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REGULATION OF INSULIN RESISTANCE AND NEUROTRANSMISSION BY PROTEIN PHOSPHORYLATION

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The Examination takes place at the Library of Department of Physiology, Faculty of Medicine, University of Debrecen 15th of May 2023. 11:00 AM

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Introduction

Posttranslational modifications (PTMs) regulate all the cellular functions in living organisms. One of the most important and common PTMs is reversible protein phosphorylation and dephosphorylation. Protein phosphorylation enables cells to respond to numerous extracellular stimuli. Protein phosphorylation is carried out by protein kinases, by attaching the γ-phosphate from mostly adenosine triphosphate (ATP) to amino acids containing hydroxyl groups (serine/Ser, threonine/Thr, tyrosine/Tyr). Some of the proteins can be phosphorylated on multiple sites by different protein kinases, this way cells become capable to integrate different physiological signals. Protein phosphatases are responsible for the reversibility of this modification by catalyzing the hydrolysis of the phospho-group. The number of genes encoding protein kinases is far larger than protein phosphatases, but the significance of these enzymes is widely accepted today. In living, organisms phosphorylation is mainly carried out on Ser/Thr residues. Catalyzing dephosphorylation on these sidechains is mostly related to the PP1 and PP2A enzymes which are part of the phosphoprotein phosphatase (PPP) enzyme family.

PP1c enzymes and myosin phosphatase

All eukaryotic cells express PP1c (protein phosphatase 1 catalytic subunit) enzymes, differences in expression can only be found between different isoforms. These enzymes are playing a crucial role in various cellular pathways such as cell adhesion, cell cycle regulation, gene expression, neurotransmitter release, and apoptosis. PP1c enzymes are sensitive to some xenobiotics, like microcystin-LR, or tautomycetin (TMC). These enzymes are 35-38 kDa in molecular weight and are highly conserved in their sequence (~90%).

These protein phosphatases are not functioning in monomeric form, they are interacting with different partners to achieve specificity, this way these enzymes consist of catalytic and regulatory subunits forming holoenzymes. The interaction is achieved by special binding surfaces found on PP1c, and by short linear motifs (SLiMs) found in the interactive partners. The interacting proteins can be classified as substrate targeting/activating, or substrate specificity/inhibitory.

One of these interacting partners which are responsible for substrate specificity is the myosin phosphatase targeting subunit 1 (MYPT1), which is a 110-133 kDa protein. Myosin phosphatase (MP) is composed of the MYPT1 subunit, a PP1c subunit (PP1cδ isoform), and a 20/21 kDa M20/21 subunit with unknown function. MYPT1 contains an RVxF (KVKF) motif,

which is essential for PP1c binding and contains a MyPhoNE (myosin phosphatase n-terminal element) SLiM for secondary interactions. Ankyrin repeats can be found at the N-terminal region of MYPT1, which are important in PP1c binding, and these repeats are acting as an interaction platform with many different other proteins. MYPT1 also contains an acidic region (D/E – aspartic acid/ glutamic acid), and two phosphorylation sites (Thr^{696}, Thr^{850}) which are critical in the regulation of the holoenzyme. MYPT1 contains a domain for the M20/21 subunit binding, and the C terminal part contains a leucine zipper motif which is responsible for further interactions.

Functions of myosin phosphatase

MP was first isolated from chicken gizzard and described as an enzyme that counteracts the effect of myosin light chain kinase (MLCK) and affects the contractility of cells. Further investigations showed that this enzyme has much more functions in regulating cellular processes. This was achieved by its diverse substrate specificity and wide range of subcellular localizations. MP can dephosphorylate ERM (ezrin, radixin, moesin) proteins regulating cell adhesion and cell migration. MP also plays a role in regulating microtubule-associated proteins (MAP proteins) such as Tau, or adducin. Also, MP is a key regulator in endothelial cells by affecting the activity of eNOS and the release of NO.

MP also plays a role in gene expression regulation via the dephosphorylation of histone deacetylase 7 (HDAC7) by which, this enzyme can recirculate into the cell nucleus. MP can also dephosphorylate protein arginine methyltransferase 5 (PRMT5) on its Thr^{80} sidechain which results in the downregulation of the gene expression of tumor suppressors.

MP is also present in neuronal cells and the interaction between MP and neuronal proteins such as synaptophysin, a typical post-synaptic protein, has been previously established. According to mass spectrometry analysis, MYPT1 interacts with various neuronal proteins, such as synapsin-I, Calcineurin A subunit, Ca^{2+}-calmodulin dependent protein kinase II, and SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) complex elements such as syntaxin-I, and SNAP-25 (Synaptosomal-Associated Protein of 25kDa). Further investigation also showed that Rho-associated kinase (ROK) and MP counteract each other's effect in synaptosomes. Treating synaptosomes with TMC increased the depolarization-induced exocytosis while Y27632 (trans-4-[(1R)-1-Aminoethyl]-N-4-pyridinyl cyclohexane carboxamide dihydrochloride) compound (ROK inhibitor) decreases exocytosis.

