Hypoxia-induced inhibition of the endocannabinoid system in glioblastoma cells

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Abstract. The endocannabinoid system plays an important role in the regulation of physiological and pathological conditions, including inflammation and cancer. Hypoxia is a fundamental phenomenon for the establishment and maintenance of the microenvironments in various physiological and pathological conditions. However, the influence of hypoxia on the endocannabinoid system is not fully understood. In the present study, we investigated the effects of hypoxia on the endocannabinoid system in malignant brain tumors. We subjected U-87 MG cells, derived from malignant glioblastoma, to hypoxia (1.5% O_2) for 3 days, and evaluated their viability and expression of endocannabinoid-related genes. Hypoxia decreased the expression of cannabinoid receptor 1 and the astrocyte marker glial fibrillary acidic protein, and increased the expression of vascular endothelial growth factor and cyclooxygenase-2, the enzyme responsible for the metabolism of endocannabinoids, in U-87 MG cells. Although cannabinoid receptor (CB) engagement induces cell death in U-87 MG cells in normoxic conditions, CB agonist-induced death was attenuated in hypoxic conditions. These results suggest that hypoxia modifies the endocannabinoid system in glioblastoma cells. Hypoxia-induced inhibition of the endocannabinoid system may aid the development of glioblastoma.

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Introduction

The endocannabinoid system, which includes the G-protein-coupled cannabinoid receptors 1 and 2 (CB1 and CB2) and their endogenous ligands (endocannabinoids), participates in a variety of physiological and pathological conditions, including inflammation and cancer (1-3). CB1 is abundantly expressed in the central nervous system, while CB2 is predominantly expressed by immune cells. Endogenous and exogenous cannabinoids exert anti-inflammatory and antitumor effects (1,4-9).

Glioma, the most common type of brain tumor, originates from differentiated glial cells of the central nervous system. Glial fibrillary acidic protein (GFAP) is a marker of glial differentiation (10). Low-grade malignancies have moderate expression of the protein, while high-grade malignancies have weak GFAP expression (11). Glioblastoma, a poorly differentiated type of glioma, is highly malignant and exhibits aggressive invasive growth. Existing treatments, including surgery, radiotherapy and chemotherapy, are largely unable to cure glioblastoma, which leads to death in most cases (12,13). Therefore, new therapeutic approaches for treating glioblastoma are urgently needed. Recently, many synthetic agonists for cannabinoid receptors, which act on the endocannabinoid system, have been developed. Of these synthetic agonists, WIN 55,212-2 and KM-233 mimic the effects of endogenous cannabinoids and decrease cell viability in both glioma and glioblastoma (14,15).

Various microenvironmental stresses modulate cell signaling in physiological and pathological conditions. Hypoxia, a type of stress that is fundamental in the microenvironment of a variety of solid tumors (16), has been implicated in the persistence and development of malignancies. Hypoxic stimuli prevent apoptosis and promote tumor invasion, metastasis and angiogenesis by regulating a variety of genes, including vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1α (17). Hypoxic regions are frequently found in glioblastoma (18,19) and extensive hypoxia is associated with a worse prognosis (16).

In the present study, we examined the effects of hypoxia on the endocannabinoid system in U-87 MG human malignant glioblastoma cells. We found that hypoxia suppressed

the expression of the genes encoding GFAP and cannabinoid receptors; increased the expression of the gene encoding cyclooxygenase (COX)-2, the enzyme that metabolizes endocannabinoids (20,21), and attenuated cannabinoid receptor agonist (WIN 55,212-2)-induced cell death. Our findings provide insight into novel mechanisms by which hypoxia inhibits the endocannabinoid system and promotes the malignant phenotype of glioblastoma.

Materials and methods

Chemicals. WIN 55,212-2 and Dulbecco's modified Eagle's medium (DMEM) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA).

