Hyperoxia Resensitizes Chemoresistant Glioblastoma Cells to Temozolomide Through Unfolded Protein Response

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Abstract. *Background: Intratumoural hypoxia is associated with chemoresistance in glioblastoma multiforme (GBM), a highly malignant brain tumour. Adaptive response to endoplasmic reticulum stress induced by temozolomide is a major obstacle in recurrent GBM. We investigated whether hyperoxia resensitizes temozolomide-resistant GBM cells to temozolomide by abrogating the hypoxia-induced, unfolded protein response (UPR)-related protective mechanisms. Materials and Methods: We examined changes to key UPR modulators in temozolomide-sensitive and -resistant human GBM cells (D54 and U87) treated with/without temozolomide at different oxygen concentrations using western blotting, and cytotoxic benefits of overexpressing key chaperone, P4HB, in GBM cells (U87 and U251) under normoxia and hyperoxia. Results: Hyperoxia, alone or synergistically with temozolomide, activated the UPR in sensitive and resistant D54 and U87 cell lines. Hyperoxia also reduced survival benefit of U87 and U251 cells with P4HB overexpression through the UPR. Conclusion: Hyperoxia enhanced GBM cell sensitivity to temozolomide, likely through UPR, highlighting an important treatment modality targeting chemosensitive and -resistant GBM.*

Glioblastoma multiforme (GBM) is a highly malignant form of primary brain tumour arising from glial tissues of the central nervous system (1). GBM is classified as a grade IV malignant glioma by the World Health Organization, and is characterized as being infiltrative, rapid-growing, and refractory to conventional treatment (2). Despite the current multimodality therapy of maximal surgical resection, chemoirradiation and adjuvant chemotherapy, prognosis remains poor (3). GBM has one of the worst outcomes in human cancer, with median survival ranging from 16.2 to 21.2 months from diagnosis (4). Infiltration into the surrounding brain, rapid tumour re-growth, and difficulties with achieving complete resection render it one of the most challenging diseases to treat (5).

Temozolomide, an orally-administered and well-tolerated chemotherapeutic agent, is the standard treatment for GBM, used post-surgically with radiotherapy (6). As a DNA-methylating agent, temozolomide triggers apoptosis of GBM cells by transferring O^6-methylguanine and activating the mismatch repair mechanism. Resistance to temozolomide, however, is common and responsible for the majority of disease relapses (7). In addition to the well-known DNA repair mechanism by the alkylated DNA repair enzyme methyl-guanine methyltransferase, there are other mechanisms underlying temozolomide-resistance that remain incompletely understood (8).

Hypoxia is common in solid tumors, including GBM. In GBM, hypoxia enhances angiogenesis (9), increases the expression of stem cell markers (10), and promotes temozolomide resistance (11). Hyperoxic treatment, on the other hand, is known to potentiate the effects of chemotherapeutics in non-GBM cancer by inhibiting cell proliferation and enhancing drug uptake (12, 13). Its use as a chemotherapy adjunct has been studied in a variety of malignancies, such as breast and lung cancer (14, 15). Therapeutics using hyperoxygenation that specifically address this phenomenon of intratumoural hypoxia may potentially overcome chemoresistance in GBM while being relatively harmless to normal healthy brain cells (16).

Under hypoxic stress, several signalling pathways are activated for the maintenance of internal homeostasis (17). Pathways such as the endoplasmic reticulum stress response (ERSR) are activated during long-term adaption to low-grade hypoxic stress, and are potentially protective against chemotherapy. In particular, accumulation of chaperone

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protectors such as glucose-regulated protein 78 (GRP78) and prolyl 4-hydroxlase, beta polypeptide (P4HB) in the lumen of the endoplasmic reticulum (ER) is believed to have cytoprotective effects (7, 18, 19). We previously reported that hyperoxia potentiated the anti-tumour effect of temozolomide *via* apoptosis-induced cell death (20). Moreover, P4HB and its related ERSR signalling pathways were identified to be intimately related to temozolomide resistance (7). Overexpression of P4HB abrogated the effect of temozolomide in GBM; its inhibition had the opposite effect, inducing ERSR-mediated apoptosis. The proposed mechanism is illustrated in Figure 1. However, the connection between hyperoxygenation, ERSR and temozolomide-resistance is unclear. In the present study, we aimed to unveil the chemosensitizing mechanism of hyperoxia on different GBM cell states. We surmised that the protective role of hypoxia in temozolomide-resistant GBM cells would be mediated through the ERSR pathway and that hyperoxia may modulate sensitivity to temozolomide by suppressing P4HB-mediated responses.

Materials and Methods

Glioma cell culture and temozolomide-resistant subclones. Human glioma cell line D54-MG (obtained from Duke University Medical Centre, Durham, NC, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco; Invitrogen, Carlsbad, CA, USA), while U87-MG, and U251-MG (American Type Culture Collection, Manassas, VA, USA) were cultured in Minimum Essential Medium (MEM)-α. All were supplemented with 10% heat inactivated foetal bovine serum (FBS), and 1% penicillin and streptomycin (Gibco; Invitrogen). Cells were cultured in humidified incubator at 37°C with 5% CO_2. Isogenic temozolomide-resistant D54-R and U87-R subclones were derived after chronic exposure of D54-S and U87-S cells to temozolomide (Temodal, Schering-Plough, Whitehouse Station, NJ, USA) as previously described by our group (21).

