Original Article

Proteome Analysis of Mouse Brain Exposed to Chronic Hypoxia

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Abstract

Objective: Chronic hypoxia exists in many diseases, including cancer. The subject of our study is analysis of molecular pathways affected in the chronically hypoxic mouse brain.

Materials and Methods: Using the emPAI protocol, we performed a quantitative proteomic approach to characterize the global proteome in the mouse brain exposed to 7% O_2 for 48 hours.

Results: Utilizing the emPAI protocol to estimate protein abundance and assign molar concentrations to all proteins, we were able to identify 33 proteins with significant changes in their expression.

Conclusion: Deregulated proteins were mainly involved in cell metabolism, apoptosis, Ca^{2+} signaling, pentose phosphate pathway, 14-3-3 protein mediated signaling cascades and protein degradation. The obtained data will provide some clues for understanding mechanisms with which cells respond and adapt to chronic hypoxia.

Keywords: Chronic Hypoxia, Brain, Mouse, Proteomics, GeLC-MS/MS

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Introduction

Molecular oxygen, due to its critical role as an electron acceptor during adenosine 3 phospate (ATP) production via oxidative phosphorylation, is essential for the survival of higher organisms. Hypoxia, i.e. inadequate oxygen availability to cells and tissues, is a pervasive physiological stress affecting all organisms in unique ways (1). Hypoxia is an important pathophysiological factor in human diseases such as pulmonary diseases, altitude sickness, suffocation and cancer. Additionally, hypoxia is considered the principal pathogenic factor in stroke (2).

Cells response differently to hypoxia in a complex matter. The response is characterized by an alteration in the expression of several genes, including hypoxia-related genes and the corresponding proteins that maintain homeostasis (3). Although mRNA changes during hypoxia have been extensively investigated, hypoxia-induced changes in the proteome are in early phases of investigation. A large number of studies have focused on the influence of hypoxia on expression and post-translational modifications of single proteins or on a subset of functionally related proteins; however, only a limited number have examined the proteome-wide alterations during hypoxia, with most focusing on cell lines (4-7). Furthermore, a search in available scientific literature shows that very few long-term effects of hypoxia have been reported (8).

The mammalian brain is an organ with a high oxidative metabolic rate and although it constitutes only a small fraction of the total body, it accounts for a disproportionally large percentage of bodily oxygen consumption (9).

Given the importance of brain oxygen homeostasis, the aim of the presented study was to investigate changes in the protein profile of whole mouse brains exposed to chronic hypoxia.

We used a label free peptide centered quantitative proteomic approach, the emPAI protocol, to map protein expressions at a global level in chronic hypoxia. We applied the emPAI protocol because with mass spectrometry a relative protein concentration is automatically and quickly available for identified proteins without the need of any additional experimental setup (10).

Materials and Methods

Reagents

Sequencing-grade modified trypsin was purchased from Roche Diagnostics GmbH (Penzberg, Germany), LC-MS grade acetonitrile (ACN) and water from Biosolve b.v. (Valkenswaard, The Netherlands) and trifluoroacetic acid (TFA) from Merck KGaA (Darmstadt, Germany).

Induction of hypoxia

Swiss CD-1 mice (n=3) were kept in a chamber containing 7% O_2 (premixed gasses: 7% O_2, 93% N_2 from Messer Group GmbH, Griesheim, Germany). The mice were provided with food and water and allowed to adjust to the hypoxic environment by gradually decreasing O_2 from 21 % (normoxia) to 7% (hypoxia) during an adaptation time of 1 hour. After 48 hours of hypoxic exposure, the animals were euthanised and their dissected brains were frozen in liquid nitrogen and stored at –80 °C until further processing. All procedures were approved by the ethics committee at the University of Antwerp and conformed with the European Community regulations.

