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Biliverdin reductase plays a crucial role in hypoxia-induced chemoresistance in human glioblastoma

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ABSTRACT

Hypoxia-induced alterations in the cellular redox status play a critical role in the development of hypoxia-induced chemoresistance in cancer cells. Human biliverdin reductase (hBVR), an enzyme involved in the conversion of biliverdin into bilirubin in heme metabolism, was recently identified as an important cytoprotectant against oxidative stress and hypoxia. However, the role of hBVR on hypoxia-induced drug resistance has not been previously investigated. Using human glioblastoma cell lines, we evaluated the potential role of hBVR in hypoxia-induced drug resistance. We found that hypoxia caused a significant increase in hBVR expression in glioblastoma cells that was accompanied by chemoresistance. We also observed that siRNA-based targeting of hBVR genes attenuated the hypoxia-induced chemoresistance. Furthermore, knocking down hBVR induced a marked increase in the levels of intracellular reactive oxygen species under hypoxic conditions, and the chemosensitizing effect of hBVR depletion was reversed by pretreatment with the antioxidant *N*-acetylcysteine. These findings suggest that hBVR significantly contributes to the modulation of hypoxia-induced chemoresistance of glioblastoma cells by adjusting their cellular redox status.

1. Introduction

Glioblastoma multiformes (GBMs) are the most common malignant primary brain tumors and are among the most hypoxic tumors known. Although chemotherapy plays an important role in combination treatments to manage recurrent GBMs, these tumors typically acquire resistance to most drugs administered, and the majority of patients die following tumor recurrence [1]. Multiple mechanisms including augmented DNA repair activities, dysregulation of the apoptosis pathway, overexpression of drug efflux transporter pumps, and physiological conditions such as hypoxia and oxidative stress appear to be involved in the development of drug resistance in tumor cells [2]. In solid tumors, hypoxia plays an important role in the resistance to chemotherapy. Hypoxic microenvironment is frequently found in solid tumors and contributes to the development of aggressive and poor prognostic phenotype with high metastatic rates and chemo- or radio-resistance [3]. Due to the inefficient microcirculation in GBMs and the poor maintenance of the blood–brain barrier, GBMs are more prone to chronic hypoxia [4].

It has been reported that hypoxia results in an increased generation of reactive oxygen species (ROS), which are important mediators of the hypoxia-induced cellular response [5,6]. A growing body of evidence suggests that modulation of ROS levels can affect the hypoxia-induced chemoresistance [7,8]. Therefore, the antioxidant system may play a critical role in the hypoxia-induced drug resistance seen in GBMs.

Human biliverdin reductase (hBVR) is an evolutionarily conserved enzyme, converting biliverdin to bilirubin in the heme-degradation pathway. Recently, hBVR has been identified as a potent antioxidant responsible for the maintenance of intracellular redox homeostasis [9,10]. It was also recently reported that exogenous BVR can enhance drug resistance in normal mouse fibroblasts [11]. Although many studies of drug resistance have focused on the antioxidant defense system, the role of hBVR on chemoresistance in cancer, particularly hypoxia-induced chemoresistance, has not been previously investigated. In this study, we used human-derived glioblastoma cell lines to evaluate the role of hBVR in modulating hypoxia-induced chemoresistance.

Abbreviations: hBVR, biliverdin reductase; GBM, Glioblastoma multiforme; TMZ, temozolomide; PTX, paclitaxel; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; HO, Heme oxygenase.

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2. Materials and methods

2.1. Cell culture and reagents

Human GBM cell lines, U87 and U251 (American Type Culture Collection, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, USA). These were supplemented with 10% heat inactivated fetal bovine serum (Invitrogen, Carlsbad, USA) and 2 mM L-glutamine (Hyclone, Logan, USA). All cell cultures were periodically tested for Mycoplasma contamination using the Mycoplasma Stain Kit (Sigma, St. Louis, USA). For hypoxic conditions, cells were incubated at 37 °C containing 1% O_2, 5% CO_2, and balance N_2 in a humidified incubator. All drugs were purchased from Sigma unless specified otherwise.

