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Chronic hypoxia alters mitochondrial composition in human macrophages

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ABSTRACT

Hypoxia inducible factors (HIFs) are important mediators of the cellular adaptive response during acute hypoxia. The role of HIF-1 and HIF-2 during prolonged periods of hypoxia, i.e. chronic hypoxia is less defined. Therefore, we used human THP-1 macrophages with a knockdown of either HIF-1α, HIF-2α, or both HIFα-subunits, incubated them for several days under hypoxia (1% O_2), and analyzed responses to hypoxia using 2D-DIGE coupled to MS/MS-analysis. Chronic hypoxia was defined as a time point when the early but transient accumulation of HIFα-subunits and mRNA expression of classical HIF target genes returned towards basal levels, with a new steady state that was constant from 72 h onwards. From roughly 800 spots, that were regulated comparing normoxia to chronic hypoxia, about 100 proteins were unambiguously assigned during MS/MS-analysis. Interestingly, a number of glycolytic genes were up-regulated, while a number of inner mitochondrial membrane proteins were down-regulated independently of HIF-1α or HIF-2α. Chronic hypoxic conditions depleted the mitochondrial mass by autophagy, which occurred independently of HIF proteins. Macrophages tolerate periods of chronic hypoxia very well and adaptive responses occur, at least in part, independently of HIF-1α and/or HIF-2α and comprise mitophagy as a pathway of particular importance.

1. Introduction

Immune competent cells such as macrophages are often recruited to sites of inflammation, where they adapt to the local microenvironment to execute their functions. Accompanying inflammation, an increased metabolic demand from immune cells and pathogens provokes the local depletion of oxygen, resulting in hypoxia [1]. Periods of long lasting oxygen deprivation can also occur in association with chronic inflammatory conditions such as diabetes, atherosclerosis, Q2 tumor associated anemia, compromised perfusion of leaky microvessels or are noticed in individuals living at high altitude and thus, are permanently exposed to a low O_2 partial pressure [2–6]. Conditions of long lasting hypoxia are often termed chronic hypoxia (CH), although the term is not well defined, neither in respect to the hypoxic period, the severity of hypoxia, nor underlying proteome changes.

Macrophages are actively involved not only in the onset but also in the resolution phase of inflammation, tissue regeneration, and remodeling. Differentiation of monocytes under hypoxia elicits a distinct macrophage phenotype that at least in part is characterized by an increased protein amount of HIF-1α [7]. HIF is important to coordinate hypoxic responses and consists of a constitutively expressed β-subunit and an oxygen-regulated α-subunit. Both are members of the helix–loop–helix/Per, ARNT, SIM (PAS) transcription family [1,8]. Among the α-subunits HIF-1α and HIF-2α are best characterized. Both contain an oxygen-dependent degradation domain (ODD) with two conserved prolyl residues [9,10] that are hydroxylated by prolyl hydroxylases (PHD) 1–3 when sufficient oxygen is available. Once hydroxylated the α-subunits are recognized by the tumor suppressor protein von Hippel–Lindau (pVHL), marked for ubiquitination followed by proteasomal degradation. PHD activity is impaired under hypoxia, which in turn causes accumulation and translocation of HIF-α into the nucleus. The α-subunit forms a heterodimer with the β-subunit and binds to hypoxia-responsive elements (HRE) in regulatory regions of target genes. By recruiting cofactors like p300 or CBP the HIF proteins enhance transcription of about 400 target genes [11,12]. Besides protein stability, regulation of HIF-1α mRNA transcription and/or translational regulatory pathways is established as well, although the protein amount of the HIF-α subunits is mainly regulated at the protein level via PHD activity and subsequent 26S proteasomal degradation. Interestingly, PHD enzymes themselves are under the transcriptional control of HIF-1α. Thus hypoxia itself initiates a negative feedback loop upregulating

