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# BIOSYNTHESIS OF SPHINGOLIPIDS: DIHYDROCERAMIDE AND NOT SPHINGANINE IS DESATURATED BY CULTURED CELLS

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**Summary:** Radioactively labeled N-[1-$^{14}$C]-octanoyl-sphinganine and D-erythro-[3-$^{3}$H]-sphinganine were administered in parallel experiments to neuroblastoma cells B 104. A time dependent formation of ceramide with a double bond in its sphingoid backbone was observed in both cases. In the presence of fumonisin $B_1$ (25 $\mu$M), a strong inhibitor of sphinganine N-acyltransferase, desaturated ceramide was formed only when cells were fed with N-[1-$^{14}$C]-octanoyl-sphinganine but not with [3-$^{3}$H]-sphinganine. Thus, the introduction of the double bond occurs only at the level of dihydroceramide, after N-acylation of sphinganine. It is now obvious that sphingosine is not a biosynthetic intermediate but exclusively a catabolic product of cellular sphingolipids. 

Sphingoid bases are structural constituents of all sphingolipids. Although the presence of free long chain bases in different cells and tissues has been reported(1-4), these substances occur in rather low amounts, representing intermediates of sphingolipid metabolism. The most frequent long chain base backbones of sphingolipids in mammalian tissues are sphingosine and to a lesser extent dihydrosphingosine (sphinganine)(5). These two sphingoid bases differ only in the 4,5-trans double bond in the alkyl chain of the base.

Sphingolipid biosynthesis starts with the condensation of serine and palmitoylCoA, which is catalyzed by the pyridoxal phosphate dependent serine palmitoyltransferase (EC 2.3.1.50) yielding 3-dehydrosphinganine. A rapid reduction of this ketone by a NADPH

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**Abbreviations:** BSA = bovine serum albumin; Cer = N-acyl-D-erythro-sphingosine; $FB_1$ = fumonisin $B_1$; st-$G_{M3}$ = semitruncated-$G_{M3}$ = NeuAcα2-3Galβ1-4Glcβ1-1-st-Cer; Sa = D-erythro-sphinganine; st-$H_2$SM = semitruncated-dihydrosphingomyelin; st-SM = semitruncated-sphingomyelin; So = D-erythro-sphingosine; st-Cer = semitruncated-ceramide = N-[1-$^{14}$C]-octanoyl-D-erythro-sphingosine; st-$H_2$Cer = semitruncated-dihydroceramide = N-[1-$^{14}$C]-octanoyl-D-erythro-sphinganine; TLC = thin layer chromatography.

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dependent reductase leads to the formation of sphinganine (6, and references therein). There is no conclusive information in the literature about the next step of sphingolipid biosynthesis. It was suggested that sphinganine is directly desaturated generating sphingosine which is then acylated to form ceramide (reviewed in 6). On the other hand Ong and Brady(7), Stoffel and Bister(8) and later the group of Merrill(6,9) published indirect evidence that the introduction of the 4,5-trans double bond might not occur before addition of the amide-linked fatty acid. However, they could not prove this directly since at that time they had no possibility either to block intermediate acylation of sphingoids or to avoid hydrolysis of ceramides. The discovery of fumonisin $B_1$ as a strong inhibitor of N-acylation of sphingoid bases(9) allowed us to carry out direct studies on the desaturation of sphingolipids. We have therefore synthesized N-[1-$^{14}$C]-octanoyl-sphinganine and D-erythro-[3-$^{3}$H]-sphinganine and fed both of them for the first time to cultured cells in the absence and presence of fumonisin $B_1$.

## Materials and Methods

**Materials.** [1-$^{14}$C]-octanoic acid (4.4 Ci/mol), fumonisin $B_1$, sodium borate and bovine serum albumin (BSA) were purchased from Sigma (München FRG). Thin layer Silica Gel 60 plates, Silica Gel 60 and palladium(II)acetate were supplied by Merck (Darmstadt FRG). D-erythro-[3-$^{3}$H]-sphinganine (3000 Ci/mol) and the semitruncated N-[1-$^{14}$C]-octanoyl-sphinganine (st-$H_2$Cer) (4.4 Ci/mol) were synthesized as described(10-12). Sphingomyelinase (EC 3.1.4.12) was isolated from human urine as previously described(13). Culture medium (Dulbecco's modified Eagle medium) was from Boehringer Mannheim (FRG), foetal bovine serum from Gibco (Karlsruhe, FRG) and cell culture plastic dishes from Falcon (Heidelberg, FRG).

