5 mM EDTA, while the reaction mixture contained 50 μM pyridoxal phosphate (PyrP), 150 μM palmitoyl-coenzyme A (PCoA), 1.0 mM [G-$^3$H]L-serine (sp act 45,000–50,000 dpm/nmol), and 50–200 μg of microsomal protein in 0.1 ml total assay volume. The assay mix (protein, buffer, PyrP) was preincubated for 10 min (37°C), and the reaction was initiated by simultaneous addition of PCoA and [$^3$H]L-serine, and incubated at 37°C for 10 min. Reactions were terminated by the addition of 0.2 ml of 0.5 N $NH_4OH$ and immediately cooling on ice. Organic-soluble products were isolated by addition of 1.5 ml chloroform–methanol 1:2, 25 μg sphingosine base (as carrier), and 2.0 ml 0.5 N $NH_4OH$. Tubes were vortexed, centrifuged for 5 min to achieve phase separation, and the aqueous phase was removed. The organic phase was washed twice with 2.0 ml of deionized water (made basic with $NH_4OH$), vortexed, and centrifuged for 5 min. The washed organic phase was sampled (0.8 ml), dried under a stream of nitrogen, and counted using a Beckman LS-1800 scintillation counter. Enzyme specific activity is expressed as pmoles of 3KDS formed per min per mg of protein. Total SPT activity was obtained by multiplying the specific activity by the total protein (total pmol 3KDS formed/min).

### Inhibition studies

The sensitivity of keratinocyte SPT to known suicide inhibitors was assessed to determine whether the catalytic mechanism for keratinocyte SPT is similar to that proposed for SPT from other tissues (13). Inhibition studies were performed by preincubating the microsomes plus 50 μM PyrP with either β-chloro-L-alanine or L-cycloserine for 10 min at 37°C prior to initiation of assay with [G-$^3$H]L-serine and PCoA. Stock inhibitor solutions of 1.0 mM (L-cycloserine) and 10 mM (β-chloro-L-alanine) along with serial dilutions were prepared in assay buffer (pH 8.3, 25°C) immediately prior to use. Final microsomal protein concentration was between 0.75 and 1.0 mg/ml in the assay mixture. Results are expressed as the percent of control SPT activity with no added inhibitor (i.e., % of total pmoles formed in the 10-min assay).

Statistical analysis was performed using the one- or two-tailed *t*-test where appropriate.

## RESULTS

### Subcellular localization of SPT activity

In order to localize SPT activity within CHK, cells were grown to 1 week post-confluence, homogenized, subjected to differential centrifugation, and the SPT activity

was compared in the combined plasma membrane, mitochondrial, microsomal, versus cytosolic fractions. The percentage of total cellular activity for each fraction is shown in **Table 1**, which demonstrates that the majority of subcellular activity (≈80%) remained associated with the microsomal fraction.

TABLE 1. Subcellular localization of keratinocyte SPT

| Subcellular Fraction | Specific Activity[a] | % of Total Cellular Activity[b] |
|---|---|---|
| | *pmol/min/mg* | |
| 800 *g* Pellet | 32.2 ± 6.9 | 11.1 ± 2.4 |
| 10,000 *g* Pellet | 60.6 ± 1.0 | 5.3 ± 0.5 |
| 100,000 *g* Supernatant | 5.7 ± 1.6 | 4.4 ± 1.2 |
| 100,000 *g* Pellet | 206.0 ± 10.0 | 79.3 ± 3.8 |

[a]Specific activity (SA) is given as pmoles of 3KDS formed per min per mg protein (± SD, n = 3).
[b]Total activity = (SA × total protein). Percent of total cellular activity = total SPT activity per fraction/sum of total SPT activity in all fractions (± SD, n = 3).