Regulation of myosin phosphatase

Several factors can alter the activity of MP affecting either the catalytic subunit (PP1c), holoenzyme specific or both. The previously mentioned small inhibitor molecules TM, TMC, and MC-LR can bind to the catalytic center of PP1c enzymes, therefore, inhibiting these enzymes. There are inhibitory proteins that are expressed in eukaryotic cells that can bind to the active center of PP1c. These proteins are the inhibitor-I and inhibitor-II proteins. Inhibitor-I requires phosphorylation for inhibition. There are also inhibitory proteins that can decrease the activity of both PP1c and MP. One of these proteins is the CPI-17 (C-kinase potentiated Protein phosphatase-1 Inhibitor of 17 kDa) or KEPI (kinase-enhanced protein phosphatase type 1 inhibitor). Both CPI-17 and KEPI need to be phosphorylated for the potent inhibitory effect. MP can also be regulated via phosphorylation sites on consensus sequences located on the MYPT1 subunit. The Thr^{696} and Thr^{850} residues are phosphorylated by ROK kinase which inhibits the activity of MP. The Thr^{696} sidechain of MYPT1 can also be phosphorylated by ILK (integrin-linked kinase), MDPK (myotonic dystrophy protein kinase), and p21-activated protein kinase.

Further research showed another inhibitory protein called smoothelin-like 1 protein (SMTNL1) that hampers the activity of MP holoenzyme by binding to its regulatory subunit.

Molecular mechanism of neurotransmitter release

Communication between neuronal cells is mostly carried out via neurotransmitters/neuromodulators. The basis of this process is that in neuronal cells an action potential travels through the axon of the cell and at the terminal of the cell causing a Ca^{2+} influx into the cell body. This influx causes the cell to release neurotransmitters (e.g.: amino acids, glutamate, aspartate, monoamines: dopamine, epinephrine) into the synaptic cleft which diffuses to the postsynaptic cell membrane and bounds to receptors which causes the postsynaptic cells to depolarize. The molecular apparatus behind the neurotransmitter release is the SNARE complex. This complex consists of synaptobrevin, which is associated with the synaptic membrane, syntaxin-I, and SNAP-25. The latter two are associated with the postsynaptic plasma membrane. All three proteins contain a SNARE motif which is about 65 amino acids long. The syntaxin-I and synaptobrevin contain 1-1 motif while SNAP-25 contains 2 SNARE motifs. The assembly of this complex is essential for normal neuronal transmission.

SNAP-25

The central element of the SNARE complex, SNAP-25 contains several phosphorylation sites which enable the fine-tuning of exocytosis. Various research showed the importance of the Thr^{138} and Ser^{187} phosphorylation sites. These two side chains are mainly phosphorylated by protein kinase A and C (PKA, PKC). PKA and PKC show preference towards the Thr^{138} and Ser^{187} sidechains, respectively. The elevation of Ser^{187} phosphorylation triggers exocytosis, while the data on Thr^{138} is not consistent. Some of the research data showed an inhibitory effect between SNAP-25 and syntaxin-I interaction when SNAP-25 was phosphorylated on Thr^{138}. Scientists taught that this is because this phosphorylation site is in the region responsible for the SNAP-25 –syntaxin-I binding. On the other hand, PKA activation in some cases increased the catecholamine release from cells. Currently, the physiological standpoint is that Thr^{138} influences the releasable pool of vesicles ("slowly releasable pool", and „readily releasable pool"), and the Ser^{187} phosphorylation affects vesicle recruitment. Dephosphorylation of SNAP-25 is mainly catalyzed by PP1 and PP2A enzymes. According to *in vitro* results PP1 has a preference towards the Thr^{138} sidechain however, PP1 enzymes can dephosphorylate all four identified phosphorylation sites.

SMTNL1

SMTNL1 is a member of the smoothelin protein family, and it was first isolated from rabbit ileum. This family has two other members SMTN-A and SMTN-B which are encoded on the same *smtn* gene. SMTNL1 is expressed in vascular and striated muscle, as well. Its functional characterization results revealed its effect on muscle adaptation. SMTNL1 KO mice showed higher scores in endurance training compared to wild-type littermates. The KO mice showed an exercise-adapted phenotype. SMTNL1 Ser^{301} phosphorylation site is phosphorylated by PKA and PKG. This phosphorylation causes the protein to translocate to the nucleus where it interacts with the progesterone receptor affecting gene expression. SMTNL1 also contains a calponin homology (CH) domain which is essential for MP binding. Experiments with SMTNL1 KO mice showed impaired glucose tolerance and the regulation of the expression of insulin signaling components. In the SMTNL1 KO mice, IRS1 showed elevated and GLUT4 transporter decreased gene expression. These mice also showed decreased metabolic activity. These findings suggest that SMTNL1 influences the molecular mechanisms which cause insulin resistance.