**Incubation of B104 cells with radiolabeled lipids.** Neuroblastoma cells B104(14) were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat inactivated foetal bovine serum. For the metabolic studies cells were seeded in Petri dishes (8 $cm^2$) and grown to approximative 700 $\mu$g cell protein/dish.

Cells were cultured in the absence or presence of fumonisin $B_1$ (25 $\mu$M) for 24 h. Then labeled lipids were added as complexes with defatted BSA(15) (0.125 $\mu$Ci N-[1-$^{14}$C]-octanoyl-sphinganine or 0.22 $\mu$Ci D-erythro-[3-$^{3}$H]-sphinganine per ml medium). After the indicated times cells were washed three times with phosphate buffered saline, scraped off the dish and centrifuged at 6000 rpm for 5 min. Total lipids were extracted from the pellets with 6 ml chloroform/methanol/water (10:5:1, by volume) at 50°C, overnight.

**Lipid analysis.** Ceramides were separated from the other lipids by thin layer chromatography using diethylether/methanol (99:2, by volume) as the developing system. Two separate spots of radiolabeled lipids were detected on the chromatogram by radioactive scanning.

1. The ceramide area with an $R_f$ value of approximative 0.22 was scraped from the TLC plate and reextracted with chloroform/methanol (1:1, by volume). The extracts were evaporated under a stream of $N_2$. Saturated ceramide was separated from unsaturated ceramide by TLC plates coated with Silica Gel G 60/$Na_2B_4O_7$*10$H_2O$ (14:0.8, by weight) developed two times in chloroform/methanol (9:1, by volume)(16). Radiolabeled ceramide bands were detected by radioactive scanning, scraped from the TLC plates and quantitatively evaluated by liquid scintillation counting.

2. The other area containing mainly st-sphingomyelins (st-SM, st-$H_2$SM) ($R_f$ 0.0) was also scraped from the TLC plates and eluated with chloroform/methanol (1:2, by volume).

The eluate was applied to a new TLC plate, which was developed in chloroform/methanol/water (65:25:4, by volume). Both radioactive bands characterized as st-sphingomyelins on the basis of their $R_f$ values were subjected to sphingomyelinase degradation, using incubation conditions described in detail before(13).

To liberate free sphingosine and sphinganine, radiolabeled lipid extracts were hydrolyzed in 0.5 ml methanolic HCl saturated with argon at 65°C after adding 100 $\mu$g of unlabeled ceramide. The reaction was terminated by neutralisation with KOH and the solution was desalted by reversed phase chromatography(17). Then samples were applied to TLC plates and developed in chloroform/methanol/2.5 M $NH_4OH$ (20:10:1, by volume). Radioactively labeled sphingoid spots were visualized by fluorography.

**Other methods.** Cellular protein was measured by the method of Lowry(18) with BSA as standard.

**Presentation of data.** All data presented are means of at least duplicate determinations. Most points are means of 3-5 separate determinations. In this case individual values were all within a range of ± 15% of the mean.

## Results and Discussion

Neuroblastoma cells represent an excellent model for studying the first steps of sphingolipid biosynthesis because of their high rate of metabolism and the absence of

