59.6 Identification Of Brain Ischemia-Induced Changes In Protein Profiles By Quantitative Proteomics

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INTRODUCTION

- Molecular mechanisms that underlie brain ischemia-induced neuronal cell death are not well understood.
- Brain ischemia causes changes of multiple cellular pathways; a quantitative proteomic approach is most appropriate in revealing such changes.
- In this study, a labeling-free, quantitative proteomic system, the so-called *Protein Expression System* (Waters) was employed in analyzing proteins extracted from control and ischemic rat brains.

MATERIALS & METHODS

Brain ischemia model in rats

Transient focal brain ischemia was induced in male rats by 100-min occlusion of middle cerebral artery, followed by 24-hour reperfusion.

Protein extraction and tryptic digestion

Three sets of samples were prepared and analyzed.

Analysis-a	Cortices were homogenized in a buffer containing 50 mM Tris, pH 7.4, 10% glycerol, 1% TX-100, 2 mM EDTA plus protease inhibitors. One-hundred μg proteins were digested *in-solution* with trypsin, and subjected to MS analysis.
Analysis-b	Proteins from the afore-mentioned homogenates were first fractionated by SDS-PAGE, digested *in-gel* with trypsin, and then analyzed by MS.
Analysis-c	Cortices were processed as in Analysis-a, except that an additional 150 mM NaCl was added to the homogenization buffer.

Labeling-free, quantitative mass spectrometry

Protein Expression System

The system consists of a nanoACQUITY LC™ separation platform with a Micromass™ Q-Tof API US mass spectrometer (Waters). Accurate mass was obtained by using a nanolockspray source.

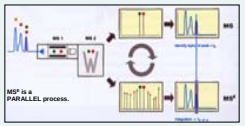


Fig. 1 Protein Expression System.
The collision cell alternates between low (MS) and elevated (MS^E) energy, fragmenting all of the precursors at the same time. The MS scans are used for quantitation while the MS^E scans are used for identification.
www.waters.com

Informatics

Protein Expression Informatics (Waters ProteinLynx Global Server version 2.2).

The differences between control and ischemic samples were determined by comparing chromatograms of each individual peptide. An Ischemic:Control (Isc:Ctr) ratio of ≤ 0.7 or ≥ 1.3 is defined as a difference. Each sample was analyzed in triplicates. Only proteins that were identified in at least two of the three runs are considered.

Bioinformatics

The function, cellular processes, pathways, signaling network and localization of identified proteins were assigned primarily by searching public databases (NCBI/PubMed, GO), and using the MetaCore™ platform (GeneGo).

RESULTS

Summary

At 24-hour reperfusion after focal brain ischemia, the predominant change of protein expression levels in the cortex was an up-regulation. This trend of change agrees well with published genomic studies on ischemic rodent brains.

The Isc:Ctr ratios of identified proteins range from 0.4 to 11.4.

Table 1 Ischemia-induced changes in protein expression.

	Total numbers	Up-regulated	Down-regulated
Analysis-a	521	142 (27.3%)	23 (4.4%)
Analysis-b	402	39 (10.3%)	17 (4.5%)
Analysis-c	146	45 (30.8%)	6 (4.1%)

Data evaluation

Chromatography – highly reproducible.

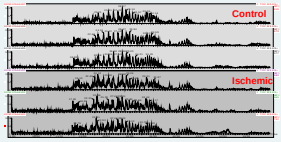


Fig. 2 LC chromatograms of triplicate runs of the same samples.

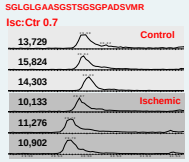


Fig. 3 Extracted ion chromatograms of a tryptic peptide in triplicate runs of the same samples.

Coverage – average 4 peptides per protein; even intra-molecule distribution of identified tryptic peptides (Fig. 4).

Quantitation – multiple entries per protein (Table 2).

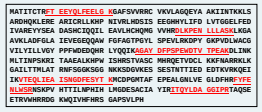


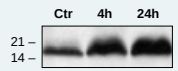
Fig. 4 Tryptic peptides (red) within a protein.

Table 2 Calcium calmodulin dependent protein kinase type II

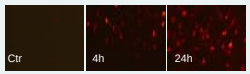
Peptide	Average Mass	Isc:Ctr
VTEQLIEAISNGDFESYTK	2144.043	0.52
FTEEYQLFEELGK	1632.783	0.45
ITQYLDAGGIPR	1303.701	0.28
DLKPENLLLASK	1340.779	0.68
FYFENLWSR	1261.602	0.38
AGAYDFPSPEWDTVTPEAK	2080.952	0.40
		0.42

Table 2 Isc:Ctr ratios of multiple peptides within a protein.

Validation



VILIP-1
calcium sensing protein
Isc:Ctr = 1.7 at 24h by MS



Arcadlin
synaptic reorganization
Ischemic brain-only

Fig. 5 Western blotting (left) and immunocytochemistry (right) analyses of ischemic brains.
The results validate mass spectrometric data.

Ctr: control; *4h* and *24h*: ischemic brains at 4-hour and 24-hour, respectively, reperfusions. The antibody against arcadlin was a kind gift of Dr. Kanato Yamagata of TMIN, Japan.

BIOINFORMATICS

Clustering

At our discretion, 165 ischemia-altered proteins identified in Analysis-a are clustered into 9 groups (Table 3, Fig. 6). Among them, secretory proteins and ion transporter proteins are two major up-regulated groups.

Table 3 Clustering of ischemia-altered proteins.

	Group total	Up-regulated	%	Down-regulated	%
Seretory	14	13	9.2	1	4.3
Ion pumps, transporters	14	13	9.2	1	4.3
Neurotransmission (channels)	5	5	3.5	5	21.7
Transmission/synapse/secretion activity	19	14	9.9	5	21.7
Mitochondrial	5	4	2.8	1	4.3
Neurogenesis/remodeling	6	6	4.2	0	0.0
Other membrane receptor/protease	10	9	6.3	1	4.3
Lipid metabolism	9	7	4.9	2	8.7
Transcription	10	7	4.9	3	13.0
Others	72	64	45.1	4	17.4

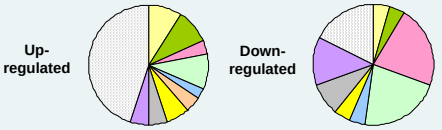
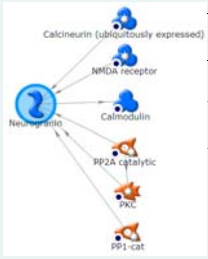


Fig. 6 Differential changes after ischemia.

Regulatory network identification

Using bioinformatic program MetaCore™, proteins that are known to function in the same pathways or processes are identified. Examples include: mitochondrial oxidative stress response, secretory vesicle trafficking, and synapse reorganization. Fig. 7 exemplifies a simple network involving neurogranin.



Identified in present study

Interactive proteins or homologues identified

Fig. 7 Examples of ischemia-regulated proteins of the same network.

Neurogranin is known to act as a "third messenger" substrate of protein kinase C-mediated molecular cascades during synaptic remodeling. It was down-regulated after ischemia in the present study. This network is retrieved with MetaCore™ using neurogranin as the root protein.

CONCLUSION

- Non-labeling quantitative proteomics is highly effective in revealing proteomic changes.
- Brain ischemia challenges expressions of proteins in the secretory pathway.

Acknowledgement

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