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The dopamine metabolite aminochrome inhibits mitochondrial complex I and modifies the expression of iron transporters DMT1 and FPN1

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Abstract Hallmarks of idiopathic and some forms of familial Parkinson's disease are mitochondrial dysfunction, iron accumulation and oxidative stress in dopaminergic neurons of the substantia nigra. There seems to be a causal link between these three conditions, since mitochondrial dysfunction can give rise to increased electron leak and reactive oxygen species production. In turn, recent evidence indicates that diminished activity of mitochondrial complex I results in decreased Fe–S cluster synthesis and anomalous activation of Iron Regulatory Protein 1. Thus, mitochondrial dysfunction could be a founding event in the process that leads to neuronal death. Here, we present evidence showing that at low micromolar concentrations, the dopamine metabolite aminochrome inhibits complex I and ATP production in SH-SY5Y neuroblastoma cells differentiated into a dopaminergic phenotype. This effect is apparently direct, since it is replicated in isolated mitochondria. Additionally, overnight treatment with aminochrome increased the expression of the iron import transporter divalent metal transporter 1 and decreased the expression of the iron export transporter ferroportin 1. In accordance with these findings, cells treated with aminochrome presented increased iron uptake. These results suggest that aminochrome is an endogenous toxin that inhibits by oxidative modifications mitochondrial complex I and modifies the levels of iron transporters in a way that leads to iron accumulation.

Keywords Dopamine metabolism · Complex I activity · DMT1 isoforms · Ferroportin 1 · Iron accumulation

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized in its late phase by the sustained loss of dopaminergic neurons from substantia nigra pars compacta (SNpc) and other brainstem regions (Braak et al. 2004). Factors involved in the immediate neuronal death of SNpc neurons are dysfunction of mitochondrial complex I, increased oxidative stress and increased iron content in affected neurons (Schapira et al. 1990; Sian-Hulsmann et al. 2011).

The association between PD and decreased complex I activity was discovered when drug users developed acute and irreversible parkinsonian symptoms traced to

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the consumption of synthetic heroin contaminated with 1-methyl-4-phenyl-l,2,3,6-tetrahydropyridine (MPTP) (Langston 1985). The PD-complex I link was further strengthened when analysis of substantia nigra post-mortem tissue of patients with Parkinson's disease revealed reduced NADH-ubiquinone reductase activity when compared with matched controls (Schapira et al. 1990; Haas et al. 1995; Krige et al. 1992).

The causes for complex I dysfunction in sporadic PD is a matter of active research. Exogenous toxins can be of relevance, given the existing correlation between PD and the exposure to toxins like rotenone, paraquat, fenazaquin, annonacin and Guadeloupe PSP-PDC (reviewed in Schapira 2010). Endogenous toxins could also cause mitochondrial dysfunction, as reported for nitric oxide (Clementi et al. 1998; Broom et al. 2011), 4-hydroxynonenal (Humphries et al. 1998; Castellani et al. 2002) and advanced glycosylated end products (Munch et al. 2000; Remor et al. 2011). An endogenous source of free radicals proper to dopaminergic neurons derives from the non-enzymatic oxidation of dopaminoquinone, the oxidation product of dopamine to dopamine o-quinone that at physiological pH spontaneously cycles to *o*-quinone aminochrome (Arriagada et al. 2004; Zoccarato et al. 2005). A one-electron gain by aminochrome results in the highly reactive leukoaminochrome *o*-semiquinone radical that reacts with oxygen to generate $O_2^{\bullet -}$ (Segura-Aguilar et al. 1998). Moreover, aminochrome seems to mediate the transfer of electrons from respiration-active complex I into oxygen, thus increasing the production of $O_2^{\bullet -}$ and H_2O_2 (Zoccarato et al. 2005). The increase in reactive oxygen species has direct consequences on mitochondrial electron transport chain. Both H_2O_2 and the hydroxyl radical inhibit complex I activity and decrease ATP production (Sims et al. 2000; Zhang et al. 1990; Bautista et al. 2000; Chinopoulos and Adam-Vizi 2001).