Insulin signaling

Insulin is a hormone secreted into the bloodstream by the β cells of the pancreas. Cells that are sensitive to insulin (e.g.: muscle cells) carry specific receptors that bind to insulin. Upon hormone binding, the insulin signaling cascade is initiated by the autophosphorylation of the insulin receptor followed by the activation of IRS (insulin-responsive elements) proteins. IRS is phosphorylated on tyrosine residues and these phospho-sites provide interacting surfaces to downstream insulin signaling elements with src-homology-2 domains (SH2) such as phosphatidyl inositol-3 kinase (PI3K). The final step in insulin signaling is the translocation of GLUT4 transporters into the plasma membrane. Abnormalities in insulin signaling lead to insulin resistance where insulin-sensitive cells do not respond to the hormone which leads to more insulin secretion by the pancreas. The molecular basis of insulin resistance can be attributed to multiple defects in the signaling cascades. One of the components is the IRS1 protein which has an altered phosphorylation profile compared to the physiological: instead of tyrosyl phosphorylation, seryl residues are over-phosphorylated. In normal cells, this mechanism shuts down insulin signaling, however, in insulin resistance this feedback loop overcoming normal insulin signaling. We hypothesize that SMTNL1 could act as a positive effector this way to some extent it can restore normal insulin sensitivity.

Aims

(1) Role of myosin phosphatase in neurotransmitter release:

- Investigation of the interaction between SNAP-25 and MYPT1 using surface plasmon resonance (SPR) measurements. *In vitro* phosphorylation of SNAP-25 with ROK. Western-blot analysis of the ROK-SNAP-25 phosphorylation with phospho-specific antibodies.
- Gene silencing of MYPT1 in B50 neuroblastoma cell line. Cell viability, protein phosphatase assay, and Western blot analysis to assess SNAP-25 regulation in siMYPT neuroblastoma cells.
- Treatment of cortical synaptosomes with TMC and H1152 inhibitors to investigate the phosphorylation level of SNAP-25 Thr^{138}. Immunoprecipitation of syntaxin-I–SNAP-25 complex to examine the effect of phosphorylation on the interaction between the two proteins.
- Confocal microscopy on mouse brain slices after TMC and H1152 treatments.

(2) Role of SMTNL1 in induced insulin resistance

- Establishing an *in vitro* insulin resistance model using C2C12 differentiated mouse myoblast/myotubule cell line using hyperinsulinaemic/hyperglycemic conditions to investigate the effects of SMTNL1 overexpression and MPA treatments, comparing to samples with empty plasmid transfected cells.
- Verification of our model with Proteome Profiler Analysis, and Western blot experiments on insulin signaling-specific components. Measurements of the energetic parameters of insulin-resistant cells with Seahorse XF96 instrument. Assessing the glucose uptake, and measurement of PI3K activity.

Materials and methods

Surface plasmon resonance

Interactions of MYPT1 and its truncated mutants with SNAP-25 were analyzed by surface plasmon resonance (SPR) using a Biacore 3000 instrument. Anti-GST antibody was coupled on the surface of a CM5 chip by amine-coupling, then full-length GST-MYPT1 WT or its C-terminal fragment (GST-MYPT1$^{667–1004}$) was captured on the surface. His-tagged MYPT1 proteins (His-MYPT1$^{1–296}$ or His-MYPT1$^{1–633}$) were immobilized directly on CM5 sensor chips by amine-coupling. Kinetic parameters and the dissociation constant (K_D) values were extracted from the sensorgrams with BIAevaluation 3.1 software using a 1:1 interaction model.

Protein production and purification

Flag-SNAP-25 plasmid (WT and Thr^{138A}) were transfected into *tsa201* cells using polyethylenimine (PEI) transfection reagent. The plasmids (15 μg) and PEI (30 μl) were diluted in 150 mM sterile NaCl solution and incubated for 30 minutes. One ml transfection mixture was added to 4 ml serum-free Dulbecco's modified Eagle's medium (DMEM) and pipetted onto the adherent *tsa201* cells (50–60% confluency). Six hours later 5 ml of 20% fetal bovine serum (FBS) containing DMEM was added to the cells, which were grown for an additional 18 hours before collecting for analysis.

Invitro Rho A kinase assay

The lysate of *tsa201* cells expressing Flag-SNAP-25 was incubated with Anti-Flag M2 affinity gel to pull down Flag-SNAP-25. Beads were washed with TBS and incubated with ROK (final concentration: 20 ng/μl) and ATP (final concentration: 0.5 mM) for 30 min at 30℃ in the presence of 1 μM of microcystin-LR in ROK assay buffer. Control samples were handled in the same way without the addition of ROK. After washing with TBS, beads were incubated with SDS-PAGE sample buffer at 100℃ for 5min. Phosphorylation and dephosphorylation of SNAP-25 were analyzed by Western blot using SNAP-25p Thr^{138} phospho-specific antibody and anti-SNAP-25 for loading control.

B50 cell culture

B50 neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (V/V) FBS and 2 mM L-glutamine in a 5% CO_2-humidified atmosphere at 37℃.

