Analyses of the effect of monensin on glycosphingolipid metabolism

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The effect of monensin on glycosphingolipid metabolism was reinvestigated. It was found that monensin increased the uptake of [³H]galactose by human fibroblasts, causing an enhanced metabolic labelling of glycosphingolipids. The preferential incorporation of radioactivity into ceramide monosaccharide in the presence of monensin was accompanied by an equally increased rate of degradation. However, using radiolabelled precursors of the ceramide moiety, a generally diminished uptake of radioactivity into all glycosphingolipids was observed under the influence of monensin, with the exception of mono- and di-hexosylceramide. The enhanced incorporation of radiolabel by these two glycolipids, as well as their increased cellular chemical content, may reflect early synthesis in a monensin-insensitive pre-Golgi compartment after disruption of the cellular Golgi transport system by monensin.

INTRODUCTION

Analogously to the post-translational modification of glycoproteins, the metabolic carbohydrate processing of glycosphingolipids appears to be intimately connected with the intracellular communication of membrane material. After initiation of glycosphingolipid (GSL) synthesis in the region of the endoplasmic reticulum by transfer of the first, and possibly the second, monosaccharide unit, a crucial step in the biosynthesis of the higher GSL homologues is the transport of ceramide mono- and di-saccharide to Golgi membranes for further carbohydrate-chain elongation (i.e. ceramide tri- and tetra-saccharide, and the gangliosides). Interference with this process by lowering the temperature [1a,2] and by drugs that either inhibit the energy metabolism {ouabain [2], antimycin A [3] or retinoic acid (H. Wiegandt, unpublished work)} or act as ionophores (monensin [4–6] or ionophore A23187 [7]), causes a block in GSL biosynthesis at the level of ceramide mono/dihexoside (CMH/CDH) formation. In both instances, i.e. in the presence of retinoic acid [8,9] or monensin, the impairment of the biosynthesis of the higher GSL is accompanied by a cellular increase in the chemical quantity of CMH and CDH, as well as a dramatic enhancement in their metabolic radiolabelling by precursor [³H]galactose/[³H]glucosamine monosaccharides [4–8]. These changes in the radiolabelling and component distribution caused by monensin could originate from a net accumulation of precursor GSL merely due to an impairment of degradation at this level. In order to investigate this possibility, the influence of monensin upon GSL metabolism in confluent serum-starved quiescent human foreskin fibroblasts (HFF) was determined.

It was found that monensin indeed increased the chemical amount of cellular CMH and CDH and, with respect to its catabolism, a drastically increased rate of degradation of CMH was detected. This was taken as an indication of a hitherto-unobserved, possibly non-lysosomal, route of intracellular CMH processing. The increased formation of lower GSL effected by monensin coincided with a reduction in the biosynthesis of sphingomyelin, suggesting that a diversion of precursor ceramide from the anabolism of sphingophospholipid to that of the sphingoglycolipid may have occurred.

MATERIALS AND METHODS

Cells

HFF were used between passages 20 and 25 for all experiments. Cells were cultivated in Dulbecco's minimal essential medium (MEM; Seromed), supplemented with 200 units of penicillin, 120 µg of rebofacin/ml and 10% (v/v) heat-inactivated fetal-calf serum (FCS; Gibco). HFF cells were grown as monolayers in 75 cm² Falcon dishes (5 × 10⁶ cells). At 3 days after the monolayers were confluent, cells were partially arrested by serum starvation (0.2% FCS) for 72 h. To observe the effects of monensin (Calbiochem), HFF cells were incubated with monensin (3 µg/ml) in the culture medium (0.2% FCS) for 24–48 h.

³H labelling of cells

For radiolabelling, the respective ³H-labelled precursors were added to the culture medium at a concentration of 2.5 µCi/ml from 24 to 48 h after treatment with monensin. The following precursors, all purchased from Amersham-Buchler (Braunschweig, Germany), were used: [*methyl*-³H]choline chloride (sp. radioactivity 75 Ci/mmol), D-[6-³H]galactose (sp. radioactivity 30.3 Ci/mmol), [9,10(n)-³H]palmitic acid (sp. radioactivity 52 Ci/mmol) and L-[3-³H]serine (sp. radioactivity 30 Ci/mmol). When labelling was carried out with [³H]galactose, glucose in the culture medium was replaced by 10 mM-sodium pyruvate. Incorporation of [³H]serine was performed in the presence of 10%-FCS-containing culture medium.

