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The synthetic cannabinoid WIN 55,212-2 increases COX-2 expression and PGE_2 release in murine brain-derived endothelial cells following Theiler's virus infection

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Abbreviations:
2-AG, 2-arachidonoyl glicerol
Abn-CBD, abnormal cannabidiol
AEA, anandamide
CB, cannabinoid
CB1, cannabinoid receptor 1
CB2, cannabinoid receptor 2
COX-2, cyclooxygenase-2
IL-1β, interleukin-1 beta
IL-12, interleukin-12
LDH, lactate dehydrogenase
MOI, multiplicity of infection
MS, multiple sclerosis
PGs, prostaglandins
PGE_2, prostaglandin E_2
SR1, SR141716A
SR2, SR144528
TMEV, Theiler's virus

ABSTRACT

Brain endothelial cells infection represents one of the first events in the pathogenesis of TMEV-induced demyelination disease (TMEV-IDD), a model of multiple sclerosis (MS). The fact that cyclooxygenase-2 (COX-2) expression in brain endothelium mediates a wide variety of actions during CNS inflammatory diseases such as MS, and that cannabinoids ameliorate the progression of TMEV-IDD, lead us to investigate the role of cannabinoids on COX-2 expression on murine brain endothelial cell cultures subjected or not to TMEV infection. Murine brain endothelial cells (b.end5) express both cannabinoid receptors CB1 and CB2. However, treatment of b.end5 with the cannabinoid agonist WIN 55,212-2 resulted in up-regulation COX-2 protein and PGE_2 release by a mechanism independent on activation of these receptors. Other cannabinoids such as 2-arachidonoyl glycerol (2-AG) or the abnormal cannabidiol (Abn-CBD) failed to affect COX-2 in our conditions. TMEV infection of murine brain endothelial cell cultures induced a significant increase of COX-2 expression at 8 h, which was maintained even increased, at 20 and 32 h post-infection. The combination of TMEV infection and Win 55,212-2 treatment increased COX-2 expression to a greater amount than was seen with either treatment alone. 2-AG and Abn-CBD did not modify COX-2 expression after TMEV. COX-2 synthesis involved different signaling pathways when was induced by WIN 55,212-2 and/or by TMEV infection. WIN 55,212-2-induced COX-2 up-regulation involves the PI_3K pathway, whereas COX-2 induction by TMEV needs p38 MAPK activation too. Overexpression of COX-2 and the subsequent increase of PGE_2 could be affecting flow blood and/or immune reactivity.

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TMEV-IDD, Theiler's virus-induced demyelination disease
TRPV1, vanilloid receptor

1. Introduction

CNS inflammation is a key feature associated with neurodegenerative disorders including multiple sclerosis (MS). MS is a chronic inflammatory demyelinating disease characterized by perivascular infiltration of lymphocytes and macrophages into the brain parenchyma. Cyclooxygenase-2 (COX-2) immunoreactivity has been found in MS and in animal experimental models specially associated to macrophages and endothelial cells [1,2]. Cyclooxygenases catalyse the first step in the synthesis of prostanoids, a large family of arachidonic acid metabolites, including prostaglandins (PGs), prostacyclin and thromboxanes. The inducible isoform COX-2 is involved in the mediation of inflammation, immunomodulation, blood flow, apoptosis and fever [3,4]. COX-2 is rapidly expressed *on* several cell types in response to growth factors, proinflammatory molecules and cytokines [5–7]. Proinflammatory cytokines such us IL-1β and TNF-α, increase the expression of COX-2 in brain microvessel endothelial cells and this has been related to increases in permeability *of* the cerebral microvasculature [4,8]. A relationship between cannabinoids and PGs has been established by several lines of evidence. In particular, dilation of cerebral arterioles or reduction of intraocular pressure has been associated with increased generation of PGs [9–11] *as* important regulators of blood flow. The endogenous cannabinoid anandamide (AEA) has been reported to cause vasodilation in different preparations through both endothelium-dependent and independent mechanisms [12,13]. In addition, the cannabinoid analogue abnormal cannabidiol (Abn-CBD) induced vasorelaxation in rat isolated mesenteric artery segments by a mechanism that is endothelium-dependent [14]. Cannabinoids have been reported to be of potential therapeutic value in the treatment of several neuroinflammatory diseases including MS [15,16]. The production of several PGs, secondary to induction of COX-2, by the cells lining the blood–brain barrier which may diffuse to the brain parenchyma may have important consequences in brain inflammatory processes by modulating blood flow but also the intracerebral immune responses. The synthetic cannabinoid WIN 55,212-2 has been shown to attenuate the progression of clinical disease symptoms in the Theiler's murine encephalomyelitis virus model of MS [17,18]. This protective effect has been associated, at least in part, to the ability of WIN 55,212-2 to diminish neuroinflammation. Recently, it has been reported that WIN 55,212-2 inhibited the adhesion and entry of leukocytes in the brain in experimental allergic encephalomyelitis (EAE) MS model [19]. However, the possible cellular targets and the mechanisms responsible for the WIN 55,212-2 effects were not addressed and remain still unclear. Virus infection of brain endothelial cells may represent one of the first events in the pathogenesis of TMEV-induced demyelinating disease. The purpose of the present work was to study whether WIN 55,212-2 modifies COX-2 expression in cerebral endothelial cells in resting conditions and/or after the infection with TMEV. The participation of CB1 and CB2 receptors as well as the effects of other cannabinoid ligands, such as the endocannabinoid, 2-arachidonoyl glycerol (2-AG) and the cannabidiol analogue Abn-CBD on COX-2 expression were also addressed.

2. Materials and methods

2.1. Materials

WIN 55,212-2 (*R*-(+)-2,3-dihydro-5-methyl-3-4-morpholinyl-methyl-pyrrolo-(1,2,3,-de)-1-4 benzoxazinyl 1-naphthalenyl methanone mesylate) was purchased from Tocris Cookson Ltd. (UK). SR141716A (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazol-carboxamide and SR144528 (*N*-[1*S*)-endo-1,3,3,-trimethylbicyclo [2.2.1]heptan-2-yil-5-(4-chloro-3methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) were gift from Sanofi Recherche (Montpellier, France). 2-Arachidonoyl glycerol and trans-4-3-methyl-6-(1-methylethenyl)-2 cyclohexen-1yl-5 pentyl-1,3-benzenediol (Abn-CBD) were from Tocris Cookson Ltd. (UK). Capsazepine was obtained from Alexis Biochemicals. The specific COX-2 inhibitor, NS-398, the MEK inhibitor PD98059, and the inhibitor of p38 MAPK, SB203580 were obtained from Calbiochem Novabiochem (UK) and the PI_3K kinase inhibitor LY294002 was purchased by Cell Signaling Tech (Beverly, MA, USA).

