Neuroprotective effect of nicotine against 3-nitropropionic acid (3-NP)-induced experimental Huntington’s disease in rats

Mohammad Tariq a,∗, Haseeb Ahmad Khan b, Ibrahim Elfaki a, Saleh Al Deeb a, Khalaf Al Moutaery a

a Neuroscience Research Group, Armed Forces Hospital, P.O. Box 7897 (W-912), Riyadh 11159, Saudi Arabia
b Department of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia

Received 23 April 2005; received in revised form 14 June 2005; accepted 16 June 2005
Available online 26 July 2005

Abstract

Nicotinic acetylcholine receptors (nAChRs) are regarded as potential therapeutic targets to control various neurodegenerative diseases. Owing to the relevance of cholinergic neurotransmission in the pathogenesis of Huntington’s disease (HD) this investigation was aimed to study the effect of nicotine, a nAChR agonist, on 3-nitropropionic acid (3-NP)-induced neurodegeneration in female Wistar rats. Systemic administration of 3-NP in rats serves as an important model of HD. The animals received subcutaneous injections of nicotine (0, 0.25, 0.50 and 1.00 mg/kg) daily for 7 days. 3-NP (25 mg/kg, i.p.) was administered daily 30 min after nicotine for the same duration. One additional group of rats served as control (vehicle only). On day 8, the animals were observed for neurobehavioral performance (motor activity, inclined plane test, grip strength test, paw test and beam balance). Immediately after behavioral studies, the animals were transcardially perfused with neutral buffered formalin (10%) and brains were fixed for histological studies. Lesions in the striatal dopaminergic neurons were assessed by immunohistochemical method using tyrosine hydroxylase (TH) immunostaining. Treatment of rats with nicotine significantly and dose-dependently attenuated 3-NP-induced behavioral deficits. Administration of 3-NP alone caused significant depletion of striatal dopamine (DA) and glutathione (GSH), which was significantly and dose-dependently attenuated by nicotine. Preservation of striatal dopaminergic neurons by nicotine was also confirmed by immunohistochemical studies. These results clearly showed neuroprotective effect of nicotine in experimental model of HD. The clinical relevance of these findings in HD patients remains unclear and warrants further studies.

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Keywords: Nicotine; Huntington’s disease; 3-Nitropropionic acid; Neuroprotection

1. Introduction

Huntington’s disease (HD) is a chronic progressive autosomal dominant neurodegenerative disorder that is characterized by a striatal-specific degeneration [67]. The pathological changes manifest clinically in midlife as a triad of cognitive decline, psychiatric disturbance and impairment of motor function. Several attempts have been made to develop experimental model of HD. In the most widely used animal models of HD, excitotoxic amino acids, such as kainic, quinolinic and ibotenic acids, are stereotactically injected into specific region of the brain. Besides being difficult, there is always a chance of error to inject the drugs into the target cells. The most recent model of HD is based on systemic injections of 3-nitropropionic acid (3-NP), a mitochondrial toxin that causes striatal neuropathy similar to that seen in clinical HD [4,8]. A major advantage of 3-NP model over other model of HD is that the lesions produced are bilateral, striatal specific and develop spontaneously after systemic administration of 3-NP. In spite of extensive research this devastating hereditary disease remains incurable, warranting further studies to determine the causes and cure of HD.

The natural alkaloid nicotine present in Nicotiana tabacum is by far the most widely studied substance that originates
from tobacco smoke and exhibits widespread pharmacological effects. During the last decade, there has been a rapid explosion of publications reporting the neuroprotective activity of nicotine. Recent studies clearly suggest that nicotinic acetylcholine receptor (nAChR) in the CNS is a new potential therapeutic target for the management of neurodegenerative diseases [34]. O’Neill et al. [38] have suggested that neuronal nAChRs agonists could provide motor improvement and retard the progressive course of various diseases including Alzheimer’s disease and Parkinsonism. Furthermore, nicotine has been shown to protect various neurons against a variety of neurotoxins via neuronal nAChRs in vivo [12,27,36,40,41,50,62] as well as in vitro [23,55,56,60,61]. In the present study, we demonstrate the protective effects of nicotine (0.25–1 mg/kg) against 3-NP-induced behavioral, biochemical and histological changes in a rat model of HD.

