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J Korean Neurosurg Soc 42 : 470-474, 2007

10.3340/jkns.2007.42.6.470

Laboratory Investigation

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Identification of Novel Metabolic Proteins Released by Insulin Signaling of the Rat Hypothalmus Using Liquid Chromatography-Mass Spectrometry (LC-MS)

Objective : The brain is dependent on glucose as an energy source. Intricate homeostatic mechanisms have been implicated in maintaining the blood glucose concentration in the brain. The aim of this study is to find the way to identify the metabolic proteins regulating the glucose in rat hypothalamus.

Methods : In this study, we analysed the secretome from rat hypothalamus in vivo. We introduced 500 nM of insulin into the rat hypothalamus. The chromatographic patterns of the secretome were identified, after which Mass Spectrometry-Mass Spectrometry (MS-MS) analysis was performed.

Results : In Liquid Chromatography-Mass Spectrometry (LC-MS) analysis, 60 proteins were identified in the secretome. Among them, 8 novel proteins were unveiled and were associated with the energy metabolism of insulin signaling in mitochondria of rat hypothalamic neuron. Nineteen other proteins have unknown functions. These ligands were confirmed to be secreting from the rat hypothalmus on insulin signaling by western blotting.

Conclusion : The hypothalamus is the master endocrine gland responsible for the regulation of various physiological and metabolic processes. Proteomics using LC-MS analysis offer a efficient means for generating a comprehensive analysis of hypothalamic protein expression by insulin signaling.

KEY WORDS : Insulin · Energy metabolism · Hypothalamus · Liquid chromatography · Mass spectrometry.

INTRODUCTION

Recently, it has become known that insulin and its related proteins are produced in the brain as well as in peripheral tissues like the pancreas[12]. Insulin and its growth factors are necessary for the survival of brain cells. When insulin is significantly reduced in the hypothalamus, it is found that neuronal cells degenerate[10].

In the hypothalamus, the eating behavior control system consists of the ventral medial hypothalamus (VMH) and periventricular nucleus (PVN). The PVN represents the central nucleus of the brain's stress systems, the hypothalamus-pituitary-adrenal (HPA) axis. Activation of the sympatico-adrenal system inhibits glucose uptake by peripheral tissues; this inhibits insulin release and induces insulin resistance as well as increasing hepatic glucose production. A decrease in brain glucose can activate glucose-sensitive neurons in the lateral hypothalamus (LH) with the release of orexigenic peptides which stimulate food intake. If the energy supply of the brain depends on the activation of the LH, an increase in body weight is inevitable. An increase in fat mass will generate feedback signals as insulin. Activation of LH in turn will stimulate the activity of the PVN. The activity of the PVN in turn is the consequence of a balance of low-affinity and high-affinity glucocorticoid receptors. This set-point can permanently be displaced by extreme stress situations, starvation, exercise, hormones, drugs or by endocrine-disrupting chemicals[4].

This study is aimed to find new secretory proteins which are produced by insulin signals involving body homeostasis in mitochondrial energy metabolism as proteins from the rat 'secretome' of the hypothalamus[1].

• Received : August 17, 2007
• Accepted : November 12, 2007
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MATERIALS AND METHODS

Materials

5-hydroxytryptamine was purchased from Sigma Aldrich. RPMI 1640 medium for cell culture were obtained from Dulbecco's modified Eagle's medium (DMEM).

Animal preparation and *in vivo* experiments

Male Wistar rats weighting 100 g (6 weeks-old) were housed in air-conditioned animal quarters, with lights on, and given food and water ad libitum. We grasped the tail and induced cervical herniation to terminate the animals and cut the head with a guillotine. We scraped the scalp from the mastoid area and exposed the skull. The skull was incised along the external auditory meatus and the brain removed. The borders of the excised hypothalamus were delineated by the anterior margin of the optic chiasm, the posterior margin of the mammillary bodies, and laterally by the hypothalamic sulci. The separated hypothalamuses were split into two pieces each and washed with PBS 3 times, and then with RPMI medium. The fragile tissue was placed in an iced cold 48 well plate and incubated in at 37°C with 5% CO_2. The stabilized tissue was finally treated with 500 nM insulin for 2 hours at 37°C with 5% CO_2.

