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The Effects of Exogenous Sphingosine on Neuro2a Cells Are Strictly Related to the Overall Capacity of Cells to Metabolize Sphingosine[1]

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Neuro2a cells were exposed to different doses (1-40 nmol/10^6 cells) of [C3-^{3}H]sphingosine and the relationship between metabolism and biological effects of sphingosine was investigated. Sphingosine appeared to be rapidly taken up and metabolized. The incorporation of sphingosine was not merely dependent on its concentration but primarily on the dose per cell of administered sphingosine. At low doses, [^{3}H]sphingosine represented a minor portion of the cellular radioactivity, and *N*-acylated metabolites, particularly ceramide, largely prevailed over degradation products. Concomitantly with ceramide increase, Neuro2a differentiation took place. With increasing exogenous sphingosine/doses, the acylation process reached saturation. From this point on, [^{3}H]sphingosine started accumulating and eventually cell toxicity occurred. In conclusion, the biological effects exerted by exogenous sphingosine on Neuro2a cells are not merely dependent on the long-chain base concentration in the culture medium, but are strictly related to the cellular dose of exogenous sphingosine and to the capacity of cells to metabolize sphingosine.

Key words: ceramide, Neuro2a cells, sphingosine, sphingosine effects, sphingosine metabolism.

Sphingosine (4*E*,2*D*-amino-1,3*D*-octadecenediol) is a naturally occurring long-chain base that, when *N*-acylated, constitutes the most abundant backbone of mammalian sphingolipids. Small but definite amounts of Sph can be detected in cells, its main origin being catabolic. In fact, dihydro-sphingosine (sphinganine), the product of *de novo* biosynthesis of long chain bases, is dehydrogenated at C4-C5 only after *N*-acylation (*1*), whereas degradation of sphingomyelin and glycosphingolipids produces Cer, which, by the action of ceramidase, is further split into Sph and fatty acid (reviewed in Refs. *2* and *3*). The terminal catabolism of Sph occurs through a two-step process that involves phosphorylation at C1, followed by a lytic cleavage to ethanolamine phosphate and trans-2-hexadecenal (reviewed in Refs. *4* and *5*). Alternatively Sph can be used as an intermediate metabolite, mainly for the biosynthesis of Cer and complex sphingolipids (reviewed in Ref. *6*). In some cell types and tissues the conversion of Sph to DMS has also been documented (*7, 8*).

Convincing evidence has been provided that Sph, as well as its *N*-acylated and 1-phosphorylated derivatives, are able to act as intracellular bioregulators. In particular, it is well established that different stimuli, applied to cultured cells or animals, elicit the production of distinct sphingoid molecules which, in turn, act on different biochemical targets with the eventual result of regulating multiple biological functions like cell growth, differentiation, stress, and apoptosis (reviewed in Refs. *9-12*). Hence the metabolic routes of biomediators of sphingoid nature and their connections to cell functions have been the object of intense investigations. Despite these efforts, however, no precise information is available on the metabolic fate of Sph taken up from the extracellular milieu, and how Sph uptake and metabolism are related to cell responses. The present study deals with the metabolism and the effects of exogenous Sph, administered at different doses, in cultured neuroblastoma cells. This study was also prompted by the evidence of Cer involvement in neuronal cell differentiation (*13, 14*) and by the suggestion that Sph metabolism plays a role in the generation of bioactive Cer (*13, 15*).

MATERIALS AND METHODS

Chemicals—All reagents were of analytical grade, and solvents were redistilled before use. DMEM, FCS, and bovine serum albumin were from Sigma (St. Louis, MO, USA); HPTLC silica gel plates from Merck (Darmstadt, Germany); *Escherichia coli sn*-1,2-diacylglycerol kinase

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Abbreviations: Cer, ceramide; DETAPAC, diethylenetriamine pentacetic acid; DMEM, Dulbecco's modified Eagle's medium; DMS, *N,N*-dimethylsphingosine; FCS, fetal calf serum; Glc-Cer, glucosylceramide; SM, sphingomyelin; Sph, D-erythrosphingosine; Sph-1-P, sphingosine-1-phosphate.

from Calbiochem (La Jolla, CA, USA). D-Erythrosphingosine, isotopically tritiated at the C3 ([^{3}H]Sph), was prepared and purified as previously described (*16*). Its specific radioactivity was 1.88 Ci/mmol and the radiochemical purity, assessed by HPTLC and autoradioscanning, better than 98%. Standard [^{3}H]sphingolipids were obtained as previously reported (*16-18*). Standard DMS and Sph-1-P were generously provided by Prof. Richard R. Schmidt (Faculty of Chemistry, University of Konstanz, Konstanz, Germany).