2. Materials and methods

2.1. Animal treatment

Adult female Wistar rats (230–280 g) grown in our animal breeding facility were used. The animals were housed in a temperature-controlled room (24 °C) with 12-h light/12-h dark cycle. Standard laboratory food and water were available ad libitum throughout the study. The experimental protocol of this study was approved by the Institutional Research and Ethics Committee. The rats of matching weights were randomly divided into five groups of eight animals each. The rats in group 1 served as control and received vehicles only, whereas rats in group 2 received 3-NP (25 mg/kg, i.p.) daily for 7 days. The animals in groups 3–5 were treated with 3-NP similarly as in group 2, in addition they received nicotine (subcutaneously) in the doses of 0.25, 0.5 and 1 mg/kg, respectively, daily 30 min before 3-NP for 7 days. Twenty-four hours after the last dose of 3-NP the rats were tested for behavioral parameters followed by transcardiac perfusion with 10% neutral buffered formalin (NBF) and fixation of the brains for immunohistochemical studies. A separate batch of animals was used for biochemical studies.

2.2. Behavioral studies

The animals were subjected to following behavioral tests by a person unaware of the treatment protocol.

2.2.1. Motor activity

Motor activity was measured using Optovarimex activity meter (Columbus Instruments, USA). The horizontal motor activity was detected by two perpendicular arrays of 15 infrared beams located 2.5 cm above the floor of the testing area. Each interruption of a beam on the x- or y-axis generated an electric impulse, which was presented on a digital counter. Similarly, the vertical motor activity was recorded using the two additional rows of infrared sensors located 12 cm above the floor. Each animal was tested separately and the motor activity was measured for a period of 2 min.

2.2.2. Balance beam test

The balance beam test was used to measure the ability of rats to traverse a horizontal narrow beam (1 cm × 100 cm) suspended 1 m above a foam-padded cushion [20,53]. During testing, the rats were given 2 min to traverse the beam. If they did not complete the task or if they fell off the beam, the trial was ended and the rats were placed back into their home cages. For successful performers, the latency to cross the beam was recorded.

2.2.3. Limb withdrawal test

In this behavioral test, the animal was placed on a 20 cm high 30 cm × 30 cm Perspex platform containing four holes, two holes of 5 cm diameter for the hind limbs and two holes with a diameter of 4 cm for the forelimbs. The rat was placed on the platform by positioning first the hind limbs and then the forelimbs into the holes. The times taken by the animal to retract its first hind limb and the contralateral hind limb were recorded. The difference between the retraction times of both hind limbs was determined. This is considered to be an important parameter to measure functional abnormalities of the hind limbs, which are indicative for the extent of striatal degeneration [63]. The test was performed three times with a 45 min interval and the average values were reported.

2.2.4. Inclined plane test

Inclined plane test, as described by Rivlin and Tator [49], was used to assess motor function in rats. The inclined plane apparatus consists of two rectangular boards connected to each other by a hinge. A rubber mat with ridges 0.6 cm in height was fixed to the movable plane and two protractor-like plywood side panels with degrees (0–90) marked on their faces were fixed on the base. The maximum inclination at which a rat could maintain itself for 5 s with the body axis perpendicular to the axis of the plane was considered as the ‘capacity angle’ for the animals. The angle was increased or decreased by a margin of 0.5° gradually until the rat could maintain its position on the inclined plane for 5 s without falling.

2.2.5. String test for grip strength

The rat was allowed to hold with the forepaws a steel wire (2 mm in diameter and 35 cm in length), placed at a height of 50 cm over a cushion support. The length of time the rat was able to hold the wire was recorded. This latency to the grip loss is considered as an indirect measure of grip strength [53].

2.3. Biochemical studies

After 24 h from the last dose of 3-NP the rats were sacrificed and the brains were rapidly dissected on a pre-cooled Petri dish. The isolated striata were quickly frozen in liquid
nitrogen and transferred in pre-cooled vials and then stored at –80 °C until analyzed.

