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N-glycans Profiling in Pilocarpine Induced Status Epilepticus in Immature Rats

Original Paper

Kapoor S.[1,3]✉, Nemcovic M.[2], Folbergrova J.[3], Kala D.[3], Svoboda J.[3], Otahal J.[3], Brnoliakova Z.[1]

[1]*Centre of Experimental Medicine, Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovak Republic*
[2]*Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic*
[3]*Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic*

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Abstract Status epilepticus (SE) is a common neurological emergency in children and a well-known epileptogenic insult. Neonates are extremely susceptible to seizures in the neonatal period due to the higher vulnerability. Neonatal SE is associated with significant mortality and morbidity. There is an evident need for attention on neonatal SE in research due to the incredibly limited diagnostic and treatment options in current neonatology, and its serious long-term consequences. The aim of the present study was to characterize the glycoprofiles in the pilocarpine-induced SE model in immature rats to assess the overall N-glycans composition. To induce lithium-pilocarpine (Li-Pilo) SE male Wistar rat pups were pretreated with lithium chloride (127 mg/kg, n=11) on the 11th postnatal day. After 24 hours, the lithium pre-treated pups were administered either with pilocarpine intraperitoneally (i.p.) (35 kg/g, n=6) or saline (n=5) in the control group (Control). On the 19th postnatal day, serum was collected and the analytical procedures were done by mass spectrometry (MS) analytics on matrix-assisted laser desorption/ionization in combination with a time-of-flight detector (MALDI-TOF/MS). Analyzed data were processed by FlexAnalysis (Bruker Daltonics) and GlycoWorkbench software. There were 21 N-glycans that were identified, appointed, and sorted with special emphasis on their structure. We have demonstrated the significant changes in terms of N-glycans sialylation in Li-Pilo compared to the Control. We also observed some other remodelation trends in different portions of relative intenstities of N-glycan clusters according to their glycan type. Our preliminary findings have laid the foundation for additional investigation into glycosylation alterations in the SE in immature rats.

Keywords *N-glycans – MALDI-TOF/MS – Status epilepticus – Sialic acid – New-born rats*

INTRODUCTION

Status epilepticus (SE) is a critical neurological condition that may lead to persistent seizures in newborns due to the immature brain's proclivity for seizure activity (Farmania et al., 2020). The most common aetiology of SE in neonates is hypoxic-ischemic encephalopathy (HIE), which accounts for around 60% of seizures during the neonatal period (Lawrence and Inder, 2010). SE is linked to brain damage and elevates risk of developing epilepsy, as well as cognitive and functional impairments (Kubová et al., 2001). Due to the significant long-term consequences, it is a socioeconomic burden (Allers et al., 2015; Penberthy et al., 2005). There is an urgent need for attention on neonatal SE in research due to the obviously limited diagnostic and therapeutic options available in modern neonatology (Farmania et al., 2020). Glycosylation is the enzymatic attachment of oligosaccharides to proteins and lipids. Aberrant glycosylation is found in a variety of pathophysiological diseases, including cancer, inflammation, autoimmune disease, and aging (Ohtsubo and Marth, 2006). Glycosylation plays an important role in brain development, physiology, and functioning, such as modulating neuronal transmission and regulating synaptic processes. As a result, dysregulated glycosylation can lead to a variety of neurological disorders (Hasan et al., 2021). N-glycosylation impacts glycoproteins in brain development and also has modulatory effects on neural transmission and neural circuit excitability (Scott and Panin, 2014). An N-glycan profile of rat serum is appropriate for pathological and pharmacological studies (Clerc et al., 2016; Gao et al., 2015). The aim of the present study was to assess the overall N-glycans composition from the glycoprofiles in the pilocarpine induced SE model in immature rats.

* *E-mail: sonam.kapoor@savba.sk*

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MATERIAL AND METHODS

In this study, immature male Wistar rat pups (weighing 18 g) were used (N=11). The animals were divided into two groups at random: control (n=5) and Li-Pilo (n=6).

Status epilepticus model

To induce the Li-Pilo SE model, male Wistar rat pups were pre-treated with lithium chloride (127 mg/kg, n=11) on the 11th postnatal day. After 24 hours, the lithium pre-treated pups were administered either with pilocarpine (Li-Pilo) intraperitoneally (i.p.) (35 mg/kg *b.w.*, n=6) or saline (0,9% NaCl, n=5) in the control group (Control). Serum was collected on the 19th postnatal day (Folbergrová et al., 2021), frozen under -80 ˚C, and analysed. The protocol of the experiment was approved by the Animal Care and Use Ethics Committee at the Institute of Physiology, Czech Academy of Sciences.