Preparation of primary rat glial cells. All experiments were performed in accordance with the guidelines set by the Animal Welfare Committee of Kanazawa University. Primary glial cells were prepared from newborn Sprague-Dawley rats as previously described (22-24). Briefly, following isoflurane anesthesia, rats were decapitated and cells were mechanically dissociated from the hippocampi and plated onto culture dishes (35 mm).

Cell culture. U-87 MG human malignant glioblastoma cells were provided by Dr Nakata (Kanazawa University, Kanazawa, Japan). U-87 MG and primary rat glial cells were maintained in DMEM containing 10% FBS at 37°C in 5% CO_2. Then, cells were divided into 2 groups. The cells in the normoxia group were incubated in 5% CO_2 and 20% O_2, while the cells in the hypoxia group were maintained in 5% CO_2 and 1.5% O_2.

Reverse transcription polymerase chain reaction (RT-PCR). To evaluate the expression of human CB1 (*CNR1*), rat CB1 (*Cnr1*), human CB2 (*CNR2*), rat CB2 (*Cnr2*), human COX-1 (*PTGS1*), human COX-2 (*PTGS2*), human VEGF (*VEGFA*) and human GFAP (*GFAP*) mRNA in the cells, RT-PCR was performed as follows. Briefly, RNA was extracted from the cells and cDNA was generated using the reverse transcriptase ReverTra Ace® (Toyobo, Tokyo, Japan). We performed PCR-based, subtype-specific gene amplification with LA Taq (Takara, Tokyo, Japan) using the primers specified in Table I.

Cell proliferation and viability assays. Cell proliferation and viability were analyzed using the Cell Counting Kit-8 (CCK-8; Wako) as previously described (25). U-87 MG cells were seeded in 96-well plates at a density of $1x10^2$ cells/well. After a 24-h incubation period, the cells were divided into 2 groups. The cells in the normoxia group were incubated in 5% CO_2 and 20% O_2, while the cells in the hypoxia group were maintained in 5% CO_2 and 1.5% O_2. After 3 days of continuous treatment, cells were allowed to recover in 5% CO_2 and 20% O_2 for ~30 min. Then, the cells were incubated with WST-8 (10 μl of WST-8 in 100 μl of medium) for 3 h at 37°C. The absorbance of the colored formazan product, generated by mitochondrial dehydrogenases in metabolically active cells, was recorded at 450 nm. Cell proliferation and viability were expressed as a ratio of the absorbance in treated wells relative to that in untreated control wells.

Table I. Nucleotide sequences of the PCR primers used for amplification of the genes encoding human CB1 (*CNR1*; NM_001160226), rat CB1 (*Cnr1*; NM_012784), human CB2 (*CNR2*; NM_001841), rat CB2 (*Cnr2*; NM_001164142), human COX-1 (*PTGS1*; NM_000962), human COX-2 (*PTGS2*; NM_000963), human VEGF (*VEGFA*; NM_001025366), human GFAP (*GFAP*; NM_001131019), human β-actin (*ACTB*; NM_001101) and rat β-actin (*Actb*; NM_031144).

