Avoidance of Aβ[25-35] / (H_2O_2)-Induced Apoptosis in Lymphocytes by the Cannabinoid Agonists CP55,940 and JWH-015 *via* Receptor-Independent and PI3K-Dependent Mechanisms: Role of NF-κB and p53

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Abstract: Cannabinoids have been suggested as potential neuroprotective compounds in Alzheimer's disease (AD). Despite intense investigation, the detailed intracellular mechanism(s) involved in cannabinoids survival effect remains to be elucidated. The present study shows that CP55,940 (a CB1 and CB2 agonist) and JWH-015 (a CB2 agonist) protect and rescue peripheral blood lymphocytes (PBL) from (10 μM) Aβ[25-35] and (50 μM) H_2O_2-induced apoptosis by two alternative mechanisms: (1) receptor-independent pathway, as demonstrated by no-dihydrorhodamine oxidation into fluorescent rhodamine 123 (R-123) as a result of cannabinoid inhibition of Aβ-generated H_2O_2; (2) receptor-dependent pathway through NF-κB activation and p53 down regulation involving phosphoinositide 3-kinase (PI-3K), as demonstrated by using either (25 μM) LY294002 (a PI-3K inhibitor), (50nM) pifithrin-α (PFT, a specific p53 inhibitor) or by using immunocytochemistry detection of NF-κB and p53 transcription factors activation. Importantly, cannabinoid agonists and PFT were able to protect and rescue lymphocytes pre-exposed to toxicants-, even when the three compounds were added up-to 12 h post-Aβ[25-35]/(H_2O_2) exposure. These results suggest that CP55,940/(JWH-015) protection/rescue of PBL from noxious stimuli is determined by p53 inactivation. These findings may contribute to a better understanding of the role played by cannabinoids as neuroprotective agents to target and interrupt molecular signaling that induce damage in AD disorder.

Key Words: Beta-amyloid, CP55,940, JWH-015, PI-3K, NF-κB, p53.

1. INTRODUCTION

Cannabinoids are a group of C_{21} terpenophenolic compounds produced by the glandular hairs of *Cannabis sativa* [1] which exert their effects by binding to specific plasma membrane G-protein-couple receptors, termed CB1 [2] and CB2 [3] receptors. Activation of these receptors has been shown to trigger several $G_i/_o$-protein-mediated signaling pathways (for a review see ref. [4]). Although, it is currently accepted that CB1 receptors are specially abundant in basal ganglia, hippocampus, cerebellum, and cortical structures; and CB2 receptors are restricted to cell types related to the immune function such as spleen macrophages, tonsils, B cells and natural killer cells, monocytes, neutrophils, and T cells (for a review see ref. [5]), it has also been demonstrated the existence of CB2 receptors in purkinje cerebellar neurons [6], microglia [7], oligodendrocytes [8] and brainstem neurons [9]. Moreover, both receptors elicit similar signaling pathways such as inhibition of adenylate cyclase, stimulation of extracellular-signal-regulated kinase [4] and activation of phosphoinositide 3-kinase/PKB [8, 10-12]. The physiological significance of these common characteristics is still unknown.

Cannabinoids have been proposed as potential therapeutic agents thanks to their involvement in control of cell death/survival decision and in neuroprotection. However, the mechanism of both actions by cannabinoids is far from clear. In fact, there are numerous, sometimes contradictory, reports of cannabinoids effects on cell fate [13]. Moreover, cannabinoids have been shown to function as antioxidant compounds *via* receptor-independent [14-16] or receptor-dependent mechanisms [17,18]. Although CB antagonists (v. gr. SR141716A) have been used to elucidate the neuroprotective mechanism of cannabinoids, they have not been conclusive (see ref. [16] versus [17,18]). Therefore, the molecular mechanism(s) of cannabinoids effect on cells is a complex and still controversial issue.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by severe neural depopulation in the hippocampus, insoluble amyloid-β[$Aβ_{1-42}$] and tau protein deposits, and severe gliosis (for a review see ref [19]). The hypothesis that Aβ might be the major trigger for neurodegeneration by oxidative stress is supported by ample evidence from both *in vivo* and *in vitro* studies [for review see refs. 20, 21]. Interestingly, McLellan and colleagues [22] have shown H_2O_2 and free radicals in *in vivo* and *ex-vivo* mouse AD brains. Additionally, Garcia-Ospina and coworkers [23] have demonstrated NF-κB, p53 and c-Jun transcription factors activation in *in situ* AD brains. In agreement with these observations, it has been demonstrated that the cytotoxic domain of Aβ[$Aβ_{1-42}$] i.e. Aβ[25-35]-induces apoptosis in peripheral blood lymphocytes (PBL) by an oxidative stress mechanism involving the generation of H_2O_2, and en-

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1573-4064/06 $50.00+.00

suing activation of the nuclear factor (NF)-κB, p53, c-Jun transcription factors, mitochondria depolarization and caspase-3 activation [24]. These data highlight the reliability of potential to use lymphocytes as non-neural cell model to directly monitor intracellular signalling mechanism(s) leading to death and/or survival responses to different oxidant stress stimuli. In this regard, lymphocytes cells share similar biochemical and functional features with neurons such as expression of catecholaminergic [25], serotonergic [26], cholinergic [27], glutamatergic [28, 29], noradrenergic [30] and gabaergic [31] systems, but most importantly, both cells respond similarly (i.e. morphologically and biochemically) to oxidative stress stimuli (e.g. H_2O_2, DA, metals [32 and references within]) and express CB2 receptors [3]. Consequently, lymphocytes might be useful to unravel the cannabinoids survival/death signalling mechanism(s) in response to Aβ-toxicity.

