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# Sulfatide is associated with insulin granules and located to microdomains of a cultured beta cell line

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**Previous studies using pancreas from various mammals and freshly isolated islets from rat pancreas have provided evidence supporting possible involvement of the glycosphingolipid sulfatide in insulin processing and secretion. In this study, sulfatide expression and metabolism in the beta cell line RINr1046-38 (RIN-38), commonly used as a model for beta cell functional studies, were investigated and compared with previous findings from freshly isolated islets. RIN-38 cells expressed similar amounts (2.7 $\pm$ 1.1 nmol/mg protein, $n = 19$) of sulfatide as isolated rat islets and also followed the same metabolic pathway, mainly through recycling. Moreover, in agreement with findings in isolated islets, the major species of sulfatide isolated from RIN-38 cells contained C16:0 and C24:0 fatty acids. By applying subcellular isolations and electron microscopy and immunocytochemistry techniques, sulfatide was shown to be located to the secretory granules, the plasma membrane and enriched in detergent insoluble microdomains. In the electron microscopy studies, Sulph I staining was also associated with mitochondria and villi structures. In conclusion, RIN-38 cells might be an appropriate model, as a complement to isolated islets where the amount of material often limits the experiments, to further explore the role of sulfatide in insulin secretion and signal transduction of beta cells.**
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***Abbreviations:*** **C/M/W: chloroform/methanol/water; DIMs: detergent-insoluble membrane domains; ESI–MS: electrospray ionisation–mass spectrometry; HPTLC: high performance thin-layer chromatography; PBS: phosphate buffered saline; TLC-ELISA: thin-layer chromatography-enzyme-linked immuno-sorbant assay; PI: protease inhibitors.**

## Introduction

Sulfatide (3′-sulfogalactosyl-ceramide) is a glycosphingolipid, which has been found to be of interest regarding beta cell function, specifically insulin processing and secretion [1–3]. Sulfatide is localised to the plasma membrane and the secretory granules of the insulin producing beta cell [4]. Insulin and sulfatide were also found to follow the same intracellular trafficking route [2], starting with synthesis in endoplasmic reticulum, followed by the transport of sulfatide and proinsulin from trans-Golgi into secretory granules. In general, the majority of the insulin-containing granules are never secreted [5] but are stored in the beta cells or transported to the lysosome, where insulin is fully degradated and sulfatide associated with granules partly degradated and reused for sulfatide synthesis [2].

Further support for the involvement of sulfatide in insulin processing and secretion has come from various *in vitro* studies [3], in which sulfatide was found to block the binding of an A8–A10 insulin domain specific antibody, and a model for the interaction was depicted. The same study also showed that sulfatide preserves insulin crystals at pH 5.5, the pH in the secretory granules, and mediates the conversion of insulin hexamers to monomers at pH 7.0, the pH when granules fuse with the plasma membrane of the beta cells. Sulfatide was also shown to promote folding and oxidation of proinsulin. Recently, Buschard et al. [1] showed in patch clamp studies using freshly isolated beta cells that exogenous sulfatide reduced

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insulin secretion, which they proposed to be mediated by activation of ATP-sensitive $K^+$-($K_{ATP}$)-channels in a glucose- and concentration-dependent manner. Thus, sulfatide released from secretory granules might have a negative feedback mechanism on insulin secretion by such channel activation. These results lent support to the hypothesis that there is a potential functional association between sulfatide and insulin, which might also be important in intra-cellular events, for instance when insulin is processed and secreted.

Evidence has been accumulating that glycosphingolipid-enriched domains, known as DIMs (detergent-insoluble membrane domains), in the plasma membrane are important for numerous cellular events related to membrane trafficking and signal transduction [6,7]. The importance of the glycosphingolipids in these domains has also been mentioned [8–10], possibly in terms of facilitating receptor signalling and protein trafficking. The location of sulfatide in such domains in beta cells has not yet been elucidated. Our previous studies using immunohistochemistry and electron microscopy have indicated that sulfatide has an intragranular location, probably in association with insulin, as well as at the cell surface.

Several insulin-secreting beta cell lines have been established in an effort to provide a tool for molecular studies of beta cell function and dysfunction [11]. These cell lines all have some of the characteristics of immature beta cells referring to glucose sensitivity, insulin metabolism and secretion, and other metabolic events. They also resemble immature beta cells ultrastructurally. One such cell line is RINr1046-38 (RIN-38), an insulin-secreting beta cell line derived from radiation-induced insulinomas in the rat [12,13]. RIN-38 cells retain the ability to secrete insulin in response to glucose at low passages (passage $<20$). The aim of this study was to evaluate the usage of this beta cell line, as a complement to isolated islets where the amount of material often limits the experiments, in further studies concerning the role of sulfatide in insulin processing and secretion.

## Materials and methods

### Materials

Sulfatide and galactosyl ceramide used as standards were purified from pig brain and sulfated lactosylceramide was prepared from post mortem kidney tissue from a patient with metachromatic leucodystorphy, according to a procedure previously described [14]. Seminolipid (1-alkyl-2-acyl-glycerol form) as standard was a gift from Dr. Ineo Ishizuka, Department of Biochemistry, Teikyo University School of Medicine, Tokyo, Japan. Other lipid standards were purified from human tissue [15,16]. [U-$^{14}$C]serine (150 mCi/mmol) and $^{35}$S-labeled sulfate (carrier free) were purchased from Amersham, Life Science (UK). The production and characterization of the monoclonal antibody Sulph I has previously been described [17]. The antigens recognized are sulfatide, sulfated lactosylceramide and seminolipid. The fluorescein (FITC)-conjugated donkey anti-mouse IgG and Texas red-conjugated donkey anti-guinea pig IgG were purchased from Jackson Laboratories (West Grove, PA, USA). Mouse IgG control antibody, the polyclonal guinea pig anti-swine insulin antibody (cross-reacts with insulin from several mammalian species including rat) and mounting medium were purchased from DAKO (Glostrup, Denmark).

