Ganglioside incorporation and release by the isolated perfused rat liver

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The fate of exogenous ganglioside G_{M1} labelled in the sphingosine moiety, [$Sph-^3H$] G_{M1} , administered as a pulse, in the isolated perfused rat liver was investigated. When a non-recirculating protocol was employed, the amount of radioactivity in the liver and perfusates was found to be dependent on the presence of BSA in the perfusion liquid and on the time elapsed after the administration of the ganglioside. When BSA was added to the perfusion liquid, less radioactivity was found in the liver and more in the perfusate at each time tested, for up to 1 h. The recovery of radioactivity in the perfusates followed a complex course which can be described by three pseudo-first-order kinetic constants. The constants, in order of decreasing velocity, are interpreted as: (a) the dilution of the labelled G_{M1} by the constant influx of perfusion liquid; (b) the washing off of G_{M1} loosely bound to the surface of liver cells; (c) the release of gangliosides from the liver. Process (b) was found to be faster in the presence of BSA, probably owing to the ability of BSA to bind gangliosides. The [$Sph-^3H$] G_{M1} in the liver underwent metabolism, leading to the appearance of products of anabolic (G_{D1a} , G_{D1b}) and catabolic (G_{M2} , G_{M3}) origin; G_{D1a} appeared before G_{M2} and G_{M3} but, at times longer than 10 min, G_{M2} and G_{M3} showed more radioactivity than G_{D1a} . At a given time the distribution of the radioactivity in the perfusates was quite different from that of the liver. In fact, after 60 min G_{D1a} was the only metabolite present in any amount, the other being G_{M3} , the quantity of which was small. This indicates that the liver is able to release newly synthesized gangliosides quite specifically. When a recirculating protocol was used, there were more catabolites and less G_{D1a} than with the non-recirculating protocol. A possible regulatory role of ganglioside re-internalization on their own metabolism in the liver is postulated.

INTRODUCTION

Gangliosides are sialic-acid-containing glycosphingolipids of eukaryotic organisms. Although the majority of gangliosides reside in the outer leaflet of the cell surface, some are found in a non-cell-associated form in blood serum and other body fluids where, presumably, they are present as protein-bound complexes or membrane fragments.

Our group and others have already reported the role of the liver in the incorporation and metabolism of exogenously added gangliosides *in vivo*. It was shown that exogenous gangliosides are mainly taken up by the liver and exhaustively metabolized along both catabolic and anabolic pathways (Ghidoni *et al.*, 1986; Ogura & Handa, 1988). It was also reported that gangliosides in the liver produce catabolites that are recycled for biosynthetic purposes (Ghidoni *et al.*, 1987; Trinchera *et al.*, 1990a,b). These investigations were conducted through the intraperitoneal or intravenous administration of radiolabelled gangliosides to rodents. Although the experiments provided extensive information on the metabolic processes of the given compound, they were limited in that they gave no answer to the uptake kinetics of the compound by the liver. Short-term experiments are, in fact, difficult to design in that such an '*in vivo*' approach, owing to the prolonged permanence of gangliosides in the bloodstream, results in the overlapping of different kinetics, e.g. the half-life in blood, the actual liver uptake and consequent metabolism. Moreover, the possibility exists that some compounds produced in the liver or other organs and, starting from the injected precursor, are released into the

bloodstream and re-internalized, for further metabolism, into the liver.

To overcome the above difficulties and to provide answers to the questions raised, we have undertaken a systematic investigation of ganglioside incorporation, metabolism and release by the isolated perfused rat liver after pulse administration of exogenous G_{M1} radiolabelled on the sphingosine moiety ([$Sph-^3H$] G_{M1}). This model has proved to be both suitable and useful in providing information on short-time uptake, metabolism, the release of specific gangliosides and the possible regulatory effect of BSA on these processes.