Several reports have documented a substantial effect of dopamine metabolites on mitochondrial function. Dopamine oxidation products cause a significant increase in swelling of brain and liver mitochondria, probably as a result of opening of the permeability transition pore (Berman and Hastings 1999). Mitochondria oxygen consumption is inhibited by dopamine and related phenylethylamines through MAO-dependent and independent mechanisms (Gluck and Zeevalk 2004). Importantly, inhibition of electron transport chain activity by dopamine is apparently mediated by quinones derived from oxidized dopamine rather than by H_2O_2 and related ROS, and it is partially prevented by GSH (Khan et al. 2005; Jana et al. 2007).

A number of neurodegenerative disorders of the central nervous system evidence iron accumulation in distinct areas of the brain. In particular, in Parkinson's disease a large body of evidence indicates that iron accumulates in the dopaminergic neurons of the SNpc (Youdim et al. 1989; Hirsch et al. 1991; Gorell et al. 1995; Vymazal et al. 1999). Because of iron's capacity to catalyze the production of free radicals (Núñez et al. this issue), it is highly likely that iron accumulation contributes to neuronal death precisely through the fostering of free radical-mediated oxidative damage. A mechanistic link between inhibition of complex I and iron homeostasis was recently reported. Complex I inhibition results in decreased iron-sulphur cluster synthesis, the constitutive activation of IRP1, and increased cytoplasm labile iron pool (Mena et al. 2011).

In this report, we tested the hypothesis that the dopamine metabolite aminochrome is an endogenous toxin that through inhibition of mitochondrial function induces deregulation of iron homeostasis.

Materials and methods

Cells

Human neuroblastoma SH-SY5Y cells (CRL-2266; American Type Culture Collection, Rockville, MD) cultured as described (Aguirre et al. 2007) were differentiated to a dopaminergic phenotype by treatment for 4 days with 10 μM retinoic acid followed by treatment for 3 days with 25 ng/ml BDNF (brain-derived nerve factor) in MEM/F12 medium containing 0.5% FBS (Encinas et al. 2000).

Cell viability

Cell viability was assessed in 96-well microplates by a LDH activity assay (Aras et al. 2008).

Antibodies

Polyclonal anti-DMT1 and monoclonal anti-FPN1 antibodies were used as previously described (Núñez

et al. 2010). Anti-β actin antibody was from Sigma Chem. Co.

Aminochrome

Aminochrome was prepared and used within 4 h by reacting dopamine with tyrosinase as described (Paris et al. 2010).

Isolation of mitochondria

Differentiated SH-SY5Y cells were removed from culture wells by incubation for 5 min with 1 mM EDTA in 100 mM NaCl, 50 mM Tris–Cl, pH 7.4. Cells were sedimented, the pellet was resuspended in mitochondria suspension buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.4, 0.5 mg/ml BSA) and homogenized with a Glass/Teflon Potter homogenizer. Mitochondria were isolated by differential centrifugation as described (Bulteau et al. 2003).

Complex I activity measurement and ATP determination

Complex I activity was calculated as the difference between the initial rates of NADH oxidation in the absence and the presence of rotenone using decyl-ubiquinone as electron acceptor (Mena et al. 2011). ATP levels were determined with a luminescence assay (Kit A2206, Invitrogen) according to the manufacturer's instructions.

RT-PCR and immunodetection

Semi-quantitative PCR for FPN1 and the 1B, +IRE and −IRE isoforms of DMT1 were performed as described (Mena et al. 2008) using the following primers: FPN1: Forward (5′-CCAAGATACCTGAAATTACAAC-3′); Reverse (5′-CCAGCCATTTATTGGAATTCTGCAGTAC-3′), DMT1 (1B): Forward (5′-CGGGGGCGGCGTGT-3′); Reverse (5′-AGGGATTACTATAGGCAGGGTTGAT-3′), DMT1(+)IRE: Forward (5′-TGGGCATACGGTAAGCATCTCT-3′); Reverse (5′-TCCCACCCTAATCCAGTTCTAAG-3′), DMT1(–)IRE: Forward (5′-TCTGAACACCATGGACGCTGACT-3′); Reverse (5′-CCATCAGAGGCCAATCGTTTAACT-3′), Housekeeping gene primers GADPH: Forward (5′-TGGGTGTGAACCATGAGAAG-3′); Reverse (5′-CCATCACGACACAGTTTCC-3′).