Because CB2 cannabinoid receptor is linked to activation of PI3K [12] and cannabinoids might be potential therapeutic use against AD [33], in the present study we have used the non-classical cannabinoid (-)-CP55,940, which shows high affinity and stereo-selectivity to both cannabinoid receptors [34], and the selective CB2 receptor JWH-015, aimed at a better understanding of the molecular signalling downstream of CB2 receptor linked to the role played by NF-κB and p53 in survival and death-signalisation against $A\beta_{[25-35]}$ and H_2O_2 stimuli.

2. MATERIALS AND METHODS

Amyloid β-protein fragment 25-35 (Cat # A4559; $A\beta_{25}GSQKAIIGLM_{35}$), and other reagents if not otherwise specified were purchased from Sigma (St. Louis MO, USA) and were of analytical grade or better.

2.1. Isolation of Lymphocytes

Peripheral blood lymphocytes (PBL) from venous blood of healthy adult male (range age 30-40 years old) were obtained by gradient centrifugation (Lymphocyte separation medium, density: 1.007 G/M; Bio-Whittaker). Isolated PBL was washed thee times with PBS (10 mM sodium phosphate, 160 mM NaCl, pH=7.4) and finally suspended in RPMI 1640 (GIBCO laboratories, NY, USA) plus 10% foetal calf serum (FCS, GIBCO laboratories). The PBL in suspension were cultured in RPMI1640 supplemented with 10% foetal calf, 2mM L-glutamine, 100-U/ml penicillin and 100-μg/ml streptomycin. The PBL were plated in 24-wells ($1x10^6$ cells/ml/well).

2.2. Experiments with Peripheral Blood Lymphocytes

2.2.1. Assessment of Apoptotic Indexes

PBL were pre-incubated for 30 min at 37°C in culture medium containing either (1, 10, 100nM) (-)-CP55,940 or JWH-015, or co-incubated with (10μM) $A\beta_{[25-35]}$ fragment, or (50μM) H_2O_2 in the absence or presence of other products of interest for 24 h. PBL was then used for parallel microscopic examinations such as viability studies, determination of ROS, rescue experiments and Immunocytochemical staining. Since Aβ promotes cellular clusters avoiding an accurate morphological evaluation, PBL cells treated with Aβ were disaggregated by gentle mechanical up-and–down micropipetting. Then, to perform viability studies 95μl either untreated (control) or treated cells were mixed with 5μl (0.1 mg/ml) acridine orange/ethidium bromide (AO/EB) and 5μl of the suspension was placed onto a slide and examined under fluorescence microscope (Zeiss Axiostart 50 microscope). Based on the differential uptake of the fluorescent DNA binding dyes AO/EB, normal PBL cells (NL, bright green chromatin) can be differentiated from early apoptotic cells (EA, bright green highly condensed or fragmented chromatin), late apoptotic cells (LA, bright orange highly condensed or fragmented chromatin) and necrotic cells (N, bright orange chromatin) [35]. Quantification of apoptotic morphology was done by counting a minimum of 300 total cells as follows: % apoptotic cells = 100X (total number of early and late apoptotic cells/ total number of cells counted). Necrotic cells were not detected under the present experimental conditions. Assessment of apoptotic indexes was repeated 3 times in independent experiments.

2.2.2. Determination of H_2O_2

Detection of H_2O_2 in cellular systems can be obtained by the use of the sensitive uncharged and nonfluorescent dihydrorhoramine 123 (DH) according to ref. [36]. Briefly, PBL ($1x10^6$ cells/ml) were incubated with 10 μM $A\beta_{25-35}$ in the presence or absence of (1, 10, 100nM) CP55,940 or JWH-015 for 24 h, respectively. To evaluate H_2O_2 generation, aliquots of 100 μl untreated and treated PBL were incubated in the presence of 1μM DH (20 mM stock solution in DMSO) for 15 min at 24 h. DH is oxidized to the cationic green fluorescent dye rhodamine-123 which accumulated intracellularly owing to the electrically negative cytoplasmic and mitochondrial membrane potential. The quantification of fluorescent cells under a fluorescence microscope (Zeiss Axiostart 50) was performed by counting a minimum of 300 total cells as follows: % fluorescent cells (observed as completely green bright fluorescent cells) = 100X (total number of green fluorescent cells/ total number of cells counted (green fluorescent cells + non-fluorescent cells observed as faded fluorescent cells and/or scarce cytoplasmic fluorescent mitochondrial dots)) compared with untreated control. In parallel, the apoptotic index was assessed to correlate H_2O_2 generation with $A\beta_{25-35}$ toxicity and fluorescent cells were counted blind. Experiments were performed in 3 separate and independent settings.

