Activity of 3-ketosphinganine synthase during differentiation and aging of neuronal cells in culture

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Abstract Changes in the enzyme 3-ketosphinganine synthase activity in rat cerebellar granule cells in culture were studied during differentiation and aging. The enzyme activity with palmitoyl-CoA and stearoyl-CoA, precursors of, respectively, C18-sphinganine and C20-sphinganine, was studied on the total cell homogenate using radioactive serine. The enzyme assay was performed by thin-layer chromatography (TLC) separation of the enzyme reaction mixture, and the resultant radioactive 3-ketosphinganine was identified by chromatographic comparison with a chemically synthesized 3-ketosphinganine, and quantified by determination of the TLC radioactivity distribution on the basis of the radioactivity content of cell lipid extract that was determined by scintillation counting. Using palmitoyl-CoA, the enzyme activity progressively increased from 40 to 54 pmol of 3-ketosphinganine/mg cell DNA per min in the first 8 days and then progressively decreased, and was 39 pmol of C18-(3-ketosphinganine)/mg cell DNA per min at day 22 in culture. For stearoyl-CoA the enzyme activity was very low at day one and then increased to a constant value of about 15 pmol of C20-(3-ketosphinganine)/mg cell DNA per min. These results are in good agreement with the finding that the ganglioside species that contain C18-sphingosine increase during cell differentiation and remain constant during cell aging, while the ganglioside species that contain C20-sphingosine continuously increase during both cell differentiation and aging.—**Chigorno, V., E. Negroni, M. Nicolini, and S. Sonnino.** Activity of 3-ketosphinganine synthase during differentiation and aging of neuronal cells in culture. *J. Lipid Res.* 1997. **38:** 1163–1169.

Supplementary key words gangliosides • biosynthesis • sphinganine • sphingosine

Gangliosides, a family of compounds that differ in both the oligosaccharide and the ceramide moieties (1), are particularly abundant in the cell membranes of the nervous system. The ganglioside species containing C18- and C20-sphingosine undergo marked quantitative changes during nervous system development, suggesting a direct involvement in the processes of neural cell differentiation and aging (2–7). Even today's current knowledge of ganglioside biosynthesis fails to explain how the sphingosine content is modulated (8, 9). In any case it is known that the first step is the biosynthesis of C18- and C20-sphinganine. This is then followed by *N*-acylation to dihydroceramides that are, in turn, oxidized to ceramides (10). Furthermore, it has been suggested that the availability of C18- and C20-sphinganine, present in cells as free molecules in a ratio similar to that of C18- and C20-sphingolipids (7, 11), is related to the biosynthesis of ganglioside species containing C18- and C20-sphingosine. Thus the C18- and C20-sphinganine distribution could be regulated through the enzymatic properties of 3-ketosphinganine synthase (EC 2.3.1.50) for palmitic acid and stearic acid, which are, together with serine, the respective precursors of C18-sphinganine and C20-sphinganine.

In non-nervous system cells it has been found that the activity of 3-ketosphinganine synthase is higher for palmitolyl-CoA than for stearoyl-CoA (12–14); this explains the predominance of C18-sphingosine in complex sphingolipids. The enzyme activity in nervous system microsomal membranes was 30–40 pmol/mg cell protein per min using palmitoyl-CoA (13, 15), but no data are available during the development of the nervous system.

The aim of this work has been to study the activity of 3-ketosphinganine synthase on palmitoyl-CoA and ste-

Abbreviations: C18-sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene; C20-sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-eicosene; C18-sphinganine, (2*S*,3*R*)-2-amino-1,3-dihydroxy-octadecane; C20-sphinganine, (2*S*,3*R*)-2-amino-1,3-dihydroxy-eicosane; C18-(3-ketosphinganine), (2*S*)-2-amino-1-hydroxy-3-keto-octadecane; C20-(3-ketosphinganine), (2*S*)-2-amino-1-hydroxy-3-keto-eicosane; C18-gangliosides, gangliosides containing C18-sphingosine; C20-gangliosides, gangliosides containing C20-sphingosine; C18-sphingolipids, gangliosides and sphingomyelin containing C18-sphingosine.