Using an Oasis HLB cartridge and washing the tissue with 0.5% acetonitrile and 0.1% tetrafluoroacetic acid, the hydrophobic impurities were removed as much as possible.

Sample preparation

The purified sample was dried in speedvac for 2 hours and eluted in distilled water to 100 $\mu\ell$ total volume with about 100 μg/ml concentration of protein. The sample was stored at -20°C overnight and 90 $\mu\ell$ of it was measured with chromatographic analysis.

Chromatographic analysis and calculation

The chromatographic analysis was performed on an AKTA FPLC system (Amersham Bioscience, UK) consisting of an isocratic pump, an autosampler with injector, columns, tubing and an ultraviolet detector controlled by Unicorn systems. The prepared 90 $\mu\ell$ samples was injected onto a C-4 narrow analytic column and eluted with 0.1% acetonitrile and tetrafluoroacetic acid or distilled water at a flow rate of 1 mL/min. The absorbance at 215 nm of the elutant was monitored. Finally, we washed out the column with 20% ethanol for 1 hour to remove the remnants.

Denaturation, reduction, alkylation and trypsin digestion

Ten micrograms of the secretome were taken and denaturated at 90°C for 20 minutes. In addition, the samples were reduced by adding 10 mM of dithiothreitol (DTT), and incubated at 56°C for 20 minutes. The samples underwent alkylation for another 20 minutes in a dark room adding 100 mM of iodoacetamide. Finally, the samples were digested by 0.1 μg of trypsin overnight.

Liquid Chromatography-Repeated Mass spectrometry (LC-MS/MS) and Database analysis

For these experiments, the mass spectrometer was connected on-line with an AKTA Basic liquid chromatograph (GE Healthcare, NJ) by using an RP-C18, 300 m ID 5 cm column (Dionex, CA). Peptides were eluted from the column using a linear gradient of acetonitrile with 0.2% formic acid from 5 to 60% for 50 min, at a flow rate of 3 L/min. To acquire the LC-MS/MS spectra the first quadrupole was used to select the precursor ion within a window of 4 Th. A pressure of -3×10^{-2} Pa collision gas (argon) was used in the hexapole collision cell to yield the fragment ions. The doubly- and triply- charged precursor ions to be fragmented were selected automatically once their intensity rose above a defined threshold (8 conts s^{-1}). The instrument reverted to MS mode once the total ion count decreased below 2 count sec^{-1} or when the MS/MS mode had been maintained for 4 s. Data acquisition and processing were performed using a MassLynx system (version 3.5) from Waters (MA). Protein identifications were based on manual interpretation of the MS/MS spectra[6,7].

Computer programs

The program Selestact (CIGB, Havana, Cuba) coded in C for console use was developed to scan the Swiss-Prot sequence database, and calculate the number of proteins : 1-18 of the average number of peptides per protein that could be selectively isolated. The counting was constrained to those peptides of mass which comprised between 9 and 290 KDa. The software is available through the Internet (http://ca.exPAsy.org).

