Neuroprotective Antioxidants from Marijuana

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ABSTRACT: Cannabidiol and other cannabinoids were examined as neuroprotectants in rat cortical neuron cultures exposed to toxic levels of the neurotransmitter, glutamate. The psychotropic cannabinoid receptor agonist Δ9-tetrahydrocannabinol (THC) and cannabidiol, (a non-psychoactive constituent of marijuana), both reduced NMDA, AMPA and kainate receptor mediated neurotoxicities. Neuroprotection was not affected by cannabinoid receptor antagonists, indicating a (cannabinoid) receptor-independent mechanism of action. Glutamate toxicity can be reduced by antioxidants. Using cyclic voltammetry and a fenton reaction based system, it was demonstrated that Cannabidiol, THC and other cannabinoids are potent antioxidants. As evidence that cannabinoids can act as antioxidants in neuronal cultures, cannabidiol was demonstrated to reduce hydroperoxide toxicity in neurons. In a head to head trial of the abilities of various antioxidants to prevent glutamate toxicity, cannabidiol was superior to both α-tocopherol and ascorbate in protective capacity. Recent preliminary studies in a rat model of focal cerebral ischemia suggest that cannabidiol may be at least as effective in vivo as seen in these in vitro studies.

INTRODUCTION

Cannabinoid components of marijuana are known to exert behavioral and psychotropic effects but also possess therapeutic properties including analgesia, ocular hypotension, and antiemesis. This report examines another potential therapeutic role for cannabinoids as neuroprotectants and describes their mechanism of action in rat cortical neuronal cultures. During an ischemic episode, large quantities of the excitatory neurotransmitter, glutamate, are released in the brain. This event causes neuronal death by over-stimulation of NMDA (NMDAr), AMPA and kainate type receptors, which massively increase intracellular calcium, resulting in metabolic

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stress and production of toxic reactive oxygen species (ROS). Antioxidants such as α-tocopherol,\textsuperscript{4,5} can prevent this toxicity by reducing the ROS formed during ischemic metabolism. Cannabinoids such as (-)Δ⁹-tetrahydro-cannabinol (THC) and its psychoactive analogues have previously been suggested to reduce glutamate toxicity,\textsuperscript{6} although this effect was apparently cannabinoid receptor mediated,\textsuperscript{6,7} perhaps through inhibition of voltage sensitive calcium channels.\textsuperscript{7,9} Our study examines cannabinoids as \textit{in vitro} neuroprotectants, and focuses on the non-psychoactive cannabinoid, cannabidiol. As with THC, cannabidiol is a natural component of the marijuana plant, \textit{Cannabis sativa}, although unlike THC, cannabidiol does not activate cannabinoid receptors in the brain and so is devoid of psychoactive effects. This presentation will demonstrate that cannabinoids are potent antioxidants which can protect neurons from ischemic injury without psychoactive side-effects.

**CANNABIDIOL BLOCKS NMDA, AMPA, AND KAINATE RECEPTOR-MEDIATED NEUROTOXICITY**

Glutamate neurotoxicity can be mediated either by NMDA, AMPA or kainate receptors. We therefore compared the ability of cannabinoids to prevent neurotoxicity mediated by all three types of glutamate receptors. To examine NMDAr mediated toxicity, rat cortical neuron cultures were exposed to glutamate for 10 min in a magnesium free medium. After this time, the culture media was replaced and the cells incubated for 20 hours at 37°C. In order to examine AMPA / kainate receptor mediated toxicity, neurons were incubated with glutamate for 20 hours in the presence of MK-801 (an NMDAr antagonist) and an agent to prevent receptor desensitization. To study AMPA or kainate receptors individually, glutamate was replaced with a specific receptor agonists (fluorowillardiine or 4-methyl-glutamate respectively). At the end of the incubation period, in both NMDA and AMPA / kainate models, toxicity was assessed by examination of lactate dehydrogenase (LDH) released into the media by dying cells.

