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Short communication

CANNABINOID RECEPTOR ACTIVATION INHIBITS CELL CYCLE PROGRESSION BY MODULATING 14-3-3β

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Abstract: Cannabinoids display various pharmacological activities, including tumor regression, anti-inflammatory and neuroprotective effects. To investigate the molecular mechanisms underlying the pharmacological effects of cannabinoids, we used a yeast two-hybrid system to screen a mouse brain cDNA library for proteins interacting with type 1 cannabinoid receptor (CB1R). Using the intracellular loop 3 of CB1R as bait, we identified 14-3-3β as an interacting partner of CB1R and confirmed their interaction using affinity-binding assays. 14-3-3β has been reported to induce a cell cycle delay at the G_2/M phase. We tested the effects of cannabinoids on cell cycle progression in HeLa cells synchronized using a double-thymidine block-and-release protocol and found an increase in the population of G_2/M phase cells. We further found that CB1R activation augmented the interaction of 14-3-3β with Wee1 and Cdc25B, and promoted phosphorylation of Cdc2 at Tyr-15. These results suggest that cannabinoids induce cell cycle delay at the G_2/M phase by activating 14-3-3β.

Keywords: Cannabinoids, Cdc2, Cdc25B, Cyclin B, G_2/M phase, GIPs, HeLa, Phosphorylation, Wee1, Yeast-two hybrid

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Abbreviations used: CBR – cannabinoid receptors; CB1R – type 1 CBR; CB2R – type 2 CBR; GIPs – GPCR-interacting proteins; GPCR – G protein-coupled receptor; GPR55 – G protein-coupled receptor 55; GST-14-3-3β – glutathione-*S*-transferase-tagged 14-3-3β; HA-CB1R – 3xHA-tagged CB1R; IL3 – intracellular loop 3 domain; PBS – phosphate-buffered saline; PI – propidium iodide; PPAR-γ – peroxisome proliferator-activated receptor-γ; SDS – sodium dodecyl sulfate

INTRODUCTION

Cannabinoids are a major component of *Cannabis sativa* Linnaeus. They have various recognized pharmacological activities, including tumor regression, pain control, neuroprotective, and anti-inflammatory effects [1, 2]. Cannabinoids have been found to induce cell cycle arrest and cell death in several cancer cell lines, and to inhibit tumor growth in vivo [3–8].

Cannabinoid signals are mediated by cannabinoid receptors (CBR), which are members of the G protein-coupled receptor (GPCR) family. Two types of CBR have been identified in mammalian tissues. Central type 1 CBR (CB1R), cloned in 1990 [9], is predominantly expressed in the brain [10], whereas peripheral type 2 CBR (CB2R), cloned in 1993 [11], is mainly expressed in cells of the immune system [12].

Cannabinoid signals are also medicated by other receptors, including vanilloid receptors, peroxisome proliferator-activated receptor-γ (PPAR-γ) and G protein-coupled receptor 55 (GPR55) [13–15]. Anandamide, one of the endocannabinoids, can specifically activate vanilloid receptors and modulate channel activity. Anandamide also binds to PPAR-γ, which regulates various cellular responses, such as cell differentiation and immune function [16]. Recently, GPR55 has been identified as a putative cannabinoid receptor that is involved in modulating energy homeostasis [17].

It is well known that the signals of activated GPCRs are transmitted into the intracellular compartment by G proteins. However, many studies suggest that GPCR-interacting proteins (GIPs), which form functional complexes with GPCRs, are also involved in GPCR signaling [18, 19]. GIPs are involved in various processes, including the biosynthesis, subcellular localization, signal modulation, and recycling of GPCRs [20]. Whether GIPs are involved in mediating the cell cycle modulatory effects of CBRs is not known.

