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The cannabinoid agonist WIN 55,212-2 inhibits TNF-α-induced neutrophil transmigration across ECV304 cells

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Abstract

Cannabinoids are known to possess both anti-inflammatory and neuroprotective effects. In the present study, we have investigated the ability of cannabinoids to inhibit the transmigration of neutrophils in response to chemotaxic stimuli. The cannabinoid receptor agonist WIN 55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) significantly decreased the number of migrating neutrophils across a monolayer of tumour necrosis factor alpha (TNF-α) activated ECV304 cells at concentrations ≥1 μM. In contrast, the agonists HU210 and CP 55,940 (0.01–1 μM) and the endocannabinoid anandamide (0.1–10 μM) were without significant effect on the response to TNF-α. The ability of WIN 55,212-2 to reduce the neutrophil transmigration was still seen in the presence of the cannabinoid CB_1 receptor antagonist/inverse agonist AM251 (0.1–1 μM) and the cannabinoid CB_2 receptor antagonist/inverse agonist AM630 (0.1–1 μM). TNF-α treatment of ECV304 cells caused release of interleukin-8 (IL-8), but WIN 55,212-2 did not affect either the ability of neutrophils to migrate across chemotaxis plates in response to an IL-8 stimulus, or to change the percentage of CXC 1 and CXC 2 receptors expressed by the neutrophils. WIN 55,212-2 at a concentration of 1 μM, but not at lower concentrations, produced a significant inhibition of IL-8 release from ECV304 cells in response to TNF-α-stimulation. Thus WIN 55,212-2 reduces the transmigration of neutrophils across a monolayer of TNF-α-activated ECV304 cells by an indirect action upon the release of IL-8 and/or other chemokine release from the ECV304 cells, and that this effect is brought about mainly by a cannabinoid CB receptor-independent mechanism.

Keywords: Neutrophil; Cannabinoid; Transmigration; Chemotaxis; Interleukin 8

1. Introduction

It is now well established that Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive ingredient derived from the plant *Cannabis sativa*, possesses a variety of pharmacological actions in biological systems that are brought about mainly (but not exclusively) via two G-protein-coupled cannabinoid (CB) receptors, termed CB_1 and CB_2. Cannabinoid CB_1 receptors are essentially neuronal in localisation, whereas cannabinoid CB_2 receptors are confined to peripheral immune cells and central activated microglia (for review, see Howlett, 2002). Compounds able to activate cannabinoid CB_1 and CB_2 receptors include the synthetic cannabinoids WIN 55,212-2, HU210 and CP 55,940 and the endogenous fatty acid amide arachidonoylethanolamide (anandamide) (Howlett, 2002), although evidence is emerging to show that Δ^9-THC, anandamide and WIN 55,212-2 can also produce pharmacological actions via cannabinoid CB_1 and CB_2 receptor-independent pathways (for review, see Begg et al., 2005). The current interest in cannabinoids is due not the least to the identification of possible therapeutic areas where such compounds may be useful (see Baker et al., 2003; Di Marzo et al., 2004, for reviews). In this respect, cannabinoids have been reported to have neuroprotective effects, although the mechanisms underlying these effects are not entirely clear (Berger et al., 2004; Conti et al., 2002; Muthian et al., 2004; Nagayama et al., 1999; Smith et al., 2001b; van der Stelt et al., 2001).

Most studies concerning neuroprotection have focused on excitotoxicity and its modulation by cannabinoids. However, one important feature that contributes to nerve damage following an ischemic stroke is the infiltration of leukocytes,

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primarily neutrophils, into the brain during the reperfusion phase. Inappropriate activation of neutrophils can cause damage to the surrounding tissue by release of reactive molecules normally involved in rendering pathogens harmless (for review, see del Zoppo et al., 1991; Dirnagl et al., 1999). The local inflammation produced by dying cells increases the release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 1β. The release of pro-inflammatory substances produces a cytokine and chemokine cascade that attracts neutrophils from the blood stream and triggers transmigration largely stimulated by increased expression of intercellular adhesion molecule 1 (ICAM-1) and with interleukin 8 (IL-8) acting as a chemotactic cue (Kuijpers et al., 1992; Yoshimura et al., 1987). Experiments using cytokine antagonists and ICAM antibodies have provided data that show a decreased infarct volume along with a decreased number of leukocytes in the brain parenchyma in neurodegenerative animal models (Nawashiro et al., 1997; Zhang et al., 1994). Several studies employing mouse models of multiple sclerosis have reported positive effects of cannabinoids on both symptom management and disease progression (Baker et al., 2000; Pryce et al., 2003). This is interesting in the current context of neutrophil migration since multiple sclerosis is considered an autoimmune neuroinflammatory disease where leukocytes, primarily monocytes, crossing the blood-brain barrier are a crucial part of the pathology, see reviews (Raivich and Banati, 2004; Ransohoff, 2002).