Gene silencing

The siRNA delivery was carried out with a suspension transfection protocol into B50 cells. Cells at 80% confluency were washed with sterile 1x PBS, trypsinized, and transfected with 100 nM double-stranded siRNA consisting of a pool of 4 different siRNAs targeting the endogenous MYPT1 subunit of MP. For the transfections, DharmaFECT 2 transfection reagent was used according to the manufacturer's instructions. After 48 hours, cells were either subjected to MTT or phosphatase activity assays or lysed and prepared for Western blot analysis. Gene knockdown experiments were carried out in 6-well plates ($1x10^5$ cells/well) for Western-blot and phosphatase activity assays and in a 96-well format for MTT assays (~$3x10^3$ cells/well).

Site-directed mutagenesis

Bacterial expression vector pReceiver-M13, which encodes wild-type SNAP-25, was purchased from GeneCopoeia. Point mutation of Thr^{138} of SNAP-25 to alanine was performed using QuickChange II XL Site-Directed Mutagenesis Kit. Newly synthesized mutant DNA was transformed into XL10-Blue super competent cells and mutant plasmids were purified using EZ-10 Spin Column Plasmid DNA Minipreps Kit. The sequence of the mutant was analyzed and verified by DNA sequencing.

Western blotting

Solubilized proteins from cells or synaptosomes were incubated at 100 ^{o}C for 5 minutes with 5x SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) sample buffer (50% glycerol, 10% SDS, 0.31 M Tris, 100 mM dithiothreitol, 0.01% bromophenol blue) and proteins were separated by SDS-PAGE on 12% (w/V) polyacrylamide gel. After gel electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated with blocking solution (5% bovine serum albumin (BSA) in Tris-buffered saline (TBS: 136 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl pH 7.4) containing 0.1% Tween-20 (TBST) for 1hour at room temperature, and then with the primary antibodies overnight at 4℃.

Membranes were washed with TBST and incubated with secondary antibodies for 1–2 hour at room temperature. Primary and secondary antibodies were diluted in TBST containing 0.5% BSA. Immunoreactions were developed by enhanced chemiluminescence and visualized using FluorChem FC2 Imager and Bio-Rad ChemiDoc Touch instruments. Densitometry analysis of immunoreactive bands was carried out using Image J and ImageLab software.

MTT assay

Cells were synchronized in serum-free DMEM medium and were treated with TMC and H1152 as detailed before. Ten µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in PBS) solution (5 mg/ml) was added and the cells were incubated in a 5% CO_2 humidified atmosphere at 37℃ for 1 hour. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was determined by a photometer at 540 nm wavelength.

Assay of protein phosphatase activity

B50 neuroblastoma cells were treated with TMC (5 µM) or H1152 (10 µM) inhibitors for 1 hour and 30 mins, respectively, and then lysed. Protein phosphatase activity in the lysates was measured using ^{32}P-labelled 20 kDa myosin light chain (^{32}P-MLC20) substrate in TM buffer. The reaction was initiated by the addition of ^{32}P-MLC20 at a final concentration of 1 µM. After 5min of incubation at 30℃ the reaction was terminated by the addition of 200 µl 10% trichloroacetic acid (TCA) and 200 µl 6mg/ ml BSA. After centrifugation, the $^{32}P_i$ content of the supernatant was measured in a Tri-Carb 2800TR scintillation counter.

Synaptosome preparation and treatment

Synaptosomes were prepared from C3H mouse cerebral cortex. Mice (3-5/experiment) were euthanized by carbon dioxide inhalation. After decapitation, the cortex was removed and immersed in homogenization buffer (0.32 M sucrose, 1.0 mM EDTA, 0.25 mM DTT, pH 7.4) in a ratio of 10 ml ice-cold buffer to 1g of cortex. Tissue was homogenized in a glass potter, and the homogenate was centrifuged at 1000 g for 10 min at 4℃ using an Allegra X-12R centrifuge. The supernatant was collected into a new tube, the volume was increased to 12 ml with homogenization buffer, and two 2ml volumes of the diluted supernatant were added slowly to the top of a Percoll gradient (2–2 ml of 23; 15; 10; 3% Percoll/homogenization buffer) in polycarbonate tubes. Tubes were centrifuged at 32500 g for 5min at 4℃ using Beckman L7-55 ultracentrifuge. Fractions between 10/15 and 15/23% phases were collected, diluted with a four-

times volume of Krebs buffer (118 mM NaCl, 5mM KCl, 25 mM $NaHCO_3$,1mM $MgCl_2$,10 mM D-glucose, pH 7.4), and centrifuged at 12600 g for 25 min at 4℃. The synaptosome pellet was resuspended in 3ml of Krebs buffer and $CaCl_2$ was added at a final concentration of 1.2 mM, then incubated at 30℃ for 1 hour in the presence or absence of inhibitors (5 μM TMC or 10 μM H1152) and KCl.