2.2. Cell cultures

Murine brain endothelial cells (b.end5), which are recognized to present brain endothelium like properties, were obtained from ECACC (UK). This cell model is an appropriate choice to study blood–brain barrier function [20]. The cells were plated in (10^5 cells/ml) into 12-well plates and allowed to grow in Dulbeccos's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (BSA) and 1% antibiotic penicillin and streptomycin. Cells were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. The cells were allowed to achieve confluence typically in two days. The cannabinoids, WIN 55,212-2, 2-AG or Abn-CBD were dissolved in DMSO (the final concentration of DMSO was 0.1% (v/v). Primary brain endothelial cells were cultured according to a previously described method [21] with slight modifications. Cells were isolated from the brain of SJL/J mice (10–12 pups) of 7 days old. Endothelial cells in the cultures were characterized by immunostaining with antibodies for the endothelial marker PECAM-1 (CD-31).

2.3. Cell cultures infection

The Daniel's (DA) strain of TMEV was plaque purified on BHK-21 cells, and viral titer was determined by standard plaque

assay on BHK-21 cells. Cell cultures were infected with the DA strain of TMEV at a multiplicity of infection (MOI) of 2 plaque format units (PFU) per cell. Brain endothelial cell cultures were washed twice to remove serum components and 0.25 ml of appropriately diluted virus stock solution was added to each well. After adsorption of the virus for 2 h at 37 °C we added 0.75 ml of new medium containing only 2% FBS.

2.4. Cell cultures immunocytochemistry

For immunostaining of brain endothelial cell cultures, cells plated onto coverslips were fixed with 4% paraformaldehyde for 30 min. After being washed with PBS, the fixed cells were incubated overnight at 4 °C with the primary antibodies against platelet endothelial cell adhesion molecule (PECAM-1, CD31, monoclonal antibody; BD Pharmigen, San Diego, CA, USA) at a dilution 1:500, CB1 cannabinoid receptor (Calbiochem, Darmstad, Germany) at a dilution 1:100, CB2 cannabinoid receptor (Affinity BioReagents, Golden Co., USA) at a dilution 1:100, and COX-2 murine polyclonal antibody (Cayman Chemical, MI, USA) at a dilution 1:500. After being rinsed with PBS, the cells were incubated for 1 h at RT with secondary anti-rat and anti-rabbit antibodies IgGs conjugated with Alexa 488 or Alexa 594, respectively (Molecular Probes, Eugene, OR, USA). Coverslips were mounted on glass slides with fluorescent mounting medium. In all cases, specificity of staining was confirmed by omitting the primary antibody or by incubating with the specific immunogen.

2.5. Western blot analysis

Cells were lysed in 200 μl of Tris-buffered saline (TBS) pH 7.6 containing 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml leupeptin, 10 μg/ml aprotinin, 5 mM benzamidine, 1 mM sodium orthovanadate, 2 mM NaF and 5 mM DTT. Whole-cell lysates were mixed with 5× Laemmli sample buffer and boiled for 5 min. Equal amounts of protein (30 μg) were resolved in 10% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose at 4 °C. The membrane blots were blocked for 1 h with 5% dry milk in TBS containing 0.1% Tween-20 and then incubated overnight at 4 °C with the primary antibody anti-COX-2 (Cayman Chemicals, MI, USA), at a dilution 1:2000. Then, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT and visualized by chemiluminescence using an ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech). The blots were stripped using a 2% SDS and 0.7% (-mercaptoethanol solution in 62.5 mM Tris buffer, pH 6.8 and reprobed with α-Tubulin (Sigma Aldrich Inc., MI, USA) at a dilution 1:40,000.

2.6. PGE_2 measurement

The levels of PGE_2 in the supernatants of endothelial cell cultures were measured by Competitive Enzyme Immunoassay EIA, using a monoclonal PGE_2 antibody (Cayman, Ann Arbor, MI, USA). The minimum detectable dose of PGE_2 was less than 15 pg ml^{-1} and the intra and inter coefficient of variation were 4.9 ± 0.6 and 11.2 ± 1.3, respectively.

2.7. Cell viability analysis

The effect of TMEV infection or the treatment with WIN 55,212-2 on b.end5 cell viability was assessed using a colorimetric cytotoxicity assay based on the quantitative measurement by spectrophotometric analysis of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released following cell lysis. Control and treated cells were evaluated for LDH in the medium for different periods of time. Data are expressed relative to total LDH activity in the cells measured in supernatants from untreated cells previously lysed in Triton X-100 according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Absorvance data were obtained using a 96-well plate reader with a 490 nm filter.

2.8. Statistical analysis

Results are presented as mean ± S.E.M. of at least three independent experiments; triplicate determinations were performed in each experiment. One-way ANOVA, followed by a posteriori Tukey's multiple comparison test was used to examine the statistical significance; P-values < 0.05 were considered significant.

3. Results

3.1. Murine brain endothelial cell line (b.end5) expressed CB1 and CB2 receptors

The expression of CB1, CB2 and TRPV1 receptors has been described on human cerebrovascular endothelial cells (HBEC) [22]. Here, CB1 (Fig. 1A and B) and CB2 (Fig. 1C and D) are expressed in the murine brain endothelial cell line b.end5 as examined by immunocytochemistry. Western blot detection confirmed the above data as shown in Fig. 1E. CB1 receptor protein was evidenced by the presence of a prominent band with a molecular mass of 60 kDa, consistent with other reports [23]. The CB2 receptor appeared as a band with a molecular mass around 32 kDa [24]. In order to ensure that brain endothelial cells from SJL/J mice expressed CB receptors, we performed immunocytochemical analyses using primary brain endothelial cell cultures and confirmed the presence of CB1 and CB2 receptors in these cells (data not shown).

