

Glucosylceramide synthase inhibitors D-PDMP and D-EtDO-P4 decrease the GM3 ganglioside level, differ in their effects on insulin receptor autophosphorylation but increase Akt1 kinase phosphorylation in human hepatoma HepG2 cells

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Gangliosides function as modulators of several cell growth related receptors. It was shown for caveolin-rich adipocytes, that GM3 ganglioside binds to insulin receptor (IR), dissociates its complex with caveolin, and thus lowers IR autophosphorylation following insulin treatment. We extended those studies into human hepatocyte-derived HepG2 cells, characterized by a high level of IR but low of caveolin. To lower the glycosphingolipid content, estimated by GM3 concentration, two glucosylceramide synthase inhibitors *D-threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) and *D-threo*-1-(3,4-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (D-EtDO-P4) were used. D-PDMP at 40 μM or D-EtDO-P4 at 1 μM concentrations in culture medium decreased the GM3 content to 22.3% (17.8–26.1%) and 18.1% (13.7–24.4%), respectively, of the control value. The reduction of GM3 obtained with D-PDMP was accompanied by a 185.1% (153.5–423.8%) significant increase in the level of IR autophosphorylation following cell stimulation with 100 nM insulin. The effect of D-EtDO-P4 on IR autophosphorylation was smaller amounting to an increase by 134.8% (111.3–167.8%) of the control level and statistically non-significant. The effects of D-PDMP and D-EtDO-P4 could also be detected at the level of Akt1 kinase. In cells grown in the presence of D-PDMP the level of phosphorylated Akt1 was 286.0% (151.4%–621.1%) of that in the control. In this case the effect of D-EtDO-P4 was similar: 223.0% (181.4–315.4%) significant increase in phosphorylated Akt1. We assume that glycosphingolipid depletion in HepG2 cells may affect not only IR autophosphorylation but also, independently, the phosphorylation of Akt1, by modifying the membrane microenvironment of this kinase.

Key words: Akt1, GM3 ganglioside, glycosphingolipid depletion, insulin receptor

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INTRODUCTION

Glycosphingolipids (GSLs) and especially their sialic acid containing variety, gangliosides, are involved in a number of cellular activities such as cell growth, differentiation, and transmembrane signaling (Lopez & Schnaar, 2009). Originally Nojiri *et al.* reported that 3'-sialosyl-neotetraosylceramide and GM3 ganglioside attenuate autophosphorylation of insulin receptor (IR) (Nojiri *et al.*, 1991). Recently, Inokuchi and colleagues summarized data indicating that type 2 diabetes could be a GM3-

associated membrane microdomain disorder (Inokuchi, 2014). Briefly, autophosphorylation of insulin receptors (IRs), following insulin binding, is enhanced by their interaction with caveolin within caveolae, a membrane microdomains engaged in a number of cellular activities including signaling (Delos Santos *et al.*, 2015). According to Inokuchi, GM3 ganglioside binds to and displaces IRs out of the caveolae thus preventing their interaction with caveolin (Inokuchi, 2014). Decreasing the GM3 content should increase IRs autophosphorylation and improve the efficiency of insulin signaling pathway. The simplest procedure to decrease the cellular GSLs level, including GM3, is to inhibit the activity of glucosylceramide synthase (GlcCerS, UDP-glucose:ceramide glucosyltransferase, E.C. 2.4.1.80), which initiates the biosynthesis of the majority of animal GSLs (Sandhoff & Kotler, 2003). Therefore, the GlcCerS inhibitors have been used to reduce the GSLs level in studies on glycosphingolipid functions and also as medication in diseases like diabetes (Langeveld & Aerts, 2009).

Most of the investigations on the relation between GM3 ganglioside and IR autophosphorylation were performed on caveolin-rich adipocytes and muscle cells (Yamashita *et al.*, 2003; Inokuchi, 2014). No experiments on the effects of reducing GSLs level on insulin signaling were performed with caveolin-poor cell lines. In our studies we used human hepatoma HepG2 cells, chosen since they contain high number of IRs but very low level of caveolin (Cokakil *et al.*, 2009; Stralfors, 2012). Moreover, GM3 is the major ganglioside in these cells (Spitalnik *et al.*, 1989). Therefore, the results obtained could not be interpreted as due to the lack of the key ganglioside.

