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Interleukin-1β promotes hypoxia-induced apoptosis of glioblastoma cells by inhibiting hypoxia-inducible factor-1 mediated adrenomedullin production

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Glioblastoma is the most common brain tumor in adults. Advanced glioblastomas normally contain hypoxic areas. The primary cellular responses to hypoxia are generally mediated by the transcription factor hypoxia-inducible factor 1 (HIF-1). Interleukin-1β (IL-1β) is a cytokine that is often present in the glioblastoma microenvironment and is known to be a modulator of glioblastoma progression. However, the role of IL-1β in regulating glioblastoma progression is still controversial. In this study, we found that in the human glioblastoma cell lines U87MG and U138MG, IL-1β inhibits the transactivation activity of HIF-1 by promoting the ubiquitin-independent proteasomal degradation of the oxygen-labile α-subunit of HIF-1 and downregulates the expression of the HIF-1 target gene adrenomedullin (AM). Apoptosis and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays showed that AM protects glioblastoma cells against hypoxia-induced apoptosis in a dose-dependent manner. Thus, in the presence of IL-1β more glioblastoma cells undergo hypoxia-induced cell death. Our findings suggest that when estimating the influence of IL-1β on the prognosis of glioblastoma patients, factors such as the degree of hypoxia, the expression levels of HIF-1 and AM should be taken into consideration. For the AM-producing glioblastoma cells, IL-1β represents a potent apoptosis inducer.

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Glioblastoma (glioblastoma multiforme, GBM) is the most common and most aggressive primary brain tumor in adults. The median survival time of patients with glioblastomas is <2 years even after optimal treatment.[1] Because of the rapid cell proliferation and inadequate vascularization, glioblastomas mostly contain areas with insufficient oxygen supply.[2] The primary cellular responses to oxygen deprivation (hypoxia) are mainly mediated by the transcription factor hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimeric protein consisting of an oxygen-labile α-subunit (HIF-1α) and a stable β-subunit (ARNT). Both subunits are part of the basic Helix-Loop-Helix PER-ARNT-SIM (bHLH-PAS) family of transcription factors. In the presence of oxygen, HIF-1α is hydroxylated at certain proline residues by prolyl hydroxylases (PHDs), which labels HIF-1α for rapid ubiquitination and proteasomal degradation. In hypoxia, the activity of PHDs is decreased through various mechanisms. As a result, HIF-1α is stabilized, dimerizes with ARNT and transactivates a variety of genes involved in the cellular adaptation to hypoxia by binding to the hypoxia-response elements (HREs).[3–5]

Adrenomedullin (AM) is a 52-amino acid peptide originally isolated from pheochromocytoma and mediates a multifunctional response in cell culture and animal systems.[6,7] Besides pheochromocytoma, AM is expressed in a number of human tissues including glioblastoma.[8] Hypoxia upregulates the expression of AM in glioblastoma cells.[9] The analysis of the AM gene identified at least eight putative HREs. Genomic knockout of HIF-1α abolishes the hypoxic induction of AM.[10] RNA interference and drug inhibition of HIF-1α cause a marked decrease in AM expression, indicating that AM is a target gene of HIF-1.[10,11] *In vivo* neutralization of AM leads to enhanced glioblastoma cell apoptosis and suppressed xenograft tumor growth.[12] Therefore, AM is supposed to be an auto-/paracrine anti-apoptotic factor in glioblastoma.

The microenvironments of glioblastomas contain various growth factors and cytokines.[13] Interleukin-1β (IL-1β) is one of the cytokines that are commonly present in glioblastoma. The main source of IL-1β is supposed to be the glioblastoma cells.[14] However, the M1 tumor-associated macrophages and the non-neoplastic brain cells are also able to produce IL-1β.[15,16] *In situ* hybridization of human glioblastoma tissue sections revealed expression of IL-1β and interleukin-1 receptor types I and II in the majority of cases.[17] There is growing evidence that IL-1β modulates the glioblastoma progression by interacting directly with the tumor cells. However, previous findings showed that IL-1β activates diverse intracellular pathways with distinct impacts on the glioblastoma progression. It has been controversial whether IL-1β promotes or suppresses glioblastoma progression.[17–22]

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Abbreviations: AM, adrenomedullin; GBM, glioblastoma multiforme; HIF-1, hypoxia-inducible factor 1; IL-1β, interleukin-1β; PI3K, phosphoinositide 3-kinase
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To provide more insights into the interaction between IL-1β and glioblastoma cells, we studied the influence of IL-1β on the adaptation of glioblastoma cells to hypoxia with focus on the HIF-1/AM axis. The human glioblastoma cell lines U87MG and U138MG were used as models because they produce AM in an oxygen-dependent manner and react to human recombinant IL-1β. We found that AM protects glioblastoma cells against hypoxia-induced apoptosis in a dose-dependent manner. IL-1β inhibits HIF-1 mediated AM production by promoting the proteasomal degradation of HIF-1α and consequently promotes the apoptosis of glioblastoma cells in hypoxia. Our findings show that IL-1β represents an effective apoptosis inducer for the AM-producing glioblastoma cells. To estimate the influence of IL-1β on glioblastoma progression, it is necessary to take factors such as the degree of hypoxia and the expression levels of HIF-1 and AM into consideration.

Results

HIF-1/AM axis protects glioblastoma cells against hypoxia-induced apoptosis. Glioblastoma cells were transfected with HIF-1α siRNA. The knockdown efficiency was confirmed by immunoblotting (Figure 1a). Cell apoptosis was estimated using DNA fragmentation ELISA. As shown in Figure 1b, HIF-1α knockdown led to increased apoptosis in hypoxia.

