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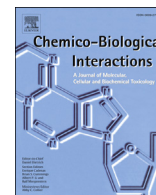

The cannabinoid beta-caryophyllene (BCP) induces neuritogenesis in PC12 cells by a cannabinoid-receptor-independent mechanism

Neife Aparecida Guinaim Santos, Nádia Maria Martins, Flávia Malvestio Sisti, Laís Silva Fernandes, Rafaela Scalco Ferreira, Osvaldo de Freitas, Antônio Cardozo Santos*

Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

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Beta-caryophyllene (BCP) is a phytocannabinoid whose neuroprotective activity has been mainly associated with selective activation of cannabinoid-type-2 (CB2) receptors, inhibition of microglial activation and decrease of inflammation. Here, we addressed the potential of BCP to induce neuritogenesis in PC12 cells, a model system for primary neuronal cells that express trkA receptors, respond to NGF and do not express CB2 receptors. We demonstrated that BCP increases the survival and activates the NGF-specific receptor trkA in NGF-deprived PC12 cells, without increasing the expression of NGF itself. The neuritogenic effect of BCP in PC12 cells was abolished by k252a, an inhibitor of the NGF-specific receptor trkA. Accordingly, BCP did not induce neuritogenesis in SH-SY5Y neuroblastoma cells, a neuronal model that does not express trkA receptors and do not respond to NGF. Additionally, we demonstrated that BCP increases the expression of axonal-plasticity-associated proteins (GAP-43, synapsin and synaptophysin) in PC12 cells. It is known that these proteins are up-regulated by NGF in neurons and neuron-like cells, such as PC12 cells. Altogether, these findings suggest that BCP activates trka receptors and induces neuritogenesis by a mechanism independent of NGF or cannabinoid receptors. This is the first study to show such effects of BCP and their beneficial role in neurodegenerative processes should be further investigated.

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1. Introduction

The degeneration of axons is an important finding in many neurodegenerative conditions including stroke, glaucoma, motor neuropathies, amyotrophic lateral sclerosis (ALS), Alzheimer's, Parkinson's and Huntington's diseases [10,39]. An important therapeutic approach in neurodegenerative conditions is axonal regeneration. Neurite outgrowth and neuronal differentiation play a key role in the development of the nervous system. The growth of axonal and dendritic extensions is critical for neuronal connectivity and alterations in this process can lead to cognitive deficits. The axonal growth and the formation of synaptic vesicles is modulated by the expression of neuronal proteins such as the axonal growth associated protein (GAP-43) and synaptic proteins (synaptophysin, synapsin I and synapsin II). The synaptic proteins increase the number and density of neuronal synapses, regulate synaptic vesicle fusion and neurotransmitter release [5,9,11,12,38,40]. Neuronal cells are able to respond to the extracellular signaling and induce neurological regeneration in pathological conditions such as traumas, ischemia or neurodegenerative diseases. The formation and growth of neurites in cultured neurons indicate the regenerative potential of neurons [8,15,21]. The drugs used to treat neurodegenerative diseases are not able to reverse the reduction of the neuronal network, which encourages the search for pharmacological agents with neurorestorative potential and ability to cross the blood-brain barrier [15]. The present study addresses the neuroprotective potential of beta-caryophyllene (BCP), a sesquiterpene abundantly found in the essential oils of spice (cinnamon, oregano and black pepper) and several plants, particularly *Cannabis sativa* and *Copaifera* spp. It is commonly ingested with vegetable food and, due to its aromatic characteristics, BCP is commercially used as food additive and in cosmetics [13,19,25]. Studies have suggested that BCP has anti-inflammatory, anti-carcinogenic,

* Corresponding author. Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Avenida do Café s/n, 14040-903 Ribeirão Preto, SP, Brazil.
E-mail address: acsantos@fcfrp.usp.br (A.C. Santos).

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antibiotic, antioxidant, anxiolytic, antidepressant, anti-alcoholism, analgesic and local anesthetic effects, and besides that, it is not mutagenic, carcinogenic or cytotoxic in cell culture [1,3,7,20,27,29,30,35]. The molecule of BCP is lipophilic and is able to cross the blood-brain barrier [16]. Studies have also suggested that BCP and essential oils containing BCP have neuroprotective potential [7,23]. Currently, the neuroprotective action of BCP has been mainly associated with antioxidant and anti-inflammatory mechanisms, both events mediated by the selective activation of CB2 receptors. BCP is not a ligand of CB1 receptors and therefore, has no psychoactive effects [2,7,19,23,26]. This is the first study to address the neuritogenic potential of BCP as a possible cannabinoid-receptor-independent mechanism of neuro-protection. The induction of neuritogenesis, and the effects on the expression of marker proteins for axonal growth (GAP-43) and synaptogenesis (synapsin I and synaptophysin) were evaluated in rat pheochromocytoma cell line (PC12 cells), a model for neurobiological and neurochemical studies [22]. PC12 cells express trkA, respond to NGF stimulation and differentiate in neuron-like cells. To investigate the involvement of NGF and NGF-induced pathway, the expression of NGF and the neuritogenic potential of BCP in the presence of k252a (trkA inhibitor) were evaluated. To investigate the involvement of other members of trk family, the neuritogenic potential of BCP was evaluated in a second neuronal model, neuroblastoma SH-SY5Y cells, which have a different profile for neurotrophin receptors of trk family, i.e., do not express trkA receptors nor respond to NGF.