| Gene	Forward (5'→3')	Reverse (5'→3')	Products (bp)
Human CB1 (*CNR1*; NM_001160226)	CAG GCC TTC CTA CCA CTT CAT	ACC CCA CCC AGT TTG AAC AGA	141
Rat CB1 (*Cnr1*; NM_012784)	CAG GCC TTC CTA CCA CTT CAT	ACC CCA CCC AGT TTG AAC AGA	141
Human CB2 (*CNR2*; NM_001841)	AAG CCC TCA TAC CTG TTC AT	ACA GAG GCT GTG AAG GTC AT	164
Rat CB2 (*Cnr2*; NM_001164142)	AAG CCC TCG TAC CTG TTC AT	ACA GAG GCC GTG AAG GTC AT	164
Human COX-1 (*PTGS1*; NM_000962)	AAT GCC ACC TTC ATC CGA GA	TGG GTG AAG TGT TGT GCA AAG	311
Human COX-2 (*PTGS2*; NM_000963)	CAG CAA ATC CTT GCT GTT CC	GTG AAG TGC TGG GCA AAG AAT	526
Human VEGF (*VEGFA*; NM_001025366)	CCT TGC CTT GCT GCT CTA CCT	GAT GTC CAC CAG GGT CTC GAT	151
Human GFAP (*GFAP*; NM_001131019)	GAT GAT GGA GCT CAA TGA CCG	CAG CCT CAG GTT GGT TTC ATC	274
Human β-actin (*ACTB*; NM_001101)	ATG GTG GGC ATG GGT CAG AAG	CTG GGG TGT TGA AGG TCT CAA	262
Rat β-actin (*Actb*; NM_031144)	ATG GTG GGT ATG GGT CAG AAG	CTG GGG TGT TGA AGG TCT CAA	262
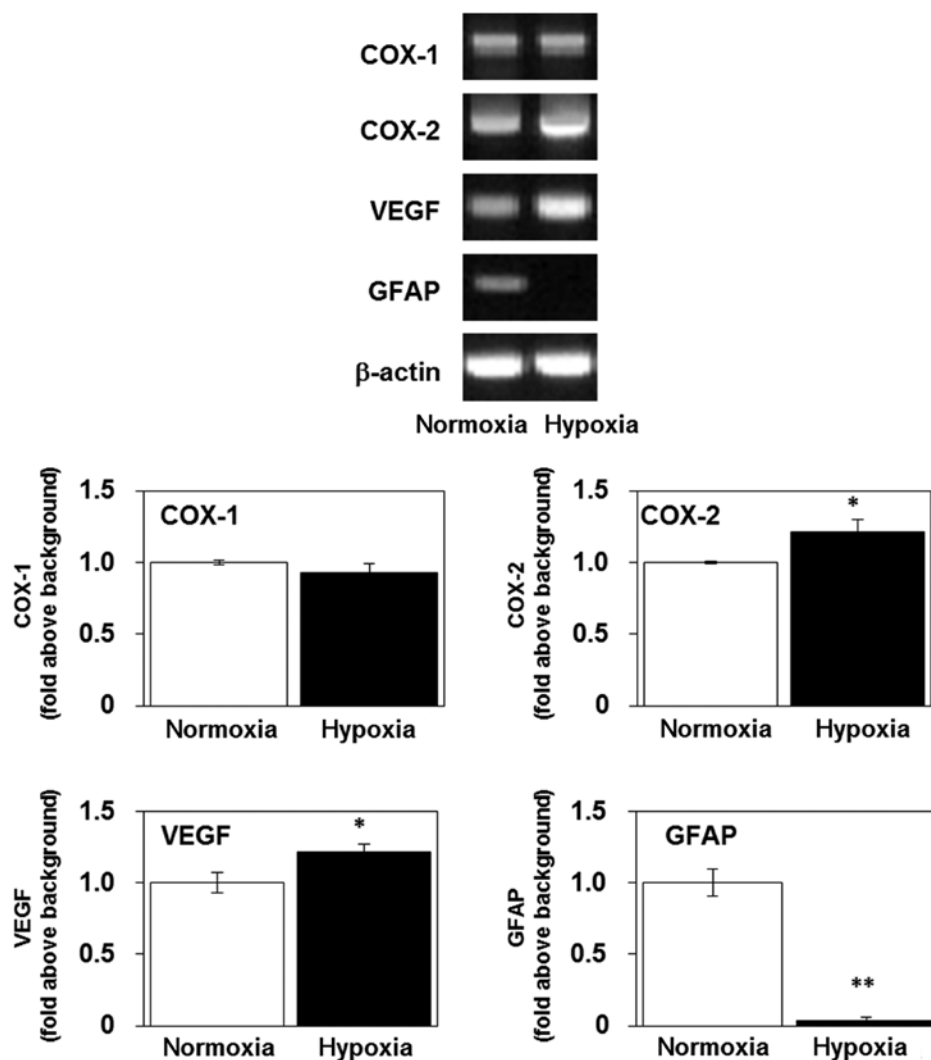
Figure 1. Hypoxia increases the expression of mRNA encoding *COX-2* and *VEGF*, and decreases expression of *GFAP* mRNA, in U-87 MG cells. Cells were incubated in 20% O_2 (normoxia) or 1.5% O_2 (hypoxia) for 3 days. We evaluated the expression patterns of COX-1 (*PTGS1*), COX-2 (*PTGS2*), VEGF (*VEGFA*) and GFAP (*GFAP*) by RT-PCR. The densities of the bands corresponding to COX-1, COX-2, VEGF, GFAP and β-actin were quantified by densitometry. Graphs depict mean ± SEM from 3 independent experiments. Statistical analysis was conducted using Student's unpaired t-test; *P<0.05 and **P<0.01, compared with normoxic controls.