2.2. Cell viability assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Rockville, USA). Human GBM cell seeding a 96-well plate, subjected to the indicated treatment, and then 10 μl of CCK-8 solution was added to each well, and plates were incubated at 37 °C for an additional 2 h. Plates were read on microplate reader at 450 nm with a reference wavelength at 630 nm. IC_{50} values were calculated by log expression using the software of Microsoft Excel 2003. Relative reversal rate = (IC_{50} A−IC_{50} B)/(IC_{50} A−IC_{50} C), where IC_{50} A was IC_{50} values of resistant cells before RNAi, IC_{50} B was IC_{50} values of resistant cells after RNAi, IC_{50} C was IC_{50} values of sensitive GBM cells [12].

2.3. Immunoblot analysis

Cells and tissues were solubilized with a lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% SDS, protease inhibitor cocktail (Roche, Indianapolis, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, and 1 mM sodium orthovanadate. The protein content was determined using the Bradford assay. Protein (40 lg) of each sample was resolved by SDS–PAGE, transferred onto nitrocellulose membranes, and blocked with TBST in 5% skim milk. The membranes were incubated with the primary antibodies at 4 °C overnight. Secondary antibodies were added for 1 h at room temperature. The antibody–antigen complexes were detected using the ECL detection system (Pierce, Rockford, USA).

2.4. Enzyme assay

Biliverdin reductase assay – The enzyme activity was determine by measuring the rate of bilirubin formation. hBVR activity was measured in U87 or U251 cells plated in 60 mm plastic dishes and pooled samples of cells using a colorimetric reaction to measure the formation of bilirubin, as described by manufacturer (Sigma, St. Louis, USA).

2.5. Visualization of DAPI-labeled nuclei

Cells were seeded on glass coverslips in 24-well culture plates. 24 h later, cells were incubated under normoxia or hypoxia with or without TMZ for 36 h. Medium was then removed and cells were fixed for 10 min with PBS containing 4% paraformaldehyde and then washed 3 × 5 min in PBS. Cells were then permeabilized with 0.2% Triton X-100 in PBS. After 5 min, 5 μl of DAPI (10 μg/ml) (Sigma, St. Louis, USA) were added in well. After 15 min of incubation in dark at 37 °C, cells were washed with PBS. Then, the coverslips were mounted in Mowiol (Sigma, St. Louis, USA) and observed with a fluorescence microscope at 352 nm.

2.6. Analysis of apoptosis by caspase-3 activity assay

Caspase-3 activity was examined by cleavage of chromogenic caspase substrate, Ac-DEVD-pNA (ace-tyl-Asp-Glu-Val-Asp p-nitroanilide). Sample supernatants containing 50 μg of total protein were added to a reaction buffer containing Ac-DEVD-pNA (2 mM) and incubated for 4 h at 37 °C, and then the absorbance of yellow pNA cleavaged form was measured by a spectrometer at 405 nm.

2.7. Analysis of apoptosis by annexin V staining

The amount of phosphatidylserine (PS) on cell surfaces was determined using an Annexin V-FITC apoptosis detection kit (Abcam, Cambridge, USA), according to the manufacturer's instructions.

2.8. RNA interference and transfection

The specific siRNA for hBVR was purchased from BIONEER Co. RNA interference (RNAi) of the hBVR transcript was performed. Briefly, cells were plated in a 100 mm dish, transfected with 50 nmol of siRNA and Oligofectamine™ reagent in serum-free medium and incubated for 4 h at 37 °C in a CO_2 incubator. Following incubation, the cells were supplied with growth medium containing 10% fetal bovine serum. The efficiency of gene silencing was confirmed by real-time RT-PCR and Western blot analyses.

2.9. Measurement of intracellular ROS level

The cellular levels of ROS were determined using dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma, St. Louis, USA). Cells were stained with 50 μM of DCF-DA for 30 min and then harvested. The fluorescent intensities were quantified using an cytometer (Becton Dickinson FACSorter). To examine the effect of *N*-acetylcystein (NAC) (Sigma, St. Louis, USA), cell were treated with 20 mM of NAC for 24 h.