Analysis of serum N-glycoprofile by MALDI-TOF/ MS

Blood serum of the amount 10 µl was premixed with 40 µl 10 mM Tris, pH 7.5 in 0.1% s*odium* dodecyl sulfate and incubated with dithiothreitol and i*odoacetamide* according to standard protein reduction and alkylation protocols (Shevchenko et al., 2007). To release the N-glycans, serum was incubated with 1 enzyme unit (U) of PNGase F (peptide-N-glycosidase F, Roche) at 37 °C overnight. Isolation of N-glycans was performed by porous graphitic carbon s*olid phase extraction (P*GC SPE, 100 mg Supelclean ENVI-Carb, Supelco) as described previously (Pažitná et al., 2020) by 60% *acetonitrile* in 0.1% *trifluoroacetic acid*. To increase the signal intensities and stabilize the sialic acid, N-glycans were subjected to permethylation (Bua et al., 2021). Permethylated N-glycans were analysed by matrix assisted laser desorption/ionization mass spectrometer in combination with time-of-flight detector (UltrafleXtreme MALDI-TOF/MS, Bruker Daltonics) in reflectron positive ion mode with 20 mg/ml 2,5-d*ihydroxybenzoic* acid in 30% *acetonitrile in* 1 mM sodium hydroxide as the matrix solution. The measurement on MALDI-TOF/MS itself was recorded five times for each analytical sample. Analysed data were processed and interpreted by FlexAnalysis (Bruker Daltonics) and GlycoWorkbench (Ceroni et al., 2008) software. Obtained mass spectra of permethylated N-glycans were compared and evaluated with special focus on their N-glycan type.

Statistics: Data were evaluated using GraphPad Software. Unpaired *t*-test with two tailed analysis was used to evaluate the difference among the experimental groups. The level of $p < 0.05$ was considered statistically significant difference. Data were expressed as means ± SEM.

RESULTS

Alterations in N-glycans were evaluated in pilocarpine induced SE model in new-born's rat sera. The cluster of 21 N-glycans was appointed and sorted with special emphasis on their structural type (Table 1).

Table 1. The list of detected N-glycans sorted according to their structural type.

Glycan type	m/z
High-Man	1579.8; 1783.5; 1988.1; 2192.2; 2396.4
C-Bi	2227.4; 2431.5; 2736.8; 2792.7
C-Bi-Fuc	1836.1; 2040.2; 2605.7; 2966.9
C-Tri	3154.1; 3603.2
C-Tri-Fuc	2081.2; 3328.1; 3777.4
C-Tetra	3964.3
C-Tetra-Fuc	4138.4
Hybrid	2390.4

Explanatory notes: m/z – mass-to-charge ratio, m/z values are experimental [M + Na]$^+$; High-Man – high-mannose type of N-glycan; C-Bi – complex bi-antennary; C-Bi-Fuc – complex bi-antennary with Fucose; C-Tri – complex tri-antennary; C-Tri-Fuc – complex tri-antennary with Fucose; C-Tetra – complex tetra-antennary; C-Tetra-Fuc – complex tetra-antennary with Fucose; Hybrid – hybrid type of N-glycan.

The significant changes between experimental groups (Li-Pilo *vs* Control) were observed in relative intensities of sialylation in N-glycans, as well as some remodelation trends in variations of relative intensities within N-glycan clusters according to their glycan type.

In the Li-Pilo group, we found significantly lower portion of relative intensities of non-sialylated N-glycans (m/z: 1579.8; 1783.5; 1836.1; 1988.1; 2040.2; 2081.2; 2191.2; 2396.4) compared to the Control group (Figure 1a). There were no statistically significant differences within the clusters of mono- (m/z: 2227.4; 2390.4; 2431.5; 2605.7), di- (m/z: 2736.8; 2792.7; 2966.9), and tetra-sialylated (m/z: 3964.3; 4138.4) N-glycans. However, we detected significantly higher level of relative intensities within the tri-sialylated (m/z: 3154.1; 3328.1; 3603.2; 3777.4;) N-glycan cluster in Li-Pilo group compared to the Control group. *The overall relative intensities in tri-sial clusters of N-glycans in Control (29.4%) were augmented by approximately one fifth in Li-Pilo (36.4 %).*

There were no statistically significant changes in N-glycans relative intensities based on their glycan types between Li-Pilo and Control groups, even though there are noticeable remodelation trends in high-mannose, complex-tri-fucose, and hybrid N-glycan types in Li-Pilo compared to the Control (Figure 1b).

