Published in final edited form as:

Neurotox Res. 2012 August ; 22(2): 177–180. doi:10.1007/s12640-012-9326-7.

Protective Effects of Nicotine Against Aminochrome-Induced Toxicity in Substantia Nigra Derived Cells: Implications for Parkinson's Disease

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Abstract

Parkinson's disease is a debilitating progressive neurodegenerative disorder that results from the loss of or damage to dopaminergic cells containing neuromelanin in the substantia nigra (SN). The underlying neurodegenerative mechanism(s), however, remain elusive. Aminochrome, the precursor of neuromelanin is an endogenous substance capable of inducing selective neurotoxicity to dopaminergic neurons in SN. Nicotine, on the other hand, may offer protective effects against dopaminergic cell damage induced by various neurotoxins including MPTP and salsolinol. In this study, we sought to determine whether nicotine may also protect against aminochrome-induced toxicity in SN derived RCSN-3 cells. Exposure of RCSN-3 cells to a combination of aminochrome (50 μ M) and dicoumarol (50 μ M) for 48 h induced approximately 70 % cell death. Pretreatment

with nicotine, dose-dependently blocked this toxicity. The effects of nicotine in turn were dose-dependently blocked by mecamylamine, a non-selective nicotinic receptor antagonist. These results suggest involvement of nicotinic receptors in protective effects of nicotine against aminochrome-induced toxicity and provide further evidence for possible therapeutic effects of nicotine or nicotinic agonists in Parkinson's disease.

Keywords

Aminochrome; Nicotine; RCSN-3 Cells; Neurotoxicity; Neuroprotection; Parkinson's disease

Considerable effort is dedicated towards development of novel pharmacotherapies for Parkinson's disease (PD) and understanding the cellular and molecular mechanisms leading to selective neurodegeneration of dopaminergic neurons containing neuromelanin in substantia nigra (SN) that are responsible for motor symptoms in PD. Although, the molecular basis of such neurodegeneration remains unclear, it is generally accepted that various mechanisms including mitochondrial dysfunction, aggregation of proteins such as alpha-synuclein, dysfunction of protein degradation systems, oxidative stress, and neuroinflammation can be major contributory factors (Dehay and Bezard 2011; Schapira and Jenner 2011). Various in vivo and in vitro models have been developed to facilitate screening compounds or to understand the cellular/molecular mechanisms responsible for dopaminergic deficiency associated with nigro-striatal pathway. However, majority of these studies utilize neurotoxins that: (i) are of exogenous origin, (ii) induce extensive acute cell death (e.g. MPTP induces severe Parkinson symptoms in only few days while PD degeneration occurs over years, and (iii) each neurotoxin may induce damage by a different mechanism (Paris et al. 2007). In this study we used aminochrome which is produced endogenously during dopamine oxidation and is the precursor of neuromelanin. Aminochrome is involved in nearly all mechanisms that are proposed to be involved in the degenerative process in PD including (i) mitochondria dysfunction (Van Laar et al. 2009; Aguirre et al. 2012; Muñoz et al. 2012), (ii) aggregation of proteins such as alpha-synuclein (Norris et al. 2005) or actin causing disruption of cytoskeleton and axonal transport (Paris et al. 2010), (iii) protein degradation dysfunction (due to inactivation of the proteasomal system when aminochrome forms adducts with Parkin, a ubiquitin ligase (LaVoie et al. 2005) or inhibiting the fusion between autophagy vacuoles and lysosomes by forming adducts with beta-tubulin (Paris et al. 2010, 2011), and (iv) oxidative stress where aminochrome is reduced by one-electron to leukoaminochrome o-semiquinone radical resulting in generation of superoxide radicals, hydrogen peroxide and hydroxyl radicals (Arriagada et al. 2004).

Nicotine, on the other hand, has been shown to offer protective effects against dopaminergic cell damage induced by various neurotoxins including MPTP and salsolinol (Copeland et al. 2005, 2007; Quik et al. 2008; Ramlochan Singh et al. 2011). Here, we were interested in determining whether nicotine may also protect against aminochrome-induced toxicity and if so, whether this action is mediated through nicotinic receptors.