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aroyl-CoA, thus to correlate it to the developmental changes of the C18- and C20-ganglioside species, in rat cerebellar granule cells in culture. Such cells that have been used successfully to study ganglioside metabolism and ganglioside properties (6–8, 15–20) are virtually homogeneous neurons that undergo differentiation for 7–8 days and then age in a regular fashion (21).

EXPERIMENTAL PROCEDURES

Materials

Commercial chemicals were the purest available, common solvents were distilled before use, and water was purified with the MilliQ system (Millipore). L-Serine, methyl orange, trypsin, crystalline bovine serum albumin, fetal calf serum (FCS), and all the reagents for cell culture were from Sigma Chemical Co. The basal modified Eagle's medium was from Irvine Scientific. Ferric chloride, ammonium thiocyanate, dithiothreitol, HEPES, and HPTLC Kieselgel 60 precoated plates (250 μm thickness; 10 × 20 cm) were from Merck. L-[3-^{3}H]serine (32 Ci/mmol) was from Amersham Life Science.

Chemical preparation of 3-ketosphinganine

Figure 1 shows the scheme of the reactions for the preparation of 3-ketosphinganine from sphingosine. C18-sphingosine was prepared from cerebroside (22) and 10 mg was dissolved in 3 mL of 10% (w/v) triethylamine in dehydrated dimethylformamide; 11 mg of di-*t*-butyl dicarbonate was added under vigorus stirring. After 40 min at 45°C, the solution was dried under reduced pressure and the residue was dissolved in 3 mL of ice-cold hydrochloric acid, pH 2.5, and immediately partitioned with 4 mL of ethyl acetate (3 times). The organic phase was dried over sodium sulfate, filtered, and dried again. The residue, **1**, (Fig. 1) (yield, 100%) was dissolved, under nitrogen, in 1 mL of dehydrated chloroform, and 35 mg of MnO_2 was added. After 36 h under stirring at room temperature, the reaction mixture was applied to a short column of celite, and compound **2** was eluted with 5 mL chloroform (yield, 90–95%). The crude product **2** was dissolved in 2 mL ethyl acetate and hydrogenated in the presence of 0.5 mg *tris*-(triphenylphosphine)Rhodium(I) chloride. After 24 h at room temperature, the solvent was evaporated under reduced pressure leaving compound **3** that was then purified by flash chromatography using the solvent system *n*-hexane–2-propanol 95:5 (by vol) (yield, 90%). Compound **3** was dissolved under nitrogen at 0°C in 0.3 mL of trifluoroacetic acid. After 10 min the solution was evaporated leaving compound **4** that was then treated with 1 mL of Amberlite IRA 400 (OH^- form) in 0.3 mL of methanol, filtered, and stored in methanol at −80°C (yield, 100%).

Cell cultures

Granule cells, obtained from the cerebellum of 8-day-old Sprague-Dawley rats (Charles River), were prepared and cultured as described (21, 23). The cell culture contained >90% granule cells, ≈5% GABA-ergic neurons, and <5% glial cells. Replication of non-neuronal cells was prevented by adding cytosine β-D-arabinofuranoside (2.5 μg/mL) to the culture medium. The cells were cultured for up to 22 days in 100-mm dishes with 10 ml basal modified Eagle's medium containing 10% fetal calf serum, and 100 μL of glucose solution (100 mg/mL) was added every 3 days. Viability, assayed by the trypan blue absorption method, was >95% until 22 days.

Control experiments were carried out to establish that the cells would be in good nutritional condition even after 22 days in culture. The control cells were cultured in dishes with 10 mL BME containing 10% FCS and every 3 days we added 100 μL glucose solution (100 mg/mL), 100 μL glutamine solution (200 mM), 10 μL vitamin-B_6 solution (1 mg/mL), and fatty acids (palmitic and stearic) that had been dried and resuspended in the cell medium (200 nmol/dish).

