

Alpha-7 nicotinic acetylcholine receptor agonist treatment in a rat model of Huntington's disease and involvement of heme oxygenase-1

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Abstract

Neuroinflammation is a common element involved in the pathophysiology of neurodegenerative diseases. We recently reported that repeated alpha-7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR) activations by a potent agonist such as PHA 543613 in quinolinic acid-injured rats exhibited protective effects on neurons. To further investigate the underlying mechanism, we established rat models of early-stage Huntington's disease by injection of quinolinic acid into the right striatum and then intraperitoneally injected 12 mg/kg PHA 543613 or sterile water, twice a day during 4 days. Western blot assay results showed that the expression of heme oxygenase-1 (HO-1), the key component of the cholinergic anti-inflammatory pathway, in the right striatum of rat models of Huntington's disease subjected to intraperitoneal injection of PHA 543613 for 4 days was significantly increased compared to the control rats receiving intraperitoneal injection of sterile water, and that the increase in HO-1 expression was independent of change in $\alpha 7$ nAChR expression. These findings suggest that HO-1 expression is unrelated to $\alpha 7$ nAChR density and the increase in HO-1 expression likely contributes to $\alpha 7$ nAChR activation-related neuroprotective effect in early-stage Huntington's disease.

Key Words: alpha 7 nicotinic receptor; PHA 543613; quinolinic acid; cholinergic anti-inflammatory pathway; neuroinflammation; neurodegenerative disease

Introduction

Epidemiological studies have shown that smokers have a lower risk of neurodegenerative diseases than non-smokers. These effects seem to be related to the activation of nicotinic receptors by nicotine, which is a nonselective agonist of alpha 7 nicotinic receptor ($\alpha 7$ nAChR) (Gotti and Clementi, 2004; O'Reilly et al., 2005; Thacker et al., 2007). Several studies have reported the beneficial effects of $\alpha 7$ nAChR activation on neuronal survival and neuroinflammation in animal models of neurodegenerative diseases (Medeiros et al., 2014; Sérière et al., 2015). These homopentameric ligand-gated cation channel receptors are widely expressed on neurons and non-neuronal cells (microglia, astroglia, oligodendrocytes and endothelial cells) (Bertrand et al., 2015). In peripheral macrophages, cholinergic anti-inflammatory mechanisms through stimulation of $\alpha 7$ nAChR are well documented (Egea et al., 2015; Han et al., 2017). Shytle et al. (2004) reported that both activated microglia and macrophages can mediate the inhibition of lipopolysaccharide-induced tumor necrosis factor-alpha (TNF- α) release. Accordingly, it was hypothesized that the cholinergic anti-inflammatory pathway (CAP) identified in the periphery has a brain counterpart in the central nervous system (CNS) that could regulate microglial activation.

Referring to the CNS, it has been previously stated that $\alpha 7$ nAChR stimulation was associated with the activation of

the Jak2/PI3K/AKT cascade, which promotes translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) to the nucleus (Parada et al., 2010). By analogy with the mechanism observed in periphery, Nrf2 activation could promote the overexpression of phase II antioxidant enzymes such as heme oxygenase-1 (HO-1). The end products of HO-1 activity are known for their ability to reduce the inflammatory response (Egea et al., 2015). To date, there is little evidence related to the participation of this CAP in the brain. Additional experiments are necessary to confirm this hypothesis.

We have recently shown that repeated administrations of a potent agonist of $\alpha 7$ nAChR, PHA 543613, decreased microglial activation in a dose-dependent manner and significantly improved neuronal survival in an *in vivo* neuroinflammatory excitotoxic rat model (Foucault-Fruchard et al., 2017). PHA 543613, also known as [N-(3R)-1-azabicyclo[2.2.2]-Oct-3-yl-furo [2,3-c]pyridine-5-carboxamide hydrochloride], is characterized by rapid brain penetration (Acker et al., 2008). Published data about this compound provide additional support for the hypothesis that it represents a potential drug in the management of neurodegenerative diseases. This agonist was shown to improve cognitive function in a model of Schizophrenia (Wishka et al., 2006). It has also demonstrated neuroprotective and anti-inflammatory effects in different intracerebral haemorrhage models and in neurodegenerative rodent models such