The insulin signaling pathway starts from the hormone binding to IRs, which results in receptor autophosphorylation. Activated IRs phosphorylate insulin receptor substrates, the scaffolding proteins. Next, through the recruitment to the plasma membrane of PI3K kinase, synthesis of PIP3 followed by the recruitment of PDK-1 and Akt kinases, the latter kinase is phosphorylated (Cohen, 2008; Guo, 2014). Activated Akt phosphorylates a number of its protein substrates thus initiating the multiplicity of responses (Schultze *et al.*, 2011). Akt kinase

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Abbreviations: GlcCerS, glucosylceramide synthase; D-PDMP, *D-threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; D-EtDO-P4, *D-threo*-1-(3,4-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; GM3, NeuAc3Gal β 4Glc β 1Cer; IR, insulin receptor

occurs in three isoforms. In human hepatoma HepG2 cells the Akt1 is expressed at the highest level (Santi & Lee, 2010). Therefore, for our studies we selected this particular isoform.

In the current study, we set out to determine if for the caveolin-poor HepG2 cells decreasing glycosphingolipid level, as measured by GM3 ganglioside content, affects IRs autophosphorylation and Akt1 kinase phosphorylation. The GlcCerS inhibitors selected were D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) (Inokuchi & Radin, 1987), a commercial reagent used in numerous publications with all the effects of its activity well recognized (Liour & Yu, 2002), and D-*threo*-1-(3,4-ethylenedioxy)phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (D-EtDO-P4) (Lee *et al.*, 1999). The D-EtDO-P4 was chosen since at the inception of our work it was the most effective GlcCerS inhibitor with no apparent side effects. It was synthesized in our laboratory as described (Lee *et al.*, 1999).

MATERIALS AND METHODS

Cell culture and treatment with insulin. Human hepatoma HepG2 cells obtained from ATCC (Manassas, VA, USA) were cultured at 37°C under 5% CO₂ in Dulbecco's modified Eagle medium with GlutaMax (Invitrogen Corp., Camarillo, CA, USA) supplemented with penicillin (0.25 mg/ml), streptomycin, (0.25 mg/ml) and neomycin (0.5 mg/ml) (Gibco, Carlsbad, CA, USA) and containing 10% heat inactivated fetal bovine serum (Biosera, Bousses, France) (complete medium). HepG2 cells were plated at density of 2×10⁶ cells for a 25-cm² and 7.5×10⁶ cells for a 75-cm² culture flask. To grow as monolayers, the cells were cultured with or without inhibitors for 96 h. D-PDMP was purchased from Matreya (Pleasant Gap, PA, USA) while D-EtDO-P4 was synthesized in our laboratory as described (Lee *et al.*, 1999). Its ESI-MS spectrum and HPLC characteristics are presented in Supplementary material at www.actabp.pl. The inhibitors were added from a stock solution in DMSO to serum-containing media. Final DMSO concentration in all cell cultures was 0.1%. The media were changed daily. Before treatment with insulin cells were washed twice with PBS and maintained under culture conditions for 2 h in a serum free medium. Insulin was added from a stock solution to the final concentration of 100 nM. In experiments on IR autophosphorylation cells were treated with insulin at 37°C for 5 min while in studies on Akt1 kinase phosphorylation, for 15 min, and were washed thrice with cold PBS. For cell lysis, to 75-cm² flasks 2.5 ml of a buffer containing 50 mM Hepes, pH 7.4; 150 mM NaCl; 10% glycerol; 1% TritonX-100; 1.5 mM MgCl₂; 100 mM NaF; 10 mM sodium pyrophosphate; 1 mM Na₃VO₄ and "cOmplete" protease inhibitors (Roche, Basel, Switzerland) was added. The flasks were kept for 30 min on ice, cells were scraped, homogenized in the same buffer, centrifuged at 1500×g for 10 min and the supernatants were frozen at -70° C.

