

Egr-1 is not upregulated in response to hypoxic and oxygenation conditions in human glioblastoma *in vitro*

HARUN M. SAID¹, BUELENT POLAT¹, CARSTEN HAGEMANN², GILES H. VINCE², JELENA ANACKER³,
ULRIKE KÄMMERER³, MICHAEL FLENTJE¹ and DIRK VORDERMARK^{1,4}

Departments of ¹Radiation Oncology, ²Neurosurgery, ³Gynecology and Obstetrics, University of Würzburg, Würzburg;
⁴Department of Radiation Oncology, Martin Luther University of Halle-Wittenberg, Germany

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Abstract. The early growth response factor 1 (Egr-1) gene (also known as krox24, NGFI-A, TIS8 or zif268) belongs to a family of immediate early response genes. This family of proteins contains a conserved zinc finger DNA-binding domain and can bind to a GC-rich sequence in the promoter region of target genes. Egr-1 expression is rapidly and transiently activated in many different cell types during development. In adult tissues, a variety of signals, including serum, growth factors, cytokines and hormones, stimulate Egr-1 expression. In several studies, it was demonstrated that the transcription factor Egr-1 is regulated by hypoxia, and it is hypothesized that Egr-1 is responsible for the hypoxia-induced regulation of the N-Myc downregulated gene 1 (NDRG1) in human tumor cells. In the present study, Egr-1 regulation was examined in the human glioblastoma cell lines U373, U251, GaMG and U87-MG under extreme hypoxic aeration conditions (0.1% O₂) for 1, 6 and 24 h, 24-h extreme hypoxia with reoxygenation for 24 and 48 h, respectively, as well as oxygenated conditions (21% O₂ and 5% CO₂) *in vitro*. Protein and mRNA levels were detected in the lysates by Western blotting and RT-PCR, respectively. Egr-1 expression under hypoxic conditions was compared with the well-known and characterized hypoxia-induced gene regulator hypoxia-inducible factor-1 α (HIF-1 α) in parallel experimental sets. Cells incubated for 24 h with 100 μ M desferrioxamine served as a positive control for hypoxia, and β -tubulin and β -actin were used as loading controls. The experimental data indicate that Egr-1 was not upregulated under extreme hypoxic conditions (0.1% O₂) or by reoxygenation after hypoxia in different glioblastoma cells *in vitro*. In conclusion, the regulation of Egr-1 in reaction to hypoxic

development, at both the protein and mRNA levels, is not a general phenomenon. In contrast to previously published data, no Egr-1 regulatory events were observed in glioblastoma under hypoxic conditions *in vitro*. We suggest that Egr-1 regulation in human tumors in reaction to hypoxia could be a cell-specific post-translational event. Therefore, at least in glioblastoma, HIF-1 α can be considered a major regulator of NDRG1 under hypoxic conditions. Further extensive analysis of tumor cells from different origins under similar physiological conditions is necessary to increase our knowledge of the conditions and functional role of Egr-1 in the regulation of hypoxia-induced gene expression.

Introduction

Early growth response factor 1 (Egr-1) is a transcription factor that triggers the transcription of downstream genes within 15-30 min of various stimulations (1). It has been reported in several publications (2,3) that Egr-1 is regulated by hypoxia, and it is hypothesized that Egr-1 is responsible for the hypoxia-induced regulation of N-myc downregulated gene 1 (NDRG1) in human tumor cells. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor consisting of two subunits, HIF-1 α and HIF-1 β , which are basic-helix-loop-helix proteins containing a PAS domain (4). In normoxia, the von Hippel-Lindau tumor suppressor, which is the recognition component of an E3 ubiquitin ligase complex, targets HIF-1 α (5,6), leading to its ubiquitylation and consequent proteasomal degradation (6-10). HIF-1 α is responsible for the regulation of several hypoxia-induced genes. These genes are expressed rapidly by HIF-1-specific promoter activation. HIF-1 binds to the hypoxia responsive element (HRE) and thereby mediates cell growth and angiogenesis (11,12). Due to HRE positioning in the distal promoter region, HIF-1 also acts as a transcriptional enhancer, as hypothesized in a similar gene expression model (4). During hypoxia, the HIF-1 α / β heterodimer binds to the core pentanucleotide sequence (RCGTG) in the HREs of target genes. HIF-1 β subunits are non-oxygen responsive nuclear proteins that also play various roles in transcription (13). In contrast, HIF-1 α subunits are highly inducible by different oxygenation conditions in human glioblastoma cells. There is a clear enhanced binding of HIF-1 α from nuclear extracts of glioblastoma cell samples exposed to extreme hypoxic conditions. Upregulation of HIF-1 α under these con-

