Abstract. The endocannabinoid system regulates physiological and pathological conditions, including inflammation and cancer. Recently, emotional and physical stressors were observed to be involved in impairing the endocannabinoid system, which was concomitant with an increase in serum corticosteroids. However, the influence of corticosteroids on the endocannabinoid system has yet to be completely elucidated. The present study investigated the effects of corticosterone, one of the corticosteroids, on the endocannabinoid system in malignant glioblastoma cells in vitro. U-87 MG cells derived from malignant glioblastoma were subjected to corticosterone stimulation and their viability, signal transduction, and endocannabinoid-related gene expression were examined. Corticosterone decreased the mRNA and protein expressions of cyclooxygenase-2. Of note, although endocannabinoids decreased cell viability, corticosterone inhibited the cannabinoid receptor agonist-induced decrease in cell viability by downregulating the mRNA and protein expressions of cannabinoid receptor 1 (CB1) in glioblastoma cells. These results suggest that corticosteroids modify the endocannabinoid system in glioblastoma cells, and a reduction in the beneficial anti-tumor effects of endocannabinoids through downregulation of the CB1 receptor by corticosterone may promote the malignant phenotype of glioblastoma.

Introduction

The endocannabinoid system, including the seven transmembrane cannabinoid receptors 1 and 2 (CB1 and CB2) and their endogenous ligands, is involved in a wide variety of pathological situations such as inflammatory and oncogenic processes (1-3). Endogenous and exogenous cannabinoids are known to exert anti-inflammatory and anti-tumor effects (1,4). Recently, we reported that hypoxic stress modifies the endocannabinoid system to promote malignancy in glioblastoma cells through downregulation of the CB1 receptor and upregulation of cyclooxygenase-2 (COX2) (5), the enzyme responsible for metabolizing endocannabinoids (4). This result indicates that hypoxic stress aggravates cancer by inhibiting the endocannabinoid system.

Emotional and physical stressors typically induce the release of adrenal corticosteroid hormones such as corticosterone and cortisol in rodents and human, respectively (6). Corticosteroid hormones inhibit immune responses by downregulating the activity of nuclear factor-xB (NF-xB) signaling (7) and the production of several cytokines, such as tumor necrosis factor-x or interleukin (7). Interestingly, emotional and physical stresses have been reported to impair the endocannabinoid system concomitant with an increase in serum corticosteroids (8). In the present study, we examined the effects of corticosterone, which is one of the corticosteroids (6), on the endocannabinoid system in malignant glioblastoma cells. We found that corticosterone inhibits the expression of the genes encoding CB1 and attenuates cannabinoid receptor...
agonist (WIN 55,212-2)-induced decrease in glioblastoma cell viability. Our findings reveal the mechanisms by which stress hormones inhibit the endocannabinoid system through downregulation of the CB1 receptor, thus reducing the beneficial anti-tumor effects of endocannabinoids and consequently promoting the malignant phenotype of glioblastoma.

Materials and methods

**Chemicals.** Corticosterone (CORT), WIN 55,212-2, and Dulbecco’s modified Eagle’s medium were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum was obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-cannabinoid receptor 1 (CB1) rabbit antibody (ab137410) and anti-glucocorticoid receptor (GR) (EPR4595) rabbit antibody (cat. no. ab109022) were obtained from Abcam (Tokyo, Japan). Anti-GAPDH rabbit antibody (cat. no. 2118) and horseradish peroxidase-conjugated anti-rabbit IgG (cat. no. 7074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-COX2 antibody (cat. no. 160106) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA).

**Cell culture.** U-87 MG malignant glioblastoma cells (glioblastoma of unknown origin; ATCC® HTB-14™) were provided by Prof. Nakata (Kanazawa University, Kanazawa, Japan). U-87 MG cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C and 5% CO₂.

**Cell viability assay.** Cell viability was analyzed using the Cell Counting Kit-8 (Wako Pure Chemical Industries) as previously described (5,9). U-87 MG cells were seeded into 96-well plates at a density of 1x10³ cells/well. After a 24-h incubation period, the cells were stimulated with several concentrations of CORT. On day 2 after stimulation, 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 viability was expressed as a ratio of the absorbance in treated wells relative to that in untreated control wells.

**Western blotting analysis.** Western blotting was performed as described previously (10). Briefly, proteins were extracted from cells, and protein concentrations were determined by a protein assay. Equal amounts of protein (30 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred onto polyvinylidene fluoride membranes, which were incubated with primary antibodies (1:1,000), followed by incubation with horseradish peroxidase-linked secondary antibodies (1:2,000).