Determination of the 3-ketosphinganine synthase activity

The cell medium was carefully removed and the cells were rinsed twice with 5 mL of 0.05 M potassium phosphate, pH 7.4, 0.15 M NaCl, 10 mM EDTA solution; they were then scraped off, suspended at 4°C in 0.5 mL 0.05 M HEPES, pH 8, 10 mM EDTA, 5 mM dithiothreitol, transferred to a small tube, sonicated three times for 5 sec, and immediately assayed for 3-ketosphinganine synthase activity (12). Experiments to determine enzyme features were carried out under the following conditions: incubation time, 10–20 min; 1 mM serine and 0.05–0.4 mM acyl-CoA; 0.15 mM acyl-CoA and 0.1–2 mM serine; 50–100 μg cell proteins derived from cells at days 2 and 8 in culture. The experimental conditions to determine the developmental profile of the enzyme activity were: 10, 15, and 20 min of incubation time, using 1 mM serine and 0.1, 0.15, 0.2 mM palmitoyl-CoA or stearoyl-CoA, and 50–100 μg cell proteins. The experiments were carried out on a pool of 8–10 cell dishes. Control experiments were carried out on sonicated cells maintained for 30 min at 90°C. At the end of the incubation time, standard 3-ketosphinganine was added to the enzymatic reaction mixture and purified by partitioning the total lipid extract (24); after TLC separation the plate was subjected to digital radiochromatoscanning (Digital Autoradiograph, Berthold;