Establishment of stable P4HB-overexpressing cell lines. To study whether the effect of hyperoxia was P4HB-mediated, we overexpressed P4HB in U87-MG and U251-MG cells (all from American Type Culture Collection). Briefly, P4HB cDNA clone (OriGene Technologies, Inc., Rockville, MD, USA) in pcDNA3.1/His© A expression plasmid (Invitrogen), and pcDNA3.1/His© A empty vector were transfected into U87-S and U251-S cells using FuGENE 6 Transfection Reagent according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA). Stable clones with high P4HB expression construct and empty vector were selected using neomycin.

Oxygenation treatment. Cells were exposed to different oxygen concentrations at a normobaric pressure *in vitro,* as previously described (20). OxyCycler C42 chamber (BioSpherix, Lacona, NY, USA) was employed for culturing cells at low (hypoxic, 1% O_2) and high (hyperoxic, 40% O_2) oxygen conditions. The normoxic (21% O_2) condition was achieved by using a standard humidified incubator. Cells were incubated under different oxygen conditions with (250 μM) or without temozolomide treatment. Alterations in ERSR signaling were then tested at various time points.

Western blotting. Details of the procedure have previously been described (21). Briefly, proteins were extracted as whole-cell lysates using RIPA Buffer (Cell Signaling Technologies, Inc., Danvers, MA, USA). Protein lysates (20 μg) were separated on 12% Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% non-fat milk in TBS/T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6), the membrane was probed with one of the following primary antibodies (1:1000 dilution) at 4˚C overnight: rabbit monoclonal antibodies against C/EBP homologous protein (CHOP), GRP78, inositol-requiring kinase 1 (IRE1α), protein kinase-like endoplasmic reticulum kinase (PERK), P4HB, and β-actin (all from Cell Signaling Technologies), followed by one-hour incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at 1:10,000 dilutions (Santa Cruz Biotechnology). Immunoreactive signals were detected by using Immobilon Western HRP Substrate (Merck Millipore, Billerica, MA, USA).

Cytotoxicity assay. Cell viability was measured using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). Cells were first seeded onto 96-well plates at an initial density of 5000 cells per well. Seeded cells were cultured in different concentrations of temozolomide (0, 62.5, 125, 250, 500, 1000, 2000, and 3000 μM) under different oxygen concentrations. Absorbance (optical density) was measured at 595 nm. The percentage of viable cells relative to that of controls was then calculated. The half maximal inhibitory concentration (IC_{50}) values were derived from the line of best fit. Experiments were performed in triplicates.

Results

Activation of ERSR after 24 h of hyperoxia. D54-MG and U87-MG cells were incubated at three oxygen levels (hypoxia, 1%; normoxia, 21%; and hyperoxia, 40%). The expression levels of the key modulators (GRP78, P4HB, PERK, IRE1α and CHOP) of the ERSR pathway were quantified and compared across different time points (24, 48 and 72 h) by western blotting. Overall, alterations in ERSR signalling were observed with increased oxygenation, indicated by a fall in GRP78 and P4HB expressions from hypoxia to hyperoxia. These proteins would normally serve as protectors against ER stress. With prolonged hyperoxia, their down-regulation then led to the induction of two transmembrane sensors, PERK and IRE1α. This in turn enhanced the expression of the downstream pro-apoptotic protein, CHOP. Figure 2 illustrates the de-regulations of these signalling molecules after 24 h of high oxygen (40%) incubation in both D54 (Figure 2a) and U87 (Figure 2b) cells. Due to the high heterogeneity of GBM cells, we found that D54-MG was less responsive to hyperoxgenation than U87-MG in terms of the duration of treatment required. D54-MG exhibited the most significant ERSR upon 72 h of hyperoxia, whereas similar effects were seen in U87 cells at 48 h. Densitometric data were then calculated on the expression of each ERSR signaling molecule and compared