FPN 1 and DMT1 proteins were detected by Western blot analysis as described (Aguirre et al. 2005). The immunoreactive bands were developed with a peroxidase-based SuperSignal chemiluminescence assay kit (Pierce) and quantified with the Quantity One software (Bio-Rad). Cell ferritin was quantified by a sandwich enzyme-linked immunosorbent assay as described (Arredondo et al. 1997). Polyclonal anti-ferritin and anti-ferritin-peroxidase were from Rockland Immunochemicals.

^{55}Fe uptake

Cells were incubated for 20 h in DMEM, 10% FCS plus varied concentrations (0–20 μM) of aminochrome in the absence or presence of 1 mM *N*-acetylcysteine (NAC). After incubation, cells were challenged with 1 μM $^{55}FeCl_3$, 100 μM ascorbate in Hank's buffered salt solution (HBSS) pH 7.0, for 1 h at 37°C. After washing, cell-associated ^{55}Fe radioactivity was determined as described (Núñez et al. 2010).

Data analysis

The data are presented as the mean ± SEM. Differences were analyzed by one-way ANOVA. Dunnett's post hoc test was used for comparison using InStat, GraphPad Software. P values <0.05 were considered significant.

Results

Dopamine, at sub-millimolar concentrations, inhibits electron transport chain complex IV, and, to a lesser extent complex I, in isolated rat brain I mitochondria (Khan et al. 2005). Considering that one of the characteristic of PD neurons is diminished activity of complex I, we assessed if the dopamine metabolite aminochrome has complex I inhibitory activity in dopaminergic SH-SY5Y neuroblastoma cells. Incubation of cells with 5–20 μM aminochrome for 20 h resulted in a significant inhibition ($P < 0.01$) of complex I activity, which compared favourably with inhibition by 100 μM 1-methyl-4-phenylpyridinium (MPP+), a known inhibitor of complex I (Fig. 1a).

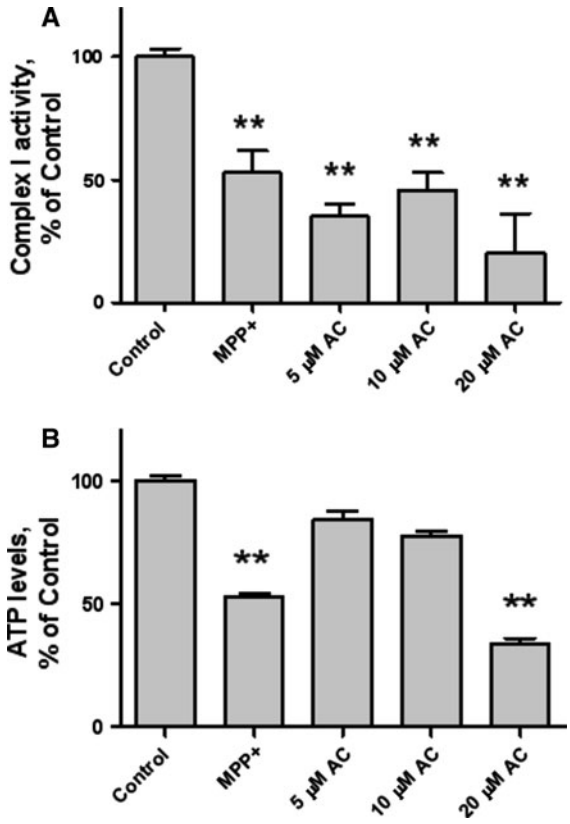

Fig. 1 Micromolar concentrations of aminochrome inhibit complex I activity in SH-SY5Y cells. **a** SH-SY5Y cells differentiated to a dopaminergic phenotype were treated with different concentrations of aminochrome for 20 h prior to determination of complex I activity as described in "Materials and Methods". Complex I activity is expressed in nmoles/min/mg. Results represent mean ± SEM of three separate experiments. **b** Determination of ATP levels in SH-SY5Y cells treated with different concentrations of aminochrome for 20 h. $*P < 0.05$ and $**P < 0.01$ compared with the control condition

Concentrations of aminochrome above 30 μM resulted in significant loss of cell viability (Supplemental Fig. 1).

