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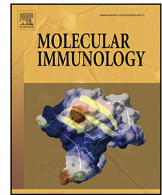

Regulation of the HIF-system in human macrophages – Differential regulation of HIF-α subunits under sustained hypoxia

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ABSTRACT

Macrophages are often associated to pathophysiological processes and were found at hypoxic areas. However, cell adaption greatly depends on hypoxia-inducible factors (HIF). Activation of these transcription factors is induced by heterodimerization of an α-(HIF-1α, -2α, -3α) and HIF-1β subunit. The main regulatory pathway is represented by α-subunit stability. Beside, little is known about the exact mechanisms of fine-tuning in Hif-regulation. The present study characterizes the hypoxia-induced regulation of HIF-1α and -2α in human macrophages. The hypoxic increase of both subunits is initially mediated by protein stabilization. Sustained hypoxia caused a distinct regulation of HIF-1α and -2α. The striking increase of HIF-2α protein expression was contrasted by a dramatic decrease of HIF-1α. The long-term downregulation of HIF-1α is due to downregulation of its mRNA. This decrease was accompanied by increased expression of ahif, a natural cis-antisense transcript of HIF-1α. The ahif-transcript was strongly inducible by hypoxia and rapidly degraded under reoxygenation. Using an adenoviral overexpression and siRNA silencing approach revealed that the targeted regulation of ahif is mediated by the HIF-system itself. Furthermore it could be shown that ahif indeed is able to modulate the hypoxic expression of HIF-1α and influences the expression of the HIF-target gene Enolase-2.

Taken together, this study characterizes a new regulation process of the HIF-transcription factor-system in human macrophages under hypoxia. For the first time evidence is provided that ahif is regulated by the HIF-system and influences HIF-1α expression in primary human macrophages.

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1. Introduction

The hypoxia-inducible factors play a major role in the adaptation to low oxygen tensions. Therefore they are indispensable for a multitude of cellular functions like survival, angiogenesis, apoptosis and glucose metabolism.

The HIF transcription factor complex consists of a heterodimer formed by one HIF-α (HIF-1α, -2α, -3α) and the β-subunit (HIF-1β or ARNT). Whereas the β-subunit is constitutively and ubiquitously expressed, the α-subunits are tightly regulated by oxygen. Under normoxic conditions the α-subunits are continuously hydroxylated by HIF-prolylhydroxylases (PHD 1–3). This hydroxylation is required for the binding of the von-Hippel–Lindau tumor suppressor protein to the α-subunits, which leads to subsequent ubiquitination and proteosomal degradation (Semenza, 2003). The PHDs belong to the enzyme family of dioxygenases. They need in addition to the co-factors Fe^{2+}, ascorbate and 2-oxogluterate, oxygen as substrate. Therefore, reduced oxygen tension leads to an inactivation of these enzymes, which consequently results in a stabilization of the HIF-α-subunits. The α-subunits translocate into the nucleus, where they dimerize with the β-subunit and bind to the hypoxia response element (HRE) (Bruick, 2003). Besides this posttranscriptional regulation of the HIF-system, another regulatory mechanism, meanwhile called the antisense effect, was described by the group of Tartof in 1999. They characterized a transcript, which is partly complementary to the 3′-UTR of HIF-1α and named it natural antisense transcript of HIF-1α (ahif) (Thrash-Bingham and Tartof, 1999). A further report correlated the expression of ahif with the prognosis in breast cancer (Cayre et al., 2003). Uchida and his colleagues showed that the transcript itself is regulated by hypoxia in the A549 lung epithelial cell line. Furthermore, they show an involvement of the HIF-system within this regulation (Uchida et al., 2004). But the different impact of HIF-1 and HIF-2 in the hypoxic ahif regulation remains unclear. In addition, until now, there is no direct evidence for a regulation of HIF-1α by ahif.

The HIF-system has exceeding importance for the development of the cardiovascular system and in pathophysiological processes:

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knock-out models for HIF-1α and HIF-2α are lethal due to defects within the cardiovascular system during the embryonic development (Kotch et al., 1999; Peng et al., 2000). The progression of the atherosclerotic plaque is greatly influenced by hypoxia and especially the HIF-1α transcription factor (Bjornheden et al., 1999; Murdoch et al., 2004).

Macrophages have been shown to play a pivotal role in the pathogenesis of atherosclerosis and other diseases. Moreover they home to the hypoxic sites of the plaque (Murdoch et al., 2004; Sluimer et al., 2008) and express large amounts of HIF-1α and HIF-2α under these conditions (Sluimer et al., 2008; Vink et al., 2007). In contrast to other cells, macrophages are one of the few cell types expressing both, HIF-1α and -2α (Elvidge et al., 2006; Lofstedt et al., 2007) but not HIF-3α (Augstein et al., 2011). Furthermore, it was shown that elements of the HIF-system are involved the regulation of crucial cellular functions in macrophages. This includes for example vascular remodelling (Nakayama et al., 2013), cell survival (Poitz et al., 2011), leukocyte adhesion (Kong et al., 2004) and the lipid uptake (Crucet et al., 2013). However little is known about the distinct regulation processes of the HIF-system under hypoxia including the antisense regulation in macrophages. The present study focusses on the hypoxic regulation of HIF-1α within primary human macrophages.

