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CHANGES IN LIVER GANGLIOSIDES IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Diabetes mellitus is associated with various structural and functional liver abnormalities that affect the glycogen and lipid metabolisms. The effects of streptozotocin-induced diabetes and of insulin supplementation to Sprague–Dawley diabetic rats on ganglioside patterns in liver were determined. Diabetic livers showed a tendency to hepatomegaly 3 weeks after STZ-induction of diabetes. The concentration of total gangliosides in diabetic and non-diabetic livers was similar, but the concentration of total gangliosides in the liver of insulin-stabilized rats was slightly increased. Bidimensional TLC chromatographic analysis of gangliosides isolated from normal diabetic and insulin-stabilized diabetic livers showed quantitative and qualitative changes. In comparison with normal controls, the densitometric analyses of diabetic liver ganglioside patterns had increased amounts of GM3, GM1, GD1b, and GT1b gangliosides, while GM2 could not be detected. The hepatic ganglioside pattern of insulin-stabilized diabetic rats was partially restored, resembling the profile of normal rats. The activity of GalNAcT, GalT-2 and SialT-4 transferases was measured in liver microsomal fractions of the different groups of animals. Diabetic rats showed an increased activity of GalNAcT and a decrease in the activity of GalT-2 and SialT-4 compared with the controls. The enzymatic activities found in insulin-treated rats showed a tendency to return to the values observed in normal control animals. The results evidenced that streptozotocin-induced diabetes affects the liver ganglioside pattern and the ganglioside synthesis enzyme activity. The alterations found in ganglioside metabolism could represent one of the earliest changes associated with the diabetic pathology.

KEYWORDS: diabetes; gangliosides; rat; liver; streptozotocin.

ABBREVIATIONS: STZ, streptozotocin; EGF, epidermal growth factor; TLC, thin layer chromatography; NANA, N-acetyl neuraminic acid; ip, intraperitoneal; PBS, phosphate buffer saline; GalNAcT, UDP-GalNAc:GM3 N-acetylgalactosaminyltransferase; GalT-2, UDP-Gal:GM2 galactosyltransferase; SialT-4, CMP-NeuAc:GM1 sialyltransferase; SPG, sulfoparagloboside.

INTRODUCTION

Gangliosides, which are constituents of the plasma membrane of vertebrate cells, are composed of a hydrophilic sialic-acid-containing oligosaccharide chain and a ceramide hydrophobic tail (Wiegandt, 1985). They are inserted into the outer leaflet of the plasma membrane through their ceramide moiety, which seems to govern some properties of the membrane (Yohe *et al*., 1976; Masserini and Freire, 1986). Ganglioside biosynthesis, which occurs in the Golgi apparatus, starts with glucosylceramide by the sequential addition of galactose, N-acetyl galactosamine, and sialic acid to the growing oligosaccharide chain. These reactions are catalysed by specific glycosyltransferases, many of which were studied and partially characterized in the rat liver Golgi apparatus (Trinchera and Ghidoni, 1989). Gangliosides are thought to be involved in cell–cell interactions, cell adhesion and recognition

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(for review see Kopitz, 1997). Glycosphingolipids appear to be capable of modulating transmembrane signal transduction. An example of this ability would be the modulation of cell growth by modification of epidermal growth factor (EGF) receptor activity (Zhou *et al*., 1994). Numerous studies have shown ganglioside changes during embryonic differentiation and aging of the nervous system (Rösner, 1993) as well as after oncogenic transformation (Kopitz, 1997). There are very few published reports concerning changes in gangliosides in extraneural tissues, mainly because of the fact that gangliosides are few, heterogeneous and possess structures that are difficult to determine. Nevertheless, several studies were performed in the liver of humans (Riboni *et al*., 1992), mice (Lorke *et al*., 1990) and rats (Bouhours and Bouhours, 1991), revealing complex ganglioside patterns. The exact role of hepatic gangliosides has not been completely defined in either healthy or diseased livers. Altered ganglioside patterns have been reported in cirrhosis and hepatocellular carcinoma in human livers (Tanno *et al*., 1988), in the serum of rats (Senn *et al*., 1990*a*), and in patients with liver diseases of different etiology (Senn *et al*., 1990*a*, 1991), but data concerning the role of hepatic gangliosides during diabetes are lacking. Diabetes is one of the most common diseases in humans and a major contributor to mortality and morbidity. The main metabolic complications of diabetes mellitus consist of retinopathy, nephropathy, peripheral neuropathy, and vasculopathy. However, symptoms of altered digestive functions are not uncommon among the diabetic population (Feldman and Schiller, 1983).