Cell Cultures—The murine neuroblastoma cell line clone NB2a (Neuro2a, CCL-131, American Cell Type Culture Collection, Bethesda, MD, USA) was cultured in DMEM supplemented with 10% FCS and grown in a humidified 5% CO_2 incubator at 37°C. Cell survival was evaluated by trypan blue exclusion. Cell differentiation was evaluated and quantified as cells bearing neurite-like processes as previously described (*13*).

Administration of [^{3}H]Sph to Neuro2a Cells—At the time of the experiments, the medium was removed from the plates and increasing amounts of Sph were added to cells in FCS-containing medium for different times. In each treatment the same amount of [^{3}H]Sph (75 nCi/ml) was added as a tracer, thus obtaining a diminution of Sph specific radioactivity with increasing Sph amount. At the end of the pulse period, medium was carefully collected and cells were rapidly washed with cold PBS and harvested by use of a rubber scraper. The pulse medium was centrifuged and stored at −20°C, until processed.

Lipid Extraction and Quantification—Total lipids were extracted from cells and culture media at 4°C, as previously described (*16*). After partitioning, the organic phase was subjected to a mild alkaline hydrolysis. The obtained aqueous and methanolysed organic phases were counted for radioactivity and analyzed by HPTLC. The following solvent systems (by volume) were used: (A) chloroform/methanol/water (55:20:3); (B) chloroform/methanol/32% NH_4OH (40:10:1); (C) chloroform/methanol/0.2% $CaCl_2$ (55:45:10); (D) *n*-butanol/acetic acid/water (3:1:1). For the quantification of [^{3}H]Sph-1-P, aliquots of the lipid extract were directly applied to silica gel HPTLC plates and developed in solvent system D. The recognition and identification of [^{3}H]Cer, [^{3}H]SM, [^{3}H]Glc-Cer, and [^{3}H]gangliosides were performed as previously described (*16, 17*). [^{3}H]Sph-1-P was identified by co-migration with standard Sph-1-P in different chromatographic systems (*19*).

Assay for Cer—The content of Cer in Neuro2a cells was measured enzymatically using the diacylglycerol kinase assay (*20*), properly optimalised. In particular, aliquots (corresponding to 2×10^5 and 4×10^5 cells) of the metanolysed organic phase were dried in Pyrex glass tubes under nitrogen and solubilized in 20 μl of 7.5% octyl β-D glucopyranoside, 5 mM cardiolipin, 1 mM DETAPAC, pH 7.0. Standards of ceramide (50-1,000 pmol) were prepared in the same way. The tubes were then sonicated for 20 s and incubated at room temperature for 15 min. Thereafter, 50 μl of 2×reaction buffer (containing 100 mM imidazole-HCl, 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM EGTA, pH 6.6), 10 μl of freshly prepared 20 mM dithiothreitol in 1 mM DETAPAC, pH 7.0, and 10 μl of *E. coli* diacylglycerol kinase in 10 mM imidazole and 1 mM DETAPAC, pH 6.6 (0.5 mg protein/ml, 3 U/ml) were added. After mixing, the reaction was started by addition of 10 μl of 10 mM [γ-^{32}P]-ATP (3×10^5 dpm/nmol). Samples were then vortexed and incubated at 25°C for 45 min. The reaction was then stopped and phases separated as previously described (*20*). After washing of the lower phase, the products were separated by HPTLC using chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1, by volume). The position of the spots corresponding to ceramide [γ-^{32}P]phosphate was determined by autoradiography. The spots were scraped into vials and the radioactivity was determined by liquid scintillation counting. The quantity of ceramide was calculated by reference to the appropriate standard curves. Using these conditions, exogenous ceramide standards were quantitatively converted to ceramide-1-phosphate; in the range 50-1,000 pmol of substrate, the reaction was linear and the linearity was not affected by the addition of the methanolysed chloroform phase from 4×10^5 Neuro2a cells. Contrary to a recent report (*21*), no fluctuation in the activity of the diacylglycerol kinase was observed.