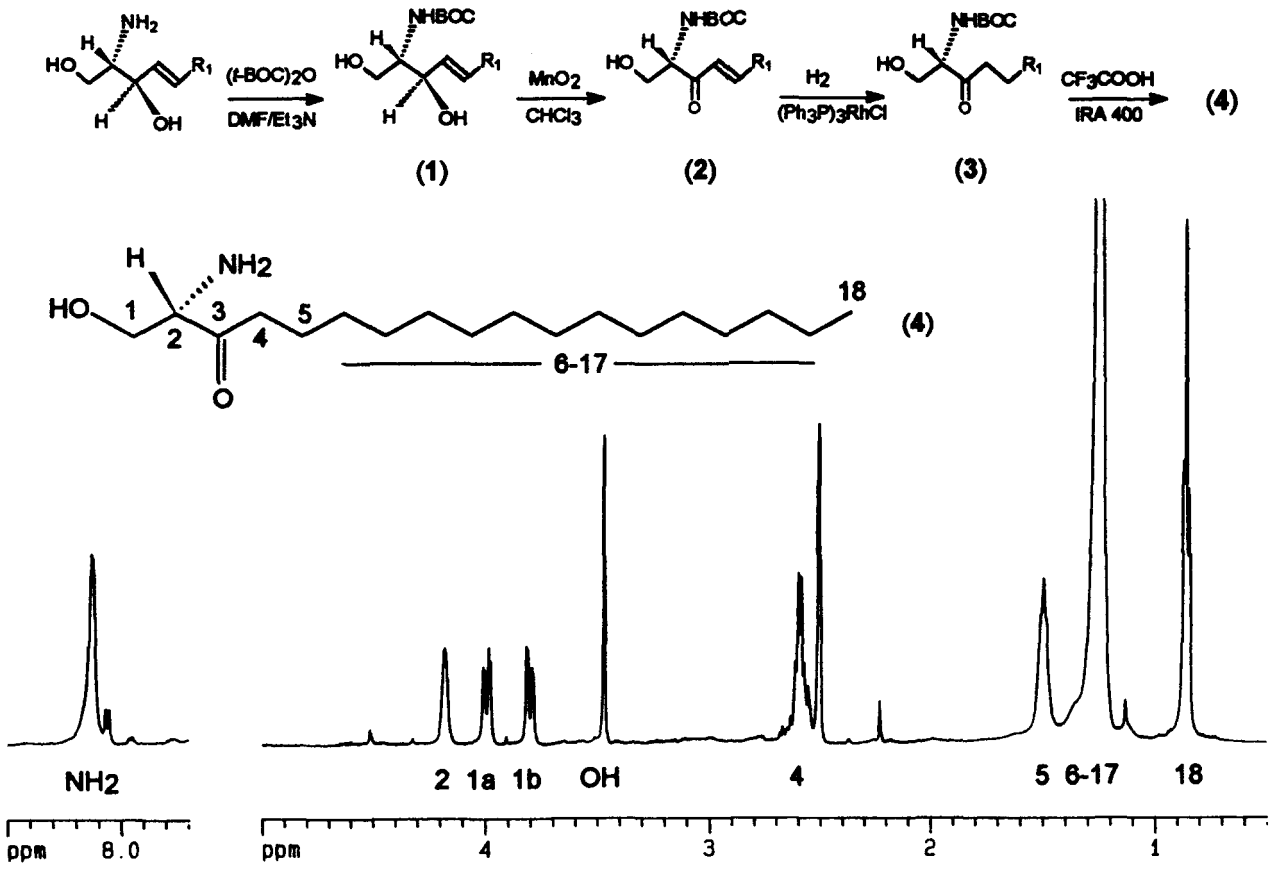

Fig. 1. Reaction scheme for the synthesis of C18-(3-ketosphinganine), and 500 MHz ^{1}H-NMR spectrum of the chemically synthesized compound dissolved in $(CD_3)SO$. Proton chemical shifts were referenced to the signal of residual pentadeuterated dimethylsulfoxide set at 2.49 ppm.

counting SD <1.5%). Thus the radioactivity of 3-ketosphinganine was calculated on the basis of the TLC radioactivity percent distribution and of the radioactivity of total lipid extract (see below).

Three sets of experiments, each one performed in triplicate, with three acyl-CoA concentrations in each experiment were carried out.

Recovery of 3-ketosphinganine

The recovery and stability of 3-ketosphinganine during extraction and partition was determined after adding 10–50 nmol of standard C18-(3-ketosphinganine) to the enzyme reaction mixture by TLC analysis followed by colorimetric detection and densitometric quantification.

Stability of palmitoyl-CoA and stearoyl-CoA under assay conditions

At the end of the incubation time, the reaction mixture was centrifuged and the supernatant was analyzed for acyl-CoA and fatty acid contents by TLC analysis followed by colorimetric detection and densitometric quantification.

Other analytical procedures

Radioactivity of total lipid extract was determined by liquid scintillation counting. HPTLC was performed with the following solvent systems: chloroform–methanol–2 N NH_4OH 40:10:0.6, by vol, for the separation of 3-ketosphinganine; chloroform–methanol–water 110:40:6, by vol, for the separation of acyl-CoA and fatty acids; *n*-hexane–propanol 9:1, by vol, or chloroform–methanol–2 N NH_4OH 80:20:2, by vol, for compound **1**; *n*-hexane–propanol 9:1, by vol, for compounds **2** and **3**. Lipids were stained with ninidrine or with anisaldehyde reagent (25).

The structural characterization of chemically synthesized C18-(3-ketosphinganine) was determined by ^{1}H-NMR on a Bruker AM500; proton assignment was made by chemical shift correlation spectroscopy; protein content was determined using the Pierce protein assay kit (26). The DNA content was determined according to Burton (27).