Cannabidiol prevented cell death equally well with an EC50 of 2-4 μM in both NMDA and AMPA / kainate toxicity models (Fig. 1). Similar data was also observed when glutamate or AMPA-specific or kainate receptor specific ligands were used (data not shown). These results demonstrate cannabidiol protect equally regardless of whether toxicity is mediated by NMDA, AMPA or kainate receptors. This suggests glutamate receptors are probably not the site at which cannabidiol acts, protection is more likely to be due to a mechanism that occurs downstream of the initial glutamate receptor activation event.

**NEUROPROTECTION BY TETRAHYDROCANNABINOL**

Unlike cannabidiol, THC is a ligand for the brain cannabinoid receptor\textsuperscript{10} and this action has been proposed to explain THC’s ability to protect neurons from NMDAr

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\textsuperscript{4}Abbreviations: AMPA, 2-amino-3-(4-butyl-3-hydroxyisoxazol-5-yl)propionic acid; BHT, Butylhydroxy-toluene; NMDA, N-methyl-D-aspartate; NMDAr, NMDA receptors; ROS, reactive oxygen species; THC, -Δ⁹-tetrahydrocannabinol.
FIGURE 1. Effect of cannabidiol on NMDAr (A) and AMPA/kainate receptor (B) mediated neurotoxicity. Data shown represents mean values ± SEM from a single experiment with four replicates. Each experiment was repeated on at least four occasions with essentially the same results. Cannabinoids were present during (and, in the case of NMDAr mediated toxicity, after) the glutamate exposure periods. See text for further experimental details.

FIGURE 2. Effect of THC, cannabidiol and cannabinoid receptor agonist on glutamate induced neurotoxicity. Neurons exposed to glutamate in an AMPA/ kainate receptor toxicity model, were incubated with 10 μM cannabidiol or THC in the presence or absence of SR141716A (500 nM). See text for experimental details. Data represents mean values ± SEM from four experiments each with three replicates.
toxicity in vitro. However, THC and cannabidiol were similarly protective in AMPA/kainate receptor toxicity assays, suggesting that cannabinoid neuroprotection does not involve cannabinoid receptor activation. This was confirmed using the cannabinoid receptor antagonist, SR-141716A (Fig. 2). Neither THC or cannabidiol neuroprotection was affected by cannabinoid receptor antagonist indicating their action is not cannabinoid receptor–mediated.

Cannabinoids as Antioxidants

Easily oxidizable compounds such as glutathione, ascorbate and α-tocopherol, are used by living cells as disposable antioxidants which protect vital membranes and proteins from ROS damage. This type of ROS damage has previously been demonstrated to be a factor in glutamate neurotoxicity. To investigate whether cannabinoids might possess antioxidant abilities and so protect neurons by absorbing the ROS formed following glutamate receptor activity, the antioxidant properties of cannabidiol and other cannabinoids were assessed by both cyclic voltammetry and in a Fenton reaction system (iron catalyzed ROS generation). Cyclic voltammetry, which examines the ability of a compound to accept or donate electrons under a variable voltage potential, was used to measure the oxidation potentials of both natural and synthetic cannabinoids (Fig. 3). All of the cannabinoids tested (cannabidiol, cannabinsol, THC, naboline, HU-211 and levanatrodol), yielded electron donation profiles similar to that of the known antioxidant, butylhydroxy-toluene (BHT). Anandamide, an endogenous cannabinoid receptor ligand that is structurally unrelated to cannabinoids and is not a good electron donor, was included as a negative control. The antioxidant properties of cannabinoids were also examined in a Fenton reaction system using the lipid and water-soluble compound, tert-butyl hydroperoxide as a substrate.

