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WIN55,212-2 Inhibits Production of CX3CL1 by Human Astrocytes: Involvement of p38 MAP Kinase

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Abstract

CX3CL1 (fractalkine) has been shown to be neuroprotective but also may play a role in human immunodeficiency virus (HIV)-1-associated neuropathogenesis. In this study, we found that production of CX3CL1 by human astrocytes stimulated with interleukin (IL)-1 β was inhibited in a concentration-dependent manner following pretreatment with the synthetic cannabinoid WIN55,212-2. The CB₂ receptor selective antagonist SR144528 significantly inhibited WIN55,212-2-mediated suppression of CX3CL1 suggesting a CB₂ receptor-related mechanism. IL-1 β triggered the activation of p38 and ERK1/2 (p44/42) MAP kinase (MAPK) signaling pathways, but WIN55,212-2 mainly inhibited p38 MAPK phosphorylation. This finding was mirrored in experiments using known inhibitors of these MAPKs suggesting that the suppression of CX3CL1 production by WIN55,212-2 involves inhibition of signaling via p38 MAPK. Our results support the concept that synthetic cannabinoids have anti-inflammatory properties and that these agents may have therapeutic potential for certain neuroinflammatory disorders.

Introduction

Expression of CX3CL1 in the brain is abundant where it is mainly found in neurons and astrocytes (Harrison et al. 1998). Upregulation of CX3CL1 expression was found in the brains of AIDS patients with human immunodeficiency virus type-1 (HIV-1)-associated dementia (HAD) and was mainly detected in astrocytes (Pereira et al. 2001). Marked upregulation of CX3CL1 has been observed in neurons and neuropil in brain tissue from pediatric patients with HIV-1 encephalitis (HIVE) (Tong et al. 2000), and increased CX3CL1 levels in cerebrospinal

fluid of HIV-1-infected, cognitively impaired patients has also been reported (Erichsen et al. 2003). These findings suggested a possible role of CX3CL1 in HIV-1 neuropathogenesis.

Cannabinoids have been shown to alter immune cell functions (Klein et al. 2003), including certain properties of the resident macrophages of the brain parenchyma, i.e. microglia (Cabral et al. 2008). These activities are mediated through either cannabinoid receptors (CB₁ or CB₂) or via non-cannabinoid receptor-mediated mechanisms. The synthetic cannabinoid agonist WIN55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)-methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate) is a CB₁/CB₂ agonist that has been shown to have beneficial effects in animal models of the neuroinflammatory disorder multiple sclerosis (Croxford and Miller 2003).

Astrocytes play a pivotal neuroprotective role but also have been implicated in neurodegenerative processes. These glial cells respond robustly to interleukin (IL)-1 β , a proinflammatory cytokine produced by activated microglia (Hu et al. 1999). Binding of IL-1 β to its receptors (mainly type I, IL-1R1) initiates downstream mitogen-activated protein kinase (MAPK) signaling pathways and upregulates many transcription factors which lead to a cascade of events culminating in regulation of gene expression.

As our laboratory has been interested in the anti-inflammatory activity of cannabinoids, this study was undertaken to determine whether WIN55,212-2 treatment would affect production of CX3CL1 by IL-1 β -stimulated human astrocytes and to investigate the signaling mechanism involved in WIN55,212-2's effect on CX3CL1 production.

Materials and Methods

Reagents

The following reagents were purchased from the indicated sources: recombinant human CX3CL1 and antibody to human CX3CL1 (R&D Systems, Minneapolis, MN); anti-p38 and -extracellular signal-regulated kinase 1 and 2 (ERK1/2 or p44/42) MAPK antibodies (Cell Signaling, Beverly, MA); SB203580 (an inhibitor of p38 MAPK), SB202474 (negative control of SB203580), U0126 (an inhibitor of MAP kinase kinase [MEK]1/2, upstream of ERK1/2) (EMD Biosciences, La Jolla, CA); gentamicin, Fungizone[®] and SuperScript[™] III reverse transcriptase (Invitrogen, Carlsbad, CA); WIN55,212-2, WIN55,212-3 (S(-)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate), Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salts (HBSS), penicillin, streptomycin, trypsin, Tween 20, phosphate buffered saline (PBS), poly-L-lysine, Tris, bovine serum albumin (BSA), random hexamer, primers and 3,3'-diaminobenzidine, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO); acrylamide/bis-acrylamide gel and protein assay (Bio-Rad, Hercules, CA); CDP-Star substrate (Applied Biosystems, Foster City, CA); K-Blue substrate (Neogen, Lexington, KY); heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT); SR141716- rimonabant [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] and SR144528 [*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide] (supplied by National Institute on Drug Abuse [NIDA], Bethesda, MD).