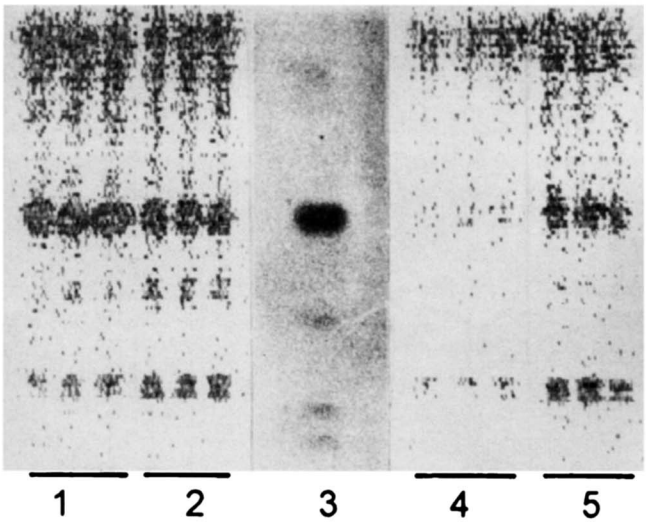

Fig. 2. 3-Ketosphinganine synthase activity with palmitoyl-CoA and stearoyl-CoA in rat cerebellar granule cells in culture: HPTLC separation of the enzyme reaction products. 1, 2, 4 and 5, digital autoradiographic detection; 3, colorimetric detection; 1, enzyme activity with 0.15 mM palmitoyl-CoA at day 1 of cell culture, in triplicate; 2, enzyme activity with 0.15 mM palmitoyl-CoA at day 8 of cell culture, in triplicate; 3, standard 3-ketosphinganine; 4, enzyme activity with 0.15 mM stearoyl-CoA at day 1 of cell culture, in triplicate; 5, enzyme activity with 0.15 mM stearoyl-CoA at day 8 of cell culture, in triplicate. Other experimental conditions: 1 mM serine, 15 min incubation time.

RESULTS

3-Ketosphinganine synthase activity, determined as the production of 3-ketosphinganine using radioactive serine and either palmitoyl-CoA or stearoyl-CoA, was assessed by TLC separation of the enzyme reaction products and radiochromatoscanning determination of the TLC radioactivity distribution (**Fig. 2**), an assay procedure that gives contaminant-free results. In fact, the radioactive organic phase obtained by partitioning had an overall radioactive background and contained some unknown contaminants. Under our TLC experimental conditions, the biosynthesized radioactive C18- and C20-(3-ketosphinganine) showed identical chromatographic behavior and were identified by TLC comparison with C18-(3-ketosphinganine) chemically synthesized from C18-sphingosine. Adding a known amount of cold C18-(3-ketosphinganine) to the enzymatic reaction mixture led to a ≥90% recovery of radioactive 3-ketosphinganine. The structure of C18-(3-ketosphinganine) was confirmed by NMR (Fig. 1). Thus the chemical synthesis of C18-(3-ketosphinganine) that we developed requires only a few days work, has high yield, and gives a >95% pure product.

Experiments carried out with 0.15 mM acyl-CoA and varying concentrations of serine, using undifferentiated or differentiated cells (days 2 or 8 in culture), showed similar qualitative enzyme behavior with palmitoyl-CoA and stearoyl-CoA, but the enzyme activity, expressed per mg of cell protein, was much higher with palmitoyl-CoA under all the experimental conditions (**Fig. 3**). Similar results (Fig. 3) were obtained with varying concentrations of acyl-CoA and constant serine concentration.

There were no significant differences in acyl-CoA (and fatty acid) contents at the end of incubation of 2-day or 8-day cultured cells with either palmitoyl-CoA or stearoyl-CoA. Note that palmitoyl-CoA and stearoyl-CoA were in large excess in the reaction mixture. This results excludes the possibility that the different enzyme activity with palmitoyl-CoA versus stearoyl-CoA is related to some hydrolase activities or to a different stability of the two activated fatty acids under the assay experimental conditions.

Thus, in agreement with previous suggestions (12, 15), the developmental profile of the enzyme activity was studied using 1 mM serine, 0.1–0.15–0.2 mM acyl-CoA and an incubation time of 15 min. Using three acyl-CoA concentrations in each experiment, performed in triplicate, the results were very similar and within a SD of ±20% determined over three separate experiments.