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as models of Parkinson's and Alzheimer's diseases (Krafft et al., 2012, 2013, 2017; Sadigh-Eteghad et al., 2015; S erri ere et al., 2015). Therefore, the purpose of the present study was to assess the participation of HO-1 in the modulation of neuronal loss and neuroinflammation mediated by $\alpha 7$ nAChR activation in a rat model of brain excitotoxicity. The model of acute neuroinflammation chosen, admitted as an animal model mimicking the early-stage Huntington's disease, is obtained by unilateral striatal injection of quinolinic acid (QA). QA is an agonist of glutamate N-methyl-D-aspartate (NMDA) receptors with excitotoxic properties. This heterocyclic amino acid increases the expression of various enzymes (proteases, lipases, and endonucleases) that leads to neuronal death (Schwarcz and Kohler, 1983; Estrada Sanchez et al., 2008). Dysfunction of neuronal activity related to the QA injection induces a pro-inflammatory environment leading to the activation of surrounding microglial cells (Estrada Sanchez et al., 2008).

Material and Methods

Animals

Experiments were conducted on 10-week-old normotensive male Wistar rats ($n = 12$) (Janvier Labs, Le Genest-Saint-Isle, France), housed in a temperature ($21 \pm 1^\circ\text{C}$)- and humidity-controlled ($55 \pm 5\%$) environment in a 12-hour light/dark cycle (food and water *ad libitum*). All procedures were carried out according to the European Community Council Directive 2010/63/EU for laboratory animal care and the experimental procedure was approved by the Regional Ethical Committee (Authorization N 2015022011523044).

Excitotoxic neuroinflammation model mimicking early-stage Huntington's disease

Rats were anesthetized with isoflurane (4% for induction and 2% for maintenance, gas anesthetizing box, AerraneTM, Baxter, France) and placed in a stereotaxic David Kopf apparatus (Phymep, Paris, France) to be lesioned in the right striatum with QA (150 nmol, 2 μL , Sigma Aldrich, Lyon, France) at the following stereotaxic coordinates according to the Atlas of Paxinos and Watson (Paxinos and Watson, 2008): anterior-posterior (AP): +0.7 mm; medial-lateral (ML): -3 mm; dorsal-ventral (DV): -5.5 mm from bregma.

PHA 543613 injection

Lesioned rats randomly received intraperitoneal injection of PHA 543613 hydrochloride (ICOA, Orl ans, France) at 12 mg/kg (QA-PHA group, $n = 6$) or vehicle (sterile water, QA-vehicle group, $n = 6$) 1 hour before surgery and then twice a day until sacrifice at day 4 post-lesion (12 hours after the last PHA 543613 injection).

Western blot assay

On day 4, the rats were killed by decapitation and both ipsilateral and contralateral striata were dissected from brain tissue. These hemispheres were homogenized with lysis buf-

fer and supplemented with sodium fluoride (NaF), phenylmethane sulfonyl fluoride (PMSF), protease and phosphatase inhibitor cocktails (Couturier et al., 2012). Lysates were centrifuged at $15,000 \times g$ for 15 minutes at 4°C . The resulting supernatants were collected to measure the quantity of total protein using the Bradford method. After denaturation (100°C , 5 minutes), beta mercaptoethanol and bromophenol blue were added to 30 μg of samples. Proteins were separated on a SDS gel electrophoresis (40 minutes, 200 V) and were transferred onto a nitrocellulose membrane (Biorad, Marnes-la-Coquette, France). The blots were blocked for 3 hours at room temperature with 5% (v/v) nonfat dried milk in Tris-buffered saline containing 0.05% Tween 20 and then incubated with primary antibodies anti-HO-1 (1:300, rabbit antibody, ab68477, Abcam, Paris, France) or anti- $\alpha 7$ nAChR (1:200, rabbit antibody, ab10096, Abcam, Paris, France) in blocking buffer overnight at 4°C . Membranes were incubated with a horseradish peroxidase-conjugated secondary polyclonal antibody at room temperature (1:7500, goat antibody, 111-033-144, Jackson ImmunoResearch, West Grove, PA, USA) for 2 hours. Mouse polyclonal antibody against β -actin was used as housekeeping protein (Sigma Aldrich, Saint-Quentin Fallavier, France). Immunoreactive proteins were exposed to the enhanced chemiluminescence western blotting detection system and the signals were captured using the Gbox system and the GeneSys image capture software (Syngene, Ozyme, Saint Quentin en Yvelines, France). The densitometry relative difference between HO-1/ $\alpha 7$ nAChR and β -actin was analyzed with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The expression levels of HO-1 and $\alpha 7$ nAChR proteins in all rats were quantified independently of each other on the same nitrocellulose membrane. Each protein was quantified in all rats ($n = 12$) under the same condition and at the same time.

