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Nascent proteomes of ischemic-injured and ischemic-tolerant neuronal cells

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Abstract

In a recent study on ischemic rodent brains, we quantitatively characterised and compared brain proteomes under ischemic-preconditioned or injured or tolerant conditions. We discovered an enriched presence of repressive transcriptional regulator proteins with essential roles as epigenetic regulators in ischemic-tolerant brains (Stapels et al., 2010). We further showed their robust, dynamic and differential changes under different ischemic conditions in brains and in cultured neuronal cells. In the present work, using neuronal cell cultures, we aimed to characterise the nascent proteome, the proteome that presents early when the cells receive an ischemic insult. These would be the proteomic changes of newly synthesised proteins. Identification of effectors of this phase of response to ischemia bears the best promise of identifying therapeutic targets for treating acute stroke when patients present to hospital. We compared these nascent proteomes across different ischemic conditions using bioinformatic tools.

Keywords

nascent proteome; ischemic injured; ischemic tolerant; neuronal cells; quantitative proteomics; epigenetic regulators; click chemistry; newly synthesised proteins; bioinformatics; computational biology

1 Introduction

We hypothesise that endogenous neuroprotection against ischemic brain injury is induced by epigenetic regulator proteins rendering genomic reprogramming and proteomic reconfiguration, and that this regulation is achieved by quantitative alteration of the components of epigenetic regulator protein complexes. Such changes result from alterations in biosynthesis of multiple proteins. To test the hypothesis, among stroke models, demands technologies that can:

- quantitatively describe and compare changes of multiple proteins simultaneously (quantitative proteomics)

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- distinctively determine changes in new protein biosynthesis proteome-wide (*nascent proteomes*).

Both are technically extremely challenging with the latter practically impossible until very recently.

Traditionally, studies of protein biosynthesis rely on metabolic labelling with radioactive isotope-labelled amino acid(s), which is prohibitive in many experimental settings and ineffective in high throughput proteomic analyses. 'Click chemistry' is an approach in which two small molecules (e.g., an azide and an alkyne) can be efficiently and readily joined. In the latest development of Click protein biochemistry, an azide-containing amino acid is incorporated into newly synthesised proteins, which then can be detected or purified by various alkyne-conjugated probes. This new approach, when combined with high throughput quantitative Mass Spectrometry (MS), makes it possible to characterise newly synthesised proteins proteome-wide (Dieterich et al., 2007; Deal et al., 2010).

Here we identify and compare nascent proteomes in cultured neuronal cells that are subjected to three different ischemia-induced conditions: ischemic-preconditioned, ischemic-injured and ischemic-tolerant. The inclusion of all three conditions allows identification of condition-specific proteomic changes.

2 Methods

2.1 Simulated ischemia in neuronal cultures

Simulated ischemia was induced in cultured neuronal cells (differentiated, mouse brain-derived NS20Y cells) by Oxygen-Glucose Deprivation (OGD) for defined periods of time. Our previous work (Stapels et al., 2010) has established that, in NS20Y cells, 120 min OGD causes 40–60% cell death when examined 24 h after the OGD (i.e., injurious), whereas 30 min OGD does not substantially injure but will prepare (precondition) the cells to become resistant to 120 min OGD, a condition termed ischemic tolerance. In this work, three groups of cells were subjected to 30 min OGD (preconditioned) or 120 min OGD (injurious) or 120 min OGD preceded by 30 min OGD (tolerant). Control cells underwent incubations of matched periods of OGD treatment times in the control medium (Stapels et al., 2010).

2.2 Click metabolic labelling

After OGD treatments (30 min preconditioning OGD) or 120 min injurious OGD or 120 min injurious OGD preceded by 30 min preconditioning OGD (Stapels et al., 2010), cells were incubated in complete media under aerobic condition for 1 h to allow protein synthesis recovery, followed by a 30 min incubation in a methionine-free medium to deplete the intracellular methionine pool. Then cells were incubated for 2 h with 50 μ M L-Azidohomoalanine (AHA) (labelling), an azide-containing surrogate amino acid replacing methionine. At the end of labelling, cellular proteins were extracted. AHA-labelled, newly synthesised proteins (the nascent proteome) were separated from previously synthesised proteins (not labelled by AHA) by Click reaction with a commercial kit (Invitrogen, Carlsbad, CA).