3.2. WIN 55,212-2 induces an increase on COX-2 expression by murine brain derived endothelial cells

We initially evaluated the effects of the mixed CB1/CB2 synthetic agonist WIN 55,212-2 on the expression of COX-2. Western blot analysis showed that COX-2 protein was up-regulated, in a dose dependent manner, in murine brain derived endothelial cells, b.end5, treated with WIN 55,212-2 for 20 h (Fig. 2A). The lowest effective dose in inducing COX-2 expression was 100 nM and the higher dose used (1 μM) was as effective as the dose of 100 nM. WIN 55,212-2 at 100 nM was also able to increase mRNA expression of COX-2 in brain endothelial cell cultures obtained from SJL/J mice (data not shown). We next examined whether WIN 55,212-2 *induces*

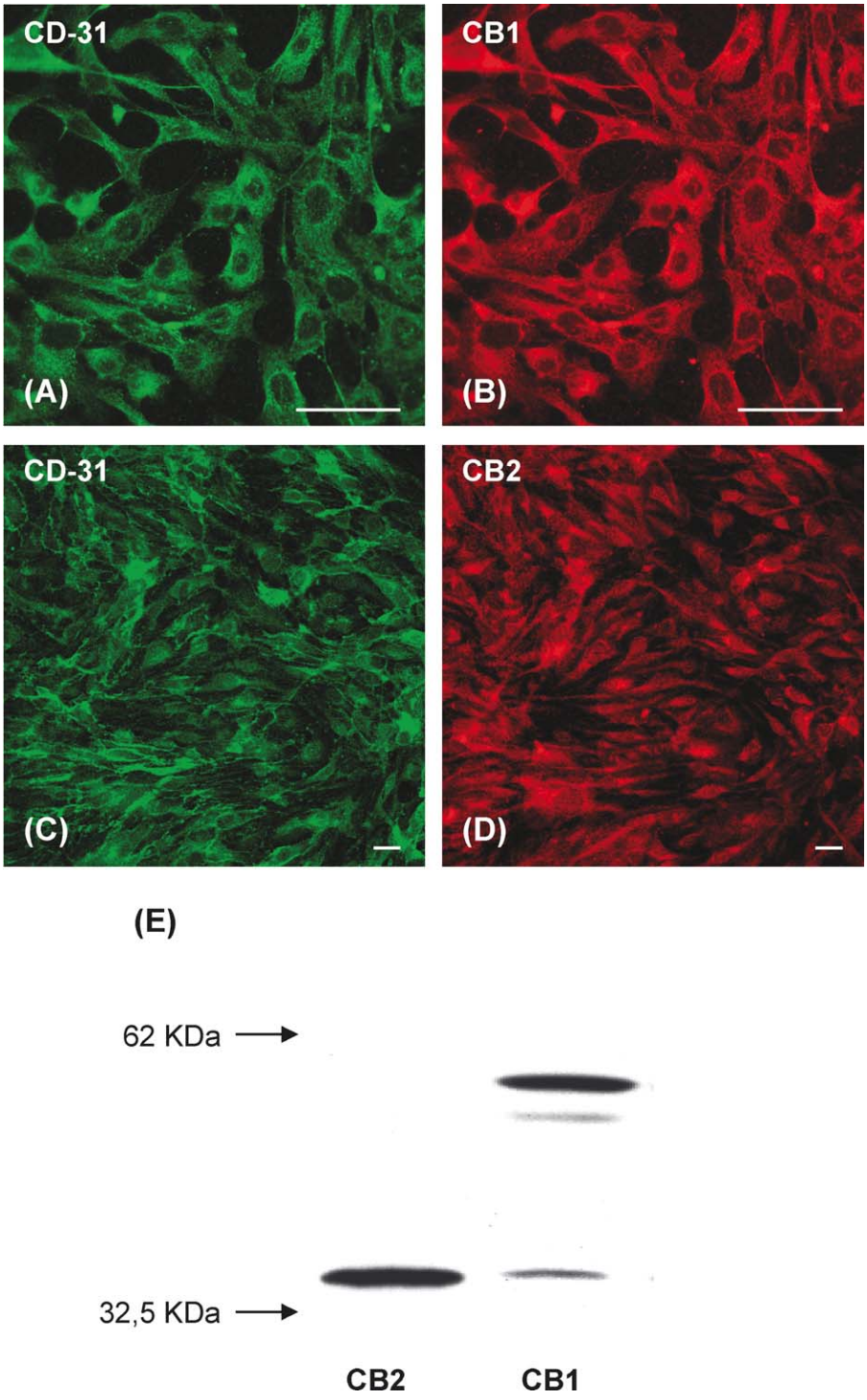

Fig. 1 – Murine brain derived endothelial cells express CB1 and CB2 receptors. b.end5 cells were double stained by immunofluorescence labelling with anti-CB1 or anti-CB2 (1:100 dilution) antibodies (red) (B and D, respectively) and anti-CD31 (1:500 dilution) antibody (green) (A and C). Non-specific interactions of secondary antibodies were verified by omitting the primary antibodies. Scale bar (40 μM). Whole protein of cell-lysates of brain derived endothelial cells were used to detect CB1 and CB2 receptors by Western blotting as it has shown in panel E.

COX-2 protein in the cell line b.end5 was dependent on activation of CB1 or CB2 receptors by using the selective receptor antagonists, SR141716A and SR144528, respectively (Fig. 2C). The effect of WIN 55,212-2 (100 nM) on COX-2 expression was not significantly blocked by the presence of the CB1 antagonist SR141716A (1 μM) neither by the CB2 antagonist SR144528 (1 μM), nor by simultaneous addition of both antagonists pointing to the lack of involvement of both