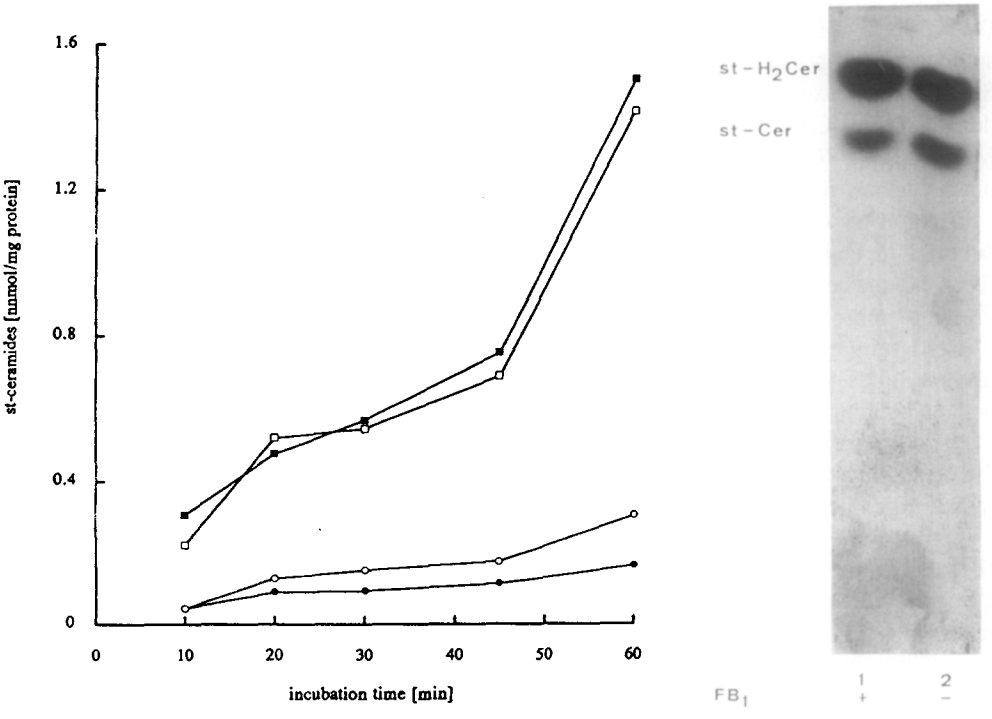


**Fig.1. The effect of fumonisin $B_1$ on the desaturation of the exogenously added dihydroceramide, st-$H_2$Cer in cultured B104 cells**
Neuroblastoma cells were cultured for 24 h in the presence (lane 1, full symbols) or absence (lane 2, open symbols) of fumonisin $B_1$ (25 $\mu$M). Then st-$H_2$Cer (0.125 $\mu$Ci) was added to the medium. Cells were harvested either after 60 min (fluorogram) or after the indicated times (graphic). Lipids were extracted and ceramides analysed by TLC and fluorography as described in Materials and methods. The developing solvent used for TLC was chloroform/methanol (9:1, by volume). □ ■ st-$H_2$Cer; ○ ● st-Cer

complex gangliosides in their sphingolipid pattern(19). To clarify if sphinganine or N-acyl-sphinganine is subjected to the desaturase reaction, we synthesized a suitable N-acyl-sphinganine, a semitruncated N-[1-$^{14}$C]-octanoyl-sphinganine (st-$H_2$Cer). By virtue of its short-chain fatty acid, its uptake by cells is considerably higher while its degradation is lower than that of N-stearoyl-sphinganine (data not shown).

When added to the culture medium in the presence or absence of an excess of fumonisin $B_1$, an inhibitor of sphinganine N-acyltransferase (EC 2.3.1.24), st-$H_2$Cer was rapidly taken up by neuroblastoma cells and desaturated, yielding N-[1-$^{14}$C]-octanoyl-sphingosine (st-Cer) (Fig.1). The radioactive material marked st-Cer in Fig.1 was scraped from the TLC plate, extracted and subjected to FAB-mass spectrometry. Characteristic peaks, m/z 448 for $MNa^+$ and m/z 408 for $MH^+$-$H_2O$, observed were identical to those obtained for synthetic st-Cer. In addition, the extracted st-Cer could be chemically reduced by $H_2$ (1 torr) and $Pd(OAc)_2$ in methanol for 1 h to regain st-$H_2$Cer.

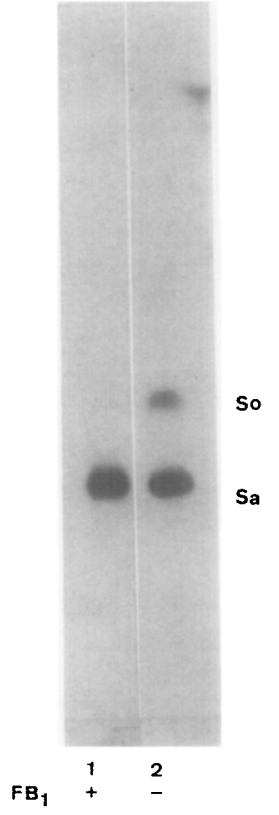


**Fig.2. The effect of fumonisin $B_1$ on the metabolism of exogenously added D-erythro-[3-$^3$H]-sphinganine in cultured B104 cells**
Neuroblastoma cells were cultured for 24 h in the presence (lane 1) or absence (lane 2) of fumonisin $B_1$ (25 $\mu$M). Then D-erythro-[3-$^3$H]-sphinganine (0.22 $\mu$Ci) was added to the medium. After 3 h cells were harvested. Lipids were extracted, acid-hydrolysed, desalted and separated by TLC using chloroform/methanol/2.5 M $NH_4OH$ (20:10:1, by volume) as solvent and visualized by fluorography. The data presented have been taken from one representative experiment.

The possibility that st-$H_2$Cer is degraded in the lysosomes releasing free [1-$^{14}$C]-octanoic acid, which might have been used for N-acylation of endogenous sphingosine could be excluded since no radioactive ceramide was formed when cells were fed with 50 $\mu$M [1-$^{14}$C]-octanoic acid in the absence of fumonisin $B_1$. These findings demonstrate the direct desaturation of st-$H_2$Cer and simultaneously exclude the formation of st-Cer from endogenous sphingosine and labeled octanoic acid.