### Cell culture

RINr1046-38 (RIN-38) cells were studied between passages 11–17. Cells were cultured in RPMI 1640 (ICN Biomedicals. Inc., Ohio, USA), 10% fetal calf serum, 1% L-Glutamine, 1% penicillin-streptomysin (5000 IU/mL-5 mg/mL, ICN Biomedicals) and 11.1 mM glucose in a humidified atmosphere of 95% air-5% $CO_2$ at 37°C. Cells were grown in 25 cm$^2$ flasks for the metabolic studies, and in 175 cm$^2$ flasks for the isolation of granules and DIMs fractions. Confluent cells were washed with 0.53 M Versene and harvested by trypsinization (0.25 g/L in Versene). Cells were studied at a density of approximately $10^5$ cells/cm$^2$.

### Extraction and separation of lipids

Cells, harvested as described above, were suspended in ultrapure water (Millipore, Bedford, MA, USA) and freezing/thawing/ultrasonication (1 min, 47 kHz ± 6%, Branson 2200) was repeated 4 times. The total amount of protein was determined using the BCA Protein Assay Reagent method (Pierce, Rockford, USA). Lipids were extracted using chloroform/methanol/water C/M/W (4:8:3 by vol.) [18] and as a separation step, silica gel-60 chromatography was performed as previously described [2]. Briefly, sulfatide, sulfated lactosylceramide, seminolipid, glycerophospholipids and sphingomyelin were eluted with C/M/W (65:25:4 by vol.) and gangliosides with C/M/W (30:60:20 by vol.). For lipids extracted from DIMs (see below) an additional elution-step was included; the first step being C/M (9:1 v/v) to elute cholesterol. For sulfatide and sulfated lactosylceramide quantifications and metabolic studies, the "C/M/W 65:25:4 fraction" was saponified in methanol:1 M KOH (1:1 v/v), 37°C overnight to remove glycerophospholipids. Then 0.5 mL methanol and 2 mL chloroform were added and the tube centrifuged (1000 × g, +21°C, 5 min) whereafter the upper phase was removed. The lower phase, containing sulfatide and sulfated lactosylceramide, was washed by repeated partitioning by adding 0.5 mL methanol and 0.5 mL 0.9% NaCl twice. Quantification of lipid phosphorus and seminolipid was performed on the same fraction before saponification.

### Quantification of glycolipids

Sulfatide and sulfated lactosylceramide were identified and quantified by a previously described TLC-ELISA method [19], and seminolipid according to the method described by Pernber et al. [20], using the Sulph I antibody [17] and purified standards of these antigens. All steps were performed at room

temperature. The intensity of the developed colour was determined with the ImageMaster™ 1D (Pharmacia & Upjohn, Uppsala, Sweden). GM1 was identified and quantified by TLC-ELISA using the beta-subunit of cholera toxin, as previously described [19].

### Analysis of lipid phosphorus, sphingomyelin and cholesterol

Lipid phosphorus was assayed using a modified Fiske-Subbarow method [21]. Lipid phosphorus measurements include the sum of glycerophospholipids and sphingomyelin. Quantitative data for glycerophospholipids, given throughout the study, were obtained by subtracting the content of sphingomyelin from the lipid phosphorus value. For sphingomyelin quantification, saponified lipid fractions were applied as 7 mm wide bands to HPTLC plates (Merck, Darmstadt, Germany) and chromatographed (C/M/W, 65:25:4 by vol.). For cholesterol quantification, aliquots from the C/M 9:1 (v/v) fraction eluted from Silica-gel column, as described above, were applied as 7 mm wide bands to HPTLC plates (Merck) and chromatographed (C/M, 95:5 v/v). After drying, the plates were dipped in 0.001% primulin (w/v) in acetone/water 4:1 [22]. The fluorescence was recorded with a Camag TLCII scanner with excitation at 365 nm and emission cut off filter at 400 nm. Samples in the range 0.1–0.5 nmol and 0.2–1.0 nmol were analysed for sphingomyelin and cholesterol, respectively.

### Electrospray ionisation–mass spectrometry

The purification of the sulfatide fraction has been described [23]. Semi-quantitative data concerning the fatty acid composition of sulfatide was obtained by using ESI–MS. Samples were dissolved in C/M/W (30:60:20, by vol.) at a concentration of 10 $\mu$M before mass spectrometry. ESI–MS in the negative mode was performed on a quadropol-time of flight mass spectrometer (Q-TOF, Micromass, Manchester, UK) equipped with a nanospray ion source. For MS–MS the gas cell was pressurized with argon gas and fragmentation of the precursor ion was affected at a collision energy of 60–70 eV.

### Immunocytochemistry

Double immunolabelling and nuclear staining were performed according to the method of Berntson et al. [24]. RIN-38 cells (passage 12) were cultured on cover slips, fixed for 10 min in 4% paraformaldehyde and permeabilised with 0.05% saponin in PBS (10 mM sodium-phosphate buffer, containing 0.15 M sodium chloride, pH 7.4) with 1% BSA (PBS-BSA). The cells were incubated with the primary Sulph I antibody (2.6 mg/mL). As a second step, incubation with the secondary antibody (FITC-conjugated anti-mouse IgG antibody, diluted 1:700) was performed. Cells were then incubated with the primary insulin antibody (0.2 mg/mL, diluted 1:50) followed by incubation with the detecting antibody (Texas red-conjugated anti-guinea pig IgG antibody, diluted 1:600) together with Hoechst 33258 (0.05 $\mu$g/ml, Sigma, St. Louis, MO), which has a high specificity for double-helical DNA and emits blue fluorescence when excited by ultraviolet light. Antibodies were all diluted in PBS-BSA with 0.05% saponin. All incubations were performed at room temperature for 40 min, and rinsing with PBS-BSA was performed between each incubation step. Negative controls were performed by incubation with a mouse IgG1 (directed towards *Aspergillus niger* glucose oxidase, 2.5 $\mu$g/mL) instead of Sulph I, in combination with omitting the primary insulin antibody. The cover slips were mounted on microscope slides and viewed with Nikon microphot-FXA epifluorescence equipment. Immunofluorescent pictures were captured using a Hamamatsu C 5810 colour chilled 3CCD camera.