Statistical analysis

Results were analyzed using GraphPad Prism software v.5, San Diego, California, USA and expressed as the mean \pm SEM (Standard error of the mean). Comparisons between groups were performed using the Mann-Whitney *U* test and comparisons between ipsilateral and contralateral striata were conducted using the Wilcoxon one-tailed test. The level of significance was $P < 0.05$.

Results

Effect of PHA 543613 treatment on HO-1 expression in the striatum

HO-1 expression was evaluated in ipsilateral and contralateral striata in the QA-vehicle ($n = 6$) and QA-PHA ($n = 6$) groups. The results are illustrated in **Figure 1**. Western blot assay results revealed that HO-1 protein expression was significantly decreased in both groups ($P < 0.05$), and there was a significant difference in the decrease of HO-1 protein expression between the ipsilateral and contralateral striata

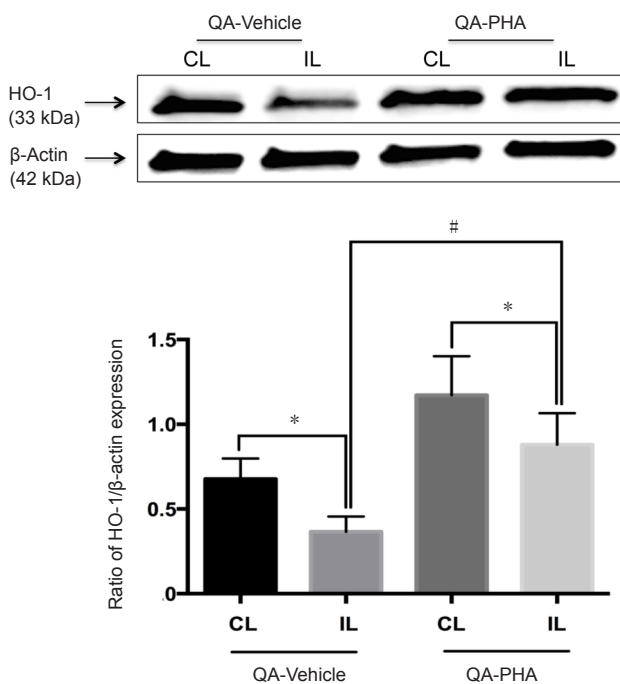


Figure 1 Effects of PHA 543613 treatment on HO-1 expression in the striatum of rats using western blot assay

Rats were intrastrially lesioned with QA and treated twice a day for 4 days with PHA 543613 (QA-PHA) or sterile water (QA-vehicle) ($n = 6$ rats for each). Data are expressed as the mean \pm SEM of the densitometry relative difference between HO-1 and β -actin expression. * $P = 0.03$ (Wilcoxon one-tailed test), # $P = 0.02$ (Mann-Whitney U test). HO-1: Heme oxygenase-1; QA: quinolinic acid; CL: contralateral; IL: ipsilateral.

in each group (HO-1/ β -actin ratio in the QA-vehicle group: 0.69 ± 0.13 in the contralateral striatum vs. 0.37 ± 0.09 in the ipsilateral striatum; HO-1/ β -actin ratio in the QA-PHA group: 1.20 ± 0.20 in the contralateral striatum vs. 0.91 ± 0.18 in the ipsilateral striatum; $P = 0.03$). However, HO-1 expression in the ipsilateral striatum of rats in the QA-PHA group was significantly higher than in the QA-vehicle group (+146%; $P = 0.02$). HO-1 expression level in the contralateral striatum was also higher in the QA-PHA group than in the QA-vehicle group (+74%, not statistically significant).