2.10. Data analysis

Significance was determined using one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. For all data sets comparing the mean of only two groups, an unpaired Student's *t*-test was employed.

3. Results

3.1. Hypoxia protected GBM cells against temozolomide- or paclitaxel-induced apoptosis

In order to examine the effects of hypoxia on chemoresistance in GBM cell lines, we first determined the half-maximal inhibitory concentration (IC_{50}) values for the current standard chemotherapy against GBMs, temozolomide (TMZ), and paclitaxel (PTX) after pre-exposure to hypoxic or normoxic conditions. Treatment of U87 and U251 GBM cells with TMZ after 24 h of incubation under hypoxic conditions resulted in an IC_{50} value that was 3.2- and 2.5-fold higher, respectively, than that for cells under normoxic conditions (Table 1). The IC_{50} values for PTX in both cell lines were increased approximately 2.1- and 1.8-fold under hypoxic conditions (Table 1).

Table 1
Anticancer drug resistance induced by hypoxia against glioblastoma cell lines.

Drug	Cell lines	IC_{50}[a]		Degree of resistance
		Normoxia	Hypoxia	
Temozolomide	U87	103.62[b]	331.58	3.2
	U251	231.43	578.52	2.5
Paclitaxel	U87	7.24[c]	15.23	2.1
	U251	9.83	17.61	1.8

[a] Mean of at least three separate experiments.
[b] IC_{50} (μmol/L).
[c] IC_{50} (nmol/L).

We then explored the effects of hypoxia on TMZ-and PTX-induced apoptosis. We assessed Annexin V expression and caspase-3 activity in U87 cells incubated with IC_{50} concentration of TMZ or PTX under normoxic and hypoxic conditions. Exposure of U87 cells to hypoxic conditions (1% O_2) for 24 h prior to drug treatment led to a significantly increased resistance to anticancer drug-induced apoptosis for both TMZ and PTX relative to cells maintained under normoxic conditions (20% O_2; Fig. 1A,B). Similar results were obtained with human U251 glioma cells. (Supplementary Fig. 1A). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining revealed that normoxic U87 cells had more nuclei fragmentation than did hypoxic cells after treatment with TMZ (Fig. 1C). These data demonstrate that pre-exposure of GBM cells to hypoxic conditions prior to drug treatment leads to resistance to TMZ- or PTX-induced apoptosis.

3.2. Hypoxia increased the expression of human biliverdin reductase in GBM cells

To determine whether the expression of hBVR was affected by hypoxic conditions at different time points, we induced hypoxia in vitro by incubating U87 cells in 1% O_2 from 8 to 48 h prior to performing western blot and real time-PCR. We found that after exposure to hypoxic conditions for 8, 12, 24, or 48 h, the mRNA and protein levels of hBVR were increased, with the highest levels for each being approximately 3.6-fold higher than the levels found in U87 cells after a 24 h incubation under normoxic conditions (Fig. 2A,B). Similar results were obtained with human U251 glioma cells (Supplementary Fig. 1B). Consistent with the increased protein and mRNA expression levels seen, hypoxic conditions induced a significant time-dependent increase in hBVR activity (Fig. 2C).

3.3. Inhibition of hBVR sensitizes GBM cells to hypoxia-induced drug resistance

To explore the possible role of hBVR in hypoxia-induced drug resistance, we first evaluated the knockdown efficiency of hBVR siRNAs. Transfection of any of the three different hBVR siRNAs into U87 cells effectively reduced the expression of hBVR when compared with that in the cells transfected with non-targeting scramble siRNA (control siRNA) (Supplementary Fig. 2A). Owing to its strong inhibitory effect, as demonstrated in Supplementary Fig. 2A, siRNA1 was used further to examine the impact of hBVR knockdown on the hypoxia-induced resistance to TMZ or PTX.

