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Short Communication

Therapeutic action of cannabinoid on axonal injury induced by peroxynitrite

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ABSTRACT

This study examined whether the potent cannabinoid HU210 ameliorates axonal injury through its indirect action to stimulate the secretion of corticosterone. We observed that HU210 dramatically reduced peroxynitrite-induced axonal injury in rats receiving adrenalectomy and corticosterone replacement treatment. These results suggest that the ameliorating effects of cannabinoids on axonal injury associated with multiple sclerosis are achieved by its direct action, but not by its indirect action to elevate the serum corticosterone levels.

Multiple sclerosis (MS) is a disorder that begins in early adulthood with an autoimmune inflammatory strike against components of the myelin sheath, resulting in injury and degeneration of axons in various brain regions (Steinman, 2001). A number of partially effective drug treatments are available. Corticosteroids are the cornerstone of therapy, but their efficacy is limited. The lack of an effective drug treatment has led patients to search for additional therapies, one of which is cannabis. Cannabis is estimated to be taken by up to 15% of patients with MS (Linassi and Hader, 2003). Several clinical trials have indeed shown the beneficial effects of cannabinoids for the relief of MS symptoms in humans (Brenneisen et al., 1996; Clifford, 1983; Petro and Ellenberger, 1981; Ungerleider et al., 1987; Zajicek et al., 2003). Recent animal studies further demonstrated the therapeutic effects of cannabinoids for the reduction of inflammation (Arevalo-Martin et al., 2003; Baker et al., 2000; Lyman et al., 1989; Wirguin et al., 1994) and axonal injury (Arevalo-Martin et al., 2003) in animal models of MS. It is undetermined, however, if the therapeutic action of cannabinoid on axonal injury is produced by its direct action or indirect action to stimulate the secretion of corticosterone.

It is now known that cannabinoids or cannabis can exert their action by activating the cannabinoid CB1 receptors (Matsuda et al., 1990) and CB2 receptors (Munro et al., 1993), which are distributed mainly in the brain and immune system, respectively. In light of accumulating evidence suggesting an important role of peroxynitrite in producing axonal injury in the development of MS, we used the rat model of peroxynitrite-induced axonal injury to test the hypothesis that cannabinoid may inhibit axonal injury by its direct action on the brain CB1 receptors but not through its indirect action to stimulate the secretion of corticosterone. We examined the effects of the potent cannabinoid HU210 on the extent of axonal injury produced by administration of the peroxynitrite donor 3-morpholinosydnonimine into the rat corpus

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callosum, according to Touil et al. (2001). To avoid the confounding problem of the increase of corticosterone secretion by HU210 (Martín-Calderón et al., 1998; Rodriguez de Fonseca et al., 1996), we employed adrenalectomy and corticosterone replacement strategy.

Adult male Wistar rats weighing 250–280 g were used. All procedures were in accordance with the guidelines established by the Canadian Council on Animal Care as approved by the University of Saskatchewan Animal Care Committee. Under isoflurance anesthesia, 16 rats received bilateral adrenalectomy as described (Marinelli et al., 1994). Rats were given 0.9% NaCl after adrenalectomy to compensate for loss of salt. Then, rats were placed onto the stereotaxic instrument, followed by injection of 0.5 μl solution into the corpus callosum with the co-ordinates of 0.4 mm posterior, 2.3 mm lateral and 2.8 mm deep. As summarized in Table 1, the first group of 4 control rats received an intra-corpus injection of 3-morpholinosydnonimine (100 mM) that had been kept for 3 days at room temperature to destroy formed peroxynitrite. The remaining 12 rats were given an intra-corpus injection of fresh 3-morpholinosydnonimine solution at 100 mM diluted in saline and then divided into 3 groups of 4 rats each (Table 1) receiving twice daily i.p. injections of vehicle (group 2), HU210 (50 μg/kg) (group 3) and HU210 (50 μg/kg) plus AM281 (3 mg/kg) (group 4) for 5 days. We chose 50 μg/kg of HU210 because this dose approximately equals to 5 mg/kg of Δ^9-tetrahydrocannabinol (Δ^9-THC, the psychoactive constituent of marijuana) in rats, which has been used in a previous study involving the principal rat model of MS, experimental autoimmune encephalomyelitis (Lyman et al., 1989). The selective CB1 receptor antagonist AM281 was used because HU210 could act on both CB1 and CB2 receptors. The dose of 3 mg/kg of AM281 was chosen given that this dose could effectively and specifically block the CB1 receptors in the rat brain (Cui et al., 2001). After surgery, all rats with adrenalectomy received a corticosterone replacement treatment that mimics the circadian rhythm of circulating adrenal steroids. This treatment consisted of (i) s.c. implantation of a corticosterone pellet (40 mg, adjusted to 100 mg with cholesterol) to mimic the basal level of corticosterone in the diurnal cycle (Meyer et al., 1979) and (ii) corticosterone in their drinking water at night to mimic the nocturnal rise in corticosterone (25 μg/ml corticosterone in 0.9% NaCl with 0.15% ethanol). This corticosterone replacement treatment has been shown to normalize circulating levels of corticosterone (Marinelli et al., 1994; Rodriguez et al., 1998).

