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Effect of U18666a on Beta-Glucosidase, Sphingomyelinase, and Beta-Galactosidase Activities in Astrocytes of Young Rats

Daniela Copetti Santos · Cristina da Silva Garcia · Carla Vieira de Andrade · Vanessa Vitcoski Daitx · Vitória da Costa Moraes · Francieli Rohden · Janice Carneiro Coelho

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Abstract Niemann-Pick type C disease (NPC) is a neurodegenerative genetic disorder caused by accumulation of lipids, especially cholesterol, in the perinuclear space. U18666A is a cholesterol transport-inhibiting agent, being used to mimic NPC, mainly in fibroblasts. The objective of this study was to observe the effect of the drug U18666A, which causes the accumulation of cholesterol in the cytoplasm of astrocytes from newborn rats, on some lysosomal hydrolase activities. Filipin staining and fluorescence microscopy, through CellM software, were used for visualization and quantification of cholesterol. The dose of U18666A that provided the greatest accumulation of cholesterol was that of 0.25 μg/mL in incubation for 48 h. Primary rat astrocytes incubated with the drug (NPC) showed a significantly higher amount of cholesterol than those without U18666A (controls). The measurement of activity of enzymes sphingomyelinase and beta-glucosidase in astrocytes of rats with NPC was significantly lower than that of control astrocytes, which is consistent with the disease in humans. The activity of the enzyme beta-galactosidase showed no significant difference between both groups. We concluded that U18666A appears to be an excellent intracellular cholesterol transport-inhibiting agent affecting some metabolic pathways in astrocytes of young rats, which mimics NPC in these animals. Just like the change in the activity of lysosomal enzymes has been demonstrated, other biochemical parameters of the cell can be tested with this animal model, thus contributing to a better understanding of the disease.

Keywords Niemann-Pick type C disease · Astrocytes · U18666A · Cholesterol · Lysosomal enzymes

D. C. Santos (✉) · C. da Silva Garcia · C. V. de Andrade · V. V. Daitx · V. da Costa Moraes · F. Rohden · J. C. Coelho
Lysosomal Storage Diseases Laboratory, Biochemistry Department, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
e-mail: daniela-santos@saude.rs.gov.br

J. C. Coelho
e-mail: janice.coelho@ufrgs.br

J. C. Coelho
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 anexo, Porto Alegre, RS 90035-003, Brazil

Introduction

Niemann-Pick type C disease (NPC) is a rare lysosomal storage disorder, estimated to be in 1 case per 150,000 live births (Meikle et al. 1997).

The disease was described as having a sub acute nervous system involvement, with moderate/slower course and a mild visceral storage; however, later work led to a reclassification of NPC as a cellular lipid trafficking disorder, involving more specifically endocytosed cholesterol (Pentchev et al. 1994). In NPC disease, cells fail to esterify exogenously added cholesterol. This disorder is characterized by unique abnormalities of intracellular transport of endocytosed cholesterol with accumulation of unesterified cholesterol in endosomal/lysosomal compartment and the Golgi complex (Ikonen and Holtta-Vuori 2004; Vanier 2010). Besides cholesterol sequestration, NPC cells can also accumulate other lipids: sphingomyelin, phospholipids, and glycosphingolipids accumulated in the liver and spleen (Patterson et al. 2001), and glucosylceramide, lactosylceramide, and gangliosides GM2 and GM3 accumulated in the brain of affected individuals (Zervas et al.

2001). NPC is related to a progressive neurodegenerative phenotype and in most cases is fatal (Patterson et al. 2001).

This disease has an autosomal recessive inheritance and is caused by mutations in genes, NPC1 (in 95 % of the cases) and NPC2 (in approximately 4 % of the cases) (Vanier 2010) located on chromosome 18. Mutations in NPC1 and NPC2 genes cause severe imbalance of intracellular lipid transport, leading to accumulation of unesterified cholesterol in perinuclear lysosomes (Liscum and Sturley 2004). The NPC1 and NPC2 proteins both bind cholesterol. NPC1 binds cholesterol with high affinity in a 1:1 molar ratio and NPC2 rapidly transfers cholesterol, but not glycosphingolipids, ceramide, phospholipids, or fatty acids, between phospholipid liposomes or membranes in vitro (Wang et al. 2010).

NPC clinical manifestations are heterogeneous: hepatosplenomegaly, prolonged neonatal jaundice, respiratory impairment, and secondary progressive neurological disorder. Clinical manifestations start from infancy to childhood, but occur most frequently during late childhood (Vanier 2010).