2.3 Quantitative MS analyses of AHA-labelled proteins

AHA-labelled proteins from 3 independent cultures were pooled, protein content in pooled samples assayed, samples denatured with 0.05% RapiGest (Waters Corp, Milford, MA), reduced with DTT, alkylated with iodoacetamide, and then digested with 0.05 μ g/ μ L trypsin. Following removal of detergent using the method recommended by the manufacturer, 25 μ g portions of the digests were analysed by LC-MS/MS using an Agilent 1100 series capillary LC system (Agilent Technologies Inc., Santa Clara, CA) and an LTQ Velos linear ion trap

mass spectrometer (Thermo Scientific, San Jose, CA). Electrospray ionisation was performed using a Captive Spray source (Michrom BioResources, Inc., Auburn, CA) at 10 $\mu\text{L}/\text{min}$ flow rate and 1.4 kV setting. Samples were applied for 5 min at 20 $\mu\text{L}/\text{min}$ to a trap cartridge (Michrom BioResources), and then switched onto a 0.5×250 mm Zorbax SB-C18 column with 5 μm particles (Agilent Technologies Inc.) using a mobile phase containing 0.1% formic acid and 7–30% acetonitrile gradient over 200 min. Data-dependent collection of MS/MS spectra used the dynamic exclusion feature of the instrument's control software (repeat count equal to 1, exclusion list size of 100, exclusion duration of 30 s, and exclusion mass width of -1 to $+4$) to obtain MS/MS spectra of the 5 most abundant parent ions following each survey scan from m/z 350–2000. The tune file was configured with a 275°C capillary temperature, no averaging of microscans, a maximum inject time of 50 msec and AGC target of 3×10^4 in MS mode and maximum ion time of 100 msec and AGC target of 1×10^4 in MS2 mode. Peptides were identified using the program Sequest (Thermo Scientific) using a mouse-only version of the UniProt database (Jain et al., 2009) amended by adding sequence-reversed entries. The search used trypsin specificity, 57 Da static mass increase for cysteines due to alkylation, differential mass increase of 16 for oxidised methionines, 2.0 Da parent ion tolerance, 1 Da fragment ion tolerance, and monoisotopic mass calculation. Matches to sequence reversed entries were used to estimate peptide and protein False Discovery Rates (FDRs) as previously described (Wilmarth et al., 2009). Peptides were filtered at roughly 1% FDR, and protein lists were compiled using an in-house Python program to perform peptide-subset-removal parsimony filtering. At least 2 distinct fully tryptic peptide sequences were required to match each protein identification per sample. This resulted in 524 identified proteins with 12 matches to sequence reversed entries (2.2% protein FDR).

Duplicate MS runs were performed for each of the following 4 samples (each a pool of 3 independent cultures as noted earlier):

- ischemic (OGD-treated)-preconditioned
- ischemic-injured
- ischemic-tolerant
- control.

For each identified protein, spectral counts from the duplicate runs were averaged, and used to establish ratios between an ischemic sample and the control. A $\geq 30\%$ difference between ischemic and control samples was defined as an ischemia-induced change (Stapels et al., 2010). Hence, for each ischemic condition, two datasets were established consisting up- and down-regulated proteins, respectively.

2.4 Bioinformatic analyses of regulated proteins

Bioinformatic analysis was performed on proteins that showed a change in abundance in ischemic (OGD-treated) cells with the assistance of the MetaCore program (GeneGo, Inc., St. Joseph, MI. www.genego.com). Primarily, two types of analyses were performed:

- *Enrichment Analysis* for Biological Processes (GO terms) and Process Networks (GeneGo terms) that can be recognised with up- or down-regulated proteins, for each individual dataset
- *Compare Experiments Analysis* to determine the overlap, or the lack of it (unique), among the datasets of three ischemic conditions.

Briefly, *Enrichment* analyses were performed using proteins (by their gene names) that have been experimentally verified to be associated with particular ontologies as background

(built-in in the MetaCore). The statistical relevance (p -value) of a dataset to a GO term was calculated with consideration of the size of the background, the dataset and the particular proteins/genes, as determined by the MetaCore. In *Compare Experiments Workflow* analyses, cross-dataset comparisons were performed using 'Network objects' (i.e., the name of a protein and its known attributes, as defined by the MetaCore) associated with a dataset. For the definition of 'Network Object' and its use, see Stapels et al. (2010).

3 Results

3.1 Effectiveness of AHA labelling in cultured neuronal cells

We first tested the AHA labelling on cultured neuronal cells. Figure 1 shows that differentiated NS20Y cells were effectively labelled with AHA, hence allowing proteomic analyses of newly synthesised proteins.

3.2 General description of proteomic dataset

A total of 524 proteins from cell extracts isolated with the click reaction were identified and quantified by MS. Table 1 lists numbers of proteins that demonstrated a change in abundance, under different ischemic conditions. Figure 2 presents Venn diagrams of up- and down-regulated proteins under the three different ischemic conditions.

