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Comparative proteome analysis using amine-reactive isobaric tagging reagents coupled with 2D LC/MS/MS in 3T3-L1 adipocytes following hypoxia or normoxia

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ABSTRACT

Hypoxia during the expansion of adipocytes is known to contribute both to the secretion of multiple inflammation-related adipokines as well as to obesity. We therefore investigated the nature of protein changes occurring in adipocytes during hypoxia by observation of the intracellular proteins that are expressed in 3T3-L1 adipocytes. Lysates were utilized for quantitative proteome analysis using isobaric tags for relative and absolute quantitation (iTRAQ) combined with peptide separation by multi-dimensional liquid chromatography. Antioxidants and elongation factors, as well as glycolytic enzymes were increased in hypoxic adipocytes. These changes were supported by similar changes suggested by real-time PCR. The proteins showing changes are all potential targets for revering the mechanism behind the phenomenon of induction of obese adipocytes by hypoxia. This study can therefore aid in defining the proteomic changes that occur in adipocytes in response to oxygen stress, and can further characterize adipocyte metabolism and adaptation to low oxygen conditions.

Hypoxia occurs when oxygen availability does not match the demand of the surrounding tissue, resulting in decreased oxygen for respiration [1]. Hypoxia is known to induce various responses in cells, including, for example, stoppage or slowing of the rate of progression through the cell cycle [2,3]. Low oxygen tension induces large changes in gene expression [4], which and controlled by the hypoxia-inducible factor 1 alpha (HIF-1α), a factor that is activated and binds to hypoxia-responsive elements in target genes [5,6].

Recent studies have reported that adipocytes, in addition to their well-known roles as fat storage cells, also produce and secrete a variety of bioactive molecules [7,8], that appear to be involved in obesity and other metabolic disorders, such as insulin resistance, that can be regarded as serious public health problems. Therefore, adipocytes are also of interest in biological studies due to their important roles in metabolic processes such as glucose homeostasis and energy balance [9].

Obesity is attributed to both hypertrophy (an increase in cell size) and hyperplasia (an increase in cell number) of adipocytes [7]. Hypertrophic changes in occur in adipocytes during the development of obesity and the size of these cells can increase up to 140–180 μm in diameter. Given that the diffusion limit of oxygen is considered to be 100 μm [10], it can be seen that hypertrophy combined with hyperplasia can easily lead to a hypoxic condition in the adipocytes of obese subjects.

Many studies have revealed that hypoxia induces the production of inflammation-related adipokines such as IL-6, IL-18, MIF, and PAI-1 and that it also inhibits the production of adiponectin in 3T3-L1 adipocytes [11,12]. However, the nature of the proteomic changes induced by hypoxia in adipocytes is not known. Therefore, we performed the present study to examine the hypoxic regulation of expression of various adipocyte-produced proteins in a mouse adipocyte model, the 3T3-L1 cell line, by subjecting differentiated cells to a low oxygen pressure. We analyzed quantitative proteome data using isobaric tags for relative and absolute quantitation (iTRAQ) between hypoxia and normoxia conditions in lysates obtained from hypoxic and normal adipocytes.

Materials and methods

Cell culture and sample preparation. Mouse 3T3-L1 fibroblasts were grown in subconfluent cultures in high glucose DMEM sup-

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plemented with 10% fetal calf serum and were maintained in 5% CO_2 humidified atmosphere at 37 °C A 3T3-L1 cell culture was induced to differentiate into adipocytes in a 150 mm dish. Fully differentiated 3T3-L1 adipocytes were incubated under hypoxic and (control) normal conditions. The hypoxic condition was maintained in a 1% O_2, 5% CO_2, humidified atmosphere at 37 °C After 24 h incubation, 3T3-L1 adipocytes from each growth condition were washed twice with PBS and lysed in a hypotonic lysis buffer (20 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM EDTA) followed by sonication. Insoluble materials were removed by centrifugation at 10,000*g*.

