Biochemical and Biophysical Research Communications 434 (2013) 166–172

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hypoxia-inducible factor-1β (HIF-1β) is upregulated in a HIF-1α-dependent manner in 518A2 human melanoma cells under hypoxic conditions

Markus Mandl *, Barbara Kapeller, Roman Lieber, Karin Macfelda

Department of Biomedical Research, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria

ARTICLE INFO

Article history:
Received 26 February 2013
Available online 26 March 2013

Keywords:
HIF
ARNT
Hypoxia
Melanoma
Angiogenesis
Invasion

ABSTRACT

Solid tumors include hypoxic areas due to excessive cell proliferation. Adaptation to low oxygen levels is mediated by the hypoxia-inducible factor (HIF) pathway promoting invasion, metastasis, metabolic alterations, chemo-resistance and angiogenesis.

The transcription factor HIF-1, the major player within this pathway consists of HIF-1α and HIF-1β. The alpha subunit is continuously degraded under normoxia and becomes stabilized under reduced oxygen supply. In contrast, HIF-1β is generally regarded as constitutively expressed and being present in excess within the cell. However, there is evidence that the expression of this subunit is more complex.

The aim of this study was to investigate the role of HIF-1β in human melanoma cells. Among a panel of five different cell lines, in 518A2 cells exposed to the hypoxia-mimetic cobalt chloride HIF-1β was rapidly elevated on protein level. Knockdown experiments performed under cobalt chloride-exposure and hypoxia revealed that this effect was mediated by HIF-1α. The non-canonical relationship between these subunits was further confirmed by pharmacologic inhibition of HIF-1α and by expression of a dominant-negative HIF mutant. Overexpression of HIF-1α showed a time delay in HIF-1β induction, thus arguing for HIF-1β de novo synthesis rather than protein stabilization by heterodimerization. A Hen's egg test-chorioallantoic membrane model of angiogenesis and invasion indicated a local expression of HIF-1β and implies a biological relevance of these findings.

In summary, this study demonstrates the HIF-1α-dependent regulation of HIF-1β under hypoxic conditions for the first time. The results indicate a novel cell specific mechanism which might prevent HIF-1β to become a limiting factor.

1. Introduction

The hypoxia-inducible factor (HIF) pathway mediates cellular adaptation to reduced oxygen supply, a consequence of excessive proliferation in solid tumors. HIF activity contributes to invasion, metastasis, metabolic alterations, chemo-resistance and enables the induction of angiogenesis [1–3]. Constitutive HIF activity was found in malignant melanoma [4] which represent an aggressive and heterogenous disease [5,6]. Therefore inhibition of the HIF pathway provides a treatment strategy in cancer therapy [7,8]. The HIF pathway consists of alpha and beta subunits of transcription factors belonging to the Per-ARNT-Sim (PAS) family. Under normoxic conditions HIF-1α (as well as HIF-2α) is hydroxylated on two conserved proline residues mediating its recognition by the von Hippel–Lindau tumor suppressor protein and its subsequent degradation via the proteasome. Hypoxia prevents the hydroxylation of HIF-1α leading to the accumulation of the subunit. Subsequently HIF-1α translocates into the nucleus where it dimerizes with HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) via interaction of PAS domains followed by initiation of target gene transcription (e.g. vascular endothelial growth factor, VEGF) [1,9,10].

In addition to hypoxic conditions, HIF-1α protein level is elevated by growth factors via phosphatidylinositol 3-kinase (PI3K)/Akt signaling mediating increased HIF-1α mRNA translation [11,12]. Blocking of PI3K with the classical inhibitor LY294002 therefore inhibits HIF-1α and the expression of its target genes [7,13]. Another approach for experimental HIF inhibition is the forced expression of a deletion-mutant of HIF-1α (or HIF-2α) containing a PAS domain which mediates binding to other PAS proteins and acts as a dominant-negative form (dnHIF) [14–17]. Inhibition of the pathway is therefore achieved by disturbing the formation of functional HIF complexes [14]. In contrast to the oxygen-sensitive subunit HIF-1α, HIF-1β is generally regarded as con-

Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator (HIF-1β); CAM, chorioallantoic membrane; DnHIF, dominant-negative HIF; Het-CAM, Hen's egg test-chorioallantoic membrane; HIF, hypoxia-inducible factor.

* Corresponding author. Fax: +43 1 40400 5229.
E-mail address: mmandl@mail.austria.com (M. Mandl).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.051

stitutively expressed, meaning an oxygen-independent regulation [10,12]. Further this subunit is considered to be present in excess within the cell [3]. However, there is evidence that HIF-1β is increased on mRNA as well as on protein level when cells are exposed to hypoxia or treated with the hypoxia-mimetic cobalt chloride ($CoCl_2$) [18,19].

The aim of this study was to investigate the role of HIF-1β in human melanoma cells. Among a panel of five different cell lines, in 518A2 cells exposed to the hypoxia-mimetic cobalt chloride HIF-1β was rapidly elevated at protein level. Further experiments performed under hypoxia or $CoCl_2$ exposure revealed that this effect was mediated by HIF-1α. The results indicate a novel cell specific mechanism by which HIF-1α regulates the expression of HIF-1β under hypoxic conditions. This might prevent HIF-1β from being a limiting factor.

