Published in final edited form as:

Neurosci Lett. 2005 December 2; 389(2): 99–103.

Reactive oxygen species and p38 phosphorylation regulate the protective effect of Δ^9 -tetrahydrocannabinol in the apoptotic response to NMDA

Jia Chen^{*}, Stacie L. Errico, and William J. Freed

Development and Plasticity Section, Cellular Neurobiology Research Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services, Baltimore, Maryland 21224, USA

Abstract

NMDA causes oxidative stress in neurons, and produces cell death involving elements of both necrosis and apoptosis. To examine the neuroprotective mechanism of Δ^9 -Tetrahydrocannabinol (THC) in NMDA-induced death of AF5 cells, we measured reactive oxygen species (ROS) formation after exposure to NMDA. ROS generation was increased by NMDA, and NMDA-induced ROS generation was significantly decreased by THC. Western blotting revealed an increase in phosphorylated p38 MAPK after NMDA treatment, which was also blocked by pretreatment with THC. The time course of ROS generation and activation of MAPK signaling pathways were similar. SB203580, a p38 inhibitor, partially blocked glutamate excitotoxicity in AF5 cells. The present data suggest that THC protects against NMDA-induced apoptosis in AF5 cells by blocking ROS generation and inhibiting the activation of p38-MAPK.

Keywords

THC; NMDA; Reactive oxygen species; Apoptosis; MAP kinase; Neuroprotection

Oxidative stress has been implicated in a variety of pathological processes, including several neurodegenerative diseases [1,10]. Reactive oxygen species (ROS) also play important roles in the regulation of a number of normal physiological processes, including cell proliferation, survival, senescence and apoptotic cell death [19]. N-Methyl-D-aspartate (NMDA), as one type of stimulus which induces oxidative stress, can cause cell death involving ROS [16,24]. The mechanisms of NMDA receptor-mediated cell death are complex and incompletely understood. The excitotoxic effects of glutamate are largely mediated by increased Ca^{2+} influx through activated NMDA receptors [8]. Associated with Ca^{2+} influx is an increase in ROS that appears to originate in mitochondria [17]. In some cases, preventing or inhibiting ROS generation can be neuroprotective [9,14].

Oxidative stress is known to induce apoptosis in a wide variety of cell types, apparently by modulating intracellular signaling pathways. The activity of several mitogen-activated protein kinases (MAPKs), consisting of extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal protein kinase (JNK), and p38 MAPK have been identified as being increased in nitric oxide-induced cell death [11], H₂O₂-induced apoptosis [15], and NMDA-induced apoptosis [4,13].

^{*}Corresponding author. Dr. Jia Chen, NIDA Intramural Research Program, Cellular Neurobiology Research Branch, 333 Cassell Drive, Triad Bldg, Room 3505, Baltimore, Maryland, 21224. Tel.: +1 410 550 6565 ext. 134; fax: +1 410-550-1621. E-mail address: jchen@intra.nida.nih.gov (J. Chen).

Cannabinoids can produce cell death as well as neuroprotective effects in a number of cell types and model systems. The role of the JNK or p38 MAPK in cell death and cellular protective effects of cannabinoids is controversial. Previous studies have shown that activation of the CB1 cannabinoid receptor is coupled to stimulation of JNK and p38 MAPK [6,22]. There may be a threshold above which long-term JNK activation induced by cannabinoids leads to neural cell death. Therefore, activation of JNK and p38 MAPK may be responsible for some of the cellular responses elicited by the CB1 receptor, such as THC-induced cell death [22]. However, exogenous cannabinoids can also produce cellular protection by inactivation of JNK or p38 MAPK, with concomitant activation of other signaling pathways, either independently or coordinately [7].

In a previous study we found that THC, a CB1 cannabinoid receptor agonist, can protect AF5 cells from NMDA-induced toxicity, an effect apparently related to the antioxidant properties of this compound [3]. In order to further investigate the protective mechanisms of THC, ROS and downstream signaling pathways in NMDA-receptor-mediated apoptosis were examined.

Experiments were performed on the AF5 cell line, which was immortalized using a fragment of the SV40 large T antigen gene known as T155g, and has been described previously [23]. For THC treatment (3 μ M, Sigma), the cells were first incubated with THC in serum-free DMEM/F12 for 6 h, and then the media were replaced with media containing 7.5 mM NMDA in HBSS for 30 min. After exposure, cells were cultured in fresh serum-free DMEM/F12 media for 16–18 h.

Relative levels of cellular peroxides were quantified using the dye 2, 7-dichlorofluorescein diacetate (DCF, Sigma) as described previously [20]. After exposure to NMDA for 30 min, AF5 cells were incubated with 100 μ M of DCF for 15–30 min at 37°C and were then washed with PBS. To obtain dissociated AF5 cells for the ROS assay, the culture medium was first removed, then cells were lysed with 1% Triton X-100 in PBS. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (Techan Genios).