Trypsin digestion and iTRAQ labeling. One hundred micrograms of dried proteins from each cell line were dissolved in 20 μl 500 mM triethyl ammonium bicarbonate (TEAB) dissolution buffer and 1 μl denaturant. The samples were chemically reduced and alkylated using 2 μl reducing reagent and 1 μl cysteine blocking reagent, as described and supplied in the iTRAQ kit (Applied Biosystems iTRAQ™ Reagents). A vial of trypsin in 25 μl Milli-Q Water was added to each sample and the trypsin digestion was carried out at 37 °C overnight according to the standard protocol supplied by the manufacturer. Normoxic cell lysates were labeled with iTRAQ reagents 114 and 116 and hypoxic cell lysates were labeled with iTRAQ reagents 115 and 117 for 1 h. Each reagent labeled digest was then combined into one sample and vacuum dried.

2D LC/MS/MS analysis. The LC/MS/MS was performed by a MALDI-TOF/TOF spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Framingham, MA) equipped with an integrated Famos autosampler, Switchos switching pump, and Probot MALDI spotting device (LC packings, Amsterdam) with SCX/RP columns. The iTRAQ labeled peptide mixtures were separated on a column packed with SCX resin (3 cm) in I.D. 250 μm silica tubing and a C18 RP column packed with C18 resin (10 cm) in I.D. 75 μm silica tubing. A six-salt step was performed using 0, 25 mM, 50 mM, 125 mM, 250 mM, and 500 mM ammonium acetate. At each step, a salt plug was loaded onto the SCX column for peptide elution. Peptide fractions were then captured by the trap_column for preconcentration and desalting. The mobile phases A and B were 0% and 80% ACN containing 0.1% TFA, respectively. Every fraction was collected at 20 s intervals directly onto 6 of 576 wells MALDI plates, and 5 mg/mL of CHCA in 70% ACN/0.1% TFA was added by a Probot MALDI spotting device. Mass spectra were obtained using a Nd:YAG laser (355 nm, 200 Hz).

Database search and statistical analysis. A database search for the iTRAQ experiments was performed with GPS software (version 3.6, Applied Biosystems) to identify and quantify the raw MS/MS data from the MALDI-TOF/TOF mass spectrometer. For protein identification, the MS/MS data were analyzed using the IPI mouse database (version 3.40) with the MASCOT search engine (version 2.1, http://www.matrixscience.com). The parameters for identification of peptides were 0.1 Da for MS and 0.5 Da for MS/MS, allowing up to one missed cleavage. Variable modifications considered were oxidation of methionine, *s*-methylmethanethiosulfonate (MMTS) alkylation of cysteine, iTRAQ labeled N-terminal, and iTRAQ labeled lysine. We performed a decoy database search to reveal the false discovery rate.

We converted peak area value of identified peptides to $\log_2$ value and normalized with MA plot. Also, we calculated distribution of peptides with $\log_2$ 116/114, 115/114, and 117/114.

For data integration, we applied category analysis. The validated proteins were classified with regard to their assumed biological function and molecular function using information from FatiGO, a web-based program for functional profiling (http://www.babelomics.org) [13].

Real-time PCR. For real-time PCR, gene sequences were searched in the mouse species database of NCBInr. Specific primers were designed using primer design software (Primer 3) and their sequences are listed in Supplemental Table 1. cDNA (1 μg) was reverse-transcribed from total cellular RNA prepared using TRIzol reagent (Invitrogen, Carlbad, CA). PCR amplification mixtures (20 μl) contained 10 μl of 2× SYBR Green I Premix Ex Taq (TAKARA