2. Materials and methods

2.1. Materials

Cobalt chloride ($CoCl_2$) and LY294002 were purchased from Sigma–Aldrich® (Steinheim, Germany). The anti-HIF-1α antibody (clone mgc3) was obtained from Thermo Scientific (Pierce Biotechnology, Rockford, USA), anti-HIF-2α (clone ep190b) as well as anti-HIF-1β (clone 2B10) antibodies were purchased from abcam® (Cambridge, UK). The anti-β-actin antibody (clone AC-15) was bought from Sigma–Aldrich® (Steinheim, Germany). siRNA Transfection Reagent and Medium as well as all siRNA's (HIF-1α: sc-35561, HIF-2α: sc-35316, Arnt1: sc-29733, FITC-conjugated control: sc-36869) were purchased from Santa Cruz Biotechnology® (Szabo-Scandic HandelsgmbH, Vienna, Austria). The HIF-1α expression plasmid was obtained from OriGene (Rockville, USA). The eGFP-encoding plasmids pGFP-N3 and pEGFP-C1 were kindly provided by Dr. Grusch (Medical University of Vienna, Vienna, Austria).

2.2. Cell culture

Human melanoma cell lines 518A2, A375, SK-MEL-28 and MEL-JUSO were kindly provided by the Department for Dermatology (Medical University of Vienna, Vienna, Austria). 607B cells were a gift from Dr. Wacheck (Medical University of Vienna, Vienna, Austria). All cells were maintained in DMEM high glucose with L-glutamine (Sigma–Aldrich®, Steinheim, Germany) supplemented with 10% FCS (PAA, Linz, Austria) and 1% Penicillin/Streptomycin (Gibco®, Auckland, New Zealand). Cells were grown at +37 °C in a humidified atmosphere containing 5% CO_2. For hypoxic exposure, cells were incubated at 1% O_2 and 5% CO_2.

2.3. Cobalt chloride-induced chemical hypoxia

Cells were seeded at a density of 1.2×10^5 cells/well on 12-well plates and allowed to adhere overnight. Next day, the medium was removed and chemical hypoxia was induced by treatment with 100 μM $CoCl_2$. After defined time points (0, 2, 4, 8 and 24 h) supernatant was discarded and cells were washed twice with PBS. Cells were lysed by the use of 50 μl 2× sample buffer [125 mM Tris (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol, bromophenol-blue] per well and proteins were subjected to Western Blot analysis.

2.4. Knockdown of HIF subunits

Cells were seeded on 6-well plates at a density of 2×10^5 cells/well in antibiotic-free DMEM supplemented with 10% FCS. Cells were grown overnight before transfection with siRNA (Santa Cruz Biotechnology®) according to the manufacturer's protocol. Briefly, for each reaction 4 μl siRNA were diluted in 100 μl siRNA Transfection Medium and mixed with 6 μl siRNA Transfection Reagent diluted in 100 μl siRNA Transfection Medium. For knockdown of two subunits, 4 μl of each appropriate siRNA were used. After transfection, cells were stimulated with 100 μM $CoCl_2$ for 24 h. For exposure to hypoxia (1% O_2, 5% CO_2, 6 h), knockdown procedures were up-scaled and performed in 12.5 cm^2 flasks. Thereafter supernatants were collected and stored at −80 °C for ELISA. Cells were washed with PBS and lysed by using 100 μl 2× sample buffer to gain proteins for Western Blotting.

2.5. Construction of a dominant-negative form of HIF-1α (dnHIF)

The HIF-1α deletion-mutant acting as dominant-negative form was constructed as described in literature [14]. Briefly, the appropriate sequence was obtained by PCR using cDNA from HEK293 cells as template and the following primer pair: for 5′-TTTGGATCCACCATGCGAAGTAAAGAATCTG-3′; rev 5′-TTTGGATCCTTAAGCGTAATCTGGAACATCGTATGGGTATTTGTCAAAGAGGCTACT-3′ Subsequently the PCR product was cloned into the vector pEGFP-C1. Finally, sequencing of the resulting eGFP–dnHIF fusion gene confirmed the presence of a PAS domain.

2.6. Plasmid transfection procedure

Cells were seeded on a 12-well plate at a density of 1.2×10^5 cells/well and allowed to adhere overnight. Cells were transfected either with the HIF-1α expression plasmid or pGFP-N3 which served as control (1 μg/well). Transfection was performed using Exgen500 (Fermentas, St. Leon-Rot, Germany) according to the supplier's instructions. After 24 h, successful transfection was verified by eGFP expression of control cells using fluorescence microscopy. Stable transfection of cells was achieved by selection with 400 μg/ml G418 (Calbiochem®, Merck, Darmstadt, Germany). Chemical hypoxia was induced as described above and cell lysates containing proteins were subjected to Western Blotting.

2.7. Reverse transcription (RT)-PCR of stable transfected cells

RNA was isolated using GenElute™ Mammalian Total RNA Kit (Sigma–Aldrich®, Steinheim, Germany) according to the supplier's guidelines. cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, Darmstadt, Germany) as described in the manufacturer's instructions. Transgene expression was determined using the following primer: dnHIF: for 5′-TCTGGGTTGAAACTCAAGCAACTGTC-3′, rev 5′-AAGC GTAATCTGGAACATCGTATGGG-3′; eGFP: for 5′-GCCATGCCCGAAG GCTACG-3′; rev 5′-GCCCTTCAGCTCGATGCGG-3′. PCR was performed according to the supplier's guidelines using GoTaq® PCR reagents (Promega, Madison, USA). PCR products were dissolved on a 2% agarose gel containing ethidiumbromide and visualized using the GeneFlash gel documentation system (Syngene, Cambridge, UK).