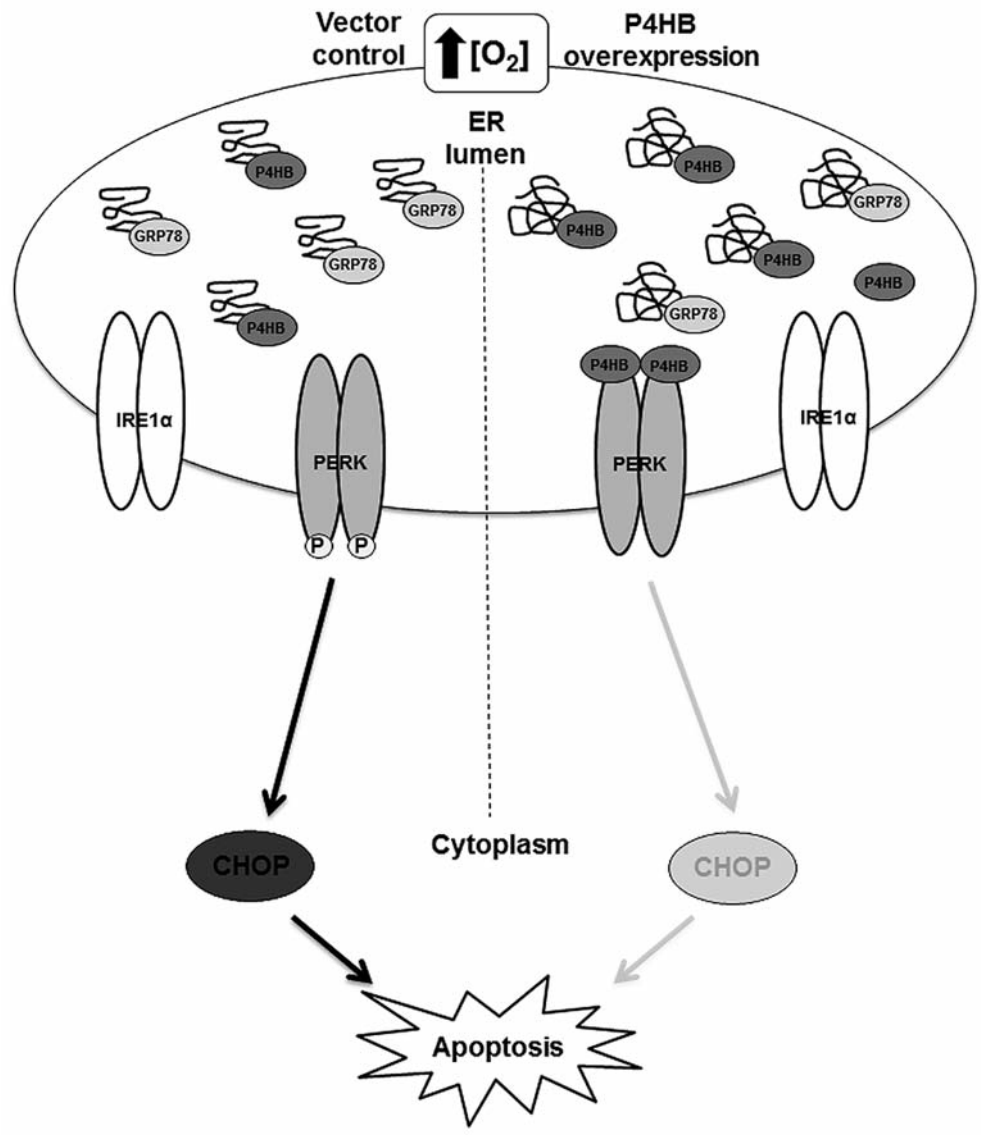

Figure 1. *Under stressful conditions, such as hyperoxia, disruption of normal protein folding would result in the unfolded protein response (UPR). In vector control cells (left panel), molecular chaperones glucose-regulated protein 78 (GRP78) and prolyl 4-hydroxlase, beta polypeptide (P4HB) would attend to the unfolded protein in the endoplasmic reticulum (ER) lumen by dissociating from the transmembrane receptors protein kinase-like endoplasmic reticulum kinase (PERK) and inositol-requiring kinase 1 (IRE1α). As a result, oligodimerization and autophosphorylation of the latter receptors would activate the downstream pro-apoptotic mediator C/EBP homologous protein (CHOP) and initiate cell death. In glioblastoma cells with overexpression of P4HB (right panel), excessive P4HB would attend to the unfolded proteins, while remaining bound to PERK and IRE1α to maintain their inactivity. As a result, oligomerization of the transmembrane receptors, hence cell death, does not occur.*

between hypoxia, normoxia and hyperoxia at the optimal time point for each cell line (D54 at 72 h and U87 at 48 h) (Figure 2c and d). The results were in line with our previous findings that hyperoxia-induced growth inhibition was cell line-dependent, and that D54 cells were less sensitive to hyperoxia than U87 cells (20). The overall results support the hypothesis that hyperoxia can trigger cell death *via* down-regulation of the ERSR chaperone protectors.

Hyperoxia ameliorates GBM cell resistance to temozolomide via ERSR. Our previous findings suggest that hyperoxia could enhance chemosensitivity of temozolomide-resistant GBM cells and act as an effective adjunct to temozolomide (20). We then examined if these effects were dependent on the ERSR pathway. The same mediators of ERSR were examined for their altered expressions under different treatment conditions with concomitant temozolomide given