Entry into mitosis, and thus initiation of the cell cycle, is dependent on the formation of a complex between Cdc2 and cyclin B [21]. A positive regulator of this process is the phosphatase Cdc25, which dephosphorylates Cdc2 at both its Tyr-14 and Tyr-15 residues, leading to Cdc2/cyclin B complex formation [22]. The tyrosine kinase Wee1 opposes the actions of Cdc25. It prevents inappropriate entry into the cell cycle by maintaining the tyrosine-phosphorylated form of Cdc2 [23]. Both Cdc25 and Wee1 are regulated by interactions with 14-3-3β, a member of the 14-3-3 family of adapter/scaffolding proteins that interact with and regulate the functions of a large number of proteins [24]. 14-3-3β binding prevents Cdc25 from dephosphorylating Cdc2, but enhances the activity of Wee1 by increasing its intracellular stability [25]. Both of these actions of 14-3-3β thus inhibit cell cycle progression.

Here, using yeast two-hybrid assays, we screened a mouse brain cDNA library for novel GIPs that interact with CB1R and modulate cell proliferation. The intracellular loop 3 domain (IL3) of CB1R, one of the major binding sites for GIPs on GPCRs [20], was used as bait. These screens identified 14-3-3β as

a CB1R-interacting partner, an interaction that was confirmed using affinity-binding assays.

Because 14-3-3β is involved in the G_2/M phase transition through the modulation of Wee1 and Cdc25 functions, we investigated the effect of cannabinoids on cell cycle progression in HeLa cells. Notably, treatment with a cannabinoid agonist induced a significant cell cycle delay at the G_2/M phase. This effect was achieved by modulating 14-3-3β-mediated regulation of Wee1 and Cdc25. Our findings demonstrate a novel molecular mechanism underlying the inhibitory effects of cannabinoids on cell cycle progression. This mechanism may account for cannabinoid effects on tumor cell growth.

MATERIALS AND METHODS

Construction of plasmids

The 3xHA-tagged CB1R (HA-CB1R) expression plasmid, pcDNA3.1-3xHA-CB1R, was obtained from the Missouri S&T cDNA resource center. The bait plasmid, pHybTrp/Zeo-CB1R-IL3, encoding the IL3 domain of CB1R and used for yeast two-hybrid screening, was generated by PCR amplification using pcDNA3.1-3xHA-CB1R plasmid as a template and the primer pair 5'-GGA AGG CTC ACA GCC ACG CC-3' and 5'-ATC TCG AGC TAC TTG GCT AAC C-3'. Amplified PCR products and pHybTrp/Zeo plasmid [3] were digested with the restriction enzymes *Eco*RI and *Xho*I, and ligated into the corresponding enzyme sites of the plasmid. A FLAG-tagged 14-3-3β plasmid (pcFLAG-14-3-3β) was generated by first amplifying 14-3-3β cDNA from the pACT2-14-3-3β prey plasmid employed in yeast two-hybrid screens using the primer pair 5'-ATG ACC ATG GAT AAG AGT-3' and 5'-TTA GTT CTC TCC CTC TCC-3', and then inserting the amplified products into the pGEM-T/Easy plasmid (Promega). In the final step, the pGEM-T/Easy-14-3-3β plasmid was digested with *Eco*RI and ligated into the corresponding sites of pcFLAG to generate pcFLAG-14-3-3β. A pGEX-5X-2-14-3-3β plasmid encoding glutathione-*S*-transferase-tagged 14-3-3β (GST-14-3-3β) was generated by PCR amplification using pACT2-14-3-3β plasmid as the template and the primer pair 5'-AAA AAT CAA TGA CAT GAT AGA TGA-3' and 5'-AAA CTC GAG TTA GTT CTC TCC CTC TCC AG-3'. The amplified PCR products were inserted into the pGEX-5X-2 plasmid (GE Healthcare) using *Eco*RI and *Xho*I restriction enzymes.