Analysis of glycolipids, ceramide and phospholipids (PC and SM)

Cells were harvested by scraping from 75 cm² culture flasks and the total lipids extracted. Isolation of glycosphingolipids and component separation by t.l.c. was performed as described previously [7]. After labelling with [³H]palmitic acid or [³H]serine, [³H]SM was separated from the neutral lipid fraction on a Florisil column by the method of Laine *et al.* [10]. [³H]SM was identified from its *R_f* value on t.l.c. and revealed with the Dittmer/Lester spray reagent [11]. [³H]Ceramide was analysed in the neutral fraction by t.l.c., chloroform/methanol/acetic acid

Abbreviations used: GSL, glycosphingolipid(s); MEM, (Dulbecco's) minimal essential medium; FCS, fetal-calf serum; CDH, ceramide dihexoside; CMH, ceramide monohexoside; CTetH, ceramide tetrahexoside; CTH, ceramide trihexoside; gangliosides of the sialoganglio series are abbreviated as described in [1]: a suffix to G designates the neutral carbohydrate portion, e.g. G_{lac} (lactose), G_{tri} (gangliotriose) or G_{tet} (gangliotetraose); the number of sialic acid residues is given in arabic numerals and positional sialoisomers by the addition of a, b or c; HFF, human foreskin fibroblasts; PC, phosphatidylcholine; SM, sphingomyelin.

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(90:2:8, by vol.) being used as a running solvent. Ceramide was revealed on t.l.c. by exposure to I_2 vapour. After labelling with [3H]choline the total lipid extract was separated from non-lipid [3H]labelled contaminants by Sep-Pak C_{18} cartridges as described by Williams & McCluer [12]. SM and PC were separated by t.l.c. using the running solvent chloroform/methanol/water (65:25:4, by vol.). For analysis of the radioactivity incorporated into each lipid, the labelled lipids were separated on t.l.c. plates as described above, and the pattern of radioactivity was detected by direct radioscanning with a t.l.c. linear analyser (Berthold).

Chemical quantification of glycosphingolipids, SM and PC

All analyses were performed on cells partially arrested by serum starvation and incubated in the presence or absence of monensin for 48 h. For estimation of the chemical quantity of neutral glycosphingolipids, the t.l.c. plates were stained with 2% orcinol/ H_2SO_4 [13] at 110 °C for 10 min. The spots were analysed at 440 nm on a Shimadzu dual-wavelength t.l.c. scanner (CS 910; Chromopac-R-IB) and quantified by calculation from standard curves [14]. Gangliosides were quantified densitometrically on t.l.c. plates at 580 nm by using a resorcinol reagent [15]. The amount of SM and PC was analysed chemically by two methods, after extraction from the total lipid, as described by Saito *et al.* [16]. Method (a) is based on the procedure described by Macala *et al.* [17]. After separation on t.l.c., the lipids were charred for densitometry with Fewster's reagent ([18]; 3% cupric acetate (w/v) in 8% phosphoric acid (v/v) solution) at 180 °C for 15 min, and the t.l.c. plates scanned at 350 nm as described for glycolipids. The estimation of the chemical quantity of SM and PC by method (b) is based on the determination of phosphorus described by Bartlett [19]. For this, the lipids were separated on t.l.c. plates and revealed with iodine vapour. After scraping the relevant positions from the t.l.c. plate, the lipids were extracted with 10 ml of chloroform/methanol/water (10:10:1, by vol.) and the solvent was evaporated by flushing with N_2 at room temperature. Each sample was mineralized in 1 ml of 1.25 M- H_2SO_4 for 4 h at 50 °C. After hydrolysis, 0.5 ml of 5% (w/v) ammonium molybdate in 2.5 M- H_2SO_4 and 0.1 ml of Fiske & SubbaRow's reagent [19a] were added and the sample volume adjusted to 2.4 ml with water. The solution was heated for 10 min at 100 °C and the absorbance was read at 830 nm.

Protein

Protein was determined by the method of Lowry *et al.* [20], with BSA as standard.

RESULTS

Changes in cellular GSL, SM and PC concentration in the presence of monensin

Quiescent HFF were kept for 48 h in the presence of monensin. The subsequent quantitative analysis showed an approx. 50% increase in the total GSL content. This accumulation of GSL was mostly due to an approx. 2.5-fold increase in CMH and CDH (Table 1). By contrast, the cellular contents of SM and PC were diminished, in the presence of monensin, by roughly one-third that of controls (Table 1).