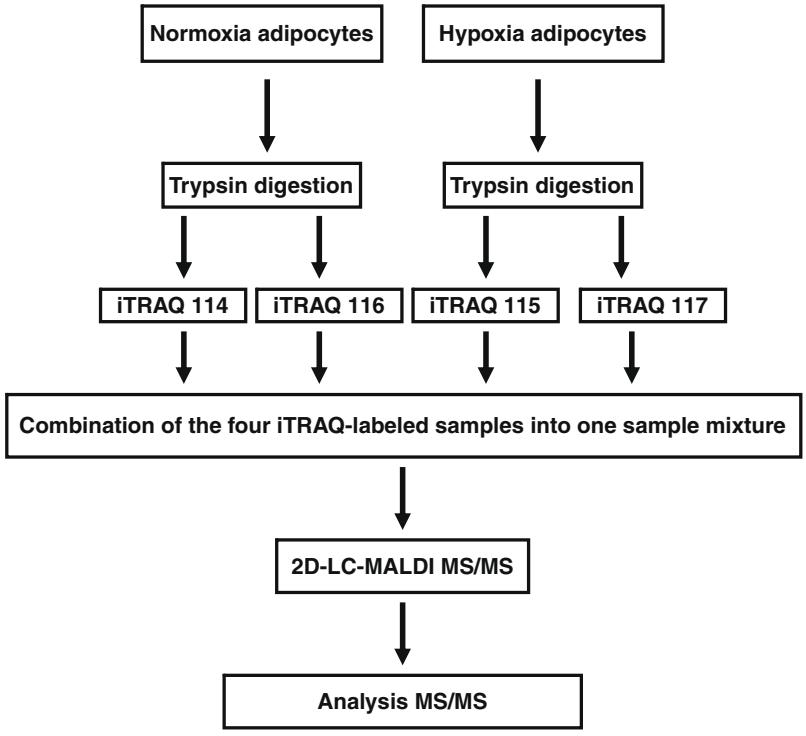

Fig. 1. Overview of iTRAQ-reagent methodology used for multiplexed comparative analysis of proteins. Flow diagram showing the steps involved in determining relative quantities of proteins between normoxic and hypoxic adipocytes.

Co., Ltd.), 2 μl of 10 μM forward and reverse primer mix and 8 μl of diluted cDNA template. Real-time quantitative PCR was performed with a Bio-Rad real-time PCR detection system iQ5, with following amplification parameters: 50 °C for 10 min, 95 °C for 5 min; then 40 repeats of the following: 95 °C for 10 s, 60 °C for 30 s. After amplification, melting curve analysis was performed as described in the manufacturer's protocol (Bio-Rad).

Results and discussion

Our study represents an investigation of the changes in protein expression occurring in 3T3-L1 adipocytes exposed to hypoxia and normoxia. We also observed the protein changes effected by hypoxia with quantitative proteome analysis. Two 50 μg samples from normoxic cell lysates and two 50 μg samples from hypoxic cell lysates were trypsinized, each sample was tagged with a specific isobaric iTRAQ reagent and then the samples were mixed for LC/MS/MS analysis (Fig. 1).

Statistical Identification and quantification of proteins

We have generated quantitative data that present a reliable tool for estimating relevant protein changes in adipocytes. For the current quantitative proteomics, we utilized two methods. The first was to perform comparative proteome analysis of hypoxic and normoxic 3T3-L1 adipocytes using iTRAQ. Since the same peptides from four samples are efficaciously merged, it allows good quality MS/MS data to be acquired from low copy number proteins. The technique offers high quantitative reproducibility with quantification of peptides at the protein level. Therefore, we can obtain greater confidence data for quantifying and identifying specific proteins. In the second method, multi-dimensional separation is used as a proteomics tools. This method allows peptide mixtures produced by crude enzymes to be separated on coupled strong cation-exchange (SCX) and reversed phase (RP) liquid chromatography columns [14,15]. The peptides are first separated by charge on the SCX column and then further fractionated by hydrophobicity on the RP column using high performance liquid chromatography. We performed this proteome analysis with matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS/MS) combined with off-line 2D-LC separation system.

The MASCOT scores were in the confidence range of 95% probability (significance threshold $p < 0.05$). From a total 2832 observed ions, 840 peptides were identified with more than 95% probability. We obtained a 9.04% false discovery rate from analysis of a decoy database. For data with less than 95% probability, we performed manual validation with MS/MS spectra, which resulted in identification of 40 additional peptides. We identified 205 proteins and performed statistical analysis with quantification data. The peak areas of each iTRAQ peak were transformed to $\log_2$ values and normalized to the peak area of $\log_2$ 114/116 and $\log_2$ 115/117 (Fig. 2A). After normalization, we regarded a significantly increased level to be higher than 0.50 of $\log_2$ ratio (1.5-fold) value with calculation of the confidence level with an *R*-square value 0.99 (Fig. 2B).

The identified proteins were classified by biological function and molecular function using information from FatiGO web-based program (Supplemental Fig. 1).

Differentially expressed proteins between normoxia and hypoxia in adipocytes

A total of 96 proteins were significantly increased in hypoxic adipocytes in our experiments, whereas the other 109 proteins showed no change or only slight changes in their cell levels. Upon clustering analysis on biological function and molecular function, the proteins that changed in highest proportion under hypoxia were linked with responses to stress, biosynthetic processes, and glycolysis. These increased proteins are shown in Supplemental Table 2, and the representative MS/MS spectra of the peptides that showed changes in expression level are shown in Fig. 3.

