BEYOND PROTEIN PROFILING: MEASUREMENT OF CHANGES IN ISOFORMS AND PHOSPHORYLATION DUE TO STROKE

Martha D. Stapels[1], Grace M. Credo[1], Jennifer Fournier[1], Martin Gilar[1], John C. Gebler[1], Aaron White[2], Giuseppe Pignataro[2], Roger P. Simon[2], and An Zhou[2]
[1]Waters Corporation, Milford, MA and [2]Legacy Research, Portland, OR

OVERVIEW

Purpose:

To measure stroke-induced changes in brain proteomes

Methods:

LC/MS^E analysis[1] of tryptic digests
Label-free quantitative proteomics
Phosphopeptide enrichment with TiO_2

Results:

Observed changes in isoforms
Western blots verified MS results
Identified and quantified phosphopeptides

INTRODUCTION

There is more to the story of a stroke than the simple changes in relative protein concentration. Many proteins exist in multiple molecular forms under normal conditions, so observing changes in isoforms is necessary. In addition, phosphorylation is a critical modification in regulating stress response. Therefore, it is desirable to monitor the changes of this modification in response to stroke. Quantitative MS methods with low protein coverage may not generate the required level of information needed to observe these changes. In this study, we employed a label-free proteomic MS approach[2] for the quantitative analysis of isoform and phosphorylation changes in ischemic rat and mouse brains as compared to controls. Western blot analyses were used to validate the observations.

METHODS

Sample Preparation:
- Stroke induced by occluding the middle cerebral artery
- Control and stroke brains pooled and homogenized
- Proteins solubilized with Rapigest™
- Reduction, alkylation, and tryptic digestion performed in-solution

Phosphopeptide Isolation
- Micro elution plate packed with TiO_2
- Enhancer™ used to reduce non-specific binding in the loading step
- Elution with 50 µL of 0.3 N ammonium hydroxide in water

INSTRUMENTATION

Samples were analyzed using a Waters nanoACQUITY UPLC™ system and a Q-Tof API US.

LC method:
- 180 µm by 20 mm trapping column with 5 µm Symmetry® C_{18}
- 75 µm by 100 mm analytical column with 1.7 µm BEH C_{18}
- 60 minute gradient from 5-30% ACN at 300 nl/min

MS Method:
- 0.6 sec MS scan with CE at 10 eV
- 0.6 sec MS^E scan with CE stepped from 23-33 eV
- Lockmass channel sampled every 30 sec

Data Analysis:
- Protein Lynx Global Server (PLGS) 2.2 with Expression Informatics
- Peptides tracked across triplicate injections
- Ions detected in 2 of 3 replicates chosen for databank search

RESULTS

Identification of Peptides

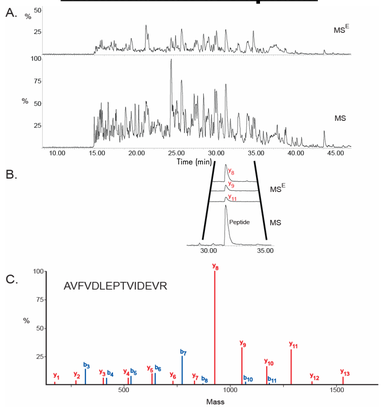

Figure 1: A. Peptides fragmented with alternating low and elevated collision energy. B. Extracted ion chromatograms show how precursors and fragment ions aligned by retention time. C. Database search result for this peptide from α-tubulin.

Quantitation of Peptides

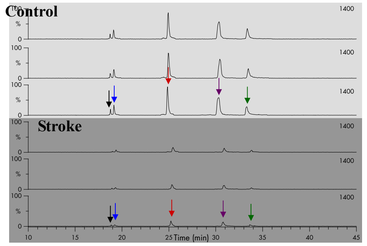

Figure 2: Extracted ion chromatograms of five peptides identified and quantified from one protein from the digestion of control and stroke mouse brains. Peptides were tracked across all injections by PLGS software. This protein was significantly underexpressed after stroke.

ISOFORM CHANGES

- Splice variants and isoforms cause complexity
- Multiple peptides should be measured for each protein in a quantitative proteomic experiment

Key:
Significantly Overexpressed (Probability 0.95 or more)
Significantly Underexpressed (Probability 0.05 or less)

14-3-3 Epsilon

Mass	Peptide	Ratio	Probability
816.4151	LAEQAER	1.6	1
907.524	NLLSVAYK	1.4	1
917.527	IISSIEQK	1.2	0.83
1189.662	DSTLIMQLLR	0.5	0
1194.603	EAAENSLVAYK	1.9	1
1237.647	HLIPAANTGESK	1.2	0.96
1256.596	YLAEFATGNDR	1.3	0.97
2087.966	AAFDDAIAELDTLSEESYK	2.0	1