SDS/PAGE and Western blotting. Cell lysates were centrifuged, diluted with Laemmli electrophoresis buffer containing 2-mercaptoethanol and boiled for 5 min. Samples containing about 50 µg of protein were analyzed by SDS/PAGE (4% stacking and 14% separating gels). Resolved proteins were transferred onto nitrocellulose (GE Healthcare Life Science, Little Chalfont, UK) and the membranes were blocked with 2% defatted milk in PBS containing 0.05% Tween 20 (PBS-T) for 30 min at room temperature. Phosphorylated IR (pIR) and β-actin

were detected with primary antibodies purchased from Abcam (Cambridge, UK) diluted 1:500 for anti-pIR, and 1:5000 for anti-β-actin. The membranes were incubated with the antibodies overnight in a cold room and washed several times with PBS-T. HRP-conjugated secondary anti-rabbit antibodies (Dako, Glostrup, Denmark) and anti-mouse immunoglobulins (Jackson Immuno Research, West Grove, PA, USA) were used at the dilution of 1:5000 and 1:10000, respectively. After addition of the secondary antibody, incubation for 1 h at room temperature and washing with PBS-T, the signal from HRP was detected with SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, PA, USA) by exposure on Kodak autoradiographic film (Sigma-Aldrich, St. Louis, MO, USA). Band intensity was analyzed with a GelScan 1.44 software and normalized against the β-actin signal.

Glycosphingolipid extraction and quantification of GM3. GM3 standard was isolated in our laboratory from canine erythrocytes by DEAE-Sephadex and silicic acid column chromatography (Pacuszka *et al.*, 1978). [³H] GM1, about 98% pure and labeled with tritium to 2.1 Ci/mmole, was prepared and characterized as described (Schwarzmann & Sandhoff, 1987; Panasiewicz *et al.*, 2003).

To cell pellets scraped from 25-cm² flasks about 100 pmoles, 250000 cpm, of [³H]GM1 was added and lipids were extracted with chloroform and methanol mixture (Svennerholm & Fredman, 1980). The extracts were dried under a stream of N₂ and, after the addition of chloroform, methanol and water separated into Folch upper and lower layers (Folch *et al.*, 1957). The lower layers were extracted twice with equal volume of chloroform-methanol-water (3:48:47, v:v). The upper layers were pooled, diluted with methanol to 5 ml and applied on Sephadex A25 (Pharmacia, Stockholm, Sweden) acetate form columns of 0.5 ml bed volume, prepared in methanol. The columns were washed with 7 ml of methanol. Monosialogangliosides (GM3 and [³H]GM1) were eluted with 5 ml of 0.05 M ammonium acetate in methanol. The ganglioside-containing fractions were dried and desalted on SepPak C18 cartridges (Schnaar, 1994). Aliquots containing 10% of the samples were treated with 2 units (1 µl) of recombinant endoglycoceramidase from *Rhodococcus* sp. (TaKaRa, Otsu, Japan) as specified by the manufacturer. The oligosaccharides released from the gangliosides were conjugated with anthranilic acid and purified on Discovery DPA-6S (Sigma-Aldrich, St. Louis, MO, USA) columns (Neville *et al.*, 2004). The fluorescent oligosaccharide conjugates were analyzed by normal-phase HPLC on a TSK gel amide-80 column, 4.6×250 mm (Tosoh Bioscience, Tokyo, Japan) as described (Neville *et al.*, 2004). A Perkin Elmer fluorescence detector set at Ex 360 nm, Em 425 nm was used. To allow the calculation of the final yields, before the HPLC analysis, the radioactivity of the samples was determined by liquid scintillation counting. During each set of endoglycoceramidase assays, a control sample containing 250 pmoles of GM3 and 250000 cpm of [³H]GM1 was processed in parallel.