Responses to stress

Proteins associated with stress responses were expressed at higher levels in hypoxic adipocytes. Heat shock proteins, including heat shock protein 60 kDa (Hsp60) and heat shock protein 80 kDa,

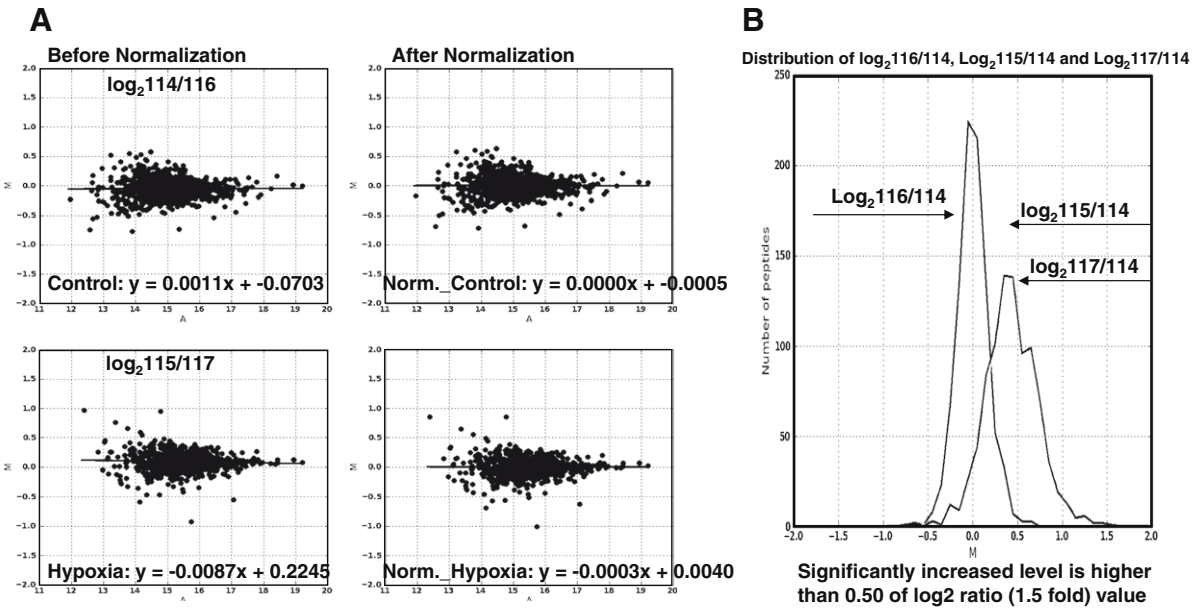

Fig. 2. Normalization of $\log_2$ 114/116 and $\log_2$ 115/117. (A) MA plot normalization. $M = \log_2 114/116 = \log_2 114 - \log_2 116$ and $\log_2 115/117 = \log_2 115 - \log_2 117$. *A* = number of peptide. (B) Distribution of $\log_2$ 116/114, $\log_2$ 115/114, and $\log_2$ 117/114. The *y*-axis indicates the number of identified peptides and *x*-axis indicates *M* value. Rsqr (*R* square, R^2) = 0.99.