2.2.3. Immunocytochemistry detection of NF-κB, and p53 Transcription Factor Proteins

Immunocytochemistry was performed according to suppliers´ protocol (Santa Cruz Biotechnology, goat ABC staining System (cat # sc-2023) using the primary goat polyclonal antibodies NF-κB p65 (C-20)-G (Santa Cruz Biotechnology cat#sc-372-G), and p53 (FL-393) (Santa Cruz Biotechnology cat #sc-6243-G). After treatments, cells were

plated on poly-L-lysine coated cover slip and fixed in 4% methanol in 0.1 M phosphate buffer, pH 7.4 for 25 min and then washed with phosphate-buffered saline (PBS). Slides were exposed to 1% hydrogen peroxide in PBS for 10 min. After several washes, cells were permeabilized with Triton X-100 solution in PBS for 5 min. Cells were incubated with primary antibodies (10 μg/ml) for 2 h at room-temperature (RT) and subsequently incubated with biotinylated antibody at RT for 1 h. Finally, the specimens were stained with the ABC enzyme kit. After staining, they were cover-slipped with cover glasses. Positive diaminobenzidine (DAB) stained nuclei (dark-brown colour) were microphotographed using a Zeiss (Axiostart 50) microscope equipped with a Canon PowerShot G5 digital camera.

2.3. CP55,940 and JWH-015 Rescue Experiments Against $A\beta_{[25-35]}$

PBL were incubated with (100nM) CP55,940; JWH-015 or p53 inhibitor (50nM) Pifithrin-α immediately or at 1, 3, 6, 12 h of (50μM) H_2O_2, 10 μM $A\beta_{[25-35]}$ post-exposure for 24 h. After this time, treated PBL were evaluated in parallel for apoptotic, and reactive oxygen species indices as described in *section 2.3.1-2.* Additionally, immunocytochemistry detection of NF-κB, and p53 transcription factor proteins were performed when either agonist or PFT was added at 12 h post- toxic- exposure as described in section 2.3.3.

2.4. Statistical Analysis

Data are means ± S.E. of three independent experiments. The difference between two groups was statistically analyzed by student's *t* test. A *p*-value of <0.05 versus control was considered significant.

3. RESULTS

3.1. The Agonist CP55,940 and JWH-015 Protect Lymphocytes Against $A\beta_{[25-35]}$ and Hydrogen Peroxide-Induced Apoptosis in a Receptor-Independent Fashion

It is well established that $A\beta_{[25-35]}$ –induces morphological changes such as highly condensed chromatin, nuclear fragmentation and apoptotic bodies, typical of apoptosis in lymphocytes [24]. Because the AO/EB assay has been validated as the most reliable method for detection and quantification of cell death when compared with other methods [35], it was used in this study to detect lymphocytes viability. Thus, treatment of PBL with 10 μM $A\beta_{[25-35]}$ provoked 13±2% of cell death. Given that CP55,940 has recently been reported to protect PC12 cells against Aβ neurotoxicity [37], we assessed whether CP55,940 could protect PBL against 10μM $A\beta_{[25-35]}$ toxic stimuli. Concentration of CP55,940 (10, 100nM), except (1nM CP55,940 + Aβ = 4 ± 1) almost abolished $A\beta_{[25-35]}$ –induced cell death to control values (i.e. CP55,940 + Aβ = <1% apoptotic index). The structurally unrelated cannabinoid receptor agonist JWH-015 also reduced $A\beta_{[25-35]}$ –noxious stimuli to control values (i. e. JWH-015 + Aβ = <1% apoptotic index). Both agonists were innocuous at the concentrations tested when incubated alone with PBL (Table **1**). Therefore, the effective protective concentration of (10nM, 100nM) CP55,940 and JWH-015 were used for further experiments.

Because it has been suggested that cannabinoids might protect cells from oxidative stress *via* receptor-independent pathway, and because it is well established that $A\beta_{[25-35]}$ produces H_2O_2, we assessed whether both agonists were able to protect lymphocytes against $A\beta_{[25-35]}$ toxicity through blocking H_2O_2 action. Table (**1**) shows that $A\beta_{[25-35]}$ generates H_2O_2 (18 ± 2%), but this effect was almost completely reduced at any of the agonist concentration tested (1,10,100nM) to control values (<1 ± 0% H_2O_2 index). To further confirm that H_2O_2 was directly involved in PBL cell death, we assessed the effect of (50μM) H_2O_2 added to lymphocytes alone or in combination with the agonists. As expected, H_2O_2 induced cell death (20 ± 2% apoptotic index) concomitantly with H_2O_2 detection (23 ± 2% H_2O_2 index). Noticeably, CP55,940 and JWH-015 reduced both apoptosis as well as H_2O_2 indices (Table **2**).

