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Stimulation of CB1 Cannabinoid and NMDA Receptors Increases Neuroprotective Effect against Diazinon-Induced Neurotoxicity

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Cannabinoids have been shown to exert a neuroprotective influence in organophosphorus-induced toxicity. In our study, we examined the effects of the cannabinoid receptor agonist WIN55,212-2 and NMDA receptor agonist NMDA on cell death in the pheochromocytoma cell line PC12 subjected to the action of an organophosphorus compound, diazinon. Diazinon decreased cell viability in a concentration-dependent manner. Following the exposure of PC12 cells to 200 μM diazinon for 48 h, reductions in cell survival and protein level of CB1 receptors were observed. Treatment of the cells with 0.1 μM WIN55,212-2 and 100 μM NMDA prior to diazinon exposure significantly elevated the cell survival level and protein level of CB1 receptors. The cannabinoid antagonist AM251 (1 μM) did not inhibit the neuroprotection effect induced by WIN55,212-2, indicating that the neuroprotective effect of this agonist was cannabinoid receptor-independent. The NMDA receptor antagonist MK-801 (1 μM) enhanced diazinon-mediated neurotoxicity suggesting that precisely NMDA receptors may play a protective role.

Keywords: cannabinoids, diazinon, neuroprotection, neurotoxicity, PC12 cells, viability.

INTRODUCTION

Diazinon (O,O-diethyl-O-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate) is an organophosphorus (OP) agent widely used as a pesticide. Diazinon can enter the organism through inhalation, ingestion, and/or skin contact. This OP is a hydrophobic molecule and, therefore, can easily penetrate biological membranes, especially via phospholipid bilayers [1]. Different studies showed that diazinon inhibits acetylcholinesterase (AChE) activity and protein synthesis in PC12 cells [2]. In other studies, it was reported that this compound disturbs DNA synthesis in and induces apoptosis of PC12 cells [3, 4]. Sublethal concentrations of diazinon inhibit the outgrowth of axon-like processes from differentiating mouse N2a neuroblastoma and PC12 cells [4, 5].

Cannabinoids are a group of C_{21} compounds occurring in the glandular hairs of *Cannabis sativa*. The well-known representative cannabinoid is Δ^9-tetrahydrocannabinol (Δ^9-THC) [6]. The endocannabinoid system includes specific cannabinoid receptors (CB1 and CB2), their endogenous ligands, and enzymatic systems of their biosynthesis and degradation [7]. Synthetic and endogenous cannabinoids were shown to exert, under certain conditions, neuroprotective effects in animals and *in vitro* models with respect to various forms of neuronal injury, such as cerebral ischemia, traumatic brain injury, toxicity, and neurodegenerative disorders [8, 9]. *In vitro* and *in vivo* experiments demonstrated that neuroprotective effects of cannabinoids are probably not mediated via CB1 receptors [10, 11].

It is well established that NMDA and endogenous cannabinoids display complex interactions in their control of synaptic plasticity [12]. In particular, CB1 receptors are located presynaptically on glutamatergic projections to the hippocampus. Thus, CB1 receptors and glutamate receptors are expressed on the same neuronal elements. These two receptor types were found to display a complex signaling interaction in primary hippocampal neuron cultures [13].

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PC12 cells (a neuronal model cell line) are widely used as an *in vitro* model in investigations of development, pathogenesis, and toxicity. PC12 cells were shown to express cannabinoid CB1 receptors [14], although some reports suggest that the cells are not endowed with this receptor type [15]. PC12 cells also seem to be a suitable system for studying important features of NMDA receptors [16].

In our study, a toxicity model with diazinon as a toxic agent was examined under *in vitro* conditions. Then, the neuroprotective effects of the CB1 receptor agonist WIN55,212-2 and NMDA receptor agonist NMDA were assessed against diazinon-induced toxicity in PC12 cells. Also, the CB1 receptor antagonist AM251 and NMDA receptor antagonist MK801 were used in our study. Finally, possible interactions between CB1 receptors and ionotropic glutamate receptors, including those of the NMDA type, were examined in PC12 cells under conditions of diazinon-induced toxicity. To date, there are no studies of interactions between these two receptor types.

METHODS

Materials. The PC12 cell line was purchased from the Pasteur Institute (Tehran, Iran). Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serums (FBS) were obtained from Gibco Life Technologies (USA). The CellTitre 96 AQueous One Solution Cell Proliferation Assay Kit was from Promega Corporation (USA). WIN55,212-2, AM251 and N-methyl- D-aspartate (NMDA) were purchased from Tocris (Great Britain); MK801, diazinon, Triton X-100, and other chemicals were from Sigma-Aldrich (USA). Rabbit polyclonal antibody directed toward the CB1 receptor (Sc-20754) and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Sc-2004) were from Santa Cruz Biotechnology (USA).