Time-dependence of metabolic carbohydrate labelling of GSL in the presence of monensin

In order to distinguish more clearly whether the enhancement of the chemical amounts of CMH/CDH by monensin resulted from anabolic changes or from an impaired catabolism, continuous labelling with [3H]galactose was performed in the presence of the drug. By 30 min, a time interval short enough that the expected effect would be predominantly that of anabolism,

uptake of [3H]galactose radioactivity into GSL already reflected the relative proportions of component labelling corresponding to later time intervals. In the presence of monensin, precursor incorporation into CMH and CDH at all times chosen showed increases of 7–10-fold that of controls (Figs. 1a and 1b).

Table 1. Cellular content of GSL, SM and PC of HFF kept in the presence of monensin for 48 h

Lipid	Content (nmol/mg of protein)	
	No monensin	Monensin for 48 h
CMH	4.55 (± 0.14)	11.05 (± 0.15)
CDH	0.8 (± 0.02)	2.2 (± 0.22)
CTH	4.15 (± 0.09)	3.85 (± 0.19)
CTetH	1.8 (± 0.15)	1.85 (± 0.2)
G _{lac} 1	3.35 (± 0.05)	4.2 (± 0.46)
G _{tri} 1	0.9 (± 0.03)	1.3 (± 0.03)
G _{tet} 1	0.3 (± 0.03)	0.35 (± 0.05)
G _{lac} 2	0.25 (± 0.03)	0.3 (± 0.02)
G _{tet} 2a	0.25 (± 0.04)	0.3 (± 0.02)
SM (M_r 702)	142.8 (± 8.8)*	99.66 (± 5.6)*
	140.0 (± 1.6)†	97.9 (± 0.9)†
PC (M_r 733)	257.1 (± 16.8)*	189.8 (± 7.5)*
	264.8 (± 2.4)†	187.5 (± 7.3)†

* Phosphate analysis by the method of Bartlett [19].

† Densitometry as described by Macala *et al.* [17].

Fig. 1. Time-dependent incorporation of [3H]galactose into GSL components

(a) Control in the absence of monensin; (b) with the addition of monensin; for conditions of labelling, see the Materials and methods section; each point represents the mean result for two parallel cultures. Symbols: ■, CMH; ▲, CDH; ●, CTH; ◆, CTetH; □, G_{lac}1; △, G_{tri}1; ◇, G_{tet}1.

Fig. 2. Time-dependent changes in the radioactivity of $[^3\text{H}]$ galactose-prelabelled total GSL of HFF in the absence of the ^3H -labelled precursor

For conditions of labelling, see the Materials and methods section; each point represents the mean result for two parallel cultures. ■, Control; ▲, monensin.

Fig. 3. Time-dependent changes in the radioactivity of $[^3\text{H}]$ galactose-prelabelled GSL components of HFF in the absence of the ^3H -labelled precursor

(a) Control without monensin; (b) in the presence of monensin; for conditions of labelling, see the Materials and methods section; each point represents the mean result for two parallel cultures. Symbols are as for Fig. 1.

Time-dependence of GSL catabolism in the presence of monensin

Further support for the assumption that CMH/CDH catabolism remained unimpaired under the influence of monensin was obtained by analysis of metabolically prelabelled GSL

Table 2. $[^3\text{H}]$ Galactose uptake by HFF in the presence of monensin: comparison of diffusible and GSL-incorporated radioactivity

Incubation was for 3 h.

	$10^{-2} \times \text{Uptake}$ (d.p.m./standard culture dish)	
	Dialysable radioactivity	Total GSL
Control	410 ± 20	124 ± 1
Monensin	3365 ± 67	1521 ± 30

under chase conditions. HFF were prelabelled with $[^3\text{H}]$ galactose for 3 h. After removal of the precursor from the medium and keeping the cells under chase conditions in the presence of monensin, the GSL component radioactivity was analysed at various time intervals.

Over an observation period of 26 h the total GSL of control cultures had lost very little of their radiolabel (Fig. 2). There was, however, an apparent shift of radioactivity with time from CMH to the higher GSL components (Fig. 3a). The loss of some 16 % of $[^3\text{H}]$ CMH label thereby equalled approximately the gain in the higher homologues. Under these control conditions, an apparent half-life of the total CMH of 19 h was calculated.