Since a decrease in complex I activity could result in decreased ATP production, inhibition of complex I activity was further explored determining ATP levels in aminochrome-treated cells (Fig. 1b). Ten micrometers aminochrome, a concentration that produced significant inhibition of complex I was not effective in decreasing ATP levels. Nevertheless, treatment with 20 μM aminochrome resulted in a significant decrease. Treatment of cells with 100 μM MPP+ also resulted in a significant decrease in ATP production (Fig. 1b).

Inhibition of complex I by aminochrome could be a primary effect on the electron transport chain or

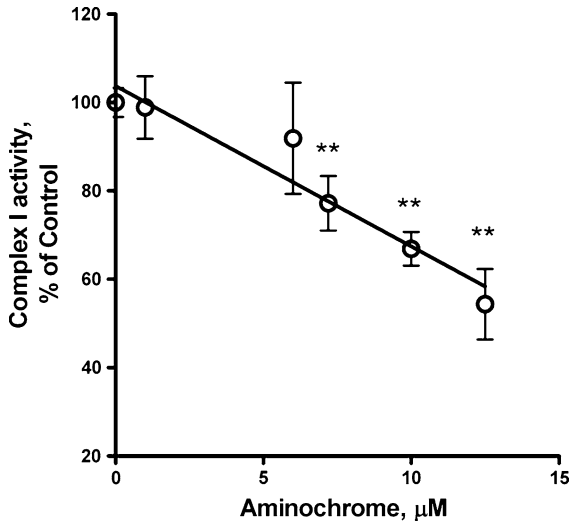

Fig. 2 Aminochrome inhibits complex I activity in isolated mitochondria. Freshly isolated mitochondria from rat liver were incubated for 10 min with aminochrome prior to determination of complex I activity. Significant decrease in complex I activity was detected after incubation with 12.5 μM aminochrome. Values represent the mean ± SEM of three separate experiments. $*P < 0.05$ compared with the control condition

secondary to the modification of an unknown cellular target. To differentiate between these possibilities, we tested the effect of aminochrome in mitochondria freshly isolated from rat liver. A significant decrease in complex I activity was detected after 10 min of incubation with 7.5–12.5 μM aminochrome (Fig. 2). This result suggests that aminochrome is a direct inhibitor of mitochondrial complex I.

Increase in the iron import transporter DMT1(+)IRE levels has been found in SNpc neurons of mice treated with MPTP and in *post mortem* tissue of PD patients (Chinopoulos and Adam-Vizi 2001, 2008). Similarly, other study reported that chronic treatment of mice with MPTP resulted in increased DMT1(+)IRE and (−)IRE isoforms and decreased FPN1 in substantia nigra neurons (Lv et al. 2011). Since aminochrome is apparently an inhibitor of complex I, we turned our attention to assess if aminochrome induced changes in iron transporters. Treatment of differentiated SH-SY5Y cells with aminochrome significantly increased mRNA level of DMT1(+)IRE isoform (Fig. 3a) without effect on mRNA levels of the DMT1(−)IRE isoform. The mRNA levels of the iron export transporter FPN1 were also modified: aminochrome treatment resulted in a significant decrease of FPN1 mRNA. Co-treatment of aminochrome and the