Since the anti-apoptotic effect of AM in glioblastoma was only observed *in vivo*, we studied the anti-apoptotic potential of AM in cell culture. Glioblastoma cells were incubated in normoxia (20% O_2) or hypoxia (1% O_2) with or without AM for 24 h. Hypoxia caused a significant increase in cell apoptosis, which was in turn suppressed by AM. In normoxia, AM had no influence on the apoptosis of glioblastoma cells (Figure 2a). In addition, AM caused a slight suppression of hypoxia-induced cell death. The viability of normoxic glioblastoma cells was not affected by AM (Figure 2b). The apoptosis suppression by AM in hypoxic glioblastoma cells was dose-dependent (Figure 2c).

IL-1β inhibits the HIF-1 pathway and downregulates the expression of AM in hypoxic glioblastoma cells. To study the influence of IL-1β on the HIF-1/AM axis, glioblastoma cells were incubated in hypoxia (1% O_2) with or without IL-1β for 2 or 4 h. The steady-state level of the oxygen-labile HIF-1α was detected by immunoblotting. The protein content of HIF-1α in hypoxic glioblastoma cells was reduced by IL-1β within 2 h (Figure 3a). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and trypan blue staining did not show any decrease in cell viability at this time (data not shown). To study whether IL-1β consequently inhibits the transactivation activity of HIF-1, reporter gene assays were performed using a luciferase reporter gene construct containing six copies of HIF-1 binding sites. IL-1β caused a decrease in luciferase activity by about 50% in hypoxic glioblastoma cells (Figure 3b).

Since the reduction in HIF-1α protein content was already observed after 2-h treatment, we studied the effect of IL-1β on the AM expression after incubating the cells for 6 h in hypoxia with or without IL-1β. As shown in Figure 3c, IL-1β significantly downregulated the expression of AM in hypoxic glioblastoma cells.

HIF-1α was nearly undetectable in normoxic glioblastoma cells (data not shown). In reporter gene assays, IL-1β did not cause any change in HIF-1 transactivation activity in normoxic glioblastoma cells (Figure 3d).

We then studied whether the AM secretion by hypoxic glioblastoma cells was inhibited by IL-1β. Glioblastoma cells were incubated in normoxia or hypoxia with or without IL-1β for 8–24 h. As shown in Figure 4, hypoxia significantly induced the secretion of AM by glioblastoma cells, which was in turn suppressed by IL-1β.

IL-1β promotes the apoptosis of hypoxic glioblastoma cells. Since AM protects the glioblastoma cells against hypoxia-induced apoptosis, we suggested that the inhibition of the HIF-1/AM axis by IL-1β would lead to an increase in cell apoptosis in hypoxia. Glioblastoma cells were incubated for 24 h in hypoxia with or without IL-1β. The fragmented DNA was fluorescent labeled instead of ELISA detection, since fluorescence detection is visible and more sensitive. Glioblastoma cells incubated with IL-1β in hypoxia showed more fluorescent-labeled fragmented DNA (Figure 5a). The fluorescence intensity of single cells was further quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). For each experiment, 150–200 cells

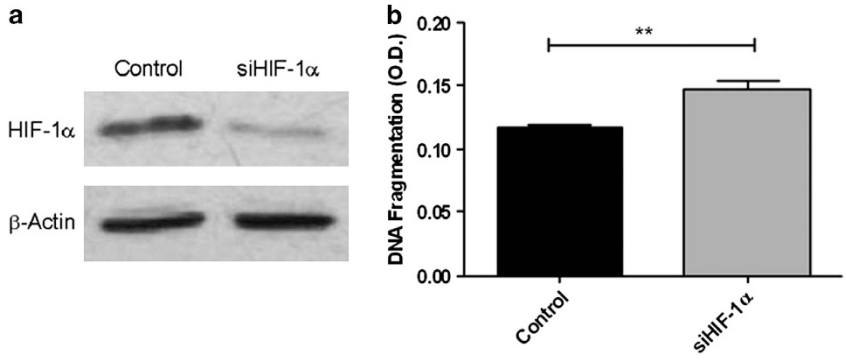

Figure 1 HIF-1 inhibits the apoptosis of hypoxic glioblastoma cells. (**a**) U87MG cells were transfected with siRNA against HIF-1α. Forty-eight hours after transfection, cells were incubated for 2 h in hypoxia (1% O_2). HIF-1α was detected by immunoblotting. β-Actin was used as a loading control. The results are representative for three independent experiments. (**b**) U87MG cells were transfected with siRNA against HIF-1α. Forty-eight hours after transfection, cells were incubated for 24 h in hypoxia (1% O_2). DNA fragmentation was analyzed using ELISA detection (O.D., optical density). The data are shown as the mean ± S.E.M. ($n=3$). $^{**}P<0.01$