Because it is known that CB2 activates phosphoinositide 3-kinase/PKB [12], we investigated whether this pathway could be involved in CP55,940/JWH-015 cytoprotection. As a first approach, we incubate (25μM) LY294002, a specific PI3K inhibitor, alone or in combination with (10μM) Aβ or (10nM) CP55,940 (/JWH-015). As shown in Table (**1**), LY294002 inhibitor alter neither apoptosis nor % H_2O_2 indices of those compounds. Unexpectedly, when LY294002 was incubated together with Aβ plus CP55,940 (/JWH-015), it was observed that both apoptosis or % H_2O_2 indices were reduced to control values. Similar results were obtained when (50μM) H_2O_2 was evaluated under analogous experimental conditions (Table **2**).

3.2. CP55,940 (/JWH-015) Protects Lymphocytes Against $A\beta_{[25-35]}$ and Hydrogen Peroxide-Induced Apoptosis in a Receptor-Dependent Fashion

The above results prompted us to test the possibility that PI3K activation could be masked by the agonists antioxidant property. We therefore assessed whether CP55,940 (/JWH-015) were able to activate NF-κB and down-regulate p53 through PI3K. As shown in Fig. (**1**), while (10μM) Aβ induces activation and nuclei translocation of both NF-κB and p53 transcription factors (**C, D**), (100nM) CP55,940 induces NF-κB activation and nuclei translocation (**E**) but not p53 (**F**) when compared to untreated PBL (**A, B**). Amazingly, when Aβ was co-incubated with CP55,940 NF-κB nuclei translocation was evidently detected (**G**), but p53 was undetectable (**H**). We further incubated (25μM) LY294002 alone or in combination with CP55,940. As expected, neither NF-κB nor p53 activation and/or nuclei translocation was visible (**I, J**). Noticeably, when LY294002 was incubated with Aβ, the inhibitor did not modify NF-κB or p53 detection patterns (i.e. staining was similar to PBL exposed to Aβ, data not shown). Results were comparable when JWH-015 was used under similar experimental conditions.

Table 1. Effect of PI3K Inhibitor and the Cannabinoid Agonists CP55,940, and JWH-015 on PBL Under Aβ[25-35] Exposure

Treatment	APO (%)	H_2O_2 (%)
Untreated	<1 ± 0	<1 ± 0
Aβ(10μM)	13 ± 2*	18 ± 2*
CP55,940 (1nM)	<1 ± 0	<1 ± 0
CP55,940 (10nM)	0	<1 ± 0
CP55,940 (100nM)	0	<1 ± 0
CP55,940 (1nM) + Aβ(10μM)	4 ± 1*	2 ± 1
CP55,940 (10nM) + Aβ(10μM)	<1 ± 0	<1 ± 0
CP55,940 (100nM) + Aβ(10μM)	0	0
LY294002 (25μM)	<1 ± 0	<1 ± 0
LY294002 (25μM) + CP55,940 (10nM)	<1 ± 0	<1 ± 0
LY294002 (25μM) + Aβ(10μM)	12 ± 1*	19 ± 2*
LY294002 + CP55,940 + Aβ	3 ± 1	<1 ± 0
JWH-015 (1nM)	<1 ± 0	<1 ± 0
JWH-015 (10nM)	0	<1 ± 0
JWH-015 (100nM)	0	<1 ± 0
JWH-015 (1nM) + Aβ(10μM)	3 ± 1*	1 ± 1
JWH-015 (10nM) + Aβ(10μM)	<1 ± 0	<1 ± 0
JWH-015 (100nM) + Aβ(10μM)	0	<1 ± 0
LY294002 + JWH-015 (10nM)	<1 ± 0	<1 ± 0
LY294002 + JWH-015 + Aβ	2 ± 1	1 ± 1

PBL were incubated for 24 h with either (10 μM) Aβ[25-35], (1, 10, 100nM) CP55,940 (/JWH-015), and (25μM) LY294002 alone or in combination as indicated. The evaluation of apoptosis and % H_2O_2 indexes were performed as described in *Materials and Methods.* Quantification of apoptosis and % H_2O_2 are expressed as a mean of percentage ± S.E. from three independent experiments. *, *p*-value of <0.05 versus control was considered significant.