3.3 Functional characteristics of the nascent proteomes of ischemic neuronal cells

Bioinformatic analyses were performed, with the assistance of the MetaCore program, for proteins that were up or down regulated, as a means of deciphering the cellular processes and networks that may mediate ischemic injury or neuroprotection.

Figure 3 demonstrates intersections of datasets involving proteins that were regulated. Tables 2-5 list the most significant Biological Processes (by gene ontology (GO) terms), as well as 'Process Networks' (a term used by the MetaCore program), that are associated with up- and down-regulated proteins under each ischemic condition. Tables 2 and 3 include proteins that show a change under more than one condition, whereas Tables 4 and 5 are from proteins that changed only (uniquely) under a specific ischemic condition.

It was evident that, for cellular processes and networks that are associated with proteins either up or down regulated, there was overlapping expression among ischemic-preconditioned, ischemic-injured and ischemic-tolerant neuronal cells. For example, enriched translational regulation processes were seen with all three ischemic conditions. At the same time, processes and networks that were unique to a specific ischemic condition were also revealed by bioinformatic analyses.

Worth-noting is the identification of chromatin and nucleosome assembly processes associated with proteins that were down regulated only in ischemic-injured cells; Table 6 lists those proteins. In this particular dataset, proteins that contributed to the recognition of chromatin/nucleosome regulation processes include chromobox protein homologue 5 (Cbx5), transcription activator BRG1 (Smarca4), and two histone proteins (histone H1.3 (Hist1h1d) and histone H2B type 1-K (Hist1h2bk)), as identified by the MetaCore program. In ischemic-injured or ischemic-tolerant cells, these proteins were either up regulated or unchanged.

Taken together, the present results demonstrate an effective incorporation of AHA into cultured neuronal cells and differential changes of the nascent proteomes under different ischemic conditions.

4 Discussion and conclusion

In our recently published proteomic characterisation of ischemic rodent brains (Stapels et al., 2010), we demonstrated that, at the time when the phenotype of ischemic injury or ischemic tolerance is fully developed and exhibited (matured), there is an enriched presence of epigenetic gene repressor proteins in ischemic-tolerant brains, and a recognition of chromatin and nucleosome remodelling processes associated with those transcriptional regulator proteins. Such proteins include polycomb group (PcG) proteins.

The present work aimed to characterise the nascent proteomes in neuronal cells at the onset of development of above-mentioned ischemia-induced conditions, and to use bioinformatic tools to identify significant cellular processes associated with those conditions. We hypothesised that the development of ischemic-injured or ischemic-tolerant phenotypes involves changes in biosynthesis of new proteins, independent of transcriptional regulation. This notion is suggested and supported by the observation that

- mRNA levels of PcG proteins undergo dynamic changes during development (Vogel et al., 2006)
- PcG protein levels increase under tolerant conditions (Stapels et al., 2010; Piper et al., 2010), and the induction of ischemic tolerance depends on new protein synthesis (Barone et al., 1998)
- temporal order of changes of PcG protein levels and biosynthesis under ischemic-tolerant conditions reveal a robust, early up regulation, without an increase in their gene transcripts (Piper et al., 2010).

Datasets from the present proteomic study revealed changes in abundance of proteins involved in translational processes under all three ischemic conditions examined. This is not surprising yet remarkable in that these were the nascent proteomes consisting of newly synthesised proteins. This suggests that when cells were subjected to different ischemic conditions, a common response is a regulation in protein synthesis, at a time when transcriptional regulations may have not occurred or may not be the prominent mechanism. What distinguishes the three nascent proteomes (ischemic-preconditioned, ischemic-injured and ischemic-tolerant) is a decrease in the abundance of several proteins associated with chromatin/nucleosome assembly processes in ischemic-injured neuronal cells (Tables 5 and 6). Of particular interest is the injury-only down regulation of chromobox protein homologue 5 and transcription activator BRG1 (gene Smarca4). Both proteins are known to interact with histone proteins and are involved in chromatin modelling. Naito et al. (2009) has reported a role of BRG1 in renal ischemic response. Given that these two proteins were up regulated in ischemic-preconditioned and ischemic-tolerant cells (Table 6), it would be of interest to further investigate their potential neuroprotective roles in brain ischemia. Equally interesting, or even more so, is the possible, *early* involvement of epigenetic regulation in the development of the injurious or tolerant phenotype, as suggested by differential changes of the above-mentioned chromatin/nucleosome remodelling proteins under different ischemic conditions. The kinetics of the nascent proteome during the development of the injurious or tolerant phenotype, over time, remains to be determined. It is not known how similar or different the nascent proteomes may be to or from the whole proteomes (without separation of the nascent and previously synthesised proteins), under the afore-studied neuronal ischemic conditions. Such comparisons can be made only when both the nascent and the whole proteomes are characterised using the same experimental settings.