2.3.1. Dopamine analysis
The analysis of dopamine in striatum was carried out according to the procedure of Patrick et al. [42]. The striata from the right cerebral hemisphere were weighed and homogenized for 10 s in 0.1 M perchloric acid containing 0.05% EDTA using Teflon homogenizer. The homogenates were immediately centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatants were filtered using 0.45 μm pore filters and analyzed by high performance liquid chromatography (HPLC). The HPLC system consisted of an electrochemical detector from Metrohm (Model 651 Herisau, Switzerland), an autoinjector (Model 712, Waters Associate Inc., Milford, MA, USA), a solvent delivery pump (Waters Model 510) and an integrator (Waters Model 745). The mobile phase consisted of a mixture of 0.1 M citric acid monohydrate, 0.1 M sodium acetate, 7% methanol, 100 μM EDTA and 0.01% sodium octane sulfonic acid and the column was C-18 Bondapak (3.9 mm × 150 mm). The flow rate was maintained at 1 ml/min and the injection volume was 20 μl.

2.3.2. Glutathione analysis
The measurement of glutathione (GSH) in striatum was carried out enzymatically according to the modified procedure of Owen [39]. The striata from the left cerebral hemisphere were homogenized in ice-cold perchloric acid (0.2 M) containing 0.01% EDTA. The homogenates were centrifuged at 4000 rpm for 10 min. The enzymatic reaction was started by adding 200 μl of clear supernatant in a spectrophotometric cuvette containing 800 μl of 0.3 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 100 μl of 6 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and 10 μl of 50 units/ml glutathione reductase (all the above reagents were freshly prepared in phosphate buffer of pH 7.5). The absorbance was measured over a period of 4 min at 412 nm at 30 °C. The glutathione level was determined by comparing the change of absorbance (ΔA) of test solution with the ΔA of standard glutathione.

2.4. Histology
The histological procedure reported earlier was used with some modifications [51]. The rats were transcardially perfused with 10% NBF till the outflow perfusate was absolutely clear and free form blood contamination. After perfusion the brains were removed and immersed in the same fixative as used for perfusion (10% NBF), for 3 days. The specimens were then processed overnight for dehydration with increasing concentrations of alcohol to water (100% alcohol, 95% alcohol, 80% alcohol and distilled water). The rehydrated specimens were immersed in citrate buffer (pH 6.0) and heated in the microwave for 5+5 min to free the binding sites for TH immunoreactivity. After cooling to room temperature, the brain sections were quenched with 3% H2O2 and allowed to react with specific monoclonal antibody against rat TH (Novocastra Laboratories Ltd., UK) in 1:20 dilution for 30 min at ambient temperature. After rinsing with tris-buffered saline (TBS), the sections were sequentially incubated with biotinylated goat antibody, strept AB complex/HRP and chromogenic substrate for peroxidase, according to manufacturer’s instructions (Dako MS, Denmark). For each TH section, an adjacent section was stained with haematoxylin for structure identification using light microscopy.

2.5. Statistical analysis
Data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Dunnett’s multiple comparison tests to determine the significance level between the experimental groups, using SPSS software (Version 10). P values less than 0.05 were considered statistically significant.

3. Results
3.1. Effect of nicotine (NIC) on 3-NP-induced behavioral deficits
3.1.1. Motor activity
Administration of 3-NP for 7 days significantly reduced the horizontal locomotor activity of rats (ANOVA F = 2.40, P < 0.05). Concomitant treatment with nicotine dose-dependently but insignificantly reversed the effect of 3-NP on horizontal locomotor activity (Table 1). 3-NP also significantly diminished the vertical locomotor activity (ANOVA F = 3.51, P < 0.05), which was significantly increased in the rats treated with the high dose of nicotine (Table 1).

3.1.2. Balance beam test
All the animals in the 3-NP alone group failed to pass the beam balance test within the cut-off time of 120 s, whereas the control rats performed this task in 7.7 ± 2.4 s (Table 1). Co-treatment with nicotine significantly and dose-dependently improved the performance of 3-NP treated rats in the balance beam test (Table 1).