Figure 4 shows the developmental profile of the 3-ketosphinganine synthase activity with palmitoyl-CoA and stearoyl-CoA in granule cells for up to 22 days of culture; the results are expressed per mg of cell protein and mg of cell DNA. Using stearoyl-CoA, the enzyme activity at the beginning of the cell differentiation process was hardly detectable but it then became much more evident; with palmitoyl-CoA it was easily detectable throughout the culture period. The data, expressed as enzyme activity per mg of cell protein, can be rather confusing as protein synthesis occurs during cell differentiation (Fig. 4) (18). However, when the data are expressed as enzyme activity per mg of cell DNA, it becomes clear that during differentiation there is an increase in enzyme activity. At the end of differentiation, the activity level for palmitoyl-CoA as substrate drops. Nevertheless, at day 22 the enzyme activity with palmitoyl-CoA was still much higher than with stearoyl-CoA.

DISCUSSION

C18-sphingosine and C20-sphingosine are the main long-chain base components of gangliosides in the nervous system (28). Of all the complex sphingolipids, the gangliosides of the nervous system are the only compounds that contain the C20-sphingosine species (28–30). In in vitro and in vivo systems (2–6, 28–32) the C18-ganglioside content increases during cell differen-

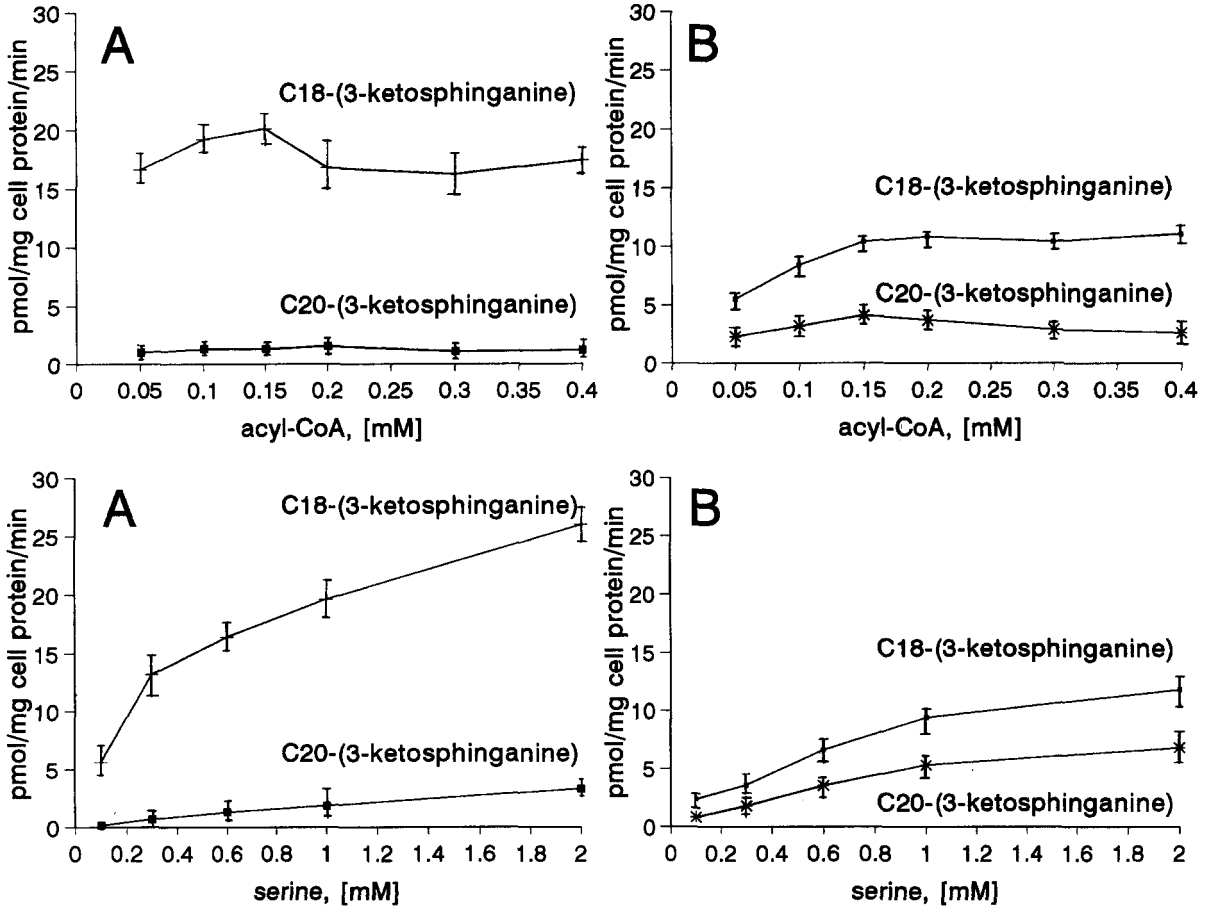

Fig. 3. 3-Ketosphinganine synthase activity with palmitoyl-CoA and stearoyl-CoA in rat cerebellar granule cells at days 2 (A) and 8 (B) in culture, as determined by HPTLC separation and quantification of the formed C18-(3-ketosphinganine) and C20-(3-ketosphinganine). The enzyme activity was determined with 1 mM serine and the reported acyl-CoA concentrations, or with 0.15 mM acyl-CoA and the reported serine concentrations. Incubation time was 15 min.