### Assay characterization

In order to determine the requirements for keratinocyte SPT activity, we compared enzyme activity under standard assay conditions and after exclusion of various constituents of the cell-free system. The incorporation of [G-$^3$H]L-serine into 3KDS during the 10-min assay was maximal with the complete assay mixture, which included the PCoA substrate, PyrP coenzyme, microsomal protein, and assay buffer (**Table 2**). Removal of PyrP coenzyme from the assay mixture resulted in a 28% reduction of SPT activity. The remaining activity (≈70%) is presumed to represent the fraction of SPT isolated in holoenzyme form (i.e., SPT:PyrP complex), and is similar to that reported in rat liver (12), and slightly larger than the 50% holoenzyme reported in *B. melaninogenicus* mid-log-phase cells (24). Pyridoxal phosphate concentrations greater than 50 μM in the assay mix did not result in increased SPT activity (data not shown). Thus, as in other tissues, keratinocyte SPT is a pyridoxal phosphate-dependent enzyme. Moreover, removal of PCoA substrate or microsomal protein or heat inactivation of enzyme reduced activity to less than 5% of the complete assay mixture (Table 2). In all subsequent experiments, the assay mixture without added PCoA was used as the control for nonspecific background incorporation and was subtracted before pmole calculations were made. Finally, the assay was also sensitive to the quantity of added microsomal protein, with maximal specific activity obtained with protein concentrations in the 0.50 to 1.0 mg/ml range (data not shown). Reduced activity at higher protein concentrations has been attributed to interference from other microsomal enzymes (12).

The incorporation of [G-$^3$H]L-serine was found to be linear with assay times up to 10 min. Thus, the 10-min incorporation time point was used for all subsequent determinations. Repeated measurements of maximal specific activity from numerous keratinocyte preparations and kinetic analysis have resulted in activities of 270 ± 20 pmol/min per mg of microsomal protein (n > 50). This high level of SPT activity found in CHK is not likely to reflect differences in tissue preparation or assay conditions, since we determined SPT activity in rat liver comparable to those reported in the literature (i.e., between 30 and 50 pmol/min per mg) (17).

The pH profile of keratinocyte SPT activity showed an alkaline pH maximum of 8.2 ± 0.4 (data not shown), comparable to that observed in rat liver (12), but higher than the pH optimum found in partially purified enzyme preparations from *B. melaninogenicus* (pH 7.0–7.6) (24).

### Kinetic analysis

A representative plot of total 3KDS product formed in the presence of varying concentrations of L-serine is shown in **Fig. 1**, with the double-reciprocal plot as an inset. The apparent $K_m$ for L-serine was determined to be 0.40 (± 0.04) mM, somewhat lower than the 0.67 mM reported in rat liver (12). The kinetic parameters for palmitoyl CoA were not determined since SPT activity decreased when PCoA concentration exceeded 0.175 mM (data not shown), an effect that has been attributed to substrate inhibition (12, 28). Thus, all subsequent assays were performed at an acyl CoA concentration of 0.150 mM.

### Substrate specificity

Because the specificity of SPT for acyl-CoA substates should determine the base structrures of newly-synthesized sphingolipids, the specificity of keratinocyte SPT was examined using various saturated and unsaturated acyl-coenzyme A derivatives. Maximal SPT activity was achieved using the palmitoyl-CoA (16:0) substrate (**Fig. 2A**). When saturated fatty acyl chain lengths were increased either by one or two carbon lengths over C16, enzyme activity was significantly greater than that observed with similarly shortened substrates. For example, the odd-chained n-heptadecanoyl-CoA (17:0) was preferred over the shorter n-pentadecanoyl-CoA (15:0) substrate (80% vs 65% of the activity with PCoA, respectively; $P < 0.025$). In addition, while the larger, even-chained stearoyl-CoA (18:0) substrate showed 33% of the activity observed with PCoA ($P < 0.0005$), the shorter myristoyl-CoA (14:0) showed less than 8% of the maximal activity ($P < 0.0005$).