### Electron microscopy procedures

RIN-38 cells (passage 13) cultured in a culture flask were washed in 0.1 M cacodylate buffer, pH 7.4, fixed overnight in 4% paraformaldehyde at 4°C and washed in cacodylate buffer ($2 \times 5$ min) and 1% PBS-BSA ($3 \times 5$ min). Cells were incubated overnight at 4°C with Sulph I, diluted 1:1000 and after washing in 1% PBS-BSA ($6 \times 10$ min), cells were incubated overnight at 4°C with 1 nm gold labelled goat anti-mouse IgG (British BioCell International, Cardiff, UK) diluted 1:300 in 1% PBS-BSA and absorbed with rat serum. Cells were postfixed after washing ($6 \times 10$ min in 1% PBS-BSA and $2 \times 10$ min in PBS) in 2% glutaraldehyde for 2 h and washed in distilled water ($4 \times 10$ min), before silver enhancement using AURION R-GENT SE-EM (Aurion, Wageningen, Holland). Cells were washed in distilled water ($4 \times 10$ min) before osmication in 1% $OsO_4$ diluted in 0.1 M cacodylate buffer. After washing in 0.1 M cacodylate buffer, the cells were scraped from the culture flask, dehydraded in alcohol and embedded in Epon Resin 812 before ultrasections were examined in a Philips 208 electron microscope. For each experiment, controls were prepared by omitting the primary antibody. These controls yielded the expected negative results.

### Effect of Fumonisin B1 and chloroquine diphosphate on the sulfatide metabolism in RIN-38 cells

These experiments were performed according to culturing conditions described by Fredman et al. [2], with a few modifications. During the experiments, confluent RIN-38 cells (passage 14) in 25 $cm^2$ flasks ($n = 3$) were incubated in Eagle MEM, sulfate free medium (ICN Biomedicals) containing 5.6 mM glucose, 10% heat inactivated fetal calf serum and 1% penicillin-streptomysin. The long-term experiment with Fumonisin B1 (18.5 $\mu$g/mL, Sigma) was performed for 25 h, including 1 h of pre-incubation, and $^{14}$C-serine (3 $\mu$Ci/mL) was used to evaluate lipid synthesis. In the experiments with chloroquine diphosphate (100 $\mu$g/mL, Sigma), $^{14}$C-serine (3 $\mu$Ci/mL) and $^{35}$S-sulfate (20 $\mu$Ci/mL) were combined. Lipid extraction, separation and saponification of the "sulfatide fraction" were performed as described above. The effect of the

various inhibitors on sulfatide synthesis was analysed using autoradiography and scintillation counting performed on scraped out gel fractions with individual lipids, as described below.

### Autoradiography and scintillation counting

This procedure has been described previously [2]. Aliquots of the saponified "sulfatide fraction", corresponding to 50 $\mu$g protein, were applied on alumina-backed HPTLC plates (Merck). The individual lipid bands after chromatography were identified by similar migration as structurally identified lipid standards and liquid scintillation was performed on scrape out fractions. A 2-fold or higher increase or a 50% or lower reduction in radioactive incorporation into lipids compared to controls was required to conclude an effect of the inhibitors on lipid metabolism. Structurally identified standards of sulfated lactosylceramide and sphingomyelin were visualized with 1% orcinol reagent and 3% copper acetate, respectively. To establish that the $^{35}$S-sulfate incorporation was responsible for the sulfatide labelling, hydrolysis of the sulfatide fraction from cells incubated with both $^{14}$C-serine and $^{35}$S-sulfate in the same experiment and subsequent autoradiography, to identity $^{35}$S-sulfate and/or $^{14}$C-galactosylceramide, were performed [2].

### Isolation of granule fractions

Granules were isolated using a modification of the procedure described by Andersson and Abrahamsson [25]. The procedure was performed at +4°C throughout. Confluent RIN-38 cells ($10^6$–$10^7$ cells) were harvested as described above (passage 15–16), washed in ice-cold PBS and suspended in 30 mL 0.25 M sucrose (Sigma) solution containing 1 mM sodium-EDTA (Merck) and 5 mM Hepes (Sigma), pH 7.0. The cells were transferred to a Teflon tube, homogenized twice for 10 s and centrifuged at 900 × g, 5 min at +4°C. The supernatant was transferred to a new teflon tube and centrifuged at 13 000 × g, 10 min at +4°C and the pellet, containing granules and mitochondria, was resuspended in 250 $\mu$L sucrose solution (as described above). This suspension was layered on a 66% Percoll gradient (0.66 mL 2.5 M sucrose (Sigma), 5.94 mL percoll (Pharmacia & Upjohn), 3.4 mL sucrose solution (as described above) in small Teflon tubes (total gradient volume was 9 mL) and centrifuged at 19 000 × g, 25 min at +4°C. The granule fraction formed a white band in the percoll gradient, and this fraction was removed (200–300 $\mu$L) and put on a 10 cm (i.d. 1 cm) Sephacryl S-1000 (Pharmacia & Upjohn) column. The column was equilibrated with approximately 30 mL sucrose solution (as described above), after which the sample was put on the column, and 18 fractions (500 $\mu$L/fraction) were eluted with the same sucrose solution. Visible granules were eluted in fractions number 5–8 (the void volume) and all eluted fractions were stored at −20°C until lipid extraction (as described above). Sulfatide, sulfated lactosylceramide and seminolipid content were analysed using TLC-ELISA, as described above. Insulin content (in isolation number 2, due to lack of material, fraction 6 could not be analysed) was measured using ELISA, as described below.