Yeast two-hybrid screening

The bait plasmid was transformed into the yeast reporter cell line L40 with mouse brain cDNA library (Clontech), as recommended by the manufacturer. The methods used to isolate positive clones were described in a previous study [3].

Cell culture and transfection

293T and HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and

100 μg/ml streptomycin. For GST pull-down assays, HeLa cells (1.5×10^6 cells/dish) were plated onto 100-mm tissue culture dishes and transfected with 10 μg of pcDNA3.1-3xHA-CB1R plasmid using Lipofectamine 2000 (Life Technologies), as recommended by the manufacturer. For co-immunoprecipitation assays, 293T cells (1.5×10^6 cells/dish) were plated onto 100-mm dishes and transfected with the indicated plasmids using the calcium phosphate method [26].

GST pull-down assay

Lysates of *Escherichia coli* BL21 cells expressing the plasmid encoding GST-14-3-3β were incubated with glutathione-Sepharose 4B beads (GE Healthcare) for 1 h at 4ºC in lysis buffer consisting of phosphate-buffered saline (PBS) containing 0.5% NP-40 and protease inhibitors (5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 mM phenylmethylsulfonyl fluoride). After extensive washing with the lysis buffer, beads were incubated with lysates (500 μg) of HA-CB1R-expressing HeLa cells for 1 h at 37ºC followed by washing with lysis buffer. The bound proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer and analyzed by immunoblotting using antibodies against CB1R (Cayman).

Immunoprecipitation assay

The interaction between CB1R and 14-3-3β was confirmed by preparing lysates of 293T cells co-expressing HA-CB1R and FLAG-14-3-3β using PBTX buffer (PBS containing 1% TritonX-100 and protease inhibitors). The interaction between 14-3-3β and Wee1 or Cdc25B was tested using PBTX extracts of synchronized HeLa cells (see below) incubated with or without the cannabinoid agonist Win 55,212-2 (Cayman) for the indicated time. For the immunoprecipitation assay, cell lysates (500 μg) of both cell types were pre-cleared by incubation with 20 μl of protein A-Sepharose CL-4B beads (10% slurry; GE Healthcare) followed by incubation with 1 μg of antibodies against CB1R or 14-3-3β (Santa Cruz Biotechnologies) with gentle rotation for 4 h at 37ºC. After incubation, 50 μl of beads was added to each reaction and the mixtures were allowed to incubate for an additional 2 h at room temperature. The beads were washed with PBTX buffer, and bound proteins were eluted with SDS sample buffer. The eluates were analyzed by immunoblotting with anti-FLAG (Sigma-Aldrich), anti-Wee1 (Santa Cruz Biotechnologies), anti-Cdc25B (Santa Cruz Biotechnologies), or anti-14-3-3β antibodies.

Cell synchronization and flow cytometry

HeLa cells were synchronized using a double-thymidine block-and-release protocol, as described previously in detail [27]. Briefly, HeLa cells were synchronized at the G_1/S border by plating cells onto a 100-mm tissue culture dish (8×10^4 cells/dish), incubating them for 18–24 h, and then treating them with 2 mM thymidine for 18 h (Fig. 2A). Cells were then released from the block by changing the medium to complete growth medium. Thymidine was

again added to the medium to a final concentration of 2 mM 9 h after release, and the cells were grown for an additional 17 h.

After the medium was again changed to complete growth medium, the cells were treated with 20 μM Win 55,212-2 for the indicated time. To analyze the protein expression levels, cells were harvested with PBTX, and cell lysates were analyzed by immunoblotting using antibodies against Wee1 or Cdc2-Tyr15P (Santa Cruz Biotechnologies). For flow cytometry, the cells were harvested by trypsinization, washed with ice-cold PBS, and fixed in 70% ethanol for 20 min. The fixed cells were stained with propidium iodide (PI; Sigma-Aldrich) solution (PBS containing 50 μg/ml PI, 0.5 μg/ml RNase A, and 3.8 mM sodium citrate) at 4ºC for 20 min. Cell cycle distribution was assessed using a FACSVantage SE system (BD Biosciences). Data from 10,000 cells per sample were collected and analyzed.