Protein extraction and SDS-PAGE

The tissues were pulverized in a mortar in liquid nitrogen and dissolved in a lysis buffer (25 mM NH_4HCO_3) (11). The total protein content was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, USA). The proteome sample (100 µg) was separated using SDS-PAGE in a 15% gel. The gel was stained with Coomassie Brilliant Blue R-250 (1g/L) and destained overnight. The samples were prepared in triplicates (three × normoxia, three × hypoxia) (12,13).

In-gel tryptic digestion

Following SDS-PAGE of the protein samples, complete lanes were cut out of the gel and sliced into 15 fractions. Each fraction was in-gel digested as described by Shevchenko et al. (14). The resulting peptide mixture was then extracted from the gel fractions and dried using vacuum centrifugation and stored at -20°C until analysed.

LC-MS/MS analysis of SDS-PAGE separated protein fractions

The dried extracted peptides were dissolved in 10 µl 0.1% formic acid (V/V). All peptide mixtures were analyzed three separate times in order to improve peptide and protein identification. A 2-µl sample was concentrated and desalted on a C18 PepMap100 precolumn (300µm I.D. × 5 mm, Dionex/LC Packings) using a FAMOS autosampler (Dionex-/LC Packings) and an ABI 140A solvent delivery system pump (Applied Biosytems) at a 20 µl/min flow rate. After valve switching, the trapped peptides were eluted towards a nano-LC C18 PepMap100 analytical column (75 µm × 150 mm, Dionex/LC Packings) using the Ultimate pump (Dionex/LC Packings) at a 0.150 µl/min flow rate. The peptides were separated using a two-step linear gradient from 0% to 50% buffer B in 35 minutes and from 50% to 100% in 15 minutes. Buffer A was 5% acetonitrile (V/V) and 0.1% formic acid (V/V) in water; and buffer B was 80% acetonitrile (V/V, from the 0% to 50% buffer B in 35 minutes) and 0.1% formic acid (V/V) in water. The separated peptides were ionized in the nanospray source using distal coated SilicaTips (New Objective, Woburn, MA, USA) with a 8 µm tip I.D. whereon 1800 V was supplied. The ionized peptides were measured with an API 2000 QTrap ESI mass spectrometer (Applied Biosystems, Carlsbad, CA, USA) in LIT mode and automatically selected for MS/MS using the IDA parameters from the Analyst 1.4 software (Applied Biosystems) as described before (15). Analyst software utilizing Mascot (version 1.9; Matrix Science Ltd, London, UK) as search engine was used for performing database searches in the mouse protein sequences stored in Swiss-Prot (March 20th 2007). Swiss-Prot with search restriction set to mouse proteins was used to identify proteins with the following parameters: mass tolerance for the precursor ion: 1.5 Da, mass tolerance for fragment ions: 0.8 Da, variable modifications, methionine (Met) oxidation, pyrrolidone carboxylic acid formation from amino- terminal glutamine, pyro-carbamidomethyl formation from amino-terminal cysteines, deamidation of glutamine or asparagine; and one missed trypsin cleavage. To establish criteria for our unambiguous protein identification, we first selected peptides for which the Mascot score was at least equal to the threshold probability score of 95% significance (this was on average 30). Furthermore, peptides having at least 3 successive amino acids covered by b or y fragmentions were manually validated.

Protein quantification and abundance measurement

The abundance of identified proteins was estimated by calculating the protein abundance index (PAI) and the exponentially modified protein

abundance index (emPAI) (10). PAI is defined as the number of detected peptides divided by the number of observable peptides per protein, normalized by the theoretical number of peptides expected via *in silico* digestion. The emPAI is an exponential form of PAI minus 1 defined as $emPAI = 10^{PAI} - 1$ and the corresponding protein content in mole percent is calculated as $mol\ \% = (emPAI/\sum emPAI) \times 100$. Microsoft Office Excel was used to calculate the mole percent. The theoretically observable peptides were determined by the *in silic*o digestion of mature proteins using GPMAW (v 6.11) (16). The observed peptides were unique parent ions including those with one missed cleavage.

Analysis with DAVID software

The regulated proteins were classified into functional groups and different pathways using the DAVID software tools (17).