Western blotting was performed as previously described [3]. Equivalent amounts of total proteins were separated by PAGE/SDS. The primary antibody concentration for anti-JNK1 was 1: 1000 (Santa Cruz); the antibody concentrations for anti- p38, anti-phospho-p38 (Cell Signaling) and anti-phospho-JNK (Santa Cruz) were 1:500, and the antibody concentration for anti- α -tubulin (Sigma) was 1:2000. Following incubation with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1: 1000 to 1: 2000, Sigma), immunoreactive bands were visualized by ECL and measured by volume densitometry using NIH image software. Cell proliferation was measured by an MTT assay [20]. Data are presented as means $\pm$ standard error of the mean (S.E.M.) and analyzed by one-way ANOVA and *post-hoc* Tukey multiple comparison tests for repeated experiments. Group differences were considered significant for $p < 0.05$.

After exposure to 7.5 mM NMDA for 30 min, ROS were increased 30 min and 60 min after removal of NMDA (Fig. 1A). Little or no increase in ROS was present 16 h after NMDA stimulation (data not shown). Pretreatment with 3 μ M THC significantly decreased NMDA-induced ROS generation (Fig. 1B). There was no change in ROS after treatment with THC alone (data not shown).

Western blotting showed that activation of phospho-p38 kinase was increased at 15 and 30 min, and started to decrease 60 min after removal of NMDA. Total p38 protein levels were unchanged (Fig. 2A; left panel). There were no differences in Phospho-JNK and total JNK protein under the various experimental conditions (Fig. 2B, left panel).

When pretreated with THC for 6 h prior to NMDA exposure, phospho-p38 kinase activity was suppressed as compared to cells treated with NMDA alone. There were no apparent changes in total p38 MAPK protein (Fig. 2A; right panel). There were no differences in Phospho-JNK or total JNK protein under the various experimental conditions (Fig. 2B; right panel).

To confirm that the p38 MAPK pathway is involved in NMDA toxicity, we examined the effect of SB203580, a selective p38 MAPK inhibitor, in AF5 cells after NMDA exposure. When pretreated with various concentrations of SB203580 (0.1–10 μ M, Sigma) for 1h, the loss of cell viability induced by 7.5 mM NMDA was partially blocked by concentrations of 0.5 – 10 μ M (Fig. 3). SB203580 alone produced no cytotoxicity (data not shown).

The present study was focused on the neuroprotective mechanisms of THC, which appears to act as an antioxidant in preventing NMDA-induced cell death in AF5 cells [3]. Although NMDA-induced apoptosis has been studied extensively [19], neuroprotective mechanisms of THC in NMDA toxicity have been less completely explored. We found that both ROS generation and phosphorylated p38 MAPK were significantly increased with a similar time course, after NMDA stimulation. At least to some degree, THC appears to utilize the same signaling pathways as NMDA-induced apoptosis in AF5 cells, and exerts its protection by blocking ROS generation, and by inhibiting activation of p38 MAPK.

In some circumstances, increases in ROS represent the first step in a series of events which result in cell death. Increased cellular ROS can arise from either intracellular sources, or can be induced by exogenous stimulation. Here, NMDA, as an exogenous stimulus, initiates an intracellular reaction which leads to increased production of ROS. Previous studies have found that during 30 min of exposure of hippocampal neurons to NMDA, intracellular superoxide production was increased significantly, and remained elevated for several hours after removal of NMDA [16]. In the present study, ROS generation was initiated after NMDA stimulation, and THC largely blocked the increase of ROS produced by NMDA. Therefore, ROS generation may be critical for NMDA-induced cell death in AF5 cells, and blocking ROS may be responsible for the protective effect of THC.

Glutamate exposure has previously been shown to transiently induce JNK-1 and p38 activation in granule cells in vitro. p38 was markedly activated 5 min after glutamate exposure and the activity remained increased above basal levels for more than 1 h. The degree of increase of JNK activation was not remarkable, possibly due to the high basal activity of JNK in granule cells [4,13]. We observed increased phosphorylated p38 MAPK after NMDA treatment, while activation of phosphorylated p38 MAPK was blocked by pretreatment with THC prior to NMDA. This reaction also takes place at an early stage after NMDA exposure. The present results suggest that there is at least a temporal correlation between ROS generation and activation of p38 MAPK signaling pathways. We cannot, however, rule out the possibility that other pathways and modulation of phosphorylation of other proteins are also involved in NMDA toxicity and THC-mediated neuroprotection.