Cell Culturing. PC12 cells were grown in the DMEM supplemented with 10% FBS + 100 units/ml antibiotic/antimycotic. These cells were then incubated in a humidified chamber containing 5% CO_2 at 37ºC. For the experiments, confluent cultures were harvested with trypsin and then re-suspended in 5 ml of the medium supplemented with FBS (to inactivate trypsin). The cells were seeded into 6 well or 96 well plates and allowed to adhere for 24 h at 37ºC in air with 5% CO_2.

Drug Exposure. Our treatment samples included the following groups: (i) PC12 cells treated with 200 μM diazinon, 0.1 μM cannabinoid receptor agonist WIN55,212-2, 100 μM NMDA receptor agonist NMDA and 1 μM MK801 in the separate groups for 48 h. Pretreatment of PC12 cells with different drugs took place 15 min before exposure to diazinon. Diazinon itself was in the well for 48 h. (ii) PC12 cells pretreated with 0.1 μM WIN55,212-2 and then exposed to 200 μM diazinon. (iii) PC12 cells pretreated with a cannabinoid receptor antagonist AM251 (1 μM); then, WIN55,212-2 and diazinon were added after another 15-min-long interval for 48 h (AM+WIN+DZ). (iv) In another group, PC12 cells were pretreated with 100 μM NMDA before being exposed to 200 μM diazinon for 48 h (NMDA+DZ). (v) PC12 cells were pretreated with 1 μM MK801; then, NMDA was added; 15 min later, diazinon was introduced for 48 h (MK+NMDA+DZ). (vi) In this group, drugs were added, respectively, in the group of WIN55,212-2, NMDA, and diazinon at 15-min-long intervals (WIN+NMDA+DZ). (vii) In this group, WIN55,212-2, NMDA, MK801, and diazinon were also added, respectively, at 15-min-long intervals (WIN+NMDA+MK+DZ). (viii) In the final group, WIN55,212-2, MK801, NMDA, and diazinon were added in the order mentioned above also at 15-min-long intervals (WIN+MK+NMDA+DZ). All drugs (WIN55,212-2, AM251, NMDA, MK801, and diazinon) were dissolved in dimethyl sulfoxide (DMSO) and diluted in the culturing medium to the desired concentration. The final concentration of DMSO was less than 0.1%, a concentration that exerted no confounding effects on the responses under study.

Measurement of Cell Viability. Cell viability was measured using the CellTitre 96 AQueous One Solution (MTS) Cell Proliferation Assay Kit. Cells (10^4 per well in 200 μl medium) were seeded in 96 well plates and allowed to adhere for 24 h at 37ºC. The medium was replaced with a fresh medium, and cells were exposed to the medium containing 200 μM diazinon or cannabinoid and to NMDA agonists and antagonists with and without diazinon (incubation for 48 h at 37°C). The treatment media were removed, and the cells were washed with phosphate buffer saline (PBS). Then, 100 μl of the serum-free culture medium containing 20 μl of CellTitre 96® AQueous One Solution Reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium] MTS was added into each well of the 96-well assay plate and re-incubated for 2 h at 37ºC. The absorbance of the sample was recorded at 490 nm using a Wallac microplate reader. The MTS

reduction values were expressed as percentage of the control (untreated cells).

Cell Lysis and Protein Assay. PC12 cells were plated in 6-well culture plates at the density of 10^6 cells/well. After 48-h-long treatment, cells were lysed by the following procedure. The medium was removed, and cells were washed twice with cold PBS. Cells were then lysed in 500 µl of the lysis buffer (Tris-HCl, 50 mM; NaCl, 500 mM; EDTA, 5 mM, and Triton X-100, 0.2% v/v (pH 7.5) supplemented with protease inhibitors. The cell lysate was sonicated for 1 min on ice. Then, the lysate was centrifuged at 10,000 g at 4ºC for 15 min. The supernatant was removed for protein assay. The protein concentration was measured using bovine serum albumin as a standard [17].

Analysis of CB1 Receptor Protein by Western Blotting. The resulting supernatants were used for quantitative analysis of the CB1 receptor amount by Western Blotting. Each sample containing 20 µg protein was separated by SDS–polyacrylamide gel electrophoresis (PAGE) on a 12% gel. After electrophoresis, the protein was transferred to a nitrocellulose membrane (20 V). Blots were blocked with 5% fat-free milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature. The nitrocellulose membrane was incubated with 1 to 200 dilutions of rabbit polyclonal CB1R antibody (sc-20754, Santa Cruz, USA) in 5% fat-free milk TBS-T for overnight at 4ºC. After washing with TBS-T, the nitrocellulose membrane was incubated with HRP-conjugated anti-rabbit IgG (1 to 10,000 dilution) for 60 min. Finally, the nitrocellulose membrane was incubated with the 3,3′,5,5′ tetramethybenzidine (TMB) liquid substrate system until protein bands appeared.