Last but not the least, the present proteomic data remain to be validated, for example, by immunochemical means using specific antibodies. The acceptance of quantitative MS findings was of relative poor reliability due to the low spectral counts for many of the

proteins and randomness of the selection process for MS/MS scans. Furthermore, the distribution of AHA-labelled proteins in different subcellular compartments remains unknown. While the present experimental protocols were beneficial in obtaining a preliminary comparison of multiple nascent proteomes of ischemic neuronal cells, a more thorough and accurate characterisation will rely on analyses of fractionated cellular components and with increased pre-MS sample fractionation steps and data screening stringency. Bioinformatic analyses that were performed on the present datasets were also limited, in that only Biological Processes and GeneGo Process Networks that are associated with particular datasets were reported, without any screening at our discretion; many of those terms are too general to be informative. Upon the validation of the present MS results, future analyses will focus on network building and pathway map construction for each specific neuronal ischemic condition.

In summary, by combining the Click chemistry-based metabolic labelling of live cells, quantitative MS and bioinformatics, the present work provides a preliminary characterisation of the nascent proteomes of neuronal cells under multiple ischemic conditions. Future studies will be directed at characterisation of nascent neuronal proteomes for the three ischemic conditions, at different post-ischemia time points, with subcellular fractionation in sample preparations, and with increased MS run repeats and data screening stringency.

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Biographical notes

An Zhou is an Associate Professor of Neuroscience at the Neuroscience Institute of the Morehouse School of Medicine. She received her PhD Degree in Physiology and Biochemistry in 1991 from the University of Copenhagen. Her research interests include proteomics of neuronal disorders, epigenetic regulation of ischemic tolerance, and biosynthesis of proteins and peptides in neuroendocrine cells. She has published 30 research articles including a recent paper on Science Signalling (Stapels et al., 2010), which reports characteristics of brain proteomes under different ischemic conditions and a gene repressor-mediated mechanism in brain ischemic tolerance.

Roger P. Simon is a Professor of Medicine and Neurobiology at the Morehouse Medical School. He received his MD Degree from Cornell and neurology training from UCSF. He published, in Science, with Brian Meldrum, the first description of glutamate blockade for brain ischemia. His studies of ischemic tolerance include the first descriptions of: tolerance to focal ischemia (Simon, 1993), epileptic tolerance (Sasahira, 1995), the genomic response to tolerance (Stenzel-Poore, 2003), the proteome of tolerant brain, with An Zhou (Stapels et al., 2010) and regulation of micro RNAs by preconditioning ischemia, with Julie Saugstad (Lusardi, 2010).

Larry David is a Professor of Biochemistry and Molecular Biology and the Director of the Proteomics Shared Resource at the Oregon Health and Science University in Portland Oregon. He received his PhD Degree in 1986 in Biochemistry from Oregon Health and Science University and his research interest is in understanding how age-related changes in crystallins, the major proteins of the lens, contribute to cataract. For the last 16 years, he has used mass spectrometry to study proteins and applies this experience to improve methods to both identify and quantify proteins and their modified forms in complex mixtures.

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Figure 1.

Dosage-dependent and time-dependent incorporation of AHA into cultured neuronal cells. Differentiated NS20Y cells were incubated under the following conditions: Control, in absence of AHA; AHA-1 and AHA-2, 25 μ M and 50 μ M AHA, respectively, for 4 h; AHA-3, 50 μ M AHA for 2 h. Top row: AHA-labelled cellular proteins were detected and visualised with an Alexa488-conjugated alkyne probe (2 μ M); bottom row: DAPI staining to reveal nuclei.

Figure 2.

Venn Diagrams of proteins that changed under different ischemic conditions
PC: preconditioned; INJ: injured; TOL: tolerant.

Figure 3.

Dataset intersections

Intersections among the three datasets (PC: preconditioned; INJ: injured; TOL: tolerant) are demonstrated by the numbers of “network objects” that are unique to a particular ischemic condition (colour-filled), associated with two of three conditions (open) or all three conditions (dashed line).

Top: up-regulated proteins; bottom: down-regulated proteins.