2.8. Western Blot analysis

Cell lysates were heated for 10 min at + 95 °C and dissolved on 10% poly-acrylamide gels. Western Blotting was performed using polyvinylidene difluoride (PVDF) membranes (Immobilion™, Millipore, Billerica, USA) for 1 h at 300 mA followed by blocking with 5% non-fat dry milk in 0.1% TBST for 1 h.

Anti-HIF-1α and anti-HIF-2α antibodies were diluted 1:2000 and 1:1000, respectively. Anti-HIF-1β and anti-β-actin antibodies were used 1:2500 and 1:200,000. PVDF membranes were incu-

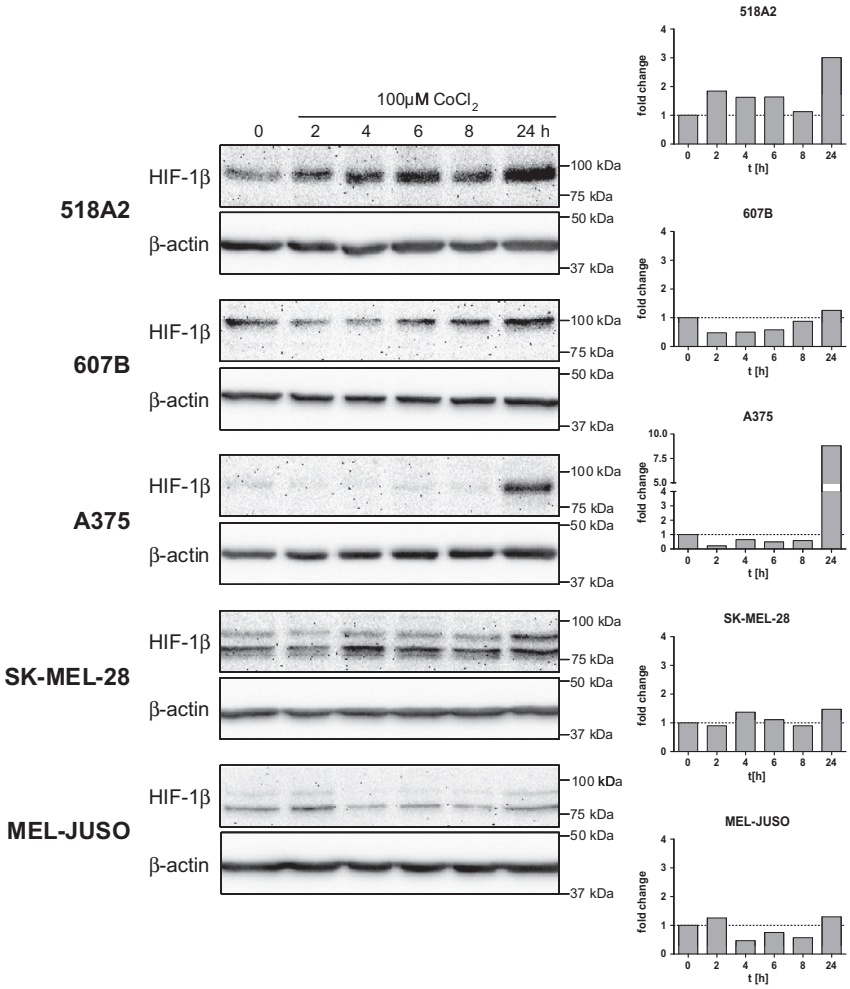

Fig. 1. Effects of cobalt chloride-induced chemical hypoxia on HIF-1β in human melanoma cells. 518A2, 607B, A375, SK-MEL-28 and MEL-JUSO cells were treated with 100 μM cobalt chloride ($CoCl_2$) as indicated or left untreated (t = 0 h). Subsequently cell lysates were subjected to Western Blot analysis. HIF-1β/β-actin ratios were calculated and normalized to untreated control cells (t = 0; dotted line; right panel).

bated with primary antibodies overnight at +4 °C. The secondary polyclonal goat anti-mouse immunoglobulins-HRP antibody (DAKO, Glostrup, Denmark) was diluted 1:5000 in 5% non-fat dry milk/TBST and incubated for 1 h at room temperature. Blots were developed using the ECL reagent [200 μM p-Coumaric acid, 1.25 μM luminol, 0.1 M Tris–HCl, pH 8.8; 3 μl 3% H_2O_2/ml added freshly] or the Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK). Finally chemoluminiscence was recorded and quantified digitally using the Chemi-Doc™ XRS$^+$ system (BioRad, Munich, Germany).

2.9. Hen's egg test-chorioallantoic membrane (Het-CAM) assay

Fertilized specific-pathogen-free eggs (*White leghorn*) were kindly provided by BAXTER Biosciences (Vienna, Austria) at embryonic day 5 (E5) and prepared for the assay as described previously [20]. At E6, 2×10^6 518A2 human melanoma cells were mixed with 20 μl Matrigel™ supplemented with human collagen IV (final 100 μg/ml, both Sigma–Aldrich®, Steinheim, Germany) to enhance polymerisation and grafted onto the chorioallantoic membrane (CAM). Cellular proliferation was observed using a stereo microscope (Nikon SMZ1000). At E11, CAM's were fixed with 4% paraformaldehyde/PBS overnight at +4 °C. Finally, CAM's were excised, dehydrated and embedded into paraffin for histological analysis.