Statistical Analysis. In the Western Blot, protein bands were quantified using densitometry by NIH Imager software and normalized with respect to the β-actin band intensity. Protein bands were expressed as percentages of the control level. Numerical data are presented below as means ± s.e.m. Statistical testing used one-way analysis of variance followed by the Tukey's test. The criterion for statistical significance of differences was $P < 0.05$ for all comparisons.

RESULTS

Cytotoxicity and Cell Viability of PC12 Cells at Different Concentrations of Diazinon. To determine whether diazinon is neurotoxic, its effect on the viability of cultured PC12 cells was examined (Fig. 1). Survival of the cells was presented as a function of the diazinon concentration after treatment, using the MTS assay as a measure of cell viability. Exposure of PC12 cells to 100 to 400 µM diazinon reduced cell viability in a dose-dependent manner. Concentrations of 300 and 400 µM were toxic to the cells, and the rate of cell death increased. At 200 µM diazinon, cell survival was reduced to 20% of the untreated cells (control) and was, therefore, used for subsequent experiments on toxic effects (a concentration that produced a submaximal effect on cell viability) (Fig. 1, $P < 0.05$, one-way ANOVA). In the previous study, different doses of WIN55,212-2 (0.1-100 µM) were assayed. WIN55,212-2 exerted a neurotoxic effect at a 1 µM dose and higher with respect to the control. WIN55,212-2 at a 0.1 µM concentration was not toxic; this concentration was, therefore, deemed appropriate for subsequent experiments on neuroprotective effects in PC12 cells [10]. In addition, the protective effect of 0.1 µM WIN55,212-2 was examined in the presence of different doses of diazinon. Any dose increase could decrease the protective ability of WIN55,212-2 at 0.1 µM (Fig. 1, $P < 0.05$, one-way ANOVA).

Protective Effects of WIN55,212-2 Applied Different Times Prior to Diazinon. The effects of WIN55,212-2 were examined in cases where it was applied at different time intervals prior to induction of toxicity. Co-treatment of WIN55,212-2 with diazinon was not effective. WIN55,212-2 application for 15 min prior to diazinon provided better cell

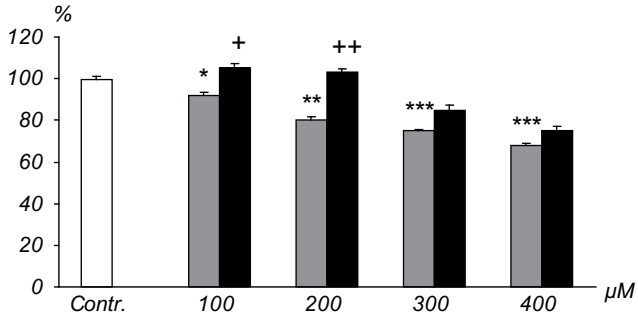

Fig. 1. Toxic effects of different concentrations of diazinon and the protective influence of 0.1 mM WIN55,212-2 on cultured pheochromocytoma PC12 cells. Concentrations of diazinon, µM, are shown below the columns; Contr. is the control. Vertical scale) Index of viability of PC12 cells, %; that in the control is taken as 100%. Gray columns, viability in the presence of diazinon; filled columns, that in the presence of both diazinon and WIN55,212-2. ** and *** indicate significant intergroup differences *vs* control with $P < 0.01$ and $P < 0.001$, respectively; + and ++ symbols indicate cases with $P < 0.05$ and 0.01 in comparison with the diazinon group. ANOVA + Tukey; $n = 6$ in all cases.

survival compared to the effect of diazinon alone. At the same time, adding WIN55,212-2 24 h before diazinon significantly reduced the protective effect with respect to diazinon. Therefore, all pretreatments with WIN55,212-2 were set to happen 15 min prior to diazinon application (Fig. 2, $P < 0.05$, one-way ANOVA).

WIN55,212-2 and Survival of PC12 Cells. Incubation of PC12 cells with 200 μM diazinon for 48 h induced cell death up to 20% ($P < 0.01$). Cell survival in the group treated with WIN55,212-2 alone was greater. The addition to 0.1 μM WIN55,212-2 significantly increased PC12 cell survival ($P < 0.01$). To determine whether the neuroprotective effect of WIN55,212-2 was CB1 receptor-mediated, the above receptors were blocked with its antagonist AM251. The cells were treated with 1 μM AM251 for 15 min prior to incubation with 0.1 μM WIN55,212-2. Under such conditions, the neuroprotective effect of WIN55,212-2 was not abolished (Fig. 3, $P < 0.05$, one-way ANOVA).

Protective Effect of NMDA on Diazinon-Induced Toxicity. The treatment with 1 μM NMDA receptor antagonist MK801 alone for 48 h somewhat worsened cell survival, though insignificantly. At the same time, cell survival in the group treated with the NMDA receptor agonist (NMDA alone) for 48 h increased the viability *vs* unexposed control cells (insignificant increase). Diazinon (200 μM) alone killed 20% of the cells. Pretreatment with NMDA not only prevented the reduction of the cell number induced by 200 μM diazinon, but it raised the viability by about 25% ($P < 0.01$). To determine whether the viability increase due to NMDA was mediated by receptors to the latter, the cells were pretreated with the NMDA receptor antagonist MK-801 followed by the addition of NMDA and diazinon for 48 h. In this group (MK+NMDA+DZ), MK-801, due to blocking of NMDA receptors, increased the neurotoxicity of diazinon by about 15%. (Fig. 4, $P < 0.05$, one-way ANOVA).