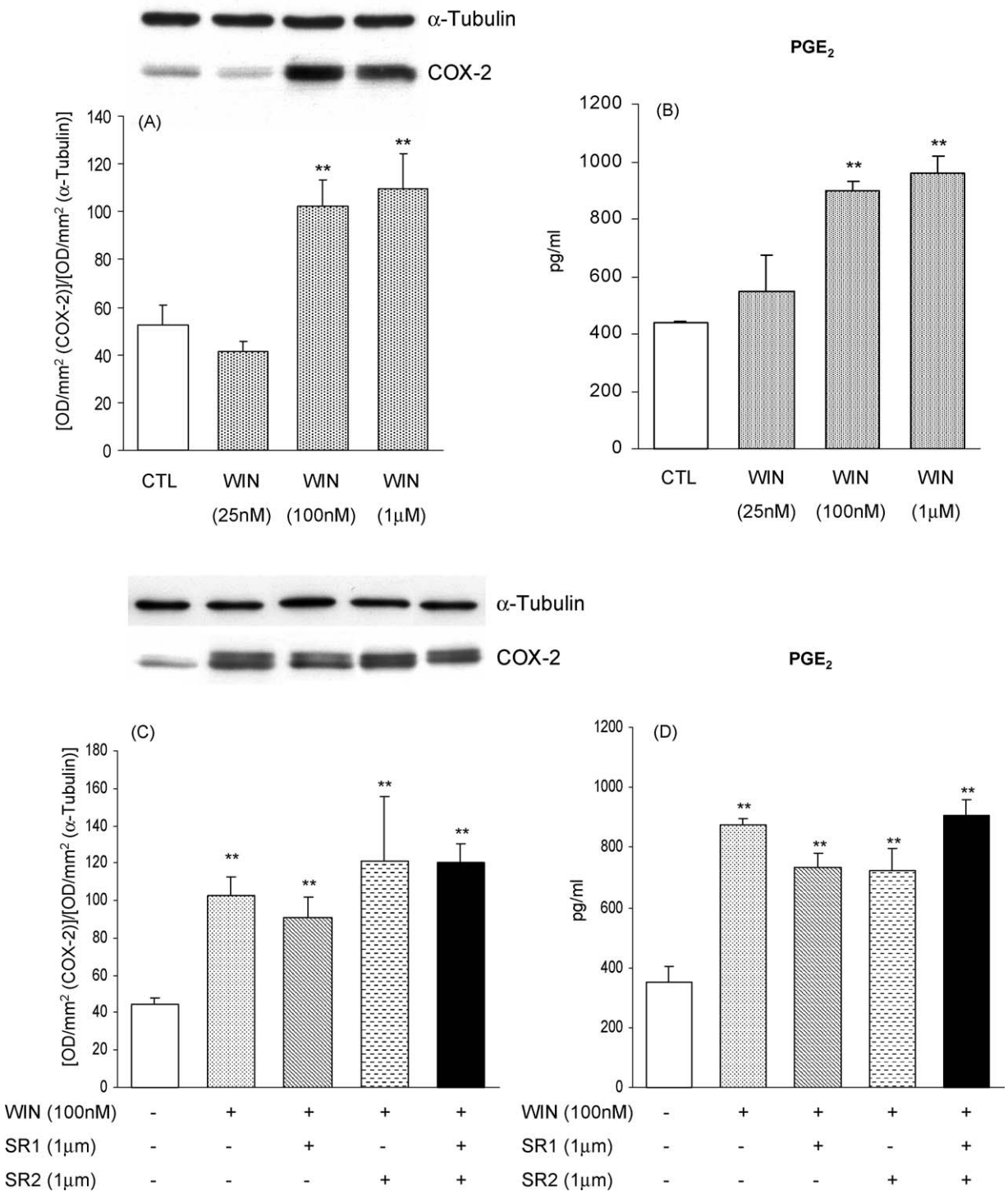

Fig. 2 – WIN 55,212-2 induce COX-2 expression by an independent mechanism of cannabinoid receptors. Confluent brain endothelial cells monolayers (b.end5) were treated for 20 h with different concentrations of WIN 55,212-2 (25 nM, 100 nM or 1 μM). Proteins of cell-lysates (30 μg) were subjected to Western immunoblot analysis with anti-COX-2 antibody and are presented as a relative ratio % where COX-2 signal, obtained by densitometric analysis, was normalized to α-Tubulin (A). PGE_2 levels were measured by ELISA in supernatants of these same cells (B). In other experiments b.end5 cells were pre-treated for 1 h with cannabinoid receptors antagonists (SR1 for CB1 receptor or/and SR2 for CB2 receptor) and results of COX-2 induction are presented like the same way as a relative ratio % (C). In supernatants of these cells PGE_2 were measured by ELISA (D). Results show the means ± S.E.M. from three independent experiments done in triplicate. $^{**}P < 0.01$ vs. CTL.

receptors. Neither SR141716A (1 μM) nor SR144528 (1 μM) modified COX-2 expression (COX-2/Tubulin: CTL: 37.46 ± 4.88; SR1: 45.81 ± 12.73; CTL: 49.98 ± 7.056; SR2: 59.97 ± 8.69). Because PGE_2 is one of the major PGs produced by cerebral vascular endothelial cells [25], PGE_2 production was measured as an index of COX activity. Cells treated with WIN 55,212-2 (100 nM) experimented about two fold increase in PGE_2 release that were not significantly modified by the selective CB1 or CB2 receptor antagonists alone or in combined treatment (Fig. 2D). Neither SR141716A (1 μM) nor SR144528 (1 μM) alone was capable of modifying PGE_2 levels (not shown). However, WIN 55,212-2 (100 nM)-induced PGE_2 production was completely inhibited by 1 μM NS-398, a selective COX-2 inhibitor (not shown). Since vanilloid receptors (TRPV1) have been described on human cerebrovascular endothelial cells [22], we evaluated whether the up-regulation of COX-2 expression by WIN 55,212-2 involves TRPV1 receptors. The pre-treatment with the TRPV1 antagonist, capsazepine (10 μM) for 1 h did not modify WIN 55,212-2-induced COX-2 expression (not shown). Therefore, although brain endothelial cells expressed both types of CB receptors, our results indicate the lack of involvement of the CB1 and CB2 receptors in mediating COX-2 responses to WIN 55,212-2.

3.3. *WIN 55,212-2 treatment did not affect the viability of murine brain derived endothelial cells b.end5*

Because cannabinoid-induced COX-2 up-regulation in human neuroglioma cells has been associated with the production of cellular death [26] we next assessed the viability of b.end5 cells after treatment with WIN55,212-2 by measuring the release of LDH. None of the doses of WIN 55,212-2 used (25 nM–1 μM) caused any detrimental effects on cellular viability within 72 h of exposure. Fig. 3 shows the cell viability results obtained after WIN 55,212-2 treatment at the dose of 100 nM.

3.4. *Effect of other cannabinoids on COX-2 expression*

The endocannabinoid AEA has been reported to be a potent vasodilator of cerebral microcirculation and to cause COX-2 expression in brain endothelial cells [27]. Therefore, we tried

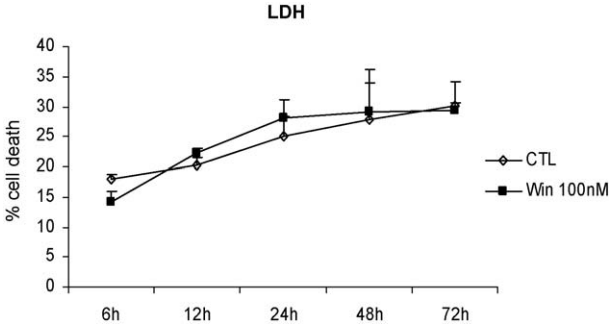

Fig. 3 – Cell viability is not modified by WIN 55,212-2 treatment. Cell death was evaluated by LDH assay, as described in Section 2, in b.end5 cells treated with WIN 55,212-2 (100 nM) for 6, 12, 24, 48, 72 h. Data are the means ± S.E.M. of three independent experiments done in triplicate.