2. Materials and methods

2.1. Cell culture and experimental design

Peripheral blood mononuclear cells (MNC) were obtained by Ficoll density gradient centrifugation (LSM 1077, PAA) with a subsequent Percoll gradient ($\rho = 1.064\,\text{g}\,\text{cm}^{-3}$, GE healthcare) of human buffy coats (DRK) from healthy donors. The mononuclear cell fraction was resuspended in Medium 199 with Earle's salts (PAA) supplemented with 10% FCS (Sigma–Aldrich), streptomycin sulphate (100 μg/ml), penicillin (100 IU/ml) and amphotericin B (0.25 μg/ml) and seeded at 1×10^6 cells per well in 6-well ultra-low attachment plates (Corning Life Science). The cells were further cultured for 12 days to allow differentiation into monocyte-derived macrophages (MDMs).

Macrophages were seeded for adherence to normal plastic plates (BD Biosciences). Adenoviral infection was done using a multiplicity of infection of 100. The infection efficiency following 48 h was nearly 100% as confirmed by lacZ-staining or GFP-expression.

Human umbilical venous endothelial cells (HUVEC) and vascular smooth muscle cells were isolated and cultured as previously described (Augstein et al., 2011).

Exposure to hypoxia was done using a hypoxic chamber (BioSpherix) within a standard incubator. The cells were exposed to 0.5% O_2 balanced with a gas mixture consisting of 95% N_2 and 5% CO_2. The normoxic controls were cultured for the indicated time in an incubator under normal culture conditions (humid atmosphere, 5% CO_2).

2.2. Protein extraction and immunoblot analysis

Antibodies and dilutions were used as follows: monoclonal mouse-anti-HIF-1α (BD Biosciences) 1:500, polyclonal rabbit-anti-HIF-2α (Novus biologicals) 1:1000, monoclonal mouse-anti-β-actin (Santa Cruz) 1:1000, sheep-anti-mouse-HRP (Amersham) 1:10,000 and goat-anti-rabbit-HRP (Santa Cruz) 1:2500.

Total cell extracts were prepared by scraping the cells from the culture plates in lysis buffer (50 mM Tris–Cl, pH 6.1, 2% SDS, 5% Glycerol). The cell extracts were heated to 95 °C for 10 min and BCA assay (Interchim) was performed in order to determine protein concentrations. SDS-PAGE gels were run and electroblotted onto PVDF membranes (Pall Fluoro Trans® Membran, Pall Corporation) using standard methods. The blots were probed with primary antibodies in 0.1% TBST/5% non-fat dried milk before detection with secondary antibody and visualization of protein bands by chemiluminescence. Densitometric analysis of protein bands was done using "QuantityOne" software (BioRad). Equal loading of proteins was determined by detection of β-actin.

2.3. RNA Isolation and real-time RT-PCR

Total RNA was isolated using the Invisorb Spin Cell RNA Mini Kit (Invitek). cDNA was synthesized with the Revert Aid™ H Minus First Strand Synthesis Kit (MBI Fermentas) from 1 to 5 μg total RNA with Random Hexamer primers. PCR was done with the iCycler real-time PCR System (BioRad) using the SYBR®Premix ExTaq™ (Lonza). The PCR conditions for all primer sets were as follows: initial denaturation at 95 °C for 1 min followed by 45 amplification cycles, each consisting of 95 °C for 20 s, 58 °C for 45 s and 72 °C for 20 s with a final extension step at 72 °C for 2 min with a subsequent melting point analysis. Identity of PCR products was proven by melting point analysis, electrophoresis and sequencing of resulting PCR products (sequencing facility, MPI CBG Dresden) Relative quantification of gene expression was calculated using the $\Delta\Delta c_t$ method using Gene expression Macro Version 1.1 (BioRad) (Table 1).

2.4. Construction and purification of adenoviruses

Recombinant replication-deficient adenoviruses were constructed using the AdenoX Expression System-1 (Clontech). In brief: the coding sequences of HIF-α-subunits and ahif were amplified from oligodT-reverse-transcribed cDNA using primers with 5′-extensions containing the recognition sequence for *Nhe*I and *Kpn*I to allow cloning into the pShuttle vector and a stop-codon in the case of dominant-negative HIF-2α (HIF-αdn). The identity of the fragments was verified by sequencing (MPI CBG Dresden). The expression cassette including the fragment of interest was cut out of pShuttle vector and cloned into pAdenoX vector, followed by linearization using *Pac*I and subsequent transfection into HEK293 cells by calcium-phosphat-precipitation using the Calciumphosphat Transfection Kit (Sigma–Aldrich). The resulting virus was collected and amplified. High-titer stocks of the recombinant adenovirus were purified by caesium chloride density gradient centrifugation. The virus-titer was determined using the AdenoX-Rapid-Titer-Kit (Clontech).

2.5. Nuclear extraction

Macrophages were seeded confluently on petri dishes (58 cm^2) and incubated for 12 h under normoxic culture conditions to allow adherence. The culture media was exchanged after adhesion. After exposure to either normoxia or hypoxia cells were lysed and processed with the Nuclear Extract Kit (Active Motif) according to the manufacturer's manual.

2.6. Estimation of DNA binding activity

Nuclear protein extraction was performed as described above. An ELISA-based Kit for detection of HIF-1 DNA binding activity (TransAM™ HIF-1) was used according to manufacturer's instructions (Active Motif).

2.7. Transfection of siRNA

Transfection of macrophages with siRNA (siMAX siRNA, MWG Biotech) was done using the Interferin™ siRNA transfection reagent (Polyplus Transfections) according to the manufacturer's

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