RESULTS AND DISCUSSION

CB1R interacts with 14-3-3β

The third intracellular loop 3 domain of CB1R was employed as bait in yeast two-hybrid screens of a mouse brain cDNA library. Several clones were obtained and sequenced. A homology search in GenBank using the BLAST

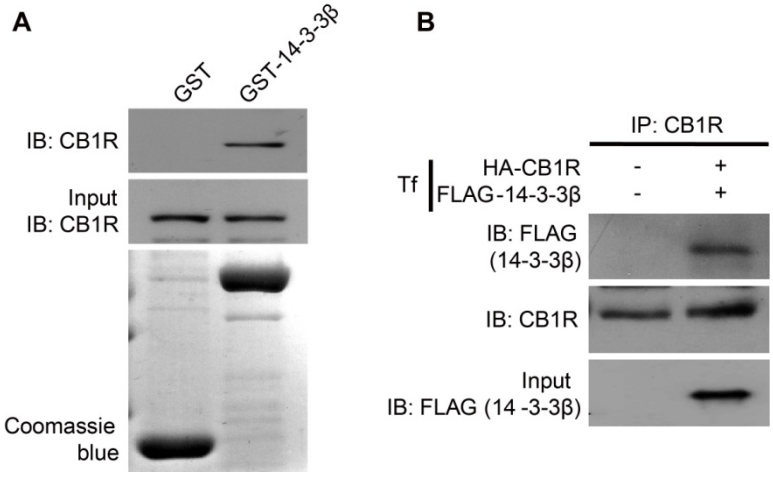

Fig. 1. Interactions between CB1R and 14-3-3β. A – Beads charged with bacterially expressed GST or GST-14-3-3β were incubated with extracts of HeLa cells transfected with HA-CB1R expression plasmid (10 μg). Bound proteins were analyzed by immunoblotting with antibodies against CB1R. Coomassie blue staining was used to estimate the levels of GST and GST-14-3-3β fusion proteins. B – Extracts of 293T cells (500 μg) transfected with expression plasmids for HA-CB1R (12 μg) and FLAG-14-3-3β (1 μg) were immunoprecipitated with 1 μg of anti-CB1R antibodies. Immunoprecipitates were analyzed by immunoblotting with anti-FLAG or anti-CB1R antibodies. Input lanes were loaded with 10% of the amount of HeLa or 293T cell extract used for each assay. The results are representative of at least three independent experiments.

program identified 14-3-3β as an interacting partner of CB1R. To confirm the specific binding of CB1R and 14-3-3β, we performed GST pull-down assays with bacterially expressed GST-14-3-3β protein or GST alone (control) using soluble proteins obtained from HeLa cells expressing HA-CB1R. As shown in Fig. 1A, CB1R was detected in GST-14-3-3β precipitates, but not in GST precipitates. To determine whether the specific binding of these two proteins occurs in a mammalian cellular context, we performed immunoprecipitation assays using 293T cells co-transfected with plasmids encoding HA-CB1R and FLAG-14-3-3β. CB1R was immunoprecipitated from cell lysates using anti-CB1R antibodies, and the precipitates were analyzed by immunoblotting using anti-FLAG and anti-CB1R antibodies. We found that 14-3-3β co-precipitated with CB1R (Fig 1B), suggesting specific binding between these two proteins in mammalian cells.