The protocol for animal handling (5/2015/DEMÁB; 6/2011/DEMÁB) was authorized by the Animal Care and Protection Committee of the University of Debrecen and following the guidelines of the European Union Council, as well as the Hungarian regulations on the use of laboratory animals. Mice were given ad libitum access to food and water and were kept at 25℃ temperature, applying a 12-hour light/12-hour dark cycle. The veterinarian at the University of Debrecen examined the health status of the mice every month, and in case of potential sickness, microbiological examinations are conducted, and the appropriate interventions (therapy, quarantine) are applied. Mice were raised in the Experimental Animal House of the University of Debrecen, Debrecen, Hungary (XV-KÁT/2000).

Preparing brain slices and treatment

Experiments were performed in artificial cerebrospinal fluid (aCSF) of the following composition: 120 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$,10 mM glucose, 1.25 mM NaH_2PO_4 2 mM $CaCl_2$,1mM $MgCl_2$ 3mM Myo-inositol, 0.5 mM ascorbic acid, and 2mM sodium pyruvate, pH 7.2. For the slice preparation, a modified aCSF (low sodium aCSF) was used where 95 mM NaCl was replaced by 130 mM sucrose and 60 mM glycerol. C3H mice (8 to 30 days old) of both sexes were used. After decapitation of the animal and removal of the brain, 200 μm thick coronal brain slices were prepared in ice-cold low sodium aCSF using a Microm HM 650 vibratome. After preparation, the slices were transferred into a Millicell CM culture plate insert filled with aCSF. The culture plate inserts were placed in an incubating chamber which was designed for fluorescent dye loading. Following the transfer, the incubating solution was slowly replaced with aCSF containing 10 μM H1152 or 5 μM tautomycetin with or without 8mM KCl. The slices were incubated for 60 min at room temperature under moderately pressurized carbogen (95% O_2 5% CO_2) conditions. After incubation, the slices were either frozen in liquid nitrogen for Western blot analysis or mounted in Mounting Medium for cryotomy. The mounted samples were sliced with Leica CM 1860 Cryotome and the 6 μm thick slices were prepared for immunohistochemical experiments.

Immunofluorescence staining and confocal microscopy

Brain slices were also prepared for immunohistochemistry. Slices were dried, then incubated in a blocking buffer consisting of 10% horse serum and 0.2% Triton X-100 in TBS for 1 hour at room temperature and the staining procedure was applied as described above using primary antibodies against SNAP-25 Thr^{138} and syntaxin.

Cell maintenance, differentiation, and insulin resistance model

The C2C12 mouse myoblast cell line was cultured in low-glucose (5.5 mM) DMEM supplemented with 2 mM L-glutamine and 10% (v/v) FBS in a 5% CO_2-humidified atmosphere at 37 ◦C. Cells were grown in collagen-coated petri dishes for differentiation and maintained in this medium (5.5 mM DMEM without phenol red, supplemented with 2 mM L-glutamine and 2% (v/v) horse serum). The medium was changed every 24 hours for 3 days.

For the insulin resistance treatment, high glucose (HG, 25 mM glucose) DMEM without phenol red was supplemented with 2 mM L-glutamine, 2% horse serum, and 100 nM of insulin (chronic insulin). The low glucose (LG, 5.5 mM glucose) DMEM without phenol red was supplemented with 2 mM L-glutamine, 2% (v/v) horse serum, and 50 pM of insulin and 10 nM of medroxyprogesterone-17-acetate (MPA) was added or not. All these treatments were done on both SMTNL1 overexpressing and empty vector-expressing cells.

For Western-blotting, Proteome Profiler, and PI3K activity experiments the media was changed to serum and hormone-free DMEM medium for 5 h, and cells were treated again for 30 min with 100 nM or 50 pM of insulin (acute insulin) before harvesting. Samples were prepared for the PI3K assay according to the supplementary protocol section for PI3K activity measurement.

Transient transfections

The full-length mouse SMTNL1 construct (pcDNA-3.1 expression vector) with N-terminal FLAG tag, referred to as FT-SMTNL1, and the empty pcDNA-3.1 plasmid as a transfection control, referred to as empty vector/control, were overexpressed in C2C12 cells on day 0 of the full-length protocol using GeneJuice Transfection Reagent. Transfection was carried out in suspension to increase transfection efficiency. In the case of 6 well plates, 3μg of plasmid - 6 μl GeneJuice ratio was used. After mixing the cells with the transfection reagent cells were plated, and the volume was adjusted to the final 2 mL containing 10% FBS. All the ratios were adjusted for 96-well plates.

Proteome profiler analysis

Phosphorylation levels of various proteins were analyzed with an R&D Systems Proteome Profiler Human Phospho-Kinase Array kit. After the previously described treatments, cells were harvested and 200 μg of protein was used for each experiment, according to the manufacturer's instructions. Chemiluminescence was detected with a ChemiDoc Touch instrument. Dot intensities were analyzed with ImageJ software and normalized to control dots on each membrane.