Estimation of total IR, Akt1, and phosphorylated Akt1 by ELISA. The levels of the proteins of interest were determined by a solid phase sandwich enzyme linked immunosorbent assay using appropriate kits, as recommended by their manufacturers. The IR (β subunit) ELISA kit was supplied by Millipore (Billerica, MA, USA) (formerly Upstate), total Akt1 as well as phosphorylated Akt1 [pS473] kits were purchased from Invitrogen Corp. (Camarillo, CA, USA). After incubations optical

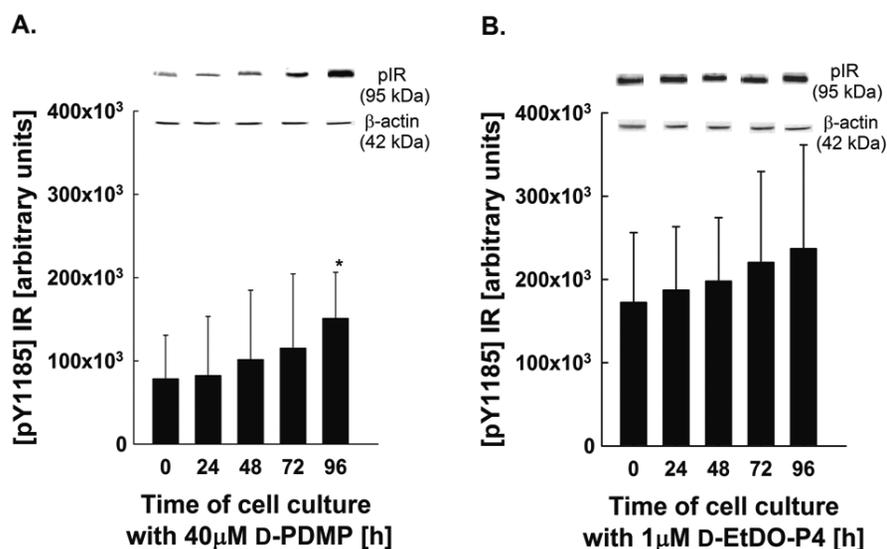


Figure 1. Effect of duration of culture with 40 μM D-PDMP (A) or 1 μM D-EtDO-P4 (B) on autophosphorylation of insulin receptor (IR) in HepG2 cells treated with 100 nM insulin.

Cells grown in complete media supplemented with 40 μM D-PDMP (A) or 1 μM D-EtDO-P4 (B) were treated with 100 nM insulin and IR autophosphorylation was estimated by Western blotting with anti-phosphorylated IR antibody as described in Methods. (For A, $n=4$, $p<0.05$; for B, $n=6$).

density was measured at 450 nm with Labsystems Multiscan RC Microplate Reader (ThermoFisher Scientific, Waltham, USA). The values obtained were calculated per milligram of protein in the samples, determined with bicinchoninic acid according to the protocol of Pierce Chemical Co. (ThermoFisher Scientific).

Other procedures. To estimate the protein content of the cells after lipid extraction, tubes were left open to dry, the residues were suspended in 2 ml of 0.1 M NaOH and left at room temperature to dissolve. Protein concentrations were determined as above.

Statistical analyses were performed with PQStat v 1.4.4 software. The normality of the data distribution was assessed with the Shapiro-Wilk test. Most data were analyzed by Anova followed by Dunnett's multiple comparison test. The significance of the differences of kinase Akt1 phosphorylation levels was verified with T-test for dependent pairs, $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

D-PDMP and D-EtDO-P4 decrease GM3 ganglioside level in HepG2 cells in a concentration- and time-dependent manner