Cannabinoids cause cells to delay in the G_2/M phase of the cell cycle

It is well known that 14-3-3β can modulate cell cycle progression at the G_2/M phase by interacting with Wee1 and Cdc25B [22, 25]. To determine whether CB1R activation modulates the cell cycle at the G_2/M phase and to assess the potential involvement of 14-3-3β in this signaling, we first investigated the effect of Win 55,212-2 on cell cycle progression in HeLa cells. Cells were synchronized at the G_1/S border using a double-thymidine block-and-release protocol (Fig. 2A). Following release, cells were incubated with or without 20 μM Win 55,212-2 for the indicated time, after which flow cytometry was performed to monitor cell cycle progression. As shown in Fig. 2B, CBR activation significantly increased the proportion of cells in G_2/M phase and induced a marked cell cycle delay. Notably, the cell population in G_2/M phase in the presence of Win 55,212-2 increased from 38.2 ± 6.1% to 64.5 ± 2.1% 10 h after release. The proportions of cells in each phase of the cell cycle became similar for cells with and without Win 55,212-2 treatment 16 h after release (Fig. 2C). These results suggest that CBR activation induces a G_2/M-phase cell cycle delay in HeLa cells. We consider that this might ultimately induce inhibition of cell proliferation, since there is evidence that cell cycle delay correlates with anti-proliferation [28, 29].

Activation of CBR induces 14-3-3β-mediated signaling

We next investigated the molecular mechanisms underlying Win 55,212-2-induced G_2/M phase delay. 14-3-3β interactions with Wee1 and Cdc25B have been shown to inhibit formation of the Cdc2/cyclin B complex required for entry into mitosis, leading to an increase in the G_2/M phase cell population [21, 25]. It was previously reported that an increase in Wee1 expression can induce cell cycle arrest at the G_2/M phase [30], so we first investigated whether Win 55,212-2 affected Wee1 expression levels. HeLa cells synchronized at the G_1/S border using a double-thymidine block-and-release protocol were incubated with or without Win 55,212-2 for 8 to 12 h. Then, Wee1 expression was analyzed via immunoblotting. As shown in Fig. 3A, Wee1 levels were significantly increased 8 and 10 h after release in cells treated with Win 55,212-2 compared to untreated control cells.