Abbreviations: 2D-DIGE, 2-dimensional differential gel electrophoresis; ACAD9, acyl-CoA dehydrogenase 9; AMPK, AMP-dependent protein kinase; ATG, autophagy-related protein; ATP5, ATP synthase subunit; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; BNIP3L, BNIP3 ligand; CAT, cathepsin; CH, chronic hypoxia; FACS, fluorescence activated cell sorting; GAPDH, glycerinaldehyd-3-phosphat-dehydrogenase; GO, gene ontology; HIF, hypoxia inducible factor; IMMT, mitochondrial inner membrane protein; LC3, microtubule-associated protein 1A/1B-light chain 3; MS/MS, tandem mass spectrometry; NAO, nonyl acridine orange; p53, tumor protein 53; PHD, prolyl hydroxylase

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PHDs, particularly PHD2, which in turn acts on HIF to lower its amount and activity [10,13–15]. The relevance of this regulatory circuit for chronic hypoxia is unknown and the contribution of HIF-1α as well as HIF-2α to chronic hypoxic responses is largely unexplored.

In addition, HIF-independent responses to hypoxic stress are known to modulate protein translation, cell metabolism and cell fate under the control of mammalian target of rapamycin (mTOR) signaling, the unfolded protein response (UPR), miRNA alterations or AMP-dependent protein kinase (AMPK) [16]. In addition PHDs and factor inhibiting HIF (FIH) have non-HIF-α substrates, which may affect some physiological functions. Under hypoxia HIF-1 mediates a transition from oxidative to glycolytic metabolism by regulating pyruvate dehydrogenase kinase 1, lactate dehydrogenase A and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) as well as BNIP3 ligand (BNIP3L). The purpose of blocking mitochondrial respiration and switching to glycolysis is to attenuate mitochondrial ROS (reactive oxygen species) formation that otherwise occurs due to a reduced efficacy of the electron transfer to oxygen under hypoxia [17,18]. Substantiating altered mitochondrial respiration, a reduced mitochondrial mass is a common adaptive response. Mitophagy, a specialized form of autophagy, involves various factors such as nucleoporin p62, beclin1, microtubule-associated protein 1A/1B-light chain 3 (LC3), BNIP3, or BNIP3L [19,20]. These factors are part of the phosphatidylinositide 3-kinase class III complex-mediated autophagosome formation. The autophagosome docks to lysosomes followed by fusion and subsequent digestion of all trapped macromolecules. Some proteins eliciting autophagosome formation like BNIP3 are HIF-regulated, suggesting that mitophagy under hypoxia is a HIF-dependent process [21–23].

Whereas gene array and chromatin immunoprecipitation (ChIP) sequencing technology described HIF-binding sites and gene expression reasonably well, only a few studies approached global proteome changes under hypoxia with even less information being available on how the distinct α-subunits affect proteome changes [24–26]. To better define the term chronic hypoxia we used a 2D-DIGE approach followed by MS analysis to identify HIF-dependent or -independently regulated proteins. We noticed increased mitophagy and glycolysis as essential adaption mechanisms under chronic hypoxia, interestingly enough, being HIF-independently regulated.

2. Material and methods

2.1. Cell culture

If not indicated otherwise, chemicals were purchased from Sigma (Steinheim, Germany), while cell lines came from ATCC (LGC Promochem, Wesel, Germany). The monocytic cell line THP-1 was incubated at 37 °C with 5% CO_2 in RPMI medium containing stable glutamine, 10% FCS and 1% penicillin/streptomycin (from PAA Laboratories, Cölbe, Germany). Monocytes were stably transduced with a lentiviral shRNA (Mission shRNA) against HIF-1α (sh1: TRCN0000003810) and HIF-2α (sh2: TRCN0000003804) or both isoforms (sh1/2) and selected using puromycin (sh1 and sh2) or a combination of puromycin/neomycin (sh1/2). Controls are control virus (CV) transduction THP-1 cells with a pLKO.1-puro vector containing a non targeted short hairpin sequence and a puromycine resistance. Monocytes were differentiated into macrophages with 10 nM TPA for 5 days.