Correspondence to: Dr Harun M. Said, Department of Radiation Oncology, University of Würzburg, Josef-Schneider-Str. 11, 97080 Würzburg, Germany

E-mail: said_h@klinik.uni-wuerzburg.de

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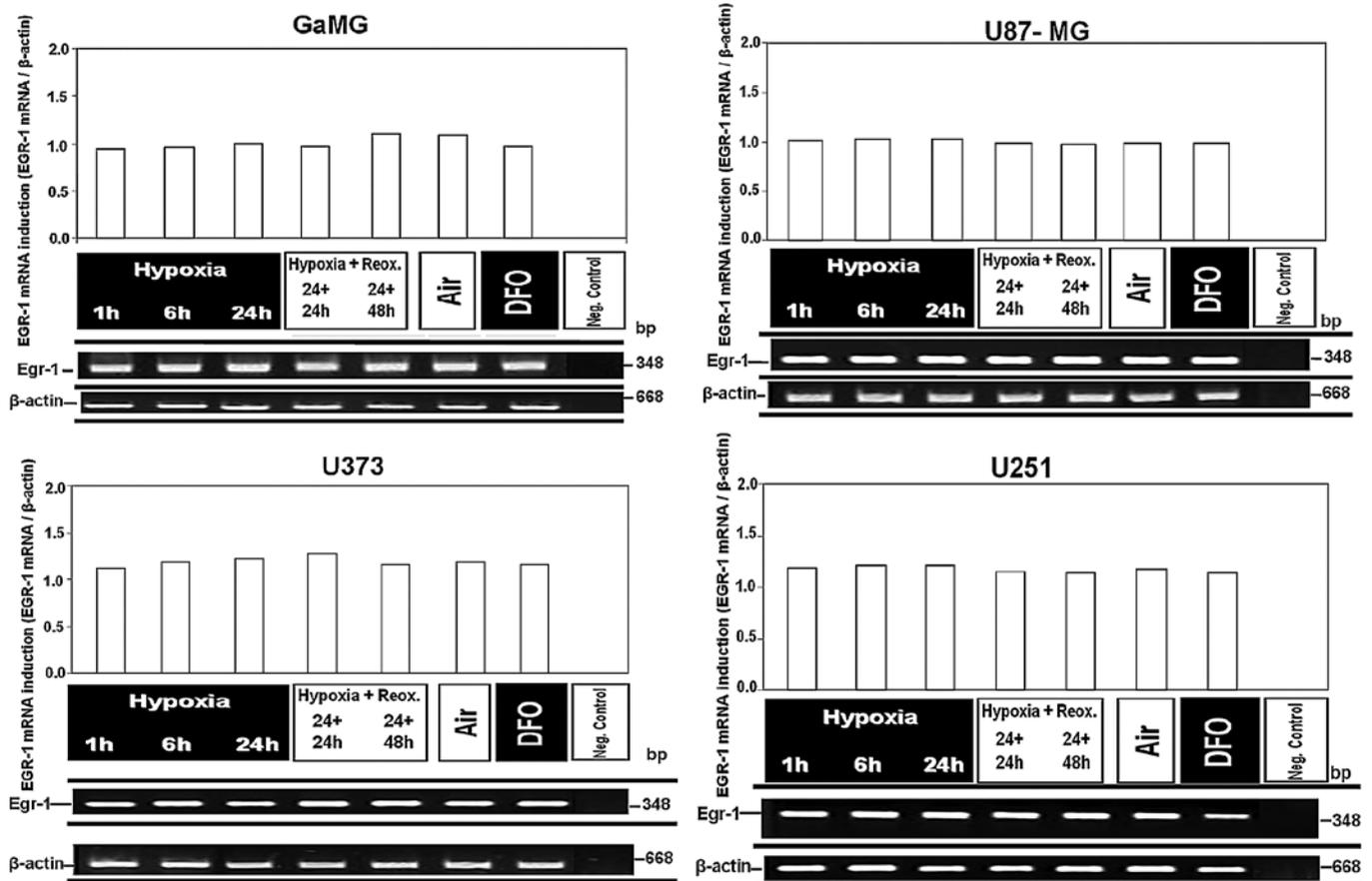