WIN55,212-2 and NMDA Interaction with Respect to the Diazinon-Induced Toxicity. When WIN55,212-2 and NMDA were used in combination, two groups (WIN+NMDA+DZ and WIN+NMDA+MK801+DZ) revealed neuroprotective effects ($P < 0.05$). At the same time, cell survival in the WIN55,212-2 + MK801+ + NMDA + diazinon group was significantly lower ($P < 0.05$). We hypothesized that NMDA may help WIN55,212-2 to protect cells against diazinon-mediated neurotoxicity. Therefore, we pretreated cultured cells with 1 μM MK801 for 15 min prior to or 15 min after the addition of NMDA. The protective effect of NMDA was completely blocked by MK-801 in this group (WIN+MK801+NMDA+DZ). It seems that NMDA can help WIN55,212-2 to protect cells

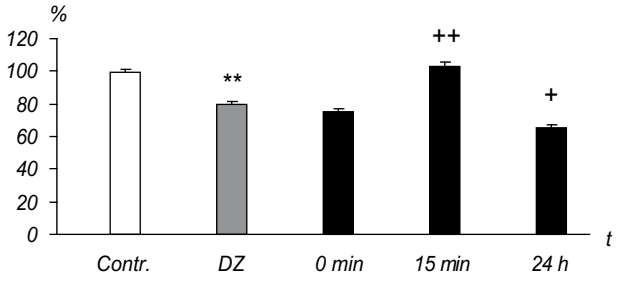

Fig. 2. Protective effect of 0.1 μM WIN55,212-2 against 200 mM diazinon (DZ)-induced toxicity, dependence on time prior to the diazinon exposure (shown below the columns). Cell viability was assessed 48 h after the beginning of incubation with diazinon. Other designations are similar to those in Fig. 1.

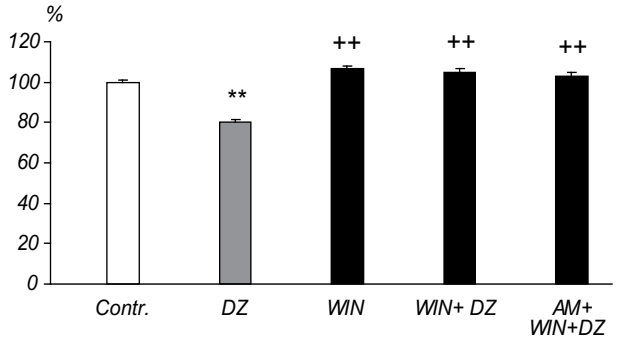

Fig. 3. Effect of 0.1 μM WIN55,212-2 and 1 μM AM251 on 200 μM diazinon-induced cell death. Cell viability was assessed 48 h after the beginning of incubation with diazinon; WIN55,212-2 and AM251 were added 15 min before diazinon. Agents added to the culture medium are shown below the columns. Other designations are similar to those in Figs. 1 and 2.

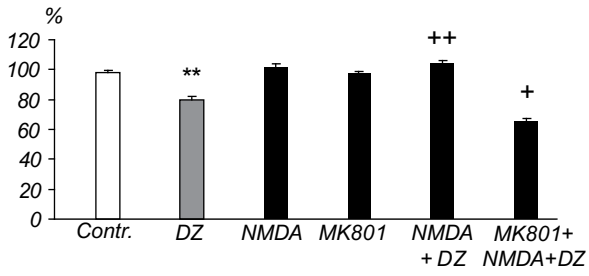

Fig. 4. Effects of a glutamate receptor agonist (NMDA) and an antagonist (MK-801) on diazinon-induced neurotoxicity. PC12 cells were treated with 200 μM diazinon (DZ), NMDA, and DZ added 15 min after NMDA in the presence or absence of MK-801. Other designations are similar to those in Figs. 1-3.

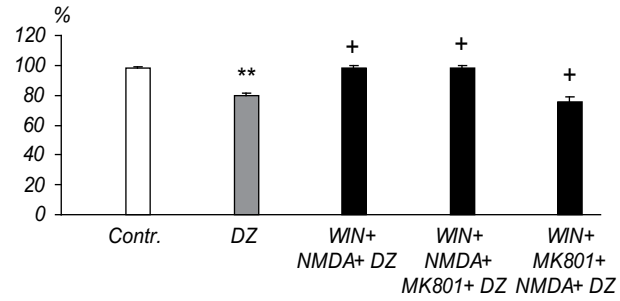

Fig. 5. Interaction between WIN55,212-2, an NMDA receptor agonist, and an NMDA receptor antagonist in the effects against diazinon-induced neurotoxicity. PC12 cells were treated by the shown agents 15 min prior to diazinon. Other designations are similar to those in Figs. 1-4.