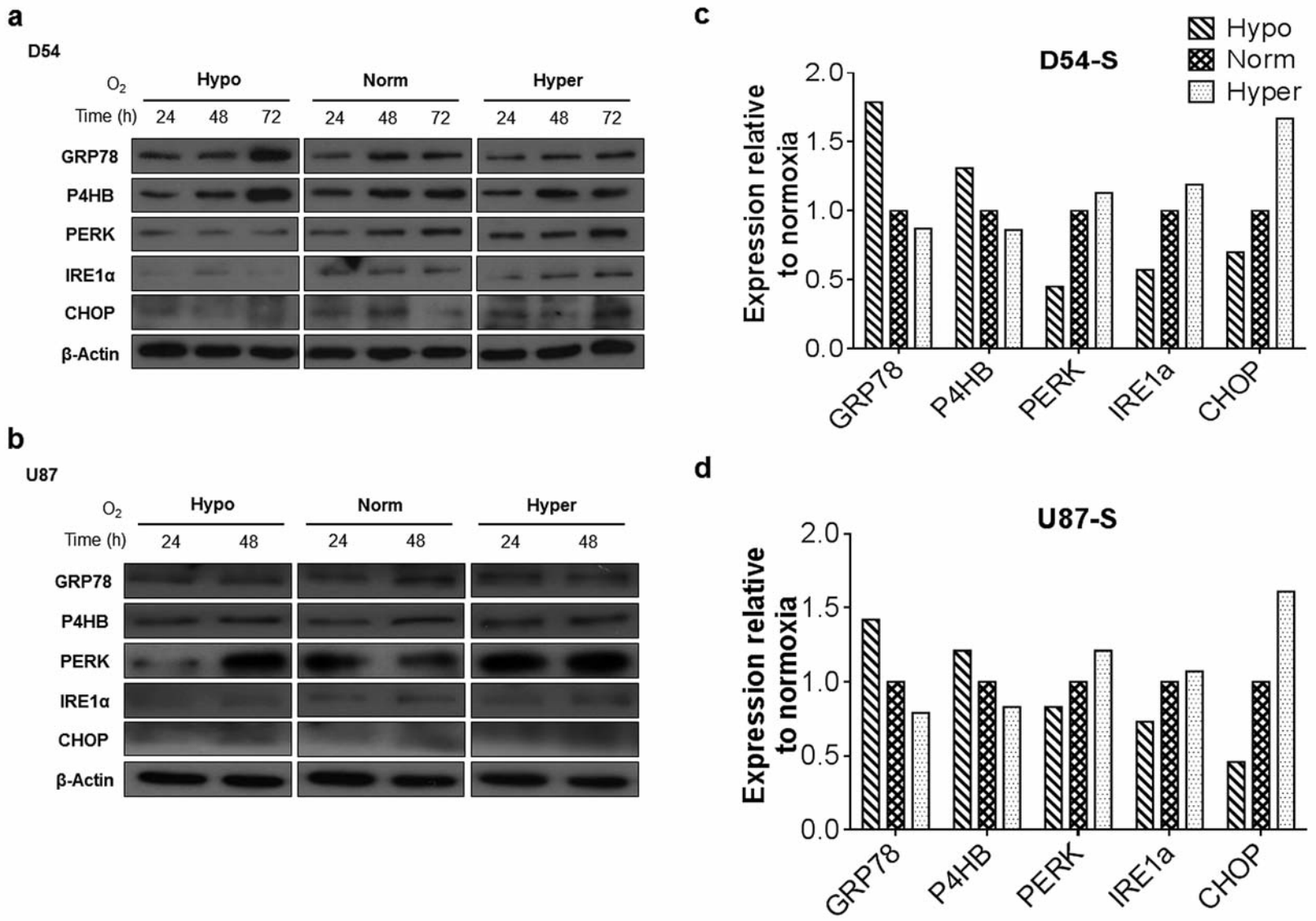

Figure 2. *Western blot of key modulators of the unfolded protein response (UPR) pathway – glucose-regulated protein 78 (GRP78), prolyl 4-hydroxlase, beta polypeptide (P4HB), protein kinase-like endoplasmic reticulum kinase (PERK), inositol-requiring kinase 1 (IRE1α), and C/EBP homologous protein (CHOP) – showing the activation of the endoplasmic reticulum stress response (ERSR) after 24 hours of oxygenation in D54 (a) and U87 (b) glioblastoma cells. A gradual decrease in the expression of GRP78 and P4HB was detected in response to increasing oxygen concentration. This led to increased expression of transmembrane receptors PERK and IRE1α, and pro-apoptotic mediator CHOP. β-Actin was used as an internal control. Densitometric measurements were performed for the two cell lines at their cell line-dependent optimal treatment time points, 72 h for D54 (c) and 48 h for U87 (d). Normoxia was used as the control for reference. Hypo, Hypoxia; Norm, normoxia; Hyper, hyperoxia.*