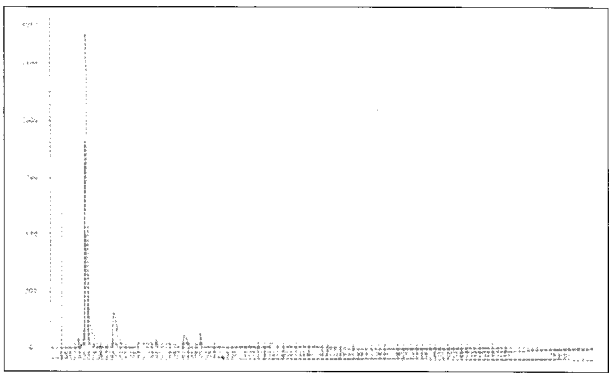

Fig. 1. Fast Protein Liquid Chromatography chromatograms of the secretome from rat hypothalamus. This shows the patterns of curves and slopes of the insulin treated secretomes. Chromatographic conditions : C-4 narrow analytic column, eluted with 0.1% acetonitrile and tetrafluoroacetic acid, flow rate of 1 mL/min, ultraviolet at 215 nm.

Table 1. 60 proteins secreted from rat hypothalamus by insuling signaling

IPI Reference	Protein	Peptide	Localization	Function
IPI00206171.1	Microtubule-associated protein 2	18	Subcellular	Stabilize microtubules
IPI00190943.1	Glial fibrillary acidic protein, astrocyte	6	Cytoplasm	Develope glial cell
IPI00211936.2	Alpha-internexin	4	Subcellular	Genesis neurons
IPI00470288.3	Creatine kinase B-type	3	Cytoplasm	Energy transduction
IPI00205036.1	Alpha-2-globulin chain	3	Subcellular	Hemoglobun
IPI00371946.3	Alanine-rich C-kinase substrate	3	Unknown	Unknown
IPI00231643.4	Superoxide dismutase	3	Cytoplasm	Destroys radicals
IPI00194281.2	81 KDa protein	3	Unknown	Unknown
IPI00324893.4	14-3-3 protein zeta / delta	4	Mitochondria	Mitochondrial imports stimulating factor
IPI00231631.11	Beta-enolase	2	Cytoplasm	Muscle development
IPI00189362.2	17 KDa protein	2	Subcellular	Zinc binding
IPI00189795.1	Tubulin alpha-1 chain	1	Subcellular	Microtubules
IPI00210119.1	Stop protein	2	Subcellular	Microtubules-stabilize
IPI00199693.2	Microtubule-associated protein 1A.	2	Subcellular	Structural protein
IPI00231767.4	Triisophosphate isomerase	5	Unknown	Unknown
IPI00231801.3	Cystatin C precursor	1	Subcellular	Enzyme activity
IPI00230835.4	14-3-3 protein gamma	2	Cytoplasm	Adapter protein
IPI00196661.1	14-3-3 protein theta	3	Cytoplasm	Adapter protein
IPI00230897.4	Hemoglobin beta-1 subunit.	2	Subcellular	Oxygen transport
IPI00231955.5	Calmodulin.	2	Unknown	Calcium transfer
IPI00190240.0	Ribosomal proteins 27A	6	Unknown	Unknown
IPI00209258.4	Spectrin alpha chain, brain	3	Cytoplasm	Interacts with calmodulin
IPI00231677.4	14-3-3 protein eta	2	Cytoplasm	Adapter protein
IPI00388666.3	Heterogeneous nuclear ribonucleoprotein A1	2	Unknown	Unknown
IPI00393340.2	Hypothetical protein	1	Subcellular	Intermediate filament
IPI00231771.4	Protein S100-B	2	Nucleus	Binds calcium
IPI00212767.1	Anionic trypsin-1 precursor	1	Subcellular	Secreted protein
IPI00230941.4	Vimentin.	1	Nucleus	Intermediate filaments
IPI00208205.1	Heat shock cognate 71 KDa protein	1	Cytoplasm	Chaperone
IPI00212478.1	Epsilon1 globin.	1	Subcellular	Oxygen transport
IPI00212969.2	32 kDa protein	1	Unknown	Unknown
IPI00189362.2	17 kDa protein	2	Unknown	Unknown
IPI00387771.5	Peptidyl-prolyl cis-trans isomerase A	1	Cytoplasm	Folding of proteins
IPI00196994.1	Rho Gdp dissociation inhibiton inhibitor alpha	1	Unknown	Unknown
IPI00211150.4	20 kDa protein	3	Unknown	Unknown
IPI00372520.1	AC2-067 heterogeneous nuclear	1	Unknown	Unknown
IPI00210212.3	Ribonucleoprotein A2 / B1	1	Unknown	Unknown
IPI00189722.1	Tropomodulin-2	1	Cytoplasm	Blocks actin filaments
IPI00198118.1	112 KDa protein	1	Unknown	Unknown
IPI00231736.8	Fructose-bisphate aldolase C	1	Cytoplasm	Catabolism
IPI00189995.1	Calretinin	1	Cytoplasm	Calcium-binding protein core component
IPI00231340.6	Germinal histone H4 gene	2	Nucleus	Nucleosome
IPI00195929.1	Cytoplasmic linker protein 2 branched-chain-amino-acid	1	Cytoplasm	Membranous organelle
IPI00215523.1	Aminotransferase, cytosolic tyrosine	1	Cytoplasm	Catabolism
IPI00230837.4	3-monooxgenase/ tryptophan 5-monooxgenase activation protein, beta polypeptide	1	Cytoplasm	Adapter protein
IPI00367553.2	Melanoma-like 3 snap-25B	1	Unknown	Unknown
IPI00204644.1	Synaptosomal-associated protein 25	1	Unknown	Unknown
IPI00421428.6	Phosphoglycerate mutase 1	2	Cytoplasm	Catabolism
IPI00360030.3	GPRIN1 protein	1	Unknown	Unknown neuroprotective
IPI00326412.3	Gamma-enolase	1	Cytoplasm	Properties