2.10. Immunohistochemistry

10 μm thick CAM sections were deparaffinised with xylene and rehydrated through a graded alcohol series. All following steps were carried out at room temperature and slides were washed with 0.05% PBST. Antigen retrieval was performed with 0.1% pepsin for 10 min followed by application of 3% H_2O_2 for 10 min. Permeabilisation was carried out with 0.1% Triton/PBST for 10 min followed by blocking of unspecific binding sites with 10% goat serum for 20 min. Anti-HIF antibodies were applied 1:50 overnight at +4 °C. Alexa488-conjugated goat anti-mouse antibody (DAKO, Glostrup, Denmark) was diluted 1:500 and incubated for 1 h. CAM sections were counterstained with DAPI and mounted in fluorescence mounting medium (DAKO, Glostrup, Denmark). Images were acquired using a BX60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Nuance™ Multispectral Imaging System (Intas, Göttingen, Germany).

2.11. Enzyme linked immunosorbent assay (ELISA)

Vascular endothelial growth factor (VEGF) within cell culture supernatants was measured using the Human VEGF Mini Development Kit from PeproTech® (Rocky Hill, USA) as described in the manufacturer's instructions. The ABTS substrate was purchased

from Sigma–Aldrich® (Steinheim, Germany). All samples were measured in triplicates.

2.12. Statistical analysis

ELISA values are presented as mean ± SEM. Statistical comparison between groups was performed using unpaired *t* test. $P < 0.05$ was considered as statistically significant. Calculations were done using GraphPad Prism® 4 software (GraphPad Software, La Jolla, USA).

3. Results

3.1. Effects of cobalt chloride-induced hypoxia on HIF-1β

HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) is considered as constitutively expressed [10,12] which means that mRNA and protein are maintained at constant levels independent of oxygen availability [21]. However, there is evidence that HIF-1β is upregulated within cells after treatment with the hypoxia-mimetic cobalt chloride ($CoCl_2$) or exposure to hypoxia on both RNA and protein levels [18,19].

To determine the inducibility of HIF-1β in human melanoma cells five different cell lines were exposed to 100 μM $CoCl_2$ for 0, 2, 4, 6, 8 and 24 h, respectively followed by Western Blot analysis. As shown in Fig. 1, HIF-1β was rapidly elevated in 518A2 cells and peaked after 24 h. In A375 cells HIF-1β protein level was increased only 24 h after stimulation. Within all other tested cell lines, HIF-1β levels compared to appropriate controls remained unchanged after 24 h. The short term induction of HIF-1β in 518A2 cells appeared to be a unique attribute of this cell type. Therefore 518A2 cells were selected for further experiments.

3.2. Knockdown of HIF-1α depletes HIF-1β on protein level in 518A2 cells

Cellular treatment with $CoCl_2$ or exposure to hypoxia elevates HIF-1α and HIF-2α protein levels due to prevention of degradation [1,10]. Therefore the upregulation of HIF-1β might be mediated by one of these subunits. To test this hypothesis, knockdown experiments in 518A2 cells were performed followed by stimulation with 100 μM $CoCl_2$ for 24 h. Subsequently, supernatants and cell lysates were collected and subjected to ELISA and Western Blot analysis respectively. As shown in Fig. 2A, silencing of HIF-1α depleted HIF-1β on protein level. The secretion of VEGF, a prominent HIF target gene, was significantly impaired when HIF-1α was knocked down (Fig. 2B). This finding highlights the predominant role of HIF-1α among other subunits in 518A2 cells. A similar correlation between the protein level of HIF-1α and HIF-1β was found when cells were exposed to 1% O_2 for 6 h (Fig. 2C).

These data indicates that HIF-1β is regulated in a HIF-1α-dependent manner in 518A2 human melanoma cells under hypoxic conditions.

3.3. Inhibition of PI3K/Akt signaling by LY294002 decreases HIF-1α and HIF-1β on protein level

HIF-1α protein levels can be elevated via PI3K/Akt signaling. Therefore blocking this pathway with the PI3K inhibitor LY294002 inhibits HIF-1α as well as the expression of its downstream targets and angiogenesis [13,22,23]. Further it was demonstrated that inhibition of HIF-1α via LY294002 has no effect on HIF-1β [23]. Thus treatment of 518A2 with this compound should decrease HIF-1α as well as HIF-1β and thereby confirm the observed non-canonical relationship between both subunits.