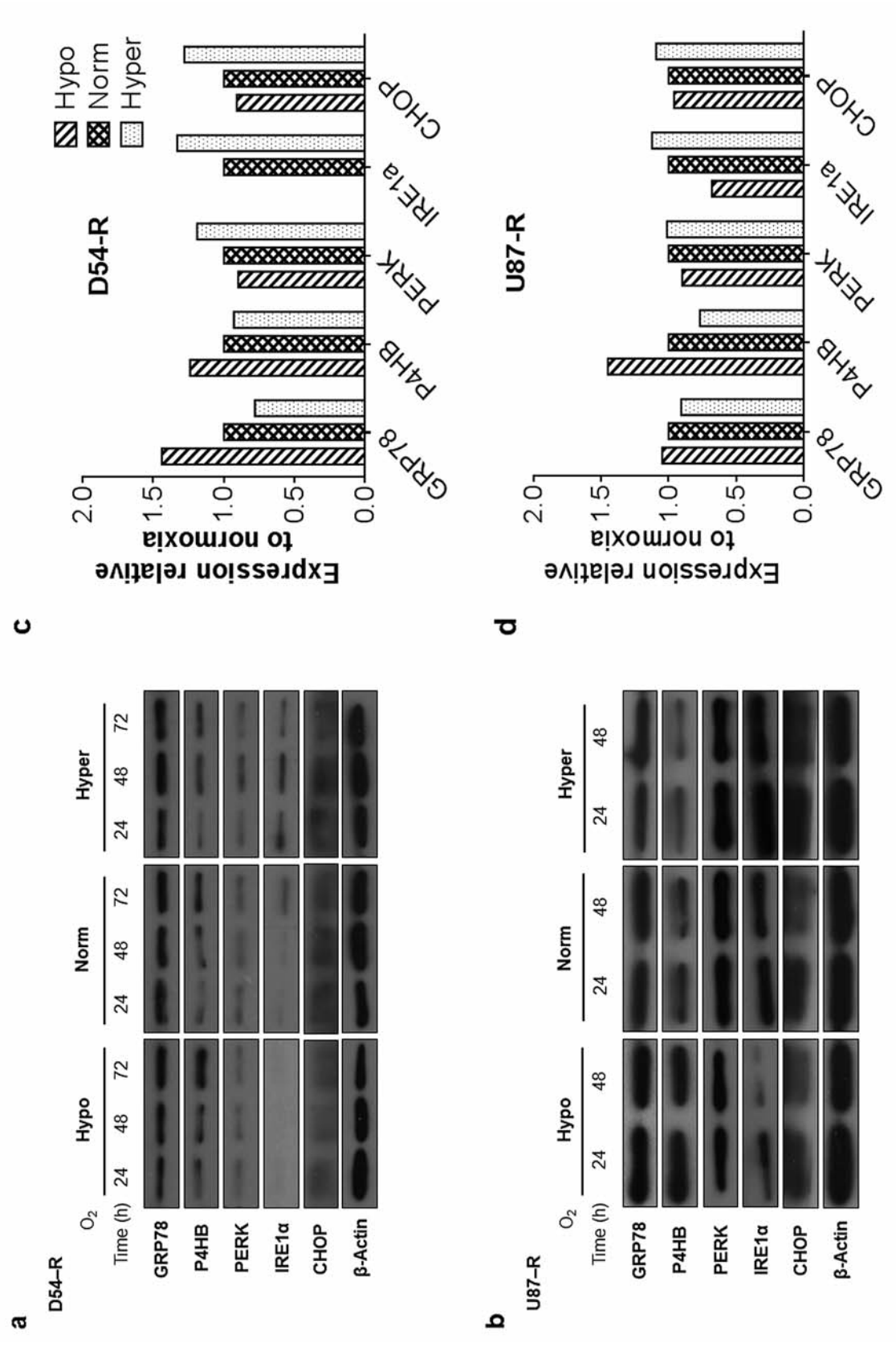

Figure 3. *Western blot examining the expression levels of key modulators of the unfolded protein response (UPR) pathway glucose-regulated protein 78 (GRP78), prolyl 4-hydroxlase, beta polypeptide (P4HB), protein kinase-like endoplasmic reticulum kinase (PERK), inositol-requiring kinase 1 (IRE1α), and C/EBP homologous protein (CHOP) in a pair of temozolomide-resistant cell lines, D54-R (a) and U87-R (b). Similar to their chemoesensitive counterparts, activation of the ERSR was observed, after 24 h of oxygenation with 250 μM of concomitant treatment with temozolomide. The expression levels of the chaperone proteins GRP78 and P4HB gradually decreased, while those of the transmembrane receptors PERK and IRE1α and pro-apoptotic mediator CHOP gradually increased in response to increasing oxygen concentration. β-Actin was used as an internal control. Densitometric measurements were performed for the two cell lines at their cell line-dependent optimal treatment time points, 72 h for D54 (c) and 48 h for U87 (d). Normoxia was used as the control for reference. Hypo, Hypoxia; Norm, normoxia; Hyper, hyperoxia.*