Figure 1. Early growth response factor 1 (Egr-1) mRNA expression under hypoxic conditions in glioblastoma *in vitro*. Examination of hypoxia-inducible regulation of Egr-1 gene expression at the mRNA level (semi-quantitative RT-PCR) *in vitro* in human glioblastoma cell lines (hypoxia time course experiments at an O_2 concentration of 0.1%). Glioblastoma cell lines were exposed to hypoxic conditions (0.1% O_2) for 1, 6 or 24 h. Total mRNA from cells subjected to 24 h under normoxia or to treatment with desferroxamine (DFO, 100 μ M) served as the negative and positive control, respectively. RT-PCR did not reveal any regulatory events under different oxygenation, hypoxia and reoxygenation conditions. Bar graphs show band intensities after densitometric evaluation and normalization to β -actin expression. A representative experiment of three is shown.

ditions was confirmed by Western blotting (14,15). Regulation of NDRG1 by HIF-1 α has also been demonstrated (16,17).

Our findings indicate that EGR-1 is not upregulated in response to extreme hypoxic conditions (0.1% O_2) or reoxygenation after hypoxia in human glioblastoma. Therefore, HIF-1 α remains one of the most promising targets for new therapeutic strategies in cancer treatment, particularly in the therapeutic modulation of the adaptive hypoxic response. Based on our own data and the research of others, we believe that Egr-1 is not suitable as a new target for the therapeutic modulation of the adaptive hypoxic response, at least not in human glioblastoma.

Materials and methods

Cell culture and hypoxia treatment. The early-passage human malignant glioma cell lines U87-MG, U251 and U373 from the American Type Culture Collection (ATCC, Rockville, MD) and GaMG, a cell line established from a patient with glioblastoma multiforme (Gade Institut of the University Bergen, Norway) (18), were grown on glass Petri dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and non-essential amino acids.

Additionally, all culture media were supplemented with penicillin (100 IU/ml)/streptomycin (100 μ g/ml) and 2 mM L-glutamine. *In vitro* hypoxia treatment of cultures was in a Ruskinn (Cincinnati, OH, USA) Invivo $_2$ hypoxic workstation as previously described (19) for 1, 6 or 24 h at 0.1% O_2 . Dishes were returned to the incubator after a 24-h hypoxia treatment for reoxygenation experiments.

Preparation of protein lysates from human tumor cells and immunoblotting. Tumor cell lysates were prepared with 0.1 ml lysis buffer [1X Tris-buffered saline (TBS), 1% Nonidet P-40 (Amresco, Vienna, Austria), 0.5% sodium deoxycholate, 0.1% SDS and the protease inhibitors pepstatin A (1.4 μ M), aprotinin (0.15 μ M), leupeptin (2.3 μ M), PMSF (100 μ M) and phosphatase inhibitor mix (Sigma, St. Louis, MO, USA)]. Whole-cell lysates (20 μ g) were separated by SDS polyacrylamide gel electrophoresis (8% gels) and transferred to a 0.45- μ m nitrocellulose membrane (Protran BA 85; Schleicher & Schuell, Dassel, Germany). Non-specific binding was blocked by 5% nonfat milk powder in TBS overnight at 4°C followed by incubation with the Egr-1 primary antibody sc-110 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:100 in 1.0% nonfat milk powder in TBS for 1 h at room