The presence of *cis*-unsaturated bonds in the fatty acid moiety of the CoA substrate resulted in greater than 90% loss of SPT activity (Fig. 2B), indicating that saturation

TABLE 2. Requirement of assay components for optimal SPT activity

| Description | CPM | % of Maximal |
|---|---|---|
| Complete assay mix | 5280 ± 280 | 100 |
| Minus pyridoxal phosphate | 3750 ± 174 | 70* |
| Minus palmitoyl CoA | 262 ± 81 | <2** |
| Minus microsomal protein[a] | 166 ± 6 | 0 |
| Protein added after assay was terminated | 263 ± 77 | <2 |
| Heat-denatured protein | 178 ± 38 | 0 |

Incorporation of [$^3$H]L-serine into organic phase with assay components eliminated as indicated. Protein concentration = 0.91 mg/ml. Each point represents mean ± SD (n = 3). Results are not corrected for background cpm. Significant differences from complete assay mix: *, $P < 0.01$; **, $P < 0.005$.

[a]Cpm obtained without added protein used as background.

of the acyl chain is the primary structural determinant of enzymatic activity. The presence of the *cis*-(n-9) unsaturated bond in palmitoleoyl CoA (16:1) decreased enzyme activity to a level comparable with shortening of the acyl chain by two carbon lengths (Fig. 2A and 2B). Yet, the preference for C16 over C18 or C14 substrates remained apparent even with unsaturated substrates. For example, enzyme activity with palmitoleoyl CoA (16:1) was approximately twofold higher than with oleoyl-CoA (18:1) ($P < 0.005$), and nearly fivefold higher than myristoleoyl-CoA (14:1) ($P < 0.005$) (cf, Figs. 2A vs 2B). Further *cis*-unsaturation to linoleoyl CoA (18:2) or arachidonoyl CoA improved enzymatic activity only slightly. Finally, as expected, keratinocyte SPT did not utilize acetyl-CoA as a substrate for the reaction (not shown). These studies demonstrate that keratinocyte SPT displays highly specific substrate preferences.

## Inhibition of SPT activity

Pyridoxal 5′-phosphate-dependent enzymes are known to be inhibited by certain mechanism-based inhibitors. To determine whether keratinocyte SPT utilizes a catalytic mechanism similar to the enzyme in other tissues, two mechanism-based, suicide inhibitors of SPT, L-cycloserine (29) and β-chloro-L-alanine (30), were added to the assay mixture. **Table 3** shows the greater than 90% inhibition of activity was achieved with concentrations of L-cycloserine and β-chloro-L-alanine of 0.05 and 0.50 mM, respectively, with approximate $IC_{50}$s of 3.0 and 25 μM, respectively. These studies provide strong evidence that the reaction mechanism for keratinocyte SPT is similar to SPT from other mammalian sources, i.e., a requirement for PyrP coenzyme complexation with L-serine prior to the decarboxylation and subsequent fatty-acyl substrate condensation steps (14).