EXPERIMENTAL

Radiolabelling of gangliosides

Ganglioside G_{M1} was 3H -labelled at the level of the C-3 of the long-chain base ([$Sph-^3H$] G_{M1}) by the 2,3-dichloro-5,6-dicyano-benzoquinone/ NaB^3H_4 method (Ghidoni *et al.*, 1981). The specific radioactivity was 1.17 Ci/mmol and the radiochemical purity, assessed by t.l.c. radiodensitometry (Ghidoni *et al.*, 1986), was over 99%. 3H -labelled gangliosides G_{M2} , G_{M3} , G_{D1a} , G_{D1b} , lactosylceramide, glucosylceramide and ceramide, used only for reference purposes, were prepared as previously reported (Ghidoni *et al.*, 1986).

Perfusion protocol

Adult male Wistar rats (Charles River, Milan, Italy), 180 g average body weight, were employed. The animals were kept,

Abbreviations used: for glycolipid and ganglioside designation, the nomenclature of Svennerholm (1964) and the IUPAC–IUB Recommendations (1977) were followed; LDH, lactate dehydrogenase; Sph, sphingosine.

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until the day before the experiment, under the following conditions: light, from 06:00 to 18:00 h; temperature, 20 °C; food and water, *ad libitum*. Animals were lightly anaesthetized with Pentothal (50 mg/kg intraperitoneally). Experiments were performed between 09:00 and 14:00 h.

The perfusion technique was a modification of that described elsewhere (Scholz *et al.*, 1973). The perfusion liquid was 4.62 mM-KCl/2.33 mM-H₂KPO₄/2.31 mM-MgSO₄/3.5 mM-CaCl₂/120 mM-NaCl/5 mM-D-glucose/19.9 mM-Hepes/0.1 % BSA (Helmy *et al.*, 1986). The perfusion medium was in a thermostatically controlled glass chamber at 37 °C, where it was gassed with O₂/CO₂ (19:1). The livers were perfused through the portal vein. When the non-recirculating procedure was followed, the effluent perfusate was collected via a plastic cannula in the vena cava connected to an automatic fraction collector with rubber tubing. At 15 min after completing all the surgical procedures, 15 µCi of [*Sph*-³H]G_{M1}, resuspended in 0.5 ml of perfusion liquid, were injected into the plastic tube just before the connection with the portal vein, at a rate of 50 µl/s.

Recirculation experiments were performed by connecting the effluent from the vena cava directly to the thermostatically controlled glass chamber, thus forming a closed circuit.

In both cases the perfusion flow was adjusted at 12 ml/min with a peristaltic pump.

Treatment of the liver and perfusate

At different times after administration only the clear yellowish-coloured livers were considered as well-perfused; those of a red appearance were discarded. The livers were homogenized at 6000 rev./min for 60 s, with a Polytron homogenizer (20 ST; Kinematica, Lucerne, Switzerland) by adding 4 vol. of perfusion liquid immediately after being excised, and ultracentrifuged at 100 000 *g* during 2 h. The precipitates and the supernatants were analysed for radioactivity.

Total lipids were extracted from the particulate fraction and partitioned by the phosphate buffer/tetrahydrofuran procedure of Tettamanti *et al.* (1973). The aqueous phase obtained, containing the gangliosides, was evaporated to dryness, resuspended, and exhaustively dialysed against distilled water and finally freeze-dried. The organic phase was evaporated to dryness before processing.

Perfusates were dialysed over 48 h against distilled water and freeze-dried. The dried residue was extracted with chloroform/methanol (2:1, v/v) and chloroform/methanol (1:1, v/v); the combined extracts were dried under vacuum and finally resuspended in chloroform/methanol (2:1, v/v).

In some experiments the perfusates were centrifuged at 100 000 *g* for 2 h and the radioactivity analysed in both the pellets and the supernatant.