This study was undertaken to examine possible alterations in the concentration and composition of hepatic gangliosides in an experimental model of streptozotocin-induced Sprague–Dawley diabetic rats, and to determine whether the changes in ganglioside patterns correlate with ganglioside biosynthetic enzyme activity in the diabetic state.

MATERIAL AND METHODS

Animals

Male Sprague–Dawley rats weighing 250–300 g at the start of the experiment were fed a standard pellet laboratory chow and were provided with water *ad libitum*.

Induction of diabetes

Diabetes was induced by the administration of streptozotocin (STZ, Sigma Chemical Co., St Louis, MO, U.S.A.) 50 mg/kg (ip) dissolved in 10 mM sodium citrate buffer (pH 4.5); control rats received only vehicle. Diabetes was achieved in the majority of animals within 24 h as determined by measuring daily fasting blood glucose and glucosuria with reactive strips (Haemoglukotest and Glucostick, respectively, Boehringer Mannheim, Germany). Only animals with glucose levels >350 mg/dl, 2 days after STZ treatment were included in the study. In order to study the effect of insulin, 1 day after STZ injection one lot of diabetic rats received insulin subcutaneously (Monotard MC Novo, Nordisk), 3–4 U in the morning and 4–5 U in the evening, to maintain the glucose level at an average of 180 mg/dl approximately.

At the end of the experimental period (3 weeks) the animals were weighed, killed by an overdose of ether, and their livers perfused *in situ* with 150 ml of cold PBS (100 mM, pH 7.4). The perfusion was carried out in antegrade direction (portal to caval vein).

Purification of gangliosides

Total glycosphingolipids were purified from perfused livers of STZ-diabetic, STZ-diabetic insulin-stabilized, and normal control animals as described in Daniotti *et al*. (1990). The liver samples were washed in cold PBS solution and the surrounding connective tissue and large blood vessels were removed and immediately homogenized with an Ultra Turrax homogenizer. Total lipids were extracted twice with 4 volumes of chloroform: methanol (C:M) 1:2 (v/v) and the residue was re-extracted with C:M 2:1 (v/v). The combined extracts were dried, resuspended in C:M:water (60:30:4.5; v/v) and desalted with a Sephadex G-25 column equilibrated with the same solvent mixture. The eluates were partitioned according to Folch *et al*. (1957) and the upper phase was completely dried, resuspended in C:M (4:1, v/v) and passed through a silicic acid column (Sigma Chemical Co.) equilibrated with chloroform. The column was washed with C:M (4:1; v/v) and gangliosides were eluted with C:M (2:3; v/v). The eluate was dried and samples containing 20–40 nmol ganglioside sialic acid were resuspended in 15 µl C:M (2:3; v/v) and spotted on silica gel thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany). Chromatograms were developed with two successive solvent systems: C:M (4:1; v/v) and C:M:0.25% (w/v) $CaCl_2$ (50:40:11, v/v/v). Gangliosides were visualized with resorcinol–HCl reagent. Neuroaminic acid bound to lipid was determined by the resorcinol method (Svennerholm, 1963).

Table 1.
Characteristics of control, diabetic, and insulin-treated rats

Rats	*Body weight (g)*	*Liver weight (g)*	*LW/BW ratio*	*Blood glucose (mg/dl)*
Control (n=12)	306 ± 44	12.2 ± 2.9	0.039 ± 0.003	168 ± 51
STZ (n=13)	195 ± 59**	9.2 ± 1.3**	0.050 ± 0.012	568 ± 179*
STZ+I (n=14)	314 ± 57	15.6 ± 2.3	0.047 ± 0.008	173 ± 97

The values represent the mean ± SD.
*P<0.0001; **P<0.01, compared with control.

Gangliosides were abbreviated according to Svennerholm (1963). Bidimensional TLC was carried out according to Gornati *et al*. (1995) using a solvent mixture C:M:0.25% (w/v) $CaCl_2$ (50:40:11, v/v/v) for the first run and n-propanol:28%NH_3:H_2O (75:5:25, v/v/v) for the second. Densitometric analyses were performed as previously described (Daniotti *et al*., 1990).