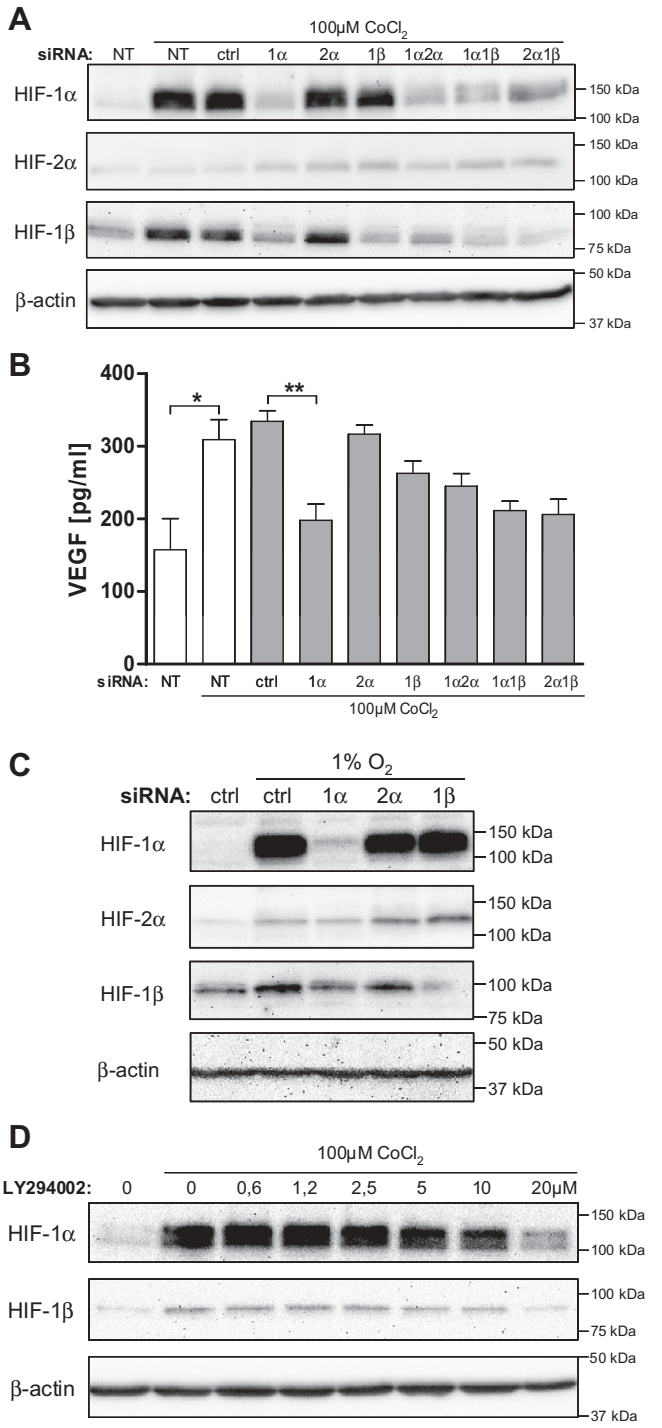

Fig. 2. Inhibition of the HIF pathway by siRNA and LY294002. (A) siRNA mediated knockdown of either one or two HIF subunits was performed and subsequently cells were stimulated with 100 μM cobalt chloride ($CoCl_2$) for 24 h followed by Western Blot analysis. (B) VEGF secretion of knockdown cells (corresponding to (A)) was measured by ELISA. Values are presented as mean ± SEM. *P*-values <0.05 were considered as significant. (C) Silencing of HIF subunits followed by exposure to 1% O_2 for 6 h. Protein levels of HIF subunits were assayed by Western Blotting. NT: not transfected (white bars); ctrl: control, scrambled siRNA transfected; VEGF: vascular endothelial growth factor; (D) 518A2 cells were exposed to 100 μM $CoCl_2$ for 24 h in the presence or absence of various concentrations of LY294002. Controls (no LY294002) were treated with an appropriate amount of DMSO as vehicle control. Subsequently HIF-1α and HIF-1β protein levels were determined by Western Blotting.

To test this hypothesis, 518A2 cells were stimulated with 100 μM $CoCl_2$ for 24 h in the presence or absence of different con-

centrations of LY294002. Subsequently HIF-1α and HIF-1β protein levels were assayed by Western Blotting (Fig. 2D). As expected, treatment of 518A2 cells with LY294002 decreased the expression of both HIF subunits in a dose-dependent manner. This finding confirms the non-canonical relationship between HIF-1α and HIF-1β within this cell type.

3.4. Ectopic expression of a dominant-negative form (dnHIF) inhibits HIF-1α and HIF-1β in 518A2 human melanoma cells

Another approach to inhibit the HIF pathway is the ectopic expression of a HIF-1α (or HIF-2α) deletion mutant acting in a dominant-negative manner [14,15,17,24] by inhibition of functional HIF complex formation [14]. Therefore the dominant-negative HIF (dnHIF) construct was generated similar as described previously [14] and cloned into the expression vector pEGFP-C1 leading to a transgene encoding an eGFP–dnHIF fusion protein.

To prevent the HIF pathway in 518A2 cells by forced expression of an inhibitor, cells were stable transfected either with the dominant-negative construct or with the eGFP-encoding vector serving as control. RT-PCR analysis revealed the expression of the appropriate transgene in transfected cells (Fig. 3A).

Inhibition of the HIF pathway in 518A2 cells by a dominant-negative form should therefore reduce the expression level of HIF-1β under hypoxic conditions and thus confirm previous results. To test this hypothesis stable transfected cells were stimulated with 100 μM cobalt chloride for 4, 8 and 24 h or left untreated ($t = 0$ h). Subsequently the protein level of HIF-1α and HIF-1β was determined by Western Blotting. As shown in Fig. 3B, HIF-1α accumulated in vector-transfected control cells in a time-dependent manner due to cobalt chloride-induced hypoxia. Surprisingly the stabilization of this subunit was delayed and less pronounced in cells expressing the dominant-negative form. HIF-1β increased on protein level over time in vector-transfected control cells. In contrast, HIF-1β expression was inhibited in 518A2 cells transfected with dnHIF compared to cells containing the empty vector.

This finding demonstrates that HIF-1β expression depends on the HIF pathway itself and is in agreement with previous results.

To test the inducibility of HIF-1β in transfectants under hypoxia, cells were exposed to 1% oxygen for 6 h or maintained under normoxic conditions. Protein levels of HIF-1α and HIF-1β were subsequently assayed by Western Blotting and compared to untransfected hypoxic and normoxic 518A2 cells. HIF-1α and HIF-1β levels were elevated in untransfected 518A2 cells when exposed to hypoxia compared to normoxic conditions (Fig. 3C). In vector-transfected cells both subunits were induced due to hypoxia. In contrast, in dnHIF-expressing cells HIF-1α stabilization was impaired compared to the vector-transfected control. HIF-1β was inducible as well in dnHIF-transfected cells under hypoxia and the protein level of this subunit was equal compared to hypoxic vector-transfected control cells.