Effect of PHA 543613 on $\alpha 7$ nAChR expression in the striatum

Quantification of $\alpha 7$ nAChR expression was performed on ipsilateral and contralateral striata in the QA-vehicle ($n = 6$) and QA-PHA ($n = 6$) groups. The results are illustrated in **Figure 2**. The overall level of $\alpha 7$ nAChR in the contralateral and ipsilateral striata was determined using western blotting (QA-vehicle group: 0.60 ± 0.05 in the contralateral striatum vs. 0.60 ± 0.09 in the ipsilateral striatum; QA-PHA group: 0.50 ± 0.01 in the contralateral striatum vs. 0.52 ± 0.06 in the ipsilateral striatum). No significant difference was observed between the animals ($P > 0.05$).

Discussion

PHA 543613 has already demonstrated neuroprotective and anti-inflammatory effects in rodent models of neuro-

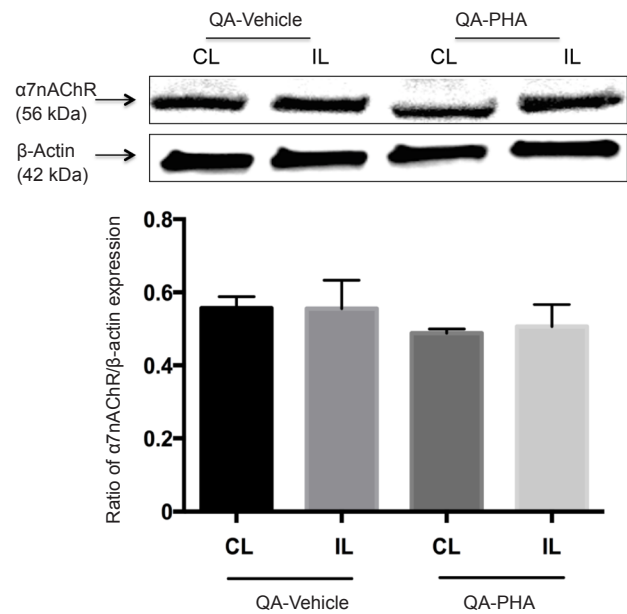


Figure 2 Effect of PHA 543613 on $\alpha 7$ nAChR expression in the striatum of rats using western blot assay

Rats were intrastrially lesioned with QA and treated twice a day for 4 days with PHA 543613 (QA-PHA) or sterile water (QA-vehicle) ($n = 6$ rats for each group). Data are expressed as the mean \pm SEM of the relative difference between $\alpha 7$ nAChR and β -actin expression. $\alpha 7$ nAChR: Alpha-7 nicotinic receptor; QA: quinolinic acid; CL: contralateral; IL: ipsilateral.

degenerative diseases such as Parkinson's and Alzheimer's diseases (Krafft et al., 2012, 2013, 2017; Sadigh-Eteghad et al., 2015; S erri ere et al., 2015). We have recently highlighted that PHA 543613 decreased microglial activation with a dose effect and improved neuronal survival in a rat model of Huntington's disease and we recently confirmed the agonist properties of PHA 543613 on $\alpha 7$ nAChR expression in neuron and astrocyte cultures (Foucault-Fruchard et al., 2017). However, the pathways activated following the stimulation of $\alpha 7$ nAChR in the brain are poorly understood. The present study aimed to add knowledge about the expression of a key component of the cholinergic anti-inflammatory pathway, HO-1, after repeated administrations of $\alpha 7$ nAChR agonist. HO-1 end products generated from heme degradation may modulate inflammation. First, carbon monoxide (CO) released from HO activity may modulate apoptotic, proliferative, and inflammatory cellular programs. CO can downregulate the production of pro-inflammatory mediators (interleukin-6, tumor necrosis factor, inducible nitric oxide synthase...) and upregulate the anti-inflammatory cytokines (interleukin-1, interleukin-10...) via the mitogen-activated protein kinase (MAPK) pathway. CO can also stimulate the production of reactive oxygen species, which can downregulate pro-inflammatory transcription (transforming growth factor- β , Egr-1...). Bilirubin, another product of heme degradation, may also exert anti-inflammatory and anti-proliferative effects. However, the degree of HO-1 activation should be regulated because a third end product of HO-1,

Fe²⁺ may be deleterious in the case of excess activation (Ryter et al., 2015).