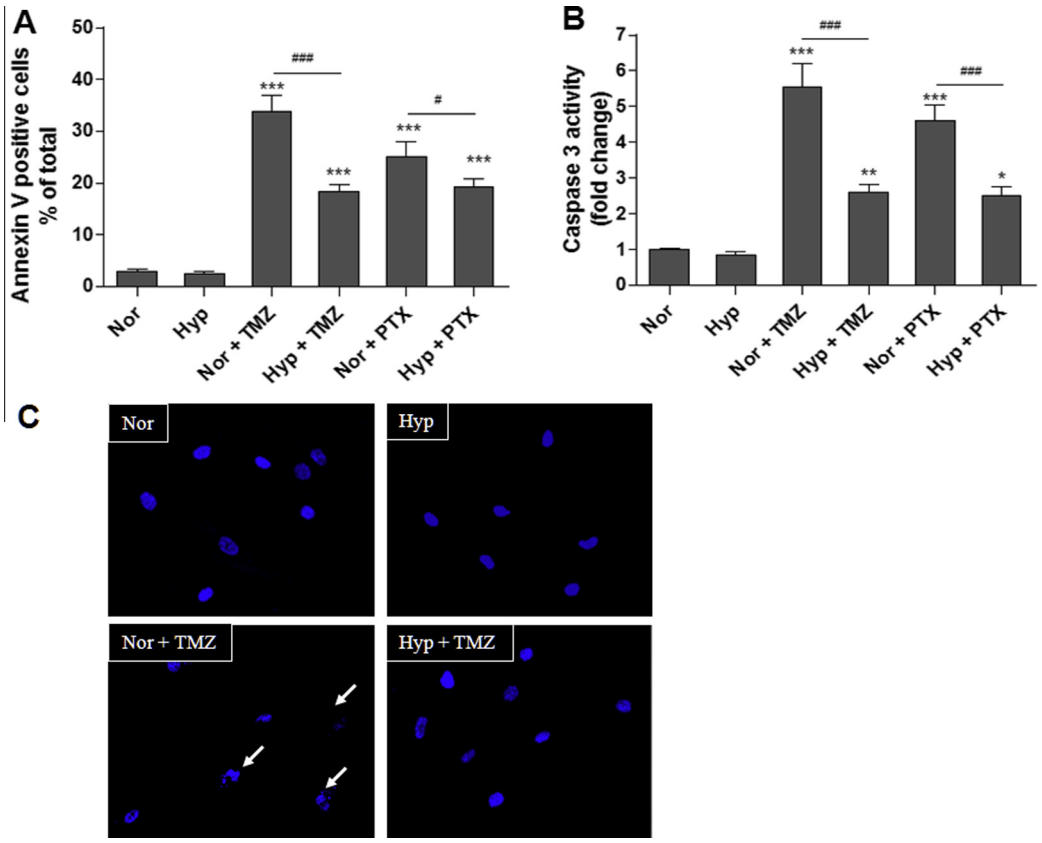

Fig. 1. Effect of hypoxia on drug-induced apoptosis in human GBM cells. (A and B) U87 GBM Cells were grown under normoxic (Nor, 20% O_2) or hypoxic (Hyp, 1% O_2) conditions for 24 h. After incubation, the cells were treated with or without 100 μM TMZ or 7 nM PTX for an additional 36 h. Assays for Annexin V staining and caspase-3 activity were then performed as described in the Section 2. The data are reported as the mean ± SE of three independent experiments. Statistical significance were determined by one-way ANOVA followed by a Dunnett's post hoc test or unpaired Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from control cells (Nor); $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, significantly different between drug-treated normoxic cells and drug-treated hypoxic cells; Nor, normoxia; Hyp, hypoxia. (C) Nuclear condensation and fragmentation were observed after nuclei staining with DAPI. Arrows point to fragmented nuclei.

