IN VITRO SYNTHESIS OF GLYCOSYLCERAMIDE IN RABBIT PLATELETS

by

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Rabbit platelets were incubated for 1 hr. in the presence of [U-^{14}C]glucose. Glycosylceramides were separated from other lipids by column and thin-layer chromatography. Of the total lipid radioactivity recovered, ca. 50% was noted in a single glycosylceramide fraction. Further identification of this fraction revealed that essentially all the radioactivity resided in the glucose portion. These data demonstrate that platelets have the capacity to synthesize glycosylceramide and that this may be a significant route of lipogenesis.

Introduction. It has been well documented that platelets have the ability to synthesize certain lipids, i.e. phospholipids and fatty acids in vitro (1-5). Recent evidence shows that glycosylceramides (6,7) and their catabolyzing enzymes (8) are normal constituents of platelets. However, it is not known to what extent platelets synthesize glycosylceramides.

This communication presents evidence which indicates that platelets have the capacity to synthesize glycosylceramide and that this may represent a major pathway of lipogenesis.

Materials and Methods. [U-^{14}C]glucose was obtained from Amersham-Searle Corp., bovine serum albumin (Type F, essentially fatty acid free) from Sigma, DEAE-cellulose from Reeve Angel Inc., silica gel G from Brinkmann Instruments Inc. and Analtech Inc., lipid standards from Supelco Inc. and Applied Science Laboratories Inc., X-ray film (Cronex 4) from DuPont, and blueprint paper (200 ss) from GAF Corp. All solvents were reagent grade.

Blood was obtained by cardiac puncture without anesthesia from female New Zealand white rabbits weighing ca. 4 kg. Nine volumes of blood were collected into a syringe containing one volume of 77 mM EDTA, pH. 7.4.

Plastic labware was used exclusively. Platelet rich plasma was obtained from whole blood by centrifugation for 15 minutes at 500 x g at room temperature. Platelets were then pelleted by centrifugation for 10 min. at 1600 x g at room temperature. The platelet pellet was resuspended in 4.0% bovine serum albumin, 154 mM NaCl, 5.0 mM KCl, and 0.55 mM glucose at pH 7.4. A final 1600 x g centrifugation for 15 seconds was included to reduce contamination by red and white blood cells. Examination of Wright stained slide preparations showed the presence of a few red cells and lymphocytes. Ten to 34 μCi of [U-^{14}C]glucose (Sp. Act. 260-297 μCi/μmole) were added to 1 ml volumes of the albumin suspended platelets (1.7-2.9 x 10^9 platelets/ml), and incubated in air for 1 hour at 37° in a shaking incubator. Reactions were stopped in a melting ice-bath and then centrifuged at 8° for 10 min. at 1600 x g. The platelet pellet was resuspended by mixing for 15-20 seconds in 2.0 ml of cold distilled water to lyse any remaining red blood cells (9). Isotonicity was restored quickly by adding 6.0 ml of 1.2% NaCl. The platelets were pelleted as above and washed twice with 0.9% NaCl. Lipids were then extracted according to Folch et al. (10). In some experiments total lipid extracts were applied directly to silica gel G (Analtech precoated plates, .25 mm layer) and developed with chloroform/methanol/water (70:30:4 v/v/v) (11). Lipids were visualized with iodine and prints of the thin-layer chromatograms were made on blueprint paper (12). The glycosylceramide fraction was then scraped into scintillation vials and the radioactivity determined using a cabosil-toluene cocktail. In other experiments the lipids were first separated using DEAE-cellulose column chromatography as described by Rouser et al. (13). The chloroform/methanol/acetic acid (9:1:.002 v/v/v) fraction was rechromatographed on silica gel G (E. Merck, .5 mm layer) as described above and analyzed by autoradiography. In some cases, the glycosylceramide fraction was scraped into scintillation vials and counted or it was scraped into Pasteur pipettes stoppered with glass wool and the lipids eluted with 5 ml each of chloroform/methanol (1:1 v/v) and