Enzyme assays

The activity of galactosyl N-acetyl transferase (GalNAcT) was assayed according to Daniotti *et al*. (1990). The incubation mixture contained the following components in 20 µl final volume: 400 µM GM3, [^{3}H]-UDP-GalNAc (100 µM, 200,000 cpm), 25 mM $MnCl_2$, 100 mM cacodylate–HCl buffer (pH 7.2), 50 µg Triton CF54:Tween 80 (2:1, v/v) and 100 µg of protein of microsomal fraction of rat liver obtained by centrifugation at 100,000 × g for 1 h at 4°C. The mixture was incubated for 1 h at 37°C. Control tubes were incubated in the absence of an exogenous acceptor, but otherwise under identical conditions to correct for incorporation into endogenous acceptor. The assay conditions for the activity of GalT-2 were similar for GalNAcT, but using GM2 (400 µM) as an acceptor substrate and [^{3}H]-UDP-Gal donor (100 µM, 200,000 cpm). The activity of SialT-4, was determined in similar conditions, using GM1 (400 µM) as a glycolipid acceptor and [^{3}H]-CMP-NeuAc (100 µM, 200,000 cpm) and cacodylate–HCl buffer (pH 6.2). Reactions were stopped by adding 500 µl of a mixture of 5% trichloroacetic acid: 0.5% phosphotungstic acid. Then lipids were extracted with C:M (2:1, v/v), evaporated in a vial and dissolved in 50 µl of 10% SDS. Radioactivity was determined in a liquid scintillation spectrometer after addition of 0.2% 2,5-diphenyloxazole and 10% naphthalene in dioxane.

Other determinations

Protein was determined according to Lowry *et al*. (1951) using bovine serum albumin as standard.

Statistical analysis

Results were statistically analysed by Student's t-test.

RESULTS

In the present study male Sprague–Dawley rats were made diabetic with a single STZ injection. After 24 h, one group of animals was treated with insulin. Daily blood glucose determinations were made and the insulin dose was adjusted to approach euglycemia. The effect of STZ on blood glucose at the end of the experimental period (3 weeks) is indicated in Table 1. The blood glucose levels increased (P<0.0001) 3.3-fold in the STZ-treated rats (568 ± 179 mg/dl), thus demonstrating the efficacy of this chemical in promoting a permanent and stable diabetic state. No difference (P=0.8) in the degree of hyperglycemia was observed between control animals (168 ± 51 mg/dl) and insulin-stabilized rats (173 ± 97 mg/dl). At the end of the 3 week feeding period, STZ-diabetic animals had lower body weights (195 ± 59 g *vs* 306 ± 11, P<0.0001) and lower liver weights than those of normal control animals (9.2 ± 1.3 g *vs* 12.2 ± 2.9 g, P<0.01). Insulin-stabilized diabetic animals evidenced an increase in the body and liver weights compared with diabetic rats (P<0.0001 and P<0.01, respectively). The liver weights of diabetic rats were larger than those of the control and insulin-treated groups when normalized with body weights. Diabetic animals and insulin-stabilized rats presented enlarged livers in relation to their body weight.

Periodic acid Schiff (PAS) staining of liver histological sections showed that glycogen levels were severely depleted in diabetic animals compared with non-diabetic ones. In insulin-stabilized diabetic animals, liver PAS staining showed that hepatocellular stores were replenished and no differences with normal control liver were found (data not shown).