Similar NPC phenotype can be induced through numerous approaches and various drugs like steroids and hydrophobic amines which have been considered to cause a cholesterol traffic imbalance similar to NPC (Lange et al. 2000; Lloyd-Evans et al. 2008). Other proteins, like CFTR (White et al. 2004), GULP (Kiss et al. 2006), Rab11 (Holtta-Vuori et al. 2002), PMD (Simons et al. 2002), CREB (Lemberg et al. 2008), MLN64 (Zhang et al. 2002), and VPS4 (Bishop and Woodman 2000), not structurally related to either NPC1 or NPC2, also produced a storage of cholesterol creating a NPC-like phenotype. On the other hand, other works demonstrated that Rab proteins 7, 8, and 9 correct the cholesterol deposition in NPC human fibroblasts (Linder et al. 2007).

The most effective and specific hydrophobic amine used to create a NPC-like phenotype is 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A), which contains a hydrophobic ring structure associated with a branched lateral structure and an amino group that inhibits the transport of intracellular cholesterol. Described 30 years ago, it inhibits the enzyme desmosterol reductase (Cenedella and Bierkamper 1979) responsible for reduction of desmosterol to cholesterol in cholesterol biosynthesis, and it also blocks the transport of LDL-cholesterol from lysosomes to the endoplasmic reticulum (Eisele et al. 1997). U18666A lowers the amount of cholesterol that reaches the endoplasmic reticulum and prevents the movement of cholesterol between lysosomal compartments and the plasma membrane, and from the plasma membrane to other intracellular compartments (Lange and Steck 1994). It can also act on the activity or synthesis of other proteins or lipids that facilitate cholesterol movement and change the cellular distribution of NPC1 protein.

The compound U18666A has been used to mimic NPC phenotype, because the cholesterol accumulated in the intracellular space in cells treated with U18666A is similar to the one observed in cells of patients with NPC (Vanier 2010; Wang et al. 2010; Neufeld et al. 1999; Lange et al. 2002). These studies on the blockade in cholesterol transport by U18666A and increased levels of cholesterol in cell cytoplasm were conducted on fibroblasts from humans (Lange et al. 2002), skunks (Jurgelski et al. 1973), newborn rats (Bierkamper and Cenedella 1978), and NPC^{-}/$^{-}$ mice (Sáez et al. 2013). The use of this compound to mimic NPC in astrocytes from wild rats, not knockout rat or mice, has not yet been reported, as well as the effect of cholesterol accumulation in astrocytes produced by U18666A on lysosomal hydrolase activities.

Therefore, the purpose of this study was to observe the effect of the drug U18666A, which causes the accumulation of cholesterol in the cytoplasm of astrocytes from newborn rats, in order to show this accumulation of cholesterol in the cytoplasm and the effect of this drug on the some lysosomal hydrolase activities.

Materials and Methods

Animals

Wistar rats (0–3 days) from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS had free access to water and a 20 % (w/w) protein commercial chow. They were kept in a room with a 12:12 h light/dark cycle and temperature of 22 ± 1 °C. The "Principles of Laboratory Animal Care" (NIH publication n°85-2, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre.

Cortical Astrocyte Culture

The rats were euthanized by decapitation and the cerebral cortex was removed. It was homogenized with CMF-BSS buffer and placed in a 24 well-plate in DMEN—Dulbecco's Modified Eagle Medium (GIBCO®), supplemented with 10 % Fetal Bovine Serum (SBF) (Invitrogen). After the cells reached confluence in culture, the medium was changed 4 h after the total incubation time was completed, every four days.

The "multiwell" plates used for the development of cultures were previously incubated with a substrate of poly-L-lysine (Sigma).

Experiments to Obtain the Time Curve with the use of the Drug U18666A

In these experiments, the cultures were incubated with U18666A at doses of 0.1; 0.25; 0.5; 1, and 2 μg/mL in periods of 24, 48, and 72 h, in order to determine the optimal dose and time of incubation of the drug in astrocytes so that cholesterol accumulation is apparent.

Filipin Staining

The technique of Kruth et al. (1986) was used for the observation of the amount of cholesterol accumulated after addition of the drug. 450 μL of medium DMEN supplemented with 5 % of lipoprotein deficient serum (LPDS) was added to each well of the culture plate, according to the time set for the dose curve above. 0.73 μg/100 μL of low-density lipoprotein (LDL) was added and subsequently the plates were again incubated for another 24 h in CO_2 incubator at 37 °C. After this period, in the dark, the cells were washed twice with 300 μL of pre-warmed PBS and the medium removed. Then the cells were fixed for 45 min with 300 μL of phosphate buffered formalin 10 % and pH 7.2 in dark room. The fixative was removed and the cells washed with PBS buffer and 0.9 % saline. Filipin staining (0.01 % in PBS) (Sigma) was used for 45 min for the staining of coverslips. Then the cells were washed with 0.9 % saline. For microscopic analysis, a 358 nm excitation and 461 nm emission filter was used, for an exhibition of 64 ms.