3.1.3. Limb withdrawal test
The difference between the retraction times of the two hind limbs was significantly higher in 3-NP alone treated rats (102.4 ± 34.4 s) as compared to control rats that were...
Table 1
Effect of nicotine (NIC) on 3-nitropropionic acid (3-NP)-induced behavioral deficits

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Motor activity (counts/2 min)</th>
<th>Balance beam test (s)</th>
<th>Limb withdrawal test (s)</th>
<th>Inclined plane test (angle°)</th>
<th>String test (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1394 ± 102</td>
<td>7.7 ± 2.4</td>
<td>10.0 ± 0.0</td>
<td>80.2 ± 1.5</td>
<td>78.0 ± 8.1</td>
</tr>
<tr>
<td>3-NP</td>
<td>288 ± 151*</td>
<td>120.0 ± 0.0</td>
<td>1024 ± 34.4</td>
<td>60.2 ± 1.5</td>
<td>4.3 ± 13#</td>
</tr>
<tr>
<td>3-NP + NIC 0.25</td>
<td>761 ± 140</td>
<td>28.3 ± 4.9</td>
<td>12.8 ± 3.8</td>
<td>75.2 ± 1.5</td>
<td>15.0 ± 4.5</td>
</tr>
<tr>
<td>3-NP + NIC 0.50</td>
<td>873 ± 203</td>
<td>168.3 ± 53.3</td>
<td>9.0 ± 3.2**</td>
<td>75.2 ± 1.5**</td>
<td>22.8 ± 6.8**</td>
</tr>
<tr>
<td>3-NP + NIC 1.00</td>
<td>882 ± 173</td>
<td>186.5 ± 71.6</td>
<td>6.7 ± 2.9**</td>
<td>75.2 ± 1.5**</td>
<td>34.5 ± 6.1**</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.
* P < 0.05.
** P < 0.001 vs. control group.
# P < 0.05.
## P < 0.001 vs. 3-NP alone group using Dunnett’s multiple comparison test.

able to quickly retract their both hind limbs (Table 1). The performance of 3-NP treated rats in the limb withdrawal test was significantly and dose-dependently improved by nicotine (ANOVA F = 7.18, P < 0.001).

3.1.4. Inclined plane test
Administration of 3-NP alone significantly impaired the motor function of animals in the inclined plane test as indicated by a lower capacity angles in this group of rats (Table 1). All the three doses of nicotine appeared to be equipotent in improving the ability of 3-NP treated rats in scoring high capacity angles (ANOVA F = 41.06, P < 0.001).

3.1.5. String test (grip strength)
The animals treated with 3-NP alone showed a significantly lower latency to the grip loss (4.3 ± 1.3 s) in the string test as compared to control rats (78.0 ± 8.1 s) (Table 1). Pretreatment with nicotine significantly and dose-dependently increased the latency time of the 3-NP treated animals (ANOVA F = 24.96, P < 0.001).

3.2. Effect of nicotine on 3-NP-induced striatal dopamine (DA) depletion
Administration of 3-NP (30 mg/kg) for 7 consecutive days significantly reduced striatal DA levels in the rats (5.12 ± 0.83 μg/g) as compared to control levels (12.20 ± 1.00 μg/g) (Fig. 1). The medium (8.68 ± 0.64 μg/g) and high (10.16 ± 0.55 μg/g) dose of nicotine significantly increased the striatal DA levels in 3-NP treated rats (ANOVA F = 12.67, P < 0.001).

3.3. Effects of nicotine on 3-NP-induced striatal glutathione depletion
There was a massive depletion of GSH in 3-NP alone treated rats as compared to control group (Fig. 2). Although the low dose of nicotine failed to modify striatal GSH levels in 3-NP treated rats, medium and high doses of nicotine significantly protected the animals against 3-
3.4. Effect of nicotine on tyrosine hydroxylase immunoreactivity

The striatum of control animals showed intense expression of TH (Fig. 3A), whereas a sharp decrease in TH immunoreactivity was observed in 3-NP treated rats (Fig. 3B). The loss of dopaminergic neurons due to 3-NP was dose-dependently reversed by nicotine showing gradually increased density of TH immunostaining of the dopaminergic neurons (Fig. 3C–E) as compared to 3-NP alone treated rats (Fig. 3B).

4. Discussion

The treatment of rats with 3-NP produced significant motor and behavioral abnormalities including bradykinesia, muscles weaknesses and rigidity (Table 1). These findings are in agreement with earlier reports who also observed a variety of neurobehavioral abnormalities and motor deficit in rats following 3-NP administration [6,7,24,54,63]. The symptoms developed by chronic administration of 3-NP are akin to juvenile onset and late hypokinetic stages of HD [9,15,28]. Treatment of rats with nicotine produced a highly significant and dose-dependent protection of animals against 3-NP-induced behavior and motor deficit (Table 1). Neuroprotective effect of nicotine has been shown against MPTP-induced striatal damage [30] as well as secondary neurodegenerative changes following spinal cord injury in rats [47]. Several distinct mechanisms can be involved in tissue sparing induced by nicotine in rats following exposure to neurodegenerative agents. Involvement of nAChRs in nicotine-induced neuroprotection against quinolinic acid [37] and MPTP [30] induced neurodegeneration has been reported earlier, suggesting a definite role of cholinergic neurotransmission in neuroprotective effect of nicotine [44,45].