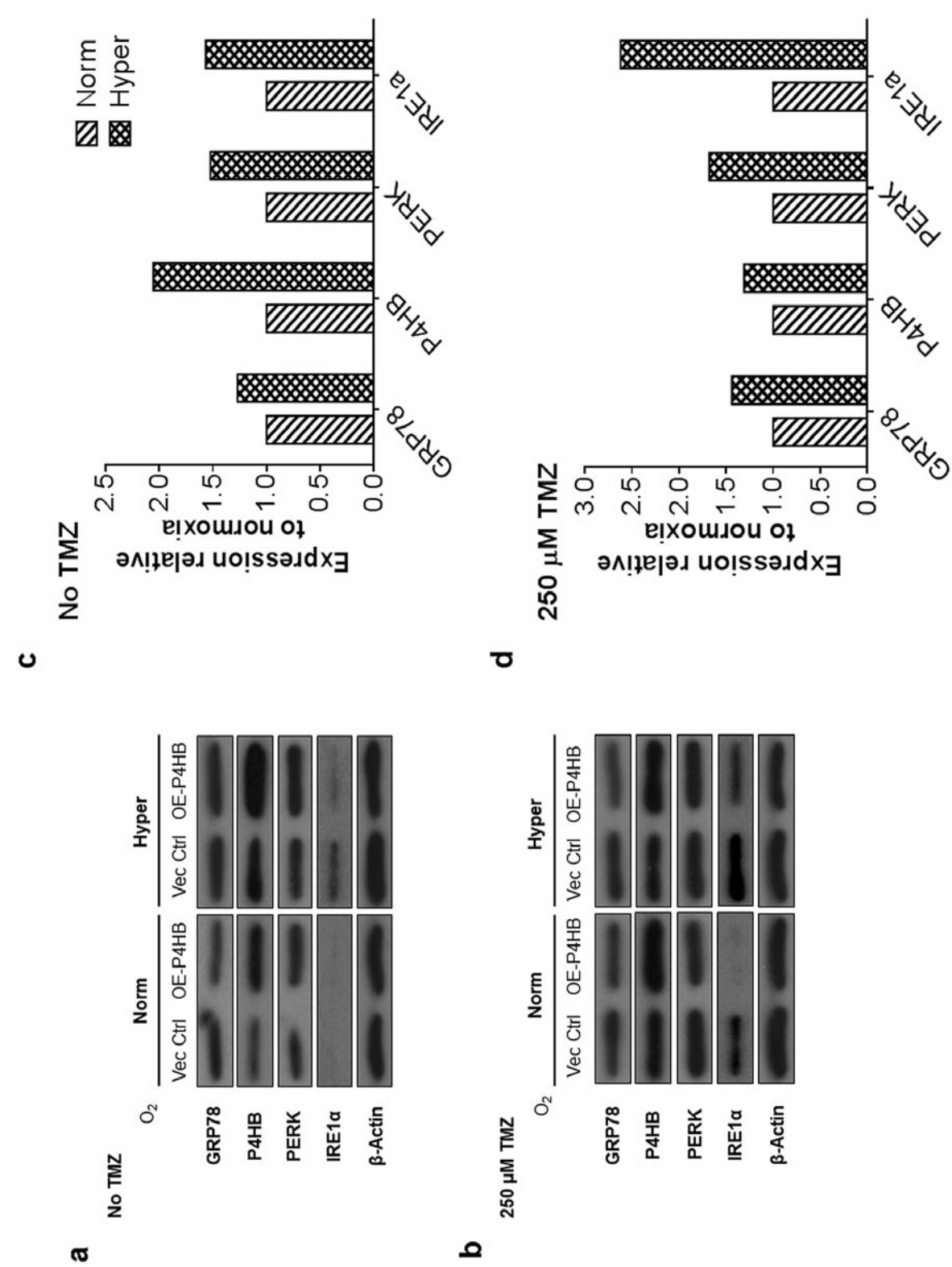

Figure 4. *Western blot comparing the prolyl 4-hydroxlase, beta polypeptide -overexpressing (OE-P4HB) U251 cell line treated under normoxic (Norm) and hyperoxic (Hyper) conditions without (a) and with (b) concomitant 250 μM temozolomide. The expression levels of selective unfolded protein response pathway modulators were higher under hyperoxia. Except for P4HB, which was overexpressed, the expression levels of other modulators were greater in vector controls cells (Vec Ctrl) in comparison to OE-P4HB cells under each oxygenation condition, suggestive of the protective role of P4HB against activation of the endoplasmic reticulum stress response. β-Actin was used as an internal control. Densitometric measurements were performed for U251 cells with OE-P4HB without temozolomide (c) and with concomitant 250 μM temozolomide (d). Normoxia was used as the control for reference.*