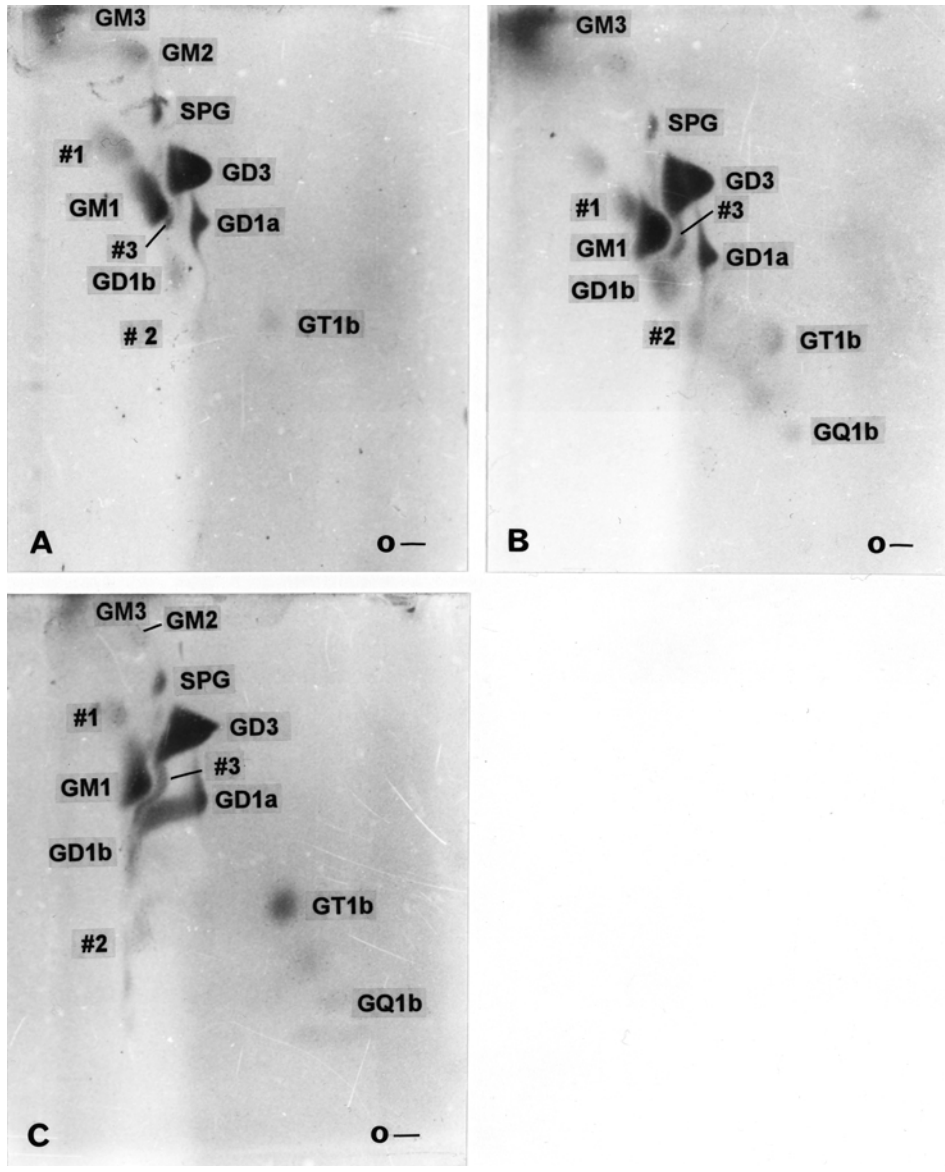

Fig. 1. Two-dimensional thin-layer chromatogram of total gangliosides obtained from normal livers (A), STZ-diabetic livers (B) and insulin-stabilized livers (C). ○, Origin. First dimension (↑), second dimension (←).

The concentration of total gangliosides in diabetic and non-diabetic liver tissues was similar (0.31 ± 0.13 nmol NANA/mg prot. and 0.264 ± 0.073 nmol NANA/mg prot., $P=0.58$, respectively), but the concentration of total gangliosides in the liver tissue of insulin-stabilized rats evidenced a slight but significant increase (0.559 ± 0.190 nmol NANA/mg prot., $P<0.05$). When results were expressed as nmol NANA/g wet mass tissue a decrease in the concentration of hepatic gangliosides of insulin-stabilized rat could be observed.

A bidimensional TLC chromatographic analysis of gangliosides isolated from normal, diabetic and insulin-stabilized diabetic livers is depicted in Figure 1. The pattern of normal and diabetic livers showed the presence of 13 ganglioside spots, 9 of which were identified as GM3, GM2, SPG, GM1, GD3, GD1a, GD1b, GT1b, GQ1b according to their relative mobility compared with reference standards. Other minor components, marked as spots #1, #2, and #3, could not be identified by any available reference material, but they are suspected to be gangliosides because of their reactivity to resorcinol–HCl reagent as well as by the procedure of isolation. Table 2 shows the densitometric analysis of gangliosides obtained from the liver of diabetic rats (Fig. 1B), demonstrating an increase in the amount of GM3, GM1, GD1b, and GT1b gangliosides in comparison with non-diabetic livers (Fig. 1A). GM2 could not be detected in diabetic

Table 2.
Ganglioside and SPG composition of normal control, diabetic, and insulin-stabilized rat livers