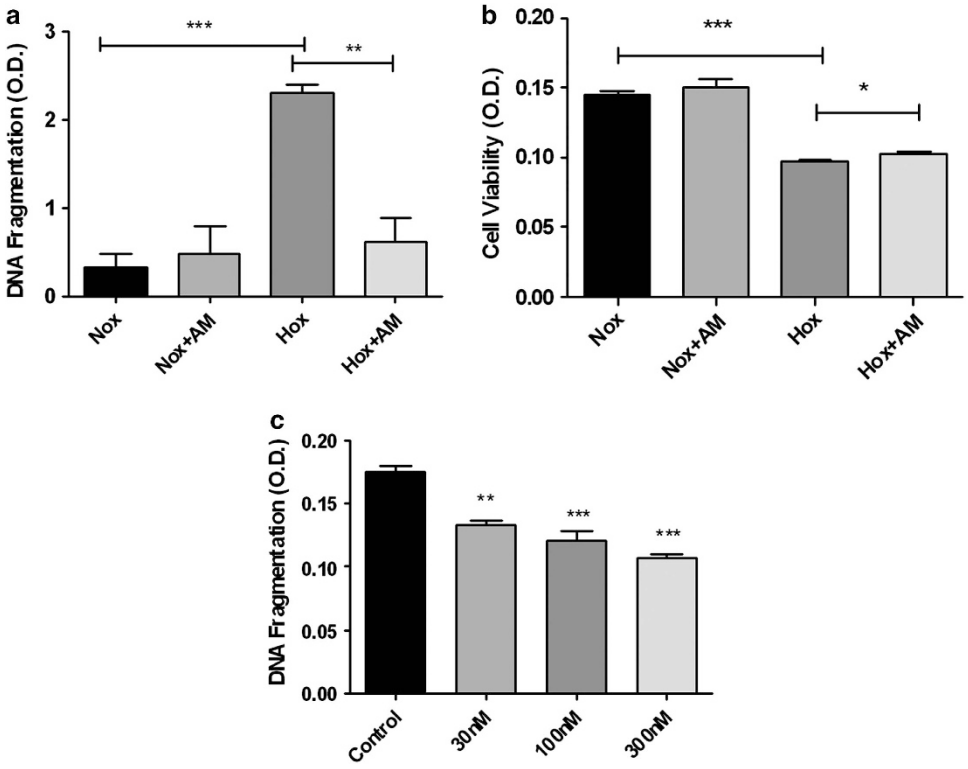

Figure 2 Adrenomedullin is an anti-apoptotic factor for hypoxic glioblastoma cells. (**a**) U87MG cells were incubated for 24 h in normoxia or hypoxia (1% O_2) with or without adrenomedullin (300 nM). DNA fragmentation was analyzed using ELISA detection. The data are shown as the mean ± S.E.M. ($n=3$). ***$P<0.001$, **$P<0.01$. (**b**) U87MG cells were incubated for 48 h in normoxia or hypoxia (1% O_2) with or without adrenomedullin (300 nM). Cell viability was determined by MTT assay. The data are shown as the mean ± S.E.M. ($n=6$). ***$P<0.001$, *$P<0.05$. (**c**) U87MG cells were incubated for 24 h in hypoxia (1% O_2) with or without adrenomedullin (30/100/300 nM). DNA fragmentation was analyzed using ELISA detection. The data are shown as the mean ± S.E.M. ($n=3$). ***$P<0.001$ *versus* control group, **$P<0.01$ *versus* control group

were analyzed (Figure 5b). The fluorescence intensity of IL-1β treated group was significantly stronger than the control group (Figure 5c).

To determine whether the increase in cell apoptosis was caused by suppressed AM secretion, we treated the hypoxic glioblastoma cells with exogenous AM in addition to IL-1β. As shown in Figure 5d, exogenous AM protected hypoxic glioblastoma cells against IL-1β induced apoptosis in a dose-dependent manner.

Proteasome inhibitor MG132 attenuates the inhibitory effect of IL-1β on the HIF-1/AM axis. We attempted to understand the mechanisms by which IL-1β inhibits the HIF-1/AM axis. Since IL-1β reduces the HIF-1α protein content in a short time period, we suggested that IL-1β promotes the proteasomal degradation of HIF-1α. The proteasome inhibitor MG132 is a well-characterized inhibitor of the proteasomal degradation of HIF-1α. MG132 inhibits the interaction between HIF-1α and the proteasome in a dose-dependent manner and markedly increases the half-life of HIF-1α.[23–26] In our work, treatment with MG132 led to an accumulation of HIF-1α in normoxic glioblastoma cells within 30 min, indicating that the proteasomal degradation of HIF-1α was efficiently inhibited (data not shown). In the presence of MG132, IL-1β had no influence on the protein content of HIF-1α (Figure 6a). IL-1β also failed to downregulate the AM expression in the presence of MG132 (Figure 6b).

The proteasomal degradation of HIF-1α is mainly mediated by the ubiquitin system.[3] The influence of IL-1β on the ubiquitination of endogenous HIF-1α was studied by immunoprecipitation. MG132 was added to protect ubiquitinated HIF-1α from proteasomal degradation. As shown in Figure 6c, the ubiquitination of HIF-1α was not affected by IL-1β.

IL-1β does not inhibit the synthesis of HIF-1α. We then studied whether IL-1β inhibits the synthesis of HIF-1α. The mRNA content of HIF-1α in hypoxic glioblastoma cells was not affected by IL-1β after 1–2 h treatment, whereas the protein content of HIF-1α was markedly reduced after 2 h treatment (Figures 3a and 7a). Since there is no specific inhibitor for HIF-1α translation, we determined the effect of IL-1β on HIF-1α translation by studying the influence of IL-1β on the global regulator of eukaryotic translation phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway, which also regulates the translation of HIF-1α.[27–29] The protein content of AKT and the phosphorylation status of AKT were used as indicators of the activity of PI3K/AKT/mTOR pathway. As shown in Figure 7b, neither the protein content of AKT nor the phosphorylation of AKT was affected by IL-1β in hypoxic glioblastoma cells.