Astrocyte cultures

Astrocytes were prepared from 16- to 22-week-old aborted human fetal brain tissues obtained under a protocol approved by the Human Subjects Research Committee at our institution. Brain tissues were dissociated and resuspended in DMEM containing penicillin (100 U/ml), streptomycin (100 μ g/ml), gentamicin (50 μ g/ml) and Fungizone[®] (250 pg/ml) and plated onto

poly-L-lysine (20 µg/ml)-coated 75-cm² flasks at a density of 80–100 × 10⁶ cells/flask and incubated at 37°C in a 6% CO₂ incubator. Culture medium was changed at a weekly interval. On day 21, flasks were shaken at 180–200 rpm for 16h followed by trypsinization with 0.25% trypsin in HBSS for 30 min. After adding FBS (final concentration 10%), centrifugation and washing, cells were seeded into new flasks with DMEM followed by medium change after 24h. The subculture procedure was repeated four times at a weekly interval to achieve highly purified astrocyte cultures (99% of cells stained with anti-GFAP antibody) which were plated onto 12-well (10⁶ cells/well) or 48-well (10⁵ cells/well) plates for RNA extraction or ELISA assay, respectively.

Cell viability assay

To determine the effect of WIN55,212-2 and MAPK inhibitors on astrocyte viability a MTT assay, which provides quantitative assessment of mitochondrial integrity, was used. After treatment of astrocytes with WIN55,212-2 and inhibitors, MTT (final concentration of 1 mg/ml) was added to cell cultures for 4h followed by addition of lysis buffer (20% SDS [w/v] in 50% N,N-dimethyl formamide, pH 4.7, adjusted with 2.5% acetic acid and 1 N HCl [32:1]) for 16h. Cell lysate was collected and absorbance was read at 600 nm (Molecular Devices, Sunnyvale, CA) to reflect uptake of MTT by live cells.

Enzyme-linked immunoabsorbent assay (ELISA)

After treatment, astrocyte culture supernatants were collected for ELISA measurement (Sheng et al. 2005) of CX3CL1. In brief, 96-well ELISA plate pre-coated with mouse anti-human CX3CL1 antibody (2 µg/ml) overnight at 4°C was blocked with 1% BSA in PBS for 1h at 37°C. After washing with PBS with Tween 20, culture supernatants and a series of dilution of rhCX3CL1 (as standards) were added to wells for 2h at 37°C. Goat anti-human CX3CL1 detection antibody was added for 90 min followed by addition of donkey-anti-goat IgG horseradish peroxidase conjugate (1:10,000) for 45 min. A chromogen substrate K-Blue was added at room temperature for color development which was terminated with 1M H₂SO₄. The plate was read at 450 nm and CX3CL1 concentration was extrapolated from the standard concentration curve.

Western Blot

Cell lysates collected after treatment were electrophoresed in 12% acrylamide/bis-acrylamide, electrotransferred onto nitrocellulose membrane and probed with antibodies for MAPKs (p38 and p44/42) followed by alkaline phosphatase-conjugated secondary antibodies with chemiluminescence detection using Kodak Image Station (Carestream Health (formerly Kodak), New Haven, CT).

Statistical analysis

Data are expressed as mean ± SD or SE as indicated. For comparison of means of multiple groups, analysis of variance (ANOVA) was used, followed by Fisher's PLSD test.

Results

Prior to assessing the effects of WIN55,212-2 on CX3CL1 production, experiments were carried out to verify that IL-1β's stimulatory effect on CX3CL1 by astrocytes is concentration- and time-dependent (data not shown), and for all subsequent experiments we stimulated astrocytes with 10 ng/ml IL-1β for 72h, which gave optimal production of CX3CL1. Pretreatment of astrocytes with various concentrations (0.3 – 10 µM) of WIN55,212-2 or the inactive enantiomer WIN55,212-3 for 1h prior to IL-1β exposure for 72h demonstrated that CX3CL1 production was significantly inhibited by WIN55,212-2 in a concentration-dependent

manner, while WIN55,212-3 exhibited no effect even at the highest concentration (10 μ M) (Fig. 1A). The concentrations of WIN55,212-2 or WIN55,212-3 used showed no toxicity to astrocytes by MTT assay (data not shown).