In our study, we observed a significant decrease of HO-1 expression in ipsilateral striatum compared with contralateral striatum in both groups (–86% and –33% in the QA-vehicle and QA-PHA groups respectively). Tasset et al. (2010) performed an *in vitro* study and demonstrated that QA exerted a pro-oxidant effect and decreased Nrf2 expression on rat striatal slices. Consequently, it is possible to speculate that this phenomenon is associated with a decrease of transcription of anti-oxidant genes such as HO-1. Colin-Gonzales et al. (2013) also investigated the effects of QA infused intrastrially on HO-1 expression in rats. Contrary to our experimentation, they observed an increase in a time-dependent manner at 1, 3, 5 and 7 days post QA lesion compared with control animals. However, it is important to highlight that the experimental procedure was different from ours. The dose of QA used (240 nmol) was higher than in our surgical lesion technique, and the stereotaxic coordinates were different (AP: +0.5 mm; ML: 2.6 mm from bregma; DV: 4.5 mm from dura). In addition, HO-1 expression was only quantified in the ipsilateral striatum of QA and control animals lesioned with isotonic saline solution.

In the present study, we revealed for the first time that repeated administrations of the $\alpha 7$ nAChR agonist, PHA 543613, significantly increased HO-1 expression in the ipsilateral striatum of the QA-PHA group compared with QA-vehicle. Increased HO-1 expression was also observed in the contralateral striatum. Several studies have already highlighted a correlation between HO-1 expression and HO-1 activity in the CNS (Colín-González et al., 2013; Lin et al., 2017). The ipsilateral side represents the region of interest in our QA lesion model. These observations correspond to a protective action of HO-1 activation as described previously (Suttner and Dennery, 1999). The dual behavior, protective (formation of anti-oxidant compounds) or toxic (production of Fe²⁺), of this enzyme is widely reported and the protein expression level depends on the neuroinflammation model and drug exposure methods (Colín-González et al., 2013; Tronel et al., 2013). Increased HO-1 expression, 10 times higher than the basal value, seems to be toxic whereas 2-fold to 10-fold increase in HO-1 expression seems to be protective (1.7-fold and 2.4-fold increases in HO-1 expression in ipsilateral striatum relative to contralateral striatum in the QA-vehicle and QA-PHA groups respectively) (Suttner and Dennery, 1999). After activation, the $\alpha 7$ nAChR theoretically undergoes rapid desensitization to limit the influx of Ca²⁺ into the cell which can lead to excitotoxicity. A compensating mechanism characterized by an increased number of $\alpha 7$ nAChR binding sites in several brain regions, particularly in the prefrontal cortex, can be initiated (Christensen et al., 2010). However, 4-day treatment with PHA 543613 did not lead to a significant modification of $\alpha 7$ nAChR expression. This finding suggests that HO-1 expression is not associated

with an increase of $\alpha 7$ nAChR density.

Increased HO-1 expression in our study seems to underlie the neuroprotective and anti-inflammatory effects associated with $\alpha 7$ nAChR activation observed under excitotoxic conditions. Other studies have supported the correlation between the neuroprotective effects and the induction of HO-1 expression in neurodegenerative models (Parada et al., 2014; Buendia et al., 2015). Taken together, these observations reinforce the hypothesis that the cholinergic anti-inflammatory pathway identified in the periphery has a brain counterpart in the CNS. However, other signaling pathways such as Nrf2 (*i.e.*, activator protein 1, nuclear factor kappa B or hypoxia inducible factor-1) can regulate HO-1 expression (Alam and Cook, 2003; Ferrándiz and Devesa, 2008) and further investigations have to be performed to confirm our hypothesis.

Author contributions: LFF contributed to the conception, design, definition of the intellectual content, literature retrieval, experimental studies, data acquisition and analysis, statistical analysis, manuscript preparation and editing and was the guarantor of the paper. CT contributed to the conception, design, definition of intellectual content, experimental studies, and manuscript review. SB, ZG and JB contributed to the experimentation. SC and DA contributed to the conception, design, and definition of the intellectual content, and manuscript review. They contributed equally to this work and approved the final version of this paper for publication.

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Research ethics: All procedures were carried out according the European Community Council Directive 2010/63/EU for laboratory animal care and the experimental procedure was approved by the Regional Ethical Committee (Authorization N°2015022011523044).

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