Table 1. Continued

IPI Reference	Protein	Peptide	Localization	Function
IPI00195851.1	Hypothetical protein aldoal 1	1	Unknown	Unknown
IPI00196508.1	Amphiphysin	1	Cytoplasm	Regulated exocytosis
IPI00551812.1	ATP synthase beta chain, mitochondrial precursor	1	Mitochondri a	Produces ATP from ADP
IPI00188688.1	Histone H2A	1	Unknown	Unknown
IPI00195813.2	SFRS 7 protein	1	Unknown	Unknown
IPI00231651.6	Brain acid soluble protein 1	3	Membrane	Elongating axons
IPI00212320.1	Neuromodunin.	2	Membrane	Nerve growth
IPI00476991.1	Neural cell adhesion molecule 1	1	Membrane	Neuro fasciculation
IPI00231801.3	Acyl-coA-binding protein	2	Membrane	GABA receptor
IPI00193173.4	Serum albumin precursor	1	Secreted	Osmoregulation

IPI : International Protein Index, KDa : Kilodalton, ATP : Adenosine Tri-Phosphate, ADP : Adenosine Di-Phosphate, GABA : Gamma Amino Butric Acid

Verification of the secreted proteins by Western blot

To verify the results obtained from LC-MS, proteins in the secretome were subjected to western blot analysis. The secretomes containing an equal amount of protein were separated by sodium dodecyl sulfate -polyacrylamide gelelectrophoresis (SDS-PAGE) and blotted to nitrocellulose membrane (Hybond-ECL, Amersham Bioscience, Buckinghamshire, England). The membrane was incubated with an antibody as specified, followed by a secondary antibody conjugated with horseradish peroxidase. Specific antigenantibody complexes were detected by enhanced chemiluminescence (Pierce Biotechnology, Inc. Rockford, IL). GAP-43 (anti-neuromodulin) and ACBP (anti-acyl CoA) antibodies (Santa Cruz, USA) were used in this study.