Quantification of Fluorescence of Filipin Staining

All slides were examined and photographed by NIS-Element AR 3.10 in Nikon fluorescence microscope (Nikon Instruments, Melville, NY). The quantification of the fluorescence of the images was analyzed for the number of pixels, using CellM Olympus software.

Cholesterol Dosage

Astrocyte intracellular cholesterol was determined by fluorometric technique using the commercial kit Amplex Red Cholesterol (Molecular Probes) according to the manufacturer's instructions. Thus, a four-point standard curve, and a H_2O_2 positive control were used, as well as the samples that were pipetted into a black 96-well fluorometric plate and flat bottom (OptiPlate^{TM}-96F, Perkin Elmer).

Fifty microliters of reaction solution W2 (300 μL Amplex Red Reagent + 2 U/mL cholesterol oxidase + 0.2 U/mL cholesterol esterase) was added to the curve and samples in all wells. The plate was kept for 30 min at 37 °C, in shaking incubator and dry heat. Afterwards, fluorescence was measured in plate reader (Spectramax M5-Molecular Devices) at 544 nm excitation and 590 nm emission. The value obtained was corrected by measuring protein levels (Lowry et al. 1951) found in the astrocytes tested and expressed in μg of cholesterol/mg protein.

Measurement of Acid Sphingomyelinase Activity (ASM)

A technique adapted from Pentchev et al. (1980) was used for measuring ASM activity. Thus, ^{14}C-10 μCi/200 μL sphingomyelin radioactive substrate was used. After incubation of the sample in substrate during 4 h while stirring at 37 °C in dry incubator (Marconi MA-127), some procedures were performed and titration of radioactivity was carried out in β Perkin Elmer's scintillation system (Liquid Scintillation Analyzer TriCarb® 2800 TR). The results were expressed in nmol/h/mg of protein.

Measurement of Beta-Galactosidase (GLB) Enzyme Activity

Miniaturized technique of Goldim et al. (2012) adapted from Suzuki and Suzuki (1970) was used for measuring the activity of beta-galactosidase (GLB): 10–20 μL of the sample diluted in 10 μL of NaCl 0.2 M elution buffer was placed in a fluorometric plate, and 20 μL of the substrate 4-methylumbelliferyl β-D-galactoside 1.33 mM. After incubation at 37 °C for 1 h, fluorescence was read in plate reader (SpectraMax M5-Molecular Devices) at 365 nm excitation and 450 nm emission. The results were expressed in nmol/h/mg of protein.

Measurement of Beta-Glucosidase (GLB) Enzyme Activity

The enzymatic activity of β-glucosidase was measured according to Goldim et al. (2012) adapted from Petters et al. (1976). 5 μL of the sample diluted in 12.5 μL of phosphate citrate buffer 0.54 M, pH 5.5 were placed in a fluorometric plate. Then 25 μL of the substrate 4-methylumbelliferyl β-D-glycoside 10 mmol/L (substrate) with 50 mmol/L of sodium taurocholate in distilled water. After incubation at 37 °C for 1 h, fluorescence was read in a plate reader (SpectraMax M5-Molecular Devices) at 365 nm excitation and 450 nm emission. The results were expressed in nmol/h/mg of protein.

Statistical Analysis

Data were compared by two-way analysis of variance (ANOVA) followed by Duncan's test when F value was

significant ($p < 0.05$). All analyzes were performed using the *Statistical Package for the Social Sciences* (SPSS) in a compatible PC-computer.

Results

Determination of U18666A Dose for the Experimental Model

According to Fig. 1, we can see that the dose of 0.25 µg/mL of U18666A in astrocytes from rats was the most appropriate regarding the increase in cholesterol compared to astrocytes from rats not treated with the referred substance.

In Fig. 1, it can also be seen that incubation of drug during 48 h was more appropriate to ensure this significant increase in cholesterol level.

Filipin staining in astrocytes indicating increase in cholesterol can be visualized in Fig. 2.

Presence of Cholesterol in Astrocytes from Rats with and Without Addition of U18666A

Control cells did showed no fluorescence in the cytoplasm, indicating no accumulation of cholesterol (Fig. 2a). In turn, cells to which U18666A (0.25 µg/mL) was added for 48 h showed an increased amount of cholesterol in the cytoplasmic space, which is indicated by the intense perinuclear fluorescence (Fig. 2b). After quantification by fluorescence using CellM software, the differences were confirmed by one-way ANOVA where it was found that the amount of cholesterol in control cells was significantly lower than that in the astrocytes with the drug ($p < 0.001$).

