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β-Carbolines induce apoptosis in cultured cerebellar granule neurons via the mitochondrial pathway

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Abstract

N-Butyl-β-carboline-3-carboxylate (βCCB) is, together with 2-methyl-norharmanium and 2,9-dimethylnorharmanium ions, an endogenously occurring β-carboline. Due to their structural similarities with the synthetic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), harman and norharman compounds have been proposed to be involved in the pathogenesis of Parkinson's disease. While also structurally related, βCCB has received much less interest in that respect although we had previously demonstrated that it induces the apoptotic cell death of cultured cerebellar granule neurons (CGNs). Herein, we have investigated the molecular events leading to CGN apoptosis upon βCCB treatment. We first demonstrated that βCCB-induced apoptosis occurs in neurons only, most likely as a consequence of a specific neuronal uptake as shown using binding/uptake experiments. Then we observed that, in βCCB-treated CGNs, caspases 9, 3 and 8 were successively activated, suggesting an activation of the mitochondrial pathway. Consistently, βCCB also induced the release from the mitochondrial intermembrane space of two pro-apoptotic factors, i.e. cytochrome *c* and apotptosis inducing factor (AIF). Interestingly, no mitochondrial membrane depolarisation was associated with this release, suggesting a mitochondrial permeability transition pore-independent mechanism. The absence of any neuroprotective effect provided by two mPTP inhibitors, i.e. cyclosporine A and bongkrekic acid, further supported this hypothesis. Together, these results show that βCCB is specifically taken up by neuronal cells where it triggers a specific permeabilization of the outer mitochondrial membrane and a subsequent apoptotic cell death.
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Keywords: Apoptosis; Mitochondrion; Caspases; β-Carbolines; Cerebellar granule neurons

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders that affect more than one percent of the population over the age of 60. PD is mainly a sporadic disease although some genetic and toxic forms have been described. The prototypic toxic

Abbreviations: βC, β-carboline; βCCB, β-carboline-3-carboxylic acid ester; $\Delta\Psi_m$, mitochondrial membrane potential; AIF, apoptosis-inducing factor; BA, bongkrekic acid; CGN, cerebellar granule neuron; CyA, cyclosporin A; IMM, inner mitochondrial membrane; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam bromide; OMM, outer mitochondrial membrane; PTP, permeability transition pore; VDAC, voltage-dependent anion channel.

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form of PD is caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a meperidine analogue. In the brain, MPTP is oxidized into MPP+ by monoamine oxidase B which is abundantly expressed by glial cells. MPP+ is subsequently and specifically taken up by dopaminergic neurons where it induces a dysfunction of the complex I of the respiratory chain. Because a deficit in complex I function has also been described in PD patients, MPP+ toxicity is widely used as a model of PD (Eberhardt and Schulz, 2003).

Some environmental pyro-indoles (carbolines) are structurally close to MPTP and MPP+ when they are *N*-methylated. Among carbolines, β-carbolines (βCs), including harman and norharman compounds, have received most attention in the field of PD research (Collins and Neafsey, 2002). Some studies indeed suggest that βCs could, as MPTP, be metabolized in vivo and, hence, lead to the formation of a toxin similar to MPP+. Moreover, βCs are toxic for dopaminergic neurons and reproduce PD-like symptoms when injected into rodents (Matsubara et al., 1998). In humans, the concentration of two βCs, i.e. 2,9-dimethylnorharmanium and 2-monomethylated norharmanium, seems to be increased in the CSF of patients with idiopathic Parkinson's disease compared to control subjects (Matsubara et al., 1995). This led to the emergence of a theory according to which exposure to some environmental toxins, including βCs, would contribute to the pathogenesis of PD.

βCs structurally related to harman and norharman compounds also deserve other function in the nervous system. For example, some are well characterized negative modulators at inhibitory ionotropic receptors, such as the type A receptor for γ-aminobutyric acid receptor ($GABA_AR$) (Rigo et al., 1994) or the strychnine-sensitive glycine receptor (Rigo et al., 2002). Among these, *N*-butyl-β-carboline-3-carboxylate ester (βCCB), has also been shown to occur in mammalian tissues (Braestrup et al., 1980), including the brain (Pena et al., 1986, 1988). Relation between βCCB and neuronal degeneration has never been investigated in vivo, but we have previously shown that, in vitro, βCCB was able to induce neuronal apoptosis (Malgrange et al., 1996).

Since βCCB is a potentially endogenous compound structurally related to MPP+ and is able to induce an apoptotic death in cultured cerebellar granule neurons (CGNs), we decided to decipher the molecular pathway leading to apoptosis in this model. CGNs are among the most homogenous neuronal cultures that can be obtained from the rat central nervous system since they consist of a homogenous neuronal subtype, i.e. cerebellar granule neurons, and since their overall glial content was found not to exceed 5% (Lefebvre et al., 1987). CGNs have not only been widely used as a model to study neuronal apoptosis induced by various toxins, but were also found to adhere to all the criteria established for studying PD-related toxins, including the MPP+ (Marini et al., 1989; Gonzalez-Polo et al., 2001; Kalivendi et al., 2003). They were also successfully used to study apoptotic mitochondrial events, including mitochondrial membrane potential changes (Gonzalez-Polo et al., 2003).

From a molecular point of view, apoptosis is a complex type of cell death which can be triggered by numerous stimuli acting at as many death sensors. It is usual to distinguish the extrinsic pathway from the intrinsic pathway. The former is initiated through the activation of membrane death receptors by specific ligands (Ashkenazi and Dixit, 1999), which in turn triggers the activation of caspase 8 (Earnshaw et al., 1999). Activation of the intrinsic pathway, on the other hand, requires an increase in the mitochondrial membrane permeability that leads to the release of mitochondrial pro-apoptotic factors, including cytochrome *c* and apoptosis inducing factor (AIF) (van Loo et al., 2002). Once in the cytosol, cytochrome *c* binds to the scaffolding protein apaf-1 to form the apoptosome which activates caspase 9 (Adams and Cory, 2002). Regarding AIF, it translocates to the nucleus where it contributes to apoptotic DNA fragmentation (Cande et al., 2002). Finally, both initiator caspases, caspase 8 and caspase 9, activate the effector caspase 3, which eventually initiates the machinery leading to apoptotic cell death (Fischer et al., 2003).

In this work, we have investigated the molecular events leading to neuronal apoptosis upon βCCB treatment. We first demonstrated that βCCB-induced apoptosis occurs in neurons only, most likely as a consequence of a specific neuronal uptake. Then we observed that βCCB-induced neuronal death was dependent upon caspase 9 and caspase 3 activation. This pattern of caspase activation suggested a mitochondrial implication. This was confirmed by the finding that two mitochondrial pro-apoptotic factors, i.e. cytochrome *c* and AIF, were released into the cytosol. Interestingly, the mitochondrial release of these factors was not associated with any $\Delta\Psi_m$ loss suggesting a selective OMM permeabilization.

2. Material and methods

2.1. Chemicals

Butyl β-carboline-3-carboxylate (βCCB) was purchased from Tocris (UK). Pancaspase inhibitor Boc-D-FMK, caspase 9 inhibitor *Z*-Leu-Glu(Ome)-His-Asp(Ome)-fluoromethylketone, caspase 3 inhibitor *Z*-Asp-Glu(Ome)-Val-Asp(Ome)-fluoromethylketone and caspase 8 inhibitor *Z*-Ile-Glu(Ome)-Thr-Asp(Ome)-fluoromethylketone were all from Calbiochem, San Diego,

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