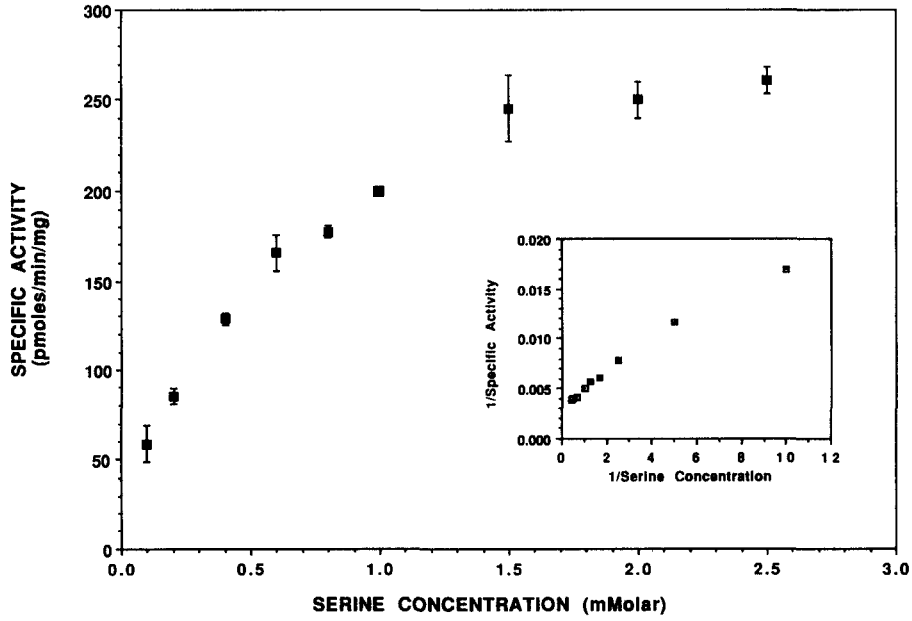


**Fig. 1.** Specific activity (SA) versus serine concentration. Representative plot of the change in SA as [G-$^3$H]L-serine concentration was varied from 0.10 to 2.5 mM. Protein, palmitoyl-CoA, and pyridoxal phosphate concentrations were 0.75 mg/ml, 150 μM, and 50 μM, respectively (n = 3, ± SD). Representative Lineweaver-Burk plot for L-serine concentration $(mM)^{-1}$ versus SPT activity (total pmol/10 min)$^{-1}$ is shown as inset.

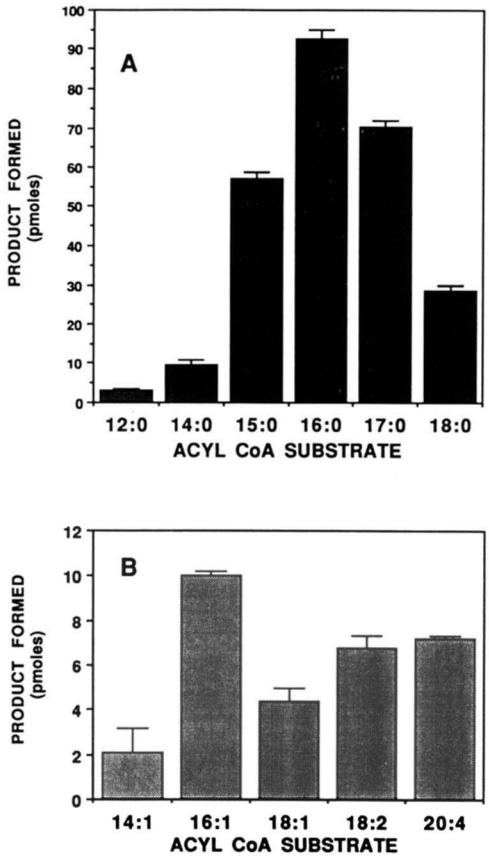


**Fig. 2.** Substrate specificity. Various acyl-CoA substrates were substituted for PCoA in the assay mixture at concentrations of 0.150 mM. The total lipid-soluble product was isolated and counted for tritium incorporation from [$^3$H]L-serine. The acyl-coenzyme A derivatives were prepared in assay buffer just prior to use. 2A: pmoles incorporated with saturated acyl CoA substrates. 2B: pmoles incorporated with unsaturated acyl CoA substrates. Note the change in y-axis in Fig. 2A versus 2B.

## DISCUSSION

The epidermis contains large quantities of sphingolipids, which are concentrated in the upper, differentiated cell layers, where they are thought to participate in the maintenance of the cutaneous permeability barrier (31, 32). Major changes in lipid composition occur as epidermal keratinocytes migrate from the basal, dividing layer outward to the fully differentiated, anucleate corneocytes, including a depletion of phospholipids coupled with a significant increase in sphingolipid content (31–33). Despite the known ability of cultured keratinocytes to synthesize sphingolipids from radiolabeled acetate, serine, and fatty acids (6–8), the enzymes responsible for their synthesis have not been identified in either cultured cells or epidermis. The initial characterization of SPT from CHK demonstrates that CHK contain abundant SPT activity (270 ± 20 pmol/min per mg) when compared with other mammalian tissues. This level is five- to sevenfold greater than the activity previously reported in various rat tissues (**Table 4**) (17). Although the high enzymatic activity in keratinocytes may reflect the requirement of the epidermis to produce large quantities of sphingolipids for permeability barrier function, the levels of activity found in vivo may be quite different. In fact, we found the SPT activity of murine epidermis to be nearly 50% less than that observed in CHK cells (Table 4). This difference may be due to a number of factors. First, some enzymatic activity may be lost during the isolation of epidermis from whole skin. Second, the lower levels of activity in epidermis may indicate that specific control mechanisms are operative in vivo, which are not observed in cultured cells. Finally, the activity differences observed may reflect a more homogeneous, enzyme-enriched subpopulation of epidermal cells present in culture, which is diluted by other enzyme-poor cells in a heterogeneous tissue, such as the epidermis.