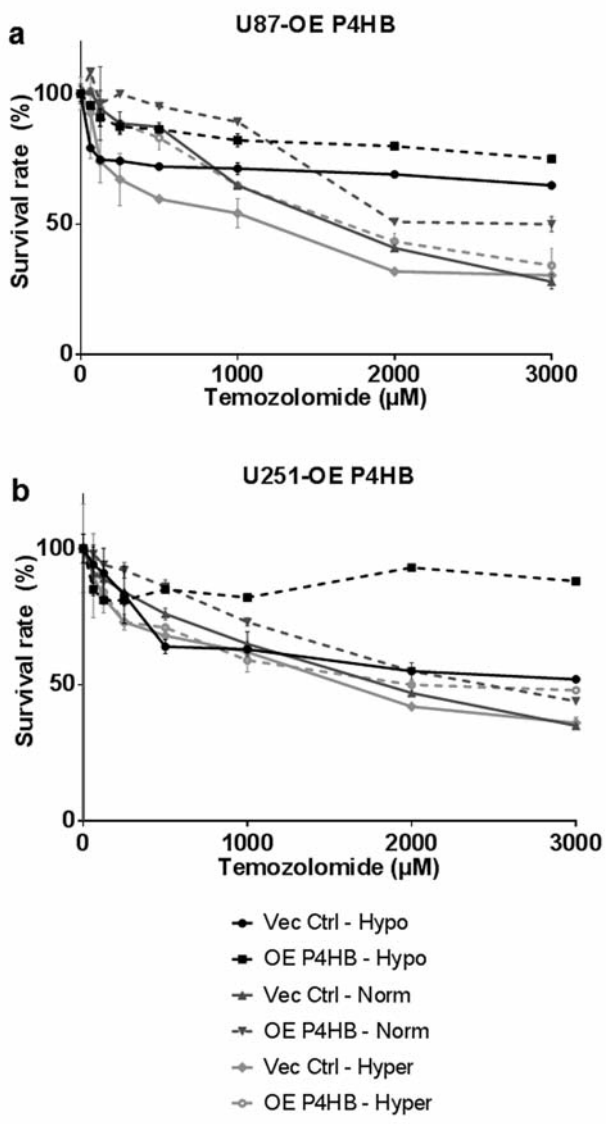

Figure 5. *In vitro cytotoxicity in a pair of P4HB-overexpressing (OE-P4HB) cell lines U87 (a) and U251 (b), and their respective vector controls (Vec Ctrl) under different oxygenation conditions. Survival benefits in glioblastoma cells treated with hyperoxygenation was observed as half maximal inhibitory concentration (IC_{50}) decrease with greater oxygen concentration (Hyper vs. Norm vs. Hypo) in OE-P4HB cells and their respective vector controls for both cell lines. Hypo, Hypoxia; Norm, normoxia; Hyper, hyperoxia.*

at 250 μM, a concentration which was not cytotoxic but which still enhanced signaling alteration when combined with oxygenation (Figure 3) (7). Notable induction of ERSR signaling as a result of hyperoxia with concomitant temozolomide treatment was observed in temozolomide-resistant D54-R (Figure 3a) and U87-R (Figure 3b) cells. The results were similar to those of their temozolomide-sensitive counterparts (D54-S and U87-S) as showed decreased expression of the ER chaperone protector GRP78, and P4HB, coupled with an increased expression of the ER stress activators PERK and IRE1α, as well as the pro-apoptotic protein CHOP.

ERSR signalling was involved in hypoxia-induced chemoresistance and tumor maintenance. Elevated P4HB expression occurs in hypoxic tumor cells. It is the key chaperone protein that has been found to be significantly dysregulated during the development of temozolomide resistance in GBM cells (7).

Hyperoxia restored chemosensitivity to temozolomide in P4HB-overexpressing cells via the ERSR. We then hypothesized that disruption of the ERSR pathway *via* direct or indirect interruption of P4HB could ultimately re-sensitize temozolomide-resistant GBM cells to temozolomide. We proceeded to examine whether the effects of forced up-regulation of P4HB could attenuate the effect of hyperoxia and whether the restoration of temozolomide sensitivity in P4HB-overexpressing (OE-P4HB) cells was mediated by ERSR pathways. Western blotting was performed on OE-P4HB cells with and without treatment with temozolomide under normoxia and hyperoxia (Figure 4). Comparing the expression levels of OE-P4HB cells with their vector controls, P4HB expression level was predictably higher in OE-P4HB cell line. The expression levels of other ERSR members, including the transmembrane receptor proteins PERK and IRE1α, were lower, if not the same, in the OE-P4HB cell line, when compared to the vector control (Figure 4a). While similar trends were observed under normoxic and hyperoxic conditions, the expression levels were found to be higher under hyperoxia, as well as under chemotherapy insult (temozolomide at 250 μM) (Figure 4b) which is suggestive of the fact that hyperoxygenation, to some degree, induces the activation of the UPR as a result of ER stress induction, as opposed to normoxia.

An interesting trend was also observed for the expression levels of GRP78 and P4HB. While the up-regulation of P4HB compared to vector controls was expected in OE-P4HB cells, the opposite was found for GRP78 expression. The two molecular chaperones displayed reciprocity in their expression levels (Figure 4). More specifically, up-regulation of P4HB (*i.e.* as in OE-P4HB cells) led to a comparatively down-regulated level of expression of GRP78, while a lower expression level of P4HB in the vector control (compared to OE-P4HB) yielded a higher expression of GRP78. Although GRP78 and P4HB are both molecular chaperones, our results suggest that they perhaps serve distinct and possibly opposing functions under certain conditions. Moreover, acquired temozolomide resistance in GBM cells may be chaperone-specific.

We then performed *in vitro* chemosensitivity assays with P4HB-overexpressing GBM cells (U87 OE-P4HB and U251 OE-P4HB) to further show that they had a survival benefit

at all levels of oxygenation (hypoxia, normoxia, and hyperoxia) (Figure 5a and b). The IC_{50} value for temozolomide in U87 OE-P4HB, and U251 OE-P4HB cells under different oxygen states were greater than those of the respective vector control cells (Table I). The findings suggested that up-regulation of P4HB might confer resistance to temozolomide in GBM cells.

Next, we compared the chemosensitivity of GBM vector control and OE-P4HB cell responses to different oxygen treatments. Cells with overexpression of P4HB showed diminished response to treatment with hyperoxia (Table I). In contrast, a decrease in IC_{50} values of temozolomide were recorded for both U251 and U87 cells treated under induced oxygenation (Table I). The IC_{50} values of the corresponding cells were the greatest after treatments under hypoxia, followed by a gradual decrease under normoxia, and to a far lesser extent under hyperoxia.