Results

To investigate the potential impact of hypoxia on global protein expression, mice were exposed to 48 hours of hypoxia (7% O_2). Proteomic analysis of the whole brain tissue extracted from normoxic and hypoxic mice was performed using GeLC-MS/MS. This multidimensional analysis involved the proteome of the brain being first separated by one-dimensional SDS-PAGE followed by RP-C18 column separation and protein identification by mass spectrometry as described in material and methods. This methodology allowed the identification of more than 600 proteins from the normoxic and hypoxic samples. Quantitative analyses were only performed for the proteins identified in both samples. This resulted in a list of 190 proteins which were further considered.

The proteins identified within the two conditions were quantified using the exponentially modified protein abundance index (emPAI) (supplementary material). Based on emPAI, the fold change in expression level of proteins identified under both conditions could be estimated, thus giving further insights into the cellular process. In the comparative two-dimensional PAGE technique, a $\geq 50\%$ change was considered significant and sufficient to take account of systematic errors, therefore, fold changes of ≥ 1.5 or ≤ 0.5 were significant, with a fold change value of 1 representing no difference in the proteins between the two states (13).

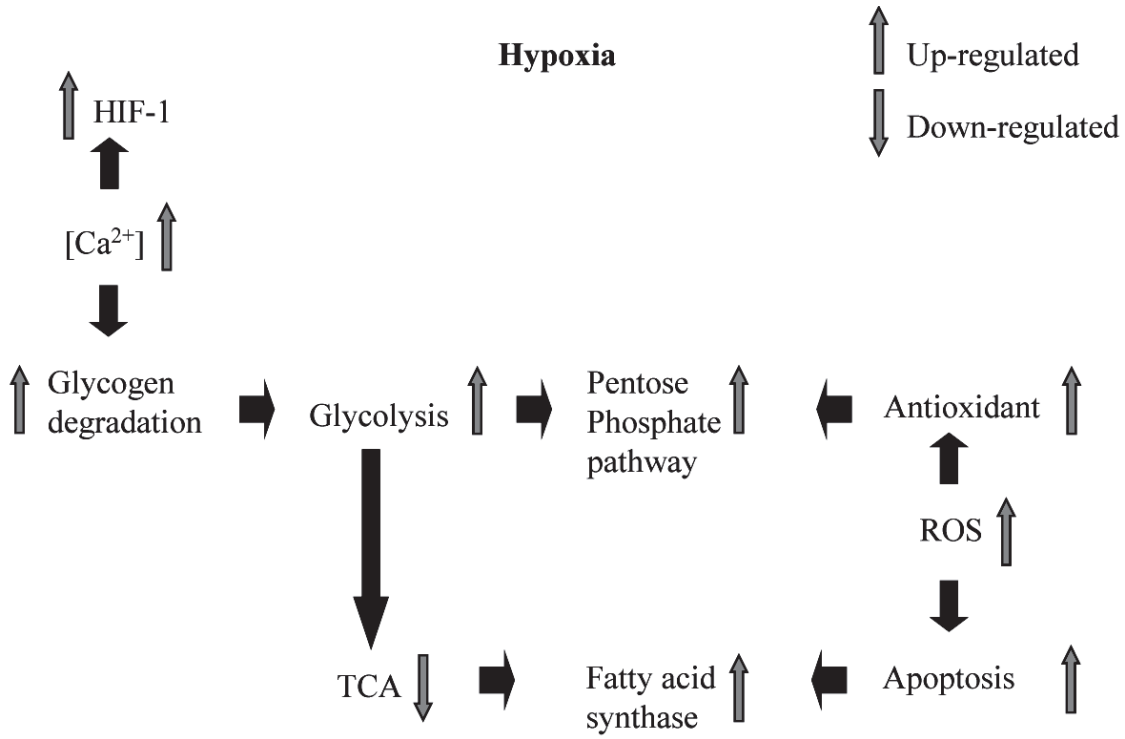

Fig 1: Effect of chronic hypoxia in mouse brain under 7%O_2 (48 hours). Ca^{2+}: calcium, ROS: reactive oxygen species. HIF-1: hypoxia inducible factor-1. Communications between different molecular pathways are indicated by black arrows. Green arrows indicate up- or down- regulation of specific molecular pathways. For example, we assume ROS level is increased because proteins identified in the antioxidant group were up-regulated, etc.