This result demonstrates the oxygen-dependent regulation of HIF-1β in 518A2 wildtype and transfected cells. In addition it indicates that the HIF pathway inhibition by a dominant-negative form can be overcome to restore HIF-1β levels under hypoxia.

3.5. Overexpression of HIF-1α in 518A2 cells excludes heterodimerization per se as the major cause of HIF-1α-dependent inducibility of HIF-1β

HIF-1β might be elevated on protein level due to dimerization with HIF-1α and will be protected thereby against a putative degradation. Overexpression of HIF-1α should therefore result in a similar expression pattern of both subunits.

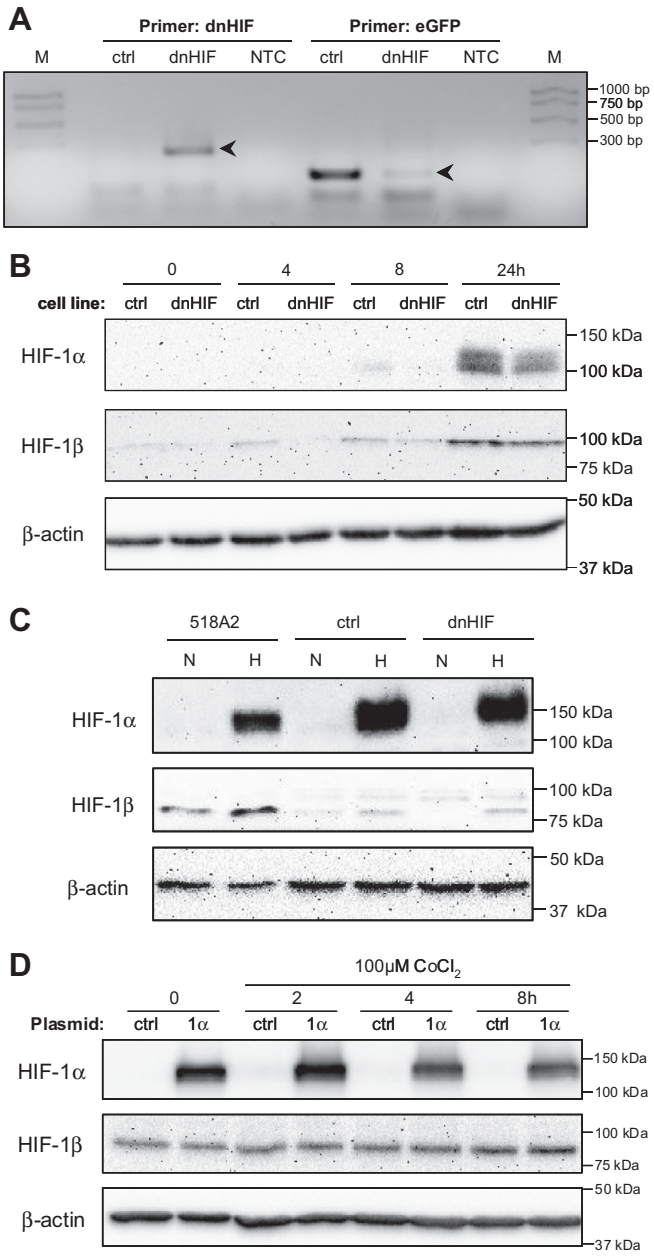

Fig. 3. Overexpression of dnHIF and HIF-1α. (A) RT-PCR analysis of stable vector-transfected 518A2 control cells (ctrl) expressing eGFP and cells stable transfected with the dominant-negative form of HIF-1α (dnHIF). Arrow heads indicate the expression of the dominant-negative form of HIF-1α fused to eGFP in dnHIF transfected cells. M: size marker; NTC: no-template control; (B) stable-transfected cells were exposed to 100 μM $CoCl_2$ as indicated or left untreated ($t = 0$ h). HIF-1α and HIF-1β protein levels were determined by Western Blotting. (C) 518A2 wildtype and stable-transfected cells expressing either the empty vector (ctrl) or dnHIF were exposed to 1% oxygen for 6 h or maintained under normoxic conditions. Afterwards Western Blotting was conducted to assay protein levels of HIF-1α and HIF-1β. N: normoxia; H: hypoxia; (D) cells were transfected with the HIF-1α expression plasmid or with an eGFP-encoding vector (control, ctrl). Thereafter cells were stimulated with 100 μM $CoCl_2$ as indicated or left untreated ($t = 0$) and subjected to Western Blotting.

To test this assumption HIF-1α was transient overexpressed in 518A2 cells followed by stimulation with 100 μM $CoCl_2$ for 2, 4 and 8 h, respectively or left untreated ($t = 0$ h). HIF-1α and HIF-1β levels were subsequently determined by Western Blotting. In HIF-1α-overexpressing cells the expression of this subunit peaked after 2 h whereas HIF-1β reaches its maximum after 8 h (Fig. 3D). Thus heterodimerization of both subunits could be excluded to be per