**FIGURE 3.** A comparison of the oxidation potentials of cannabinoids and the antioxidant, BHT. The oxidation profiles of (750 μM) BHT, cannabinoids and anandamide, were compared by cyclic voltammetry. Anandamide, a cannabinoid receptor ligand with a non-cannabinoid structure was used as non-responsive control. Experiments were repeated three times with essentially the same results. See text for experimental details.
FIGURE 4. Effect of cannabidiol and THC on dihydrorhodamine oxidation. Cannabinoids were compared with BHT for their ability to prevent t-butyl hydroperoxide induced oxidation of dihydrorhodamine. See text for experimental details. Data represent mean values ± SEM from a single experiment with three replicates. This experiment was repeated four times with essentially the same results.

FIGURE 5. The effect of cannabidiol on oxidative toxicity in neuronal cultures. Toxicity was induced by addition of 250 μM t-butyl hydroperoxide in the presence or absence of cannabidiol. Each experiment represents the mean of four replicates, repeated on three occasions.
Dihydrorhodamine, an oxidation sensitive fluorescent dye, served as the target (and indicator) of oxidation in this reaction. Cannabidiol and THC both prevented dihydrorhodamine oxidation in a concentration dependent manner similar to that of the antioxidant, BHT (Fig. 4).

**CANNABINOIDS PREVENT OXIDANT TOXICITY IN NEURONAL CULTURES**

The ability of cannabinoids to prevent ROS toxicity in cultured neuron preparations was also examined. (Fig. 5). Tertbutyl hydroperoxide was again used as the oxidant, because its solubility in both aqueous and organic solvents, facilitates oxidation in both cytosolic and membrane delimited cellular compartments. As previously shown with glutamate toxicity studies, cannabidiol protected neuron cultures well against hydroperoxide toxicity (in a dose dependent manner), so that 30 μM cannabidiol was able to rescue 75% of neurons from 250 μM peroxide (a dose calculated to have maximal lethal effect).

**CANNABIDIOL IS A POWERFUL ANTIOXIDANT IN NEURONAL CULTURES**

The protective capacity of cannabidiol was compared with more familiar antioxidants in an AMPA / kainate toxicity model where neurons were exposed to both glutamate and equal concentrations (5 μM) of cannabidiol, α-tocopherol, BHT or ascorbate (Fig. 6). While all of the antioxidants attenuated glutamate toxicity to varying degrees, cannabidiol was 30–50% more protective than either α-tocopherol or ascorbate.

![Figure 6. Comparison of antioxidants and cannabidiol for their ability to prevent glutamate toxicity in neurons. The effects of cannabidiol, BHT, ascorbate and α-tocopherol (10 μM) were examined in a model of AMPA/kainate receptor dependent toxicity. All drugs were present throughout the glutamate exposure period. Each experiment represents the mean of four replicates, repeated on three occasions. See text for further experimental details. Significant differences between cannabidiol and other antioxidants are indicated with an asterisk.](image-url)
PRELIMINARY IN VIVO STUDIES OF CANNABIDIOL

The efficacy of cannabidiol as an antiischemic agent has recently been examined in a rat stroke model. In anesthetized Wistar rats, a suture was fed through the carotid artery up into the middle cerebral artery (MCA). The suture prevented blood flow and was left in place for 90 minutes, after which time it was removed. The animals were allowed to recover for 48 hours and then a six point battery of neurological tests was performed. After these tests, the animals were sacrificed and their brains were fixed, sliced and the area of infarct calculated by computer imaging. At the onset of ischemia, either 5 mg/kg of cannabidiol or vehicle was intravenously administered to the animals using a “blinded” protocol. A second 20 mg/kg dose was administered by intra-peritoneal injection 12 hours after surgery. Forty-eight hours after surgery the animals were sacrificed and their brains perfused with a 2% solution of triphenyltetrazolium chloride. The samples were then fixed, sliced and the infarct volume calculated by computer imaging. Representative images of brain slices taken from a typical control and cannabidiol treated animal are presented in Figure 7. In this study cannabidiol reduced infarct size by 60% by comparison with vehicle treated animals. Behavioral parameters were also significantly improved ($p < 0.016$, $n = 7$) by cannabidiol treatment although the drug had no significant effect on blood pressure, glucose levels, blood gases or rectal temperature.