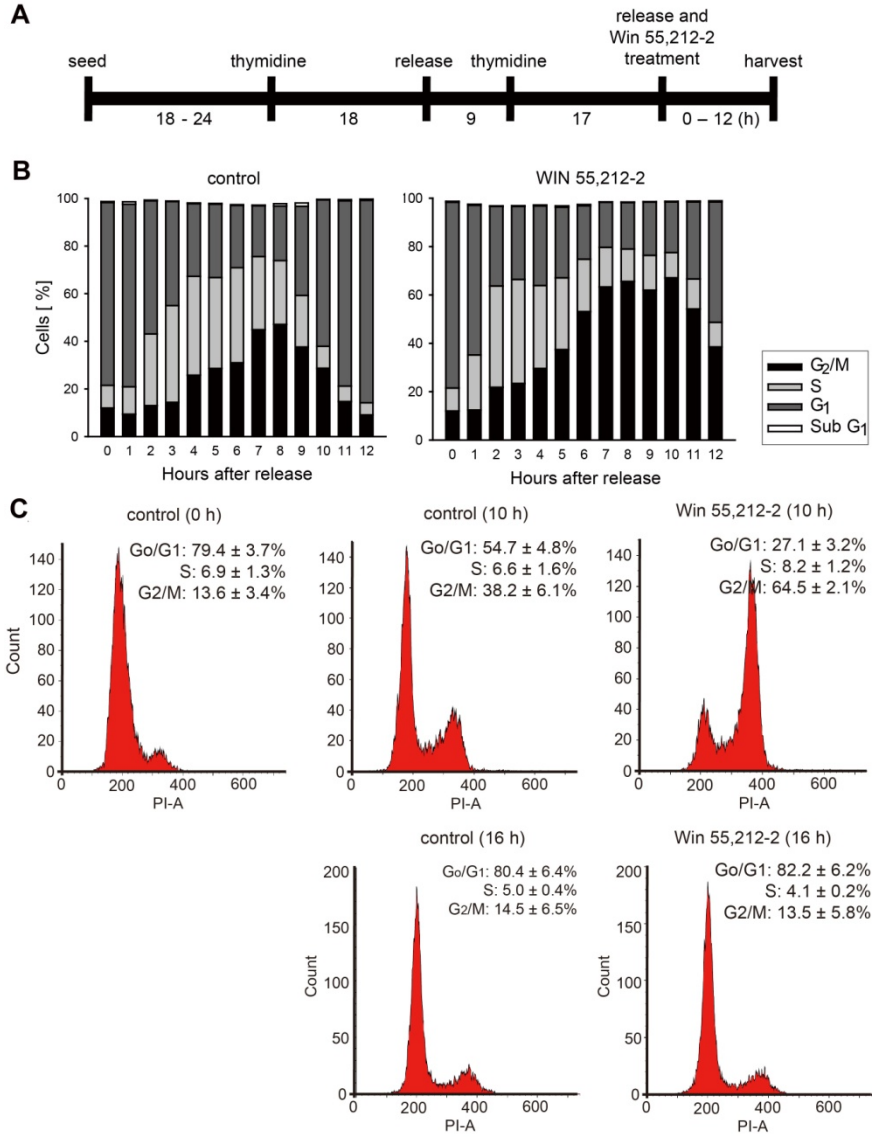

Fig. 2. The effect of Win 55,212-2 on cell cycle progression in HeLa cells. A – The double-thymidine block-and-release protocol was used to synchronize the HeLa cells, as indicated (for details, see the Materials and Methods section). B – Synchronized cells were treated with 20 μM Win 55,212-2 for the indicated time. After staining with PI, cell cycle phases were analyzed by flow cytometry. C – The cell populations in each cell cycle are shown in the absence and presence of Win 55,212-2 at 0, 10 and 16 h, as indicated. Results are from two or three independent experiments, and the indicated values are the means ± S.E.