Analysis of radiolabelled lipids

The individual lipids contained in both the aqueous and organic phases were separated by t.l.c. using chloroform/methanol/aq. 0.3 % CaCl₂ (60:42:11, by vol.) (eluting solvent system for radiolabelled gangliosides) or chloroform/methanol/water (55:20:3, by vol.) (eluting solvent system for the other lipids). Radiolabelled gangliosides were identified by a comparison of their *R_f* values with those of reference compounds of known structure and, after purification, by sensitivity to *Vibrio cholerae* sialidase (*N*-acetylneuraminyl hydrolase, EC 3.2.1.18) (Ghidoni *et al.*, 1987) and β -galactosidase (β -galactoside galactohydrolase, EC 3.2.1.23) (Cahan *et al.*, 1982). In all cases, reference gangliosides were processed in parallel. Non-ganglioside lipids were identified by a comparison of their *R_f* values with those of authentic standards.

Determination of radioactivity

Liquid-scintillation counting in solution was performed using Ultima Gold (Packard, Meriden, CT, U.S.A.) as the liquid-scintillation cocktail. Radiolabelled lipids were revealed by fluorography and quantified by radiodensitometry using a RITA t.l.c. analyser (Raytest, Straubenhardt, Germany).

Lactate dehydrogenase (LDH; L-lactate:NAD⁺ oxidoreductase, EC 1.1.1.27) assay

LDH activity was measured in perfusates according to MacIntosh & Plummer (1976).

Statistics

The radioactivity decay curves were analysed by using a non-linear-regression data-analysis program for IBM-PC computers.

Histological analysis

Specimens from the livers of rats of each group were fixed in 10 % (v/v) formalin and Bouin's fluid and embedded in paraffin. Sections 5 µm thick were stained with haematoxylin/eosin, Gomori's reticulin stain and Masson's trichrome stain.

RESULTS

Tissue integrity during the perfusion protocol

The integrity of the liver throughout the perfusion was tested by the LDH activity in the perfusion liquid and by histological observation. Except for a rapid increase immediately after surgery, most likely due to damage of some cells in the course of operation, the LDH activity value remained constant for up to 1 h from the beginning of the perfusion (Table 1). The histological observation of the tissue performed at 30, 40 and 60 min after

Table 1. LDH activity in the perfusate at different times after surgery

Time after surgery (min)	LDH activity (nmol/h per ml)
10	3.15
20	1.03
30	1.05
40	1.04
50	1.05
60	1.04

Fig. 1. Time course of total radioactivity incorporation in the liver in the presence or absence of BSA in the perfusion liquid

Results are the mean values for three experiments. S.D. values were less than 15 % of the mean values. The dose was 15 µCi. ●, Absence of BSA; ▲, presence of BSA.

surgery showed a preserved hepatic architecture without evidence of flogosis or hepatocellular damage. The same results were obtained in each of the protocols followed, i.e. the non-recirculating perfusion in the presence or absence of BSA and the recirculating perfusion protocol with BSA.

Time course of radioactivity incorporation in the liver homogenates

The incorporation of radioactivity in the liver as a function of the time after administration of [*Sph*-³H]G_{M1}, when the non-recirculating protocol was employed, is shown in Fig. 1. When BSA was present in the perfusion fluid, the radioactivity decreased slightly for up to 1 h after administration, while in its absence a larger incorporation, with a marked decrease during the perfusion time, was observed.

When the liver homogenates were submitted to ultracentrifugation at 100 000 *g* for 2 h, at least 95% of all the incorporated radioactivity was in a particulate form at each investigated time, regardless of the presence of BSA.