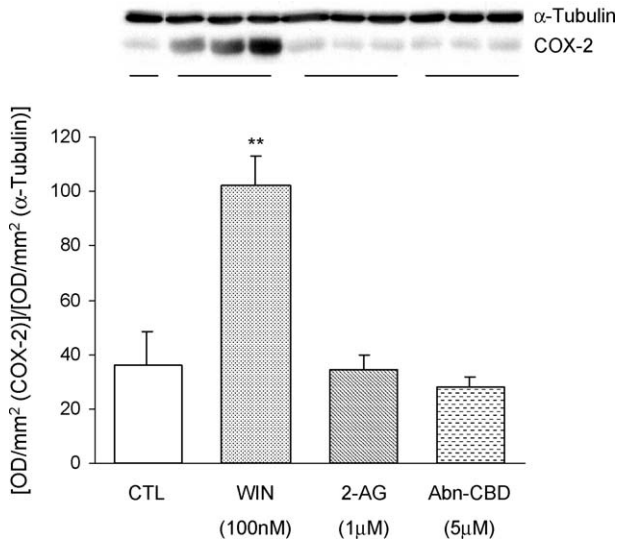

Fig. 4 – 2-AG and Abn-CBD have no effect on COX-2 expression. Brain endothelial cells (b.end5) were treated for 20 h with different cannabinoid ligands, WIN 55,212-2 (100 nM), 2-AG (1 μM) or Abn-CBD (5 μM). Densitometric analysis of cell-lysates proteins (30 μg) obtained by Western immunoblot are presented as a relative ratio % where COX-2 signal was normalized to α-Tubulin signal. Results show the means ± S.E.M. from three independent experiments done in triplicate. $^{**}P < 0.01$ vs. CTL.

to test whether the endocannabinoid, 2-arachidonoyl glycerol increases COX-2 expression too. Dose response studies (not shown) of 2-AG up to 1 μM indicated a lack of effect of this endocannabinoid on the expression of COX-2 protein in our cell cultures. As an example, Fig. 4 shows the data obtained after 2-AG exposure of b.end5 cells for 20 h at the dose of 1 μM. We also examined the effects of the compound abnormal cannabidiol, a structural analogue of the marijuana constituent cannabidiol which does not bind to CB1 or CB2 receptors but acts on another yet undefined CB receptor on endothelial cells [14]. Our results indicated that Abn-CBD at doses up to 5 μM did not modify COX-2 expression in our cells. Neither 2-AG (up to 1 μM), nor Abn-CBD (up to 5 μM) were capable to increase PGE_2 release from brain endothelial cells (not shown).

3.5. *WIN 55,212-2 enhances TMEV-induced COX-2 expression in brain endothelial cells*

Evidence suggests that Theiler's virus may enter the CNS by infection of cerebrovascular endothelial cells. Therefore, infection of endothelial cells may represent one of the first events in the pathogenesis of TMEV-induced demyelination. The persistence in vitro of TMEV in cloned mouse cerebrovascular endothelial cells supports this concept [28]. In fact, no appreciable cytopathic effect of TMEV infection was observed in brain endothelial cells at least until the time of 32 h post-infection (not shown). Cerebral endothelial cells infected with DA strain of TMEV at a MOI of 2 showed time dependent up-regulated expression of the COX-2 protein after 8, 20 and 32 h post-infection (Fig. 5A). WIN 55,212-2 (100 nM) pre-treatment

for 1 h significantly ($P < 0.01$) enhanced this effect about a 50% as indicated the densitometric analysis of western blot results from whole cell lysates collected at 20 h post-infection (Fig. 5B). However, COX-2 protein expression did not show significant changes in TMEV-infected cells when were pre-treated with 2-AG (1 μM) or Abn-CBD (5 μM) for 1 h too. The up-regulation of COX-2 expression *was* accompanied by increases in PGE_2 levels as showed in Fig. 5C. Thus, infected cells pre-treated with WIN 55,212-2 released more PGE_2 (about 30%) than TMEV-infected cells pre-treated with the vehicle. However, TMEV-infected cells when pre-treated with 2-AG or Abn-CBD did not experiment significant changes (Fig. 5C). Therefore, neither in resting nor under stimulated conditions, like TMEV infection, 2-AG or Abn-CBD modified the expression of COX-2.

3.6. WIN 55,212-2 and TMEV induce changes in COX-2 expression and cellular localization

We then addressed the localization of COX-2 protein in brain endothelial cells infected by Theiler's virus or treated with WIN 55,212-2 (100 nM) by using confocal microscopy. Control resting cells showed a weak COX-2 labelling mainly within the cell nucleus (Fig. 6B). In WIN 55,212-2-treated cells a strong perinuclear COX-2 immunofluorescence signal and weak granular labelling diffusely distributed throughout the cytoplasm was observed (Fig. 6C). Some cells showed an intense granular staining at the nuclear periphery. In endothelial cells infected with TMEV, COX-2 immunofluorescence was detectable in the perinuclear zone, but mainly in the cytoplasm, showing a fibrillar pattern with fibrils spreading from the nucleus toward the cell periphery (Fig. 6D). Non-specific interactions of secondary antibodies were verified by omitting the primary antibodies (Fig. 6A).

3.7. WIN 55,212-2-induced COX-2 and PGE_2 release requires PI_3K activity

The involvement of different routes in the signaling pathways driven by TMEV infection and/or WIN 55,212-2 treatment on COX-2 induction was then analysed in *b.end5*. The pathways signaling of p38 and p42/p44MAPK have been involved in