Table 1: Regulated proteins identified in normoxic and hypoxic mouse brains with their DAVID analysis and emPAI calculations. Only proteins with significant differential ratio changes of ≥ 1.5 or ≤ 0.5 are listed.

Type, accession number	Name	pI / MW	emPAI		Protein content (molar %)		Differential ratio (H/N)
			Normoxia	Hypoxia	Normoxia	Hypoxia	
TCA Cycle							
CISY_MOUSE	Citrate synthase	8.72 / 51703	0.139	0.044	0.311	0.098	0.3
Glycolysis							
G6PI_MOUSE	Glucose-6-phosphate isomerase	7.75 / 62728	0.169	0.314	0.377	0.694	1.8
Glycogen degradation							
PYGB_MOUSE	Glycogen phosphorylase, brain form	6.28 / 96668	0.039	0.100	0.087	0.222	2.6
Pentose phosphate pathway							
6PGL_MOUSE	6-phosphogluconolactonase	5.55 / 27237	0.128	0.438	0.288	0.968	3.4
Ca^{2+}-Signaling							
KCC2A_MOUSE	Calcium/calmodulin-dependent protein kinase type II alpha chain	6.61 / 54081	0.051	0.284	0.114	0.627	5.5
SYNJ1-MOUSE	Synaptojanin-1	6.45/172509	0.043	0.072	0.096	0.159	1.7
Antioxidant							
PRDX1_MOUSE	Peroxiredoxin-1	8.26/22162	0.359	0.584	0.803	1.291	1.6
GSTP1_MOUSE	Glutathione S-transferase P1	7.68/23594	0.550	0.931	1.230	2.055	1.7
LGUL_MOUSE	Lactoylglutathione lyase	5.24/20796	0.211	0.333	0.427	0.736	1.7
Signal cascade							
1433G_MOUSE	14-3-3 protein gamma	4.8/28285	0.467	0.136	1.045	0.301	0.3
1433Z_MOUSE	14-3-3 protein zeta/delta	4.73/27754	0.832	0.438	1.861	0.968	0.5
1433T_MOUSE	14-3-3 protein theta	4.69/27761	0.145	0.225	0.324	0.497	1.5
Apoptosis							
ANXA5_MOUSE	Annexin V	4.83/35730	0.148	0.259	0.331	0.572	1.7
FAS_MOUSE	Fatty acid synthase	6.13/272257	0.021	0.043	0.047	0.094	2
KCY_MOUSE	UMP-CMP kinase	5.68/21151	0.160	0.249	0.358	0.551	1.5
HPRT_MOUSE	Hypoxanthine-guanine phosphoribosyltransferase	6.21/24555	0.166	0.259	0.371	0.571	1.5
NDKA_MOUSE	Nucleoside diphosphate kinase A	6.84/17197	0.311	0.501	0.695	1.107	1.6
AN32A_MOUSE	Acidic leucine-rich nuclear phosphoprotein 32 family member A	3.99/28520	0.194	0.304	0.433	0.671	1.5
HMGB1_MOUSE	High mobility group protein B1	5.62/24878	0.202	0.318	0.452	0.703	1.6
TERA_MOUSE	Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit	5.14/89525	0.095	0.173	0.213	0.382	1.8
Cytoskeleton							
SPTB2_MOUSE	Spectrin beta chain, brain 1	5.4/274052	0.040	0.068	0.090	0.151	1.7