Cannabinoid receptor agonist treatment. Cells were treated with the cannabinoid receptor agonist WIN 55,212-2 in 5% CO_2 and 20% O_2 following a prior 3-day exposure to normoxia or hypoxia. After 2 days of agonist treatment, cell viability was analyzed using CCK-8 (Wako), as described above.

Statistical analysis. Data are presented as the mean ± standard error of the mean (SEM) from at least 3 independent experiments. Statistical analysis was performed using Student's unpaired t-test or Kruskal-Wallis non-parametric analysis of variance (ANOVA) followed by a Bonferroni post hoc test, and results were considered statistically significant at P<0.05.

Results

Hypoxia increases the levels of mRNA encoding COX-2 and VEGF, and decreases the level of mRNA encoding GFAP, in U-87 MG cells. We examined the effects of continuous exposure (3 days) to hypoxia on the expression of the genes for

COX-1, COX-2, VEGF and GFAP. Hypoxic exposure increased the levels of the mRNA encoding COX-2 and VEGF, but not COX-1, in the U-87 MG cells (Fig. 1), indicating that hypoxia activated inflammatory and angiogenic responses (26,27). Notably, *GFAP* mRNA expression decreased over 72 h of hypoxia (Fig. 1), indicating that the cells had begun to dedifferentiate. Inflammation, angiogenesis and dedifferentiation are predictors of poor antitumor treatment responses and have previously been reported to potently activate tumor progression and invasion (28,29).

Hypoxia does not affect cell viability and induces lamellipodia formation in U-87 MG cells. We previously showed that a 3-day exposure to hypoxia inhibited cell proliferation and viability in mouse fibroblast cells (30). In the present study, we investigated the effect of sustained exposure to hypoxia on U-87 MG cell proliferation and viability. We found that proliferation and viability were not significantly altered by hypoxia (Fig. 2), indicating that U-87 MG cells, unlike mouse fibroblast cells, are tolerant to hypoxia.
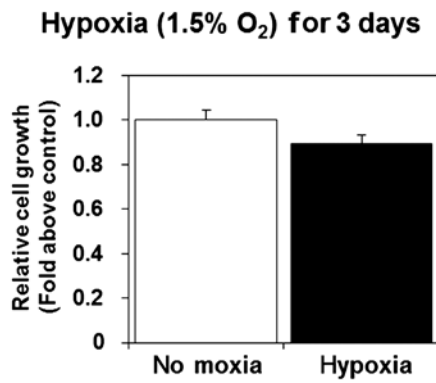
Figure 2. U-87 MG proliferation is unaltered by culture in sustained hypoxic conditions. Cells were incubated in 20% O_2 (normoxia) or 1.5% O_2 (hypoxia) for 3 days. Cell proliferation and viability were analyzed using the Cell Counting Kit-8. Bars depict mean ± SEM from 3 independent experiments. Statistical analysis was conducted using Student's unpaired t-test.