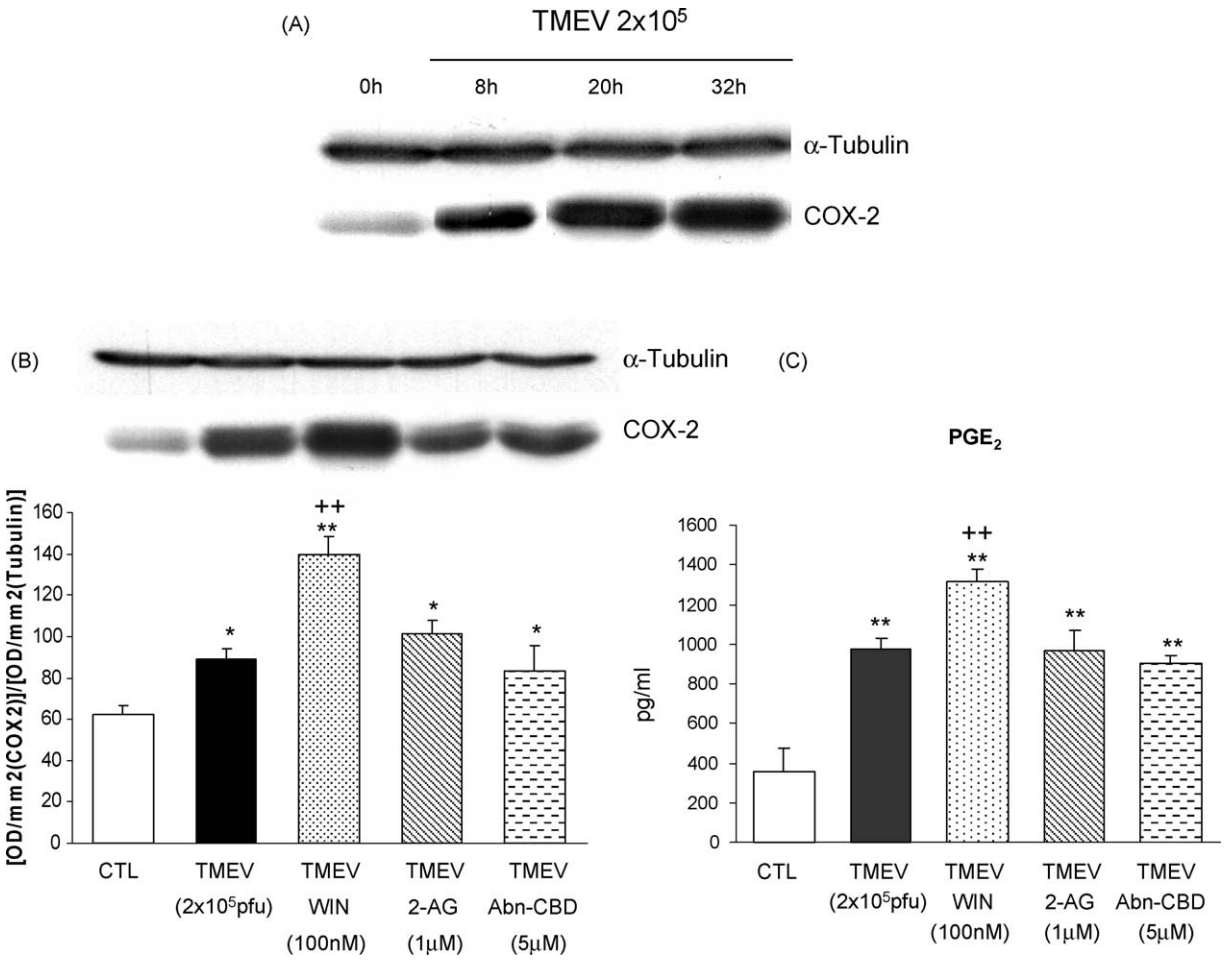

Fig. 5 – WIN 55,212-2 enhances TMEV-induced COX-2 expression in brain endothelial cells. COX-2 expression were detected by Western immunoblot in b.end5 cells that were infected by Theiler's virus (2×10^5 pfu) for 8, 20 and 32 h (A), and in infected cells pre-treated with cannabinoid ligands, WIN 55,212-2 (100 nM), 2-AG (1 μM) or Abn-CBD (5 μM), for 20 h (B). Results show the means ± S.E.M. of relative ratio % of COX-2 signal normalized to α-Tubulin from three independent experiments done in triplicate. PGE_2 levels were measured by ELISA in supernatants of these same cells monolayers (C). $^{*}P < 0.05$ vs. CTL; $^{**}P < 0.01$ vs. CTL; $^{++}P < 0.01$ vs. TMEV.

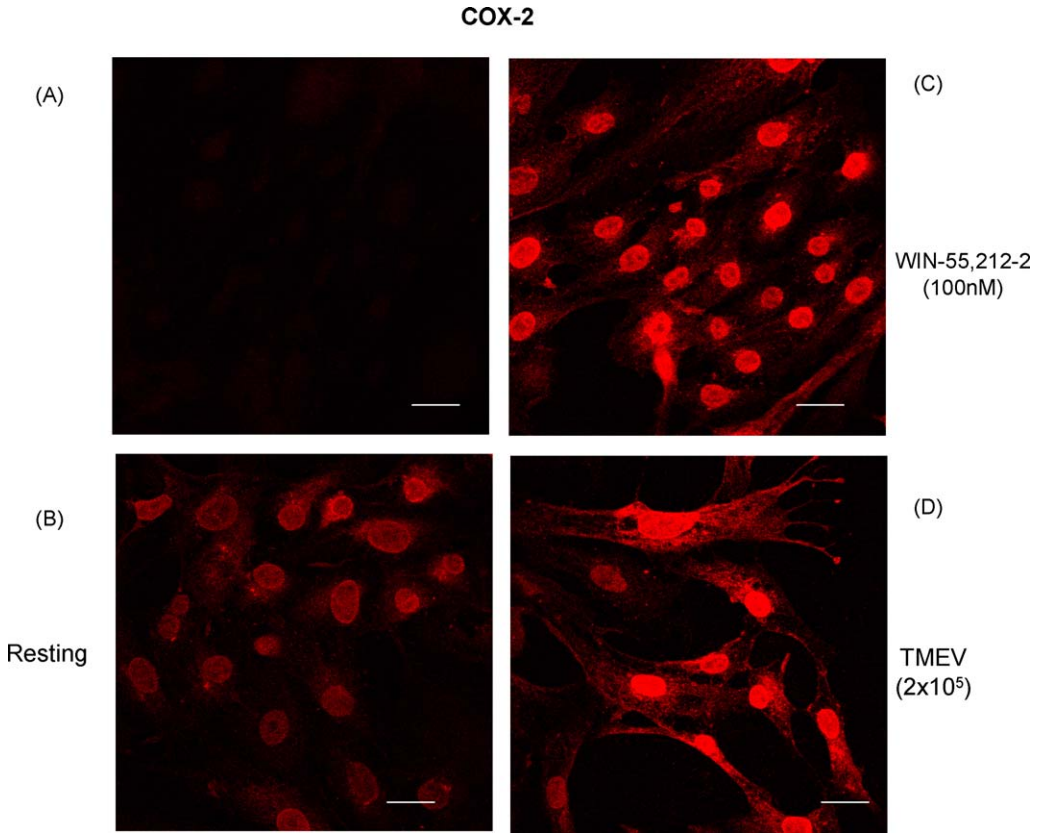

Fig. 6 – Variations in COX-2 location in virus infected brain endothelial cells. Resting b.end5 cells (B), WIN 55,212-2 (100 nM) treated cells (C) or Theiler's virus infected cells (D) were stained by immunofluorescence labelling with anti-COX-2 antibody (1:500 dilution). Non-specific interactions of secondary antibodies were verified by omitting the primary antibodies (A). Scale bar (20 μM).