TBB5_MOUSE	Tubulin beta-5 chain	4.78/49639	0.400	1.322	0.896	2.919	3.3
DNM1L_MOUSE	Dynamin-1-like protein	6.61/82606	0.147	0.061	0.330	0.134	0.4
Protein degradation							
CAND1_MOUSE	Cullin-associated NEDD8-dissociated protein 1	5.52/136245	0.052	0.107	0.116	0.236	2
UBE1X_MOUSE	Ubiquitin-activating enzyme E1 X	5.43/117734	0.191	0.350	0.428	0.773	1.8
UBE2N_MOUSE	Ubiquitin-conjugating enzyme E2 N	6.13/17127	0.259	0.412	0.578	0.911	1.6
UBP5_MOUSE	Ubiquitin carboxyl-terminal hydrolase 5	4.89/95772	0.054	0.082	0.120	0.180	1.5

Type, accession number	Name	pI / MW	emPAI		Protein content (molar %)		Differential ratio (H/N)
			Normoxia	Hypoxia	Normoxia	Hypoxia	
Other							
DHE3_MOUSE	Glutamate dehydrogenase 1	8.05/61298	0.124	0.060	0.276	0.132	0.4
KCRU_MOUSE	Creatine kinase	8.39/46974	0.139	0.044	0.311	0.098	0.3
PSA_MOUSE	Puromycin-sensitive aminopeptidase	5.61/103286	0.073	0.073	0.163	0.052	0.3
AP2B1_MOUSE	AP-2 complex subunit beta-1	5.22/104516	0.053	0.053	0.118	0.178	1.5
CAH2_MOUSE	Carbonic anhydrase 2	6.52/29073	0.0585	0.259	1.307	0.572	0.4
ATPB_MOUSE	ATP synthase subunit beta	5.19/56265	0.094	0.145	0.211	0.320	1.5

The fold change (differential ratio) in the protein expression between proteins from normoxia and hypoxia conditions can be seen in table 1.

Taking the ≥ 50% cut off value, 33 proteins significantly changed in expression between normoxia and hypoxia, of which 25 were up-regulated and 8 were down-regulated. The regulated proteins were analyzed using the DAVID software; they were involved in glycolysis, TCA cycle, apoptosis, Ca^{2+} signaling, pentose phosphate pathway, signaling cascade, protein degradation and cytoskeletal proteins.

The molecular pathways affected by hypoxia are shown in figure 1. Although, we can not directly quantify Ca^{2+} and reactive oxygen species (ROS) using proteomics approaches, proteins affected by these could be identified.

Discussion

Hypoxia is one of the key factors influencing stroke, as well as tumor growth and progression. Generally speaking, hypoxia is considered a global phenomenon and is defined as an overall reduction in oxygen availability or its partial pressure below critical levels. The underlying mechanism of hypoxia is still unclear and remains under investigation.

Understanding the effect of hypoxia has been mainly investigated at the mRNA level and only a few proteomics analyses have been done so far.

In the presented study, we used the GeLC-MS/MS based proteomics to examine the impact of hypoxia on global protein expression of the whole brain tissue in mice. We chose brain tissue because of its sensitivity to oxygen deprivation.

The proteins identified within the two conditions (normoxia, hypoxia) were quantified using emPAI and are reported in table 1. The GeLC-MS/MS method provided sufficient separation and generated data allowing the identification and quantification of individually identified proteins; it also yielded the Mascot output information which was used to calculate an emPAI value. The emPAI value was then used to estimate the protein content within the sample mixture in molar fraction percentages. In addition, the fold change (differential ratio) in the expression level of proteins identified under both conditions were estimated, giving further insights into cellular processes.

Thirty-three proteins, such as antioxidants, glycolysis proteins, TCA-cycle proteins, cytoskeleton proteins and other components, were found to be differentially expressed under chronic hypoxia (Table 1). Based on these proteins, an analysis of the effect of hypoxia was made (Fig 1).