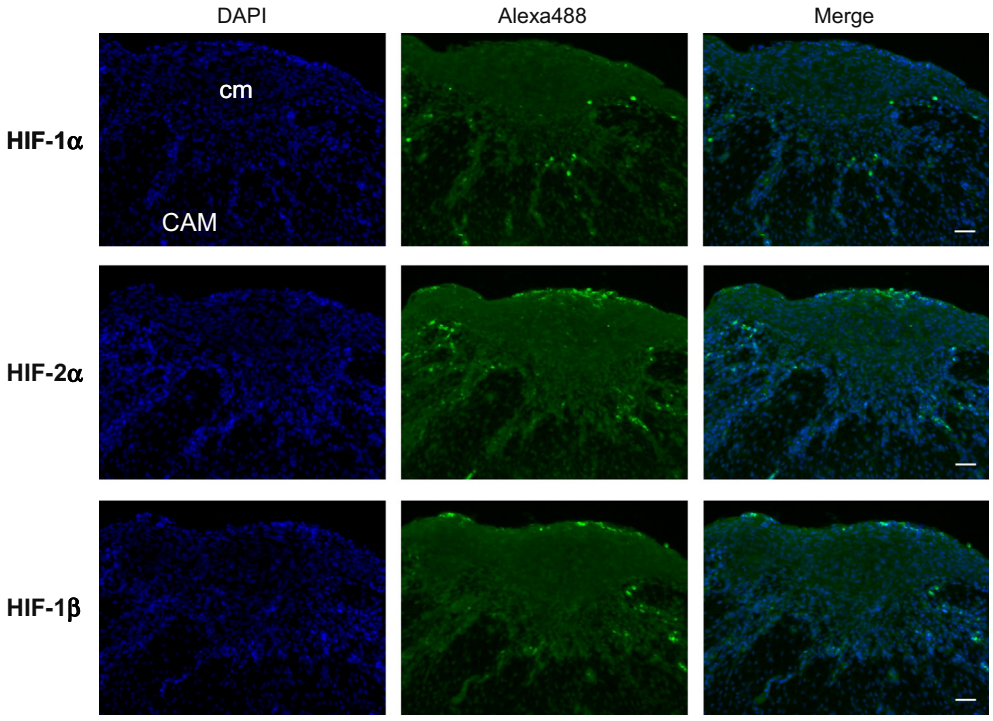

Fig. 4. 518A2 human melanoma cells in a Hen's egg test-chorioallantoic membrane (Het-CAM) model of angiogenesis and invasion. Cells were mixed with Matrigel™ and applicated onto the chorioallantoic membrane (CAM) of fertilized chicken eggs on embryonic day 6. After 5 days of incubation, CAMs were fixed with paraformaldehyde, excised and embedded into paraffin. HIF-1α, HIF-2α and HIF-1β were stained in serial sections (10 μm) using an appropriate Alexa488-conjugated secondary antibody. DAPI was used for counterstaining. Magnification 400×; scale bar 25 μm; CAM: chorioallantoic membrane; cm: cell mass.

se the major cause of HIF-1α-dependent upregulation of HIF-1β. Further the observed time delay in HIF-1β induction argues for de novo synthesis of this subunit under cobalt chloride-induced hypoxia.

In another set of HIF-1α-knockdown experiments, treatment of 518A2 cells with the proteasome inhibitor MG-132 could not protect the beta subunit from a putative degradation and reverse the non-canonical relationship between HIF-1α and HIF-1β (data not shown).

Taken together, these results indicate that heterodimerization of both subunits is not sufficient to upregulate HIF-1β under cobalt chloride-induced hypoxia.

3.6. HIF-1β is locally expressed in 518A2 cells in a Het-CAM model of angiogenesis and invasion

To evaluate the inducibility of HIF-1β in a cancer-related model Het-CAM assays were performed. Oxygen-dependent regulation of HIF-1β as indicated by previous results would lead to augmented expression in hypoxic areas of the cell mass whereas a homogenous expression pattern would suggest a constitutive regulation.

To test this assumption 518A2 cells were mixed with Matrigel™, seeded onto the chorioallantoic membrane (CAM) and incubated for 5 days. As shown in Fig. 4. 518A2 human melanoma cells were able to invade the CAM. Further, the expression of HIF-1α, HIF-2α as well as HIF-1β was locally detected within the cell mass indicating an oxygen-dependent regulation. These finding demonstrates that HIF-1β inducibility is a biological relevant effect during cellular adaptation processes to the microenvironment.

4. Discussion

This study demonstrates that HIF-1β is upregulated in a HIF-1α-dependent manner under hypoxic conditions for the first time. In general, HIF-1β is considered as constitutively expressed and not influenced by hypoxia [10,12]. However, Wang et al. described the inducibility of this subunit on RNA as well as on protein levels due to hypoxic stress or cobalt chloride exposure [19]. Another study conducted by Chilov et al. provides evidence for the upregulation of HIF-1β under hypoxia in some cell lines [18]. In contrast, Huang et al. reported that HIF-1β protein levels remained constant regardless of pO_2 [25].

The results presented in this study are in line with the reports mentioned above supporting the concept that HIF-1β is obviously a hypoxia-responsive protein only in a limited number of cell types/lines. This is of great importance when HIF-1β is considered as loading control. In addition, the data raises the question how HIF-1α can regulate HIF-1β. It is known that HIF-1α can initiate the expression of hypoxia-inducible genes either directly or indirectly (e.g. via forced expression of other transcription factors). Further, it was demonstrated that HIF-1α exerts co-activator activities independent of HIF-1β [12]. However, the exact mode how HIF-1β is controlled by HIF-1α remains to be elucidated.

In conclusion, the results indicate a novel HIF-1α-dependent mechanism regulating HIF-1β. This might prevent HIF-1β to become a limiting factor under hypoxic conditions and highlights new challenges in HIF biology.

Acknowledgments

Thanks to Severin Muehleder and Anna Mandlmayr for technical help as well as Walter Miklos for providing the hypoxic incubator. Special thanks to Dr. Volker Wacheck, Dr. Michael Grusch and BAXTER Biosciences for providing materials.

References

[1] F. Dayan, N.M. Mazure, M.C. Brahimi-Horn, J. Pouyssegur, A dialogue between the hypoxia-inducible factor and the tumor microenvironment, Cancer Microenviron. 1 (2008) 53–68.