IL-1β and LY29004 reduce the steady-state level of HIF-1α synergistically. The translation of HIF-1α could be suppressed by the PI3K inhibitor LY29004. However,

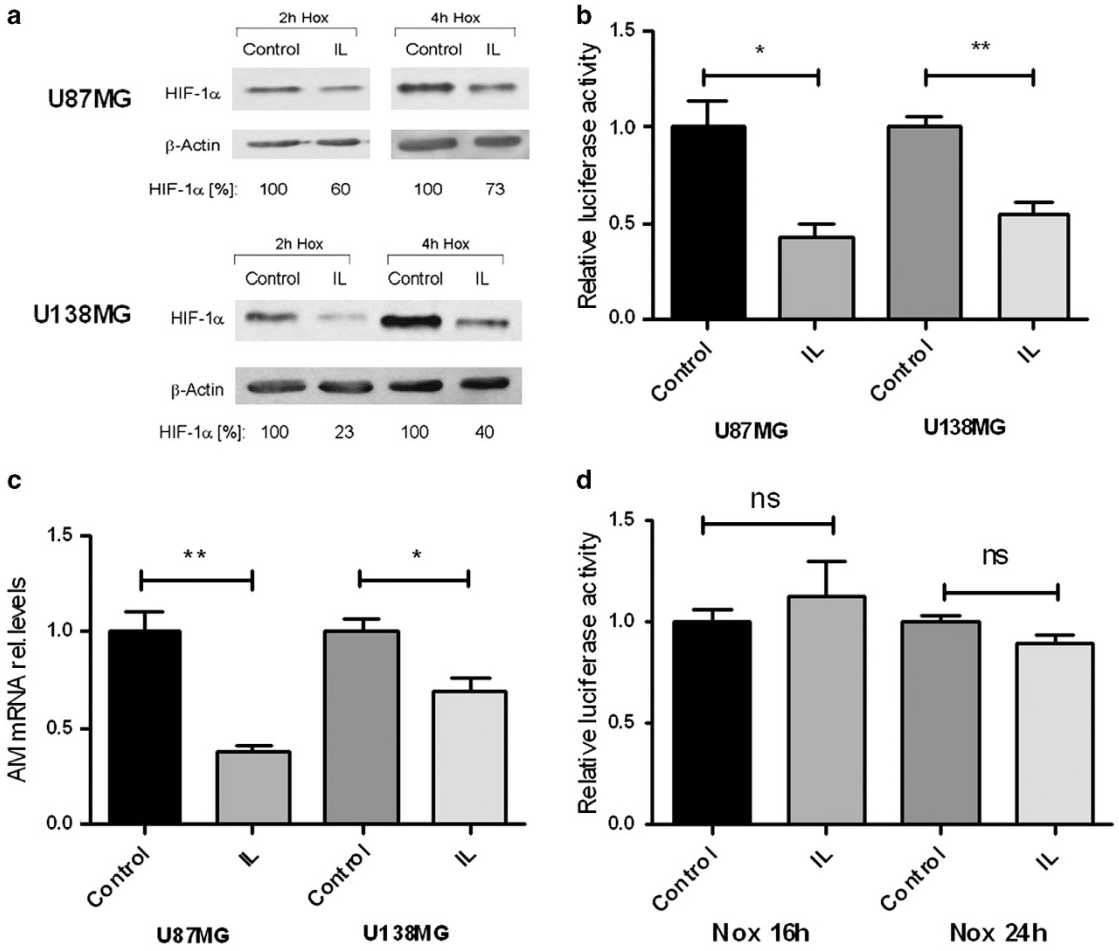

Figure 3 Interleukin-1β inhibits HIF-1/AM axis in hypoxic glioblastoma cells. (**a**) U87MG and U138MG cells were incubated for 2 or 4 h in hypoxia (1% O_2) with or without interleukin-1β (10 ng/ml). HIF-1α was detected by immunoblotting. β-Actin was used as a loading control. Densitometry analysis was performed using Aida 2D Densitometry (Raytest). Protein expression of HIF-1α was normalized of β-actin. The results are representative for three independent experiments. (**b**) U87MG and U138MG cells were co-transfected with hypoxia responsive firefly luciferase plasmid and Renilla luciferase plasmid. Twenty-four hours after transfection, cells were incubated for 16 h in hypoxia with or without interleukin-1β (10 ng/ml). Firefly luciferase activities were normalized to Renilla luciferase activities. The data are shown as the mean ± S.E.M. ($n=3$). $^{**}P<0.01$, $^{*}P<0.05$. (**c**) U87MG and U138MG cells were incubated for 6 h in hypoxia with or without interleukin-1β (10 ng/ml). Total mRNA was analyzed for adrenomedullin (AM) and ribosomal protein L28 expression by qRT-PCR. Normalized AM/L28 ratios are shown. The data are shown as the mean ± S.E.M. ($n=3$). $^{**}P<0.01$, $^{*}P<0.05$. (**d**) U87MG cells were co-transfected with hypoxia responsive firefly luciferase plasmid and Renilla luciferase plasmid. Twenty-four hours after transfection, cells were incubated for 16 or 24 h in normoxia with or without interleukin-1β (10 ng/ml). Firefly luciferase activities were normalized to Renilla luciferase activities. The data are shown as the mean ± S.E.M. ($n=3$). ns, not significant

LY29004 was not able to reduce the steady-state level of HIF-1α in hypoxic glioblastoma cells after being added 10 min in advance to hypoxic incubation (Figure 8), although the phosphorylation of AKT was efficiently inhibited (data not shown). In the presence of IL-1β, the HIF-1α steady-state level was markedly decreased by LY29004 (Figure 8).