Next, we examined the morphology of U-87 MG cells after sustained exposure to hypoxia. We found that lamellipodia formation occurred at the cell edges (Fig. 3), which is known to facilitate cell migration (31).

Hypoxia decreases the level of mRNA encoding CB1 in U-87 MG cells. Next, we examined the expression of CB1 (*CNR1*) and CB2 (*CNR2*) mRNA in U-87 MG cells using RT-PCR. The cannabinoid receptors facilitate the transduction of extracellular signals to the cytoplasm (2). The gene product encoding CB1 was detectable in U-87 MG cells, but the level of mRNA encoding CB2 was below the limit of detection (Fig. 4). Sustained 3-day exposure of U-87 MG cells to hypoxia decreased their expression of mRNA encoding CB1 (Fig. 4). These results indicate that hypoxia may regulate the cannabinoid system by modulating the expression of CB1.

Hypoxia attenuates cannabinoid receptor agonist-induced cell death in U-87 MG cells. Recently, cannabinoid receptor agonists have been shown to mimic the effects of endogenous cannabinoids and cause cell death in glioblastoma (15). Given the hypoxia-induced downregulation of cannabinoid receptor expression that we observed, we next examined the effects of sustained hypoxia on cannabinoid receptor agonist-induced cell death. We treated the cells with WIN 55,212-2 (0.1, 1.0, or 10 μM) after an initial 3-day exposure to hypoxia (1.5% O_2) or normoxia (20% O_2). We analyzed cell proliferation and viability 2 days after treatment with WIN 55,212-2. WIN 55,212-2 treatment significantly reduced the viability of the cells under normoxic conditions in a dose-dependent manner, compared to the viability of the untreated normoxic controls (Fig. 5). However, the low doses of WIN 55,212-2 (0.1 or 1.0 μM) failed to induce cell death in the hypoxia group (Fig. 5). These results suggest that hypoxia confers tolerance to cannabinoid receptor agonist-induced cellular toxicity in glioblastoma.
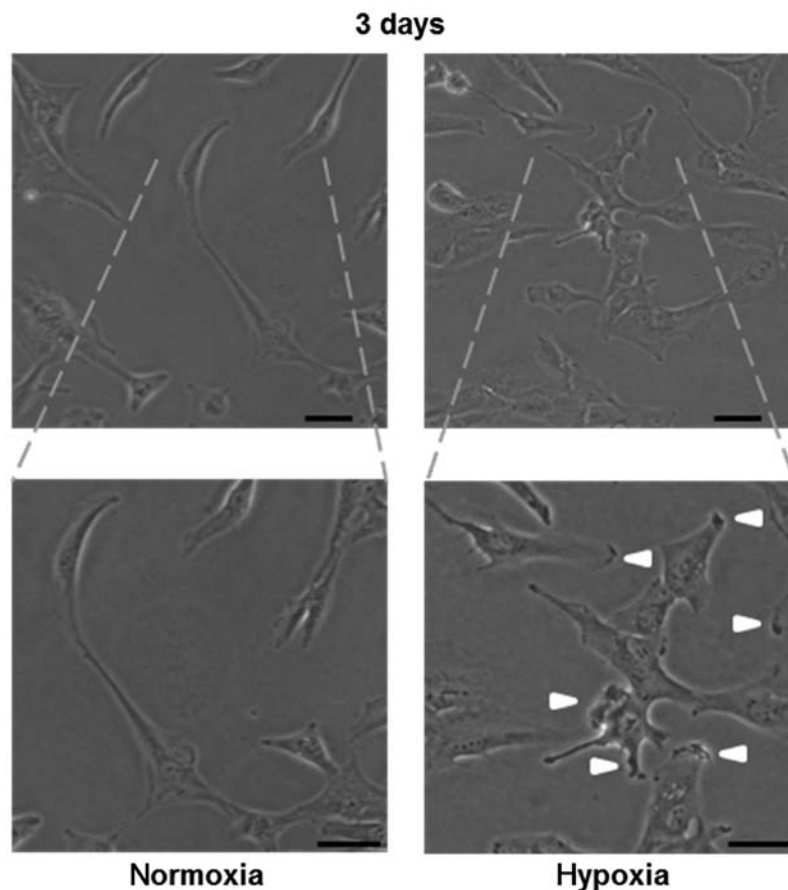