[2] E.B. Rankin, A.J. Giaccia, The role of hypoxia-inducible factors in tumorigenesis, Cell Death Differ. 15 (2008) 678–685.
[3] G.L. Semenza, Hypoxia-inducible factors in physiology and medicine, Cell 148 (2012) 399–408.
[4] S. Kuphal, A. Winklmeier, C. Warnecke, A.K. Bosserhoff, Constitutive HIF-1 activity in malignant melanoma, Eur. J. Cancer 46 (2010) 1159–1169.
[5] V. Gray-Schopfer, C. Wellbrock, R. Marais, Melanoma biology and new targeted therapy, Nature 445 (2007) 851–857.
[6] A. Sekulic, P. Haluska Jr., A.J. Miller, J. Genebriera De Lamo, S. Ejadi, J.S. Pulido, D.R. Salomao, E.C. Thorland, R.G. Vile, D.L. Swanson, B.A. Pockaj, S.D. Laman, M.R. Pittelkow, S.N. Markovic, Malignant melanoma in the 21st century: the emerging molecular landscape, Mayo Clin. Proc. 83 (2008) 825–846.
[7] M. Milani, A.L. Harris, Targeting tumour hypoxia in breast cancer, Eur. J. Cancer 44 (2008) 2766–2773.
[8] W.R. Wilson, M.P. Hay, Targeting hypoxia in cancer therapy, Nat. Rev. Cancer 11 (2011) 393–410.
[9] G.L. Semenza, Hydroxylation of HIF-1: oxygen sensing at the molecular level, Physiology (Bethesda) 19 (2004) 176–182.
[10] A. Zagorska, J. Dulak, HIF-1: the knowns and unknowns of hypoxia sensing, Acta Biochim. Pol. 51 (2004) 563–585.
[11] J. Karar, A. Maity, PI3K/AKT/mTOR pathway in angiogenesis, Front. Mol. Neurosci. 4 (2011) 51.
[12] G.L. Semenza, Oxygen homeostasis, Wiley Interdiscip. Rev. Syst. Biol. Med. 2 (2010) 336–361.
[13] N. Pore, Z. Jiang, H.K. Shu, E. Bernhard, G.D. Kao, A. Maity, Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycin-independent pathway, Mol. Cancer Res. 4 (2006) 471–479.
[14] J. Chen, S. Zhao, K. Nakada, Y. Kuge, N. Tamaki, F. Okada, J. Wang, M. Shindo, F. Higashino, K. Takeda, M. Asaka, H. Katoh, T. Sugiyama, M. Hosokawa, M. Kobayashi, Dominant-negative hypoxia-inducible factor-1 alpha reduces tumorigenicity of pancreatic cancer cells through the suppression of glucose metabolism, Am. J. Pathol. 162 (2003) 1283–1291.
[15] Y. Higashimura, R. Yamaji, N. Harada, M. Nakao, Y. Nakano, H. Inui, Construction of a dominant negative form of human hypoxia-inducible factor-2alpha, Biosci. Biotechnol. Biochem. 74 (2010) 2100–2102.
[16] E.J. Park, D. Kong, R. Fisher, J. Cardellina, R.H. Shoemaker, G. Melillo, Targeting the PAS-A domain of HIF-1alpha for development of small molecule inhibitors of HIF-1, Cell Cycle 5 (2006) 1847–1853.
[17] T. Uchida, F. Rossignol, M.A. Matthay, R. Mounier, S. Couette, E. Clottes, C. Clerici, Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha, J. Biol. Chem. 279 (2004) 14871–14878.
[18] D. Chilov, G. Camenisch, I. Kvietikova, U. Ziegler, M. Gassmann, R.H. Wenger, Induction and nuclear translocation of hypoxia-inducible factor-1 (HIF-1): heterodimerization with ARNT is not necessary for nuclear accumulation of HIF-1alpha, J. Cell Sci. 112 (Pt 8) (1999) 1203–1212.
[19] G.L. Wang, B.H. Jiang, E.A. Rue, G.L. Semenza, Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension, Proc. Natl. Acad. Sci. USA 92 (1995) 5510–5514.
[20] E. Falkner, C. Eder, B. Kapeller, W. Froschl, C. Schmatz, K. Macfelda, U.M. Losert, The mandatory CAM testing of cells and scaffolds for tissue engineering: benefits for the three Rs of cooperation with the vaccine industry, Altern. Lab. Anim. 32 (2004) 573–580.
[21] Q. Ke, M. Costa, Hypoxia-inducible factor-1 (HIF-1), Mol. Pharmacol. 70 (2006) 1469–1480.
[22] L.Z. Liu, C. Li, Q. Chen, Y. Jing, R. Carpenter, Y. Jiang, H.F. Kung, L. Lai, B.H. Jiang, MiR-21 induced angiogenesis through AKT and ERK activation and HIF-1alpha expression, PLoS One 6 (2011) e19139.
[23] H.D. Skinner, J.Z. Zheng, J. Fang, F. Agani, B.H. Jiang, Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1alpha, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling, J. Biol. Chem. 279 (2004) 45643–45651.
[24] A.H. Licht, F. Muller-Holtkamp, I. Flamme, G. Breier, Inhibition of hypoxia-inducible factor activity in endothelial cells disrupts embryonic cardiovascular development, Blood 107 (2006) 584–590.
[25] L.E. Huang, Z. Arany, D.M. Livingston, H.F. Bunn, Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit, J. Biol. Chem. 271 (1996) 32253–32259.