MDDREDLVYQAK**LAEQAER**YDEMVESMKKVAGMDVELTVEER**NLLSVAYK**NVIGARRASWRI**ISSIEQK**EENKGGEDKLKMIREYRQMVETELKLICCDILDVLDK**HLIPAANTGESK**VFYYKMKGDYHRY**LAEFATGNDR**K**EAAENSLVAYK**AASDIAMTELPPTHPIRLGLALNFSVFYYEILNSPDRACRLAK**AAFDDAIAELDTLSEESYK**D**STLIMQLLR**DNLTLWTSDMQGDGEEQNKEALQDVEDENQ

33% Coverage, 8 peptides
Protein Expression ratio: 1.5 with 21% CV
DSTLIMQLLR in 7 isoforms, not included in ratio

STOP (Stable Tubule Only Polypeptide) Protein

Mass	Peptide	Ratio	Probability
774.434	VKPTSDK	1.0	0.51
829.459	AVADALNR	1.0	0.58
897.510	DSVPLAPAK	0.8	0.1
1053.597	DLGPVAPASVK	1.1	0.62
1082.515	ATGPAPGPSGDR	0.9	0.17
1107.586	DPGPTAPDPLK	1.2	0.79
1108.593	GQDPVVPAPTK	1.9	0.99
1122.660	GQDPIIPALAK	1.2	0.84
1123.607	GQGPAVQEPLK	1.2	0.71
1128.604	DQGAVLLGPMK	0.9	0.47
1130.575	NEAPVASESVK	1.5	1
1138.589	DQGPMVPGLPK	0.9	0.31
1177.596	DQNASIMASLK	1.6	0.98
1192.663	AQSPLLPEPLK	1.2	0.74
1219.675	NQDPVIPVPLK	0.9	0.35
1253.625	DPEGAGGAGVPAAGK	0.8	0
1313.724	VEKPSVQSSKPK	1.1	0.68
1344.634	EEVTSTVSSSYR	1.2	0.82
1368.696	DQSFPAPAPTPLK	0.9	0.15
1812.903	AVAIETQPAQGESDAVAR	Control only	
2021.962	SGLGLGAASGSTSGSGPADSVMR	0.7	0

MAWPCITRACCIARFWNQLDKADIAVPLVFTKYSEATEHPGAPPQPPAPPQPGLAPPSR**AVAIETQPAQGESDAVAR**A**TGPAPGPSGDR**ETAAAPGR**SGLGLGAASGSTSGSGPADSVMR**QDYRAWKVQRPEPSCRPRSEYQPSDAPFERETQYQKDFRAWPLPRRGDHPWIPKPVQIPATSQPSPPVLGMPKRRPQSQERGPIQLSADAR**DPEGAGGAGVPAAGK**ASGADQRDTRRKAGPAWMVTRTEGHEEKPLPPAQSQTQEGGPAAGKASGADQRDTRRKAGPAWMVTRTEGHEEKPLPPAQSQTQEGGPAAGKASGADQRDTRRKAGPAWMVTRTEGHEETPLPPAQSQTQEGGPAAGKASGADQRDTRRKAGPAWMVTRTEGHEETPLPPAQSQTQEGGPAAGKASGADERDTRRKAGPAWMVRRSEGHEQTTAAHAQGTGPEGGKGR**AVADALNR**QIR**EEVTSTVSSSYR**NEFRAWTDIKPVKPIKAKPQYKPPDDKMVHETSYSAQFKGEASKPTTADNKVVDRRRIRSLYSEPFKESPK**VEKPSVQSSKPK**KTSTSQKPLRKAKDKQVASGQAAKKKTTESPSATKPDDKEQSKEMNNKLAEAKESR**VKPTSDK**NQGPVAKEPHKDQGPVAPGLPK**GQGPAVQEPLKDQGPMVPGLPK**DQAPVVPGSLKGQSPTAPGPPK**DQGAVLLGPMKDLGPVAPASVK**DQDHMASELLKNK**DSVPLAPAKAQSPLLPEPLK**NQSPVVPARAK**DQSFPAPAPTPLK**DPGPVIPEPEKDGAPMVPERRK**DQNASIMASLKNEAPVASESVK**NQGLGGPEPAKDTGTDLKGHGSVFVAPVKSQGPVVPEPTK**GQDPIIPALAK**DQGPILPEPPKNQGPPVVLGPIK**NQDPVIPVPLK**G**QDPVVPAPTK**D**PGPTAPDPLK**SQGPRGPQLPTVSPSPPVMIPTVPHAEYIEGSP

25% Coverage, 21 peptides
Protein Expression ratio: 1.0 with 24% CV
N-terminus ↓ and C-terminus ↑

Western Blot

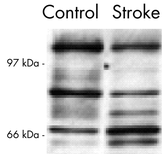

Figure 3: Western blot of STOP protein confirms that overall level remains constant, but molecular forms change during stroke. There are at least 5 STOP-related proteins, some that change during stroke.

PHOSPHOPEPTIDES

- Relative concentration of phosphopeptides were measured in the original tryptic digests
- Phosphopeptide identifications were verified by analyzing enriched samples

Syntaxin 1B

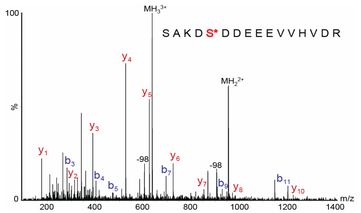

Figure 4: MS^E spectrum of a phosphopeptide from syntaxin 1B.