Figure 3. Hypoxia induces lamellipodia formation in U-87 MG cells. Cells were incubated in 20% O_2 (normoxia) or 1.5% O_2 (hypoxia) for 3 days. Hypoxia-induced lamellipodia formation in cells. Results are representative of 3 independent experiments. White triangles, lamellipodia. Black scale bar, 25 μm.
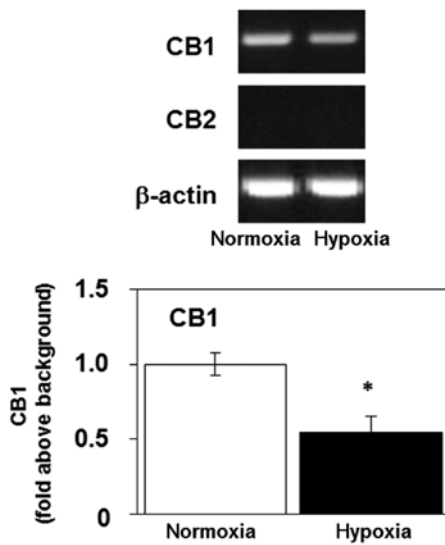
Figure 4. Hypoxia decreases the expression of mRNA encoding CB1 in U-87 MG cells. Cells were incubated in 20% O_2 (normoxia) or 1.5% O_2 (hypoxia) for 3 days. The expression patterns of CB1 (*CNR1*) and CB2 (*CNR2*) were evaluated by RT-PCR. The densities of bands corresponding to CB1 and β-actin were quantified by densitometry. The graph presents mean ± SEM from 3 independent experiments. Statistical analysis was conducted using Student's unpaired t-test; *P<0.05, compared with normoxic control.
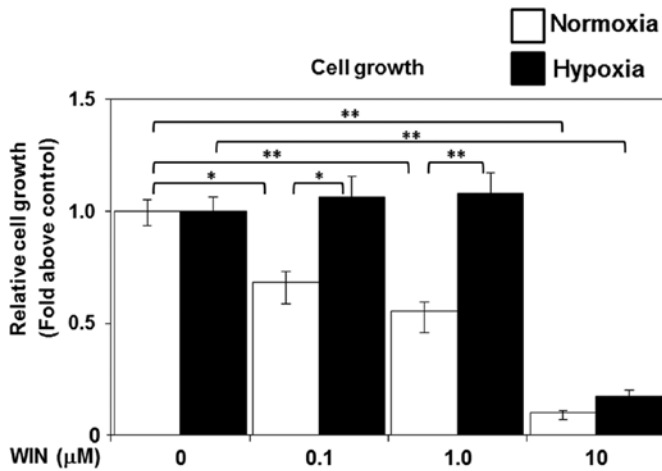

Figure 5. Effects of WIN 55,212-2 on the growth of U-87 MG cells with prior exposure to sustained normoxia or hypoxia. Cells were maintained in incubators under normoxic (5% CO_2 and 20% O_2) or hypoxic (5% CO_2 and 1.5% O_2) conditions. After 3 days of sustained treatment, cells were allowed to recover and cultured under normoxic conditions (5% CO_2 and 20% O_2) in the presence of the indicated doses of WIN 55,212-2 (0, 0.1, 1.0 or 10 μM) for 2 days. Then, cell proliferation and viability were analyzed using Cell Counting Kit-8. Bars represent mean ± SEM. Statistical analysis was conducted using a Kruskal-Wallis non-parametric ANOVA followed by Bonferroni post hoc test; *P<0.05, **P<0.01.