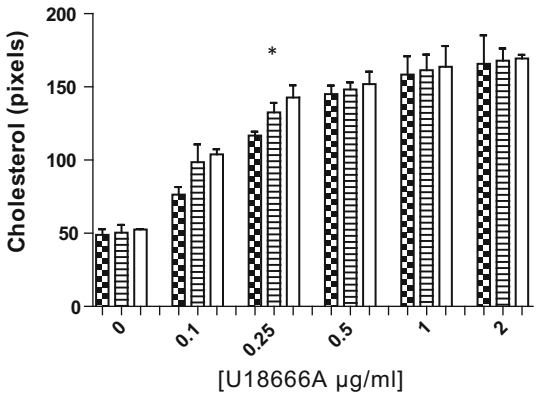

Fig. 1 Cholesterol concentration depending on the dose and time of incubation of U18666A in astrocytes from rats. (▩) 24 h; (▤) 48 h; (▥) 72 h of incubation. Cholesterol was measured using CellM Olympus software and was expressed as pixels of fluorescence. Data are expressed as mean ± standard deviation. (*) statistically significant difference from the group not treated with the drug (control) and the group with U18666A 0.25 µg/mL ($p < 0.0001$)

Amount of Intracellular Cholesterol in Astrocytes From Rats With and Without the Addition of U18666A

In an assay for quantification of intralysosomal unesterified cholesterol accumulation using Amplex Red kit, a significant difference ($p < 0.05$) was observed between control samples and the astrocytes incubated with U18666A (Fig. 3). The cholesterol level of astrocytes that received the drug (54.2 µg of cholesterol/mg of protein) was two times higher than that in control astrocytes (25.7 µg of cholesterol/mg of protein).

A significant correlation was obtained when we compared the Filipin quantitation method of cholesterol determination using CellM software and the measurement of cholesterol using the Amplex Red kit (controls: $r = 0.9973$, $p < 0.04$ and astrocytes incubated with U18666A: $r = 0.99$, $p < 0.0051$.

Measurement of the Activity of Lysosomal Hydrolases in Astrocytes From Rats that Underwent Treatment with U18666A in the NPC Model

Sphingomyelinase (ASM)

When ASM enzyme activity in astrocytes (Fig. 4) was compared between the control samples and the samples that mimic NPC, a significant difference was observed ($p < 0.0209$), that is, astrocytes in contact with the drug showed an enzymatic activity (42.50 ± 3.21) significantly lower than that of control cells (65.68 ± 7.83).

β-Glucosidase (GBA)

The activity of enzyme GBA in control astrocytes was significantly greater (701.8 ± 78.24) than that of NPC astrocytes (366.9 ± 120.2) ($p < 0.0369$). These results can be seen in Fig. 5.

β-Galactosidase (GLB)

According to the analysis of enzyme GLB activity in astrocytes from control samples and samples positive for the accumulation of cholesterol, no significant difference ($p > 0.1035$) was observed between these groups (137.1 ± 18.0; 187.5 ± 15.70, respectively), although according to Fig. 6, the enzymatic activity in NPC astrocytes may seem greater than that of the controls.

Discussion

Cholesterol is an essential component of the Central Nervous System, and growing evidence suggests an

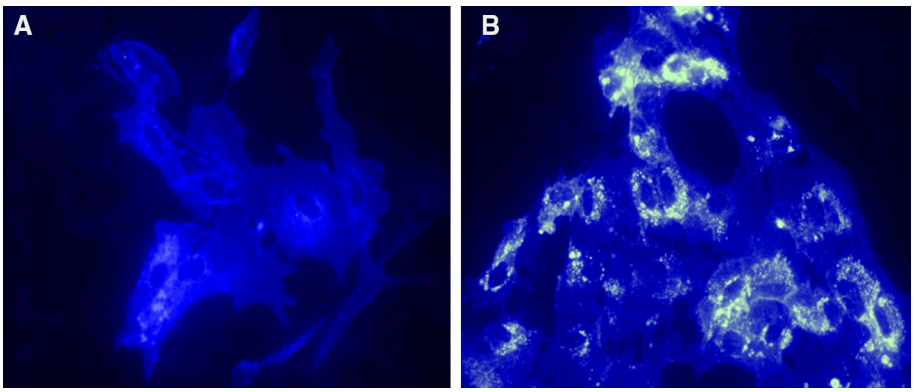

Fig. 2 Astrocytes from rats incubated without (**a**) and with (**b**) U18666A 0.25 μg/mL for 48 h. Astrocytes are visualized after Filipin staining technique