methanol. The eluted glycosylceramide was hydrolyzed in 2N HCl at 100° for 3 hr. and the hydrolysate extracted twice with petroleum ether (14). The water soluble fraction was chromatographed by thin-layer chromatography using silica gel G (Brinkmann precoated plates #5763) with 1-butanol/pyridine/0.1 N HCl (100:60:40 v/v/v) as a developing solvent (15). Standards of glucose and galactose were chromatographed simultaneously in separate lanes. Standards were detected using a phthalic acid/p-anisidine spray (16) and the radioactive zones were detected by autoradiography.

Results. The uptake of radioactive glucose into glycosylceramide by platelets is shown in Table 1. Of the total lipid radioactivity extracted, ca. one-half was noted in the glycosylceramide fraction (Table 1 A). Essentially the same result was obtained when the total lipid extract was first chromatographed on DEAE-cellulose and the resulting fraction chromatographed by thin-layer chromatography (Table 1 B). Thin-layer chromatography of the

Table 1

In vitro incorporation of [U-^{14}C]glucose into glycosylceramide by platelets

Fraction	% of Total Radioactivity*	
	A	B
Glycosylceramide	47.10 ± 4.10	46.49 ± 3.73
Phospholipids and other (non-identified lipids)	52.9	----

[U-^{14}C]glucose was incubated with platelets for 1 hr. For each experiment in column A, three separate incubations were extracted and the lipids pooled for chromatography. The total lipid extract was applied directly to thin-layer chromatography (Analtech precoated plates) as described in "Materials and Methods". In column B, the total lipids from two to four separate incubations in each experiment were extracted and pooled as above for chromatography. The lipids were separated first on DEAE-cellulose prior to thin-layer chromatography on silica gel G (E. Merck) as described in "Materials and Methods". Solvent systems for A and B were chloroform/methanol/water [70:30:4 and 70:30:3 (v/v/v)] respectively.

*Mean ± SEM of 3 experiments analyzed separately for each group.

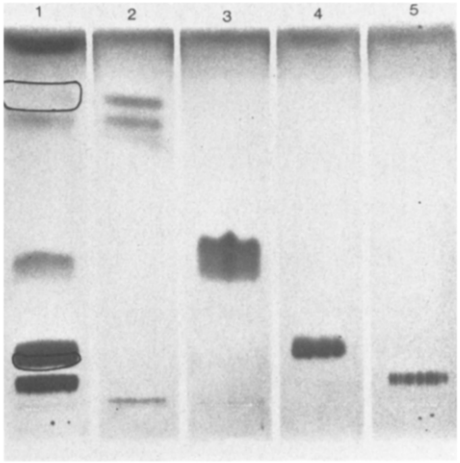

Figure 1. Autradiography of lipids separated by thin-layer chromatography. Lipids, from platelets incubated with [U-^{14}C]glucose and separated as in Table 1 B, were subjected to thin-layer chromatography as described in "Materials and Methods". The lipids were detected with iodine and then exposed to autoradiography for 4 days. The lanes are numbered at the solvent front: lane 1 is the lipid obtained from the chloroform/methanol/acetic acid (9:1:.002 v/v/v) fraction from DEAE-cellulose; lane 2, glycosylceramides; lane 3, phosphatidylethanolamine; lane 4, phosphatidylcholine; and lane 5, sphingomyelin. The radioactive zones are encircled.

total lipid extract revealed that about 53% of the total lipid radioactivity was distributed in phospholipids (41%) and uncharacterized lipids (12%).