DISCUSSION

The purpose of this study was to examine N-glycans changes as the result of metabolic alterations in rodents after

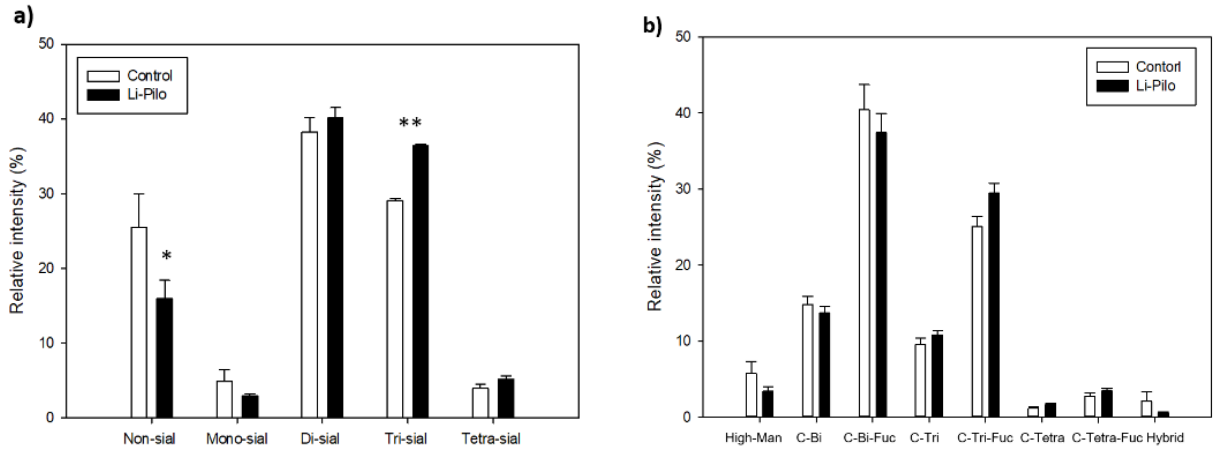

*Figure 1. The relative intensities of N-glycans in the experimental model of lithium pilocarpine (Li-Pilo) induced status epilepticus (SE) in blood sera in immature rats. N-glycans were assorted according to (a) sialylation and non-sialylation; (b) their glycan type. Explanatory notes: High-Man – high-mannose type of N-glycan; C-Bi – complex bi-antennary; C-Bi-Fuc – complex bi-antennary with Fucose; C-Tri – complex tri-antennary; C-Tri-Fuc – complex tri-antennary with Fucose; C-Tetra – complex tetra-antennary; C-Tetra-Fuc – complex tetra-antennary with Fucose; Hybrid – hybrid type of N-glycan. Data were evaluated using GraphPad Software. Unpaired t-test with two tailed analysis was used to evaluate the difference among the experimental groups. The statistically significant difference are marked as * for $p < 0.05$ and ** for $p < 0.01$. Data were expressed as means ± SEM.*

epileptogenic insults, such as Li-Pilo-induced SE. N-glycans are crucial entities for brain functions such as neurogenesis, neurite outgrowth, synapse function, and memory formation (Gaunitz et al., 2021). Severely aberrant glycosylation during early development can lead to protein misfolding, which may play a role in the development of neurological deficits (Coss et al., 2014). Moreover, different neuro-pathological symptoms such as mental retardation, seizures, and epilepsy can be induced by N-glycan alterations in the nervous system (Samal et al., 2020).

Our data indicated that blood sera from rats with Li-Pilo-induced SE revealed a significantly lower portion of relative intensities of non-sialylated N-glycans, and higher level of tri-sial N-glycans in compared to the Control groups. N-glycan clusters based on their different glycan types also revealed some remodeling variations within high-mannose, complex-tri-fucose, and hybrid N-glycan types. This research established the framework for using "brain-type" glycans like sialylation (mono-, di-, and tetra-) as biomarkers or therapeutic targets, as well as the potential for modulating their levels within neurodegenerative disorders (Samal et al., 2020).

Moreover, former preclinical studies suggest that the MALDI-TOF-based glycoprofiling method has previously been used to identify and quantify N-glycan structures in glycomic studies, as well as to detect specific N-glycan biomarkers in plasma or serum of patients with various types of disorders (Fogli et al., 2012).

N-linked glycosylated proteins play a variety of roles in the brain, including electrical gradients and neurotransmission. How alterations in N-linked glycosylation affect and may even induce attributes of neurological disorders in not well understood yet (Conroy et al., 2021). To date, very little is known about the precise neurobiological functions of individual N-glycans (Klarić and Lauc, 2022). N-glycosylation has a multifaceted role at the synapses, as sialylated N-glycan structures are ranked among the most powerful modulators of cell excitability, affecting voltage-gated Na^+ and K^+ channels particularly (Scott and Panin, 2014). According to Yu et al., (2017), they revealed that improperly expressed glycans were associated with mitochondrial dysfunction in ischemic brain injury. Boll et al. (2020), found that sialylation is a key dynamic modification for synaptic depolarization-dependent processes which are involved in synaptic plasticity and memory formation. In another study, Rebelo et al. (2021) showed that mice lacking sialyltransferases lead to motor and cognitive impairments as well as increased dysmyelination, demonstrating the relevance of sialylation.

CONCLUSIONS

In conclusion, in our study, we have demonstrated the significant changes in terms of N-glycans sialylation in Li-Pilo compared to the Control. We also observed some other remodelation trends in different portions of relative intenstities of N-glycan clusters according to their glycan type. Our preliminary findings have laid the foundation for additional investigation into glycosylation alterations in SE in immature rats.

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