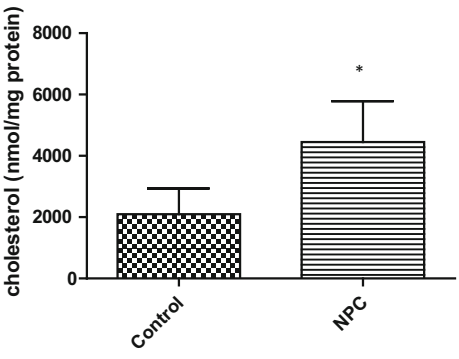

Fig. 3 Amount of intracellular cholesterol (μg/mg of protein) in astrocytes measured with a laboratory KitAmplex® Red Cholesterol. Astrocytes from rats were incubated without (control) and with U18666A 0.25 μg/mL for 48 h. Data are expressed as mean ± standard deviation. (*) statistically significant difference from control group ($p < 0.05$)

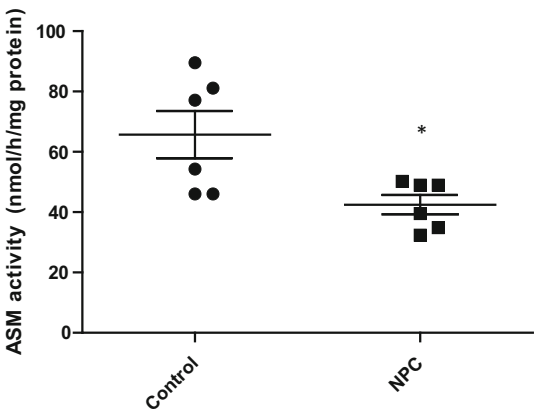

Fig. 4 Activity of enzyme ASM (nmol/h/mg of protein) in astrocytes from rats incubated without (control) and with U18666A 0.25 μg/mL for 48 h. Data are expressed as mean ± standard deviation. (*) statistically significant difference from the control group ($p < 0.02$)

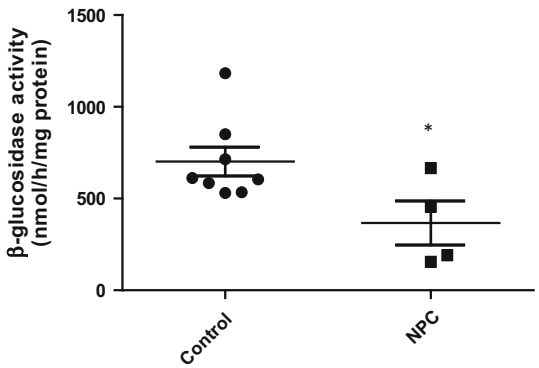

Fig. 5 Activity of enzyme GBA (nmol/h/mg of protein) in astrocytes from rats incubated without (control) and with U18666A 0.25 μg/mL for 48 h. Data are expressed as mean ± standard deviation. (*) statistically significant difference from the control group ($p < 0.03$)

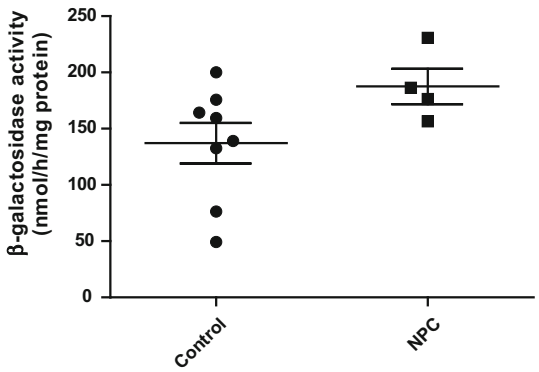

Fig. 6 Activity of enzyme GLB (nmol/h/mg of protein) in astrocytes from rats incubated without (control) and with U18666A 0.25 μg/mL for 48 h. Data are expressed as mean ± standard deviation

association between dysfunction in the brain cholesterol metabolism and the onset of neurodegenerative disorders (Dietschy and Turley 2004). Some studies suggest that in vivo, cholesterol in the Central Nervous System is derived from astrocytes and then sent to mature neurons (Nieweg et al. 2009).

The main purpose of this study was to establish a Niemann-Pick type C, disease with drug U18666A, in cultured astrocytes from neonatal rats (in vitro study), and thereafter

perform biochemical analyzes such as checking the activity of the main lysosomal enzymes associated to this disorder.

The accumulation of cholesterol was inducted in cultured astrocytes from rats aged 0–3 days through the addition of U18666A. This drug is widely used to mimic NPC disease in animal models, though in other cells (Liscum and Faust 1989, Lange et al. 1998; Underwood et al. 1996) and in the increase of cholesterol levels in NPC$^{-/-}$ mice (Sáez et al. 2013).