For this purpose we utilized RCSN-3 cells, a cell line derived from rat SN with catecholaminergic features including synthesis and release of dopamine, expression of VMAT-2, dopamine, norepinephrine and serotonin transporter as well as production of neuromelanin (Paris et al. 2008). RCSN-3 cells grow in monolayer with a doubling time of 52 h at a plating efficiency of 21 % and a saturation density of 56,000 cells/cm² when kept in normal growth media composed of: DME/HAM-F12 (1:1), 10 % bovine serum, 2.5 % fetal bovine serum, 40 mg/l gentamicine sulfate (Arriagada et al. 2004; Paris et al. 2008, 2010; Muñoz et al. 2012). Cultures were kept in an incubator at 37 °C with 100 % humidity and an atmosphere of 10 % CO₂. RCSN-3 cells in passage 8–10 were exposed to a

combination of aminochrome and dicoumarol (Sigma Chemical Co., St Louis, MO, USA) to induce maximal cell damage during the 48 h incubation. The concentrations used here were based on previous studies using the same cell line (Paris et al. 2010,2011). Aminochrome was synthesized by oxidizing dopamine with tyrosinase in buffer 20 mM MES at pH 6.0 for 15 min at room temperature as described by Paris et al.2010. Dicoumarol (10^{-2} M) was dissolved in 0.1 N NaOH and diluted (10^{-3} M) directly in distilled water. Cell death was measured by counting live and dead cells with flow cytometry after staining with 0.5 μ M calcein AM and 5 μ M ethidium homodimer-1 for 45 min at 37 °C, using 510–560 nm (excitation) and LP-590 nm (emission) for ethidium homodimer-1 and 450–490 nm (excitation) and 515–565 nm (emission) for Calcein AM. Dicoumarol is an inhibitor of diaphorase, the enzyme involved in protection against aminochrome (Arriagada et al. 2004; Paris et al.2010). Cells were pretreated with various concentrations of nicotine prior to such combination. Once the protective effects of nicotine were established, various concentrations of mecamylamine, a non-selective nicotinic receptor antagonist were applied prior to nicotine to determine the extent of nicotinic receptor involvement in neuroprotective effects of nicotine. The concentrations of nicotine and mecamylamine (both purchased from Sigma, St. Louis, MO) were based on previous studies using these agents in similar in vitro studies (Copeland et al. 2005; Ramlochansingh et al.2011). All data ($n = 5$) are expressed as mean $\pm$ SEM. The statistical significance was assessed using analysis of variance (1-way ANOVA) followed by Tukey's post hoc test. Significance was set a priori at $p < 0.05$.

Figure 1 shows toxicity induced by aminochrome (AM) is significantly enhanced by addition of dicoumarol (DIC). Whereas 50 μ M aminochrome alone resulted in approximately 35 % toxicity, addition of 50 μ M dicoumarol (which had no effect of its own) increased the toxicity to approximately 70 %. Hence this combination was used in subsequent experiments to determine the protective effects of nicotine.

Figure 2 shows dose-dependent protective effects of nicotine against AM + DIC. Nicotine at 20 μ M concentration totally blocked the toxicity induced by AM + DIC. Nicotine at concentrations used (1–20 μ M) had no effect of its own on cell viability (data not shown). Moreover, in an initial study it was shown that nicotine also significantly attenuated the modest toxicity induced by aminochrome only.

Figure 3 shows the effects of various concentrations of mecamylamine alone on cellular toxicity. Mecamylamine at 5 and 10 micromolar (μ M) concentrations did not have any effect of its own on cellular toxicity. However, at concentrations of 50 and 100 μ M resulted in significant toxicity (25 and 30 %, respectively). Hence the lower non-toxic doses of mecamylamine were used to block nicotine's effect.

Figure 4 shows that mecamylamine dose-dependently blocked the protective effects of nicotine against AM + DIC. At 5 μ M, mecamylamine partially blocked nicotine's effect. However, at 10 μ M, mecamylamine completely blocked the effect of nicotine.

It is likely that loss of dopaminergic neurons containing neuromelanin in PD involves aminochrome neurotoxicity as this metabolite of dopamine oxidation has been implicated in practically all mechanisms proposed to be related to neurodegeneration of dopaminergic neurons including mitochondria dysfunction, protein aggregation, dysfunction of protein degradation and oxidative stress (Segura-Aguilar et al. 1998; LaVoie et al. 2005; Norris et al. 2005; Zafar et al. 2006; Van Laar et al. 2009; Paris et al. 2010; Aguirre et al. 2012; Muñoz et al. 2012). That nicotine can protect against such toxicity provides further evidence for possible usefulness of nicotine or nicotinic agonists as a therapeutic intervention, particularly in prevention or slowing the progression of PD. This contention is also

supported by our findings that the effects of nicotine could be blocked by the nicotinic receptor antagonist, mecamylamine.