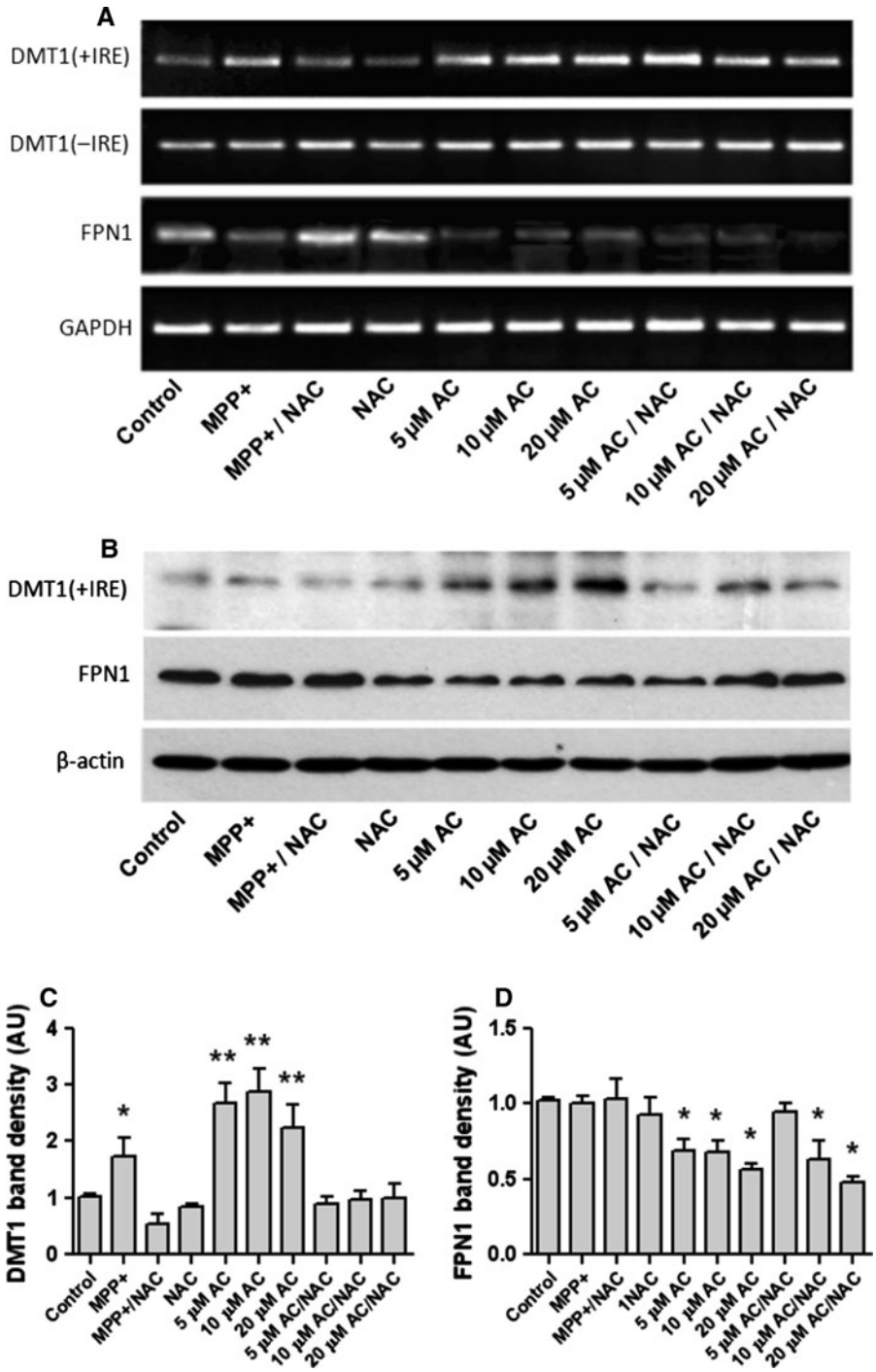

Fig. 3 Aminochrome treatment modifies the expression of iron transporters. Differentiated SH-SY5Y cells were exposed for 20 h to different concentrations of aminochrome or 100 μM MPP+ in the absence or presence of 1 mM NAC. **a** mRNA levels of DMT1(+)IRE, DMT1 (−)IRE and FPN1 determined by semi-quantitative PCR. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as a loading control. **b** Western blot of cell extracts. β-actin antibody was used as loading control. **c** Densitometry of the DMT1 bands, with mean ± SEM for three separate experiments. **d** Densitometry of the FPN1 bands, with mean ± SEM for three separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the control condition

anti-oxidant NAC partially blocked the effect of aminochrome. Protein levels were also affected by aminochrome treatment: an increase in DMT1(+)IRE and a decrease in FPN1 were observed (Fig. 3b). Quantification of band densities revealed significant increases in DMT1(+)IRE after treatment with 5–20 μM aminochrome and with 100 μM MPP+ (Fig. 3c). The increase was abrogated in cells co-treated with aminochrome plus NAC. A mild but significant decrease in FPN1 protein levels was

detected in protein levels of FPN1 after aminochrome but not MPP+ treatment (Fig. 3d). This decrease was reverted by co-treatment with NAC when cells were treated with 5 μM aminochrome but not in co-treatments with 10 or 20 μM aminochrome.