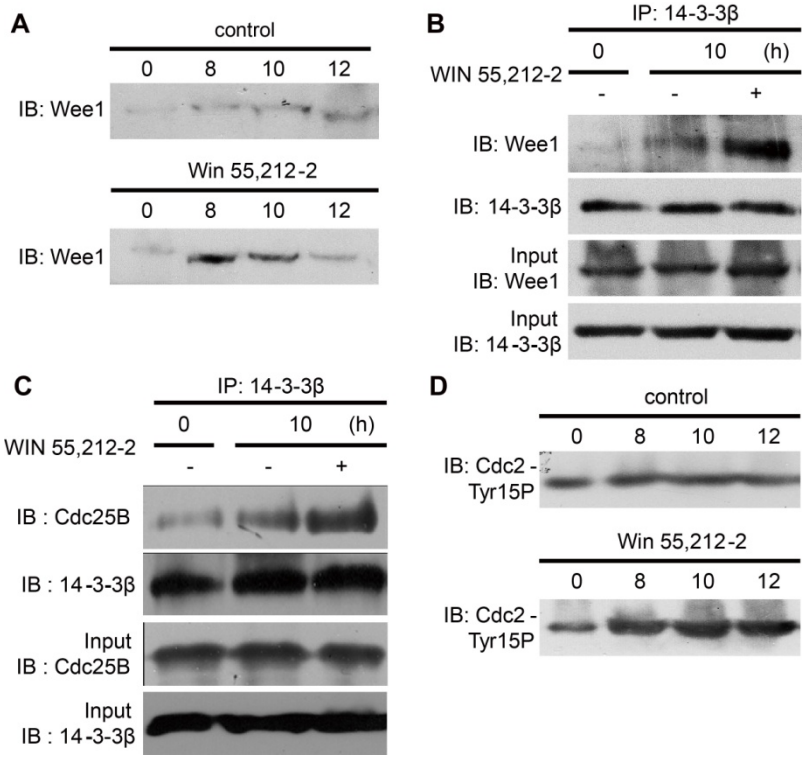

Fig. 3. The effects of Win 55,212-2 on 14-3-3β-mediated signaling. Synchronized HeLa cells were incubated with or without 20 μM Win 55,212-2 for the indicated time. After harvesting, the cells were analyzed by immunoblotting with anti-Wee1 (A) and anti-Cdc2-Tyr15P (D) antibodies. Synchronized cells were incubated with or without 20 μM Win 55,212-2 for 10 h, as indicated. Cell lysates (500 μg) were immunoprecipitated with antibodies against 14-3-3β. Immunoprecipitates were analyzed by immunoblotting with anti-Wee1 and anti-14-3-3β antibodies (B), or anti-Cdc25B and anti-14-3-3β antibodies (C). Input lanes were loaded with 10% of the amount of HeLa cell extract used for the immunoprecipitation assay. The results are representative of at least three independent experiments.

It has been reported that 14-3-3β increases the population of cells in the G_2/M phase of the cell cycle by interacting with Wee1 [25]. Therefore, we determined whether cannabinoids enhanced the interaction between 14-3-3β and Wee1. After treating synchronized HeLa cells with or without Win 55,212-2 for 10 h, we immunoprecipitated cell lysates with antibodies against 14-3-3β and analyzed the resulting immunoprecipitates via immunoblotting with anti-Wee1 and anti-14-3-3β antibodies. As shown in Fig. 3B, HeLa cells treated with Win 55,212-2 for 10 h exhibited an increase in the interaction between 14-3-3β and Wee1 compared to the control.

14-3-3 also binds Cdc25, inhibiting Cdc25-mediated dephosphorylation of Cdc2 Tyr-14 and Tyr-15 residues and preventing formation of Cdc2/cyclin B

complexes, thereby blocking entry into mitosis [22, 31, 32]. To investigate whether CBR activation enhances the interaction of 14-3-3β with Cdc25B, we treated synchronized HeLa cells with Win 55,212-2 and analyzed the cell lysates for 14-3-3β–Cdc25B binding using immunoprecipitation assays. Lysates were immunoprecipitated with antibodies against 14-3-3β, and the resulting immunoprecipitates were analyzed via immunoblotting using anti-Cdc25B and anti-14-3-3β antibodies. As shown in Fig. 3C, the interaction between 14-3-3β and Cdc25B increased significantly after treatment with Win 55,212-2 for 10 h.

Interactions of 14-3-3β with Wee1 and Cdc25B ultimately induce the inactivation of Cdc2 by maintaining the phosphorylation status of Cdc2 at Tyr-15 [25, 33, 34]. To test the effect of CBR activation on the phosphorylation of Cdc2 at Tyr-15, we treated synchronized HeLa cells with Win 55,212-2 and performed immunoblot analyses using antibodies against Cdc2-Tyr15P. Treatment with Win 55,212-2 for 8–12 h significantly increased Cdc2 phosphorylation on Tyr-15, suggesting that Win 55,212-2 induces the inactivation of Cdc2.

We showed that cannabinoid receptor activation can affect signaling of G_2/M phase regulating proteins in non-transfected HeLa cells but we did not confirm the interaction between CB1R and 14-3-3β in non-transfection condition. Therefore, further studies will be needed to confirm their interaction in HeLa cells which are expressed both proteins as normal components.

14-3-3 contributes to cell cycle arrest at G_2/M phase through two different pathways [25]. The first is a Wee1-mediated pathway in which 14-3-3β interacts with and stabilizes Wee1, thereby increasing Wee1 protein levels and kinase activity in the cell. As a result, tyrosine phosphorylation of the Wee1 target Cdc2 at Tyr-15 is increased, thereby inhibiting Cdc2/cyclin B complex formation. Notably, co-expression of 14-3-3β and Wee1 significantly increases cell cycle arrest at the G_2/M phase [25]. The second pathway is a Cdc25-mediated pathway in which 14-3-3β interacts with the phosphatase Cdc25B and sequesters it in the cytoplasm. Because active nuclear Cdc25B is the key executor of Cdc2/cyclin B complex formation, this interaction with 14-3-3β also inhibits cell cycle progression at the G_2/M phase [22, 35].