Time course of radioactivity in perfusates

The time course of the radioactivity recovered in the perfusate is shown in Fig. 2. When BSA was present, the total radioactivity in the collected perfusate (Fig. 2a) increased very sharply in the first 5 min after administration, and much more smoothly thereafter. In the absence of BSA the increase was more gradual from the beginning, the absolute values always being maintained lower than those observed in the presence of BSA. These two curves were complementary to those of the radioactivity incorporation in the liver (Fig. 1). In fact, for each time, the sum of the radioactivity recovered in the liver and in the perfusates was constant, regardless of the presence of BSA, and approached (95–99%) the value of injected radioactivity. This evidence confirmed that, under our experimental conditions, no dissipation of radioactivity occurred in locations other than the liver and the perfusate. Contrary to what was observed in the liver, the radioactivity in the perfusates was in a soluble form for at least 95% of the total at each investigated time, regardless of the presence of BSA. When the above data were expressed as the radioactivity in each fraction and reported on an exponential scale (Fig. 2b), the behaviour of both profiles was curvilinear, suggesting the presence of different kinetic components. For both curves we calculated three different constants of pseudo first order (each providing a $t_{1/2}$ value) and the relative pool sizes by extrapolation to zero time. The $t_{1/2}^I$ and $t_{1/2}^{III}$ (about 0.65 ± 0.05 min and 23 ± 2 min respectively) were found not to depend on the presence of BSA. Vice versa, the $t_{1/2}^{II}$ was found to depend significantly on the presence of BSA (2.2 ± 0.2 min and 4.5 ± 0.3 min with and without BSA respectively). All the $t_{1/2}$ values are expressed as the means $\pm$ s.d. for three different experiments. In the presence of BSA, the pool size of the slowest component was 12 times smaller (40 000 d.p.m. as against 470 000 d.p.m.) than that of the second component. The pool sizes in the absence of BSA were much higher (1 200 000 d.p.m. and 3 500 000 for the third and second components respectively).

Time course of radioactivity distribution in the individual gangliosides in the liver and perfusate

Using the non-recirculating protocol the incorporation of radioactivity into the individual gangliosides was concurrently investigated in both the liver and the perfusate after the administration of [*Sph*-³H]G_{M1} (Table 2 and Fig. 3). The total lipid-associated radioactivity values (Table 2, last column) are the same as those used for constructing the curves reported in Fig. 1 (liver) and Fig. 2a (perfusates) in the intervals 10–15, 30–35 and 60–65 min; the large difference between BSA and non-BSA

Fig. 2. Time course of total radioactivity recovery in the perfusate in the presence or absence of BSA

Each curve refers to one single animal and is representative of three different experiments. (a) Total recovered radioactivity; (b) radioactivity in each collected fraction. The dose was 15 μ Ci. ●, Absence of BSA; ▲, presence of BSA.

Fig. 3. Time course of radioactivity distribution in individual gangliosides from the liver and the perfusate

Values are the same as in Table 2 and are expressed as percentage of total ganglioside radioactivity. Key to histogram bars: ■, 10 min; ▨, 30 min; ▩, 60 min.

experiments depends on the kinetics of the liver incorporation and release as reported in the previous section. In the presence of BSA and at given time intervals the distribution of radioactivity into the gangliosides of the liver was markedly different from that of the gangliosides of the perfusate (Fig. 3). In fact, from the very first time investigated, we found in the liver metabolites of both anabolic (G_{D1a}) and catabolic (G_{M2} and G_{M3}) origin, whereas in the perfusates only G_{D1a} was present; at 60 min of perfusion

Table 2. Radioactivity distribution of individual gangliosides in the liver and perfusate

Results are mean values for three experiments.

(a) Liver	Perfusion time (min)	$10^{-3} \times$ Radioactivity (d.p.m./g of liver)					
		G_{M2}	G_{M3}	G_{D1a}	G_{D1b}	Residual G_{M1}	Total lipid
+BSA	10	19.5	19.5	101	—	3721	3900
	30	251	71.8	115	—	3152	3602
	60	574	102	132	66.0	2300	3430
-BSA	10	17.0	—	119	—	16950	17118
	30	191	8.0	151	—	9630	10052
	60	468	148	201	53.3	4910	5920

(b) Perfusate*		$10^{-3} \times$ Total radioactivity (d.p.m.)†					
		G_{M2}	G_{M3}	G_{D1a}	G_{D1b}	Residual G_{M1}	Total lipid
+BSA	10	—	—	5.6	—	176	182
	30	—	—	4.5	—	58	63
	60	—	0.2	3.8	—	30	34
-BSA	10	—	—	11.9	—	3929	3949
	30	—	—	24.0	—	1574	1602
	60	—	—	40.1	—	628	668

* The time values (10, 30 and 60 min) correspond to the fractions collected between the intervals 10–15, 30–35 and 60–65 min respectively.