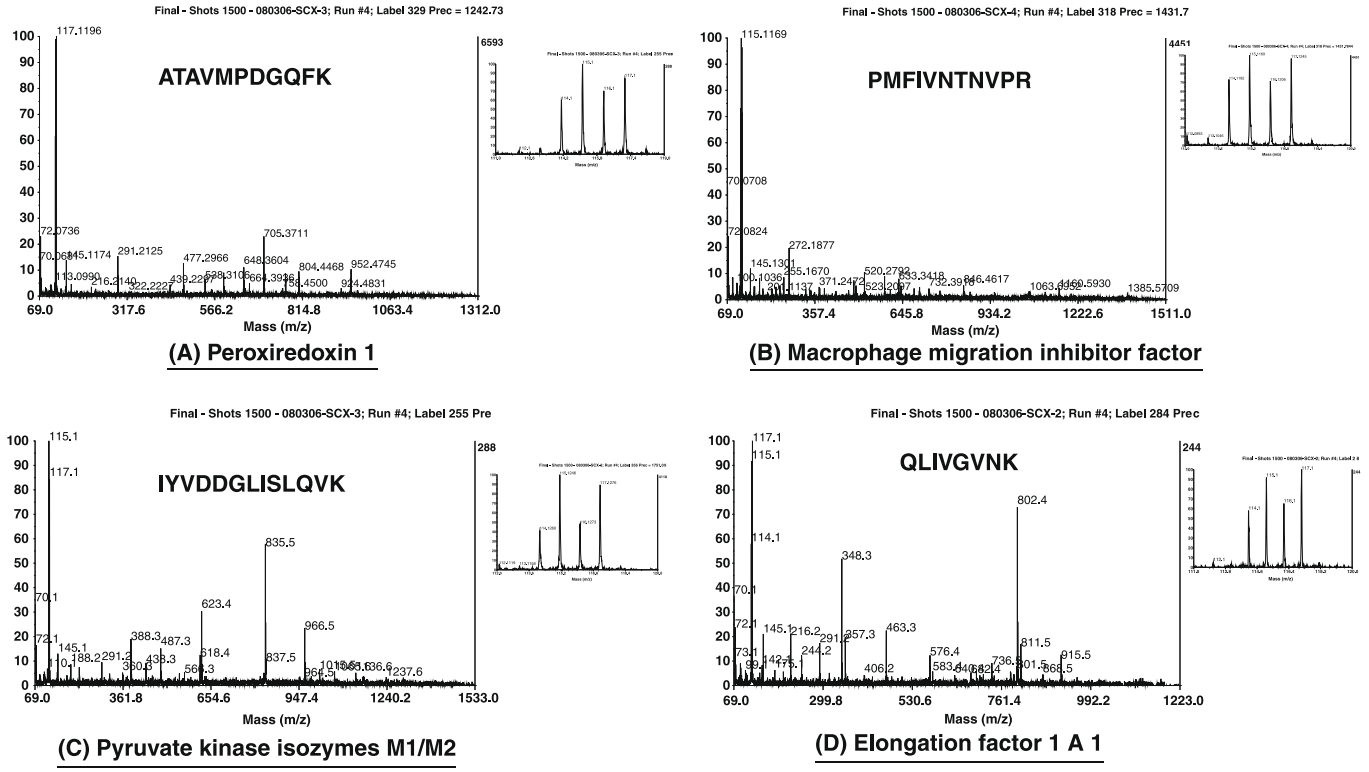

Fig. 3. Representative MS/MS spectra. The peptide shows the level changes of 115, 117 labels assigned with hypoxia compared with control 114, 116 labels assigned with normoxia. (A) the spectrum of peroxiredoxin 1; (B) the spectrum of macrophage migration inhibitor factor; (C) the spectrum of pyruvate kinase isozymes M1/M2; (D) the spectrum of elongation factor 1 A 1.

were significantly increased following hypoxic stress. Hsp60 is known to protect cells from injuries related to endogenous and exogenous oxidative stress [16]. Macrophage migration inhibitory factor (MIF) was up-regulated by hypoxia. MIF belongs to a family of proinflammatory cytokines, which are known to induce production of tumor necrosis factor TNF-α in adipocytes [17]. It also possesses thiol-protein oxidoreductase activity and has pleiotropic effects, including regulation of cellular redox homeostasis [18]. From the RT-PCR result, the mRNA level for MIF was increased in hypoxic adipocytes. Aconitate hydratase (Aco2) was also increased by hypoxia. This enzyme catalyses the stereospecific isomerisation of citrate to isocitrate via cis-aconitate, anonredox active process [19]. It is known that Aco2 is highly sensitive to inhibition by reactive oxygen species (ROS) [20,21]. Peroxiredoxins were also increased by hypoxia in our study. Peroxiredoxins are known to function in regulation of ROS damage [22]. Peroxiredoxin 1 (Prdx1) belongs to the antioxidant family and is associated with various biological processes, such as oxidant detoxification, cell proliferation, differentiation and apoptosis [23]. Prdx1 is up-regulated in lung cancer cells following hypoxia, which protects cells against hypoxia-induced stress [24]. The antioxidant enzyme, superoxide dismutase (SOD2), removes ROS in mitochondria, protecting the mitochondria from oxidative insult. SOD2 catalyzes the dismutation of two superoxide radicals, yielding hydrogen peroxide and oxygen [25].