2. Materials and methods

2.1. Chemicals

All chemical reagents used were of the highest purity (analytical grade minimum) and purchased from Sigma–Aldrich®, unless specified differently. Cell culture media were purchased from Life Technologies (Carlsbad, CA). Western blot reagents were purchased from Bio-Rad®. Type I water (ultra-pure) was obtained in the purification system by reverse osmosis, Rios DI-3, followed by purification in Milli-Q Gradient system (Millipore, Bedford, USA). BCP was dissolved in PBS (stock solution) and stored in freezer (−20 °C). Working solutions were prepared in DMEM without phenol prior to assays.

2.2. Cell culture

2.2.1. PC12 cells

PC12 cells, obtained from the American Type Culture Collection (ATCC), were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco™) supplemented with 5% heat inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum and 1% PSN antibiotic (5 mg/ml penicillin, 5 mg/mL streptomycin and 10 mg/mL neomycin). Cells were kept at 37 °C in humidified atmosphere containing 5% CO2 and 95% air. Medium was renewed every 3 days. Cells were split (2:20) every 7–9 days. Confluent cultures were washed with pre-warmed Phosphate Buffered Saline (PBS), detached with trypsin/EDTA solution (Gibco™), centrifuged and subcultured. Prior to assays, cells were seeded in poly-L-lysine coated plates at the following densities: 2.0×10^6 cells/well in 12-well plates for LDH assay and 2.0×10^5 cells/well in 24-well plates for neurite outgrowth, axonal proteins and NGF assays. Then, cells were incubated for 24 h, treated with different concentrations of BCP and incubated for 72 h.

2.2.2. SH-SY5Y cells

SH-SY5Y cells, obtained from the American Type Culture Collection (ATCC), were grown in F12 nutrient mixture (F12 HAM; Sigma Cell Culture, St. Louis, MO) supplemented with 15% fetal bovine serum (GIBCO) and 1% PNS. Cells were cultured in 75 cm^2 tissue-culture flasks at 37 °C under a humidified atmospheric condition of 5% CO2 and 95% air. Medium was replaced every day. Confluent cultures were detached with trypsin/EDTA solution (Gibco®), inactivated with growth medium, centrifuged, and subcultured (1:2; every 2–3 days). Third-passage cells with 80% confluence were used in the experiments [18].

2.3. LDH release

PC12 cells (1.0×10^6 cell/well) were treated with different concentrations of BCP (1–50 μM) and incubated for 24 h, 48 h and 72 h. The medium was collected in order to analyze the activity of the cytoplasmic lactate dehydrogenase (LDH) that leaked from cells due to membrane integrity loss. The assay was performed according to the instructions of the manufacturer (*In Vitro* Toxicology Assay Kit, Lactic Dehydrogenase based TOX7, Sigma-Aldrich®, St. Louis, MO, USA).

2.4. Neurite outgrowth: quantitative assay in PC12 cells

Assays were carried out as previously described in Ref. [14]. PC12 cells were incubated in 24-well plates (2×10^5 cells/well) for 24 h for adhesion. Then, the medium was replaced by Ham's F–12 K (Kaighn's) Medium supplemented with 1% horse serum and 1% antibiotic mixture (Penicillin/Streptomycin/Neomycin, PSN GIBCO®). Cells were then incubated (37 °C, 72 h) with one of the following additions: BCP (10 and 50 μM) or NGF ("Nerve Growth Factor from Vipera lebetina venom", 100 ng/ml). Untreated cells were used as controls. Neurite outgrowth was assessed by inverted phase contrast microscopy (Carl Zeiss Axio Observer A1 inverted microscope, 400x magnification). Phase-contrast photomicrographs of 4 fields per well were taken after incubation with BCP for 24 h, 48 h and 72 h. At least 100 cells per group were counted. The percentage of cells with neurites was determined in digitized images by using the Image J open source software [33]. Only those cells with at least one neurite with a length equal to or greater than the diameter of the cell body were considered differentiated [12].

2.5. Neurite outgrowth: quantitative assay in SH-SY5Y neuroblastoma cells

Assays were carried out as previously described in Ref. [14]. Cells were incubated in 12-well uncoated plates (2×10^5 cells/well) in F12 HAM supplemented with 15% fetal bovine serum (FBS) and 1% antibiotic mixture (penicillin/Streptomycin/Neomycin, PSN GIBCO®) for 24 h for adhesion. After this period, the medium was replaced by F12 HAM supplemented with 1% FBS and 1% PSN and retinoic acid 10 μM, and cells were incubated for 7 days, during which the medium was renewed every 2 days. Then, cells were treated with BCP (10 μM) and incubated at 37 °C for 72 h. A set of experiments were also assayed in the absence of retinoic acid. Untreated cells were used as controls. Neurite outgrowth was assessed by inverted phase contrast microscopy (Carl Zeiss Axio Observer A1 inverted microscope, 400× magnification). Phase-contrast photomicrographs of 4 fields per well were taken after incubation with BCP for 24 h, 48 h and 72 h. At least 100 cells per group were counted. The percentage of cells with neurites was determined in digitized images by using the Image J open source software [33]. Only those cells with at least one neurite with a length equal to or greater than the diameter of the cell body were considered differentiated [12].

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