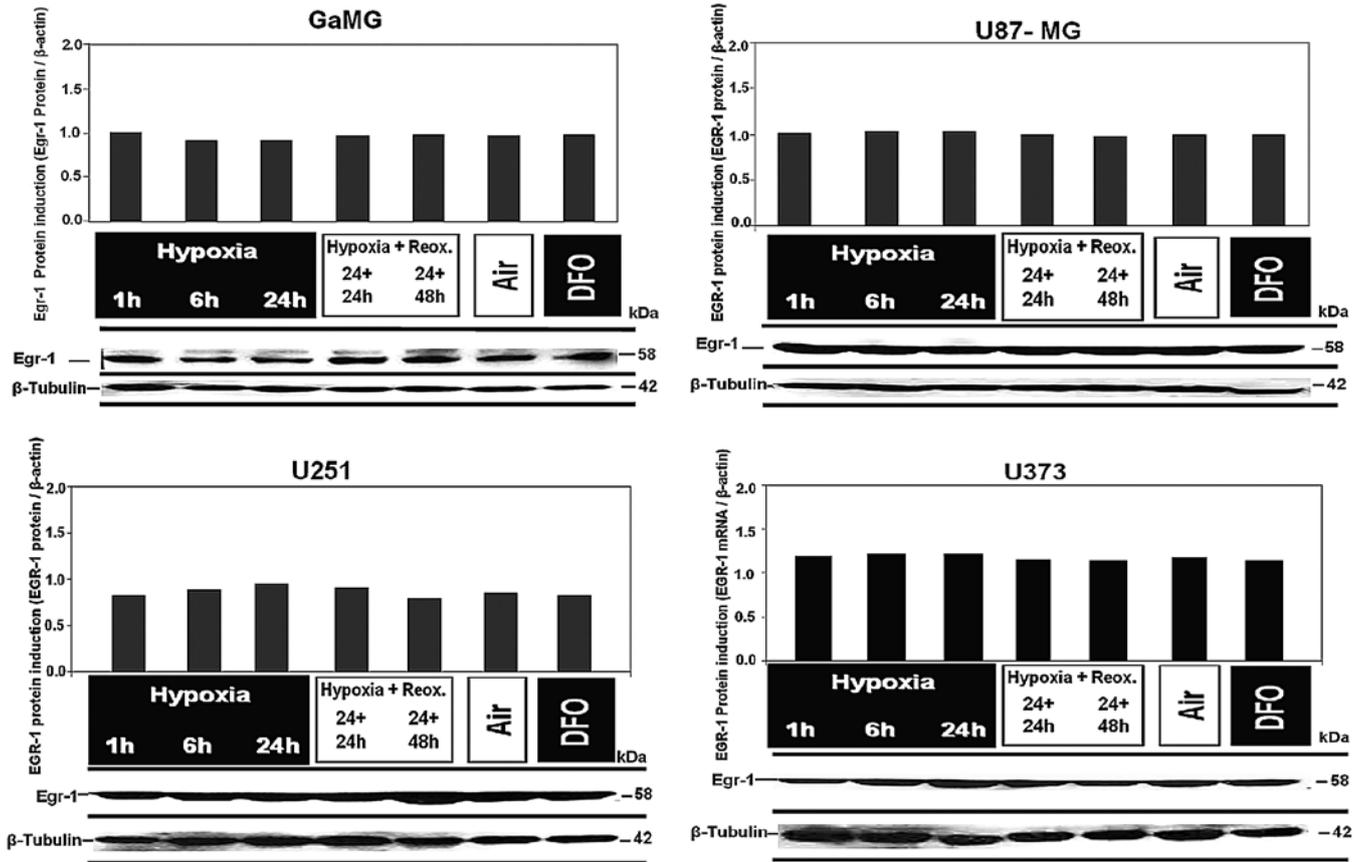


Figure 2. Early growth response factor 1 (Egr-1) protein expression under hypoxic conditions in glioblastoma *in vitro*. Regulation of Egr-1 expression at the protein level *in vitro* in human glioblastoma cell lines exposed to hypoxic conditions (0.1% O₂) for 1, 6 or 24 h. Protein lysates subjected to 24 h under normoxia or to treatment with desferrioxamine (DFO, 100 μM) served as the negative and positive control, respectively. A representative experiment of three is shown.

temperature. Blots were washed twice in TBS/0.05% Tween-20 (Bio-Rad, Munich, Germany) and subsequently 3 times in TBS for 5-10 min each. The secondary antibody, goat anti-rabbit horseradish peroxidase (stock solution 400 μg/ml; DakoCytomation, Denmark), was incubated at a dilution of 1:1000 for 1 additional hour at room temperature followed by 5 washing steps as described above. Detection of bound antibodies was accomplished by membrane development using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Cambridge, UK) for 5 min, with subsequent development of the Hyperfilm ECL (Amersham). All expression data were confirmed in at least three independent experiments.

Preparation of nuclear extracts. According to previous applied protocols (19) with minor modifications, 5x10⁷ cells/ml, were scratched from Petri dishes by the addition of 10 ml phosphate-buffered saline (PBS). A cell line pellet was obtained by centrifugation (Beckman CS-6R) for 4 min at 800 rpm. After 2 washing steps with PBS, cells were re-suspended in 1 ml PBS, transferred into a 1.5-ml tube and centrifuged at 4°C for 45 sec at 14,000 rpm. The cell pellet was resuspended in 400 μl ice cold buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 10 μl complete protease inhibitor cocktail (Roche)

and 1 mM DTT] and incubated on ice for 15 min. Cells were lysed by the addition of 25 μl of 10% NP-40 and homogenized with 10 strokes in a dounce homogenizer at 4°C followed by centrifugation for 1 min at 14,000 rpm for nuclei sedimentation. Supernatants were carefully removed and regarded as cytoplasmic fractions. Nuclear proteins were extracted by the addition of 50 μl of buffer C [20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.1 μl protease inhibitor cocktail (Roche)] and extensive shaking of the tubes for 20 min at 4°C in a tube shaker followed by centrifugation at 14,000 rpm and 4°C for 5 min. The supernatant was removed and stored in aliquots at -80°C. All steps were performed on ice.