Table 1

Numbers of regulated proteins

	<i>PC</i>	<i>INJ</i>	<i>TOL</i>
Up regulated	117	240	235
Uniquely up regulated*	31	31	32
Down regulated	146	181	170
Uniquely down regulated*	31	37	41

PC: preconditioned; INJ: injured; TOL: tolerant

* Uniquely regulated: proteins that showed a change only under a specific condition.

Table 2

Bioinformatic characteristics of up regulated proteins

	<i>GO biological processes</i>	<i>p-value</i>	<i>GeneGo process networks</i>	<i>p-value</i>
PC	Translation	5.14E-32	Translation_Translation initiation	1.47E-19
	Translational elongation	4.52E-31	Translation_Elongation-Termination	1.53E-19
	Metabolic process	4.3E-24	Cell cycle_Mitosis	2.98E-05
	Cellular metabolic process	5.12E-24	Cytoskeleton_Spindle microtubules	6.95E-04
	Primary metabolic process	7.62E-22	Transcription_Chromatin modification	1.79E-03
	Cellular process	3.07E-19	Cell cycle_G2-M	1.89E-03
	Macromolecule metabolic process	3.8E-19	Cell cycle_S phase	4.20E-03
	Cellular macromolecule metabolic process	1.37E-18	Cytoskeleton_Intermediate filaments	4.91E-03
	Gene expression	1.37E-16	Protein folding_Folding in normal condition	5.66E-03
INJ	Cellular protein metabolic process	6.99E-16	Transcription_mRNA processing	6.18E-03
	Cellular process	6.17E-43	Translation_Translation initiation	1.09E-19
	Cellular metabolic process	8.69E-43	Translation_Elongation-Termination	1.56E-14
	Metabolic process	7.31E-37	Cytoskeleton_Regulation of cytoskeleton rearrangement	3.70E-14
	Primary metabolic process	5.71E-36	Cytoskeleton_Intermediate filaments	9.68E-12
	Translation	4.72E-31	Protein folding_Folding in normal condition	4.73E-08
	Cellular protein metabolic process	2.05E-26	Cell cycle_G2-M	4.31E-07
	Translational elongation	4.68E-25	Protein folding_Response to unfolded proteins	5.48E-07
	Protein metabolic process	8.1E-25	Cytoskeleton_Actin filaments	7.05E-07
TOL	Cellular macromolecule metabolic process	1.33E-23	Cell cycle_Meiosis	1.08E-05
	Macromolecule metabolic process	2.15E-23	Immune response_Phagosome in antigen presentation	3.36E-05
	Translational elongation	1.41E-47	Translation_Translation initiation	3.80E-29
	Translation	2.78E-47	Translation_Elongation-Termination	9.23E-29
	Cellular process	6.34E-31	Cytoskeleton_Intermediate filaments	1.73E-10
	Cellular metabolic process	5.03E-29	Cytoskeleton_Regulation of cytoskeleton rearrangement	8.21E-09
	Cellular protein metabolic process	5.8E-29	Protein folding_Folding in normal condition	2.13E-06
	Metabolic process	7.86E-28	Protein folding_Protein folding nucleus	1.13E-04
	Primary metabolic process	2.34E-27	Cell cycle_Mitosis	7.42E-04
TOL	Protein metabolic process	2.46E-26	Cell cycle_G2-M	8.59E-04

<i>GO biological processes</i>	<i>p-value</i>	<i>GeneGo process networks</i>	<i>p-value</i>
Cellular macromolecule metabolic process	1.66E-17	Transcription_Chromatin modification	1.76E-03
Macromolecule metabolic process	3.55E-17	Protein folding_Response to unfolded proteins	1.98E-03

P values (applicable to Tables 2-5): the statistic relevance of identified matches between a dataset and a process. The values were calculated by the MetaCore program according to the proteins and the number of proteins in a dataset. The 10 processes with the smallest *P* values are listed.