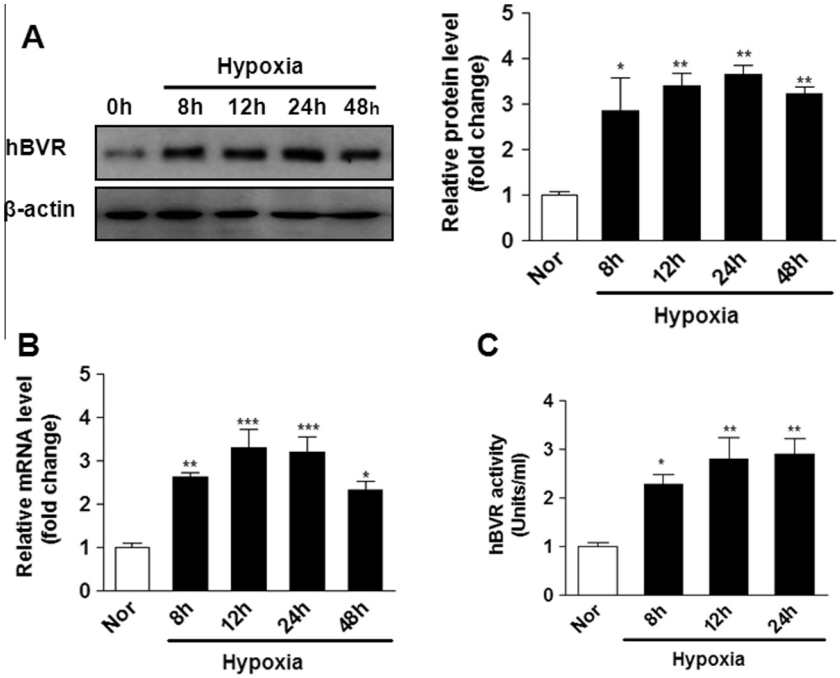

Fig. 2. Hypoxic conditions induced protein and mRNA expression of hBVR and increased hBVR activity in human GBM cells. (A) Western blot analysis of hBVR in U87 GBM cells after 12, 24, and 48 h of hypoxia. The left panel is a representative blot from three independent experiments. Protein levels were quantified by densitometry analysis (right panel, β-actin as a loading control). (B) After exposure to hypoxic conditions for the indicated times, total RNA was extracted and hBVR gene expression was determined by real-time RT-PCR. (C) After exposure to hypoxic conditions for the indicated times, the cell extract was prepared and the reductase activity was measured at pH 8.7. The activity was measured three times using separate preparations of U87 cells. The data are expressed as the mean ± SEM from three independent experiments. Statistical significance were determined by one-way ANOVA followed by a Dunnett's post hoc test; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control cells (Nor); Nor, normoxia.

Using the XTT assay, we next analyzed the IC_{50} values for TMZ and PTX in siRNA-transfected GBM cell lines. As shown in Table 2, we found that the sensitivity to TMZ and PTX was reduced in hBVR-depleted U87 cells. Importantly, this effect was remarkably more pronounced under hypoxic conditions. IC_{50} of TMZ on U87/hypoxic cells was reduced from 324.98 μmol/L (Control RNAi) to 195.88 μmol/L (hBVR RNAi). IC_{50} of PTX was reduced from 15.03 nmol/L (Control RNAi) to 11.98 nmol/L (hBVR RNAi). The relative reversal rates for U87/hypoxic cells treated with TMZ and PTX were 58.5% and 37.1%, respectively. Similar results were obtained with human U251 glioblstoma cells. The relative reversal rate of U251/hypoxic cells on TMZ and PTX were 45.5% and 33.7%, respectively (Table 2, Fig. 3A and B).

We further examined the effect of hBVR depletion on TMZ or PTX-induced apoptosis under hypoxic or normoxic conditions. Apoptosis was assessed by annexin-V staining and intracellular caspase-3 activity. Fig. 3C and D shows that knocking down hBVR markedly increased annexin V staining and caspase-3 activity when cells were treated with IC_{50} concentration of TMZ or PTX after exposure to normoxic or hypoxic conditions and this pro-apoptotic effect of hBVR depletion was remarkably more prominent under hypoxic conditions. These results strongly suggest that hBVR plays a significant anti-apoptotic role in hypoxia-induced drug resistance in glioblastoma cells.

3.4. Biliverdin reductase mediatedhypoxia-induced drug resistance occurs in a ROS-dependent manner

ROS have been shown to be involved in hypoxia-induced drug resistance. We therefore analyzed the ROS status of TMZ-treated hypoxic U87 cells. As shown in Fig. 4A, TMZ treatment resulted in a pronounced increase in ROS production under normoxic conditions, whereas much less of an increase in ROS levels was detected under hypoxic conditions. When we analyzed the ROS

Table 2
IC_{50} values of TMZ and PTX on siRNA-transfected GBM cells.