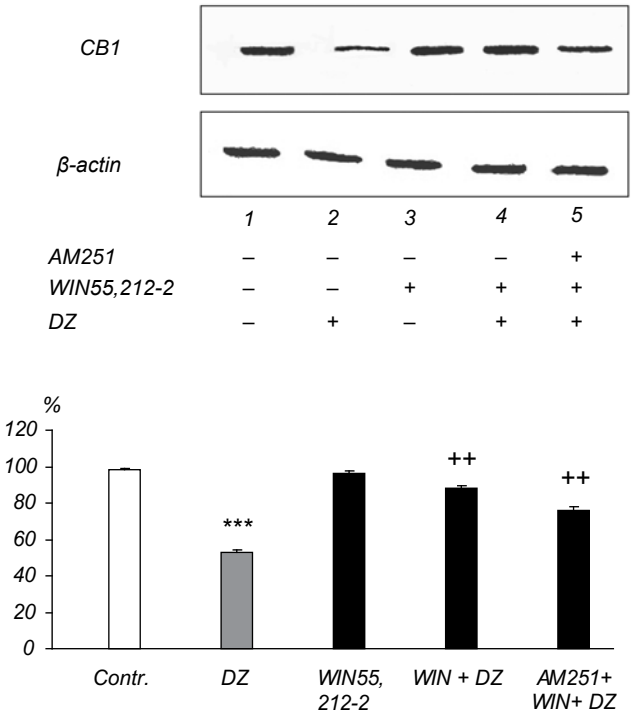

Fig. 6. Assay of CB1 receptor expression in PC12 cells. The effect of cannabinoids on the levels of CB1 receptors in PC12 cells exposed to a sublethal concentration of diazinon (200 μM). Solubilized PC12 cellular extracts were subjected to SDS-PAGE (20 μg protein per lane), blotted to nitrocellulose membrane, and incubated with rabbit antibody (Sc-20754) directed specifically against CB1 receptors and followed with HRP-conjugated goat anti-rabbit IgG (Sc-2004). A) Results of blotting; B) table of the agents acting on the samples whose analysis is shown in A, and C) mean densitometric indices of the band for CB1 (45-60 kDa) normalized with respect to the β-actin band intensity and expressed as percentage of the control level. Number of repetitions $n = 3$ in all cases. Designations on panel C are similar to those in Figs. 1-5.

when there was no MK801 or NMDA was not added before MK801. Thus, the toxic effect in the group of WIN+MK801+NMDA+DZ is significantly greater than that in the diazinon group (Fig. 5, $P < 0.05$, one-way ANOVA).

Levels of CB1 Receptor Protein in PC12 Cells Treated with Diazinon. The levels of CB1 receptor protein in unexposed control and diazinon-exposed PC12 cells were assayed by Western Blot analysis employing polyclonal anti-CB1 receptor antibody. Protein bands with a molecular mass of 45-60 kDa were observed. Compared to that in unexposed cells, the level of CB1 receptor protein decreased significantly in the cells exposed to 200 μM diazinon ($P < 0.001$). The CB1 receptor agonist alone (at a concentration used in this experiment) exerted no significant effect on the CB1 receptor protein level in PC12 cells. In the presence and absence of AM251, the level of CB1 receptor protein was significantly higher as compared with that in the diazinon alone group ($P < 0.01$). Similarly to viability experiments, AM251 did not prevent the increase of CB1 expression in the cells (Fig. 6, $P < 0.05$, one-way ANOVA).

Levels of CB1 Receptor Protein at Interaction of NMDA and WIN55,212-2. It was supposed that NMDA would increase CB1 receptor expression when applied in association with WIN55,212-2 in the absence of MK801 or in the case where the latter was applied before. In agreement with this supposition, it was found that NMDA can help WIN55,212-2 in CB1 receptor expression (WIN+NMDA+DZ and WIN+NMDA+MK+DZ). However, inhibition of NMDA receptors with MK801 application prior to NMDA decreased CB1 receptor expression (Fig. 7, $P < 0.05$, one-way ANOVA).