Table 3

Bioinformatic characteristics of down-regulated proteins

	<i>GO biological processes</i>	<i>p-value</i>	<i>GeneGo process networks</i>	<i>p-value</i>
PC	Cellular process	2.91E-26	Cytoskeleton_Regulation of cytoskeleton rearrangement	2.44E-06
	Cellular metabolic process	2.84E-24	Translation_Translation initiation	6.13E-06
	Primary metabolic process	9.66E-21	Protein folding_Folding in normal condition	3.24E-05
	Metabolic process	6.40E-20	Translation_Elongation-Termination	3.91E-05
	Cellular nitrogen compound metabolic process	8.37E-17	Transcription_Chromatin modification	6.08E-05
	Nitrogen compound metabolic process	2.04E-16	Cell cycle_G2-M	0.000184
	Cellular macromolecule metabolic process	3.34E-16	Cytoskeleton_Actin filaments	0.000195
	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1.34E-15	Transcription_mRNA processing	0.000385
	Cellular component biogenesis	1.70E-14	DNA damage_DBS repair	0.000832
	Translation	3.66E-14	Protein folding_Response to unfolded proteins	0.001096
INJ	Cellular process	2.81E-29	Translation_Translation initiation	2.21E-08
	Cellular metabolic process	1.57E-28	Cell cycle_G2-M	7.02E-08
	Primary metabolic process	5.74E-26	Transcription_mRNA processing	2.89E-07
	Metabolic process	2.57E-25	Cytoskeleton_Regulation of cytoskeleton rearrangement	3.50E-07
	Cellular macromolecule metabolic process	1.56E-19	Cytoskeleton_Intermediate filaments	1.11E-06
	Macromolecule metabolic process	8.75E-19	Protein folding_Response to unfolded proteins	1.89E-06
	Organelle organisation	3.97E-18	Transcription_Chromatin modification	3.35E-06
	Translation	1.65E-17	Cell cycle_Mitosis	6.47E-06
	Cellular protein metabolic process	3.26E-15	Protein folding_Folding in normal condition	8.73E-06
	Cellular component organisation	4.50E-14	Translation_Elongation-Termination	9.93E-06
TOL	Cellular metabolic process	8.66E-35	Translation_Translation initiation	2.23E-08
	Primary metabolic process	3.28E-31	Cell cycle_Mitosis	1.47E-06
	Metabolic process	3.84E-31	Cell cycle_G2-M	1.66E-06
	Cellular process	4.97E-31	Cytoskeleton_Regulation of cytoskeleton rearrangement	1.94E-06
	Cellular macromolecule metabolic process	3.11E-20	Transcription_mRNA processing	2.01E-06
	Cellular nitrogen compound metabolic process	2.27E-19	Cytoskeleton_Intermediate filaments	2.89E-06
	Macromolecule metabolic process	3.66E-19	Protein folding_Response to unfolded proteins	5.83E-06

<i>GO biological processes</i>	<i>p-value</i>	<i>GeneGo process networks</i>	<i>p-value</i>
Nitrogen compound metabolic process	7.82E-19	Protein folding_Folding in normal condition	1.58E-05
Cellular component biogenesis	3.26E-17	Protein folding_ER and cytoplasm	2.04E-05
Organelle organisation	3.73E-17	Transcription_Chromatin modification	3.14E-05

Table 4

Bioinformatic characteristics of proteins up regulated only under specific conditions

	<i>GO biological processes</i>	<i>p-value</i>	<i>GeneGo process networks</i>	<i>p-value</i>
PC-only	Cellular metabolic process	7.21E-10	Cell cycle_Mitosis	0.000227
	Metabolic process	6.76E-09	Translation_Translation initiation	0.002031
	Cellular process	7.69E-07	Cell cycle_G2-M	0.003922
	Embryonic cleavage	8.37E-07	Cytoskeleton_Spindle microtubules	0.005024
	DNA topological change	1.99E-06	DNA damage_DBS repair	0.006121
	Primary metabolic process	2.63E-06	Cell cycle_S phase	0.011881
	Positive regulation of retroviral genome replication	3.77E-06	Transcription_mRNA processing	0.014395
	Cell killing	5.05E-06	Signal transduction_Leptin signalling	0.044253
	Response to parathyroid hormone stimulus	1.13E-05	Cytoskeleton_Cytoplasmic microtubules	0.053011
	Osmosensory signalling pathway	2.25E-05	Cell cycle_Core	0.053011
INJ-only	Translational elongation	9.79E-18	Translation_Translation initiation	1.93E-13
	Translation	8.26E-14	Translation_Elongation-Termination	7.83E-12
	Cellular process	1.44E-09	Cytoskeleton_Macropinocytosis and its regulation	0.000236
	Actin filament organisation	8.37E-08	Cytoskeleton_Actin filaments	0.000386
INJ-only	Cytoskeleton organisation	1.38E-06	Cytoskeleton_Regulation of cytoskeleton rearrangement	0.000462
	Cellular protein metabolic process	4.06E-06	Cell adhesion_Integrin-mediated cell-matrix adhesion	0.000943
	Biosynthetic process	6.81E-06	Immune response_Phagocytosis	0.008176
	Cellular biosynthetic process	2E-05	Cytoskeleton_Cytoplasmic microtubules	0.008268
	Maintenance of protein location	2.4E-05	Immune response_Phagosome in antigen presentation	0.012133
	Actin cytoskeleton organisation	3.21E-05	Cytoskeleton_Spindle microtubules	0.060062
TOL-only	Translational elongation	1.41E-47	Cytoskeleton_Intermediate filaments	3.80E-29
	Translation	2.78E-47	Translation_Translation initiation	9.23E-29
	Cellular process	6.34E-31	Transcription_mRNA processing	1.73E-10
	Nuclear envelope reassembly	5.03E-29	Translation_Elongation-Termination	8.21E-09
	Ribosome biogenesis	5.8E-29	Protein folding_Protein folding nucleus	2.13E-06
	Ribonucleoprotein complex biogenesis	7.86E-28	Cytoskeleton_Regulation of cytoskeleton rearrangement	0.000113
	Histamine secretion involved in inflammatory response	2.34E-27	Cell cycle_G2-M	0.000742
	Histamine secretion by mast cell	2.46E-26	Cell cycle_Meiosis	0.000859