Isolation of total RNA from tumor cell lines. Total RNA was isolated from cultured tumor cells as reported previously, including the digestion of contaminating DNA with the provided DNase (20,21).

Analysis of mRNA expression levels in human GBM cell lines by semi-quantitative RT-PCR. To compare the expression of the individual genes examined, reverse transcription-polymerase chain reaction (RT-PCR) was performed using primers designed according to published information on Egr-1, β-actin and HIF-1α mRNA sequences in GenBank (accession

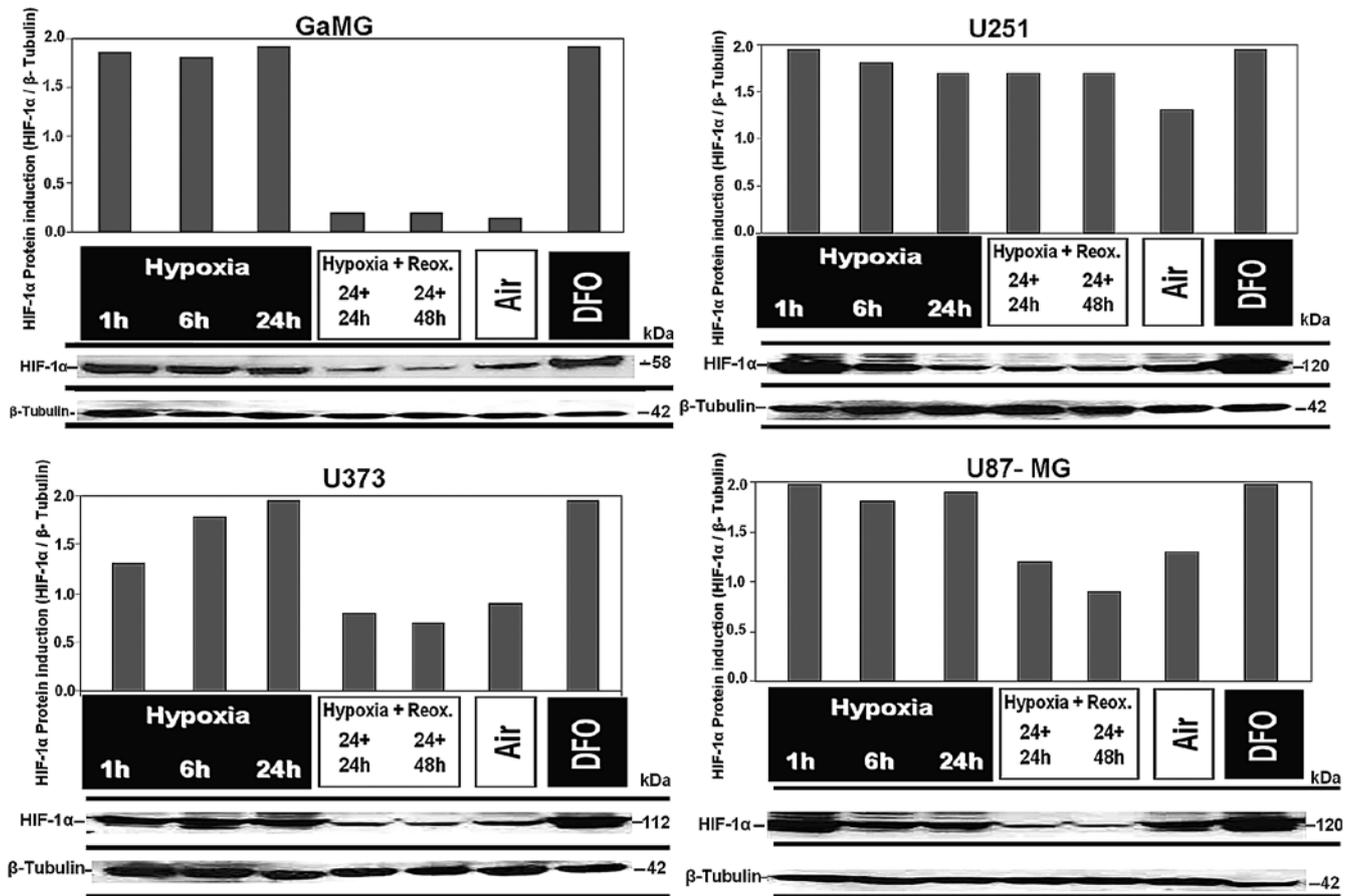


Figure 3. HIF-1 α protein expression under hypoxic conditions in glioblastoma *in vitro*. Western blot analysis showing regulation of hypoxia-inducible factor-1 α (HIF-1 α) expression at the protein level *in vitro* in human glioblastoma cell lines exposed to hypoxic conditions (0.1% O₂) for 1, 6 or 24 h. Nuclear protein extracts from cells subjected to 24 h under normoxia or to treatment with desferrioxamine (DFO, 100 μ M) served as the negative and positive control, respectively. β -tubulin served as the loading control. HIF-1 α protein expression level was shown as bar graphs after densitometric evaluation and normalization to the corresponding β -tubulin expression. HIF-1 α was strongly expressed after 1 h at 0.1% O₂, and remained increased for up to 24 h under hypoxia. A representative experiment of three is shown.

nos. NM_001964.2, NM_001101 and NM_001530.2, respectively). An aliquot of 1-5 μ g of total mRNA from human glioblastoma cell lines was transcribed at 42°C for 1 h in a 20- μ l reaction mixture using 200 U RevertAid™ M-MuLV Reverse Transcriptase (RT), oligo(dT)18 primer and 40 U Ribonuclease inhibitor (all from Fermentas, Ontario, Canada).