FIGURE 7. Cannabidiol as a neuroprotective agent in an MCA occlusion model of focal ischemia. The effects of cannabidiol were examined in Wistar rats subjected to 90 min of focal ischemia induced by occlusion of the middle cerebral artery (MCA). Forty-eight hours after surgery the animals were sacrificed and their brains perfused with a 2% solution of triphenyltetrazolium chloride. The samples were then fixed, sliced and the infarct volume calculated by computer imaging. The left-hand image is representative of brain taken from a rat treated with a vehicle control. The right image represents an animal that received 5 mg/kg (i.v.) cannabidiol (CBD) immediately prior to onset of ischemia.
DISCUSSION

The non-psychoactive marijuana constituent, cannabidiol, can prevent both glutamate neurotoxicity and ROS induced cell death. Tetrahydrocannabinol the psychoactive principle of Cannabis, also blocks neurotoxicity with a potency similar to that of cannabidiol. This neuroprotection was unaffected by cannabinoid receptor antagonist, which demonstrates that cannabinoids have effects that are independent of their involvement with cannabinoid receptors, the system responsible for cannabinoid psychoactivity.\textsuperscript{11}

Cannabidiol and THC were equally potent at blocking glutamate toxicity regardless of which glutamate receptor mediated the toxicity. This suggests that either, cannabidiol and THC antagonize three different glutamate receptors with a similar affinity or more likely, cannabinoids protect by an action downstream of the initial receptor activation event. Cannabidiol, THC and other structurally related cannabinoids were all demonstrated to be antioxidants by cyclic voltammetry. Using a glutamate neuronal toxicity model cannabidiol was demonstrated to be significantly more protective than either of the antioxidant vitamins, \(\alpha\)-tocopherol or ascorbate and comparable to the industrial antioxidant, BHT. However, unlike BHT, cannabinoids do not appear to be tumor promoters.\textsuperscript{12,13}

These properties of cannabinoids suggest they may have a therapeutic role as neuroprotectants, and the particular properties of cannabidiol make it a good candidate for such development. The lack of psychoactivity associated with cannabidiol allows it to be administered in higher doses than would be possible with psychotropic cannabinoids such as THC. It is hoped that therapeutics developed from non-psychoactive cannabinoids may also avoid the toxic side effects associated with clinical use of other promising antiischemic agents such as NMDAr antagonists.\textsuperscript{14}

Preliminary studies from a currently ongoing study using a model of focal cerebral ischemia suggest that cannabidiol may well prove to be a good protective agent in vivo. Our studies in rats have indicated that 5 mg/kg of cannabidiol (iv) reduced both infarct volume and neurological impairment by 50–60%. While it is difficult to extrapolate drug doses of given in rodent studies to humans it is worth noting that psychoactive cannabinoids produce strong physiological responses in rats when administered in the 5–20 mg/kg range (ip\textsuperscript{15}) while humans experience psychoactive effects and make physiological responses in the 10–60 mg/70kg range (po\textsuperscript{15}). This means that owing to enhanced the metabolism of rodents, 5–20\times the amount of cannabinoid must be administered comparative to a human dosage in order to achieve a similar effect. If one assumes that this ratio would also hold true for cannabidiol when used as a neuroprotectant, one might expect to achieve results similar to those seen in our rat ischemia studies at a human dose of less than 1 mg/kg. Previous clinical studies using cannabidiol in humans have already demonstrated that cannabidiol has a low toxicity, even when chronically administered to humans,\textsuperscript{16} or given in large acute doses of 10 mg/kg/day.\textsuperscript{17} From these admittedly crude calculations, it is hoped that cannabidiol may one day reach clinical trials, and if so, it may be expected that the required dose will be low, thereby reducing the chances of toxic side effects. (See Proc. Natl. Acad. Sci., July 1998, 95: 8268–8273 for the first publication of the in \textit{vitro} data presented here.)
REFERENCES