Cell lines	Drug	Oxygen status	siRNA		RR (%)
			Scramble	hBVR	
U87	Temozolomide	Normoxia	104.26[a]	92.45	58.5
		Hypoxia	324.98	195.88	
	Paclitaxel	Normoxia	6.82[b]	5.91	37.1
		Hypoxia	15.03	11.98	
U251	Temozolomide	Normoxia	231.12[a]	196.45	45.5
		Hypoxia	558.41	409.5	
	Paclitaxel	Normoxia	9.02[b]	8.17	33.7
		Hypoxia	17.21	14.45	

Each IC_{50} value is the mean of at least three separate experiments
[a] IC_{50} (μmol/L).
[b] IC_{50} (nmol/L).

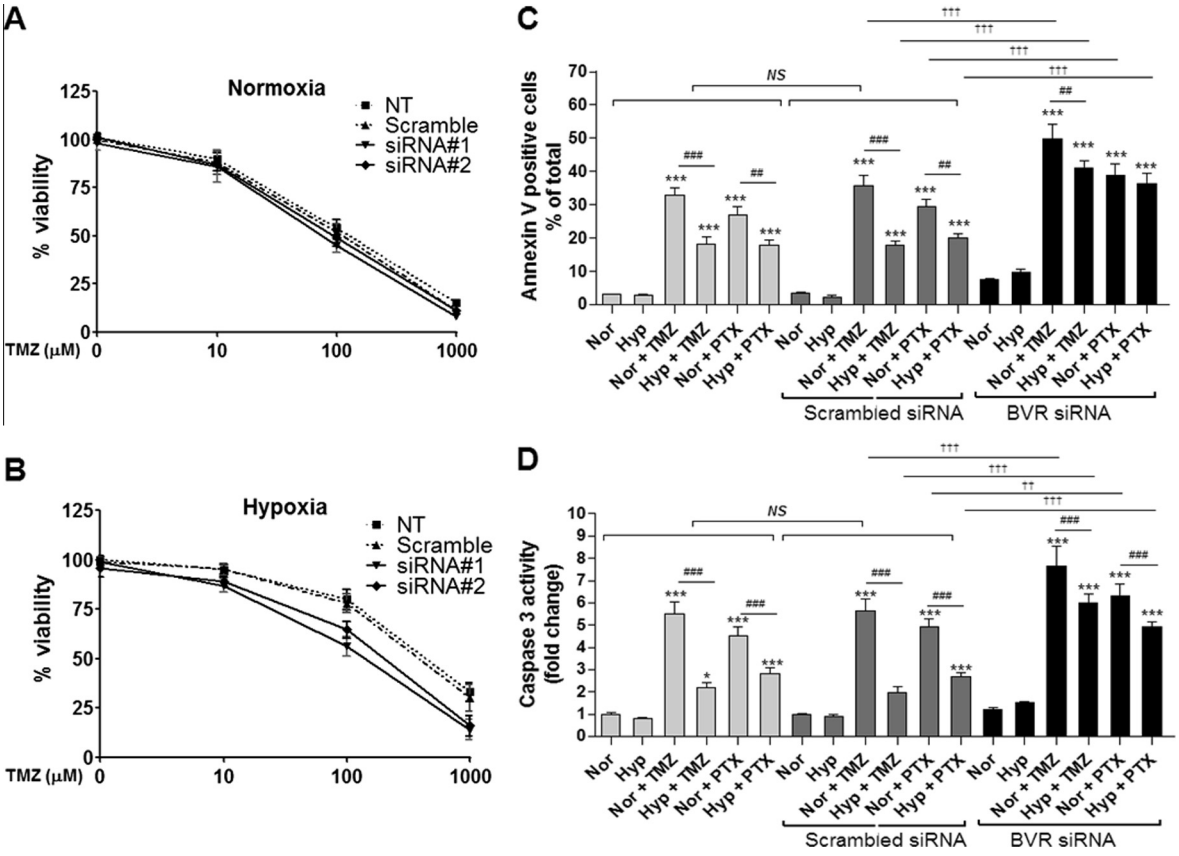

Fig. 3. Treatment of human GBM cells with siRNA targeting hBVR prevented hypoxia-induced drug resistance. (A and B) U87 GBM cells were transfected with an siRNA targeting hBVR or a scrambled control siRNA and grown under normoxic or hypoxic conditions for 24 h. After incubation, the cells were treated with or without indicated concentration of TMZ for an additional 36 h, and then an CCK-8 assay was performed as described in the Section 2. The data are expressed as the mean ± SEM from three independent experiments. NT, non-treated controls. (C and D) After transfection and incubation as described above, U87 cells were treated with 100 μmol TMZ for 36 h. Assays for annexin V staining and caspase-3 activity were then performed as described in the Section 2. Statistical analyses were determined independently for the three subgroups without siRNA, with scrambled control siRNA and with hBVR siRNA. Significance were assessed by ANOVA followed by a Dunnett's post hoc test or unpaired Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from control cells (Nor, Nor control siRNA or Nor hBVR siRNA); $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significantly different between drug-treated normoxic cells and drug-treated hypoxic cells; $^{\dagger\dagger\dagger}P < 0.001$, significantly different between control siRNA- and hBVR siRNA-transfected cells; NS, no significant difference between the non-transfected and control (scrambled) siRNA-transfected groups; Nor, normoxia; Hyp, hypoxia.