3.3. CP55,940 (/JWH-015) Protects and Rescues Lymphocytes Against Aβ[25-35] and Hydrogen Peroxide-Induce Apoptosis

We further wanted to examine whether cannabinoid agonists were able to rescue lymphocytes from Aβ[25-35] (/H_2O_2)-induced apoptosis. Therefore, PBL cells were exposed to (100nM) CP55,940 (/JWH-015) immediately (0 h) or at 1, 3, 6 and 12 h of (10 μM) Aβ[25-35] (/50μM H_2O_2) post-treatment. Whereas Aβ alone induced apoptosis and generated H_2O_2 after 24 h incubation (13±2% AO/EB apoptotic index; 18±2% H_2O_2 index), CP55,940 /JWH-105 were able to protect and rescue lymphocytes against Aβ toxicity to untreated control values (i.e. <1 ± 0% AO/EB apoptotic index) at 0, 1, 3, 6 h tested, or even if added up-to 12 h post-Aβ treatment (Table **3**). Strikingly, no H_2O_2 generation (i.e. <1 ± 0% H_2O_2 index) was detected at any interval of time evaluated. However, when (50μM) H_2O_2 was added, CP55,940 /JWH-105 were effective rescuing PBL from apoptosis up-to-3 h post-insult, but were moderately efficient to remove H_2O_2 from 6 up-to 12 h post-treatment. Additionally, immunohistochemical staining clearly showed the activation/ nuclear translocation of NF-κB when CP55,940 was added up-to 12 h post-Aβ treatment, but p53 was undetectable (comparable results to Fig. **1G, H**). To examine whether PFT, a specific inhibitor of p53, could afford a similar rescue and protective effect as cannabinoid agonists, cells were incubate with (50nM) PFT immediately (0 h) or at 1, 3, 6 and 12 h of (10 μM) Aβ[25-35] (/50μM H_2O_2) post-treatment. In fact, PFT was successful in rescuing PBL cells from Aβ[25-35]-evoked apoptosis, and % H_2O_2 generation was similar when compared with % H_2O_2 production values obtained with PBL incubate with Aβ alone (Table **3**). Of notice, PFT was also able to protect and rescue PBL against H_2O_2-induced cell death, and % H_2O_2 index were also low (Table **3**) when compared with% H_2O_2 production values obtained with PBL incubate with H_2O_2 alone.

4. DISCUSSION

The neuroprotection or neurotoxic effects of cannabinoids in human and animal studies are not yet understood, mainly because the signaling pathways that mediate both

Table 2. Effect of PI3K Inhibitor and the Cannabinoid Agonists CP55,940, and JWH-015 on PBL Under H_2O_2 Exposure

Treatment	APO (%)	H_2O_2 (%)
Untreated	<1 ± 0	<1 ± 0
H_2O_2 (50μM)	20 ± 2*	23 ± 2*
CP55,940 (1nM)	<1 ± 0	<1 ± 0
CP55,940 (10nM)	0	<1 ± 0
CP55,940 (100nM)	0	<1 ± 0
CP55,940 (1nM) + H_2O_2 (50μM)	4 ± 2*	6 ± 2*
CP55,940 (10nM) + H_2O_2 (50μM)	2 ± 2	5 ± 1*
CP55,940 (100nM) + H_2O_2 (50μM)	<1 ± 0	<1 ± 0
LY294002 (25μM)	<1 ± 0	<1 ± 0
LY294002 (25μM) + CP55,940 (10nM)	<1 ± 0	<1 ± 0
LY294002 (25μM) + H_2O_2 (50μM)	22 ± 2*	27 ± 2*
LY294002 + CP55,940 + H_2O_2	3 ± 2	4 ± 2
JWH-015 (1nM)	<1 ± 0	<1 ± 0
JWH-015 (10nM)	0	<1 ± 0
JWH-015 (100nM)	0	<1 ± 0
JWH-015 (1nM) + H_2O_2 (50μM)	1 ± 1	2 ± 1
JWH-015 (10nM) + H_2O_2 (50μM)	<1 ± 0	2 ± 1
JWH-015 (100nM) + H_2O_2 (50μM)	<1 ± 0	<1 ± 0
LY294002 + JWH-015 (10nM)	<1 ± 0	<1 ± 0
LY294002 + JWH-015 + H_2O_2	2 ± 1	4 ± 1

PBL were incubated for 24 h with either (50μM) H_2O_2, (1, 10, 100nM) CP55,940 (/JWH-015), and (25μM) LY294002 alone or in combination as indicated. The evaluation of apoptosis and % H_2O_2 indexes were performed as described in *Materials and Methods.* Quantification of apoptosis and % H_2O_2 are expressed as a mean of percentage ± S.E. from three independent experiments. *, *p*-value of <0.05 versus control was considered significant.

actions remain elusive [13]. The present study reports two major findings related to (i) the cell survival and rescue mechanism of the non-classical cannabinoid CP55,940, a specific agonist for CB1 and CB2 receptors, and JWH-015, a specific agonist for CB2 receptor, against Aβ[25-35] and H_2O_2-induced toxicity in lymphocytes, and (ii) its connection with the activation of NF-κB and down-regulation of p53 transcription factors. Specifically, we found that increasing concentrations (1, 10, 100nM) of both CP55,940 and JWH-015 provided almost 100% survival (i.e. absence of apoptotic morphology) after (10μM) Aβ[25-35] or (50μM) H_2O_2 challenge in PBL cells (Table **1**). In accordance with our data, Iuvone and colleagues [37] have shown that treatment of PC12 cells with CP55,940 (10, 100, 1000nM) significantly (v. gr. 87-91% survival) reduced (1μg/mL) Aβ-induced cell death. These results comply with the notion that cannabinoids might exert neuroprotective effects against oxidative stress through antioxidative actions. Moreover, we have been able for the first time to show that this cytoprotective action of both CB2 agonists is mainly exerted by two alternative mechanisms: by directly interacting with the Aβ by product, H_2O_2; and by triggering activation of NF-κB and p53 down-regulation mediated by PI3K. These results are supported by the following observations. Firstly, it is shown that CP55, 940 and JWH-015 agonist are potent antioxidant compounds capable of directly scavenge H_2O_2 generated by Aβ (Table **1, 2**). This observation complies with the notion that the antioxidant protection displayed by cannabinoids is either related to their chemical structure, wherein the phenolic ring is required [16], or related to their high negative oxidation potential profiles (i.e. they are able to donate electrons) when compared with butylhydroxytoluene (BHT), a common standard antioxidant compound [14]. These results suggest that CB2 receptor is not involved in the cellular antioxidant cytoprotective effects of cannabinoid. However, given that CB2 receptor is able to activate Akt/PI3K [12], a protein kinase involved in cell survival signaling [38], we evaluate the effect of LY294002, a specific inhibitor of PI3K/Akt, on ago-