Astrocyte cultures were next pretreated with selective cannabinoid receptor antagonists SR141716A (for CB₁) and SR144528 (for CB₂) for 1h prior to WIN55,212-2 treatment for 1h followed by stimulation with IL-1 β for 72h. While the CB₂ antagonist SR144528 significantly blocked (by 74%) WIN55,212-2's inhibitory effect, the CB₁ antagonist SR141716A had little effect (Fig. 1B), suggesting that WIN55,212-2 acts mainly via a CB₂-related mechanism. When astrocytes were treated with a combination of SR141716A and SR144528, blockade of WIN55,212-2's action was no greater than with SR144528 alone (Fig. 1B).

Next, we were interested to determine whether WIN55,212-2's inhibitory effect on CX3CL1 production involved an effect on a MAPK signaling pathway. As IL-1 β is known to activate both p38 and ERK1/2 MAPKs, we first wanted to assess whether production of CX3CL1 by IL-1 β -stimulated human astrocytes involves p38 and/or ERK1/2 MAPK. To do so, we pretreated astrocytes with SB203580 (an inhibitor of p38 MAPK activation) and U0126 (an inhibitor of MEK1/2, upstream of ERK1/2) for 1h prior to stimulation with IL-1 β for 72h. As is shown in Figure 2A, SB203580, but not the negative control compound SB202474, significantly suppressed IL-1 β -induced production of CX3CL1, whereas the ERK1/2 inhibitor U0126 had no effect, suggesting that IL-1 β -induced production of this chemokine by astrocytes involves p38 MAPK signaling. Treatment with these inhibitors alone (0.3-30 μ M) did not induce astrocyte toxicity by MTT assay (data not shown).

Based on these findings, we then tested the hypothesis that WIN55,212-2 would inhibit activation of p38 MAPK in IL-1 β -stimulated astrocytes. Exposure of astrocytes to IL-1 β induced the phosphorylation of p38 (p-p38) and of ERK1/2 (p-p44/42) MAPKs, while total p38 and p44/42 MAPK remained unchanged (Fig. 2B). When pretreated with WIN55,212-2 overnight, IL-1 β -induced p-p38 MAPK was most affected (71% inhibition, as determined by densitometry), whereas minimal alteration of p-p44/42 MAPK (16% inhibition) was observed (Fig. 2B), supporting the hypothesis that WIN55,212-2's inhibitory effect on CX3CL1 production involves suppression of IL-1 β -mediated p38 MAPK signaling.

Discussion

The results of this study demonstrated that the synthetic cannabinoid WIN55,212-2 is capable of inhibiting production of CX3CL1 by human astrocytes stimulated with IL-1 β through a mechanism that involves activation of CB₂ and inhibition of intracellular signaling via p38 MAPK. Taken together with a growing literature on a variety of anti-inflammatory properties of cannabinoids, these findings support the concept that synthetic cannabinoids could be exploited as potential therapeutic agents for neuroinflammatory disorders (Klein 2005). The therapeutic potential of cannabinoids in CNS diseases (Croxford 2003) has been shown in experimental models of multiple sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease and in HIV-1-associated dementia. Properties of cannabinoids that could explain their neuroprotective potential have been postulated to include anti-inflammatory and anti-oxidant effects and inhibition of calcium influx and neurotransmitter production.

Although CX3CL1 is present mainly in neurons in the normal resting CNS, under pathological conditions CX3CL1 production is upregulated in astrocytes (Pereira et al 2001). The receptor for this chemokine, i.e. CX3CR1, has been shown to be expressed in microglia and neurons and to be upregulated in microglia in the context of chronic neurodegeneration (Hughes et al. 2002). The results suggest that CX3CL1 production by reactive astrocytes could play a role in

controlling migration and functional properties of surrounding microglia (Sunnemark et al. 2005).