Glycosphingolipids	*Normal control (%)*	*Diabetic (%)*	*Diabetic+insulin (%)*
GM3	15.49 ± 0.51	19.57 ± 1.67	7.52 ± 0.80
GM2	6.06 ± 0.77	ND	3.77 ± 0.51
GM1	12.88 ± 1.71	16.66 ± 0.27	17.88 ± 1.69
GD1a	10.05 ± 0.58	8.00 ± 0.39	7.34 ± 0.59
GD1b	4.64 ± 0.95	7.48 ± 0.76	7.72 ± 0.94
GD3	24.92 ± 2.44	25.01 ± 1.88	24.38 ± 1.19
GT1b	ND	4.38 ± 0.58	8.95 ± 0.59
GQ1b	5.43 ± 0.18	2.25 ± 0.20	6.62 ± 0.61
SPG	3.83 ± 0.52	2.80 ± 0.22	2.98 ± 0.1
1	9.08 ± 1.08	4.34 ± 0.34	4.00 ± 0.55
2	3.78 ± 0.75	4.59 ± 0.41	4.73 ± 0.64
3	3.81 ± 0.47	4.87 ± 0.61	4.07 ± 0.51

Determination of ganglioside-bound sialic acid and TLC of gangliosides were carried out as indicated in Material and Methods. The percentage distribution of each ganglioside and SPG was determined by densitometric scanning of the plates. Data are the mean ± SD of three determinations.
ND, not detected.

Table 3.
Activities of transferases of control, diabetic and insulin-treated rats

Rats	*GaINAcT (pmol/mg prot/)*	*GaIT-2 (pmol/mg prot/)*	*SiaIT-4 (pmol/mg prot/)*
Control (n=12)	44 ± 3	205 ± 7	23 ± 3
STZ (n=13)	120 ± 21*	117 ± 16*	18 ± 2**
STZ+I (n=14)	57 ± 5**	144 ± 10**	20 ± 6

The values represent the mean ± SD of three determinations.
*P<0.001; **P<0.05, compared with control.

livers. GQ1b and #1 gangliosides evidenced a decrease in diabetic rat livers. The hepatic ganglioside pattern of insulin-stabilized animals was partially restored, resembling the profile of normal rats. Insulin treatment restored the GM2 ganglioside under our experimental conditions, but it failed to reach the normal control values. A similar situation was observed in the case of GQ1b. However, other gangliosides (GM1, GD1b, GD1a, and #1) were found in amounts similar to those of diabetic livers.

In order to clarify the differences observed in the expression patterns of gangliosides of diabetic and non-diabetic rats we measured the activity of the enzymes involved in the metabolism of these molecules (GalNAcT, GalT-2, and SialT-4 transferases). As indicated in Table 3, diabetic rats evidenced an increased activity of GalNAcT and a decrease in the activity of GalT-2 and SialT-4 compared with controls. The enzymatic activities found in insulin-treated rats showed a tendency to return to the values observed in normal control animals.

DISCUSSION

Diabetes mellitus is associated with various structural and functional liver abnormalities, including changes in glycogen (Van der Werve *et al*., 1984; Chatila and West, 1995) and lipidic (O'Meara *et al*., 1991; Feingold *et al*., 1982) metabolisms as well as in the antioxidant status (McLennan *et al*., 1991; Saxena *et al*., 1993). In spite of the increasing knowledge concerning diabetes, comprehensive data are lacking in the study of gangliosides in the diabetic rat model. Only the accumulation of glycosphingolipids in the renal hypertrophy of STZ-induced diabetic rats has been reported (Zador *et al*., 1993).

In the present study diabetic livers showed a tendency to a relative hepatomegaly 3 weeks after

the STZ induction of diabetes. Although insulin treatment efficiently lowered glycemia levels, it failed to restore the normal weight of the livers. This result agrees with the fact that insulin induces hepatocellular lipid accumulation by enhancing fatty acid uptake and hence triglyceride formation by hepatocytes (De Paepe *et al*., 1995). The response of diabetic livers to insulin was different from that obtained in the kidneys of insulin-treated diabetic rats. In these animals, kidney weights were similar to those of normal control animals (Zador *et al*., 1993).

We noticed that male diabetic rats slightly accumulate gangliosides in their livers. Significantly, Zador *et al*. (1993) reported an increase in GSL expression in association with renal hypertrophy in diabetic rats and correlated these changes with increases in the levels of GSL precursors. These findings suggest the importance of a quantitatively minor route of glucose utilization (pentose phosphate pathway) in hyperglycemic rats.

In insulin-stabilized rats a marked increase in the ganglioside concentration was observed. This result could be explained on the basis of insulin action as a metabolic and/or mitogenic factor in hepatocytes and hepatoma cells (Hoffmann *et al*., 1989; Thompson *et al*., 1991; Koontz and Iwahashi, 1981). Although little is known about the effect of insulin on the ganglioside metabolism in the liver; it has been reported that this hormone enhances hepatic protein expression and synthesis (Kang-Park *et al*., 1995).