### Insulin-ELISA

Flat-bottom maxi-sorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 100 $\mu$L goat-anti-guinea pig antibody (DAKO) diluted 1:1000 in PBS over night at 4°C. All subsequent procedures were performed at 20°C. The plates were prewashed 5 times in wash buffer [1L: 29.2 g NaCl, 1.15 g $Na_2HPO_4 \cdot 2H_2O$, 0.2 g $KH_2PO_4$, 0.2 g KCl, 0.01% triton X-100 (Sigma)], and then incubated with 100 $\mu$L of the insulin antibody (DAKO) at a final dilution of 1:40.000 in RIN buffer [PBS with 0.5% bovine serum albumin (Sigma) and 0.05% Tween 20 (Merck)] for 2 h, and washed 5 times. Peroxidase labelled insulin (Sigma), 80 $\mu$L, diluted 1:10000 in RIN buffer and 20 $\mu$L of the granule fractions (total volume of 500 $\mu$L) were added to each well and incubated for 2 h followed by five washes. Developing was performed using 100 $\mu$L TMB (Kem-En-Tec, Copenhagen, Denmark) for each well. After 10 min the reaction was stopped with 100 $\mu$L 0.2 M $H_2SO_4$. Plates were analysed at 450 nm and 630 nm using an ELX800 microplate reader (Bio-tek Instruments Inc., Winooski, Vermont, USA) with KC4 kinetic software.

### Isolation of DIMs fraction

DIMs were isolated with a modification of the method of Brown and Rose [26]. Confluent RIN-38 cells ($10^6$–$10^7$cells, passage 17) were washed in Versene and PBS, whereafter the cells were lysed in 5 mL 1% TritonX-Tris-NaCl-EDTA buffer (10 mM Tris, 5 mM sodium-EDTA (Merck), 150 mM NaCl, pH 7.5) containing the protease inhibitors (PI, Sigma) Leupeptine (1 mg/mL ultrapure water, diluted 1:2000 for use), Pepstatine (1 mg/mL methanol, diluted 1:1400 for use) and phenylmethylsulfonyl fluoride (10 mg/mL methanol, diluted 1:300 for use) for 20 min at 4°C. PI was added (just before use) to all solutions used in the isolation procedure. TritonX-100 (Sigma) was added to the buffer just before use. The lysed cells were scraped from the culture flask, homogenized and adjusted to 40% sucrose by adding 5 mL of an 80% sucrose Tris-NaCl-EDTA buffer. The lysate was transferred to the bottom of two ultracentrifuge tubes and on top, 25 mL 30% sucrose Tris-NaCl-EDTA buffer and 10 mL 5% sucrose Tris-NaCl-EDTA buffer were added. The sample was centrifuged at 27000 rpm (L8-70M Ultracentrifuge, Beckman Instruments), for 20 h at +4°C. About 3 mL of the gradient, containing an opaque lipid aggregate, was removed and diluted to about 40 mL with Tris-NaCl-EDTA buffer and centrifuged at 27000 rpm (L8-70M Ultracentrifuge, Beckman Instruments), for 1 h at +4°C. The pellet, containing the DIMs fraction, was stored at −20°C until lipid extraction (as described above). Sulfatide, sulfated lactosylceramide,

seminolipid, GM1, lipid phosphorus, sphingomyelin and cholesterol were analysed as described above.

### Statistics

Data are presented as mean values ± SD. The significance of difference was evaluated by using Student's *t*-test (significance level set at <0.01).

## Results

### Sulph I antigen expression in RIN-38 cells

The amount of sulfatide in RIN-38 cells (passage 11–17) was 2.7 ± 1.1 nmol/mg protein ($n = 19$), while sulfated lactosylceramide was found in approximately 1/100 of the sulfatide concentration (29 ± 2 pmol/mg protein, $n = 6$, Figure 1A). The third Sulph I antigen, seminolipid, was also found in RIN-38 cells (4 ± 2 nmol/mg protein, $n = 6$, Figure 1B) and there was no significant difference of the amount of this molecule expressed compared to sulfatide. Semi-quantitative information about the fatty acid composition of sulfatide revealed that the main species were C16:0 and C24:0 fatty acids (Figure 2), as previously described in rat islets [2,27].

Electron microscopy studies of RIN-38 cells showed apparently fewer granules as compared to beta cells in freshly isolated islets [2,4], and not all secretory granules showed Sulph I staining. In addition, the Sulph I labelling in RIN-38 cells was in the granule membrane instead of the interior of the granules as previously reported to be the primary location of sulfatide in beta cells [2,4] (Figure 3A). The plasma membrane was partly intensively stained, indicating that the Sulph I antigens are located in domains in this compartment (Figure 3B). In addition, mitochondria and villi structures of RIN-38 cells showed Sulph I staining (Figure 3C and D). Double staining of RIN-38 cells analysed by microscopy showed intense staining of Sulph I antigens (Figure 4, green) in the plasma membrane, whereas insulin was primarily intracellular (Figure 4, red). The co-localization of the antigens is supported by co-staining (yellow), which most likely represents sulfatide and insulin positive secretory granules.

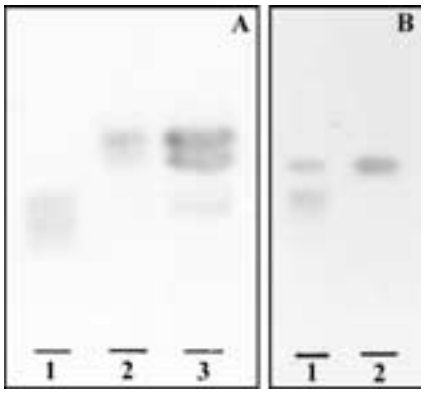


**Figure 1.** Thin layer chromatography of sulfatide, sulfated lactosylceramide and seminolipid in cultured RIN-38 cells. Culturing of cells, the lipid isolation procedure and TLC-ELISA methods are described in *Materials and methods*. A. Lane 1, sulfated lactosylceramide (20 pmol); lane 2, sulfatide (40 pmol); lane 3 sulfatide and sulfated lactosylceramide in the saponified fraction isolated from RIN-38 cells (500 μg cell protein applied). B. Lane 1, the upper band represents seminolipid isolated from RIN-38 cells (10 μg cell protein applied) and the lower sulfatide and sulfated lactosylceramide migrating together; lane 2, seminolipid (120 pmol).

### Sulfatide metabolism in RIN-38 cells

RIN-38 cells cultured with $^{14}$C-serine for 24 h showed almost no labelling of sulfatide or sulfated lactosylceramide (<1 cpm/μg protein, Figure 5A), regardless of the presence or absence of Fumonisin B1, supporting a low *de novo* synthesis rate of sulfatide. Fumonisin B1, which blocks the *de novo* synthesis of sphingolipids [28], caused a 60% decrease in radioactive incorporation in the sphingomyelin fraction and a 50% decrease in ceramide fraction compared with controls in the experiment. The band migrating just above sphingomyelin was unidentified. When the cells were cultured for 6 h with $^{35}$S-sulfate in the presence and absence of Fumonisin B1, no differences in the amount of sulfatide and sulfated lactosylceramide synthesized were found (Figure 5B).