<i>GO biological processes</i>	<i>p-value</i>	<i>GeneGo process networks</i>	<i>p-value</i>
Histamine production involved in inflammatory response	1.66E-17	Neurophysiological process_Visual perception	0.001758
Histamine secretion	3.55E-17	Inflammation_IL-6signalling	0.001978

Table 5

Bioinformatic characteristics of proteins down regulated only under specific conditions

	<i>GO biological processes</i>	<i>p-value</i>	<i>GeneGo process networks</i>	<i>p-value</i>
PC-only	Cellular process	3.02E-07	Protein folding_Protein folding nucleus	8.27E-05
	Cellular macromolecular complex subunit organisation	6.88E-07	Translation_Elongation-Termination	0.000299
	Pentose-phosphate shunt	1.76E-06	Protein folding_Folding in normal condition	0.001302
	NADPH regeneration	2.29E-06	Translation_Translation initiation	0.004868
	Cellular macromolecular complex assembly	2.88E-06	Protein folding_ER and cytoplasm	0.014383
	Cellular metabolic process	5.71E-06	Transcription_mRNA processing	0.026921
PC-only	Cellular protein metabolic process	8.27E-06	Proteolysis_Ubiquitinproteasomal proteolysis	0.030057
	N-terminal peptidyl-glycine N-myristoylation	1.24E-05	Protein folding_Response to unfolded proteins	0.032087
	Peptidyl-glycine modification	1.24E-05	Apoptosis_Apoptotic mitochondria	0.039225
	N-terminal protein myristoylation	1.24E-05	Cell cycle_Core	0.079934
INJ-only	Chromatin assembly or disassembly	1.39E-10	Translation_Translation initiation	2.09E-06
	Translation	1.75E-10	Transcription_Chromatin modification	4.56E-05
	Cellular macromolecular complex assembly	6.49E-10	Transcription_mRNA processing	0.000158
	Cellular macromolecular complex subunit organisation	2.73E-09	Translation_Elongation-Termination	0.001176
	Cellular process	4.19E-09	Cell cycle_Mitosis	0.002209
	Translational elongation	5.58E-08	Apoptosis_Apoptotic nucleus	0.009088
	Primary metabolic process	1.53E-07	Protein folding_Folding in normal condition	0.023754
	Nucleosome assembly	1.83E-07	Translation_Regulation of initiation	0.028111
	Chromatin assembly	2.31E-07	Reproduction_Spermatogenesis, motility and copulation	0.030544
TOL-only	Cellular metabolic process	2.43E-07	Protein folding_Protein folding nucleus	0.036573
	Cellular metabolic process	8.66E-35	Translation_Translation initiation	2.23E-08
	Cellular process	3.28E-31	Cell adhesion_Integrin-mediated cell-matrix adhesion	1.47E-06
TOL-only	Primary metabolic process	3.84E-31	Cytoskeleton_Spindle microtubules	1.66E-06
	Metabolic process	4.97E-31	Cytoskeleton_Cytoplasmic microtubules	1.94E-06
	Translational elongation	3.11E-20	Cytoskeleton_Regulation of cytoskeleton rearrangement	2.01E-06
	Translation	2.27E-19	Cell cycle_Meiosis	2.89E-06

<i>GO biological processes</i>	<i>p-value</i>	<i>GeneGo process networks</i>	<i>p-value</i>
Protein polymerisation	3.66E-19	Cell cycle_Mitosis	5.83E-06
Cellular component biogenesis	7.82E-19	Cytoskeleton_Intermediate filaments	1.58E-05
Microtubule-based movement	3.26E-17	Transcription_mRNA processing	2.04E-05
Cellular protein complex assembly	3.73E-17	Translation_Elongation-Termination	3.14E-05