Table I. *Half-maximal inhibitory concentration (IC_{50}) for U87 and U251 vector controls and P4HB-overexpressing cell lines under different oxygenation conditions.*

Cell line		Hypoxia (1%)	Normoxia (21%)	Hyperoxia (40%)
U87	Vec Ctrl	8574.431	1615.313	1079.566
	OE-P4HB	10680.35	2749.206	2451.146
U251	Vec Ctrl	2579.599	1841.441	1683.813
	OE-P4HB	-	2483.439	2226.272

Discussion

Our study identified the mechanism that potentially mediates hyperoxia-induced resensitization of chemoresistant GBM cells to temozolomide. The use of hyperoxia was found to activate ERSR transmembrane receptors and the pro-apoptotic UPR pathway as highlighted by the up-regulation of CHOP, a mediator of apoptosis under the ERSR (22). Overexpression of P4HB, on the other hand, diminished the effect of hyperoxia, which was consistent with our previous finding of the role of P4HB in temozolomide resistance (7). Moreover, our findings demonstrated a connection between hyperoxia-induced cell death in GBM cells and alterations in UPR.

Hypoxia exists in most solid tumours as a result of an imbalance between oxygen consumption and supply, and may account for treatment failures after conventional therapies (23, 24). Hypoxic cells are more resistant towards radiotherapy as low oxygen levels may lead to incomplete DNA damage in response to ionization (25). When compared to those under normoxia, hypoxic cells receive a poor blood supply and undergo division at lower rates, resulting in poorer drug delivery and efficacy, respectively (16). Many phenotypic changes may also occur as a result of hypoxia, including greater tumour aggressiveness, which underscores the increased difficulties in tumour treatment (26, 27). Raa *et al*. used hyperoxia to counteract the effects of tumor hypoxia in breast cancer and found growth suppression as a result of enhanced apoptosis (28). More relevant to our study is the observation that that hyperoxia caused a significant decrease in tumour growth *in vivo* by increasing apoptosis without causing significant changes in proliferation (29). Our previous study showed that hyperoxia reverses treatment failure in GBM by resensitizing it towards temozolomide in a cell line-dependent manner. D54 cells were in general less sensitive to hyperoxygenation than U87 cells. In fact, U87 were unable to withstand any increase in oxygen concentration beyond 40% (20).

Cellular stress can interrupt protein folding and processing, and trigger the UPR to alleviate any further injury to the cell. Depending on the tumor type, this process may be protective or toxic to the targeted cells – human melanocyte is a prime example of the former (30). Boelens *et al*. suggested that the ERSR may serve as a target of cancer treatment as prolonged inhibition of homeostasis may activate apoptosis *via* mitochondrial-dependent and -independent pathways (31). The present study showed that hyperoxia could indeed alter the ERSR and subsequently activate apoptotic events in GBM cells, lending support to the notion that UPR can be utilized to enhance the effect of anticancer therapeutics for glioma.

Our previous studies identified the up-regulation of P4HB to be associated with acquired resistance to temozolomide (7) and invasive phenotypes (32). P4HB may become highly expressed under hypoxic stress, and is known to be pro-survival in cancer cells when they are subjected to stressful stimuli. The up-regulation of P4HB is not exclusive to glioma; it is also found in other human cancer types, including acute myeloid leukaemia (33), lymphoma (34), and melanoma (35, 36), as well as lung (34, 37), ovarian (38), and prostate (39) cancer. Healy *et al*. suggested that inhibiting the ERSR chaperone has the potential effect of provoking apoptosis in cancer cells receiving chemotherapy (40). More specifically, the inhibition of P4HB induced apoptotic cell death through re-activation of UPR in chemoresistant GBM cells, as well as in melanoma (7, 35). Similar to Healy *et al*., we were able to show that the P4HB inhibitor bacitracin minimized resistance to temozolomide in GBM cells and increased cell death *via* the ERSR (7, 40). Our present study further demonstrates that up-regulation of P4HB improves the survival of GBM cells *in vitro* by providing some form of cytoprotection, indicating that P4HB and its related pathways may be exploited as potential targets of anticancer treatment.

Up-regulation of P4HB is related to hypoxia (41). Using *in vivo* models, Jain *et al*. reported an increase in P4HB expression levels in mice exposed to acute hypoxia. This was

also confirmed in another separate study. Moreover, increased cell death was evident when P4HB was inhibited (42, 43). In terms of translational applications, the use of specific inhibitors of P4HB has been suggested, but such use may be hindered by sub-optimal delivery due to the blood–brain barrier. The present study, therefore, provides pre-clinical evidence to support the use of hyperoxia as an alternative and effective means of reversing hypoxia-induced P4HB up-regulation by bypassing the issue of the blood–brain barrier.

Conclusion

The present study investigated the use of hyperoxia as an adjuvant to the standard use of temozolomide as a chemotherapeutic treatment for GBM. Our findings showed that hyperoxia dysregulated protein folding that in turn induced UPR-mediated apoptosis. In line with our previous studies, which showed an association between resistance to temozolomide and P4HB-upregulation in glioma, we have demonstrated that hyperoxia may be used to resensitize chemoresistant GBM cells. The current study adds new knowledge to our understanding over the relationships between hyperoxia, UPR and temozolomide resistance in glioma.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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