In contrast to the *in vivo* data, it is not known as to whether cannabinoids can influence neutrophil migration *in vitro*. Such information would provide useful mechanistic data, and in the present study we have investigated if cannabinoids can influence neutrophil migration in a simple *in vitro* model based upon monolayers of ECV304 cells (Hurst et al., 1998). The effects of various cannabinoid receptor ligands upon TNF-α stimulated neutrophil transmigration and IL-8-induced chemotaxis were examined. Furthermore, the effect of the cannabinoid receptor agonist WIN 55,212-2 on TNF-α stimulated IL-8 release from ECV304 cells was assessed.

2. Materials and methods

2.1. Chemicals

(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN 55,212-2), (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP 55,940), (6a*R*)-*trans*-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol (HU210), *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM 251) and 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone (AM 630) were obtained from Tocris, Bristol, UK. Anandamide was obtained from Cayman Chemical Company, Ann Arbor, USA. Ibuprofen and thapsigargin was obtained from Sigma, St Louis, MO, USA. 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059) was purchased from Calbiochem, San Diego, CA, USA. All cell culture media, sera and all supplements were purchased from Invitrogen, Lidingö, Sweden.

2.2. Preparation of neutrophils

Blood (30 ml) was collected in ethylendiaminetetraacetic acid (EDTA) treated tubes (Sarstedt, Landskrona, Sweden) from healthy volunteers. Briefly, after centrifugation at 400 ×*g* for 20 min the buffy coat was collected and erythrocytes were lysed at 4 °C with erythrocyte lysis buffer (42 mM NH_4Cl, 2.4 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). The cells were pelleted and resuspended in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 0.15 M NaCl, 2.7 mM Na_2HPO_4) with 0.5% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.4. For the neutrophil chemotaxis assay the neutrophils were purified in a MidiMACS LS column using $CD15^+$ antibodies for positive selection of neutrophils following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). For the transmigration assay whole blood was applied on a Polymorphprep gradient to band the neutrophils. The neutrophils were recovered and residual erythrocytes were lysed with erythrocyte lysis buffer. Following two washing steps in 10 ml PBS containing 0.5% BSA and 2 mM EDTA, pH 7.4, the neutrophil suspension was labelled with 5 μg/ml Calcein AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C and thereafter washed twice in PBS with 0.5% BSA and 2 mM EDTA, pH 7.4, and resuspended in M199 medium with 10% fetal bovine serum (FBS). The collection of blood samples from volunteers was approved by the local ethical committee at Umeå University.

2.3. Neutrophil transmigration assay

ECV304 was obtained from the European Collection of Animal Cell Cultures, Salisbury, UK. The cells (passages 182–197) were cultured in T-75 bottles in M199 medium with 10% FBS, 1% penicillin/streptomycin and 2 mM glutamine (complete medium). The cells were passaged and seeded at 500 cells/mm^2 in Transwell inserts (110 mm^2; 3 μm pore size; Corning Inc, Acton, USA). The integrity of the cell layer was monitored by measuring the transendothelial electrical resistance using an EVOM epithelial tissue voltohmmeter with an ENDOHM-12 planar electrode chamber (World Precision Instruments, Hertfordshire, UK). The transendothelial electrical resistance value reflects expression of tight junctions between the cells in the monolayer. The cells were grown on inserts 10–14 days before the assay was performed, and the transendothelial electrical resistance values were 85±22 Ω cm^2. Test compounds were added to the monolayers and incubated for 45 min at 37 °C. Thereafter, the monolayers were stimulated for 4 h at 37 °C with 20 ng/ml TNF-α dissolved in M199 medium. This 4 h incubation with TNF-α did not produce any change in the transendothelial electrical resistance values of the ECV304 monolayers, compared with controls not treated with this cytokine. In the experiments using AM251 and AM630, the compounds were added shortly (~5 min) before the incubation with WIN 55,212-2. The medium was changed to M199 without TNF-α and Calcein AM-labelled neutrophils were added and