In the continued presence of monensin there was a 30 % loss in the total GSL-bound ^3H label within the first 10 h (Fig. 2). The loss of CMH continued thereafter at a constant, but comparably lower, rate (Fig. 3b). After 26 h, some 58 % of the $[^3\text{H}]$ CMH had disappeared, whereby only some 20 % of the loss could be accounted for by incorporation into higher GSL homologues. Apparently, 38 % of the $[^3\text{H}]$ CMH had been catabolized under these conditions. In the presence of monensin, CMH showed a half-life of 10.5 h, as measured over the observation period of 26 h.

Change in $[^3\text{H}]$ galactose uptake from the medium by HFF in the presence of monensin

Cellular uptake of free $[^3\text{H}]$ galactose was measured after preincubation of HFF with the labelled sugar. Monensin caused an intracellular accumulation of diffusible radioactivity that after 3 h reached a level approx. 8-fold that of the controls in the absence of the drug. Over the same time interval a 12-fold increase in incorporation of ^3H label into GSL was measured (Table 2).

Changes in the metabolic labelling of the ceramide of sphingolipids in the presence of monensin

The results of chemical quantification of GSL and their incorporation of ^3H -labelled precursor in the HFF system are suggestive of a net increase in glycolipid formation in the presence of monensin. This implies that, in this case, additional cellular ceramide had to be provided for the increased GSL synthesis. In order to observe any such effect of monensin on ceramide biosyntheses, HFF were prelabelled with $[^3\text{H}]$ palmitic acid or $[^3\text{H}]$ serine. Uptake of label by ceramide, SM and GSL was monitored.

In principle, changes in incorporation of $[^3\text{H}]$ palmitic acid in the presence of monensin were comparable with those seen with serine as ceramide precursor, i.e. monensin generally decreased the incorporation of label into SM as well as into total GSL (Table 3).

Whereas in the presence of monensin after 24 h of prelabelling the uptake of ^3H label from palmitic acid and serine into free ceramide was not significantly affected, the incorporation into

Table 3. ^3H uptake into ceramide and sphingolipids from palmitic acid and serine in the presence of monensin

Incubation was for 24 h.

	$10^{-2} \times ^3\text{H}$ uptake (d.p.m./standard culture dish)	
	– Monensin	+ Monensin
From [^3H]palmitic acid into:		
CMH	14834	17883
CDH	792	2019
CTH	1102	529
CTetH	1113	217
Sum of neutral GSL	17841	20648
Sum of gangliosides	22249	6778
SM	148982	109504
Ceramide	7181	7177
Sum of GSL + SM + ceramide	196253	130551
From [^3H]serine into:		
CMH	782	1448
CDH	167	736
CTH	606	255
CTetH	544	180
Sum of neutral GSL	2099	2619
Sum of gangliosides	2667	92
SM	11189	4536
Ceramide	616	535
Sum of GSL + SM + ceramide	16581	7781

the ceramide of SM and into the total GSL ceramide was diminished to a comparable extent, i.e. 26.5 % for SM and 31 % for the total GSL.

Monitoring the time course of rapid [^3H]palmitic acid uptake into GSL and SM, after 30 min of incorporation, an initial increase in radioactivity of all GSL components was observed as compared with controls. This increase, most pronounced with CMH (3.2-fold) and CDH (7.2-fold), was accompanied by a strongly reduced labelling of SM (Fig. 4).

It is conceivable that the specific radioactivity of the ceramide that is used by the cells for the formation of SM would equal that of the newly synthesized GSL. If this were the case, a strong inhibition of SM biosynthesis by monensin would be observed at all times. This possibility was supported by the data of the chemical quantification of cellular SM, which showed a near-30 % decrease in the chemical amount caused by the drug (Table 1). PC was found to be decreased by monensin to a similar extent, thereby leaving the molar ratio between SM and PC of approx. 1:2 essentially unaltered (Table 1). When the metabolic uptake of radiolabelled choline into both choline phospholipids was compared, however, SM showed an incorporation that was relatively much more monensin-sensitive than that of PC (Table 4).

DISCUSSION

In a previous investigation it was observed, by metabolic labelling using [^3H]monosaccharide precursors, that the addition of retinoic acid to the culture medium of HFF caused an impairment of the ^3H -labelled precursor uptake into all higher GSL, i.e. CTH, CTetH and the gangliosides. At the same time, the lower-homologue GSLs, CMH and CDH, appeared to accumulate radioactivity [8]. However, retinoic acid caused not only a dramatic elevation of the radiolabelling of CMH and CDH, but also an increase in the chemically quantifiable (i.e.