As shown in Fig.1 the rate of desaturation followed that of incorporation of st-$H_2$Cer. Small concentrations of fumonisin $B_1$ (up to 5 $\mu$M) had no effect on the formation of st-Cer but did not completely inhibit N-acylation of sphinganine. For our studies we therefore used 25 $\mu$M fumonisin $B_1$ to be sure that inbibition of N-acylation of sphinganine is complete. Under this conditions desaturation of st-$H_2$Cer still ocurred though to a lower extent (60% of controls).

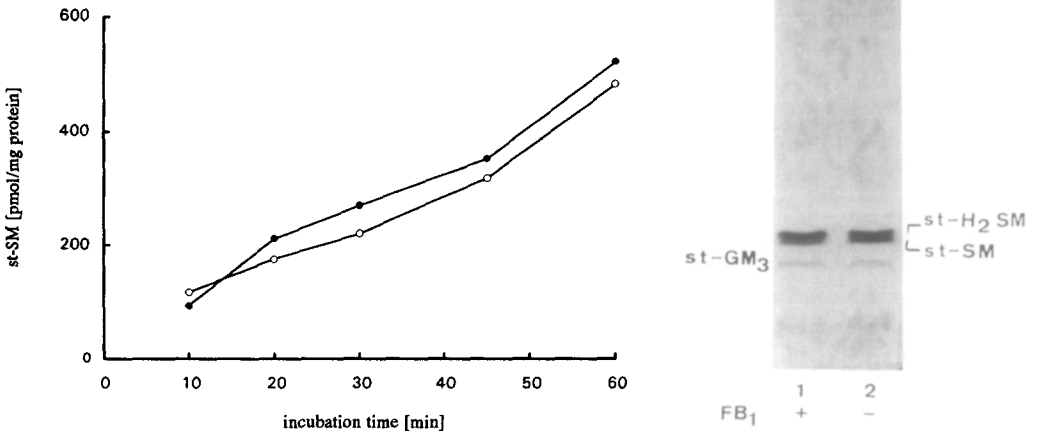


**Fig.3. The effect of fumonisin $B_1$ on the metabolism of exogenously added dihydroceramide, st-$H_2$Cer in cultured B104 cells**

Neuroblastoma cells were cultured for 24 h in the presence (lane 1, ● st-SM+st-$H_2$SM) or absence (lane 2, ○ st-SM+st-$H_2$SM) of fumonisin $B_1$ (25 $\mu$M). Then st-$H_2$Cer (0.125 $\mu$Ci) was added to the medium. Cells were harvested either after 60 min (fluorogram) or after the indicated times (graphic). Lipids were extracted, ceramides separated and remaining lipids analysed by TLC and fluorography as described in Materials and methods. The developing solvent used for TLC was chloroform/methanol/water (65:25:4; by volume).

In parallel experiments B104 cells were fed with D-erythro-[3-$^3$H]-sphinganine in the presence or absence of fumonisin $B_1$ (25 $\mu$M) (Fig.2). It is clear from this figure that the desaturation of administered D-erythro-[3-$^3$H]-sphinganine depends on its N-acylation. Thus, when cells were pretreated for 24 h with the N-acylation inhibitor, fumonisin $B_1$, the introduction of the 4,5-trans double bond was completely abolished.

When feeding st-$H_2$Cer (up to 60 min) to B104 cells, beside st-Cer two other radiolabeled sphingolipid species could be detected (Fig.3). As shown in Fig.3, semitruncated-sphingomyelins (st-$H_2$SM, upper band; st-SM, lower band) are the main metabolic products while only trace amounts of st-$G_{M3}$ could be detected. Degradation of the semitruncated-sphingomyelins by sphingomyelinase yielded both, st-Cer and st-$H_2$Cer. When cells were fed with st-Cer only the st-SM band with the $R_f$-value of the lower spot was detectable (results not shown). In the presence of fumonisin $B_1$ the amount of st-SM was decreased (Fig.3). This was not surprising because the formation of its precursor (st-Cer) was reduced accordingly by fumonisin $B_1$ (Fig.1).

Thus we have proven for the first time that dihydroceramide and not sphinganine is the substrate for the desaturation. Therefore, the enzyme catalyzing this reaction should be named dihydroceramide desaturase. From our metabolic studies in B104 cells as well as in primary cultured mouse cerebellar cells, which yielded similar results, it is obvious that free sphingosine is not an intermediate of sphingolipid biosynthesis but rather a product of sphingolipid catabolism. This is a very important finding since its function as a cellular second messenger has been proposed(20).

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