Other Methods—Sph content was determined according to Ohta *et al.* (*22*). Total proteins were assayed (*23*) using bovine serum albumin as the standard. Radioactivity was determined by liquid scintillation counting, fluorography, or radiochromatoscanning (Digital Autoradiograph, Berthold, Germany) (*16, 17*). ^3H_2O, produced during [^{3}H]Sph degradation, was determined by fractional distillation of the culture medium under carefully controlled conditions, collection of the distilled fractions, and measurement of the radioactivity by liquid scintillation counting (*24*). The volatile radioactivity of the culture medium was found to be present in the fraction distilling at 100°C and moved to the aqueous phase after partitioning with chloroform.

RESULTS AND DISCUSSION

The administration of radiolabeled Sph to Neuro2a cells is followed by a rapid and time-dependent uptake of the sphingoid base by cells (Fig. 1A). Maximum incorporation is reached after a pulse of 2-4 h and corresponds to about 45% of the administered radioactivity. In the concentration range of 0.5-20 μM (corresponding to 1-40 nmol/10^6

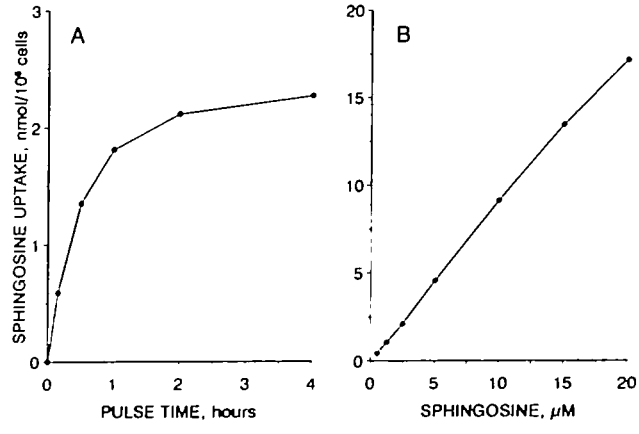

Fig. 1. **Incorporation of radioactivity into Neuro2a cells after administration of [^{3}H]Sph.** A: 2.5 μM [^{3}H]Sph was administered (2 ml/10^6 cells) for times varying from 10 min to 4 h. B: 0.5-20 μM [^{3}H]Sph was administered (2 ml/10^6 cells) for 2 h. Sph uptake was calculated summing up the values of cell-associated [^{3}H]Sph and its [^{3}H]metabolites. Data are the mean of three experiments, in which SD did not exceed 10% of the mean.

cells), extracellular Sph is taken up in a dose-dependent fashion (Fig. 1B). After 24 h under these conditions, cell survival is greater than 85% in all cases. At Sph doses higher than 40 nmol/10^6 cells, signs of cell toxicity become evident 2-4 h after exposure. Thus, the maximal amount of exogenous Sph that can be incorporated into Neuro2a cells without any significant cytotoxic effect accounts for about 20 nmol/10^6 cells. This value corresponds to about 400-fold the intracellular content of the long-chain base (0.053 ± 0.006 nmol/10^6 cells), indicating that Neuro2a cells display a remarkably high capacity to take up Sph in the absence of adverse effects.

At a fixed molar concentration of extracellular Sph, the uptake of Sph into neuroblastoma cells is inversely proportional to the cell number (Fig. 2, upper panel) and directly related to the medium volume (Fig. 2, lower panel). Therefore, the efficient Sph uptake by Neuro2a cells is not merely dependent on the Sph concentration in the medium *per se*, but primarily on the number of cells and the total amount of Sph in the medium, namely, on the dose per cell of administered Sph. These observations, although seemingly trivial, clearly show that, in order to obtain reproducible results, the experimental conditions of cell treatment with Sph should be very carefully standardized.