Table 6

Proteins that were down regulated only in ischemic-injured cells

<i>Genes</i>	<i>Proteins</i>	<i>Ratios in abundance</i>		
		<i>PC : CTR</i>	<i>INJ : CTR</i>	<i>TOL : CTR</i>
*Cbx5	Chromobox protein homolog 5 (Q61686)	2.58	0.38	1.59
Cct8	T-complex protein 1 subunit theta (P42932)	1.02	0.26	0.84
Eif2a	Eukaryotic translation initiation factor 2A (Q8BJW6)	1.01	0.38	4.09
Eif2s1	Eukaryotic translation initiation factor 2 subunit 1 (Q6ZWX6)	0.13	0.22	0.90
Eif3c	Eukaryotic translation initiation factor 3 subunit C (Q8R1B4)	0.71	0.65	1.14
Fau	40S ribosomal protein S30 (P62862)	1.46	0.22	0.21
Ganab	Neutral alpha-glucosidase AB (Q8BHN3)	1.63	0.65	2.09
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase (P16858)	1.23	0.63	0.95
Got1	Aspartate aminotransferase, cytoplasmic (P05201)	0.24	0.38	1.67
*Hist1h1d	Histone H1.3 (P43277)	0.92	0.58	0.75
*Hist1h2bk	Histone H2B type 1-K (Q8CGP1)	0.72	0.61	1.27
Hnrnpa2b1	Heterogeneous nuclear ribonucleoproteins A2/B1 (O88569)	0.81	0.56	1.43
Hnrnpc	Heterogeneous nuclear ribonucleoproteins C1/C2 (Q9Z204)	1.07	0.54	0.78
Hspa9	Stress-70 protein, mitochondrial (P38647)	0.80	0.70	0.77
Mat2a	S-adenosylmethionine synthetase isoform type-2 (Q3THS6)	1.02	0.15	1.64
Matr3	Matrin-3 (Q8K310)	1.02	0.63	1.77
Mrps5	28S ribosomal protein S5, mitochondrial (Q99N87)	1.82	0.38	1.59
Myef2	Myelin expression factor 2 (Q8C854)	2.26	0.15	1.61
Nipsnap1	Protein NipSnap homologue 1(O55125)	1.21	0.40	1.32
Pc	Pyruvate carboxylase, mitochondrial(Q05920)	4.16	0.38	2.89
Pck2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial (Q8BH04)	1.02	0.22	0.94
Prkar1a	cAMP-dependent protein kinase type I-alpha regulatory subunit (Q9DBC7)	1.50	0.12	0.85
Prpf8	Pre-mRNA-processing-splicing factor 8 (Q99PV0)	0.72	0.65	1.10
Rac1	Ras-related C3 botulinum toxin substrate 1 (P63001)	1.02	0.22	0.90
Ranbp2	E3 SUMO-protein ligase RanBP2 (Q9ERU9)	1.21	0.40	0.71
Rbm39	RNA-binding protein 39 (Q8VH51)	2.81	0.22	2.36
Rpl23	60S ribosomal protein L23 (P62830)	1.02	0.50	0.87
Rpl32	60S ribosomal protein L32 (P62911)	0.83	0.40	1.30
Rpl34	60S ribosomal protein L34 (Q9D1R9)	1.03	0.65	1.14
Rpsa	40S ribosomal protein SA (P14206)	0.79	0.50	0.87

<i>Genes</i>	<i>Proteins</i>	<i>Ratios in abundance</i>		
		<i>PC : CTR</i>	<i>INJ : CTR</i>	<i>TOL : CTR</i>
Sfrs1	Splicing factor, arginine/serine-rich 1 (Q6PDM2)	0.87	0.41	1.14
*Smarca4	Transcription activator BRG1 (Q3TKT4)	1.83	0.38	1.67
Syncrip	Heterogeneous nuclear ribonucleoprotein Q (Q7TMK9)	1.47	0.22	0.94
Tuba1a	Tubulin alpha-1A chain (P68369)	0.96	0.01	0.96
Ube2n	Ubiquitin-conjugating enzyme E2 N (P61089)	1.03	0.38	0.38
Vdac3	Voltage-dependent anion-selective channel protein 3 (Q60931)	1.50	0.50	0.87
Wdr43	WD repeat-containing protein 43 (Q6ZQL4)	1.02	0.22	0.90

* Proteins in bold type phase contribute to the recognition of chromatin or nucleosome assembly processes, as revealed by the MetaCore Program.