The observed increase in DMT1 and the decrease in FPN1 should result in increased iron retention by cells if these changes were functional. This was indeed the case as shown in Fig. 4. Aminochrome treatment induced a concentration-dependent increase in iron uptake by SH-SY5Y cells, with uptakes significantly higher that control in cells pre-treated with 10 and 20 μM aminochrome (Fig. 4a). The increase in Fe uptake was completely abrogated by the addition of NAC into the aminochrome-containing medium, an indication that oxidative stimuli may drive the increase in iron uptake. Aminochrome treatment also induced a mild increase in cell ferritin, with significant increases ($P < 0.05$) at 5 and 10 μM, while treatment with 20 μM aminochrome produced a non-significant ($P = 0.086$) increase in ferritin content (Fig. 4b). As with iron uptake, the increase in ferritin content induced by aminochrome was diminished to non-significant levels when NAC was present in the culture. The sum of the above results support a model in which inhibition of complex I by aminochrome results in changes in the expression of iron transporters that favours cell iron accumulation.

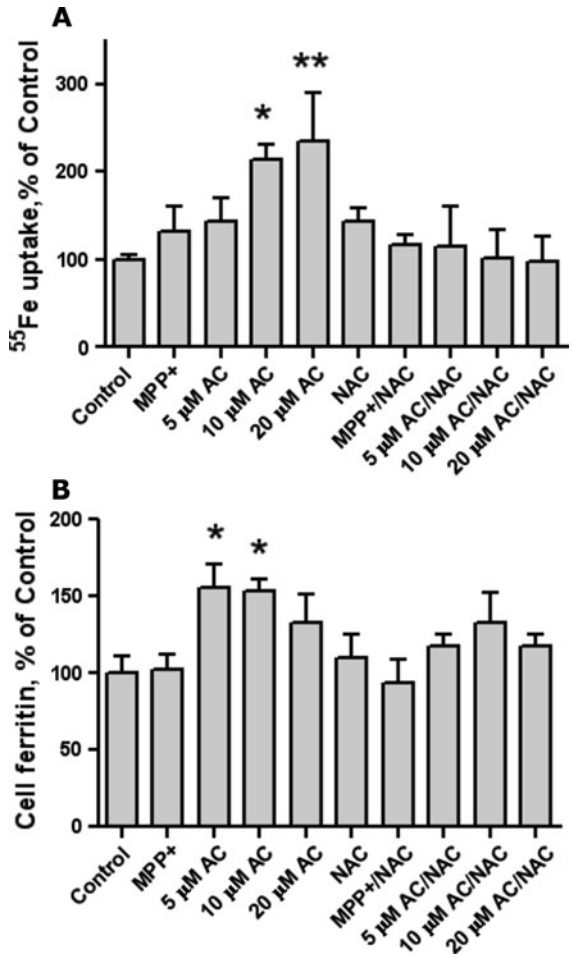

Fig. 4 Aminochrome treatment increases iron uptake and iron accumulation. **a** Cells treated for 20 h with the stated concentrations of aminochrome were incubated with 1 μM $^{55}FeCl_3$: 100 μM ascorbate in 1 mL in HBSS at 37°C for 1 h. Cells were washed twice in PBS to remove excess ^{55}Fe and detached with 100 μL Tris-saline EDTA buffer. A 10 μL aliquot was used to detect the protein concentration. 1 mL scintillation solution was added to the cell suspension to measure the ^{55}Fe radioactivity. The cellular radioactivity was defined as cellular ^{55}Fe radioactivity/protein amount (μg). The radioactivity of each group was normalized with the individual control group. **b** Cell-associated ferritin in cells treated as in (**a**). $^*P < 0.05$; $^{**}P < 0.01$ compared with control