RIN-38 cells cultured with $^{14}$C-serine and $^{35}$S-sulfate in the presence of the chloroquine for 6 h, inhibiting the recycling pathway of glycosphingolipids by affecting lysosomal enzyme activity [29], showed a 75% reduction in radioactive incorporation in the sulfatide fraction (Figure 5C) and an almost complete disappearance of the band migrating as sulfated lactosylceramide compared to controls. The reduction in radioactive incorporation of the sulfatide fraction appears to reflect the slower migration band(s) (Figure 5C), indicating that sulfatide with various fatty acids may be affected differently by chloroquine. There was no change in the incorporation of radioactivity in the ceramide, galactosylceramide/glucosylceramide and sphingomyelin fractions compared to controls. Thus, the metabolic studies support the recycling pathway as the major pathway for sulfatide and sulfated lactosylceramide synthesis in RIN-38 cells, in agreement with previous results using rat islets [2].

TLC-ELISA (Figure 1) and mass spectrometry (Figure 2) of the endogenous sulfatide showed two major sulfatide isoforms. As seen in Figure 5, there was an additional lower sulfatide band appearing in the metabolic experiments. This might represent a less hydrophobic sulfatide isoform, having similar metabolic turnover as the other isoforms, but existing in low amounts endogenously. Another possibility is that this lower band represents an unknown sulfated glycosphingolipid. Seminolipid was not determined in the metabolic experiment since it was removed during the saponification of the "sulfatide fraction", which was essential for evaluation of the experiments. Since seminolipid has not been found in beta cells *in vivo* [2] and

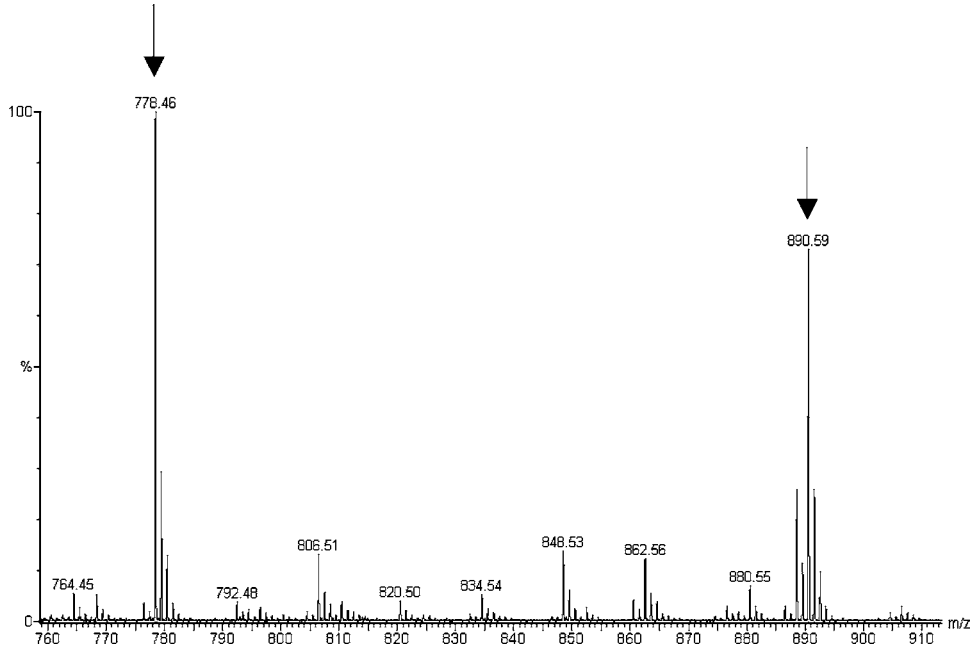


**Figure 2.** Mass spectra of sulfatide isolated from RIN-38 cells, showing the two dominant isoforms, sulfatide containing C16:0 and C24:0 fatty acids (arrows). Isolation of sulfatide and the mass spectrometric analysis are described in *Materials and methods*.