Discussion

IL-1β was initially considered to be an inhibitor of glioblastoma growth. Elevated expression of IL-1β in glioblastomas was reported to be associated with better clinical outcomes.[18] Antagonization of tumor cell-derived IL-1β promotes the colony formation of glioblastoma cells.[17] Sharma *et al.*[19] reported that chronic treatment with IL-1β induces the oxidative DNA damage in glioma stem-like cells. Castigli *et al.*[20] reported that IL-1β induces the apoptosis of glioblastoma cell line GL15 by causing an imbalance between MAPK and SAPK pathway. However, IL-1β was also reported to upregulate the expression of MMP-9 and enhance the invasiveness of a glioblastoma cell line with invasive growth pattern in 3D CL matrix.[21] Yeung *et al.*[22] showed that IL-1β stimulates the production of interleukin-6 and interleukin-8 by the glioblastoma cell line U251, which may in turn lead to unfavorable prognosis because of the potential tumor-promoting effects of interleukin-6 and interleukin-8.[30–33] These findings indicate that the influence of IL-1β on the glioblastoma progression is complex and might involve a variety of intracellular pathways.

In this study, we show that IL-1β inhibits HIF-1 mediated AM production and promotes hypoxia-induced apoptosis of the AM-producing glioblastoma cells. Our findings reveal the fact that the influence of IL-1β on glioblastoma growth is probably dependent on factors such as the degree of hypoxia and the

expression level of AM. In glioblastomas with high degree of hypoxia and high expression level of AM, IL-1β induces glioblastoma cell death rapidly and is supposed to be a potent tumor suppressor. The promoting effects of IL-1β on glioblastoma cell invasion and cytokine secretion should be less important.

The anti-apoptotic effect of AM on glioblastoma cells was only determined *in vivo* until now.[12] In our study, we could show that AM suppresses the apoptosis of glioblastoma cells in hypoxia. Interestingly, AM has no influence on glioblastoma cell apoptosis in normoxia, indicating that AM specifically suppresses hypoxia-induced apoptosis. In several other tissues, AM was also reported to suppress cell apoptosis under stressful conditions like inflammation and ischemia specifically. Different intracellular signaling molecules and pathways such as cAMP, CGRP1, MEK-ERK pathway and AKT-GSK pathway were discussed to be involved.[34–37]

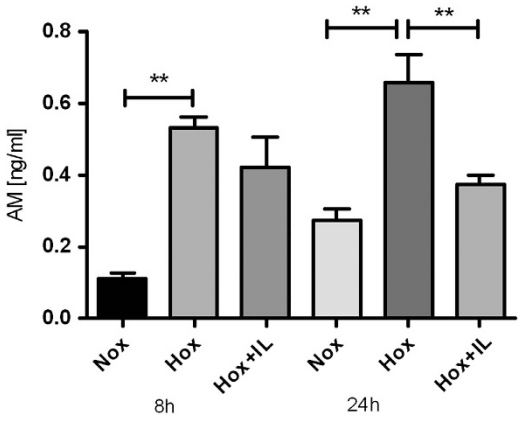

Figure 4 Interleukin-1b inhibits the adrenomedullin secretion by hypoxic glioblastoma cells. U87MG cells were incubated for 8 or 24 h in normoxia or hypoxia (1% O_2) with or without interleukin-1β (10 ng/ml). The concentration of adrenomedullin in the cell-culture supernatants was analyzed using EIA detection. Adrenomedullin concentrations were normalized to total cellular protein concentrations. The data are shown as the mean ± S.E.M. ($n=3$). $^{**}P<0.01$

Besides the anti-apoptotic effect, AM was also discussed to promote the proliferation of glioblastoma cells through the CRLR/$RAMP_2$ complex.[12] It would be worthwhile to study whether IL-1β also inhibits the proliferation of AM-producing glioblastoma cells.

The inhibition of the HIF-1/AM axis is dependent on the proteasome system. However, the ubiquitination status of HIF-1α is not changed by IL-1β. Kong *et al.*[23] reported that HIF-1α could be degraded by 20S proteasome independent of the ubiquitin system. A direct interaction between HIF-1α and a subunit of the 20S proteasome was also observed.[25] Therefore, we assume that IL-1β promotes the ubiquitin-independent proteasomal degradation of HIF-1α. Since the mechanisms involved in the ubiquitin-independent degradation of HIF-1α are still not understood, more studies need to be performed to determine whether overexpression of 20S proteasome could be considered as a new therapeutic option for glioblastoma.

The translation of HIF-1α was determined on the basis of the PI3K/AKT/mTOR pathway activity. Although the activity of PI3K/AKT/mTOR pathway seemed not to be influence by