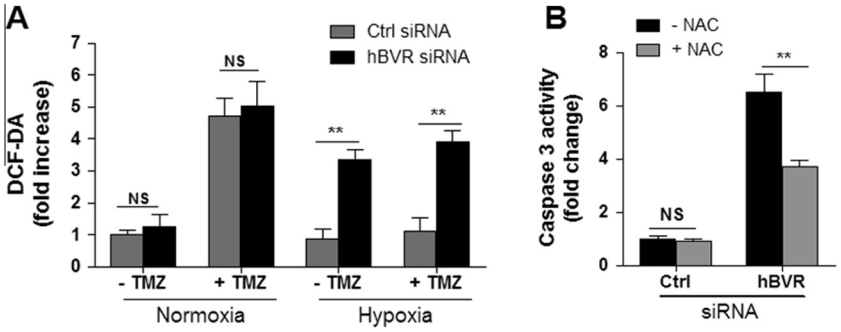

Fig. 4. Effect of hBVR knockdown on ROS production in GBM cells exposed to hypoxic conditions. (A) U87 GBM cells transfected with an siRNA targeting hBVR, or a scrambled control siRNA (Ctrl) were pre-exposed to hypoxic or normoxic conditions for 24 h. After incubation, cells were treated with or without TMZ for an additional 36 h. The cells were stained with dichlorofluorescein diacetate (DCF-DA), fixed, and immediately analyzed by fluorescence-activated cell sorting (FACS). (B) U87 GBM cells transfected with an siRNA targeting hBVR, or the scrambled control siRNA (Ctrl), were cultured under hypoxic conditions in the presence or absence of 20 mM NAC for 24 h. After incubation cells were treated with TMZ for an additional 36 h, and then caspase-3 activity assay was performed as described in Section 2. The data are expressed as the mean ± SEM from three independent experiments. Statistical significance were determined by unpaired Student's *t*-test; $^{**}P < 0.01$, significantly different from their respective untreated control cells; NS, no significant difference; Ctrl, control.

status in hBVR-knockdown cells, we observed that depletion of hBVR increased the ROS levels by more than 3.8-fold in hypoxic U87 cells, but only slightly increased the levels in normoxic cells (Fig. 4A). Interestingly, under hBVR-depleted hypoxic conditions, there was no significant difference in ROS levels between TMZ-treated and untreated cells (Fig. 4A). These results suggest that TMZ was not the main mechanism responsible for the high ROS levels observed in the hBVR-depleted hypoxic cells.