**RT-qPCR.** To evaluate the expression of human glucocorticoid receptor (GR: NR3C1), human COX2 (PTGS2), and human CB1 (CNR1) and human β-actin (ACTB) mRNAs in the cells, Real time PCR was performed as follows. Briefly, RNA was extracted from the cells, and cDNA was generated using reverse transcriptase ReverTra Ace® (Toyobo, Tokyo, Japan). We performed PCR-based, subtype-specific gene amplification with QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) using the sets of primers as follows: 5’-GGCTAGACACCCATTTTCACA-3’ and 5’-ACAGAAAGGGCTACTACAGTGC-3’ for GR (NR3C1), 5’-CAGCAATCTCTTGGTGTCC-3’ and 5’-GTGAAGTGCCTGGGCAAAGAAT-3’ for COX-2 (PTGS2), 5’-CAGGCTCTCTCCATACCCTTTCAT-3’ and 5’-ACCCCAACAGTTAACAAGA-3’ for CB1 (CNR1), and 5’-ATGGTGCGATGATGTCAGAA-3’ and 5’-CTGGGCTGTGAAAGGTCTCA-3’ for β-actin (ACTB). The quantified value of each sample was normalized with that of the β-actin figure 1. U-87MG cell viability is unaltered by corticosterone stimulation. Cells were incubated with corticosterone for 12, 24, 48, and 72 h. Cell viability was analyzed using the Cell Counting Kit-8 (Wako). Bars depict the mean ± SEM of three independent experiments. Statistical analysis was conducted using a one-way analysis of variance (ANOVA). CORT, corticosterone
value of the same sample, which was amplified simultaneously with the target gene.

**Cannabinoid receptor agonist treatment.** The cells were treated with the cannabinoid receptor agonist WIN 55,212-2 following 2-day exposure to corticosterone. After 2 days of CORT treatment, cell viability was analyzed using the Cell Counting Kit-8 (Wako Pure Chemical Industries) as described above.

**Statistical analysis.** Data are presented as the mean ± standard error of the mean (SEM) from at least three independent experiments. The Stat View statistical package (version 5.0 Japanese version, Hulinks, Japan) was used for statistical analysis. Student's t-test was used to examine the differences between the two groups (with or without CORT stimulation) in the real time PCR and western blot analyses. A one-way analysis of variance (ANOVA) was used to analyze the cell viability data. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Corticosterone does not affect cell viability in U-87 MG cells.** We investigated the effect of 12, 24, 48, or 72-h treatment with CORT (1-100 µM) on cell viability in U-87 MG cells. Cell viability was not significantly altered by CORT (Fig. 1).

**Corticosterone did not affect the levels of GR mRNA and protein in U-87 MG cells.** Next, we examined the expression of glucocorticoid receptor (GR) mRNA and protein in U-87 MG cells by real-time PCR and western blotting, respectively. GR is one of main receptors for CORT (6). GR was spontaneously detectable and the levels of GR mRNA and protein did not change by CORT stimulation in U-87 MG cells (Figs. 2 and 3). CORT may act transduction through GR receptor in U-87 MG cells.

**Corticosterone decreases the levels of COX2 mRNA and protein in U-87 MG cells.** We examined the expression of COX2 mRNA and protein in U-87 MG cells by real-time PCR and western blotting, respectively. Previously, COX2 expression was shown to be regulated by NF-κB signaling (7). COX2 was spontaneously detectable in U-87 MG cells (5), while 48-h, but not 24-h, treatment with CORT decreased the expression of mRNA encoding COX2 (Fig. 2) and COX2 protein (Fig. 3), indicating the inhibition of NF-κB signaling by CORT.

**Corticosterone decreases the levels of CB1 mRNA and protein in U-87 MG cells.** We examined the expression of CB1 mRNA...
and protein in U-87 MG cells by real-time PCR and western blotting, respectively. The cannabinoid receptors facilitate the transduction of extracellular signals to the cytoplasm (2). CB1 was spontaneously detectable in U-87 MG cells (5), while 48-h, but not 24-h, treatment with CORT decreased the expression of mRNA encoding CB1 (Fig. 2) and CB1 protein (Fig. 3). These results indicate that CORT regulates the cannabinoid system by modulating CB1 expression.