† Values of radioactivity collected in the time intervals explained in footnote *.

Table 3. Effect of recirculation on the radioactivity in individual liver lipids in the presence of BSA

Data are expressed as percentage of total radioactivity and are the mean values for three experiments.

Metabolite	Distribution of radioactivity (% of total)	
	Non-recirculating (1 h)	Recirculating (2 h 30 min)
G_{M2}	17.4	7.0
G_{M3}	3.1	6.2
G_{D1a}	4.0	1.5
G_{D1b}	2.0	tr.
Lactosylceramide	3.0	5.1
Glucosylceramide	0.2	2.8
Ceramide	0.1	5.1
Sphingomyelin	—	0.5
Others	0.5	2.1
Residual G_{M1}	69.7	67.8
$10^{-3} \times$ Total liver radioactivity (d.p.m./g of fresh tissue)	352	605
$10^{-3} \times$ Lipid-associated radioactivity (d.p.m./g of fresh tissue)	343	570

also some G_{D1b} was observed in the liver. It is noteworthy that the amount of radiolabelled G_{D1a} in the liver was much higher than that of G_{M1} catabolites (G_{M2} and G_{M3}) at 10 min. At longer times, while G_{D1a} underwent a slight increase, G_{M2} and G_{M3} were produced in greater amounts (Table 2). In the perfusate, G_{D1a} was practically the sole metabolite present, the only exception being a small amount of G_{M3} at 60 min of perfusion. The percentage of G_{D1a} in the total radioactivity was higher in the perfusate than in the liver, at each tested time (Fig. 3). In absence of BSA, a relevant amount of G_{D1a} was found as the sole

metabolite. With respect to residual G_{M1} , it should be noted that this ganglioside represents, at each time and regardless of whether BSA was present or not, at least 70 % of the total radioactivity in both the liver and perfusate. Therefore any BSA effect on liver G_{M1} incorporation is greatly reflected in the amount of total radioactivity.

Metabolism of G_{M1} with the recirculating perfusion protocol

The metabolites formed in the liver from the administered G_{M1} , when the recirculating protocol for perfusion was used, are listed in Table 3. For comparison with the non-recirculating protocol, we chose the 2 h 30 min, since at that time the percentage of residual G_{M1} over the total radioactivity was similar to that at 60 min in the non-recirculating protocol. It is evident that, with recirculation, more G_{M1} catabolites were produced. These included, besides G_{M3} and G_{M2} , also lactosylceramide, glucosylceramide and ceramide. Concurrently, compounds of biosynthetic origin (G_{D1a}) were produced to a lesser extent. In the perfusate, under recirculation, we found similar amounts of G_{D1a} and G_{M3} (each accounting for about 1.3 % of the total radioactivity), as well as G_{M1} , the major radioactive compound (results not shown).

DISCUSSION

The results indicate that the isolated perfused rat liver is a suitable model with which to investigate the uptake of exogenous gangliosides by the liver, their metabolism and their release.

In the non-recirculating protocol G_{M1} was given as a pulse immediately upstream of the liver. Under these conditions BSA notably decreased the association of gangliosides by the liver and concurrently increased their appearance in the perfusate. This effect finds a reasonable explanation in the ability of BSA to bind ganglioside micelles (Tomasi *et al.*, 1980; Venerando *et al.*, 1982) and to inhibit ganglioside association with liver membranes. The appearance of gangliosides in the perfusate differed in the presence or absence of BSA, not only quantitatively, but