COX-2 regulation by different stimuli and in different cell types [6,29,30]. Then, to examine whether phosphorylation of p38 and/or p42/44 MAPK is involved on COX-2 induction by WIN 55,212-2 or TMEV infection we used the specific inhibitors of these kinases, SB203580 (10 μM) and PD5098059 (10 μM), respectively. As shown in Fig. 7A COX-2 expression was not modified by SB203580 or PD5098059 pre-treatment, indicating that neither p38 MAPK nor p42/44 MAPK was involved in COX-2 induction by WIN 55,212-2 (100 nM) treatment. As we previously reported that WIN 55,212-2 activates PI_3K pathway [31], the effects of 1 h pre-treatment with the PI_3K inhibitor, LY204002 (10 μM) were further evaluated. In this case we observed a significant decrease (30.25 ± 0.85%) on COX-2 expression induced by WIN 55,212-2 (Fig. 7A), accompanied by a significant parallel reduction in PGE_2 release (37 ± 1.15%). We next assessed the effects of pharmacological inhibition of MAPKs and PI_3K in TMEV-infected cells (Fig. 7B). As in the case of cells treated with WIN 55,212-2, p42/44 MAPK was not implicated *on* COX-2 expression induced by TMEV infection. However, LY294002 and SB203580 were capable to inhibit COX-2 induction (39.36 ± 6.39%; 53.81 ± 2.72% reduction, respectively), indicating that PI_3K and p38 MAPK were involved on COX-2 expression. Fig. 7C shows that WIN 55,212-2-induced COX-2 up-regulation in TMEV infected cells *was* mediated by the above two pathways (Fig. 7C), since COX-2 induction was reduced 27.14 ± 6% by LY294002 and 47.27 ± 5.4% by SB203580. In addition the amount of PGE_2 measured in the supernatants of these cells cultures *was* significantly reduced 59.81 ± 4.1% by PI_3K inhibitor and 90.14 ± 1.7% by p38 MAPK inhibitor. Therefore, in mouse brain endothelial cells the induction of COX-2 expression by WIN 55,212-2 is dependent on PI_3K pathway, whereas in the case of COX-2 induction by TMEV p38 MAPK activation is necessary too.

4. Discussion

Theiler's virus infection of the CNS induces an immune-mediated inflammatory demyelinating disease in susceptible mouse strains and serves as a relevant infection model for human MS. Cannabinoids have been reported to be of potential therapeutic value in the treatment of immune-mediated neuroinflammatory diseases [15,16,32]. The cannabinoid WIN 55,212-2 was capable to diminish neuroinflammation and cell infiltrates in the spinal cord of TMEV-infected mice [17,18]. In addition, WIN 55,212-2 inhibited leukocyte entry into the brain in experimental allergic encephalomyelitis model of MS (EAE) [19]. The relevance of COX-2 and prostanoids in regulating cerebrovascular homeostasis and inflammation lead us to investigate the effects of WIN 55,212-2 on COX-2 expression and PGE_2 production *by* brain endothelial cells, under resting conditions and following TMEV infection.

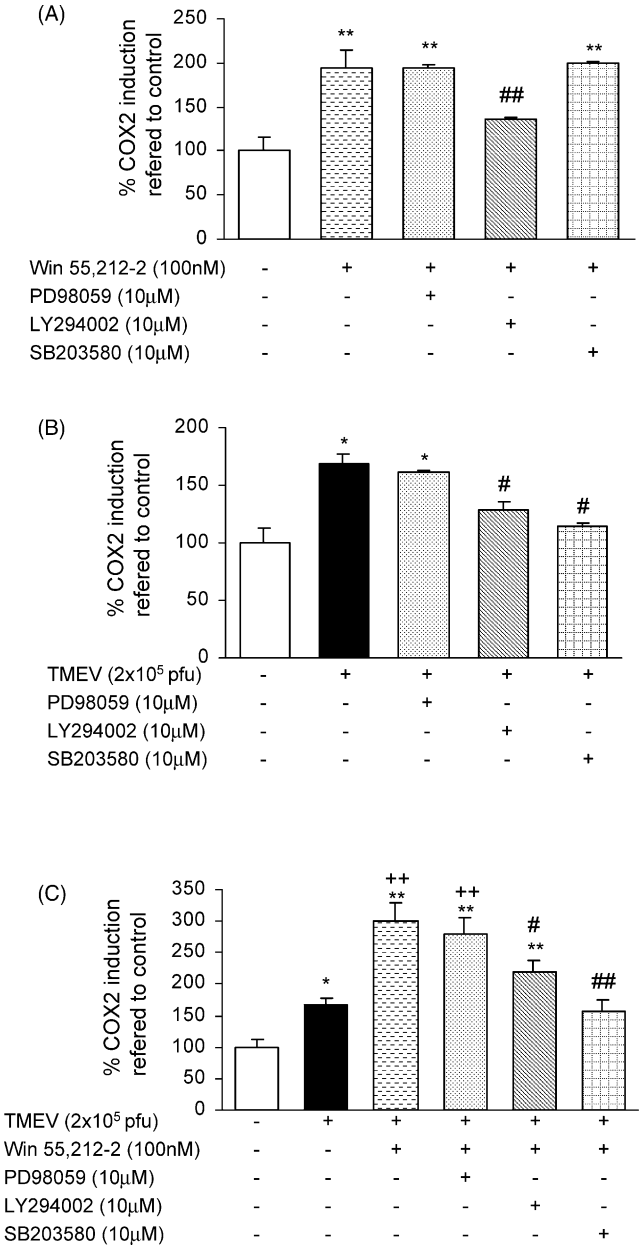

Fig. 7 – PI_3K and p38 MAPK are implicated in COX-2 induction by WIN 55,212-2 and Theiler virus. Theiler's virus cell monolayers infected (B) and/or treated with WIN 55,212-2 (100 nM) (C and A, respectively) for 20 h were pre-treatment for 1 h with the PI_3K inhibitor, LY20402 (10 μM), the p38 MAPK inhibitor, SB203580 (10 μM) or the ERK 1/2 MAPK inhibitor, PD5098059 (10 μM) as detailed in Section 2 (A). Densitometric analysis of cell-lysates proteins (30 μg) obtained by Western immunoblot are presented as a relative ratio % referred to control, where COX-2 signal was normalized to α-Tubulin signal. Results show the means ± S.E.M. from three independent experiments done in triplicate. $^{**}P < 0.01$ vs. CTL, $^{++}P < 0.01$ vs. TMEV, $^{\#}P < 0.05$ vs. TMEV + WIN 55,212-2, $^{\#\#}P < 0.01$ vs. TMEV + WIN 55,212-2.