These proteins are also known to play an important role in maintaining redox regulation and homeostasis against reactive oxygen species (ROS). ROS is known to be deleterious to proteins, lipids, and DNA [26]. There has been some debate as to whether hypoxia or anoxia result in increases in ROS [27]. However, a number of studies have suggested that a rise in ROS in response to hypoxia occurs in a variety of cells [28,29]. We hypothesize that the increase in antioxidant proteins observed here indicates that hypoxia causes oxidative damage to cell components in adipocytes and that these antioxidant proteins are then highly expressed in response to oxidative stress.

Protein biosynthesis

Elongation factors were identified in both of hypoxic and normoxic cells, but these proteins were expressed at higher levels in hypoxia. In particular, protein levels of elongation factor Tu (Tufm) were markedly increased under hypoxia, although its mRNA level was not changed. Tufm, one of the most abundant proteins in most cells, appears to be involved in polypeptide elongation during protein synthesis and therefore represents a possible target for the development of antimicrobials [30]. Also, protein expression level and mRNA expression level of elongation factor 1-α1 were increased in hypoxic condition. Elongation factor 1-α is a multiple function protein involved in protein synthesis, cell proliferation, apoptosis, and tumorigenesis [31]. However, elongation factor 2, elongation factor 1-γ and 1-β were slightly increased.

Since protein synthesis requires a great deal of cellular energy, ATP depletion will rapidly lead to inhibition of protein synthesis [32]. In general, it is thought that hypoxic cells down-regulate high-energy processes such as protein synthesis by mTOR and MAPK to conserve ATP [33]. Hypoxia also inhibits the initiation of mRNA translation and elongation [34,35]. However, in our study, we found that the family of elongation factor proteins was up-regulated in response to hypoxia, a feature that deserves further investigation. Our results indicate that even in a low oxygen condition, adipocytes can and do produce proteins, such as many adipokines, in response to hypoxia.

Glycolysis

In our results, glycolytic enzymes were increased in hypoxic adipocytes, as were levels of their mRNAs. Aldoa catalyzes the