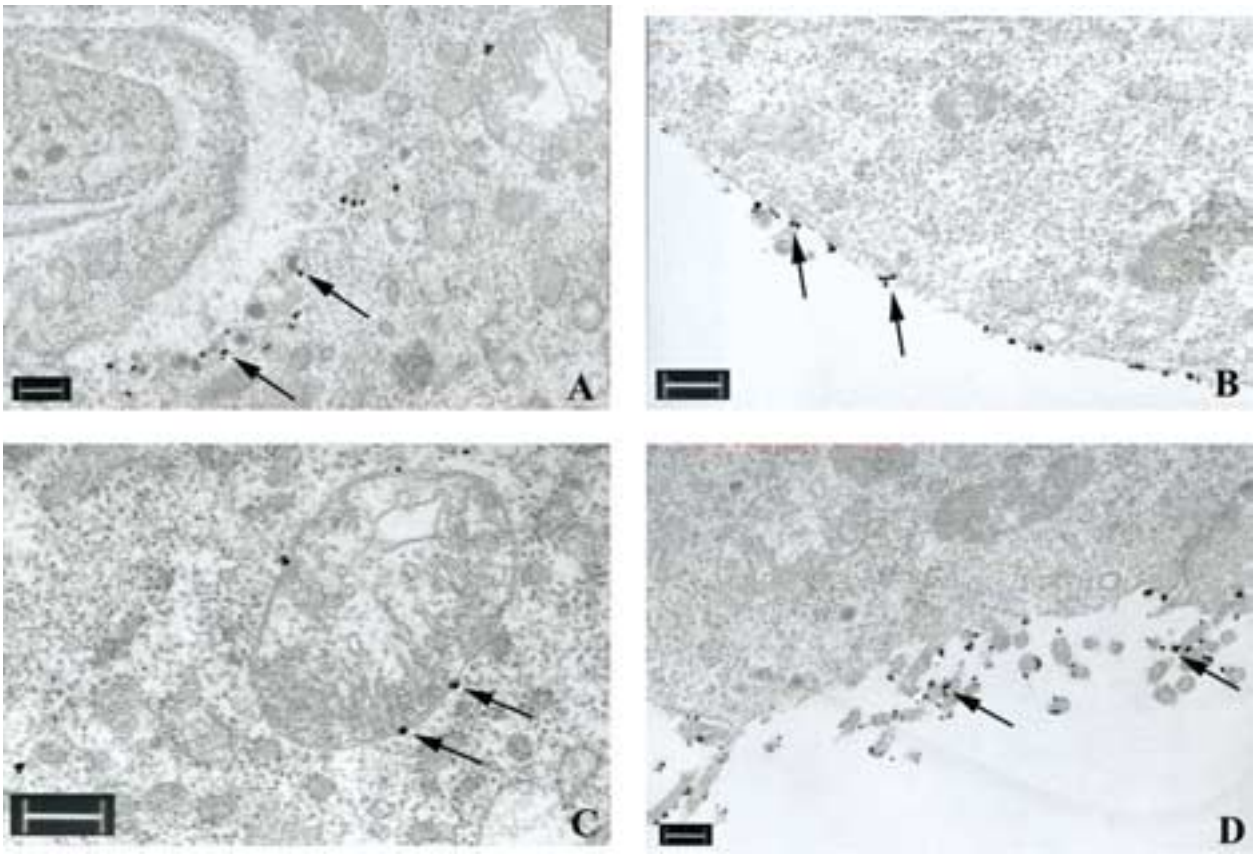


**Figure 3.** Electron micrographs illustrating parts of RIN-38 cells with immunogold labelling with Sulph I. Culturing of cells and the electron microscopy procedure are described in *Materials and methods*. Arrows indicates Sulph I positive beta cell granules (A), plasma membrane (B), mitochondria (C) and villi structures (D). Scale bar = 300 nm.

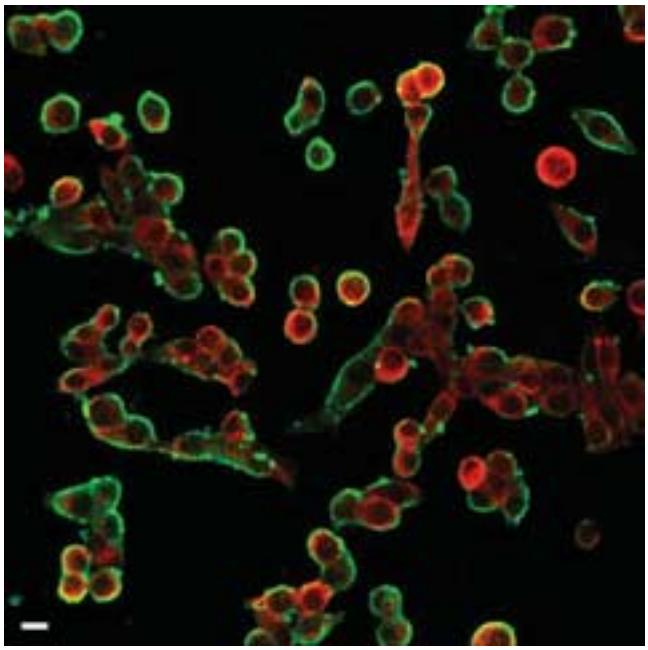

**Figure 4.** RIN-38 cells double stained with Sulph I antibody and insulin antibody. Culturing of cells and the immunocytochemical procedure are described in *Materials and methods*. Cells show Sulph I staining (green), mainly located to the plasma membrane, and insulin staining (red), which is predominantly intracellular. Double stained areas appear yellow. Scale bar = 10 $\mu$m.

thus not in focus in this study, no additional experiments to evaluate the metabolism of seminolipid in RIN-38 cells were performed.

Granule fractions isolated from RIN-38 cells

Sulfatide and insulin were found in the granule fractions eluted in the void volume (fraction 5–8, as described by Andersson and Abrahamsson [25]) (Figure 6). Sulfatide and insulin accumulated in the secretory granules, with variable amounts in the fractions eluted. The differences in the sulfatide and insulin content of the fractions in the three isolations might