In the present study, we demonstrate that WIN 55,212-2 exposure significantly increases the expression of COX-2 in murine brain endothelial cell and this, in turned increases PGE_2 production. The blockade of PGE_2 by the selective COX-2 inhibitor NS-398 indicates a critical role for COX-2 on WIN 55,212-2-induced production of PGE_2. The effects of WIN 55,212-2 on COX-2 are dose-related and mediated by a mechanism independent on the activation of CB1 and CB2 receptors in accordance with other studies in neuroglioma cells [33]. Murine brain endothelial cells expressed both CB1 and CB2 receptors, but the selective blockade of these receptors failed to modify WIN 55,212-2-*induced* up-regulation of COX-2. It is therefore conceivable that the action of WIN 55,212-2 on COX-2 induction may involve non-specific mechanisms related to its high degree of lipophility by interactions at the plasma membrane or intracellular sites, but the stereoselectivity of response as well as the dose-related effects (effective doses in the nanomolar range) argues against this possibility. It has been proposed the existence of other cannabinoid receptors, mainly on the basis of pharmacological studies with mice lacking both CB1 and CB2 receptors [34]. A receptor activated by WIN 55,212-2 has been postulated to exist in CNS of mice [35,36]. In addition, an as yet unidentified non-CB1/non-CB2 endothelial cannabinoid receptor has been suggested to be involved in the endothelium dependent vasodilator effects of certain cannabinoids [14]. Recent findings indicate that the endocannabinoid AEA increases the expression of COX-2 protein in mouse cerebral endothelial cells by a mechanism which involved, at least in part, the activation of the CB1 receptor [27]. In our study the endocannabinoid 2-AG was not capable to modify the expression of COX-2 even at high doses (1 μM) suggesting that each endocannabinoid may have distinct biological functions. In addition, Abn-CBD a structural analogue of cannabidiol that acts as a full agonist on the putative Abn-CBD sensitive receptor [12] also failed to induce COX-2 expression in our cell cultures. Abn-CBD receptors have been proposed to be present in the peripheral vascular system on the basis of the hypotensive effects displayed by Abn-CBD in CB1/CB2 receptor double knockout mice [12]. As in vitro studies with Abn-CBD were performed in HUVEC cells [14], the lack of effect of this compound upon COX-2 expression in the current work may be related to differences between peripheral and brain endothelial cells [37]. Because our main interest refers to the actions of cannabinoids in a neuroinflammatory context, and in particular in the TMEV-model of MS, we infected brain endothelial cells with DA strain of virus and assessed COX-2 protein regulation and PGE_2 biosynthesis. Results from the present study are the first to show that TMEV infection increases the expression of COX-2 on brain endothelial cells. Under resting conditions b.end5 cells express very low levels of COX-2 which is localized mainly within the cell nucleus with much less immunofluorescence in the cytoplasm but the infection with TMEV was accompanied by COX-2 trafficking between the nucleus and the cytoplasm. It is likely that COX-2 accumulation in the cytoplasm was associated with an increase in prostanoid synthesis as it has been suggested by studies in human endothelial cells activated with IL-1β [38]. In the Theiler's virus model of MS, COX-2 immunoreactivity was detected in endothelial cells and in perivascular infiltrating

macrophages (our unpublished observations). A similar restricted COX-2 expression has been described in brain tissue from MS patients [1]. However, recent studies in the TMEV-IDD model [39] showed that COX-2 expression often occurred in oligodendrocytes under apoptosis. The authors proposed that the expression of COX-2 may be functionally linked to oligodendrocytes excitotoxic death [39] as observed in neurons. The expression of COX-2 has been explored in different animal models and in MS patients, but the cellular type which expressed the enzyme varied depending on the model or the phase of the disease [2]. The exact contribution of COX-2 expression to the pathogenic events in MS is unknown, but the overexpression of COX-2 in different cell types may be specifically related to a stage of the disease and exert beneficial or detrimental effects depending on the local environment. In spite of the classical proinflammatory role of COX-2 on vascular permeability and leukocyte extravasation, mainly mediated by PGE_2, expression of COX-2 may exert some protective effects on MS. Intracerebroventricular administration of proinflammatory cytokines upregulates COX-2 expression in brain endothelial cells [4], but recent studies show that the inhibition of COX-2 increases the inflammatory response in the brain after a systemic immune activation [40]. PGE_2 has been found to be neuroprotective in several experimental settings [41,42]. Besides, PGE_2 plays an important role as modulator of several immune functions and a growing amount of data indicate that it may self-limit the inflammatory process by inhibiting the expression of proinflammatory genes, and thus, down-regulating the process of macrophage/microglia activation [43]. PGE_2 levels were elevated during recovery phase in a murine model of MS, suggesting a protective effect of PGE_2 in this model [44]. By contrast, indomethacin administration has been described to suppress active EAE [45]. This is further supported by a recent study that reported that COX-2 inhibitors ameliorated EAE by reducing IL-12 production ad Th1 cell differentiation [46]. However, COX-2 activation has been shown to suppress Th1 cell polarization and PGE_2 is a well known inhibitor of the production of IL-12 by acting at the repressor site of the promoter of IL-12p40 [47,48]. Therefore, the role played by COX-2 in regulating immune responses in MS is still controversial. In TMEV-infected cells the treatment with WIN 55,212-2 synergistically enhanced the expression of COX-2 and the production of PGE_2. The relevance of these findings remains to be investigated but it is tempting to speculate that the WIN 55,212-2 dependent increase of PGE_2 in endothelial cells may first help to further increase blood flow to favour virus clearance and second, attenuate the inflammatory pathway, as we observed in TMEV-IDD [17]. The mechanisms underlying COX-2 synthesis in endothelial cells are still an open issue. COX-2 induction is characterized by redundancy both at the extranuclear signaling level and at the level of transcriptional activation [49]. Here we showed that activation of p38 MAPK seems to be important when COX-2 is induced by TMEV infection, whereas activation of PI_3K pathway plays a major role after WIN 55,212-2 exposure. In summary, we demonstrate here that WIN 55,212-2 is capable of inducing the expression of COX-2 and the release of PGE_2 in murine brain endothelial cells by a non CB1/CB2 receptor mechanism. Infection with TMEV also elicited an up-regulation of COX-2 together with increases in PGE_2 release. The treatment with WIN 55,212-2 synergistically enhanced these effects. In this light the data presented indicate the existence of at least two pathways leading to COX-2 expression in b.end5 cells in the case of WIN 55,212-2, activation of PI3-kinase pathway is required for COX-2 induction, but p38 MAPK is necessary for COX induction by TMEV. Over expression of COX-2 in brain endothelial cells may have different functional consequences and its final outcome is likely to depend on the prevailing product of COX-2 activity, including PGE_2 that besides its known vasodilatory actions may also have a neuroprotective role in modulating the proinflammatory response occurring in several chronic inflammatory diseases of CNS like MS.

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