PI3K activity measurement

PI3K activity was measured with an ELISA kit following the manufacturer's protocol. Absorbance was measured at 450 nm. Immunoprecipitation was conducted with the p85 subunit antibody to validate the input. Liberated phosphatidylinositol (3,4,5)-trisphosphate (PIP3) values were normalized to the PI3K expression levels.

Seahorse XF96 measurements

After transfection, cells were plated, and 24 h after transfection, were treated for 72 h. Another 72 h later myoblast cells were washed, and metabolic measurements were made on Agilent Seahorse XF (ASX) 96 plates using ASX assay-medium with 5.5 mM glucose. Etomoxir (50 μM), which is an inhibitor of fatty acid oxidation (FAO) was added. Etomoxir blocks the F_0 subunit of ATP synthase. With this, we could calculate the respiratory capacity as FCCP. Oligomycin (2 μM). FCCP (4 μM, carbonyl cyanide ptrifluormethoxyphenylhydrazone) was used as a mobile ion carrier to calculate the respiratory capacity of cells since FCCP is a mitochondrial uncoupler. Antimycin A (10 μM) was applied as the inhibitor of complex III to eliminate the electron transport chain function, revealing the non-mitochondrial respiration. Finally, 100 mM 2-deoxy-D-glucose (2-DG) was added; 2-DG is a glucose analog that inhibits glycolysis through competitive binding to glucose hexokinase to confirm ECAR due to glycolysis. Five measurement points were taken at baseline and after each injection event. After the measurements, cells were solubilized in 1N NaOH and protein content was determined by BCA assay.

Glucose uptake assay

2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) uptake measurements were carried out. C2C12 cells were cultured, differentiated, and treated as

described before. The myotubes were washed with PBS buffer and incubated in glucose-free DMEM supplemented with 2% horse serum. 100 nM insulin was applied for 30 min and cells were incubated with a glucose-free medium containing 100 μM 2-NBDG for 120 min at 37 ◦C. Fluorescence was measured at an excitation of 485 nm and emission of 535 nm using a Tecan Spark Multimode microplate reader, and negative and blank controls as well as a standard curve were also set up to quantify glucose uptake. Cells were washed three times and were solubilized in 0.1 M KH_2PO_4 pH 11.0 to determine protein concentration. Fluorescent data in pmol were related to 1 mg of proteins in each case and presented as fold induction.

Statistical analysis

Statistical analysis used while investigating the role of MP in neuronal processes: student's t-test: mean ± SEM (n=5), *p<0,05; **p<0,01; ****p<0,0001, and: ANOVA: Dunett's post hoc test: mean ± SEM (n=5), *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

The effect of SMTNL1 in insulin resistance samples was tested Post hoc for two-way ANOVA. Differences were determined by Tukey's test and Sidak's test Mean ± SEM; n=3; *, p<0,05). Parametric tests were used when normalized values were normally distributed. GraphPad software was used for all analyses. The data are presented as mean ± SEM; n is the number of independently performed experiments. $p < 0.05$ was considered statistically significant. In the case of Tukey's test, different letters mean that there is a significant difference between groups.

Results

Role of myosin phosphatase in neurotransmitter release

The protein-protein interaction between SNAP-25 and myosin phosphatase was investigated using surface plasmon resonance (SPR) measurements. According to these findings, MYPT1 interacts with SNAP-25. To identify the region of MYPT1 that is responsible for the interaction with SNAP-25, we used different MYPT1 truncated mutants in these experiments. The dissociation constant values showed that the C-terminal region is responsible for the strongest interaction between SNAP-25 and MYPT1. The Thr^{138} -> Ala^{138} mutant of SNAP-25 was produced by site-directed mutagenesis. Using the wild type and mutant forms of SNAP-25 in ROK *in vitro* protein kinase assay, we identified a new ROK substrate: SNAP-25. With phospho-specific antibodies, we detected a signal on the Thr^{138} sidechain, while the mutant SNAP-25 was not phosphorylated.

To better understand the physiological role of MP in neurological processes we used siRNA-induced gene silencing. Silencing MYPT1 drastically decreased the viability of B50 cells, and we measured a significantly lower protein phosphatase activity in these cell lysates. These results suggest that MP is one of the most important PP1 enzymes in neuronal cells. With gene silencing, we also detected a lower endogenous SNAP-25 Thr^{138} phosphorylation.

Our further results also showed that the ROK and MP enzyme pair is responsible for the modulation of exocytosis via the central SNARE complex element SNAP-25. Tautomycetin (TMC), a potent PP1 inhibitor decreased the amount of SNAP-25 bound to syntaxin-I in synaptosomes. The ROK inhibitor, H1152 resulted in exactly the opposite effect: immunoprecipitated syntaxin-I bound significantly less SNAP-25. By treating synaptosomes with these inhibitor molecules we also measured a difference in the phosphorylation level of SNAP-25 at the Thr^{138} residue. ROK increased whereas TMC lowered the phosphorylation level on this residue. Interestingly, KCl-induced depolarization itself lowered the phosphorylation on this amino acid residue. The application of these inhibitors on mouse brain slices supported the previously described results by immunofluorescence and confocal microscopy. The TMC treatment decreased, while the H1152 increased the phosphorylation level of Thr^{138} of SNAP-25. The evidence we gathered strongly implies that MP and ROK are responsible for modulating neurotransmitter release via the phosphorylation of SNAP-25 Thr^{138}.