For PCR, primers were designed in flanking exons with Primer3 software (available online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). To produce a 348-bp amplification product of Egr-1, the forward primer (F1) was 5'-AGCTGGAGGAGATGATGCTG-3' (nucleotides 370-390) and the reverse primer (R1) was 5'-TGCCAGCCCCAGCGTCAAAG-3' (nucleotides 698-718). To produce a 668-bp amplification product of β -actin, the forward primer (F1) was 5'-CGTGCCTGACATTAAGGAGA-3' (nucleotides 697-716) and the reverse primer (R1) was 5'-CACCTTCACCGTCCAGTTT-3' (nucleotides 1345-1364) and to produce a 233-bp amplification product of HIF-1 α , the forward primer (F1) was 5'-TTACAGCAGCCAGACGATCA-3' (nucleotides 2516-2535) and the reverse primer (R1) was 5'-CCCTGCAGTAGGTTTCTGCT-3' (nucleotides 2729-2748). PCR was performed with 25-32 cycles with increments of 5 cycles using PCR systems and reagents acquired from Promega™ (Promega

GmbH, Mannheim, Germany) and applied according to the manufacturer's instructions. The PCR products were separated on 2% agarose gels (Sigma-Aldrich, Steinheim, Germany) and visualized by ethidium bromide staining (0.07 μ g/ml ethidium bromide; Bio-Rad).

Densitometric evaluation. Signals detected and visualized by Western blotting and RT-PCRs were analysed with 1D Kodak Image Analysis Software. Signal strength was measured in Kodak light units and divided by the corresponding signals of the loading control β -tubulin and β -actin for Western blotting and β -actin for RT-PCR, as previously described (22-25).

Results

Comparative Egr-1 mRNA expression in human glioblastoma cells *in vitro*. To investigate the effect of controlled hypoxic conditions on Egr-1 mRNA expression, we performed *in vitro* cell culture assays under an oxygen concentration of 0.1% O₂ with and without reoxygenation. No regulatory effect of these different oxygenation conditions on Egr-1 expression was detectable by semi-quantitative RT-PCR in the human glioblastoma cell lines U87-MG, U251, U373 and GaMG (Fig. 1).