After a 2-h pulse with [^{3}H]Sph, the radioactivity incorporated into cells appears to be distributed between the long-chain base and its metabolites. However, the metabolism of exogenous Sph taken up by Neuro2a cells is significantly affected by the amount of Sph taken up/cell (Fig. 3). In fact, at the lower doses used, [^{3}H]Sph represents only a minor portion of the cellular radiolabel, most of the cellular radioactivity being associated with its metabolic derivatives. By increasing cellular Sph, the percent amount of metabolized sphingoid base decreases with

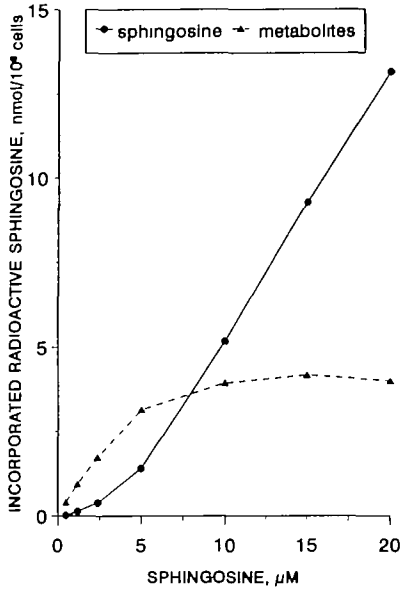

Fig. 3. **Incorporation of radioactivity into Sph and Sph-metabolites in Neuro2a cells after pulse with different concentrations of [^{3}H]Sph.** Different amounts of Sph (each containing 75 nCi/ml of [^{3}H]Sph) were administered (2 ml/10^6 cells) for 2 h. Data are the mean values of three experiments performed in duplicate, in which SD did not exceed 12% of the mean.

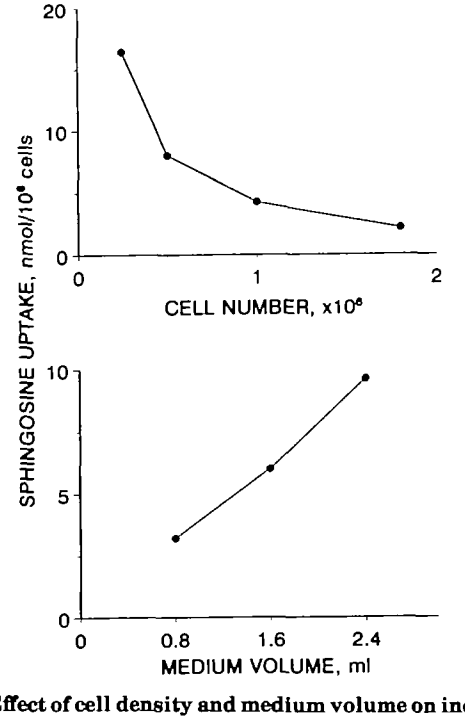

Fig. 2. **Effect of cell density and medium volume on incorporation of radioactivity into Neuro2a cells after pulse with 10 μM [^{3}H]Sph.** [^{3}H]Sph was administered in 1 ml of medium (upper panel) or to 10^6 cells (lower panel) for 2 h. Incorporated Sph was calculated as described in Fig. 1. Data are the mean values of three experiments, in which SD did not exceed 10% of the mean.

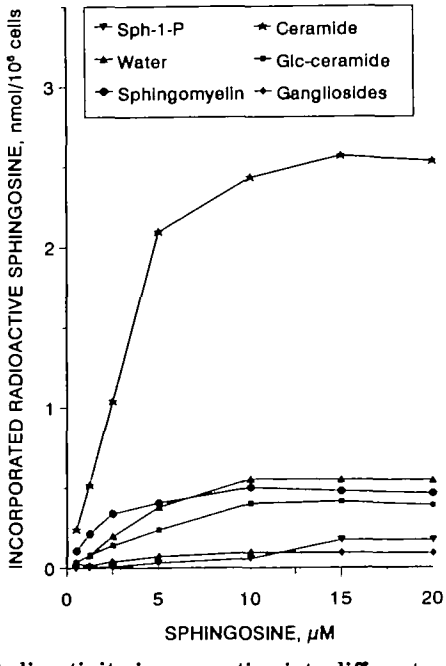

Fig. 4. **Radioactivity incorporation into different metabolites produced by Neuro2a cells after pulse with different concentrations of [^{3}H]Sph.** Different amounts of Sph (each containing 75 nCi/ml of [^{3}H]Sph) were administered (2 ml/10^6 cells) for 2 h. Data are the mean values of three experiments, in which SD did not exceed 15% of the mean.

parallel accumulation of the taken up molecule.