The duration of the cell culture experiments in the presence of inhibitors was dictated by the growth characteristics of HepG2 cells (Material and Methods). Figure 3 in Supplementary material at www.actabp.pl shows the effects of increasing concentrations of the inhibitors on the GM3 cell content after 96 h of culture. A reduction to about 35% (30.2–42%, $n=3$) of the control level was achieved with 40 μM D-PDMP (Supplementary material at www.actabp.pl, Fig. S3A). For D-EtDO-P4 a similar decrease in GM3 level was observed at about 0.1 μM to 1 μM concentration of the inhibitor (Supplementary material at www.actabp.pl, Fig. S3B). The value for 1 μM D-EtDO-P4 was 16.9% of the control (12.2–23.2%, $n=3$). For all the subsequent experiments we used D-PDMP at 40 μM and D-EtDO-P4 at 1 μM. After 96 h

of the cell culture with an inhibitor, cell GM3 content was decreased down to 22.3% (17.8–21.1%, $n=6$) for D-PDMP and 18.1% (13.7–24.4%, $n=6$) for D-EtDO-P4 (Supplementary material at www.actabp.pl, Fig. S3C).

The decline in GSLs level caused by D-PDMP is accompanied by increased autophosphorylation of IR. The effect of D-EtDO-P4 is smaller and not statistically significant.

Binding of insulin to IR at the plasma membrane initiates the insulin signaling pathway (Cohen, 2008; Guo, 2014). It was established that the interaction of IRs with caveolin increases their autophosphorylation in caveolin-rich cells (Inokuchi, 2014). The plasma membrane is a heterogeneous mosaic of microdomains, including caveolin dependent caveolae (Delos Santos *et al.*, 2015). Thus, the activity of IRs seems to be regulated by their presence in the distinct plasma membrane microdomains. However, it should be considered that caveolae are not the only membrane domains important for IR activity. Accordingly, Vainio and her associates reported IR autophosphorylation in caveolin-free human hepatoma Huh7 cells. Importantly, this reaction was strongly dependent on the presence of cholesterol, a critical component of various lipid rafts, not only of caveolae (Vainio *et al.*, 2002).

In our experiments, the effects of the two GlcCerS inhibitors studied on IR autophosphorylation were not the same (Fig. 1). The reduction of GM3 obtained with D-PDMP was accompanied by a 185.1% (153.5–423.8%, $n=4$), significant increase ($p<0.05$) in the level of IR autophosphorylation following cell stimulation with 100 nM insulin. Surprisingly, the effect of GM3 ganglioside reduction on IR autophosphorylation caused by D-EtDO-P4 was smaller. It amounted to an increase of 134.8% (111.3–167.8%, $n=6$) of the control level and was not statistically significant. The cause of these discrepant effects of the inhibitors is at present unknown. It cannot be attributed to differences in the magnitude of the GM3 reduction. Firstly, because it was rather small and secondly, because by changing the duration of the cell culture in the presence of D-EtDO-P4 inhibitor we could obtain cells with the same level of GM3 as in those grown

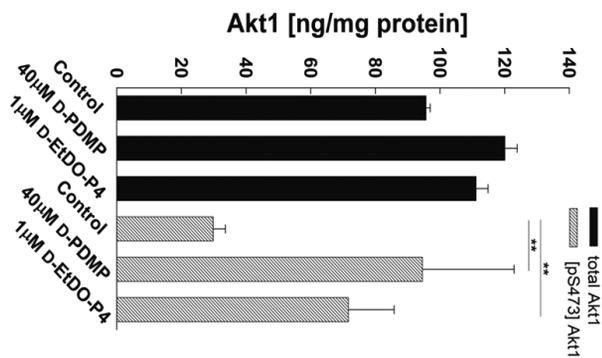


Figure 2. Phosphorylation of Akt1 kinase in HepG2 cells grown for 96 h in complete media enriched with 1 µM D-EtDO-P4 or 40 µM D-PDMP.