The presented study shows that proteins involved in glucose metabolism and biological oxidation are influenced upon exposure to 7% O_2. Under conditions of limited oxygen supply, brain tissue switches to an oxygen-independent metabolic pathway, and as expected, oxidative phosphorylation falls down while glycolysis becomes the primary method for ATP production. Accordingly, 6-phosphoglucose isomerase in the glycolytic pathway increases, similar to what is found in previous studies (18). Within the TCA-cycle enzymes, we identified citrate synthase showing decreased activity.

Studies have shown that continuous exposure to hypoxia induces membrane depolarization, resulting in increased intracellular Ca^{2+} levels (19). The Ca^{2+} signaling pathways have been shown to be required for hypoxia inducible factor 1 (HIF-1) activity (20). We were able to identify the CaMK II protein which showed the highest differential ratio of all proteins identified. The CaMK II protein is a downstream signaling molecule that participates in Ca^{2+} -mediated gene regulation. Ca^{2+} -CaMK- II activity is required for the induction of HIF-dependent gene transcription (21).

An increased level of Ca^{2+} will also increase glycogen degradation. In this study, we were able to show a significant up-regulation of glycogen phosphorylase. This increase in the enzyme activity can be caused by metabolic stress (22). Consequently, the lack of glucose supply to the brain increases glycogen degradation and protein catabolism.

Catabolism of proteins was also confirmed by the identification of proteins involved in protein-degradation. Accordingly, several ubiquitin enzymes were shown to be up-regulated. Notable is that it has been suggested that the ubiquitin-proteasome system might play an important neuroprotective role under hypoxic conditions (23).

One of the interesting proteins we identified is fatty acid synthase (FAS), a key enzyme involved in de novo fatty acid synthesis. This enzyme is up-regulated in our study. Hypoxia results in lack of building blocks involved in the synthesis of biological macromolecules. FAS is one of the key components in cell membrane biosynthesis (24). Several studies have shown that FAS is induced by hypoxia (25-27). It is speculated that FAS may play a role in neuroprotection (24). FAS over-expression is also correlated with apoptosis of cells (28). Activation of apoptosis, e.g. by a decreased level of intracellular ATP, is confirmed by the identification of several biomarker proteins. The most known apoptosis biomarker Annexin V has been identified and shown to be over-expressed (29).

Variable results have been published regarding the effect of hypoxia on the increase or decreases in intracellular reactive oxygen species (ROS) levels (30). It is also shown that an increased level of Ca^{2+} with an increased level of ROS (31, 32). ROS can be the principle mediator of cell death (33). We identified three antioxidant proteins showing increased activity. Of special interest is the GSTP1 gene which uses NADPH for removal of H_2O_2. NADPH is produced via the pentose phosphate pathway. This pathway shows an increased activity by up-regulation of 6-phosphogluconolactonase.

In addition to the proteins discussed above, proteins such as 14-3-3, those of the cytoskeleton and others were also regulated in the whole brain tissue after being exposed to hypoxia for 48 hours. Summing up, by using GeLC-MS/MS and emPAI to compare the protein expression profiling under hypoxia, this study identified a large number of proteins modulated by hypoxia in the brain. These data will provide valuable clues to aid the understanding of cell response to chronic hypoxia.

In order to increase the in-depth understanding of hypoxia, further investigation is required to determine its clinical implications.

Conclusion

Utilizing the emPAI method, we have used the proteomic information derived form the proteins detected in an increased or decreased level to determine if their protein-expression signature could predict an adaptive response to chronic hypoxia in the mouse brain. The results indicate a clear up- and down-regulation of the proteins adapted to chronic hypoxia. Although none of the proteins alone are univocally associated with chronic hypoxia, the combination of proteomic information of different proteins was sufficient to give a better understating of the molecular pathway affected by chronic hypoxia.

In conclusion, in this article we have demon-

strated the validity of the presented proteomic approach for identifying proteins with an altered amount in the mouse brain exposed to chronic hypoxia. Importantly, we report 33 proteins with altered proteomic patterns in the mouse brain affected by chronic hypoxia, opening the door to further identification of mechanisms involved in chronic hypoxia and to their potential assessment as novel biomarkers.

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There is no conflict of interest in this article.

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