The main radioactive metabolites produced after pulse with different doses of [^{3}H]Sph are represented by Cer, complex sphingolipids, Sph-1-P, and ^3H_2O. For all Sph doses, the major [^{3}H]metabolite is [^{3}H]Cer, which increases up to 2.5 nmol/10^6 cells (Fig. 4), *i.e.*, about 4-5-fold the cellular Cer (0.52 ± 0.06 nmol/10^6 cells). The radioactivity incorporated into total complex sphingolipids (which are derived from [^{3}H]Cer and are mainly represented by SM, Glc-Cer, and gangliosides) accounts for less than half than that of [^{3}H]Cer and, at the highest Sph doses, reaches values corresponding to about 1 nmol/10^6 cells. These results demonstrate that Neuro2a cells are able to *N*-acylate an amount of Sph corresponding to more than 60-fold the cellular content of Sph in 2 h. Since under physiological conditions the only origin of free Sph is the degradation of complex sphingolipids, particularly gangliosides (*18, 25*), the observed high efficiency of these metabolic pathways suggests that, in some circumstances, the turnover of these sphingolipids in Neuro2a cells may occur very rapidly and to a high extent.

At high doses of Sph (corresponding to 10-20 μM in Fig. 4), the amount of [^{3}H]Cer, and more complex sphingolipids, remains constant. This could be explained either by the saturation of the *N*-acylating process or by feed-back regulation of Cer synthesis (*26*).

Besides *N*-acylated derivatives, [^{3}H]Sph-1-P is also formed after [^{3}H]Sph administration; its content never exceeds 2% of the total incorporated radioactivity, being maximum at the highest doses (Fig. 4). In no case was the presence of [^{3}H]DMS detectable. ^3H_2O, derived from [^{3}H]-Sph degradation (*24*), was also produced and released into the medium. However, in all cases, the [^{3}H]organic metabolites were significantly more abundant than ^3H_2O (Fig. 4), and the [^{3}H]*N*-acylated metabolites were more than 80% of total [^{3}H]metabolites at all Sph doses.

We have previously demonstrated (*13*) that Sph is able to exert a differentiating effect when administered to Neuro2a cells in the μM range. The dependence of Sph uptake and metabolism on the ratio between total amount of available Sph and cell number results in different effects on Neuro2a cells. In fact, at low concentration (2 μM) and low medium volume (0.8 ml/10^6 cells), the administration of exogenous Sph does not appreciably modify the content of cellular sphingoid molecules, and no morphological effect can be detected (A in Fig. 5). If the same concentration of Sph is administered to the same number of cells but in a higher volume, the intracellular content of Sph and, much more markedly, of Cer increases, being directly proportional to medium volume, and concomitant neurite outgrowth of Neuro2a cells occurs (B in Fig. 5). The efficient metabolic processing of Sph to Cer is essential for differentiation, since Fumonisin B1 (which blocks Sph *N*-acylation) inhibits the differentiating effect of exogenous Sph (*13*).

The administration of the same amount of Sph may also result in completely opposite effects depending on cell density. As shown in Fig. 5 (lower panel), when 10 nmol Sph are administered to 10^6 cells, Neuro2a cells are able to actively metabolize Sph and to produce large amounts of Cer. Under these conditions, the morphological appearance of Neuro2a cells displays the features of differentiated cells (C in Fig. 5). When the same amount of Sph is administered