Discussion

Multiple symptoms, including iron accumulation, mitochondrial dysfunction, oxidative stress, protein aggregation and proteasomal dysfunction are involved in the pathogenesis of sporadic PD (Altamura and Muckenthaler 2009; Schapira and Jenner 2011). Indeed, inhibition of complex I by the neurotoxin MPTP produces both death of SNpc neurons and Parkinsonian symptoms in primates (Iravani et al. 2005). It is therefore possible that diminished complex I activity is a founding event in the neuronal death observed in PD. Environmental toxins that inhibit complex I may also generate the disease, as indicated by the higher incidence of PD in pesticide workers (Gorell et al. 1998; Pan-Montojo et al. 2010). Here we addressed the question whether aminochrome could be an endogenous toxin that produce parkinsonian symptoms. We found that low (5–20 μM) aminochrome concentrations inhibited complex I activity both in a whole cell system and in isolated mitochondria. In accord with complex I inhibition, aminochrome also induced a decrease in ATP levels although a higher concentration of aminochrome (20 μM) was needed to demonstrate this effect.

Excess iron is postulated as a common cause in many diseases that include Huntington, Alzheimer,

Friedreich's ataxia, Neuroferrinopathy and PD (for a recent review see Kell 2010). In particular, PD neurons show marked iron accumulation that has been traced to increased expression of DMT1 (Salazar et al. 2008; Lv et al. 2011) and decreased expression of FPN1 (Song et al. 2010). Interestingly, we found that, as with the parkinsonian toxin MPTP, aminochrome treatment increased the expression of DMT1(+)IRE while decreasing the expression of FPN1, both at mRNA and protein levels. In accordance with the above changes, we found that aminochrome-treated cells incorporated more Fe and expressed higher levels of ferritin than untreated cells. Both effects were abrogated by the anti-oxidant NAC. The observed changes suggest that aminochrome-derived oxidation products induce the transcription of DMT1 and depress the transcription of FPN1, which results in iron accumulation.

As stated above, endogenous products such as nitric oxide, 4-hydroxynonenal and advanced glycosylated end products are possible causes of mitochondrial dysfunction, although none of these toxins accounts for the particularity that only dopaminergic neurons from the SNpc undergo neurodegeneration. The results of this study support the notion that at micromolar concentrations aminochrome is an endogenous toxin that oxidatively modifies the activity of complex I and the levels of iron transporters. Since complex I inhibition results in decreased iron-sulphur cluster synthesis and the constitutive activation of IRP1 (Mena et al. 2011), it is possible that micromolar concentrations of aminochrome produce the deregulation of DMT1 and FPN1 expression through the same mechanism, i.e., IRP1 activation. A working model that summarizes of the results reported here and in recent literature evidence is shown in Fig. 5.

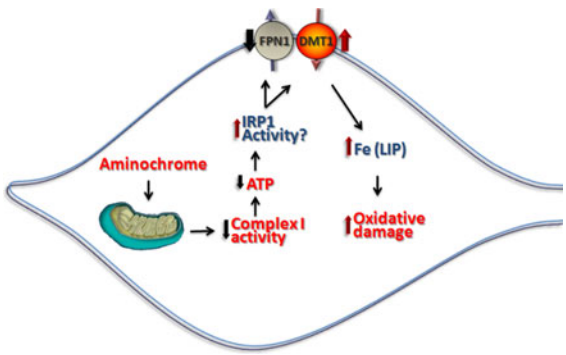

Fig. 5 Aminochrome disrupts iron homeostasis. A model is proposed in which inhibition of mitochondrial complex I by aminochrome results in decreased levels of ATP, increased expression of DMT1(+)IRE, decreased expression of FPN1 and increased uptake of iron. Thus resulting in an increase in the labile iron pool and increased oxidative stress which, among other events, can lead to cell death

In summary, our results indicate that aminochrome, at micromolar concentrations, inhibits mitochondrial complex I and induces iron accumulation by increasing the expression of DMT1 and decreasing the expression of FPN1. The relevance of aminochrome as a putative parkinsonian agent remains to be established. Unanswered questions are whether there could be conditions that result in toxic aminochrome concentrations, and if SNpc neurons, and no other dopaminergic neurons, could be selectively prone to aminochrome-induced neurodegeneration.

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