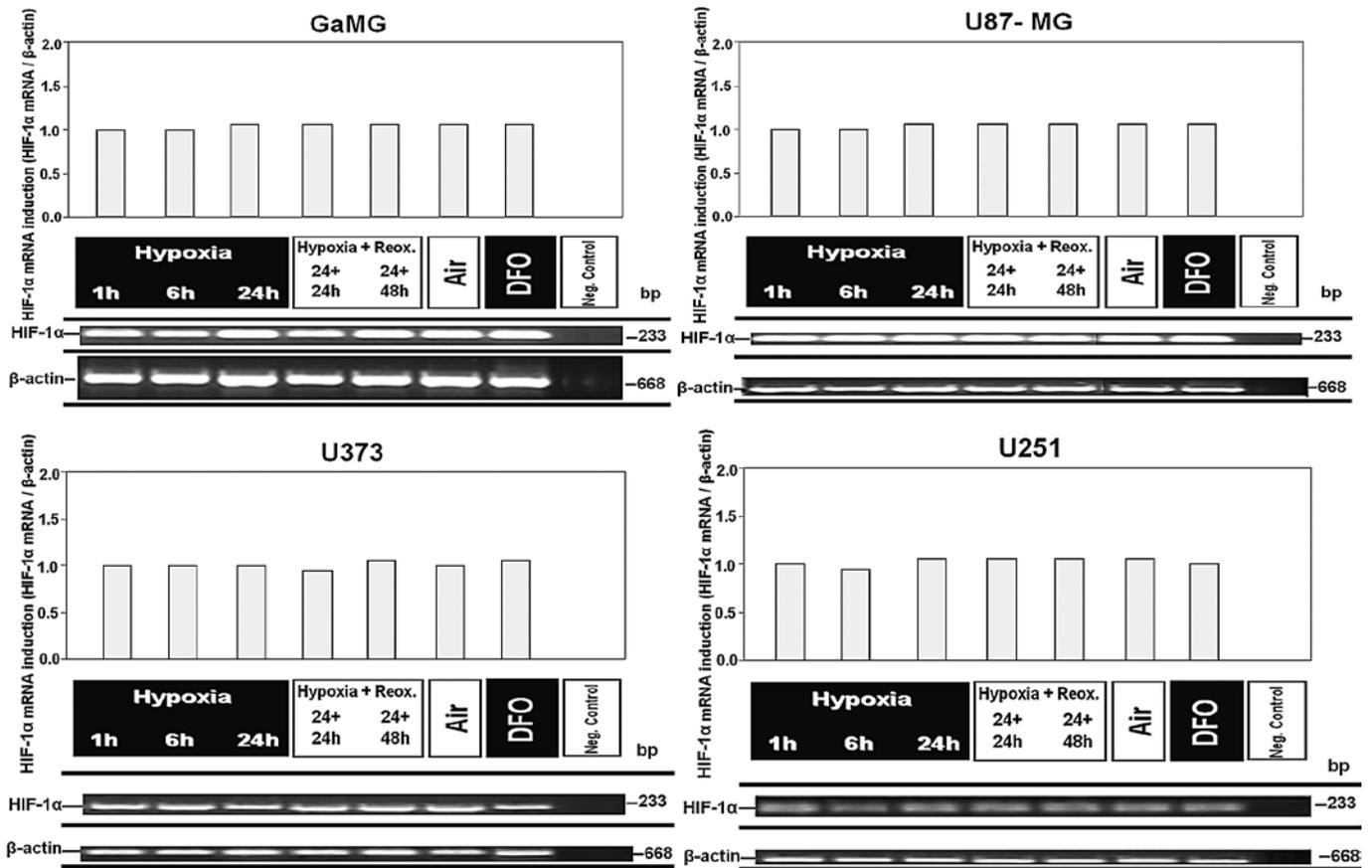


Figure 4. HIF-1 α mRNA expression under hypoxic conditions in glioblastoma *in vitro*. HIF-1 α mRNA expression in human U87-MG, U373, U251 and GaMG glioblastoma cell lines after the *in vitro* application of different hypoxic conditions. RT-PCR did not reveal any regulatory events under different oxygenation, hypoxia and reoxygenation conditions. Bar graphs show band intensities after densitometric evaluation and normalization to β -actin expression. A representative experiment of three is shown.

Densitometric evaluation confirmed these results. Together, these data suggest that the established formation of hypoxic regions within glioblastoma tumor tissue is not accompanied by an upregulation of Egr-1 mRNA.

Egr-1 protein expression in human glioblastoma cells *in vitro*. The glioblastoma cell lines GaMG, U373, U87-MG and U251 were cultured under hypoxic conditions as described above. These cells did not show any regulation of Egr-1 protein expression (Fig. 2) under the examined conditions. Egr-1 protein expression was not modified in response to different oxygenation, hypoxia or reoxygenation conditions *in vitro* in the tested glioblastoma cell lines. Densitometric evaluation of Western blotting confirmed these results.