Fig. 4. Time-dependent incorporation of [^3H]palmitic acid into GSL and SM

(a) Total ^3H uptake into sphingolipids in control (■) and monensin-treated (▲) cells; (b,c) ^3H incorporation into GSL components in controls without monensin (b) and in the presence of monensin (c). Symbols in (b) and (c) are as in Fig. 1, except: ○, G_{ter}2a; ◆, CTetH + SM. For conditions of labelling, see the Materials and methods section. Each point represents the mean result for two parallel cultures.

labelled + unlabelled) GSL content of the cells [9]. These changes in cellular GSL metabolism observed in the presence of retinoic acid were reminiscent of those induced by monensin in various cell culture systems as reported by Miller-Podraza & Fishman [1] and by Saito *et al.* [3,6]. It was shown by electron microscopy that retinoic acid, like monensin, caused a vacuolization, and possibly a disruption, of Golgi membranes [9].

The present study was initiated in order to investigate in more detail the cause of the dramatic drug-induced elevation in the radiolabelling of CMH and CDH, the increased rate of precursor

Table 4. Uptake of [³H]choline into SM and PC of HFF in the presence of monensin

Incubation was for 24 h.

	Uptake (c.p.m./ standard culture dish)*	
	– Monensin	+ Monensin
SM	7680	1600
PC	96000	64000

* Quantification by ³H scanning after t.l.c. separation.

[³H]sugar uptake by the cells and/or the impairment of the catabolic or anabolic processing of these lower GSL.

In the presence of monensin, as was found here, the cellular uptake of free [³H]sugar is strongly increased. Thus the higher intracellular levels of the precursor and the known accumulation of sugar nucleotides by dilation of Golgi vesicles, as caused by monensin [11], may account for the enhanced radiolabelling of the GSL. If in fact the enhanced availability of intracellular ³H-labelled precursor would also be reflected in a corresponding increase in specific radioactivity of newly synthesized GSL, an actual net gain in glycolipid formation effected by monensin may be calculated that amounts to some 49% of the total GSL. An increased hexose uptake by cells, as has been reported previously, may be induced by stress [21]. Therefore it is conceivable that monensin affects cellular stress mechanisms, leading to an enhanced hexose uptake in a way similar to that which has been shown with cells in culture after infection with abortive virus, by heat shock or by arsenite treatment [21,22].

Furthermore, there was no good evidence for the possibility that the increased radiolabelling of CMH and CDH by [³H]hexose precursor in the presence of monensin is due to an impairment of the catabolism of these GSL. The kinetics of cellular GSL catabolism in HFF, as shown by pulse-chase experiments, revealed a strong acceleration of the removal of GSL, in particular of CMH, by monensin (Figs. 3a and 3b).

In contrast with what was observed by the metabolic labelling of the GSL with [³H]sugar, the use of radioactive precursors of the ceramide moiety, i.e. palmitic acid or serine, showed that monensin caused a decrease in the formation of the total GSL (Table 3). At the same time, the ceramide labelling of SM was lowered to a similar extent as for GSL (Table 3). However, the great difference in sphingolipid labelling with [³H]serine or [³H]palmitate between monensin-treated and non-treated cells might be explained by the probably differently affected cellular uptake and metabolic fate of these precursor molecules. The decrease in SM formation by monensin is not due to an overall inhibition of the synthesis of all phospholipids. As shown by precursor [³H]choline incorporation, monensin caused a much greater reduction in SM labelling than in PC labelling (Table 4).

The decrease in the chemically quantifiable SM and PC of fibroblasts under the influence of monensin, without a concomitant change of the respective molar ratio of these phospholipids, is indicative of an actual loss of cellular membrane. Since at the same time the complex GSL level does not alter, and these lipids are found predominantly in the plasma membrane, this might suggest that it is intracellular membrane which is lost.

The radiolabel uptake in the formation of free ceramide was relatively insensitive to monensin. Therefore it appears even less likely that the decrease in total GSL and of SM labelling by ceramide precursors may be caused by an alteration of ceramide formation. Comparison of the ceramide-precursor labelling of single GSL components, however, reveals a strongly enhanced incorporation of label into CMH and CDH (Table 3). This increase is observed as soon as 30 min after precursor incorporation. Therefore it is likely that this effect represents changes in anabolism and a true enhancement of CMH and CDH biosynthesis (Fig. 4).

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