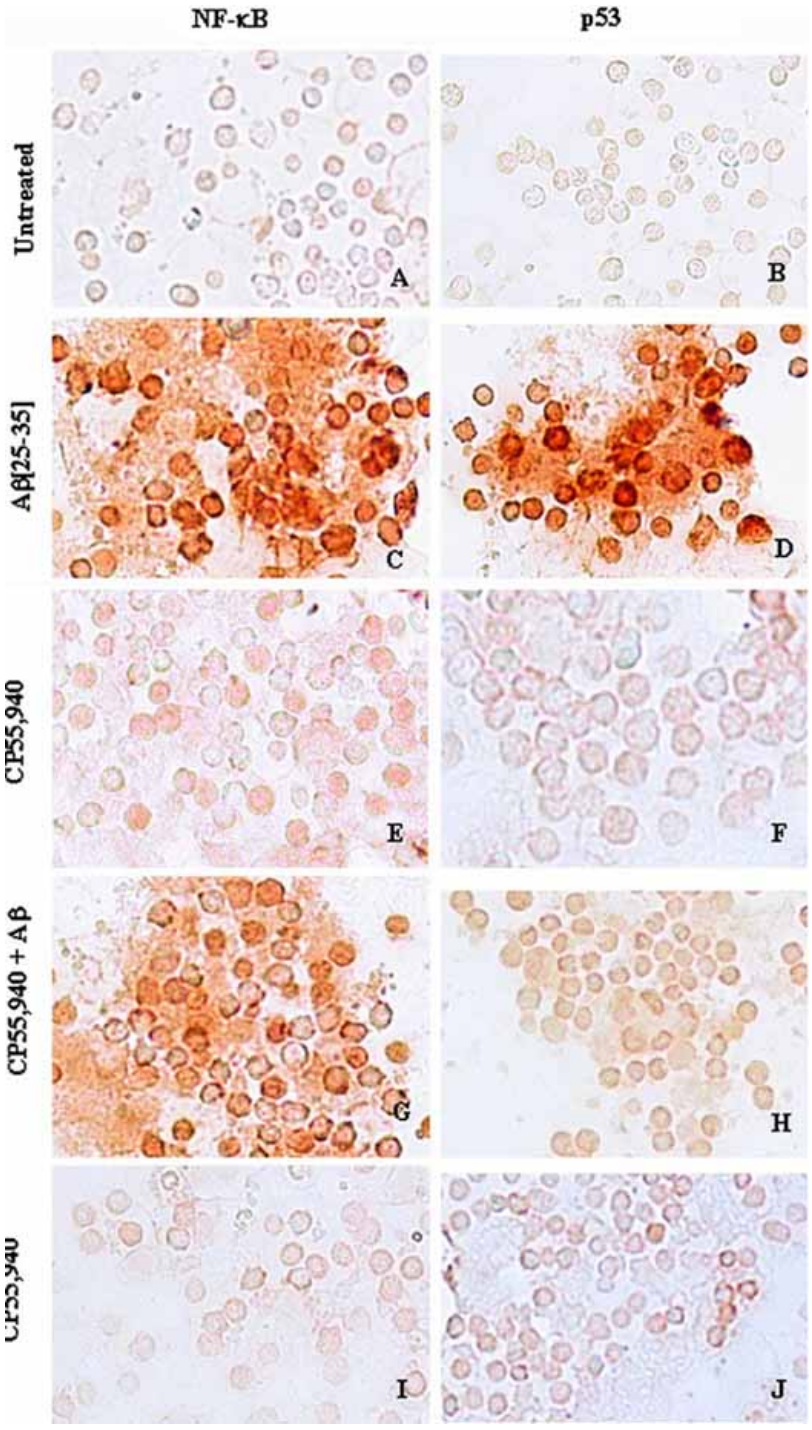

Fig. (1). CP55,940 and $A\beta_{25-35}$ induce the activation of NF-κB transcription factor in PBL.

