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ORIGINAL ARTICLE

One-Electron Reduction of 6-Hydroxydopamine Quinone is Essential in 6-Hydroxydopamine Neurotoxicity

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Abstract 6-Hydroxydamine has widely been used as neurotoxin in preclinical studies related on the neurodegenerative process of dopaminergic neurons in Parkinson's disease based on its ability to be neurotoxic as a consequence of free radical formation during its auto-oxidation to topaminequinone. We report that 50-μM 6-hydroxydopamine is not neurotoxic in RCSN-3 cells derived from substantia nigra incubated during 24 h contrasting with a significant sixfold increase in cell death (16 ± 2 %; $P < 0.001$) was observed in RCSN-3NQ7 cells expressing a siRNA against DT-diaphorase that silence the enzyme expression. To observe a significant cell death in RCSN-3 cells induced by 6-hydroxydopamine (24 ± 1 %; $P < 0.01$), we have to increase the concentration to 250 μm while a 45 ± 2 % cell death ($P < 0.001$) was observed at this concentration in RCSN-3NQ7 cells. The cell death induced by 6-hydroxydopamine in RCSN-3NQ7 cells was accompanied with a (i) significant increase in oxygen consumption ($P < 0.01$), (ii) depletion of reduced glutathione and (iii) a significant decrease in ATP level ($P < 0.05$) in comparison with RCSN-3 cells. In conclusion, our results suggest that one-electron reduction of 6-hydroxydopamine quinone seems to be the main reaction responsible for 6-hydroxydopamine neurotoxic effects in dopaminergic neurons and DT-diaphorase seems to play an important neuroprotective role by preventing one-electron reduction of topaminequinone.

Keywords 6-Hydroxydopamine · Quinone metabolism · Dopamine · Oxidation · Quinone · Parkinson's disease · Glutathione

Introduction

6-Hydroxydopamine (6OHDA) high affinity to the norepinephrine transporter and dopamine transporter (Redman et al. 2006) explain its accumulation in norepinephrine and dopamine neurons. 6-OHDA has been used as a neurotoxin to generate a preclinical experimental model for studying Parkinson disorders during long time and this is still widely used (Nowak et al. 2009; Kasture et al. 2009; Rodriguez-Pallares et al. 2009; Rauch et al. 2010; Walsh et al. 2010; Gregorio et al. 2009). The model with unilateral 6-OHDA-lesioning of rats is performed by injecting 6-OHDA directly into substantia nigra to destroy perikarya; or directly into striatum to damage dopaminergic innervations. Rats lesioned display rotational locomotor activity, circling in the direction of the lesion, while dopamine agonists produce contralateral turning owing to development of D_2 receptor super sensitization on the lesioned side. During long time has been postulated that 6-OHDA neurotoxic action depend on its ability to form free radicals and to inhibit mitochondrial respiratory chain complexes I

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and IV (Glinka et al. 1997). 6-OHDA structure is able to oxidize to a topaminequinone explaining its capacity to form free radicals. However, the question is whether 6-OHDA autoxidation in the presence of oxygen to topaminequinone with concomitant reduction of oxygen to superoxide radicals or topaminequinone one-electron reduction is the real neurotoxic step. Therefore, the aim of this study was to test the hypothesis that one-electron reduction of topaminequinone, formed during 6-OHDA autoxidation in the presence of oxygen, is the reaction involved in 6-OHDAs neurotoxicity.

Material and Methods

Chemicals

6-Hydroxydopamine, 5-hydroxydopamine, DME/HAM-F12 nutrient mixture (1:1), were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell Culture