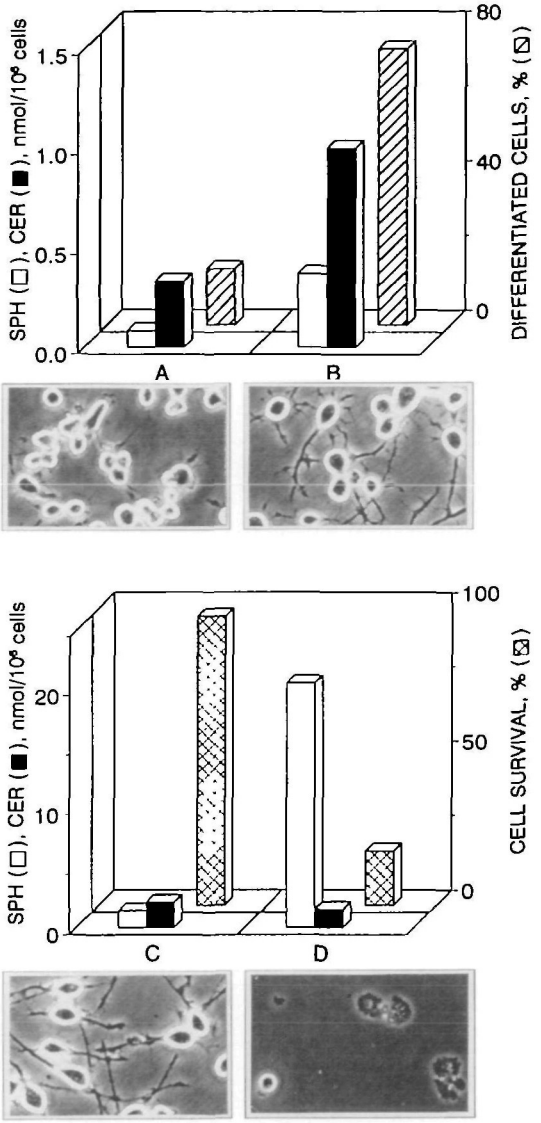

Fig. 5. **Metabolism and effects of exogenous Sph in Neuro2a cells.** Upper panel: 0.8 ml (A) or 2.4 ml (B) of 2 μM [^{3}H]Sph was administered to 10^6 cells for 2 h. Lower panel: 1 ml of 10 μM [^{3}H]-Sph was administered to 10^6 cells (C) or 0.2×10^6 cells (D) for 2 h. SPH and CER: incorporation of radioactivity into Sph and Cer, respectively. Cells bearing neurite-like processes longer than the major cell body diameter were scored as differentiated (*13*). The micrographs refer to 8 h of treatment. Data are the mean values of three experiments, in which SD did not exceed 12% of the mean.

to a lower number of cells, the saturation of the cellular metabolic machinery is accompanied by a significant accumulation of Sph in cells and, eventually, by cell death (D in Fig. 5). The amphipatic properties of Sph may be responsible for this effect by impairing cell permeability.

It has been reported that the effects exerted by exogenous long-chain bases in extraneural cells are strictly related to surface dilution (*27, 28*), which influences the amount of cellular sphingoid base. The present data demonstrate that in neuroblastoma cells Sph effects are not only related to the rate of uptake of sphingoid base/cell but are also closely dependent on the pathways of its metabolic processing and on the capacity of each pathway.

In conclusion, cultured neuroblastoma cells are able to take up and metabolize amounts of exogenous Sph that may be 100-fold higher than the endogenous content of the sphingoid base. This happens in a short time with maintenance of full cell viability. Under these conditions, Neuro2a cells actively metabolize exogenous Sph by both *N*-acylation and degradation, the former process being prevalent. Owing to this behavior, over a given range of uptake of Sph/cell (2.5-15 nmol/10^6 cells), the cellular content of Cer increases, eliciting cell differentiation. When rate of uptake of Sph/cell is higher than 18-20 nmol/10^6 cells, Sph metabolism reaches saturation, the cellular content of Sph markedly increases, and the toxic effect appears.

The results here obtained underscore the relevance of sphingosine metabolism in affecting the metabolic and functional responses of cells to exogenous Sph. Presumably, the effects of exogenous sphingosine can be different in different cell types, depending not only on the intrinsic cell sensitivity to Sph, but also on the metabolic machinery devoted to Sph processing and the prevailing metabolic route in this machinery.

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