PBL cells were left untreated (**A, B**), exposed to (10 μM) $A\beta_{[25-35]}$ (**C, D**), (100nM) CP55,940 (**E-F**),co-incubated with both (100nM) CP55,940 and (10 μM) $A\beta_{25-35}$ (**G, H**), or with 25μM LY294002 plus CP55,940 (**I, J**) for 24 h. After this time of incubation, cells were stained with anti-NF-κB-p65 (**A,C,E,G,I**), and anti-p53 (**B,D,F,H,J**) antibodies according to procedure described in *Materials and Methods.* Notice that NF-κB, and p53 positive-nuclei (dark brown colour) reflect their activation/ nuclear translocation. PBL cells treated with LY294002 alone showed similar results as in untreated cells. Magnifications 400x (**A-J**).

Table 3. The Cannabinoid Agonists CP55,940 and JWH-015 Protect and Rescue PBL Against Aβ[25-35] Toxicity

Treatment	Post-Incubation Time (hours)	None		Agonists				Inhibitor	
				CP55,940 (100nM)		JWH-015 (100nM)		PFT (50nM)	
		APO (%)	H_2O_2 (%)	APO (%)	H_2O_2 (%)	APO (%)	H_2O_2 (%)	APO (%)	H_2O_2 (%)
Aβ(10μM)	0	13 ± 2	18 ± 2	0	<1 ± 0	0	<1 ± 0	0	<1 ± 0
Aβ(10μM)	1	2 ± 1	3 ± 1	0	<1 ± 0	0	<1 ± 0	0	2 ± 1
Aβ(10μM)	3	4 ± 2	6 ± 2	0	<1 ± 0	0	<1 ± 0	<1 ± 0	4 ± 2
Aβ(10μM)	6	6 ± 1	8 ± 1	<1 ± 0	<1 ± 0	<1 ± 0	<1 ± 0	<1 ± 0	6 ± 1
Aβ(10μM)	12	7 ± 2	10 ± 2	<1 ± 0	<1 ± 0	<1 ± 0	<1 ± 0	<1 ± 0	6 ± 2
H_2O_2(50μM)	0	20 ± 2	23 ± 2	<1 ± 0	<1 ± 0	<1 ± 0	<1 ± 0	7 ± 2	18 ± 2
H_2O_2(50μM)	1	5 ± 1	13 ± 1	3 ± 1	<1 ± 0	2 ± 1	<1 ± 0	3 ± 1	12 ± 1
H_2O_2(50μM)	3	4 ± 1	15 ± 1	8 ± 2	3 ± 1	12 ± 2	1 ± 1	3 ± 1	11 ± 1
H_2O_2(50μM)	6	6 ± 2	12 ± 2	18 ± 3	5 ± 2	16 ± 3	6 ± 2	4 ± 2	14 ± 2
H_2O_2(50μM)	12	16 ± 2	11 ± 2	20 ± 3	13 ± 2	23 ± 2	10 ± 2	10 ± 2	10 ± 2

PBL were incubated with either (100nM) CP55,940 (/JWH-015) or (50nM) PFT immediately (control) or at 1, 3, 6, 12 h of (10 μM) $Aβ_{[25-35]}$ / (50μM H_2O_2) post-exposure for 24 h. After this time, treated PBL were evaluated in parallel for apoptotic indexes and % H_2O_2 production as described in *Materials and Methods.* Quantification of apoptosis and % H_2O_2 are expressed as a mean of percentage ± S.E. from three independent experiments. *, *p*-value of <0.05 versus control was considered significant.

nists action and on NF-κB activation, a transcription factor which has been demonstrated to be activated by Akt/PI3K [39]. We found that LY294002 effectively reduce apoptosis and % H_2O_2 indices when incubated with Aβ/ (H_2O_2) and CP55,940 (/JWH-015) (Table **1, 2**). These results initially corroborate the putative antioxidant action of cannabinoids. Nevertheless, since it has been demonstrated that PI3K is linked to NF-κB activation, this made us to suspect that PI3K activation could be masked by the high antioxidant capability of the agonists. In this work, we provide solid support for this alternative action mechanism. Secondly, by using immunohistochemical studies, it is shown that both CP55,940 (/JWH-015) activate the transcription factor NF-κB (Fig. **1E**), but it is undetectable when cells were exposed to a mixture of LY294002 plus agonists (Fig. **1I**). In agreement with Sanchez *et al*., [12], our observations suggest that CB2 receptor is able to trigger PI3K ensuing the activation and nuclei translocation of NF-κB (Fig. **1G**). Taken together these results comply with the notion that CB2 agonist CP55,940 and JWH-015 might protect PBL cells against Aβ and H_2O_2 by both receptor-independent [14-16] and receptor-dependent mechanisms [17,18]. Based on these findings, we hypothesize that other CB1 agonists might also be neuroprotective *in vitro* through activating PI3K as it has been shown by Molina-Holgado *et al*., [40] using the synthetic cannabinoid HU-210 in primary cortical neurons. Due to CB1 and CB2 importance in neuroprotective therapeutic approaches [41, 42], further experiments are warranted to test this assumption.