The structures of the sphingosine base molecules formed in vivo are likely to reflect the substrate specificity of SPT, along with the availability of fatty acyl-CoA substrates (34–36). In human keratinocyte SPT, we observed a substrate preference for the saturated C16 acyl-CoA substrate versus other long-chain, saturated, acyl-CoA molecules (Fig. 4). These data correlated well with the substrate selectivity of SPT from other rat tissues, where maximal activity was observed with saturated CoA thioesters possessing 16 ± 1 carbon atoms (12, 17). Moreover, keratinocyte SPT demonstrated a slight preference for longer acyl-CoA substrates (C17, C18) over the corresponding shorter chain compounds (C15, C14), similar to that observed in rat intestine and pancreas (17). These findings are important since C18 and C20 sphingosine base structures predominate in human and porcine epidermis (37–39). In addition, recent mass spectral and $^1$H-NMR analysis of O-acyl ceramides of human epidermis

TABLE 3. Inhibition of SPT with suicide inhibitors

| | % Inhibition | |
|---|---|---|
| Inhibitor Concentration | Chloroalanine | L-Cycloserine |
| *mM* | | |
| 0.0 | 0[a] | 0[a] |
| 0.0005 | ND | 9.5 ± 0.7 |
| 0.001 | ND | 20.2 ± 1.0 |
| 0.005 | ND | 59.4 ± 2.0 |
| 0.01 | 29.5 ± 0.5 | 77.5 ± 0.5 |
| 0.05 | 67.4 ± 0.5 | 98.9 ± 0.1 |
| 0.10 | 80.4 ± 0.9 | 99.0 ± 0.9 |
| 0.50 | 92.7 ± 2.2 | ND |
| 1.00 | 94.8 ± 3.1 | ND |

The percent inhibition of keratinocyte SPT activity using L-cycloserine and β-chloro-L-alanine is compared. Inhibitor concentrations in the final assay mixture ranged from 0.0005 to 1.0 mM. Each point represents the mean of duplicate values (± range); ND, not determined.

[a]Controls represents the activity of complete assay mix with no added inhibitors (cf, Table 2).

TABLE 4. Comparison of SPT activity in various tissues

| Tissue | Specific Activity |
|---|---|
| | *pmol/min/mg* |
| Human | |
| Cultured keratinocytes | 270 ± 20.0 |
| Murine | |
| Epidermis | 120.0 ± 15.0 |
| Rat | |
| Liver | 40.0 ± 4.1 |
| Liver[a] | 42.5 ± 3.7 |
| Pancreas[a] | 2.7 ± 0.6 |
| Brain[a] | 30.1 ± 4.2 |

Keratinocyte enzyme activity is compared to levels measured in rat liver and murine epidermis, as well as quantities reported previously in rat tissues.

[a]Excerpted from Merrill, A. H., et al., ref. 17.

showed the sphingoid base to be a C20 molecule, indicating that stearoyl CoA is utilized by SPT in vivo (37). Thus, since the distribution of chain lengths roughly mirrors the acyl specificity of keratinocyte SPT, it is likely that the substrate specificity of SPT plays a predominant role in determining the composition of the sphingolipid bases found in the epidermis. However, the availability of various acyl-CoA substrates within epidermal cells and their effect on sphingoid base synthesis have yet to be evaluated.

In summary, we have conclusively demonstrated that keratinocytes possess the enzymatic capacity to synthesize large quantities of sphingosine base. Whereas the enzymatic specificity and kinetic parameters for keratinocyte SPT appear to closely resemble the enzyme from other mammalian tissues, keratinocytes appear to possess an even greater enzymatic activity, consistent with the requirement of large quantities of sphingolipids for epidermal barrier function.

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