Cells were grown in complete media supplemented with 1 µM D-EtDO-P4 or 40 µM D-PDMP, treated with 100 nM insulin and phosphorylated Akt1 kinase level was determined by ELISA as described in Methods. The level of total Akt1 is included for comparison. The level of phosphorylated Akt1 without insulin was below detection limit of the procedure (n=9 **p<0.01).

with D-PDMP, but it did not affect the IR autophosphorylation. We assume that the negligible increase of IR autophosphorylation is a unique effect of D-EtDO-P4. This possibility may be supported by our preliminary experiments, where *N*-butyldeoxynojirimycin was used as an inhibitor of GlcCerS (Butters *et al.*, 2005). As could be expected, the decline in GM3 content was accompanied by a significant increase of the autophosphorylation of IR and phosphorylation of Akt1 kinase (unpublished data).

It is important that the amount of IR in HepG2 cells grown in the presence of either inhibitor was similar: 33.2 ng/mg protein ± 5.7 (n=4) was detected in cells cultured with D-PDMP versus 36.1 ng/mg protein ± 5.3 (n=4) for the control. Cells cultured with D-EtDO-P4 had IR at 34.7 ng/mg protein ± 3.5 (n=6) versus 33.9 ng/mg protein ± 3.7 (n=6) for the controls.

The decline in GSLs level caused by D-PDMP and D-EtDO-P4 is accompanied by increased phosphorylation of Akt1 kinase

Akt kinase is engaged downstream of activated phosphoinositide 3-kinase in many signaling cascades, including the insulin pathway (Kulkarni *et al.*, 2012). The effects of GlcCerS inhibitors on IR autophosphorylation and Akt phosphorylation have been studied in human adipocytes (Aerts *et al.*, 2007) however, the antibodies used in this report did not distinguish between the various isoforms of the kinase. For hepatocytes, the exact isoform-specific functions of Akt1 have not been clearly defined (Schultze *et al.*, 2011). In our experiments we selected Akt1 since, as already mentioned in the *Introduction*, of the three Akt kinase isoforms in HepG2 cells Akt1 is expressed at the highest level. In these cells Akt1 can be localized to either the cytoplasm or the plasma membrane (Santi & Lee, 2010). In the plasma membrane Akt1 kinase has been, like glycosphingolipids, detected in the detergent-resistant membrane fraction, suggesting its association with lipid rafts (Cinar *et al.*, 2007). The effects of GlcCerS inhibitors on Akt1 phosphorylation following insulin stimulation have not been studied yet.

In our investigations with D-PDMP as the inhibitor, the decrease of GM3 content was accompanied not only by a significant increase in IR autophosphorylation following insulin binding, but also by an elevated phosphorylation of Akt1 by 286.0% (151.4–625.5%, n=9) (Fig. 2). The former ob-

servation is not surprising, nevertheless the latter one differs from the results of Chavez and coworkers (2003) who used PDMP to increase ceramide level in C2C12 myoblasts. This discrepancy could result from the difference in cells used and Akt isoforms studied (Chavez *et al.*, 2014).

It is recognized that PDMP not only inhibits GlcCerS, but also elevates the level of the cell ceramide (Biebrich *et al.*, 1999). However, the latter effect is not only concentration- but also cell-type- dependent. Importantly for our studies, di Bartolomeo and Spinedi (2001) did not notice ceramide accumulation in HepG2 cells cultured with PDMP at the concentrations similar to used by us. In collaboration with researchers from another laboratory, we confirmed this observation (unpublished).

D-EtDO-P4 at 1 µM caused the highest decline in GM3 content, a non-significant increase in IR autophosphorylation and 223.0% (181.4–375.4%, n=9), significant rise in the phosphorylated Akt1 level upon insulin stimulation (Fig. 2). The simplest, though speculative, explanation for this observation would be the possibility that GSLs depletion could modify membrane microdomains in such a manner that not only IR autophosphorylation but, independently, also Akt1 phosphorylation or steps leading to it are affected.

The involvement of GM3 in insulin signaling in adipocytes is well documented and proven (Inokuchi, 2014). However, the results of Vainio *et al.*, Sasaki *et al.*, as well as our observations indicate that the effects of GSLs depletion on insulin signaling may not be limited to the GM3-regulated IR and caveolin interactions (Vainio *et al.*, 2002; Sasaki *et al.*, 2003).

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