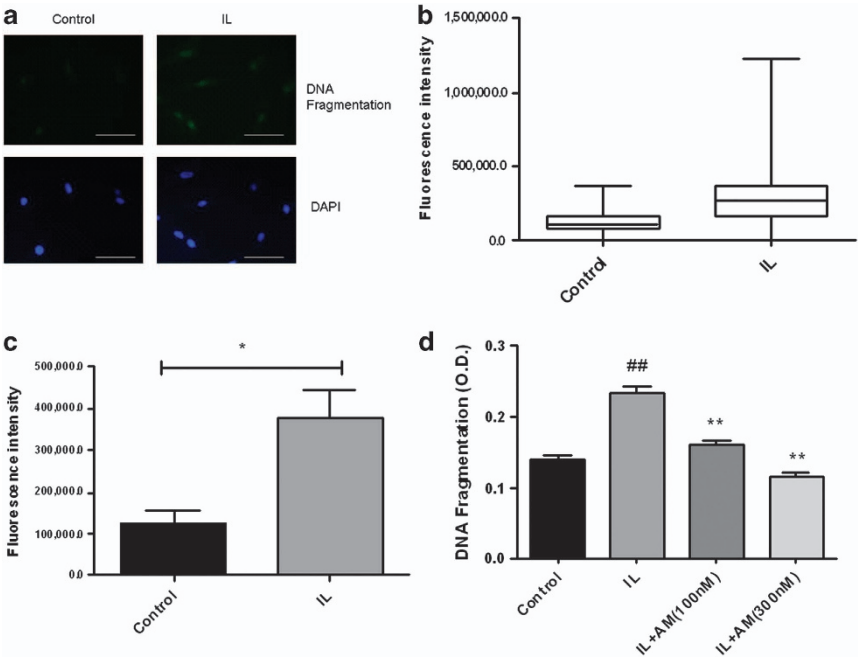

Figure 5 Interleukin-1β promotes the apoptosis of hypoxic glioblastoma cells. U87MG cells were incubated for 24 h in hypoxia (1% O_2) with or without interleukin-1β (10 ng/ml). Cell apoptosis was determined by fluorescent labeling of intracellular fragmented DNA. Representative microscopic images are shown (**a**). Scale bars: 75 μm. The fluorescence intensity of 150–200 cells was analyzed using ImgeJ software pro experiment. The distribution of the fluorescence intensity of 150–200 cells in one experiment (box plot) (**b**) and the mean of three independent experiments (**c**) are shown (mean ± S.E.M, $n=3$. $^{*}P<0.05$). (**d**) U87MG cells were incubated for 24 h in hypoxia (1% O_2) without interleukin-1β or adrenomedullin/with interleukin-1β (10 ng/ml)/with interleukin-1β and adrenomedullin (100/300 nM). DNA fragmentation was analyzed using ELISA detection. The data are shown as the mean ± S.E.M. ($n=3$). $^{\#\#}P<0.01$ *versus* control group, $^{**}P<0.01$ *versus* IL group

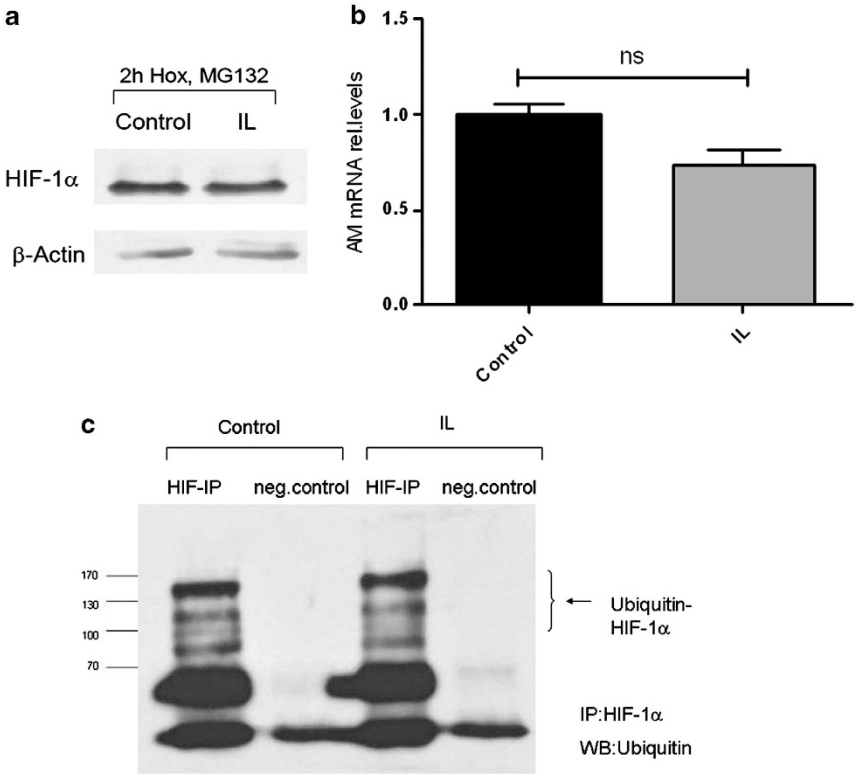

Figure 6 MG132 attenuates the inhibitory effect of interleukin-1β on HIF-1/AM axis. (**a**) U87MG cells were incubated for 2 h in hypoxia (1% O_2) with or without interleukin-1β (10 ng/ml). The proteasome inhibitor MG132 (1 μM) was added 30 min in advance. HIF-1α was detected by immunoblotting. β-Actin was used as a loading control. The results are representative for three independent experiments. (**b**) U87MG cells were incubated for 6 h in hypoxia with or without interleukin-1β (10 ng/ml). The proteasome inhibitor MG132 was added 30 min in advance. Total mRNA was analyzed for adrenomedullin (AM) and ribosomal protein L28 expression by qRT-PCR. Normalized AM/L28 ratios are shown. The data are shown as the mean ± S.E.M. ($n = 3$). ns, not significant. (**c**) U87MG cells were incubated for 2 h in hypoxia (1% O_2) with or without interleukin-1β (10 ng/ml). The proteasome inhibitor MG132 was added 30 min in advance to prevent ubiquitinated HIF-1α from proteasomal degradation. HIF-1α was precipitated by anti-HIF-1α antibody. Ubiquitin was detected by immunoblotting. Non-specific mouse IgG was used as a negative control. The results are representative for three independent experiments