tiation and remains constant during cell aging. Only very small amounts of C20-gangliosides are present at the beginning of development but during differentiation and aging these amounts increase quite markedly, though they still remain in the minority of the ganglioside mixture. Thus, in rat cerebellar granule cells from day 0 to day 22 in culture the C20-ganglioside species increase from about 0.2 nmol/mg cell protein to 3 nmol/mg cell protein, while the C18-ganglioside species increase from about 2.5 nmol/mg cell protein to 10 nmol/mg cell protein (6).

De novo biosynthesis of a sphingolipid requires the biosynthesis of sphinganine (10), and after acylation the dihydroceramide is converted to ceramide. Thus ceramide becomes available for subsequent biosynthetic steps, i.e., in the case of gangliosides, a sequential glycosylation. Moreover, sphingolipid catabolism yields sphingosine that is partly degraded and partly recycled (8, 19, 33–35). Therefore, although no information is available concerning a direct use of sphingosine or the necessity of first reducing sphingosine to sphinganine for ceramide biosynthesis, it is clear that both the catabolic sphingosine and the biosynthetic sphinganine take part in the formation of the long-chain base pool necessary to maintain the appropriate sphingolipid turnover. Thus, sphinganine biosynthesis provides not only the bases needed to increase the sphingolipid cell content but also those needed to replace the catabolic sphingosines that are degraded.

The different distribution of the C18- and C20-ganglioside species, together with the increase in sphingomyelin content during cell development (32), require a precise modulation of the sphingolipid metabolic processing. In this regard, we suggested that a predetermined distribution of C18- and C20-sphinganine plays a key role in sphingolipid biosynthesis modulation (7) and, in confirmation of this, we recently showed that the C18-/C20-sphinganine ratio is very similar to that