Hypoxia decreases the level of mRNA encoding CB1 and CB2 in primary rat glial cells. We next examined the effects of hypoxia on the expression levels of CB1 (*Cnr1*) and CB2 (*Cnr2*) mRNA in primary rat glial cells. Continuous 5-day exposure of primary glial cells to hypoxia decreased their expression of mRNA encoding both CB1 and CB2 (Fig. 6). These results indicate that hypoxia may regulate the expression levels of
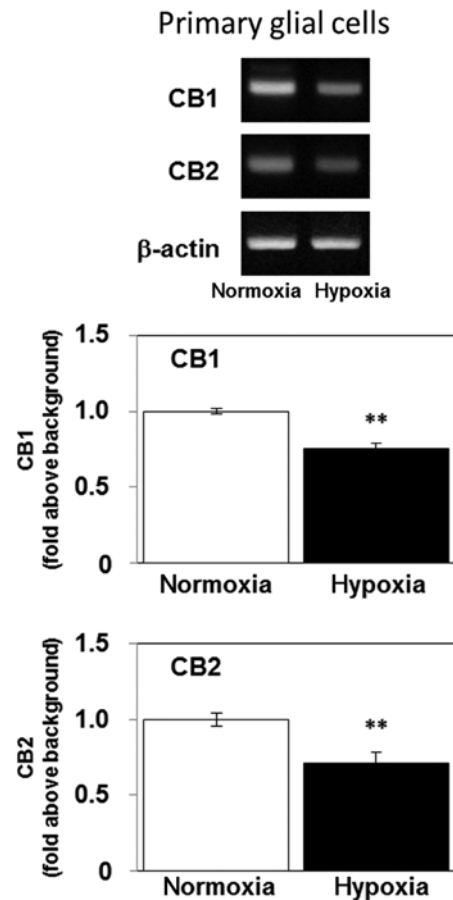

Figure 6. Hypoxia decreases the expression of mRNA encoding CB1 and CB2 in primary rat glial cells. Cells were incubated in 20% O_2 (normoxia) or 1.5% O_2 (hypoxia) for 3 days. The expression patterns of CB1 (*Cnr1*) and CB2 (*Cnr2*) were evaluated by RT-PCR. The densities of bands corresponding to CB1, CB2 and β-actin were quantified by densitometry. Bars represent mean ± SEM from 3 independent experiments. Statistical analysis was conducted using Student's unpaired t-test; **P<0.01, compared with normoxic control.

cannabinoid receptors in normal glial cells as well as glioblastoma cells.

Discussion

Several lines of evidence show that cannabinoids influence a variety of physiological and pathological conditions (2,8,32). Notably, cannabinoids can inhibit the growth of tumors, including glioblastoma, and have been proposed as potential antitumor agents (7-9,14,33,34). In contrast, hypoxia is a common feature of solid tumors, and has been implicated in the persistence and development of malignancies (16). Hypoxic regions are frequently found in glioblastoma and hypoxia promotes a more malignant phenotype (16). However, the effects of hypoxia on the endocannabinoid system are not fully understood. In the present study, we presented novel findings that hypoxia downregulates mRNA encoding CB1, upregulates mRNA encoding COX-2, and prevented cannabinoid receptor agonist-induced cell death in U-87 MG cells. Our results suggest that hypoxia may promote the progression of glioblastoma by inhibiting the endocannabinoid system.

We found that the cannabinoid receptor agonist WIN 55,212-2-induced glioblastoma cell death in a dose-dependent manner. We also observed preferential expression of mRNA encoding CB1, in the absence of mRNA encoding CB2, in U-87 MG cells. We and other investigators have previously found that cannabinoid receptor agonists (WIN 55,212-2 and KM-233) suppress cell growth via CB1, as antagonists for CB1, but not CB2, reversed cannabinoid receptor agonist efficacy in U-87 MG cells (7,14). We showed that hypoxia decreased the expression of mRNA encoding CB1 and inhibited WIN 55,212-2-induced cell death in U-87 MG cells, indicating a CB1-dependent mechanism of action. These results suggest that the activation of cannabinoid receptors in glioblastoma is essential for inhibition of tumor progression. Endocannabinoids are expressed in the brain (35), thus, it is possible that they suppress developing brain tumors via activation of cannabinoid receptors. Our present results suggest that hypoxia may counteract the beneficial antitumor effects of endocannabinoids by downregulating cannabinoid receptor expression in the brain.