Previous studies have demonstrated that (10μM) Aβ[25-35]-induces the activation and nuclei translocation of both NF-κB and p53 transcription factors concomitantly with apoptotic morphology in PBL (ref. [24] and Fig. (**1C-D**), this work). Amazingly, lymphocytes nuclei showed positive staining for NF-κB activation when treated with CP55,940 (/JWH-015) alone (Fig. **1E**) or co-incubated with Aβ (Fig. **1G**), but nuclei showed no staining for p53 protein (Fig. **1F, H**). These results suggest that NF-κB could mediate cell death (apoptosis) and cell survival in PBL cells. Which molecular mechanism(s) explain the dual role of NF-κB as an attenuator or promoter of apoptosis?. NF-κB has been reported to activate transcription of the p53 gene [24, 43-45], which in turn activates the expression of several genes that directly control or regulate the process of apoptosis such as Bax (a pro-apoptotic Bcl-2 protein family, [46,47]. Thus, one prevailing model proposes that when the molecular ratio of pro-survival (e.g. Bcl-2, Bcl-xL, Bcl-w) to pro-death Bcl-2 family members (e.g. Bax, Bad, Bak, Bid) is biased towards pro-death Bcl-2 family members (either through changes in expression level, localization or activity), this unbalance elicit the irreversible proteolysis of critical nuclear and cytoplasm constituents of the cell (for a review see ref. [48]). Accordingly, our data suggest that cannabinoid agonists might promote gene transcription of survival genes *via* NF-κB activation and suppresses gene transcription of pro-apoptotic proteins through p53 inactivation. How then p53 turn-off could be related to cannabinoids citoprotection?. Interestingly, Ogawara *et al*., [49] and Feng *et al*., [50] have shown that Akt/PKB induces phosphorylation of Mdm2 (murine double minute) at Ser^{186} and Ser^{166} /Ser^{188}, respectively, resulting in increasing of Mdm2 ubiquitinization-promoting function and stabilization. Consequently, p53 protein is reduced [49]. Based on these conjectures, it is rea-

sonable to assume that p53 is modulated by cannabinoid through PI3K-Akt pathway. Taken together our findings reveal that p53 but not NF-κB is the critical transcription factor that may possibly balances the expression of pro-death proteins towards intracellular death decision [51] under oxidative stress stimuli.

In this study, we report for the first time that CB2 agonist CP55, 940 and JWH-015 protect and rescue lymphocytes against 10μM $A\beta[_{25\text{-}35}]$ (/50μM H_2O_2)-induced apoptosis. It is evidently shown that both agonists are able to rescue lymphocytes from noxious stimuli even when added up-to 12 h post-$A\beta[_{25\text{-}35}](/H_2O_2)$ exposure for 24 h of incubation. As expected, the protective and rescue properties are directly linked to agonist capacity to either scavenging H_2O_2 (Table **3**) or activating NF-κB and p53 turn-off signalling (as demonstrated by immunohistochemical technique at 12 h post-$A\beta[_{25\text{-}35}](/H_2O_2)$. The NF-κB and p53 staining patterns are identical as in Fig. **1G, H**). Remarkably, pifithrin-α (PFT), a specific p53-inhibitor, is able to mimic the cytoprotective and rescue action of agonists against $A\beta[_{25\text{-}35}](/H_2O_2)$–induced cell death. Taken together these results suggest that protection and/or rescue of PBL from $A\beta[_{25\text{-}35}]$ toxicity is determined by p53 inactivation under agonists control.

Although CB2 cannabinoid receptors have been considered to be expressed solely in cells and organs of the immune system [3], it has also been demonstrated the existence of CB2 receptors in pukinje cerebellar neurons [6], microglia [7], oligodendrocytes [8] and brainstem neurons [9]. Moreover, CB2 receptors have been demonstrated to be present in neuritic plaque-associated astrocytes and microglia in post-mortem brains from patients with Alzheimer Disease (AD) [33, 52]. Additionally, H_2O_2 has been shown to mediate amyloid β-protein toxicity [24, 53] and has also been implicated as a pivotal molecule in AD [22, 32, 54]. These entire data highlight the importance to scrutinize CB2 receptor signalling as potential model to test different therapeutic approaches toward diminishing the devastating impact of oxidative stress damage in AD [55]. This work adds to our understanding of CB agonists in three important ways: (1) the use of human peripheral lymphocytes may contribute to a better understanding of the intracellular molecular mechanism by which CB2 cannabinoid agonists protect and promote cell survival against oxidative stress; (2) may contribute to a better understanding of the role played by cannabinoids as neuroprotective agents in contrast to the neurotoxic notion [56, 57]; (3) the ability to identify molecules, v. gr. CP55,940 and JWH-015, capable to trigger CB2 (or CB1) receptors survival signalling pathways with potent antioxidant capabilities and/or able to mimic agonist survival effects show a promise of therapeutic strategies to target and interrupt molecular damaging events that contribute to AD neurodegenerative disorder.

ACKNOWLEDGEMENTS

This research was supported by Grant #8780 from "Proyecto de Investigación en Enfermedades Neurodegenerativas" to CVelezPardo and MJDelRio.

ABBREVIATIONS

AD = Alzheimer's disease

CB = Cannabinoid

PBL = Peripheral blood lymphocytes

PFT = Pifithrin-α

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Received: 17 January, 2006 **Revised: 28 May, 2006** **Accepted: 29 May, 2006**