Table 2. Functions of 8 novel secreted proteins associated with the energy metabolism of insulin signaling in rat hypothalamus

Protein function	Energy metabolism	Mitochondrial import	Oxygen transport
1	Creatine kinase B-type	14-3-3 protein zeta / delta	Hemoglobin beta-1 subunit
2	Fructose-bisphosphate aldolase c	ATP synthase beta chain	Epsilon 1 globulin
3	Branched-chain-amino-acid aminotransferase		
4	Phosphoglycerate mutase 1		

RESULTS

Chromatography

The chromatographic separation of the secretome from the rat hypothalamus was performed with fast protein liquid chromatography (FPLC) on a C-4 narrow analytic column. The slopes and peaks of the analytic curve were prominently visible. After 2 hours in insulin containing medium, the fractionation proceeded as shown in Fig. 1.

Liquid Chromatography-Mass spectrometry (LC-MS) analysis

From MS-MS analysis, 60 individual proteins were identified (Table 1). 8 proteins were conferred to involve energy metabolism, mitochondrial import and oxygen transport in the rat hypothalamus (Table 2). Nineteen other proteins revealed have unknown functions and locations.

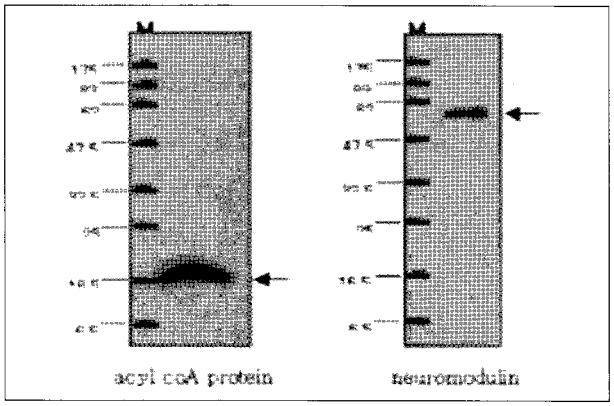

Fig. 2. In western blot analysis with secretome by insulin, 2 protein bands were identified to confirm the selected proteins.

Confirmation of the selected proteins by Western blot

Among the proteins, acyl coA binding protein and neuromodulin were identified by western blotting as 16.7 and 50 kDa bands each (Fig. 2).

DISCUSSION

The physiological signaling mechanisms link glucose sensing to the electrical activity in metabolism regulating the hypothalamus. Adenosine Tri-Phosphate (ATP) production was considered the main metabolic signal and the glucose stimulated-signal like insulin in the hypothalamic neuron is dependant on this production. In the hypothalamus insulin

is signaled on mitochondrial reactive oxygen species (mROS) in order to progress energy metabolism producing ATP[5].

In this paper, LC-MS was done and investigating the peaks in the MS graphs using Tandem MS could identify the proteins from the secretome of rat hypothalmic insulin signaling[8].

Sixty individual proteins were identified and their functions and locations were referenced from the Protein BLAST program in NCBI website (www.pubmed.com)[17]. 8 proteins that are involved in energy metabolism in mitochondria and acting as oxygen transport were identified. Among them, creatine kinase B is a target molecule of reactive oxygen species, which is signaled by insulin in the hypothalamus[14]. Some enzymes in this secretome by insulin such as fructose bisphosphate aldolase and phosphoglycerate mutase are in the glycolytic pathway to produce ATP[15,16]. The branched chain aminotransferase initiates in muscle and yields Nicotinamide Adenine Dinucleotide (NADH) and 1,5-dihydro-flavin adenine dinucleotide (FADH2) which can also be utilized for ATP generation[9,11]. Others are linked with mitochondrial import and oxygen transport[2,13].

To confirm that these proteins were from the secretome, we did western blot and obtained clear bands in the expected sizes for each[3].

CONCLUSION

This experiment was intended to evaluate the proteins in insulin signaling on the rat hypothalamus. In conclusion, LC-MS analysis is a very useful method to reveal 8 new intermediates of insulin signaling in energy metabolism.

• Acknowledgement
This article is related to basic medical research in neurology. Therefore, the author has a difficulty to find a previous related article in Journal of Korean Neurosurgical Society.

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