DISCUSSION

In our study, several concentrations of diazinon were used to determine effective amounts of the latter. Diazinon induced concentration-dependent decreases in cell viability. Diazinon in the 200 μM dose decreased cell survival and CB1 receptor expression after 48 h-long exposure. In our earlier works with OPs, we found that sublethal concentrations of diazinon inhibited the outgrowth of axon-like processes and induced apoptosis of differentiated PC12 cells [4, 18]. Other researchers also reported that diazoxon inhibited AChE activity and reduced transcripts of the α4 and β2 subunits

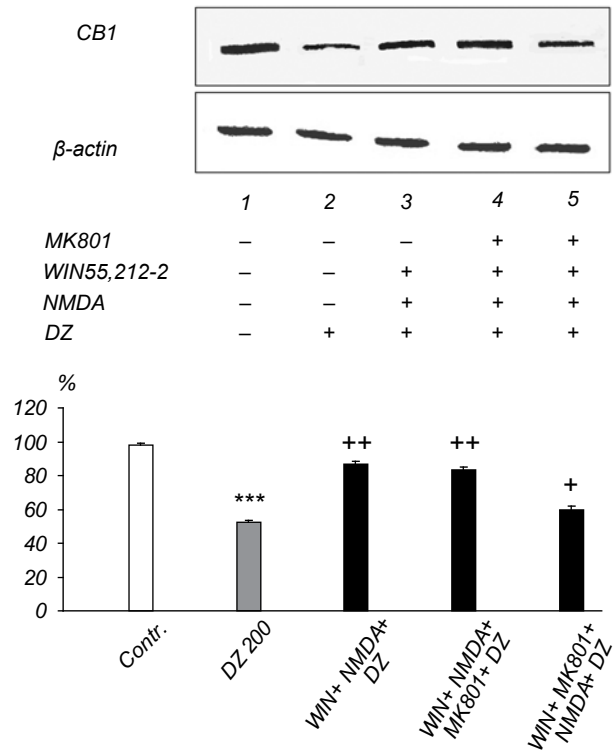

Fig. 7. Assay of CB1 receptor expression in PC12 cells. Interaction between WIN55,212-2, an NMDA agonist, and an NMDA antagonist in the effects on the levels of CB1 receptors in PC12 cells exposed to a sublethal concentration of diazinon (200 μM). Procedures of analysis are similar to those in Fig. 6; panels A-C are similar to those in this Figure. Designations in panel C are similar to those in Figs. 1-6.

of nAChRs (at the mRNA level) in PC12 cells [2]. In addition, expression of serotonergic neuronal markers was detracted in PC12 cells [19].

In this study, we hypothesized that WIN55,212-2 may protect PC12 cells against toxicity, and we checked this hypothesis under *in vitro* conditions on PC12 cells. It was found that WIN55,212-2 increases cell survival and CB1 receptor expression. So, the protective effect is obvious. According to earlier studies, WIN55,212-2 demonstrated a protective effect against toxic insults induced by glutamatergic overstimulation [20], β-amyloid [21], hypoxic/ischemic injury [22], and oxidative damage [23]. The molecular mechanisms of such neuroprotective effect are still controversial but might involve direct activation of pro-survival signalling pathways including cannabinoid receptors (mainly CB1) [24]. In general, the protective effect of cannabinoids is dependent on special conditions (such as adequate doses and short exposure times) and may be related to the enhancement of proliferation [25]. However, high doses of these agents and long exposure times inhibit cell growth and/or induce apoptosis via activation of caspases 3 and 7 [26]. Consistent with these studies, it was reported that the CB1 receptor agonist WIN55,212-2 protected PC12 cells from diazinon-induced neurotoxicity in a low dose (0.1 μM) and with a short exposure time (15 min).

Moreover, it was shown that WIN55,212-2 provided neuroprotection is not mediated via direct cannabinoid receptor activation. This finding was confirmed using the CB1 receptor antagonist AM251. AM251 did not decrease the viability and CB1 receptor expression increased by WIN55,212-2 in PC12 cells. However, in our previous work where PC12 cells were differentiated under the action of neural growth factor (NGF), AM251 inhibited the neuroprotective effect of WIN55,212-2 [4]. Hence, the reason for this disparity seems to be related to the cell differentiation process. Molderings et al. [15] reported that undifferentiated PC12 cells do not express detectable amounts of CB1 receptors. Since the level of activity is generally related to the number of active receptors, the antagonism is usually more considerable under conditions of elevated receptor expression [27].

A few mechanisms have been proposed to explain the CB1-independent neuromodulatory effect. First, cannabinoids can alter the membrane fluidity and other physiochemical properties of the membrane. Second, cannabinoids directly modulate the functional properties of voltage- and ligand-gated ion channels in the membrane [28]. Our experiments showed that activation of NMDA receptors by a subtoxic concentration of NMDA (100 μM) in the culture medium is selectively involved in the protection of the cells against diazinon, whereas the NMDA receptor antagonist MK-801 increases diazinon-mediated neurotoxicity. It seems that MK801 blocks NMDA receptors and interferes with NMDA-induced protection. Consistent with these results, other investigators reported that NMDA (100 μM) protects cerebellar granular cells from paraoxon-induced toxicity. This study suggests that NMDA (100 μM) completely blocks caspase-3 activation responsible for inducing apoptosis and protects almost all vulnerable neurons against paraoxon-induced neuronal cell death [29]. Furthermore, a few researches reported that NMDA-mediated neuroprotection is provided by distinct mechanisms. Among them, there are a calcium-dependent mechanism [30], inhibition of the pro-apoptotic JNK pathway [31], activation of

pro-survival pathways (MAPK/ERK1/2, PI3-K/Akt, PKA/CREB, and $NF_{\kappa}B$) [32], and regulation of transcription of the antioxidant system [33]. Activation of NMDA receptors is vitally important for development and survival of CNS neurons in the developing brain; this factor enhances differentiation and survival of the neurons [34].