Administration of 3-NP produced a significant depletion of striatal DA levels in rats (Fig. 1). Substantial losses in DA receptors [1] as well as DA content [3] have been suggested as the major contributory factors in HD. Studies on the transgenic models of HD clearly confirmed that decline in dopaminergic activity is accompanied by progressive disease [2,21]. Pretreatment of animals with medium and high doses of nicotine significantly and dose-dependently attenuated 3-NP-induced striatal DA depletion (Fig. 1). The results of immunohistochemistry also demonstrated protective effect of nicotine against 3-NP-induced reduction of tyrosine hydroxylase expression, which is a sensitive indicator of dopaminergic activity (Fig. 3). It is well established that activation of presynaptic nAChRs receptors results in an increased release of DA [29,43,46,65]. Nicotine has been shown to enhance
NP intoxicated rats. Chronic treatment of nicotine significantly antagonized MPTP-induced depletion of DA without altering the striatal levels of DA and DOPAC in control mice [18].

Furthermore, recent studies have shown that neurotrophic factors play a critical role in neuronal survival following exposure to neurotoxins or neurotrauma [17,68]. Nicotine stimulates the production and release of neurotrophic factors, such as brain derived neurotrophic factor (BDNF), basic fibroblast growth factor (FGF-2) and nerve growth factor (NGF) [47]. Treatment with nicotine has also been shown to upregulate NGF receptor in a variety of neuronal cells [59] and to protect against apoptosis-induced by NGF deprivation [66]. Thus, nicotine may exert its neuroprotective effect at least in part by stimulating neurotrophic factors.

There was a significant depletion of striatal GSH following exposure of animals to 3-NP (Fig. 2), clearly suggesting the role of oxidative stress in this neurodegenerative process. Maksimovic et al. [31] also observed a significant reduction in striatal GSH in quinolinic acid-induced model of HD in rats. Nicotine significantly and dose-dependently protected striatum against 3-NP-induced GSH depletion (Fig. 2). Recent studies clearly demonstrate that increased oxidative stress can be one of the major deleterious events in clinical [5] and experimentally induced Huntington’s disease [31]. Antioxidants, on the other hand, have been shown to protect nervous system against variety of toxins [35,48]. Nicotine exerts its antioxidant effect due to its free radical chain breaking properties and/or preventing the initiation of free radical generation [19]. Nicotine can bind to complex I of respiratory chain and inhibit the NADH-ubiquinone reductase activity and generation of superoxide (O2•-) anion radical [10,11]. Nicotine can also act as a scavenger of hydrogen peroxide and block the fenton reaction through binding to Fe2+ [25]. Furthermore, neurotoxicity of 3-NP is attributed to its ability to produce ischemic injury by interfering with complex II of mitochondrial respiratory chain leading to decreased ATP levels [4,32]. Neurons in brain are highly vulnerable to ischemia [22] and impairment of energy metabolism has been associated with neurodegenerative changes in brain [57]. Recently, neurodegenerative changes following exposure of rats to MPTP [14], iminodipropionitrile [58] and harmaline [57] have been shown to accompany metabolic imbalance in brain [57,58]. Nicotine has been shown to modulate cerebral energy metabolism and conserve the energy balance [16]. Intravenous injection of nicotine has been shown to increase regional cerebral blood flow (CBF) and restore energy supply in ischemic brain [13,26].

In conclusion, nicotine significantly and dose-dependently ameliorated 3-NP-induced striatal lesions and behavioral deficits in rats. The protective effect of nicotine may be attributed to its ability of restoring striatal DA levels in 3-NP intoxicated rats.

Acknowledgements

The authors wish to thank Mr. Rajakanna Jesuraja, Mr. Khalid Abdalla Elfaki and Mrs. Biju Prasad for technical assistance, as well as Miss Tess Jaime and Miss Audrey Rose Gacutan for secretarial support and typing the manuscript.

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