In the present study, we demonstrated that the quantification of fluorescence of the cholesterol levels observed in Filipin staining in the studied groups through the number of pixels in the image (CellM software), was a practical and not subjective method. This technique showed that there is a significant difference between the intralysosomal unesterified cholesterol accumulation in control cells and those containing the drug. These analyzes were one at different times and doses, and we obtained the appropriate dose and time (0.25 μg/mL in a 48-h period). After this period and with a higher dose, there is no marked accumulation of cholesterol in these cells. Moreover, according to Cheung et al. (2004), higher doses of U18666A increase the probability that the cells will suffer apoptosis. These authors used dose and incubation time similar to the ones in the present study, and there was no problem of cell death.

In the assay for quantification of intracellular cholesterol using the kitAmplex® Red Cholesterol (Molecular Probe), in astrocytes, it was demonstrated that astrocytes treated with U18666A had greater accumulation of intralysosomal unesterified cholesterol than control cells (not treated with the drug). The average cholesterol levels observed in control cells was 25.7 μg of cholesterol/mg of protein, corresponding to slightly less than twice the cholesterol levels accumulated in the cells incubated with U18666A (54.2 μg of cholesterol/mg of protein). This kit, Amplex® Red Cholesterol (Molecular Probe) was used by Tängemo et al. (2011) to quantify free cholesterol and intralysosomal unesterified cholesterol contained in fibroblasts of NPC patients with great results.

The cholesterol levels calculated with CellM software corresponded to the cholesterol levels observed in quantification using the kit Amplex® Red Cholesterol (Molecular Probe), which demonstrates that the use of an appropriate software to quantify fluorescence of Filipin test provides safe quantification of cholesterol levels, which suggests the test has the potential to support diagnosis of NPC disease based on cultured cells.

Lysosomal Storage Diseases, which include NPC, are understood as a consequence of mutations that resulted in reduced synthesis of the lysosomal enzymes, leading to accumulation of lipids within this cell organelle. In our study, we measured the activity of some lysosomal enzymes associated with degradation of lipids: sphingomyelinase, β-glucosidase, and β-galactosidase. The activities of these enzymes are changed in human NPC (Vanier et al. 1991).

We noticed that the activity of ASM in cells with higher cholesterol accumulation was reduced. These results were compatible with those where reduced ASM activity was observed in culture of fibroblasts of patients with NPC (Vanier et al. 1991; Pentchev et al. 1987; Vanier et al. 1988). The activity of enzyme GBA in astrocytes from rats was compared between control samples and samples positive for NPC, and a significant difference was found between the two groups. Samples of NPC astrocytes showed a decrease in GBA levels. This had also been observed in previous studies in fibroblasts from patients with NPC (Vanier et al. 1980; Besley and Moss 1983).

Regarding the activity of enzyme, GLB no significant difference was observed between the groups. However, in Fig. 6, we noticed that astrocytes from rats with NPC appear to have higher enzyme activity than control samples. No significant increase in the activity of GLB was described by Wenger et al. (1974) in fibroblasts from patients with Niemann-Pick type C disease.

By analyzing the changes occurred in lysosomal enzymes in our animal model and comparing them with studies carried out by other authors in fibroblasts from humans with Niemann-Pick type C disease, we once again confirm that our model is of great relevance and reliability for future research with cell cultures of astrocytes.

We concluded that U18666A appears to be an excellent inhibitor of the cholesterol transport in astrocytes of young rats and is able to mimic Niemann-Pick type C disease in these animals. This drug also alters the activity of three important enzymes in the degradation of lipids metabolism. Just like the change in the activity of lysosomal enzymes has been demonstrated, other biochemical parameters of the cell can be tested with this animal model, thus contributing to a better understanding of the disease.

References

Besley GT, Moss SE (1983) Studies on sphingomyelinase and beta-glucosidase activities in Niemann-Pick disease variants. Phosphodiesterase activities measured with natural and artificial substrates. Biochim Biophys Acta 752:54–64

Bierkamper GG, Cenedella RJ (1978) Induction of chronic epileptic form activity in the rat by an inhibitor of cholesterol synthesis, U18666A. Brain Res 150:343–351

Bishop N, Woodman P (2000) ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. Mol Biol Cell 11:227–239

Cenedella RJ, Bierkamper GG (1979) Mechanism of cataract production by 3-beta(2-diethyl-aminoethoxy)androst-5-en-17-one hydrochloride, U18666A: an inhibitor of cholesterol biosynthesis. Exp Eye Res 28:673–688

Cheung NS, Koh CHV, Bay BH, Qi RZ, Choy MS, Li QT, Wong KP, Whiterman M (2004) Chronic exposure to U18666A induces apoptosis in cultured murine cortical neurons. Biochem Biophys Res Commun 315:408–417

Dietschy JM, Turley SD (2004) Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. J Lipid Res 45:1375–1397

Eisele B, Budzinski R, Muller P, Maier R, Mark M (1997) Effects of a novel 2,3 oxi-dosqualenecyclase inhibitor on cholesterol biosynthesis and lipid metabolism in vivo. J Lipid Res 38:564–575