also qualitatively. The analysis of the recovery of radioactivity in the perfusates showed a complex time course that could be described by three kinetic constants of pseudo first order. The fastest process could be interpreted as the passage of G_{M1} , non-associated with the liver, into the perfusate. The half-life of 0.5 min is in accordance with the flow rate of the perfusion liquid and was independent of the presence of the liver in the system and of BSA in the perfusion liquid. The second process, which was, conversely, BSA-dependent, was most likely attributable to the washing off of loosely bound G_{M1} . In fact, it has been reported that gangliosides can associate with cells in culture in a loosely bound form (Callies *et al.*, 1977; Chigorno *et al.*, 1985). BSA caused a halving of the washing time, because of competition for G_{M1} with the liver cell surface. The last component, whose half-life was of 22 min, was interpreted as being due to the actual release of ganglioside by the liver. The larger sizes of the pools in the absence of BSA may reflect a relevant hepatic incorporation of G_{M1} , not rapidly available for metabolism.

Most liver radioactivity (more than 95%) was in a particulate form, from the shortest time points, suggesting that exogenous gangliosides are very rapidly inserted into the cell membrane, as already reported for cultured cells (Schwarzmann *et al.*, 1983). Once incorporated, the G_{M1} ganglioside undergoes metabolism in both the catabolic and anabolic directions, as reported in the 'in vivo' model (Ghidoni *et al.*, 1986). In fact, short-time metabolism in the liver showed the appearance of G_{D1a} earlier than catabolites as G_{M2} and G_{M3} . This suggests that, once incorporated, a portion of G_{M1} directly flows to the Golgi apparatus, where its sialylation to G_{D1a} takes place (Ghidoni *et al.*, 1986). The plasma-membrane Golgi traffic could be mediated by an endosomal pre-Golgi compartment, as in the case of low-density lipoprotein receptor (Goldstein *et al.*, 1985; Schneider, 1989).

In the light of our results, BSA seems not to affect the metabolism of gangliosides in the liver; on the other hand, the high levels of residual G_{M1} would reflect a higher proportion of the loosely bound form. The intracellular formation of G_{D1a} from G_{M1} preceded the secretion of G_{D1a} by the liver. The perfusate contained G_{D1a} as the main metabolite of G_{M1} , and BSA appears to facilitate the release of this newly biosynthesized ganglioside. The most abundant catabolites formed in the liver (G_{M2} and G_{M3}) were not detected in the perfusate, except for a very small amount of G_{M3} at 60 min after administration. This G_{M3} is likely of biosynthetic origin from some recycled G_{M1} catabolites like glucosylceramide (Trinchera *et al.*, 1990b).

The finding that newly biosynthesized gangliosides could be secreted by the liver represents the first evidence to explain, at least in part, the origin of serum gangliosides. Up to now, the most widely accepted origin for serum gangliosides resided in the 'dumping' of fragments of cell membranes by blood cells and vessels (Timofeeva *et al.*, 1987) or in secretion by intestinal cells (Bremier, 1984). The fact that the pattern of labelled gangliosides in the perfusate is different from that of the liver, at one and the same time, indicates that the release probably occurs through specific mechanisms, rather than through lysis or unspecific mechanisms.

The gangliosides in the perfusate were mostly in a soluble form. Nevertheless, the small, but significant, portion of membrane-bound radioactive gangliosides may indicate that gangliosides can be released through more than one mechanism. For instance, a shedding of gangliosides by tumour cells into the bloodstream has been supposed to be the cause of the elevation and the distribution change in the serum from patients with cancer (Dyatlovitskaya & Bergelson, 1987; Kloppel *et al.*, 1977).

To the best of our knowledge, the present results constitute the first report of a physiological release of gangliosides from one organ. Previous experiments, in fact, were carried out with cell-culture systems or in the ascitic fluid after tumour-cell transplantation (Shaposhnikova *et al.*, 1984; Ladisch *et al.*, 1983).

A final point concerns ganglioside metabolism in liver after the recirculating protocol. G_{D1a} formation was greatly reduced with a concurrent increase in the formation of catabolites (even lactosylceramide, glucosylceramide and ceramide were consistently formed). This evidence may suggest an inhibitory effect of re-internalized gangliosides on their own biosynthetic machinery with a concurrent increase in the ganglioside pool available for catabolic degradation.

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