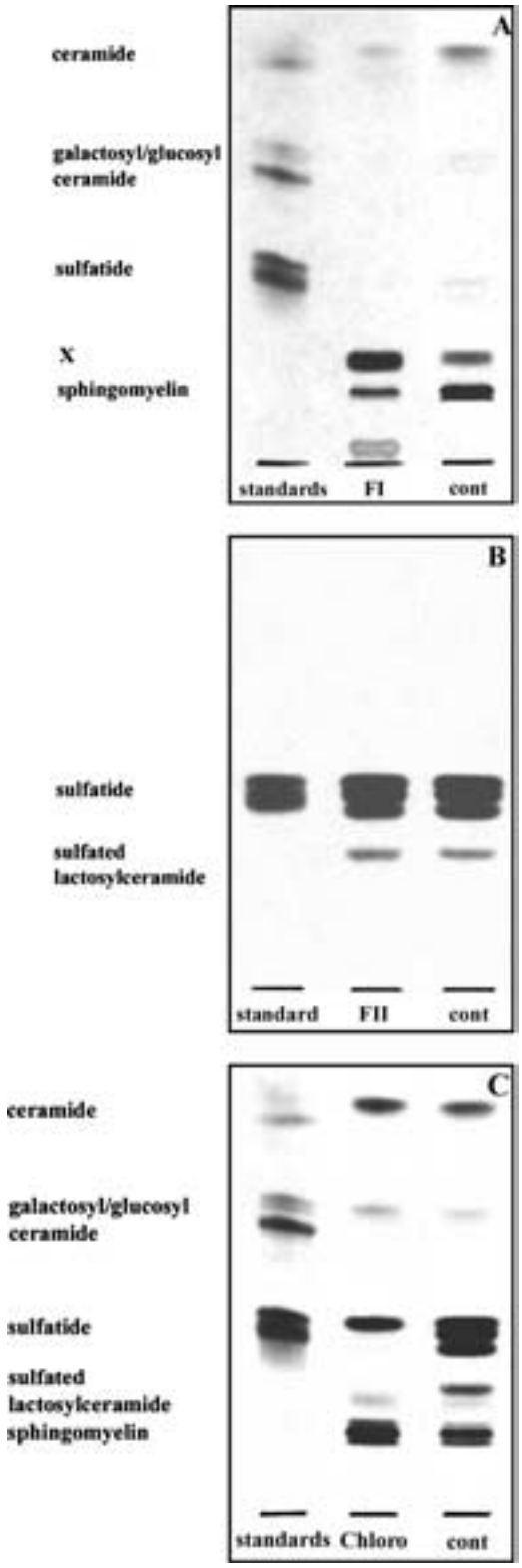


**Figure 5.** Autoradiogram of $^{35}$S-sulfate and/or $^{14}$C-serine labelled lipids in the "sulfatide fraction" from RIN-38 cells cultured in the presence and absence of Fumonisin B1 (A, B) and Chloroquine diphosphate (C). One experiment is shown. Culturing of cells and isolation of labelled lipids are described in *Materials and methods*. Lipids corresponding to 50 $\mu$g cell protein were applied. Reference lipids (standards) applied to the plates were $^{3}$H-sulfatide, $^{3}$H-galactosyl ceramide and $^{3}$H-ceramide. The double, and in some cases triple, spots reflect variation in fatty acids-the higher hydrophobicity the faster migration on the plate. (A) Autoradiogram of $^{14}$C-serine labelled lipids from RIN-38 cells cultured for 24 h with Fumonisin B1 (FI) and control (cont) ($n$ = 3 ). X corresponds to unidentified band. (B) Autoradiogram of $^{35}$S-sulfate labelled sulfatide from RIN-38 cells cultured for 6 h with Fumonisin B1 (FII) and control (cont) ($n = 3$). (C) Autoradiogram of $^{35}$S-sulfate and $^{14}$C-serine labelled lipids from RIN-38 cells cultured for 6 h with Chloroquine diphosphate (Chloro) and control (cont) ($n = 3$).

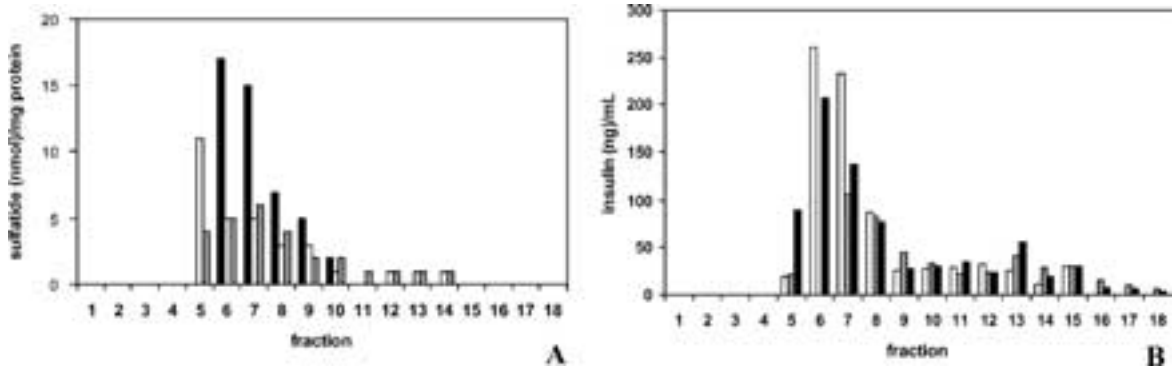


**Figure 6.** Sulfatide (A) and insulin (B) content in secretory granules isolated from RIN-38 cells ($n = 3$). Culturing of cells, the granule isolation, lipid isolation procedures as well as the TLC-ELISA and insulin ELISA methods are described in *Materials and methods*. The majority of the granules were eluted in the void volume (fraction 5–8), in which sulfatide and insulin accumulated. The different bars represent three separate granule isolations.

depend on the amount of granules received during the isolation procedure and also the number of intact granules remaining before the last isolation step, which might differ between isolations. Seminolipid was also detected in the granule fractions before saponification, but only in approximately 1/5 of the amount of sulfatide. No sulfated lactosylceramide could be detected in the granule factions.

### Detergent insoluble membrane domains (DIMs) isolation from RIN-38 cells

The DIMs fraction isolated from the RIN-38 showed significant enrichment of sulfatide and seminolipid in these domains ($n = 4$, Table 1). No sulfated lactosylceramide could be detected in the DIMs fraction. Another glycosphingolipid, GM1, was also significantly enriched in the DIMs fraction together with cholesterol and sphingomyelin, supporting a proper isolation of these domains ($n = 4$, Table 1). Quantifications of lipids in DIMs isolated from RIN-38 cells were compared to total cell extracts from RIN-38 cells cultured during the same period of time.

**Table 1.** Sulfatide, seminolipid, GM1, cholesterol and sphingomyelin content in total cell extracts ($n = 4$) compared to the isolated DIMs fraction ($n = 4$). Quantifications of lipids in DIMs isolated from RIN-38 cells were compared to total cell extracts from RIN-38 cells cultured during the same period of time. Values are presented as mean ± 50

| | Total cell extracts | DIMs fraction | *p*-value |
|---|---|---|---|
| (nmol/mg protein) | | | |
| Sulfatide | 2.0 ± 0.5 | 81 ± 19 | 0.003 |
| Seminolipid | 5 ± 2 | 332 ± 58 | 0.002 |
| GM1 | 0.05 ± 0.01 | 2.9 ± 0.3 | 0.0003 |
| Cholesterol | 78 ± 14 | 2105 ± 560 | 0.005 |
| Sphingomyelin | 13 ± 1 | 704 ± 59 | 0.0002 |
| (pmol/nmol glycerophospholipid) | | | |
| Sulfatide | 4.9 ± 0.6 | 14 ± 3 | 0.008 |
| Seminolipid | 14 ± 3 | 56 ± 7 | 0.0003 |
| GM1 | 0.13 ± 0.02 | 0.49 ± 0.04 | 0.0001 |
| Cholesterol | 193 ± 1 | 348 ± 41 | 0.005 |
| Sphingomyelin | 34 ± 6 | 119 ± 18 | 0.001 |

## Discussion

This study shows that the beta cell line RIN-38 expresses similar amounts of sulfatide as islets freshly isolated from Lewis rat pancreas, and that sulfatide follows the same metabolic pathways as in isolated islets from Lewis rats [2]. Sulfatide is located to the insulin secretory granules as previously reported for islets isolated from different mammals [2,4] and in addition, Sulph I staining was found in mitochondria and villi structures. Another information obtained in this study is that sulfatide is accumulated in the DIMs fraction in cultured beta cells, a compartment where numerous events related to signal transduction and membrane trafficking are known to occur.