Goldim M, CdaS Garcia, de Castilhos CD, Daitx VV, Mezzalira J, Breier AC, Cé J, Mello A, Andrade CV, Sartori N, Coelho JC (2012) Screening of high-risk Gaucher disease patients in Brazil using miniaturized dried blood spots and leukocyte techniques. Gene 508:197–198

Holtta-Vuori M, Tanhuanpaa K, Mobius W, Somerharju P, Ikonen E (2002) Modulation of cellular cholesterol transport and homeostasis by Rab11. Mol Biol Cell 13:3107–3122

Ikonen E, Holtta-Vuori M (2004) Cellular pathology of Niemann-Pick type C disease. Semin Cell Dev Biol 15:445–454

Jurgelski W Jr, Hudson PM, Vogel FS (1973) Induction of a chronic somatosensory epilepsy in the opossum (Didelphisvirginiana Kerr) with an inhibitor of cholesterol biosynthesis. Brain Res 64:466–471

Kiss RS, Ma Z, Nakada-Tsukui K, Brugnera E, Vassiliou G, McBride HM, Ravichandran KS, Marcel YL (2006) The lipoprotein receptor-related protein-1 (LRP) adapter protein GULP mediates trafficking of the LRP ligand prosaposin, leading to sphingolipid and free cholesterol accumulation in late endosomes and impaired efflux. J Biol Chem 281:12081–12092

Kruth HS, Comly ME, Butler JD (1986) Type C Niemann - Pick disease. Abnormal metabolism of low density lipoprotein in homozygous fibroblasts. J Biol Che 261:16769–16774

Lange Y, Steck TL (1994) Mechanism of red blood cell acanthocytosis and echinocytosisin vivo. J Membrane Biol 77:153–159

Lange Y, Ye J, Steck TL (1998) Circulation of cholesterol between lysosomes and the plasma membrane. J Biol Chem 273:18915–18922

Lange Y, Ye J, Rigney M, Steck T (2000) Cholesterol movement in Niemann-Pick type C cells and in cells treated with amphiphiles. J Biol Chem 275:17468–17475

Lange Y, Ye J, Rigney M, Steck TL (2002) Dynamics of lysosomal cholesterol in Niemann-Pick type C and normal human fibroblasts. J Lipid Res 43:198–204

Lemberg T, Parktina JR, Chai M, Schütz G, Engblom D (2008) CREB has a context dependent role in activity-regulated transcription and maintains neuronal cholesterol homeostasis. FASEB J 22:2872–2879

Linder MD, Uronen RL, Holtta-Vuori M, van der Sluijs P, Peränen J, Ikonen E (2007) Rab8-dependent recycling promotes endosomal cholesterol removal in normal and sphingolipidosis cells. Mol Biol Cell 18:47–56

Liscum L, Faust JR (1989) The intracellular transport of low density lipoprotein- derived cholesterol is inhibited in CHO cells cultured with 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one. J Biol Chem 264:11796–11806

Liscum L, Sturley SL (2004) Intracellular trafficking of Niemann-Pick C proteins 1 and 2: obligate components of subcellular lipid transport. Biochim Biophys Acta 1685:22–27

Lloyd-Evans E, Morgan AJ, He X, Smith DA, Elliot-Smith E, Sillence DJ, Churchill GC, Schuchman EH, Platt FM (2008) Niemann-Pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. Nat Med 14:1247–1255

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Meikle PJ, Brooks DA, Ravenscroft EM, Yan M, Williams RE, Jaumzems AE, Chataway TK, Karageorgos LE, Davey RC, Boulter CD, Carlssson SR, Hopwood JJ (1997) Diagnosis of lysosomal storage disorders: evaluation of lysosome-associated membrane protein LAMP-1 as a diagnostic marker. Clin Chem 43:1325–1335

Neufeld EB, Wastney M, Patel S, Suresh S, Cooney AM, Dwyer NK, Roff CF, Ohno K, Morris JA, Carstea AD, Incardona JP, Strauss JF 3rd, Vanier MT, Patterson MC, Brady RO, Pentchev PG, Blanchette- Mackie EJ (1999) The Niemann- Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. J Biol Chem 274:9627–9635

Nieweg K, Schaller H, Pfrieger FW (2009) Marked Differences in cholesterol syn- thesis between neurons and glial cells from postnatal rats. J Neurochem 109:125–134

Patterson MC, Vanier MT, Suzuki K, Morris JA, Carstea ED, Neufeld EB, Blanchette-Mackie JE, Pentchev PG (2001) Niemann- Pick disease type C: a lipid trafficking disorder. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B (eds) The metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, pp 3611–3634