A controversial situation related to receptor interactions deserves attention. We tried to reveal possible interactions between CB1 and NMDA receptors. Our findings showed for the first time that NMDA helps WIN55,212-2 to increase cell survival and to enhance CB1 receptor expression; it should be taken into account that these two receptor types are integrated together in PC12 cells. Furthermore, other studies demonstrated that mGlu1α and CB1 receptors are co-expressed in a subpopulation of interneurons in the CA1 *stratum radiatum* of hippocampal slices (double immunofluorescence staining and confocal microscopy were used). It seems that mGlu1α and CB1 receptors expressed on the same CA1 interneurons may play a cooperative role in the regulation of synaptic signalling in CA1 pyramidal cells [13]. Other studies suggested that there are strong direct and indirect interactions between cannabinoid CB1 receptors and group II metabotropic glutamate receptors signaling in layer-V pyramidal neurons in the rat prefrontal cortex [12].

Therefore, diazinon is a strong toxicant capable of damaging PC12 cells. Diazinon reduces cell viability and inhibits expression of CB1 receptors in these cells. WIN55,212-2 and NMDA noticeably protect PC12 cells against diazinon toxicity. The protective effect of WIN55,212-2 seems to be CB1 receptor-independent (considering the presence of action of AM251). However, MK801 inhibits the protective effect of NMDA. As a result, we should conclude that NMDA exerts its protective action precisely via these receptors. Finally, it is possible that WIN55,212-2 and NMDA actively interact in mediation of their protective effects.

The authors, F. Bahrami, M. Hashemi, F. Khalili, J. Hashemi, and A. Asgari, confirm that they have no conflict of interest.

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REFERENCES

1. S. J. Garfitt, K. Jones, H. J. Mason, and J. Cocker, "Exposure to the organophosphate diazinon: data from a human volunteer study with oral and dermal doses," *Toxicol. Let*., **134**, Nos. 1/3, 105-113 (2002).
2. H. Mehrani and L. Golmanesh, "Changes in mRNA and protein levels of nicotinic acetylcholine receptors in Diazoxon-exposed PC12 cells," *Toxicol. in vitro*, **22**, No. 5, 1257-1263 (2008).
3. T. L. Lassiter, E. A. MacKillop, I. T. Ryde, et al., "Is fipronil safer than chlorpyrifos? Comparative developmental neurotoxicity modeled in PC12 cells," *Brain Res. Bull*., **78**, No. 6, 313-322 (2009).
4. S. Sadri, F. Bahrami, M. Khazaei, et al., "Cannabinoid receptor agonist WIN55,212-2 protects differentiated PC12 cells from organophosphorus-induced apoptosis," *Int. J. Toxicol*., **29**, No. 2, 201-208 (2010).
5. J. Flaskos, W. Harris, M. Sachana, et al., "The effects of diazinon and cypermethrin on the differentiation of neuronal and glial cell lines," *Toxicol. Appl. Pharmacol*., **219**, Nos. 2/3, 172-180 (2007).
6. E. Nocerino, M. Amato, and A. A. Izzo, "Cannabis and cannabinoid receptor," *Fitoterapia*, **71**, S6-S12 (2000).
7. I. Svízenská, P. Dubový, and A. Sulcová, "Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures – a short review," *Pharmacol. Biochem. Behav*., **90**, No. 4, 501-511 (2008).
8. D. Baker, G. Pryce, G. Giovannoni, and A. J. Thompson, "The therapeutic potential of Cannabis," *Lancet Neurol*., **2**, No. 5, 291-298 (2003).
9. B. G. Ramírez, C. Blázquez, T. Gómez del Pulgar, et al., "Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation," *J. Neurosci*., **25**, No. 8, 1904-1913 (2005).
10. M. Hashemi, F. Bahrami, H. Sahraei, et al., "The neuroprotective effect of cannabinoid receptor agonist (WIN55,212-2) in paraoxon induced neurotoxicity in PC12 cells and N-methyl-D-aspartate receptor interaction," *Cell J*., **12**, No. 2, 183-190 (2010).
11. C. Sommer, M. Schomacher, C. Berger, et al., "Neuroprotective cannabinoid receptor antagonist SR141716-A prevents downregulation of excitotoxic NMDA receptors in the ischemic penumbra," *Acta Neuropathol*., **112**, No. 3, 277-286 (2006).
12. J.G. Barbara, N. Auclair, M. P. Roisin, et al., "Direct and indirect interactions between cannabinoid CB1 receptor and group II metabotropic glutamate receptor signaling in layer V pyramidal neurons from the rat prefrontal cortex," *Eur. J. Neurosci*., **17**, No. 5, 981-990 (2003).
13. F. Boscia, F. Ferraguti, F. Moroni, et al., "mGlu1α receptors are co-expressed with CB1 receptors in a subset of interneurons in the CA1 region of organotypic hippocampal slice cultures and adult rat brain," *Neuropharmacology*, **55**, No. 4, 428-439 (2008).
14. K. P. Sarker, and I. Maruyama, "Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid

rafts," *Cell. Mol. Life Sci*., **60**, No. 6, 1200-1208 (2003).
15. G. J. Molderings, H. Bönisch, R. Hammermann, et al., "Noradrenaline release-inhibiting receptors on PC12 cells devoid of α2 and CB1 receptors: similarities to presynaptic imidazoline and EDG receptors," *Neurochem. Int.* **40**, No. 2, 157-167 (2002).
16. M. Casado, A. López-Guajardo, B. Mellström, et al., "Functional N-methyl-D-aspartate receptors in clonal rat pheochromocytoma cells," *J. Physiol*., **490**, No. 2, 391-404 (1996).
17. M. M. Bradford, "A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem*., **72**, 248-254 (1976).
18. F. Bahrami, M. Yousefpour, H. Mehrani, et al., "Type of cell death and the role of acetylcholinesterase activity in neurotoxicity induced by paraoxon in cultured rat hippocampal neurons," *Acta Biol. Hung*., **60**, No. 1, 1-13 (2009).
19. T. A. Slotkin and F. J. Seidler, "Developmental neurotoxicants target differentiation into the serotonin phenotype: chlorpyrifos, diazinon, dieldrin and divalent nickel," *Toxicol. Appl. Pharmacol*., **233**, No. 2, 211-219 (2008).
20. J. Chen, C. Lee, S. Errico, et al., "Protective effects of Δ^9-tetrahydrocannabinol against N-methyl-D-aspartate-induced AF5 cell death," *Mol. Brain Res*., **134**, No. 2, 215-225 (2005).
21. T. Iuvone, G. Esposito, R. Esposito, et al., "Neuroprotective effect of cannabidiol, a non-psychoactive component from *Cannabis sativa*, on β-amyloid-induced toxicity in PC12 cells," *J. Neurochem*., **89**, No. 1, 134-141 (2004).
22. D. Fernández-López, J. Martínez-Orgado, E. Nuñez, et al., "Characterization of the neuroprotective effect of the cannabinoid agonist WIN55,212-2 in an *in vitro* model of hypoxic-ischemic brain damage in newborn rats," *Pediatr. Res*., **60**, No. 2, 169-173 (2006).
23. B. S. Harvey, K. S. Ohlsson, J. L. Mååg, et al., "Contrasting protective effects of cannabinoids against oxidative stress and amyloid-b evoked neurotoxicity *in vitro*," *Neurotoxicology*, **33**, No. 1, 138-146 (2012).
24. T. Gomes del Pulgar, G. Velasco, and M. Guzmán, "The CB1 cannabinoid receptor is coupled to the activation of protein kinaseB/Akt," *Biochem. J*., **347**, No. 2, 369-373 (2000).
25. M. Guzmán, C. Sánchez, and I. Galve-Roperh, "Cannabinoids and cell fate," *Pharmacol. Therapeut*., **95**, No. 2, 175-184 (2002).
26. M. Widmer, C. O. Hanemann, and J. Zajicek, "High concentrations of cannabinoids activate apoptosis in human U373MG glioma cells," *J. Neurosci. Res*., **86**, No. 14, 3212-3220 (2008).
27. V. See, A. L. Boutillier, H. Bito, and J. P. Loeffler, "Calcium/calmodulin dependent protein kinase type IV (CaMKIV) inhibits apoptosis induced by potassium deprivation in cerebellar granule neurons," *FASEB. J*., **15**, No. 1, 134-144 (2001).
28. X. Xifro, A. Falluel-Morel, A. Minano, et al., "N-methyl-D-aspartate blocks activation of JNK and mitochondrial apoptotic pathway induced by potassium deprivation in cerebellar granule cells," *J. Biol. Chem*., **281**, No. 10, 6801-6812 (2006).
29. D. Jantas and W. D. Lason, "Different mechanisms of NMDA mediated protection against neuronal apoptosis: a stimuli-dependent effect," *Neurochem. Res*., **34**, No. 11, 2040-2054 (2009).
30. J. Singh and G. Kaur, "Neuroprotection mediated by subtoxic dose of NMDA in SH-SY5Y neuroblastoma cultures: activity dependent regulation of PSA-NCAM expression," *Brain Res. Mol*., **137**, Nos. 1/2, 223-234 (2005).
31. C. C. Wang, R. G. Held, S. C. Chang, et al., "A critical role for GluN2B-containing NMDA receptors in cortical development and function," *Neuron*, **72**, No. 5, 789-805 (2011).