Notably, several studies have indicated that activation of CB2 plays a more dominant role in the inhibition of glioma and glioblastoma progression than CB1 (36,37). Although CB1 is predominantly expressed in the U-87 MG glioblastoma cells that we used in the present study, many glioma and glioblastoma cells express functional CB2 (38). To date, the effects of hypoxia on the expression of CB2 in glioma and glioblastoma cells have not been revealed. In the present study, we showed that hypoxia inhibited the expression of CB2 in normal primary glial cells. Further experiments are necessary to reveal whether hypoxia alters the expression of CB2 in glioma and glioblastoma cells.

Hypoxia increased the expression levels of mRNA encoding VEGF and COX-2, which are associated with the activation of angiogenic and inflammatory responses, in U-87 MG cells. Angiogenesis is necessary for tumor progression and inflammation induces tumor cell survival, growth and migration (39). COX-2 is known to metabolize endocannabinoids (20,21). Thus, upregulation of COX-2 may inhibit endocannabinoid signaling by decreasing the concentration of endocannabinoids. Anandamide and 2-arachidonoylglycerol are major endocannabinoids that are produced, metabolized and kept at constant concentrations in the brain (2,35). However, hypoxia upregulates the expression of COX-2, which may decrease the concentration of endocannabinoids in hypoxic regions of glioblastoma, resulting in promotion of tumor growth. Thus, hypoxia may facilitate the progression of glioblastoma by inhibiting the endocannabinoid system in two ways: i) down-regulation of cannabinoid receptors; and ii) upregulation of the endocannabinoid-metabolizing enzyme COX-2.

Notably, we detected lower *GFAP* mRNA expression after 72 h of hypoxia, indicating dedifferentiation of astrocytes. Moreover, 72 h exposure to hypoxia significantly failed to induce cell death in the U-87 MG cells. Poorly differentiated and undifferentiated carcinomas are characterized by highly malignant tumors exhibiting aggressive invasive growth (40). We previously showed that 72 h exposure to hypoxia induced-cell death in mouse fibroblast cells (30). Thus, these results suggest that U-87 MG cells, unlike mouse fibroblast cells, may have increased hypoxia tolerance.

Hypoxia concomitantly downregulates the expression of mRNA encoding GFAP and CB1, but the relationship between dedifferentiation and endocannabinoid signaling is unknown. Further experiments are necessary to reveal the relationship between them.

Lamellipodia are formed by actin assembly at the edge of a cell in the direction of migration. Phosphoinositide 3-kinase (PI3K) activation is known to induce lamellipodia formation (31). Hypoxia activates PI3K (41) and induces lamellipodia formation, as shown Fig. 3, which may facilitate tumor invasion.

Hypoxia inhibited the expression of mRNA encoding CB1 in primary glial cells, as well as in U-87 MG cells, indicating that hypoxia-induced downregulation of CB1 is common to normal glial and malignant glioma cells. Although hypoxia-induced inhibition of the endocannabinoid system is thought to promote cancer progression, its role in normal glial cells has not been revealed. Further experiments are necessary to elucidate the effects of endocannabinoid inhibition mediated by hypoxia in healthy glial cells.

In conclusion, the present study demonstrated that hypoxia downregulates CB1 receptors, upregulates COX-2 and prevents cannabinoid receptor agonist-induced cell death in U-87 MG cells *in vitro*. These results suggest that hypoxia may promote brain tumor progression by inhibiting the endocannabinoid system.

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