Pentchev PG, Boothe AD, Gal AE, Omodeo-Sale F, Fouks J, Brady RO (1980) A lysosomal storage disorder in mice characterized by the accumulation of several sphingolipids. In: 2nd International Symposium on Enzyme Therapy in Genetic Diseases. Edited by Desnick R. J., Allan R. Liss, Incorporated Scientific, Medical and Scholarly Publications, New York, 225–230

Pentchev PG, Comly ME, Kruth HS, Tokoro T, Butler J, Sokol J, Filling-Katz M, Quirk JM, Marshall DC, Patel S, Vanier MT, Brady RO (1987) Group C Niemann-Pick disease: faulty regulation of low-density lipoprotein uptake and cholesterol storage in cultured fibroblasts. FASED 1:40–45

Pentchev PG, Brady RO, Blanchette-Mackie EJ, Vanier MT, Carstea ED, Parker CC, Goldin E, Roff CF (1994) The Niemann-Pick C lesion and its relationship to the intracellular distribution and utilization of LDL cholesterol. Biochim Biophys Acta 1225:235–243

Petters SP, Coyle P, Glew RH (1976) Differentation of beta-glucocerebrosidase from beta-glucosidase in human tissues using sodium taurocholate. Arch Biochem Biophys 175:569–571

Sáez JP, Orellana JA, Vega-Riveros N, Figueroa VA, Hernández DE, Castro JF, Klein AD, Jiang JX, Zanlungo S, Sáez JC (2013) Disruption in connexin- based communication is associated with intracellular Ca^{2+} signal alterations in astrocytes from Niemann-Pick type C mice. PLoS One 15:1–12

Simons M, Kramer EM, Macchi P, Rathke-Harlieb S, Trotter J, Nave KA, Schulz JB (2002) Overexpression of the myelin proteolipid protein leads to accumulation of cholesterol and proteolipid protein in endosomes/lysossomes: implications for Pelizaeus-Merzbacher disease. J Cell Biol 157:327–336

Suzuki K, Suzuki Y (1970) Globoid cell leucodystrophy (Krabbe's disease): deficiency of galactocerebroside beta-galactosidase. Proc Nat Acad Sci USA 66:302–309

Tängerno C, Weber D, Theiss S, Mengel E, Runz H (2011) Niemann-Pick Type C disease: characterizing lipid levels in patients with variant lysosomal choles terol storage. J Lipid Res 52:813–825

Underwood KW, Andemariam B, McWilliams GL, Liscum L (1996) Quantitative analyses of hydrophobic amine inhibition of intracellular cholesterol transport. J Lipid Res 37:1556–1568

Vanier MT (2010) Niemann-Pick disease type C. Orphanet J Rare Dis 5:16

Vanier MT, Revol A, Fichet M (1980) Sphingomyelinase activities of various human tissues in control subjects and in Niemann-Pick disease: development and evaluation of a microprocedure. Clin Chim Acta 106:257–267

Vanier MT, Wenger DA, Comly ME, Rousson R, Brady RO, Pentchev PG (1988) Niemann- Pick disease group C: clinical variability and diagnosis based on defective cholesterol esterification. A collaborative study on 70 patients. Clin Genet 33:331–348

Vanier MT, Rodriguez-Lafrasse C, Rousson R, Gazzah N, Juge MC, Pentchev PG, Revol A, Louisot P (1991) Type C Niemann- Pick disease: spectrum of phenotypic variation in disruption of intracellular LDL- derived cholesterol processing. Biochim Biophys Acta 1096:328–337

Wang ML, Motamed M, Infante RE, Abi- Mosleh L, Kwon HJ, Brown MS, Golds-tein JL (2010) Identification of Surface Residues on Niemann-Pick C2 Essential for Hydrophobic Handoff of Cholesterol to NPC1 in Lysosomes. Cell Metab 12:166–173

Wenger DA, Sattler M, Clark C, McKelvey H (1974) An improved method for the identification of patients and carriers of Krabbe's disease. Clin Chim Acta 56:199–206

White NM, Corey DA, Kelley TJ (2004) Mechanistic similarities between cultured cell models of cystic fibrosis and Niemann-Pick type C. Am J Respir Cell Mol Biol 31:538–543

Zervas M, Somers KL, Thrall MA, Walkley SU (2001) Critical role for glycosphingolipids in Niemann-Pick disease type C. Curr Biol 11:1283–1287

Zhang M, Liu P, Dwyer NK, Christenson LK, Fujimoto T, Martinez F, Comly M, Hanover JA, Blanchette-Mackie EJ, Strauss JF 3rd (2002) MLN64 mediates mobilization of lysosomal cholesterol to steroidogenic mitochondria. J Biol Chem 277:33300–33310