While there are reports of neuroprotective properties of CX3CL1 against HIV-1 gp120_{III}B protein or glutamate toxicity (Meucci et al. 2000), other studies have shown that CX3CL1 was upregulated in HAD and HIVE patients (Erichsen et al. 2003) suggesting the involvement of CX3CL1 in HIV-1 neuropathogenesis. It is reasonable to postulate that multiple factors in the local environment, e.g. cytokines/chemokines as well as blood-brain barrier integrity (Cardona et al. 2006) may determine whether CX3CL1-CX3CR1 interactions have deleterious or beneficial consequences in the brain.

Previously, we have reported that astrocytes express both cannabinoid receptors CB₁ and CB₂, and that WIN55,212-2 downregulated the production of several inflammatory mediators (NO, cytokines and chemokines) in IL-1 β -stimulated human astrocytes (Sheng et al. 2005). In the present study, the inhibitory effect of WIN55,212-2 on CX3CL1 production in astrocytes was antagonized more effectively by the CB₂ antagonist SR144528 than by the CB₁ antagonist SR141716A. However, pretreatment with a combination of these antagonists was no more effective than SR144528 alone in the blockade of WIN55,212-2's inhibitory effect suggesting that the effects of WIN55,212-2 were mediated mainly by CB₂. As the inhibitory effect of SR144528 was incomplete, i.e. 74%, involvement of non-CB₁/CB₂ receptors cannot be ruled out. Because of possible psychotropic effects of WIN55,212-2 through CB₁ activation, use of highly selective CB₂ agonists would be better candidates for clinical assessment. Because of overlapping distribution and co-localization of cannabinoid and mu opioid receptors in the brain and the common responses induced by their ligands, a cross-talk between these receptors would be interesting to explore in the future.

MAPKs are required for many cellular and developmental processes. The evidence in this study that WIN55,212-2-mediated inhibition of CX3CL1 production was associated with suppression of p38 MAPK activation by IL-1 β -stimulated astrocytes suggests that inhibition of this intracellular signaling pathway plays a key role in WIN55,212-2's effect on these glial cells. Whether WIN55,212-2 also modulates other signaling cascades triggered by activation of cytokine or Toll-like receptors expressed by astrocytes remains to be elucidated.

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Figure 1.

Inhibitory effect of WIN55,212-2 on CX3CL1 production by astrocytes. (A) Concentration-response study of WIN55,212-2 and the inactive enantiomer WIN55,212-3. Astrocytes were treated with cannabinoids for 1h prior to IL-1 β exposure (10 ng/ml) for 72h. Culture supernatants were collected to measure CX3CL1 levels by ELISA. Data are representative of mean $\pm$ SD of triplicates of 2-3 separate experiments using astrocyte cultures derived from different brain tissue specimens. * p <0.05, ** p <0.01 vs. IL-1 β alone. C, untreated control. (B) Cannabinoid receptor-mediated inhibitory effects of WIN55,212-2. Astrocyte cultures were pretreated with CB₁ (SR141716A) or CB₂ (SR144528) antagonists (3 μ M) for 1h prior to WIN55,212-2 (3 μ M) treatment for 1h followed by IL-1 β exposure (10 ng/ml) for 72h. Culture

supernatants were collected to measure CX3CL1 levels by ELISA. Data are mean $\pm$ SE of triplicates of 4 separate experiments using astrocyte cultures from different brain tissue specimens. ** $p < 0.01$ vs. IL-1 β alone, $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ vs. WIN55,212-2 + IL-1 β .

Figure 2.

Involvement of p38 MAPK in (A) CX3CL1 production and (B) WIN55,212-2's inhibitory effect. (A) Astrocyte cultures were pretreated for 1h with a) p38 MAPK inhibitor SB203580 or negative control SB202474 (0.3, 3, 30 μ M) or b) MEK1/2 inhibitor U0126 (3, 10, 30 μ M) prior to IL-1 β exposure for 72h. Culture supernatants were collected to measure CX3CL1 levels by ELISA. Data are mean $\pm$ SE of triplicates of 2-3 separate experiments using astrocyte cultures from different brain tissue specimens. ** $p < 0.01$ vs. untreated control (C). (B) After replacing culture media with DMEM containing 1% serum for 24h prior to pretreatment with WIN55,212-2 (10 μ M) overnight followed by IL-1 β exposure (10 ng/ml) for 30 min, cell lysates

were electrophorezed (in 12% gel), transblotted and probed with anti-phosphorylated or -total p38 and p44/42 MAPK antibodies.