Corticosterone attenuates the cannabinoid receptor agonist-induced decrease in cell viability in U-87 MG cells. Recently, cannabinoid receptor agonists were shown to mimic the effects of endogenous cannabinoids and cause cell death in glioblastoma (5,11,12). The observed corticosterone-induced downregulation of cannabinoid receptor expression; thus, we investigated the effects of 48 h treatment with CORT on cannabinoid receptor agonist-induced decrease in glioblastoma cell viability. We treated the cells with WIN 55,212-2 (0.1 or 1.0 µM) after 48 h treatment with CORT. We analyzed cell viability 48 h after treatment with WIN 55,212-2. WIN 55,212-2 treatment significantly decreased the viability of vehicle-treated cells in a dose-dependent manner (Fig. 4). However, the doses of WIN 55,212-2 (0.1 or 1.0 µM) failed to decrease cell viability in the CORT treatment group (Fig. 4). These results suggest that CORT confers tolerance to cannabinoid receptor agonist-induced cellular toxicity in glioblastoma.
Discussion

Emotional and physical stress have been suggested to affect the development and progression of malignancies (13). Interestingly, emotional and physical stressors release corticosteroids and, in turn, impair the endocannabinoid system (8). We previously showed that hypoxia aggravates cancer by inhibiting the endocannabinoid system in malignant glioblastoma cells (5). Thus, dysfunction of the endocannabinoid system by stress-released corticosteroids may lead to the development and progression of malignancies. However, the effects of corticosteroids on endocannabinoid system-dependent malignancy are not fully understood. Endocannabinoids have been shown to influence diverse pathological processes, including malignancy (2,14,15). Endogenous and exogenous cannabinoids have anti-cancer effects; exogenous cannabinoids and many synthetic agonists for cannabinoid receptors, which mimic the effects of endocannabinoids, have been proposed as potential anti-cancer agents (14,16-20). Here, we found that corticosteroids downregulate both mRNA and protein of CB1 and attenuate cannabinoid receptor agonist-induced decrease in U-87 MG cell viability. Our results suggest that stress-released corticosteroids promote the progression of glioblastoma by inhibiting the endocannabinoid system (Fig. 5).

Corticosterone is considered an adrenal corticosteroid and functions as both a glucocorticoid and mineralocorticoid (6). Glucocorticoids are known to have anti-inflammatory and immunosuppressive potentials through inhibition of NF-κB activities (7,21,22). NF-κB signaling is involved in controlling the expression of COX2 (7). We showed that corticosterone decreases the levels of COX2 mRNA and protein (Figs. 2 and 3) in this study, indicating that corticosterone might downregulate NF-κB signaling. Interestingly, inhibition of NF-κB signaling induced downregulation of CB1 receptor.
expression (23). Thus, inhibition of NF-κB signaling by corticosterone may downregulate the expression of the CB1 receptor (Figs. 2 and 3).

Nakatani et al (24), showed that corticosterone inhibited the proliferation of C6 glioma cells. However, in our present study, corticosterone did not inhibit the cell viability in U-87 MG cells. Since U-87 MG cells are glioblastoma, a poorly differentiated type of glioma, highly malignant and exhibits aggressive invasive growth in comparison with glioma, corticosterone might not be able to inhibit the cell viability in U-87 MG cells.

Corticosterone alone downregulates the expression of CB1 (Figs. 2 and 3), but does not affect cell viability in 12, 24, 48, and 72 h after stimulation (Fig. 1). These results suggest that CB1 might not be committed to cell viability directly in U-87 cells. However, as shown in our previous study that hypoxia decreased the expression of CB1 in U-87 MG cells, the cannabinoid receptor agonist WIN 55,212-2 decreased glioblastoma cell viability, with the extent depending on the expression level of the CB1 receptor (5). In the present study, we showed that corticosterone downregulated the expression of CB1 and suppressed WIN 55,212-2-induced decrease in U-87 MG cell viability. Endocannabinoids in the brain (25) have the potential to prevent tumor development by activating cannabinoid receptors. Thus, our results suggest that corticosterone may counteract the beneficial anti-tumor effects of endocannabinoids by downregulating cannabinoid receptor expression in the brain.

The present study demonstrated that corticosterone downregulates CB1 receptors and suppresses cannabinoid receptor agonist-induced decrease in U-87 MG cell viability in vitro. These results suggest that corticosterone promotes brain tumor progression by inhibiting endocannabinoid receptor signaling.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NS designed the study and prepared the manuscript. NS, HI, and TS conducted the experiments. NS, YU, HN and AY analyzed and interpreted the data. NS, HI, and TS obtained funding. AY and TS edited the manuscript. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

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Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

References