Lipids obtained as in Table 1 B were separated by thin-layer chromatography and subjected to autoradiography (Fig. 1). Lane 1, containing the platelet lipid fraction, shows in ascending order that sphingomyelin, phosphatidylcholine, a small quantity of phosphatidylethanolamine, and a single glycosylceramide fraction were detected with iodine. An uncharacterized zone located nearest the solvent front was probably butylated hydroxy toluene which was added as an antioxidant or free fatty acids which also are eluted from DEAE-cellulose in this fraction. The radioactive zones which are encircled corresponded to phosphatidylcholine and glycosylceramide. Sphingomyelin did not produce a radioactive zone and liquid scintillation counting verified that sphingomyelin contained only 1% of the total lipid radioactivity. The distribution of radioactivity in glucose and galactose after hydrolysis of the glycosylceramide fraction is shown in Figure 2.

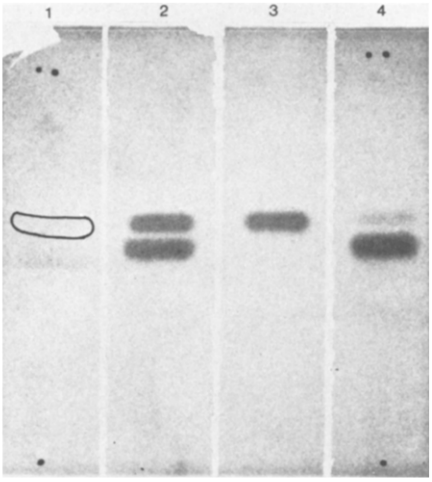

Figure 2. Autoradiography of hydrolyzed glycosylceramide separated by thin-layer chromatography. The radioactivity corresponding to glycosylceramide in lane 1 of Fig. 1 was eluted, hydrolyzed, and chromatographed. The plate was autoradiographed for 4 days, and the standards were detected as described in "Materials and Methods". The lanes are numbered at the solvent front: lane 1 is hydrolyzed glycosylceramide; lane 2, glucose and galactose; lane 3, glucose; and lane 4, galactose. The radioactive zone is encircled.

Essentially all the radioactivity was noted in the zone corresponding to the glucose standard. That glucose is not incorporated significantly into sphingosine or ceramide is based on the observation that after hydrolysis of glycosylceramide less than 1% of the radioactivity was lipid soluble.

Discussion. The data reported herein demonstrate that platelets incorporate radioactive glucose into glycosylceramide. With our conditions the glucose molecule is incorporated intact, presumably via UDP-glucose (17). Thus the appearance of radioactivity in this fraction denotes the capacity of platelets to synthesize glucosylceramide. These data, however, do not allow us to predict whether glucose is incorporated via the ceramide (15,18,19) or the psychosine (20,21) pathway.

Based on the percentage of radioactivity (Table 1) in glycosylceramide, one might conclude that this fraction is the most rapidly synthesized lipid fraction arising from glucose. However, it should be noted that almost all the radioactivity in phosphatidylcholine and phosphatidylethanolamine occurs

in the glycerol backbone portion (unpublished observation). Hence on a molar basis, the synthesis of one mole of glucosylceramide (Fig. 1 and 2) yields twice the radioactivity as that of one mole of phospholipid. Taking this into account, about one-fifth of the radioactive lipid molecules are glucosylceramide.

There are two observations which should be emphasized. Firstly, in our studies sphingomyelin was insufficiently labeled by [U-^{14}C]glucose to be detected by autoradiography (Fig. 1). Secondly, a high percentage of the radioactivity was detected in glucosylceramide. These data are at variance with those reported by Steiner (4). In his studies with platelets, 20% of the radioactivity from [U-^{14}C]glucose was recovered as sphingomyelin, second only to phosphatidylcholine (39%). Moreover, Steiner detected less than 1.5% of the radioactivity in the glycosylceramide fraction. The differences are difficult to reconcile at the present time. In light of our experiments, however, we conclude that the utilization of glucose for glucosylceramide synthesis represents a major pathway in platelets. Since glucosylceramide is common to all gangliosides (22) and since gangliosides have been observed in platelets (6,7,23) it is reasonable to suggest that this glucosylceramide may serve as a precursor for ganglioside synthesis in platelets.

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