HIF-1 α protein expression in response to *in vitro* hypoxia. HIF-1 α nuclear protein clearly responded with upregulation under hypoxic conditions and downregulation under reoxygenation or normoxic conditions in the U373, U251 and GaMG cells (Fig. 3). HIF-1 α was strongly expressed after 1 h at 0.1% O₂, remained increased after 24 h of hypoxia, and showed stable reduced expression upon reoxygenation for up to 48 h (Fig. 3). These results confirm the oxygen-dependent regulation of HIF-1 α at the protein level in the experimental models of the present study, and reaffirm that the experimental

settings for the expression analysis of Egr-1 were suitable for the evaluation of regulatory events under hypoxic conditions.

HIF-1 α mRNA expression in response to *in vitro* hypoxia. Semi-quantitative RT-PCR of the glioblastoma cell lines GaMG, U373, U87-MG and U251 cultured under hypoxic conditions as described above revealed that HIF-1 α was evenly expressed *in vitro* in GaMG, U373, U87-MG and U251 glioblastoma cells. HIF-1 α mRNA expression was not influenced by hypoxic conditions, nor was the upregulation of HIF-1 α mRNA in glioblastoma cell lines detectable (Fig. 4).

Discussion

It has been postulated that the expression of several genes, such as PAI or CEBP-1 α , is upregulated under hypoxia in tumor cells by the regulative activity of the transcription factor early growth response factor 1 (Egr-1) (26). The different origins of these tumor cells may be among the key factors that play a role during this process. The results of previous studies suggest that the transcription factor Egr-1 may be regulated by hypoxia, and that it represents the main transcription factor responsible for the hypoxia-induced regulation of the N-Myc downregulated gene 1 (NDRG1) in human

tumor cells (2,3). Other studies have identified hypoxia-induced genes that are not dependent on hypoxia-inducible factor-1 α (HIF-1 α). A large-scale microarray analysis that compared HIF-1 α and hypoxia-induced genes revealed that HIF-1 α was involved in the regulation of approximately half of the hypoxia-induced genes (27).

It has been suggested that HIF-1 α plays a key role in hypoxic response, in particular during tumorigenesis (28,29). Our previous results indicate that exposure of the four glioma cell lines U373, U251, U87-M and GaMG to hypoxic aeration conditions results in the overexpression of NDRG1 (15-17, 30,31). Comparable hypoxia-mediated Egr-1 induction and activation were observed in cultured hepatoma-derived cells deficient in HIF-1 β and in wild-type hepatoma cells, indicating that the HIF-1 and Egr-1 pathways are initiated independently in response to oxygen deprivation (31).

In the four glioblastoma cell lines examined, HIF-1 α was not regulated at the mRNA level, but was upregulated at the protein level under hypoxic conditions. However, neither Egr-1 mRNA nor Egr-1 protein showed any regulative pattern under identical aeration conditions. Therefore, HIF-1 α still represents a promising target for novel therapeutic strategies in cancer research. The discovery of new factors involved in its regulation is of great interest in the field. Based on the results which we present here for the first time, we can hypothesize that Egr-1 does not regulate NDRG1 transcription under hypoxic conditions, at least not in glioblastoma. This is in concordance with the findings of other studies. Moreover, the findings suggest that HIF-1 α plays an active role in the regulation of NDRG1 (15-17,30).

Our results suggest that Egr-1 activated by hypoxia may play an active role in the positive regulation of the activity of NDRG1 in various tumors, but not in human glioblastoma. However, based on the data presented here and on the results of prior studies, the main regulative function of Egr-1 may be carried out by the functional activities of HIF-1 α . The discovery of novel actors involved in the regulation of Egr-1 is of great interest. We propose that the activation of Egr-1 in response to hypoxia induces a different facet of the adaptive response compared to HIF-1, despite the results observed in tumor cells of other origin. Further examination of Egr-1 expression *in vivo* should lead to an extensive understanding of the functional mechanisms involved in hypoxia-induced gene regulation in human brain cancer.

In conclusion, it is likely that the Egr-1 protein is an important nuclear intermediate in the signal transduction process under hypoxia, and one of the crucial targets in human carcinogenesis in different tissues, especially those of the brain. One proposed reason could be the complex regulatory mechanism of the Egr-1 gene, which most likely involves more than one signalling pathway for the hypoxia-induced regulation of Egr-1.

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