During diabetes, the liver undergoes biochemical alterations. We described the changes in the ganglioside pattern of rat livers after 3 weeks of STZ injection. These findings suggest that diabetic liver cells have a ganglioside metabolism different from that of normal liver cells. The ganglioside pattern of diabetic livers was principally characterized by the absence of the GM2 ganglioside as well as by an increase in GM3, GM1, GD1b and GT1b. Remarkably, this altered profile was partially restored to normality after insulin treatment. In order to clarify the changes in the expression of hepatic gangliosides under the conditions in this study, we measured some of the enzyme activities involved in the biosynthesis of these molecules, whose patterns were responsive to diabetes and insulin supplementation. Although glycosyltransferases activities have been examined in mammalian and avian nervous systems (Basu and Basu, 1982; Bellman-Yip and Dain, 1970; Basu *et al*., 1995; Percy *et al*., 1991), their role in the control of the expression of hepatic gangliosides during diabetes remains unexplored. Our data on enzymatic activity were obtained *in vitro*, so that correlation with the *in vivo* system can only be tentative.

Our enzymatic studies in diabetic livers indicate a marked increase in the activity of GalNAcT and a decrease in the activities of GalT-2 and SialT-4 transferases. The discrepancy between the enzymatic activities and the contents of their biosynthetic products (GM2, GM1 and GD1a, respectively) might depend on the availability of the substrates. It is well known, in fact, that the abundance of a given product is determined not only by the enzyme activity, which can be up- or down-regulated, but also by the available amount of substrate and by the activity of the degradative enzymes (Gornati *et al*., 1997; Seyfried *et al*., 1994). Recently, Bieberich *et al*. (1998) suggested that the up-regulation of GalNAcT in conjunction with the down-regulation of SialT-4 by stimulation of phosphorylation in NG108–15 cells could serve as a physiological mechanism to increase the concentration of GM1. This assumption could explain the increased amount of GM1 ganglioside in diabetic livers. The activity of certain glycosyltransferases could be controlled by post-translational modifications such as cAMP-dependent phosphorylation/dephosphorylation (van Echten and Sandhoff, 1993; Yu, 1994; Gu *et al*., 1995) and protein kinase C-related phosphorylation systems (Bieberich *et al*., 1998). At present, it is known that diabetes produces significant alterations in the signal transduction systems, thus affecting the activity of their various components, for example, decreased autophosphorylation of the insulin receptor (Kadowaki *et al*., 1984), changes in the protein kinase activity in several body organs (Pugazhenthi *et al*., 1990; Craven and DeRubertis, 1989; Xiang and McNeill, 1992; Ishii *et al*., 1998), increased diacylglycerol kinase activity (Nobe *et al*., 1998), and changes in enzymes and metabolites of the inositol phosphate pathway (Whiting *et al*., 1979). Taking into account the observed diabetes-induced changes in the profile and metabolism of gangliosides, it may be suggested that an adequate behaviour of the signal transduction systems is necessary in order to maintain the ganglioside composition in the liver; however, further investigation is required. In relation with this point, the combination of several factors (insulin, glucagon, epidermal growth factor, glucocorticoids) was found to be necessary for maintaining activity levels of GD3-synthase in cultured rat hepatocytes (Mesaric and Decker, 1990).

Several studies demonstrated remarkable changes in the ganglioside TLC pattern in the rat liver induced by a carcinogenic chemical (Merrit *et al*., 1978), by hepatoma (Holmes and Hakomori,

1982), and by biliary cirrhosis (Senn *et al*., 1991) as well as in human liver cirrhosis (Tanno *et al*., 1988; Senn *et al*., 1990*a*). The alterations found in the pattern and metabolism of gangliosides during diabetes could represent one of the earliest changes associated with the evolution of diabetic pathology. Moreover, bearing in mind the fact that ganglioside biosynthesis in the rat liver has a differential distribution in the various cell types (Senn *et al*., 1990*b*), the observed increase or decrease in the amount of a given ganglioside might indicate the degree of compromise of the parenchymal and/or non-parenchymal cells during diabetes.

In conclusion, the results of this report represent the first direct evidence that diabetes induces changes in ganglioside metabolism in rats, which constitutes a starting point for further investigations of the involvement of gangliosides in healthy and diabetic livers.

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