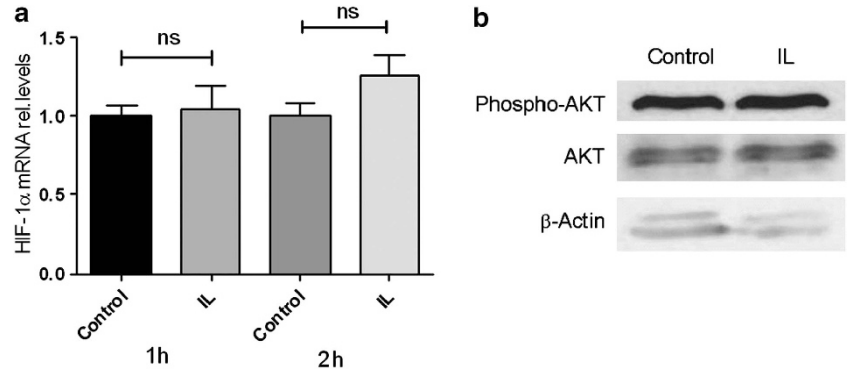

Figure 7 Influence of interleukin-1β on HIF-1α mRNA content and PI3K/AKT/mTOR pathway. (**a**) U87MG cells were incubated for 1 or 2 h in hypoxia with or without interleukin-1β (10 ng/ml). Total mRNA was analyzed for HIF-1α and ribosomal protein L28 expression by qRT-PCR. Normalized HIF-1α/L28 ratios are shown. The data are shown as the mean ± S.E.M. ($n = 6$). ns, not significant. (**b**) U87MG cells were incubated for 2 h in hypoxia (1% O_2) with or without interleukin-1β (10 ng/ml). AKT and phosphorylated AKT were detected by immunoblotting. β-Actin was used as a loading control. The results are representative for three independent experiments

IL-1β, we cannot exclude the possibility that IL-1β might inhibit the translation of HIF-1α in a proteasome-dependent manner.

Besides AM, HIF-1 activates a number of genes involved in the adaptation of tumor cells to hypoxia such as glucose transporters and angiogenic factors.[38,39] The PI3K inhibitor LY29004 is supposed to be an attractive potential anti-tumor agent because of its inhibitory effect on HIF-1α translation.[29] However, without a long-term treatment in advance to hypoxic incubation, the inhibition of HIF-1α translation by LY29004 seems to be negligible in glioblastoma cells. IL-1β enhances

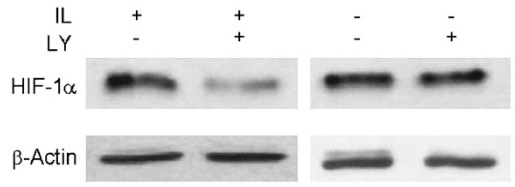

Figure 8 Interleukin-1β and LY29004 reduce the steady-state level of HIF-1α synergistically. U87MG cells were incubated for 2 h in hypoxia (1% O_2) with or without interleukin-1β and LY29004 (25 μM). LY29004 was added 10 min in advance. HIF-1α was detected by immunoblotting. β-Actin was used as a loading control. The results are representative for three independent experiments

the responsiveness of HIF-1α to LY29004, probably due to the concurrent promotion of HIF-1α degradation. This indicates that the anti-tumor potential of LY29004 in glioblastoma is dependent on the expression level of IL-1β. In glioblastomas with low expression level of IL-1β, the anti-tumor potential of LY29004 is doubtful, whereas LY29004 might represent an effective therapeutic agent in glioblastomas with high expression level of IL-1β.

In conclusion, IL-1β suppresses the survival of the AM-producing glioblastoma cells in hypoxia. The expression level of IL-1β in glioblastomas could be considered as a potential prognostic marker for glioblastomas with high degree of hypoxia and high expression level of AM. Furthermore, the expression level of IL-1β in glioblastomas may influence the selection of anti-tumor agents with inhibitory effects on HIF-1 pathway. Since all experiments were performed in cell lines, *in vivo* studies and clinical trials still need to be performed to verify our hypothesis. It would also be worthwhile to further investigate the exact mechanisms by which IL-1β promotes the proteasomal degradation of HIF-1α.

Materials and Methods

Reagents. Recombinant human IL-1β was purchased from PeproTech (Hamburg, Germany). Human adrenomedullin 52 was purchased from Sigma-Aldrich (Seelze, Germany). MG132 and LY29004 were purchased from Calbiochem (Darmstadt, Germany). AM was biologically active when injected to rodents as described by Gardiner *et al*.[40]

Cell culture. The human glioblastoma cell lines U87MG and U138MG were obtained from the American Type Culture Collection. Cells were cultured in DMEM (Gibco, Darmstadt, Germany) supplemented with 10% FCS (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories, Coelbe, Germany) in a humidified atmosphere with 5% CO_2. For hypoxic treatments, cells were incubated in the hypoxia workstation (Ruskinn invivo$_2$ 400) in the presence of 1% O_2, 5% CO_2 and 94% N_2 at 37 °C. All experiments were performed with 1% FCS or without FCS. Cells were synchronized in medium with 1% FCS 16–20 h in advance.

MTT assay. The cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) assay. Cells were incubated with MTT solution (5 g/l) for 2 h and then lysed with MTT lysis buffer (10% SDS, 1% HCl, 0.46% isopropanol). The optical density was detected with a microplate reader (Thermo Scientific, Bonn, Germany) at 570 nm.