Interestingly, some studies have reported that antioxidants, such as a combination of antioxidant vitamins (β -carotene, ascorbic acid and α -tocopherol), or α -tocopherol alone, can reduce cardiac oxidative stress and myocyte apoptosis produced by exogenous norepinephrine. These effects were associated with reversal of the changes in JNK, p38-MAPK and ERK activities, by decreasing JNK and p38 and increasing ERK activity [21]. In lipopolysaccharide-induced oxidative stress, antioxidants have been also shown to inhibit NO production by reduction of p38 MAPK activity [12]. Antioxidant neuroprotective effects of some cannabinoids have also been described. In addition to the direct antioxidant activity of phenolic cannabinoids, these compounds may further affect membrane-associated and intracellular signaling mechanisms, including the activation of the neuroprotective mitogen-activated

protein kinase signaling [2,18]. The present results are also consistent with our previous report that the protective effect of THC is related to the antioxidant properties of this compound [3].

SB203580, a specific p38 kinase inhibitor, has been reported to inhibit glutamate-induced apoptosis in rat cerebellar granule cells [13]. SB203580 inhibits p38 with an IC₅₀ of approximately 0.6 μ M, and even at 100 μ M has no effect on the activities of 12 other protein kinases tested [5]. The level of inhibition of glutamate toxicity by SB203580 has, however, varied among experiments [9]. In the present study, SB203580 partially blocked glutamate excitotoxicity in AF5 cells, as measured by an MTT assay. Therefore, the present data are consistent with the possibility that both NMDA-induced cell death and THC-induced neuroprotection in AF5 cells are mediated by generation of ROS and consequent activation of p38 MAPK.

References

1. Balyasnikova IV, Visintine DJ, Gunnerson HB, Paisansathan C, Baughman VL, Minshall RD, Danilov SM. Propofol attenuates lung endothelial injury induced by ischemia-reperfusion and oxidative stress. *Anesth Analg* 2005;100:929–936. [PubMed: 15781500]
2. Behl C, Holsboer F. The female sex hormone oestrogen as a neuroprotectant. *Trends Pharmacol Sci* 1999;20:441–444. [PubMed: 10542441]
3. Chen J, Lee CT, Errico S, Deng X, Cadet JL, Freed WJ. Δ^9 -tetrahydrocannabinol against *N*-methyl-D-aspartate-induced AF5 cell death. *Brain Res Mol Brain Res* 2005;134:215–225. [PubMed: 15836919]
4. Chen RW, Qin ZH, Ren M, Kanai H, Chalecka-Franaszek E, Leeds P, Chuang DM. Regulation of c-Jun N-terminal kinase, p38 kinase and AP-1 DNA binding in cultured brain neurons: roles in glutamate excitotoxicity and lithium neuroprotection. *J Neurochem* 2003;84:566–575. [PubMed: 12558976]
5. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 1995;364:229–233. [PubMed: 7750577]
6. Derkinderen P, Ledent C, Parmentier M, Girault JA. Cannabinoids activate p38-MAPK through CB1 receptors in hippocampus. *J Neurochem* 2001;77:957–960. [PubMed: 11331425]
7. Francisco MH, Pinteaux E, Heenan L, Moore JD, Rothwell NJ, Gibson RM. Neuroprotective effects of the synthetic cannabinoid HU-210 in primary cortical neurons are mediated by phosphatidylinositol 3-kinase/AKT signaling. *Mol Cell Neurosci* 2005;28:189–194. [PubMed: 15607953]
8. Garthwaite G, Garthwaite J. Neurotoxicity of excitatory amino acid receptor agonists in rat cerebellar slices: Dependence on calcium concentration. *Neurosci Lett* 1986;66:193–198. [PubMed: 3014386]
9. Greenlund LJ, Deckwerth TL, Johnson EM Jr. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. *Neuron* 1995;14:303–315. [PubMed: 7857640]
10. Guerin JC, Leophonte P, Lebas FX, Liard F, Terrioux P, Boulanger P. Oxidative stress in bronchopulmonary disease: contribution of N-acetylcysteine (NAC). *Rev Pneumol Clin* 2005;61:16–21. [PubMed: 15772575]
11. Han OJ, Joe KH, Kim SW, Lee HS, Kwon NS, Baek KJ, Yun HY. Involvement of p38 mitogen-activated protein kinase and apoptosis signal-regulating kinase-1 in nitric oxide-induced cell death in PC12 cells. *Neurochem Res* 2001;26:525–532. [PubMed: 11513480]
12. Hou RC, Chen HL, Tzen JT, Jeng KC. Effect of sesame antioxidants on LPS-induced NO production by BV2 microglial cells. *NeuroReport* 2003;14:1815–1819. [PubMed: 14534426]
13. Kawasaki H, Morooka T, Shimohama S, Kimura J, Hirano T, Gotoh Y, Nishida E. Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. *J Biol Chem* 1997;272:18518–18521. [PubMed: 9228012]
14. Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* 1998;60:619–642. [PubMed: 9558479]