In addition to sulfatide, the beta cell line also expresses sulfated lactosylceramide, another antigen for the Sulph I antibody, although in 1/100 of the sulfatide concentration. These results together with the absence of sulfated lactosylceramide in the granule and DIMs fractions support that the majority of the immunolabelling does not reflect this molecule. Also, the third Sulph I antigen, the glyceroglycolipid seminolipid, was detected in the cultured beta cells and in similar amounts as sulfatide. The low amounts of seminolipid compared to sulfatide in isolated granules support that the immunostaining of the granules mainly was due to sulfatide. On the other hand, the high amounts of seminolipid in DIMs support that the immunostaining seen in the plasma membrane most likely reflects both sulfatide and seminolipid. The selective presence of sulfatide in the insulin granules supports previous suggestions that sulfatide is involved in the insulin metabolism and processing [2,3]. Sulfated lactosylceramide and seminolipid has not been found in isolated rat islets [2] or in normal adult

mammalian pancreas [4] previously examined. However, sulfated lactosylceramide has been found in fetal human and newborn rat pancreatic tissue [23] and the apperance of this glycosphingolipid, and possibly also seminolipid, in RIN-38 cells might simply reflect the immature stage of an immortalized cell line.

The sulfatide content and synthesis correlate well with the results obtained from studies using isolated rat islets [2]. In addition, this study also shows that isolated granules from RIN-38 cells did contain sulfatide together with insulin. However, in contrast to previous electron microscopy results, which show sulfatide to be located to the interior of the insulin granules [2,4], secretory granules of RIN-38 cells are stained with the Sulph I antibody in the membrane of the granule itself. The reason for the absence of intra-granule labelling in the electron microscopy studies of RIN-38 cells might be due to the inability of Sulph I to reach the interior of the granules by the method used in this paper (pre-embedding). Therefore, intragranular location of sulfatide cannot be excluded. For reasons unknown, post-embedding techniques do not show any Sulph I labelling of RIN-38 cells and therefore this approach is not possible.

In addition to its presence in secretory granules, sulfatide might also be located to mitochondria and villi structures in RIN-38 cells. It is likely that the villi structures are involved during insulin secretion in RIN-38 cells, which further supports a possible role of sulfatide in this process. Sulph I staining of villi structures and mitochondria might result from seminolipid instead or together with sulfatide. The intracellular staining is observed in the absence of saponin, and this is probably due to "over-fixation" of cells that are sensitive to the fixation, which results in membrane disruption and ability for antibodies to reach intracellular compartments [30]. Using immunocytochemistry techniques, intense Sulph I staining of the plasma membrane is shown, probably reflecting sulfatide in detergent resistant microdomains, whereas insulin is primarily intracellular. The few areas double stained (yellow) in the cells might reflect sulfatide and insulin containing granules and this staining seem to be located close to the plasma membrane, probably reflecting the docking and secretion process.

As mentioned in the introduction, accumulating data support the theory that glycosphingolipids, including sulfatide [26,31], are located in detergent insoluble membrane domains (DIMs), and are a prerequisite for their function, either by themselves or in association with proteins in the same domains [8,9]. The glycosphingolipids in these domains are assumed to facilitate receptor signalling and protein trafficking across the plasma membrane. In this study, sulfatide is shown to accumulate in the DIMs compartments of RIN-38 cells. Hypothesising that these microdomains of the plasma membrane are involved in the insulin secretion process, the presence of sulfatide in DIMs might facilitate the monomerisation process, and/or contribute to the regulation of glucose-induced insulin secretion, as previously described by Osterbye et al. [3] and Buschard et al. [1]. Thus, RIN-38 cells might be a useful model to elucidate the role of sulfatide in DIMs compartments of insulin producing beta cells. Also seminolipid was associated with DIMs, but as discussed above, the absence of seminolipid in rat and human pancreas [4] oppose its involvement in insulin secretion of beta cells. Overall, the aim of analysing seminolipid was to elucidate its potential contribution to the immunostaining.

Support for the location of sulfatide in domains at the plasma membrane is given by the electron microscopy studies, showing clustering of Sulph I staining at the plasma membrane, although staining of sulfatide cannot be separated from staining of seminolipid. Furthermore, the accumulation of cholesterol and sphingomyelin in DIMs [32,33] supported the proper isolation of these domains. The glycosphingolipid GM1, suggested to be a DIMs marker [34], is also enriched in this fraction. The significant accumulations of sulfatide, GM1, cholesterol and sphingomyelin are also shown when related to glycerophospholipid content instead of proteins. The even distribution of most of the glycerophospholipids throughout the membrane in combination with the physical properties of glycerophospholipids, making these molecules less prone to form insoluble domains in the plasma membrane [35], indicates that "lipid"/glycerophospholipid might be a more reliable measurement of accumulation than "lipid"/protein.

In summary, we have shown that sulfatide is expressed in similar amounts and follows the same intracellular trafficking route in RIN-38 cells as in freshly isolated islets. Sulfatide is furthermore located to the insulin containing secretory granules in RIN-38 cells and support for possible association with villi structures and mitochondria is given. Moreover, sulfatide is also associated with detergent insoluble membrane domains. Thus, this beta cell line might be useful, in combination with freshly isolated islets, to further explore if sulfatide might be involved in insulin secretion and signal transduction of beta cells.

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