In our study, a CB1R agonist increased the levels of Wee1 protein and enhanced the interaction between 14-3-3β and Wee1. CB1R activation also promoted the interaction between 14-3-3β and Cdc25B. These results are consistent with a mechanism in which CB1R promotes cell cycle delay at G_2/M phase by enhancing 14-3-3β–Wee1 and 14-3-3β–Cdc25B interactions, which would be predicted to increase Wee1 kinase activity, reduce Cdc25B phosphatase activity, and inhibit Cdc2/cyclin B complex formation by preventing Cdc2 dephosphorylation. Additional support for this interpretation is provided by our observation that CBR activation increased phosphorylation of Cdc2 at Tyr-15. Further studies will be needed to evaluate the cellular localization of Cdc25B after cannabinoid application.

Cannabinoids have received increasing medical attention owing to their therapeutic potential to control many different diseases, including cancer.

Cannabinoids promote tumor regression by modulating signaling pathways involved in processes such as cell cycle progression, cell proliferation, cell death, angiogenesis and metastasis, migration and invasion, and epithelial–mesenchymal transition [5, 36-38]. It is well known that cannabinoids are capable of inducing cell cycle arrest in all phases [39]. In gastric cancer cells, treatment with Win 55,212-2 inhibits cell proliferation by arresting the G_0/G_1 phase and regulating the expression of various cell cycle-regulating proteins, including p27, p21 cyclin D1 and cyclin E [4, 40]. Cell proliferation is inhibited by anandamide application in hepatocellular carcinoma. Anandamide also attenuates cell cycle progression by arresting the G_1 phase [41]. 2-arachidonylglyceryl ether, a putative endocannabiniod, inhibits the cell cycle by arresting at the G_0/G_1 phase and inhibiting nuclear translocation of NF-κB in prostate carcinoma cells [42]. Treatment with delta 9-tetrahydrocannabinol induces cell cycle arrest at the G_0 phase and downregulates the expression levels of E2F1 and cyclin A in glioblastoma [43]. Anandamide also induces S phase growth arrest by regulating cell cycle-regulating proteins, including Chk1, Cdc25A and Cdk2 in breast cancer cells [44]. According to a previous study [45], treatment with delta-9-tetrahydrocannabinol, a CBR agonist, induces G_2/M cell cycle arrest in a human breast cancer cell line by increasing Wee1 protein levels and inactivating Cdc2. These results are similar to ours. During macrophageal differentiation, Win 55,212-2 alters expression and Tyr-15 phosphorylation of Cdc2 [46]. Antagonists of CBR, such as rimonabant, also modulate the cell cycle in colon and breast cancer cell lines by promoting arrest in the S phase [47]. Therefore, cannabinoids and related agents may be promising therapeutic agents for cancer treatment.

We used Win 55,212-2 to activate CB1R. Although Win 55,212-2 is known to be a high-affinity CB1R agonist [48, 49], it can also affect the signals of other receptors, including CB2R, vanilloid receptor and PPAR [50, 51]. Further studies will be needed to correlate the Win 55,212-2 effects to CB1R activation using its selective agonists or antagonists.

In this study, three major issues were addressed. First, we identified 14-3-3β as a binding partner for CB1R. Second, we showed that treatment with a CB1R agonist induces cell cycle arrest at the G_2/M phase in HeLa cells. Finally, we investigated the molecular mechanisms underlying the effect of Win 55,212-2 on cell cycle delay. Our results provide the first evidence that 14-3-3β is involved in CBR signaling in cancer cell lines.

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