Role of SMTNL1 in insulin resistance

The effect of SMTNL1 and MPA was characterized in induced insulin resistance (IR) using C2C12 cells. Using a ProteomeProfiler analysis we measured the phosphorylation levels of the elements of multiple signaling pathways in cell lysates. By overexpressing SMTNL1 STAT5a and STAT5b showed increased phosphorylation, however, the phosphorylation of STAT2, 6, CREB, and c-Jun was not affected. Phosphorylation on the Ser^{46} residue in p53 showed an increased level in the IR state. Multiple critical protein kinases showed an increase in phosphorylation in the IR model when STMNL1 was overexpressed: Thr^{172} of AMPKα2, and Ser^{473} of Akt1/2/3. In IR, multiple protein kinases showed increased activating phosphorylation like JNK1/2/3 Thr^{183}, and ERK1/2 Thr^{202}. These two are responsible for the inhibitory Ser phosphorylation of IRS1.

We further investigated the insulin signaling pathway by performing multiple Western blot experiments using phospho-specific antibodies. In IR myotubules IRS1 Ser^{307}, Ser^{318}, and Ser^{612} sites showed elevated levels. By combining SMTNL1 overexpression with MPA treatment the phosphorylation of these sites was lower compared to the empty vector-transfected cells. We also detected the lower expression of the IRS1 protein which could be the result of IRS1 degradation. MPA by itself had no effect, which means that SMTNL1 modulates insulin signaling through the regulation of gene expression.

IRS1 Ser^{307} is phosphorylated by JNK, which shows increased activity in insulin resistance. Combined with the Ser^{318} phosphorylation this triggers the dissociation of the insulin receptor and IRS1. Overexpressing SMTNL1 did not affect the expression of JNK however, the activating phosphorylation was significantly lower. The IRS1 Ser^{318} is mostly phosphorylated by PKC enzymes and gene deletion of these enzymes in some cases can promote insulin sensitivity. Our results showed that the expression of PKCε was lower when cells overexpressed SMTNL1. In the case of IRS1 Ser^{612} ERK1/2 and mTOR pathways are crucial. In some cases, by inhibiting ERK1/2 it was possible to restore insulin sensitivity, and ERK1/2 showed higher activity in IR. In our model, the activating phosphorylation of ERK1/2 was higher, but by overexpressing SMTNL1 combined with MPA treatment this phosphorylation was lower. According to our data, SMTNL1 overexpression with MPA can lower the effect of JNK and ERK1/2, this means that SMNLT1 through its effect on gene expression can lower the effects of insulin resistance. Interestingly with the Ser^{1101} phosphorylation, we also managed to observe the positive effect of SMTNL1 and MP however the high concentration of insulin lowered the phosphorylation on this sidechain.

By measuring the expression and activity of PI3K we observed a decrease in the expression of the p85 subunit, but the overexpression of SMTNL1 and MPA treatment compensated for this effect. This insulin-sensitizing effect was observed in the PI3K activity measurements as well. Based on that, SMTNL1 can keep the expression level of the p85 subunit at an elevated level even in IR.

A lower phosphorylation level of Akt1 was detected in IR myotubules. Overexpressing SMTNL1 and treating cells with MPA kept both crucial phosphorylation sites more active (Thr^{308} and Ser^{473}). Akt1 phosphorylates and inactivates the GSK-3β enzyme, which in turn activates glycogen synthase and this initiates the glycogen storages to build up. Interestingly none of the treatments had any effect on the phosphorylation of GSK-3β. Among the downstream effectors, the expression, and the level of mTOR Ser^{2448} phosphorylation were unaffected in IR cells however, SMTNL1 overexpression and MPA treatment activated the mTOR pathway. It is also important that by overexpression of SMNTL1 and combining it with MPA, we measured a higher GLUT4 expression and a higher glucose uptake.

We also measured the metabolic parameters of IR C2C12 cells with a Seahorse XF 96 (ASX) instrument. Under IR conditions the glycolytic parameters were significantly lower, however, by combining MPA treatment with the overexpression of SMTNL1, these parameters were significantly better without influencing the maximal glycolysis and glycolytic reserve parameters. The SMTNL1 overexpression and MPA treatment together restored the glycolytic parameters and elevated ATP synthesis. We measured mitochondrial activity with the oxygen consumption rate (OCR). Interestingly SMTNL1 overexpression initiated fatty acid oxidation.

Our data suggest that SMTNL1 overexpression can compensate for the effects of insulin resistance to some level, and it might be a potential therapeutical target for future therapy.