2.2. Hypoxia

Differentiated THP-1 cells were incubated for different times at 1% O_2 in a hypoxic incubator (Invivo2 400, Ruskinn Technology, Leeds U.K.). Fresh, hypoxic medium was provided to cells after 24 h and 72 h without reoxygenation. Proteins were harvested under hypoxic conditions, while RNA was harvested directly after removing cells from the incubator.

2.3. Western analysis

Cells were lysed in lysis-buffer (6.65 M urea, 10% glycerol, 1% SDS, 10 mM Tris/HCl, pH 7.4) and sonicated. After centrifugation (15,000 ×*g*, 5 min) the protein content was determined in the supernatants by a protein assay kit (Bio-Rad, Munich, Germany). For immune detection of HIF-1α and HIF-2α 100 μg protein was separated on 7.5% SDS gels and blotted on Immobilon-FL PVDF membranes (Millipore Corporation, Billerica, MA) and incubated with primary antibodies against human HIF-1α (polyclonal, Novus Biologicals Acris, Hiddenhausen, Germany) and HIF-2α (polyclonal, R&D Systems, Abingdon, UK). For validation of DIGE results 20 μg protein was loaded on a 10% gel and exposed to antibodies against acyl-CoA dehydrogenase 9 (ACAD9) (polyclonal rabbit), mouse α-tubulin, or rabbit ATP5a/b antiserum. Membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase followed by detection using Enhanced chemiluminescence (ECL).

2.4. RNA isolation from THP-1 cells

Total RNA was isolated from cell cultures using peqGold (Peqlab, Erlangen, Germany) following the manufacturer's instructions and stored at −80 °C. Concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). Reverse transcription was performed with Maxima First Strand cDNA Synthesis Kit for RT-PCR (Thermo Scientific, Karlsruhe, Germany) and cDNA was stored at −20 °C.

2.5. Quantitative real time PCR

HIF target gene expression analysis was performed using SYBR green fluorescein mix (Thermo Scientific) and a CFX96 Real Time PCR Detection System (Bio-Rad). The following primer sequences were used: TBP (Fwd: 5′-GGGCCGCCGGCTGTTTAACT-3′, Rev: 5′-AGCCCTTGAGCGTAAGGTGG CA-3′) and GLUT1 (Fwd: 5′-CCTGTGCTCCTGAGAGATCC-3′, Rev: 5′-AAG CCTGACCACGCTTTCTA-3′) and VEGF (Fwd: 5′-CAGGCTGCTGTAACGA TGAA-3′, Rev: 5′-GCATTCACATCTGCTGTGCT-3′). BNIP3 primers were purchased from Qiagen (Hilden, Germany).

2.6. Inhibition of autophagy

Differentiated THP-1 cells were treated with 10 nM bafilomycin A1 (LC Laboratories, Woburn, MA) or dimethylsulfoxid (DMSO) as a solvent control for 1 h, followed by the individual experimental set up. Medium containing 10 nM bafilomycin A1 was changed after 24 h of incubation.

2.7. FACS

Mitochondrial mass was estimated by staining with nonyl acridine orange (NAO). Cells were washed either with normoxic or hypoxic PBS and then stained with 20 nM NAO diluted in PBS for 30 min in the dark. After staining, cells were washed again and carefully removed from the dishes using accutase (PAA, Cölbe, Germany). After centrifugation and washing with PBS, cells were stained with Annexin V (ImmunoTools, Friesoythe, Germany) and propidium iodide (PI) for 15 min in the dark and measured on a LSRFortessa (BD, Heidelberg, Germany). Sample preparation for FACS analysis was performed at 4 °C, respectively on ice.

2.8. 2D-DIGE

Cells for 2D-DIGE were harvested in cooled PBS and pelleted by centrifugation (2 min) at 1000 ×*g*. Hypoxia-treated cells were harvested in hypoxic cooled PBS under hypoxic conditions. PBS was removed and pellets were stored at −80 °C. Pellets were solubilized in 20 μl DIGE-

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