15. Lee YJ, Cho HN, Soh JW, Jhon GJ, Cho CK, Chung HY, Bae S, Lee SL, Lee YS. Oxidative stress-induced apoptosis is mediated by ERK1/2 phosphorylation. *Exp Cell Res* 2003;291:251–266. [PubMed: 14597424]
16. Luetjens CM, Bui NT, Sengpiel B, Munstermann G, Poppe M, Krohn AJ, Bauerbach E, Krieglstein J, Prehn JH. Delayed mitochondrial dysfunction in excitotoxic neuron death: Cytochrome c release and a secondary increase in superoxide production. *J Neurosci* 2000;20:5715–5723. [PubMed: 10908611]
17. Malis CD, Bonventre JV. Mechanism of calcium potentiation of oxygen free radical injury to renal mitochondria. A model for post-ischemic and toxic mitochondrial damage. *J Biol Chem* 1986;261:14201–14208. [PubMed: 2876985]
18. Marsicano G, Moosmann B, Hermann H, Lutz B, Behl C. Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. *J Neurochem* 2002;80:448–456. [PubMed: 11905991]
19. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: Signaling for suicide and survival. *J Cell Physiol* 2002;192:1–15. [PubMed: 12115731]
20. Park TH, Kwon OS, Park SY, Han ES, Lee CS. N-methylated beta-carbolines protect PC12 cells from cytotoxic effect of MPP+ by attenuation of mitochondrial membrane permeability change. *Neurosci Res* 2003;46:349–358. [PubMed: 12804796]
21. Qin F, Shite J, Liang CS. Antioxidants attenuate myocyte apoptosis and improve cardiac function in CHF: association with changes in MAPK pathways. *Am J Physiol Heart Circ Physiol* 2003;285:H822–H832. [PubMed: 12714335]
22. Rueda D, Galve-Roperh I, Haro A, Guzman M. The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Mol Pharmacol* 2000;58:814–820. [PubMed: 10999952]
23. Truckenmiller ME, Vawter MP, Zhang P, Conejero-Goldberg C, Dillon-Carter O, Morales N, Cheadle C, Becker KG, Freed WJ. AF5, a CNS cell line immortalized with an n-terminal fragment of SV40 large T: Growth, differentiation, genetic stability and gene expression. *Exp Neurol* 2002;175:318–337. [PubMed: 12061863]
24. Vergun O, Sobolevsky AI, Yelshansky MV, Keelan J, Khodorov BI, Duchon MR. Exploration of the role of reactive oxygen species in glutamate neurotoxicity in rat hippocampal neurones in culture. *J Physiol* 2001;531:147–163. [PubMed: 11179399]

Fig. 1.

THC blocks ROS generation-induced by NMDA. (A). ROS generation was increased 30 and 60 min after NMDA treatment. (B). Pre-treatment with THC blocked the increase in ROS at the indicated time points. **, $p < 0.01$; ***, $p < 0.001$ compared with control; ++, $p < 0.01$; +++, $p < 0.001$ compared with NMDA at the corresponding time points. Data are means $\pm$ S.E.M.

Fig. 2.

Regulation of phosphorylation of p38 MAPK and JNK in AF5 cells treated with NMDA or NMDA plus THC. (A) Cultured cells were collected after stimulation with NMDA (7.5 mM) at the indicated times. Equal amounts of total protein were loaded in each lane. Activation of phospho-p38 kinase was increased at 15, 30 min and started to decrease 60 min after removal of 7.5 mM NMDA. Total p38 protein was unchanged (Fig. 2A, left panel). Right Panel: Cells were pretreated with THC for 6 h prior to NMDA stimulation, and samples were collected at the indicated times after NMDA exposure. Phospho-p38 kinase activity was suppressed by THC, while there were no changes in total p38 MAPK protein (Fig. 2A, right panel). (B) Cells were incubated with NMDA or THC plus NMDA. Lysates were analyzed by Western blot with specific anti-phospho-JNK and anti-JNK antibodies. There were no differences in Phospho-JNK or total JNK protein under the various experimental conditions (Fig. 2B, right panel and left panel). Blots are representative of three separate experiments.

Fig. 3.

The p38 MAPK inhibitor SB203580 attenuates NMDA-induced cell death. AF5 cells were pretreated with various concentrations of SB203580 (0.1–10 μ M) for 1h, followed by incubation with 7.5 mM NMDA for 30 min. The loss of cell viability was partially blocked by SB203580 in concentrations of 0.5–10 μ M. Pretreatment with SB203580 alone produced no cytotoxicity. *** indicates $p < 0.001$, as compared with control; ++ and +++ indicate $p < 0.01$ or $p < 0.001$ respectively, as compared with NMDA only.