We next investigated whether the chemosensitizing effect of hBVR could be reversed by pretreatment with the antioxidant, *N*-acetylcysteine (NAC). As shown in Fig. 4B, under hypoxic conditions, hBVR depletion-induced apoptosis in TMZ-treated

U87/hypoxic cells was significantly attenuated by NAC pretreatment. This suggests that hBVR contributes to hypoxia-induced chemoresistance by modulating the cellular redox status.

4. Discussion

It has now been well established that hypoxic cancer cells undergo exposure to oxidative stress, and consequently the cells develop adaptive strategies to survive in this stressful environment. One strategy for survival under oxidative and hypoxic conditions is for cells to increase their antioxidant capacity. In our study, detection of hBVR expression in U87 and U373 cells showed that hypoxia induced the expression of hBVR and that knockdown of hBVR caused a significant increase in intracellular ROS levels. These data support the hypothesis that GBM cells display a hypoxia-dependent differential modulation of hBVR and increase hBVR expression to promote cell survival under hypoxic conditions.

An intracellular ROS-adaptive response may play a pivotal role in protecting tumor cells against the cytotoxic effects of chemotherapy. Accumulating evidence suggests that modulation of ROS levels can affect hypoxia-induced chemoresistance [13]. In this study, we hypothesized that inhibition of hBVR activity could overcome hypoxia-induced chemoresistance by regulating the cellular redox status. Many antitumor agents, including TMZ and PTX, produce cytotoxic effects via the generation of ROS, which leads to oxidative stress [13,14]. Hence, those antitumor agents could activate a ROS-dependent hBVR induction, either directly or indirectly. As expected, TMZ-treated U87 cells showed a significant increase in ROS generation under normoxic conditions (Fig. 4A). However, much smaller increases in ROS levels were observed under hypoxic conditions (Fig. 4A). The lower ROS levels observed may be due to the concomitant increase in cytoprotective enzymes and antioxidants that accompanies the increased generation of ROS during hypoxia. In this study, we showed that pretreatment with an antioxidant prevented hBVR depletion-mediated chemosensitization under hypoxic conditions (Fig. 4B), which supports the notion that the antioxidant property of hBVR may play an essential role in the drug resistance of hypoxic cells.

Although the mechanism is not fully understood, there are two possible mechanisms to explain the antioxidant properties of hBVR. One is through the generation of bilirubin, a potent endogenous antioxidant, and the other is by regulation of the expression of hemeoxygenase-1 (HO-1), a well-known cytoprotective enzyme. Heme oxygenase (HO), which catalyzes the rate-limiting step in the oxidative degradation of heme to biliverdin, belongs to the heat-shock protein-32 family of proteins and has a constitutive isoform (HO-2) and an inducible isoform (HO-1) [15]. Several lines of recent evidence have shown that elevated HO-1 expression is associated with resistance to chemotherapy [16,17]. Elevated HO-1 expression is observed in many tumors, and HO-1 expression can be further increased by anticancer agents [17]. It recently has been revealed that hBVR is a leucine zipper-like DNA binding protein that binds not only to activator protein-1 (AP-1), but also to cyclic adenosine monophosphate (cAMP) response element sites [18]. Among the genes regulated by hBVR, HO plays an important role in protection against oxidative stress. Binding of hBVR to the HO-1 promoter, which is regulated by AP-1, affects the expression of HO-1 [18]. In theory, hBVR could potentiate the cellular antioxidant defense capacity against ROS-induced oxidative stress through a transcriptional regulation of HO expression. Further in vivo and in vitro studies are needed to evaluate the relationship between hBVR and HO induction in hypoxia-induced chemoresistance. The interaction of hBVR with other stress-activated signaling networks should also be examined in more detail.

In conclusion, we have shown that hypoxic conditions caused a significant increase in hBVR expression in GBM cells, which was accompanied by chemoresistance. Depletion of hBVR attenuated this hypoxia-induced drug resistance. Because of the cytoprotective nature of hBVR, elevated hBVR expression in hypoxic tumors may be a key component of the cellular adaptive response to chemotherapeutic agents. Clarifying the pathways involved in regulating hBVR expression under hypoxic conditions may provide important insights to aid in the development of effective strategies to treat refractory tumors such as GBM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.120.

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