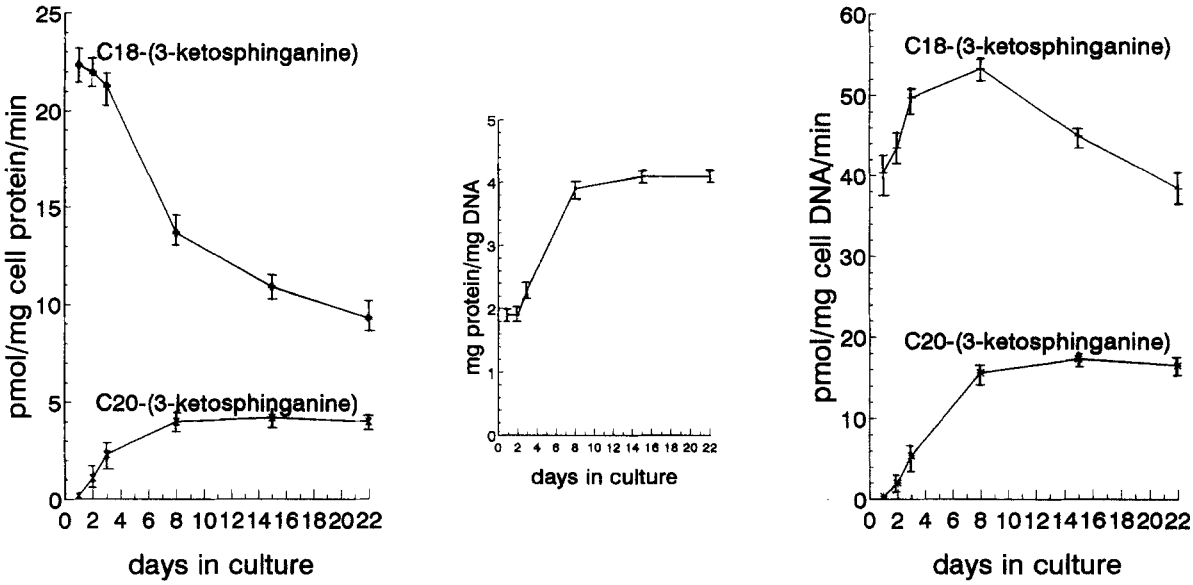

Fig. 4. Changes of the 3-ketosphinganine synthase activity with palmitoyl-CoA and stearoyl-CoA in rat cerebellar granule cells in culture, as determined by HPTLC separation and quantification of the formed C18-(3-ketosphinganine) and C20-(3-ketosphinganine). Data are related to the following experimental conditions: 1 mM serine, 0.15 mM acyl-CoA, and 15 min incubation time. Standard deviation was determined on three experiments performed in triplicate. Similar results were obtained using 0.1 or 0.2 mM acyl-CoA. The enzyme activity is expressed per mg of cell protein and per mg of cell DNA. The changes of protein content in rat cerebellar granule cells in culture are reported for better understanding of the different behavior of enzyme changes when expressed per mg of cell protein or mg of cell DNA.

of the C18-/C20-sphingolipids in neuronal cells during differentiation and aging in culture (32).

This article presents data that support the hypothesis that the enzyme 3-ketosphinganine synthase can play an important role in modulating the distribution of C18- and C20-sphingolipids. In fact, we found that the enzyme activity was higher with palmitoyl-CoA than with stearoyl-CoA and changed during cell differentiation and aging processes; furthermore, the two activated fatty acids showed quite different behavioral activity. *i)* At the beginning of cell differentiation when the C18-sphingolipid biosynthesis is already very active and the C20-ganglioside species are very scant (6, 32), the enzyme activity with palmitoyl-CoA was high and in good agreement with previous suggestions (13, 15), while that with stearoyl-CoA was hardly detectable. *ii)* During cell differentiation, the activity with both palmitoyl-CoA and stearoyl-CoA increased, in agreement with a continuous increase of both C20-gangliosides and C18-sphingolipids. *iii)* During the aging period, the activity with palmitoyl-CoA decreased, whereas with stearoyl-CoA it remained constant. Considering that after cell differentiation the C18-sphingolipids remain constant while the C20-gangliosides continue to increase (6, 32), this differing activity behavior could suggest that the sphingolipid turnover is slowed down after cell differentiation or that the recycling process of sphingosine becomes more relevant, reducing the sphinganine amount needed for sphingolipid neobiosynthesis.

Further research is needed to understand the modulation of enzyme kinetic properties on palmitoyl-CoA and stearoyl-CoA, and to determine whether or not two different enzymes can exist that are respectively specific for each of the two activated fatty acids.

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