Various in vitro and in vivo studies have shown protective effects of nicotine against various toxicants, including those selectively targeting dopaminergic neurons. Thus, nicotine blocked salsolinol-induced toxicity in SH-SY5Y cells (Copeland et al. 2005,2007; Ramlochan Singh et al. 2011) as well as MPTP-induced cell damage in non-human primates (Quik et al. 2008). Although the effects of nicotine are primarily mediated through the central nicotinic receptors, the exact nicotinic receptor subtype involvement in these protective effects of nicotine has yet to be determined. It was shown that at least in SH-SY5Y cells which are human neuroblastoma derived cells, the effects of nicotine are mediated by $\alpha 3$ and/or $\alpha 6$ containing subtypes (Copeland et al. 2007).

The damaging effects of various toxicants could be mediated through apoptotic, necrotic or a combination of the two. These processes could in turn be brought about through abnormal protein folding or protein accumulation (e.g. α -synuclein, actin, tubulin, etc.), mitochondrial damage via oxidative stress or free radical formation. It is intriguing that some of these processes could be the result of normal metabolic pathways associated with the critical neurotransmitter (e.g. dopamine). Hence, it would be of significant interest to determine the underlying mechanisms involved in aminochrome-induced toxicity and how nicotinic system specifically interacts with such processes. In this regard, it is of interest to note that some in vitro studies suggest an anti-apoptotic effect of nicotine (Copeland et al. 2007; Ramlochan Singh et al. 2011).

Since the etiology of PD remains elusive and many factors might be involved, a more comprehensive therapy might entail combination of drugs that target various aspects of the disease. Thus, nicotine or nicotinic agonists may also be useful as adjunct therapy with currently used drugs. Interestingly, it was shown that a combination of nicotine with donepezil, a cholinesterase inhibitor, used primarily in Alzheimer's disease, provided an additive protection against salsolinol-induced toxicity in SH-SY5Y cells (Das and Tizabi 2009).

In summary, our results indicate a protective effect of nicotine against aminochrome-induced toxicity in RCN-3 dopaminergic cells which is mediated by nicotinic receptors. These findings provide further support for usefulness of nicotinic interventions in PD. Further investigation on the mechanism of this neuroprotection is warranted.

Acknowledgments

This study was supported by FONDECYT #1100165 (JSA), NIH/NIGMS (2SO6 GM08016-39 (YT), and NIH-RCMI 2 G12 RR003048 (YT).

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Fig. 1.

Effects for various concentrations of aminochrome (AM) (20 and 50 μM) with and without dicoumarol (DIC) (50 μM) on % cell death in RCSN-3 cells. Cells were incubated for 48 h. Values are mean $\pm$ SEM of five independent experiments * $p < 0.05$, ** $p < 0.01$ compared to control, † $p < 0.05$, †† $p < 0.01$ compared to AM only

Fig. 2.

Effects for various concentrations of nicotine (NIC) (1–20 μ M) on toxicity induced by the combination of aminochrome (AM, 50 μ M) and dicoumarol (DIC, 50 μ M) in RCSN-3 cells. Nicotine was added 1 h before the combination of AM + DIC. Cells were incubated for 48 h. Values are mean $\pm$ SEM of five independent experiments * p < 0.05, ** p < 0.01 compared to control, † p < 0.05, †† p < 0.01 compared to AM + DIC

Fig. 3.

Effects for various concentrations of mecamylamine (MEC, 5-100 μ M) on RCSN-3 cells. Cells were incubated for 48 h. Values are mean $\pm$ SEM of five independent experiments. * p < 0.05, ** p < 0.01 compared to control

Fig. 4.

Effects for various concentrations of mecamlamine (MEC, 5 and 10 μ M) on protective effects of nicotine (NIC) (20 μ M) on toxicity induced by the combination of aminochrome (AM, 50 μ M)) and dicoumarol (DIC, 50 μ M) in RCSN-3 cells. Mecamlamine was added 1 h before nicotine which was added 1 h before the combination of AM + DIC. Cells were incubated for 48 h. Values are mean $\pm$ SEM of five independent experiments ** p < 0.01 compared to control, † p < 0.05, †† p < 0.01 compared to AM + DIC # p < 0.05, ## p < 0.01 compared to AM + DIC + NIC