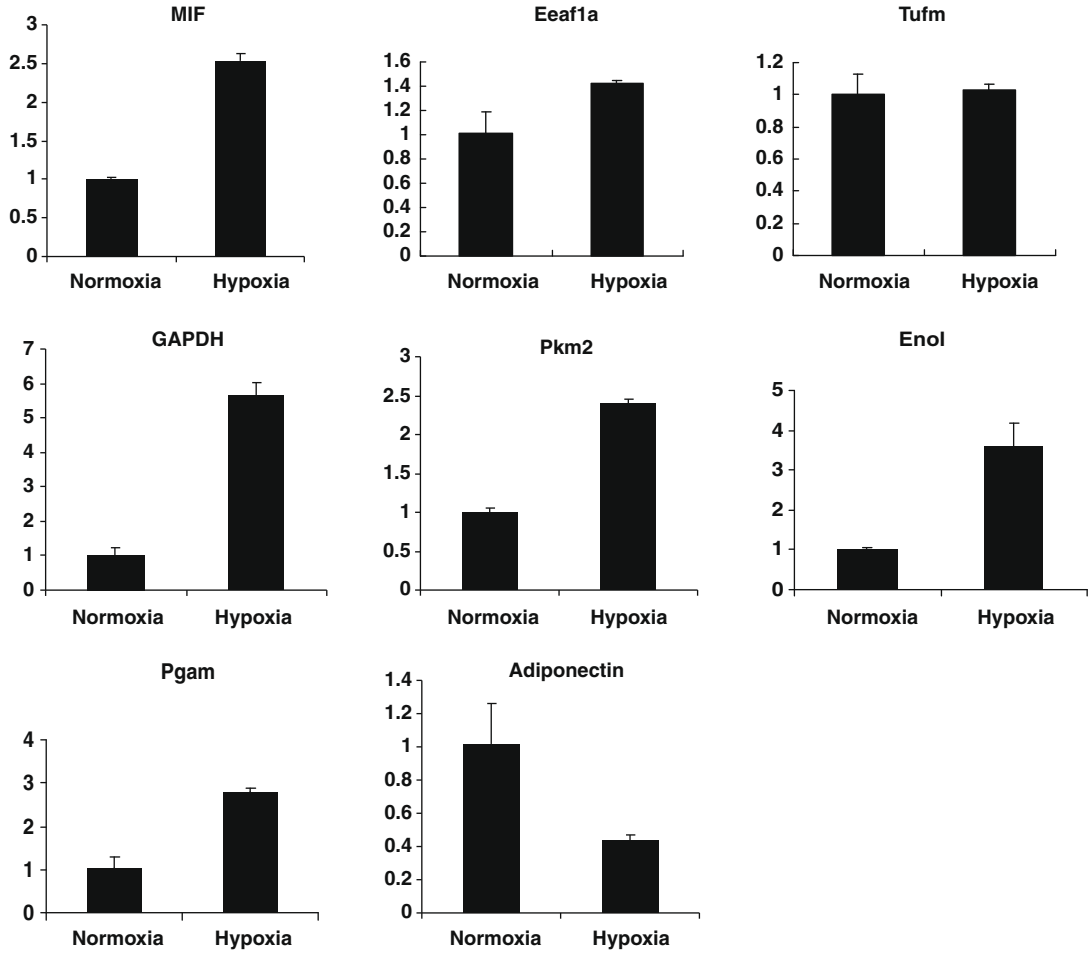

Fig. 4. Real-time PCR analysis of mRNA levels of MIF, elongation factors, glycolytic enzymes, and adiponectin under hypoxic conditions. mRNA levels were normalized by 36B4.

hydrolysis of fructose-1-6-bisphosphate into two 3-carbon products, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate [36]. GAPDH catalyzes the NAD^+-dependent oxidation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate and NADH in the second phase of glucose catabolism [36]. GAPDH and aldolase react in reverse during gluconeogenesis [36]. Pgam1 is involved in the conversion of glycerate-3-phosphate to glycerate-2-phosphate [37], and this protein expression level is increased as an effect of sustained hypoxia [37]. The mRNA levels of glycolytic enzymes were increased in hypoxic adipocytes.

In hypoxic cells, there is a need for increased glucose uptake for maintenance of ATP levels mediated by HIF, which increases expression of the glucose transporters, GLUT-1, and GLUT-3 [38,39]. HIF is known to restrict oxygen consumption and to up-regulate glycolysis. As a result, by increasing glycolytic enzyme levels following hypoxia, adipocytes can increase glucose uptake to allow anaerobic glycolysis to produce ATP molecules. We viewed the increase in glycolytic enzymes in hypoxic adipocytes, as evidence that the cells were affected by low oxygen stress imposed by the hypoxic condition.

RT-PCR analysis of genes coding for differentially expressed proteins

Significant changes ($p < 0.05$) were analyzed based on two independent experiments. The levels of mRNA were normalized relative to the amount of 36B4 mRNA [7]. Quantitative real-time PCR verified that the mRNA of MIF, elongation factors, and glycolytic enzymes was significantly up-regulated by hypoxia (Fig. 4). We further evaluated the effect of hypoxia on mRNA expression for adiponectin, which was found to decrease in hypoxic adipocytes. It has been previously reported that hypoxia or anoxia reduces adiponectin mRNA expression level [1].

In conclusion, during the development of obesity, adipocytes size and number increase in response to the diffusion limited oxygen condition. Because of this, we hypothesized that hypoxia might induce dysfunctions in adipocytes. A basic objective of our proteome study is to estimate the effects of hypoxia in adipocytes based on the large-scale differential expression of proteins between normoxic and hypoxic conditions. To our knowledge, this is the first study that has analyzed the difference in total protein expression level resulting from hypoxia in adipocytes, using quantitative proteome analysis.

We found that cellular responses to hypoxia in adipocytes are characterized by up-regulation of proteins related to stress responses, to protein synthesis, and to glycolysis. From the results of the present study, we suggest that hypoxia is a factor that can induce reactive oxygen stress responses and that hypoxic adipocytes produce proteins. Therefore, the proteins that are increasingly expressed during hypoxia may serve as features for investigation of adipocytes in stress responses such as the hypoxic condition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.03.124.

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