Summary

Our studies focused on the role of myosin phosphatase (MP) in neurotransmitter release, and SMTNL1 in the insulin resistance model using C2C12 myotubules. Myosin phosphatase holoenzyme was first described in the dephosphorylation of the myosin light chain (MLC20), thus enabling the regulation of smooth muscle contractility. In addition to the myosin light chain, several cytoskeletal targets have been identified such as ERM or Tau proteins. MP is composed of a PP1cδ catalytic, a regulatory/targeting MYPT1, and an M20/21 subunit. MP plays a role not only in the dephosphorylation of cytoskeletal elements but is expressed ubiquitously. Moreover, MP is playing an important role in several cellular processes, such as cell cycle regulation, neurotransmitter release, and gene expression regulation by dephosphorylating several key interacting proteins. Many regulatory factors can modulate the activity of MP ranging from small inhibitory molecules to interacting proteins and even MYPT1 phosphorylation via RhoA-associated protein kinase (ROK) on the inhibitory regulatory side chains. Proteins that are phosphorylated by ROK are usually dephosphorylated by MP, thus the enzyme pair counteracting each other's effect.

The interaction between SNAP-25 with MP through the MYPT1 subunit was elucidated using surface plasmon resonance (SPR) experiments. *In vitro* protein kinase assays and phosphoprotein antibodies were used to demonstrate the phosphorylation of SNAP-25 by ROK on the Thr^{138} residue. Elevated phosphorylation of the SNAP-25 Thr^{138} side chain was observed in B50 neuroblastoma cells upon siRNA interference silencing of the MYPT1 subunit, further supporting our hypothesis that the ROK/MP enzyme pair is responsible for the phosphorylation regulation of SNAP-25. Moreover, as the result of silencing, the enzyme protein phosphatase activity, and the viability of B50 cells were decreased significantly suggesting that PP1 enzymes are the dominant protein phosphatases regulating cellular processes in neuronal cells. By treating mouse cortical synaptosomes with MP and ROK inhibitors (TMC and H1152), we were able to detect the opposite effect on the SNAP-25 Thr^{138} phosphorylation (TMC increased, H1152 decreased the phosphorylation) providing further evidence for the role of this enzyme couple in neuronal processes. We obtained the same results by using immunofluorescence and confocal microscopy on synaptosomes. The role of SNAP-25 Thr^{138} phosphorylation in the formation of the SNARE complex was investigated by immunoprecipitation experiments and

revealed that the less phosphorylated SNAP-25 binds to syntaxin to a greater extent, which indicates the positive role of MP in the process of exocytosis.

An *in vitro* hyperinsulinaemic/hyperglycemic C2C12 myoblastoma/myotubule insulin resistance model has been developed to investigate the role of SMTNL1 in insulin signaling and its pathological molecular mechanisms. Using proteome profiler analysis, we got an overview of the intracellular processes during insulin resistance, and with overexpression of SMTNL1. We demonstrated the insulin-sensitizing effect of SMTNL1, mainly observed in the presence of progesterone. The insulin-sensitizing effect was detected as a decrease in the critical serine phosphorylation levels. SMTNL1 also shows additional beneficial effects on downstream elements of the insulin receptor, for example, the PI3K, the Akt, and mTOR. Our data suggest that SMTNL1 further contributes to the decrease in the phosphorylation of IRS1 Ser residues by indirectly reducing JNK and ERK1/2 activity presumably via the regulation of the expression of novel PKCε isoform. SMTNL1 was able to keep the activity of PI3K and the expression GLUT4 elevated even in insulin-resistant cells. Measuring the cellular energetic parameters revealed that SMTNL1 overexpression combined with MPA treatment restored basal glycolysis in IR to some extent. The same was also proved when mitochondrial activity was measured. SMTNL1 overexpression and MPA treatment enhanced ATP synthesis even in IR conditions. All these findings suggest that SMTNL1 might be a potential drug target in insulin resistance and related disorders. Overexpression of SMTNL1 plays a role in maintaining normal insulin sensitivity and keeping cellular metabolic pathways at physiological levels.

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List of publications related to the dissertation

1. **Tamás, I.**, Major, E., Horváth, D., Keller, I., Ungvári, Á., Haystead, T. A. J., MacDonald, J. A., Lontay, B.: Mechanisms by which smoothelin-like protein 1 reverses insulin resistance in myotubules and mice.
Mol. Cell. Endocrinol. 551, 1-12, 2022.
DOI: http://dx.doi.org/10.1016/j.mce.2022.111663
IF: 4.369 (2021)

2. Horváth, D., **Tamás, I.**, Sipos, A., Darula, Z., Bécsi, B., Nagy, D., Iván, J., Erdődi, F., Lontay, B.: Myosin phosphatase and RhoA-activated kinase modulate neurotransmitter release by regulating SNAP-25 of SNARE complex.
PLoS One. 12 (5), 1-23, 2017.
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IF: 2.766

List of other publications

3. Major, E., Győry, F., Horváth, D., Keller, I., **Tamás, I.**, Uray, K., Fülöp, P., Lontay, B.: Smoothelin-like protein 1 regulates development and metabolic transformation of skeletal muscle in hyperthyroidism.
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