Protein extraction. For the preparation of whole-cell extracts, cells were washed with ice-cold PBS and lysed with urea lysis buffer containing 10 mM Tris HCl (pH 6.8), 7.6 M Urea, 10 M Glycerin, 1% SDS, 5 mM DTT and protease inhibitor cocktail (Calbiochem). Protein concentrations were determined by Biorad protein assay (Bio-Rad, Munich, Germany) using BSA as a standard.

Immunoblot analysis. For immunoblot analysis, 60 μg protein was subjected to 7.5% SDS–PAGE and transferred onto nitrocellulose membranes (Amersham Hybond-ECL; GE Healthcare, Freiburg, Germany). Membranes were blocked at room temperature for 1 h in 3% non-fat dry milk in PBS and then incubated with primary antibodies overnight at 4 °C. Mouse monoclonal antibody against HIF-1α (1 : 1000; BD Biosciences, Heidelberg, Germany) and β-actin (1 : 2000; Applied Biological Materials, Richmond, BC, Canada), rabbit polyclonal antibodies against AKT (1 : 1000; GeneTex, Irvine, CA, USA) and phospho-AKT (1 : 1000; GeneTex) were used. After being washed with PBS-T, membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG) antibodies (1 : 2000; Dako, Hamburg, Germany). Immunoreactive proteins were detected using ECL detection reagents (Amersham ECL Western Blotting Detection Reagents; GE Healthcare) and X-ray films (Amersham Hyperfilm MP, GE Healthcare).

Immunoprecipitation. In all, 200 μg whole-cell extracts were incubated with mouse monoclonal antibody against HIF-1α (1 μg, Novus NB100-123, Cambridge, UK) or non-specific mouse IgG (1 μg, Dako) in immunoprecipitation buffer (50 mM Tris pH 7.4, 1 mM EGTA, 400 mM NaCl, 0.1% NP-40, 1 mM NaF). Antibodies and bound proteins were precipitated with Protein G Sepharose (GE Healthcare). Ubiquitin was detected by immunoblotting using mouse monoclonal antibody against ubiquitin (1 : 1000; Santa Cruz, Heidelberg, Germany).

RNA isolation and quantitative RT-PCR. Total RNA was extracted using the 6100 Nucleic Acid Prepstation (Applied Biosystems, Darmstadt, Germany) following the manufacturer's protocol. In all, 150 ng total RNA was reverse transcribed with the ThermoScript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen, Darmstadt, Germany), SuperScript III Reverse Transcriptase (Invitrogen) or Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Quantitative RT-PCR was performed in ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the SensiMix SYBR Kit (Bioline, Luckenwalde, Germany) or Taqman Gene Expression Assay (Invitrogen). The mRNA levels were normalized to human L28 mRNA. Following primers and assays were used for quantitative RT-PCR: human AM forward, 5′-GGA TGC CGC CCG CAT CCG AG-3′; human AM reverse, 3′-GAC ACC AGA GTC CGA CCC GG-5′; human L28 forward, 5′-ATG GTC GTG CGG AAC TGC T-3′; human L28 reverse, 5′-TTG TAG CGG AAG GAA TTG CG-3′; Taqman Gene Expression Assay for HIF-1α (Hs00936368_m1).

Enzyme immunoassay. The concentration of AM in cell-culture supernatants was measured by enzyme immunoassay (EIA) using the Adrenomedullin EIA Kit (Phoenix Europe GmbH, Karlsruhe, Germany) following the manufacturer's instructions. The optical density was measured at 450 nm with a microplate reader (Thermo Scientific). AM concentrations were normalized to total cellular protein concentrations.

Detection of DNA fragmentation. Intracellular DNA fragmentation was detected using the Cellular DNA Fragmentation ELISA Kit (Roche) or the DNA Fragmentation Imaging Kit (Roche) following the manufacturer's instructions. The ELISA reactions were incubated for 5–20 min. In the ELISA experiments, the optical density was measured at 450 nm with a microplate reader (Thermo Scientific). Fluorescent-labeled fragmented DNA was analyzed under a fluorescence microscope (Zeiss, Axioplan, Jena, Germany). The fluorescence intensity of single cells was quantified with the software ImageJ.[41]

Reporter gene assay. U87MG and U138MG cells were cultured to 60–70% confluence in 24-well plates and co-transfected with a hypoxia responsive firefly luciferase plasmid containing six copies of HIF-1 binding sites from the transferrin 3′ enhancer and a Renilla luciferase plasmid as a transfection efficiency control using GeneJuice (Merck, Darmstadt, Germany) according to the manufacturer's instruction. The transfection complex was removed 4 h after transfection. Twenty-four hours after transfection, the medium was changed and the cells were incubated in normoxia or hypoxia for another 16 or 24 h with or without IL-1β (10 ng/ml). After incubation, cells were washed with ice-cold PBS and lysed with passive lysis buffer (Promega, Mannheim, Germany). Luminescence was measured with Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase activities were normalized to Renilla luciferase activities.

RNA interference. U87MG cells were cultured to 60–70% confluence in 6-well plates or 24-well plates and transfected with 30 nM siRNA against HIF-1α (Invitrogen) or BLOCK-iT Fluorescent Oligo (Invitrogen) as a negative control using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction. Forty-eight hours after transfection, the medium was changed and cells were incubated in hypoxia for 2 or 24 h.

Statistical analysis. Data are shown as means ± S.E.M. of at least three independent experiments. Statistical analysis between two groups was performed by Student's *t*-test. Differences were considered as significant when $P<0.05$ ($^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$). All statistics were calculated using GraphPad Prism 5 (GraphPad Software, Witzenhausen, Germany).

Conflict of Interest

The authors declare no conflict of interest.

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