Mass	Peptide	Ratio	Probability
1035.574	LAIFTDDIK	1.20	0.89
1073.556	QALNEIETR	0.84	0.11
1490.758	QHSAILAAPNPDEK	0.84	0.06
1615.792	AIEQSIEQEEGLNR	0.98	0.38
1646.779	FVEVMTEYNATQSK	0.86	0.1
1696.761	TTTNEELEDMLESGK	1.02	0.56
1909.779	SAKDS*DDEEEVVHVDR	0.56	0

MKDRTQELR**SAKDS*DDEEEVVHVDR**DHFMDEFFEQVEEIRGCIEKLSEDVEQVK**QHSAILAAPNPDEK**TKQELEDLTADIKKTANKVRSKLK**AIEQSIEQEEGLNR**SSADLRIRKTQHSTLSRK**FVEVMTEYNATQSK**YRDRCKDRIQRQLEITGRT**TTTNEELEDMLESGKLAIFTDDIK**MDSQMTK**QALNEIETR**HNEIIKLETSIRELHDMFVDMAMLVESQGEMIDRIEYNVEHSVDYVERAVSDTKKAVKYQSKARRKKIMIIICCVVLGVVLASSIGGTLGL

Protein Expression ratio: 1.0 with 15% CV
31% Coverage with 7 peptides
Phosphopeptide Expression ratio: 0.56
Overall protein level unchanged, but phosphorylation level may have decreased due to stroke

Synapsin-1

Mass	Peptide	Ratio	Probability
945.550	AETIRSLR	1.7	1
1048.457	QAS*QAGPGPR	1.1	0.6
1052.573	QASISGPAPPK	2.1	1
1326.661	TYATAEPFIDAK	2.0	1
1453.762	EMLSSTTYPVVVK	2.3	1
1496.828	QGPPQKPPGPAGPIR	2.4	1
1561.806	GSHSQTPSPGALPLGR	2.4	1
1635.869	VLLVIDEPHTDWAK	3.7	1
1724.796	TNTGSAMLEQIAMSDR	1.7	1
2000.002	VDNQHDFQDIASVVALTK	3.3	1

MNYLRRRLSDSNFMANLPNGYMTDLQRPQPPPPPPSAASPGATPGSAAASAEASTAAPVASPAAPSPGSSGGGGFFSSLSNAVKQTTAAAAATFSEQVGGGSGGAGRGGAAAR**VLLVIDEPHTDWAK**YFKGKKIHGEIDIKVEQAEFSDLNLVAHANGGFSVDMEVLRNGVKVVRSLKPDFVLIRQHAFSMARNGDYRSLVIGLQYAGIPSVNSLHSVYNFCDKPWVFAQMVRLHKKLGTEEFPLIDQTFYPNHK**EMLSSTTYPVVVK**MGHAHSGMGKVK**VDNQHDFQDIASVVALTKTYATAEPFIDAK**YDVRVQKIGQNYKAYMRTSVSGNWK**TNTGSAMLEQIAMSDRYK**LWVDTCSEIFGGLDICAVEALHGKDGRDHIIEVVGSSMPLIGDHQDEDKQLIVELVVNKMTQALPRQRDASPGRGSHSQTPSPGALPLGRQTSQQPAGPPAQQRPPPQGGPPQPGPGPQRQGPPLQQRPPPQGGQQHLSGLGPPAGSPLPQRLPSPTAAPQQSASQATPMTQGQGRQSRPVAGGPGAPPAARPPASPSPQRQAGPPQATR**QASISGPAPPK**VSGASPGGGQR**QGPPQKPPGPAGPIR**G**QAS*QAGPGPR**TGPPTTQQPRPSGPGPAGRPTKPQLAQKPSQDVPPPIIAAAGGPPHPQLNKSQSLTNAFNLPEPAPPRPSLSQDEVK**AETIRSLR**KSFASLFSD

Protein Expression ratio: 2.4 with 28% CV
18% Coverage with 10 peptides
Phosphopeptide Expression ratio: 1.1
Overall protein level ↑, except phosphopeptide

CONCLUSIONS

- **This work illustrates the depth of information that can be obtained with label-free proteomics**
- **High sequence coverage enables measurement of changes in isoforms**
- **Selective phosphopeptide enrichment yields the identification and quantitation of phosphopeptides**

Acknowledgements

The authors wish to thank Drs. James Murphy and Keith Fadgen for assistance with instrumentation. This research was partially funded by the NIH and the Legacy Good Samaritan Foundation.

References

1. Bateman R *et al, J Am Soc Mass Spectrom.* 2002;13:792-803.
2. Silva JC *et al, Anal Chem.* 2005;77:2187-2200.

720001730EN

TO DOWNLOAD A COPY OF THIS POSTER VISIT WWW.WATERS.COM/POSTERS