Five days after surgery, all rats were sacrificed under sodium pentobarbital anesthesia (50 mg/k, i.p.) by perfusion through the left heart ventricle with 4% paraformaldehyde. Rat brains were immersed in 30% sucrose solution at 4 °C for 2 days. Brains were cut on a sliding microtome into frontal sections (30 μm), which were then processed for immunohistochemical staining to detect amyloid precursor proteins (APP) using a conventional avidin–biotin–immunoperoxidase technique as previously described (Cui et al., 2001; Zhang et al., 2002). Briefly, this procedure included incubation of sections at 4 °C for 3 days in goat anti-APP antibody (1:4000; Upstate Biotechnology, Lake Placid, NY). The primary antibody was localized using Vectastain Elite reagents (Vector Laboratories, Burlingame, CA). The reaction product was developed by incubating the sections in a solution containing diaminobenzidine and H_2O_2 for 2–5 min. The sections were mounted onto glass slides and coverslipped. Immunohistochemical controls were performed by omitting primary antibody, which produced virtually no specific staining. Quantitative assessment of APP immunoreactivity was conducted by using a computer-assisted image analysis system, i.e., Zeiss Axioskop microscope connected to a Sony RGB color camera that can measure and analyze the gray scales with a computer software (Northern Eclipse 5.0; Empix Imaging, Mississauga, ON). Statistical analysis of the data was performed using one-way ANOVA, followed by the Scheffe post hoc test.

Table 1 – Animal treatment protocols

Group	Treatment
1	Intra-corpus injection of inactivated SIN-1
2	Intra-corpus injection of SIN-1 + once daily i.p. vehicle for 5 days
3	Intra-corpus injection of SIN-1 + once daily i.p. HU210 for 5 days
4	Intra-corpus injection of SIN-1 + once daily i.p. AM281 and HU210 for 5 days

For details, see method part in the text. SIN-1, 3-morpholinosydnonimine (the peroxynitrite donor).

According to Touil et al. (2001), normal axons or nerve fibers are APP-immunonegative whereas APP-immunopositive nerve fibers are degenerating axons, which usually displayed disrupted feature and run within the corpus callosum to any direction. Group 1 control rats showed no specific immunostaining of APP throughout the rat forebrain, including the local corpus callosum where inactivated 3-morpholinosydnonimine was infused (Fig. 1A). In contrast, group 2 rats receiving active 3-morpholinosydnonimine injection displayed numerous APP-immunoreactive nerve fibers or axons around the injection site in the corpus callosum (Fig. 1B). Once daily administration of the potent cannabinoid HU210 for 5 days in group 3 rats prominently inhibited the formation of APP-immunoreactive nerve fibers or degenerating axons (Fig. 1C), whereas pretreatment with the selective CB1 receptor antagonist AM281 in group 4 rats blocked the effects of HU210 against axonal degeneration (Fig. 1D).

As shown in Fig. 2, one-way ANOVA revealed a significant overall difference among groups 1–4 in the mean density of APP immunostaining ($F_{3,15} = 308.266$, $P < 0.001$). The Scheffe post hoc test further showed that the mean density of APP immunostaining in group 1 rats receiving intra-corpus injection of inactivated 3-morpholinosydnonimine was significantly lower than that in group 2 rats receiving intra-corpus injection of active 3-morpholinosydnonimine ($P < 0.001$). While HU210 treatment in group 3 rats significantly reduced the mean density of APP immunoreactivity ($P < 0.001$), there is no statistically significant difference in the mean density of APP-immunoreactivity between groups 1 and 3 ($P > 0.05$), suggesting that HU210 effectively inhibited the neurotoxic effects of 3-morpholinosydnonimine on nerve fibers. The mean density of APP-immunostaining in group 4 rats receiving both HU210 and AM281 treatment was significantly higher than that in group 3 rats receiving HU210 treatment alone