The RCSN-3, RCSN-3pSR, and RCSN-3NQ7 cell lines (Lozano et al. 2010) grow in monolayers, in normal growth media composed of DME/HAM-F12 (1:1), 10 % bovine serum, 2.5 % fetal bovine serum, 10000 U/ml Penicillin, 10 mg/l streptomycin (Paris et al. 2008). RCSN-3 cells are catecholaminergic cell lines derived from rat substantia nigra that have expression of tyrosine hydroxylase; dopamine, norepinephrine, and serotonin transporter; monoamine oxidase-A; vesicular monoamine transporter-2 (VMAT-2); divalent metal transporter, DMT1; dopamine receptor 1 mRNA under proliferating conditions; and dopamine receptor 5 mRNA after incubation with dopamine or dicoumarol. In addition, this cell line has dopamine release and DT-diaphorase constitutes the 94 % of the total quinone reductase activity and generates neuromelanin (For review, RCSN-3 cells properties please see Paris et al. 2008). RCSN-3pSR is cell line obtained by transducing RCSN-3 cells with an empty plasmid pSuperRetro. RCSN-3NQ7 cells were obtained by transducing RCSN-3 cells with a pSuperRetro plasmid containing a siRNA against DT-diaphorase that decrease DT-diaphorase expression in 70 % (Lozano et al. 2010). The cultures were kept in an incubator at 37 °C with 100 % humidity, and the cells were grown in atmospheres of both 5 % CO_2. Fresh 6-OHDA solution containing 0.02 % ascorbic acid was used in all incubations. The RCSN-3 cell line was developed at the laboratory of Dr. Pablo Caviedes (University of Chile) that was provided through an agreement with the University of South Florida.

Determination of Cell Death

Cell death was measured by counting live and dead cells after staining with 0.5 µm Calcein AM and 5 µm ethidium homodimer-1 for 45 min at room temperature in the dark (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes). Calcein AM is a marker for live cells, and ethidium homodimer-1 intercalates into the DNA of dead cells. Cells were counted with a phase contrast microscope equipped with fluorescence using the following filters: Calcein AM 450–490 nm (excitation) and 515–565 nm (emission), and Ethidium homodimer-1, 510–560 nm (excitation) and LP-590 nm (emission).

Determination of Oxygen Consumption

The oxygen consumption was determined polarographically with a Clark electrode in a chamber at 25 °C, in a 600 µl final volume. RCSN-3 and RCSN-3NQ7 cells were trypzinized, washed and, centrifugated (2,000 rpm). The cells in 50 mM Tris–HCl pH 7.5 containing 0.08 % Triton X100 were homogenized and the protein was quantized. The incubation mixture contained 1.5 mg homogenate protein, 150 µm 6-OHDA, 500 µm NADH in50 mm Tris–HCl pH 7.5 containing 0.08 % Triton X100. The oxygen consumption rates were expressed as nmol O/min/mg protein.

Determination of Glutathione

The analyses were carried out on an HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA) equipped with a thermostatized µ-wellplate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus Mass Spectrometer (Agilent Technologies) using an electrospray ionization (ESI) interface. Standards with known concentrations of each compound (0.1, 1, 10, 25, 50, and 100 µm) were prepared in 2.5 % (w/v) MPA, 1 mM EDTA, and 0.1 % formic acid. Both standards and samples were passed through 0.22 µm filters. Then, 20 µl of each standard or sample was injected onto a Supelco Discovery C18 HPLC column (5 µm, 2.1 × 100 mm; Supelco, Bellefonte, PE) at 40 °C and eluted at a flow rate of 0.1 ml min^{-1} (Martí et al. 2012).

ATP Determination

ATP was determined using CellTiter-Glo luminescent cell viability assay where the cells were plated at 3,000 cells per well in 96-well microtiter plates and incubated overnight at 37 °C in a CO_2 incubator with 5 % CO_2. The next day the cells were incubated with 10 µm 6-OHDA during

2 h and determined according the KIT instructions (Promega Corporation, Madison, WI).

Data Analysis

All data are expressed as mean ± SE values. Statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons, and Student's t test for comparison between two given groups.

Results

We tested 6-OHDA toxicity in a cell line expressing a siRNA against DT-diaphorase to demonstrate that 6-OHDA neurotoxicity is depending on DT-diaphorase inhibition. The cells were exposed to 6-OHDA dissolved in the presence of 0.02 % ascorbic acid to prevent its auto-oxidation but as soon we dissolved the compound, we observed the appearance of a weak red color that became more intensive with the time. A significant cell death was observed when RCSN-3NQ7 were incubated with 50 μm 6-OHDA during 24 h (16 ± 2 % cell death; $P < 0.001$) in comparison with RCSN-3 wild type incubated with the same concentration of 6-OHDA (3.4 ± 2 % cell death). No significant cell death were observed in wild type RCSN-3 cells or RCSn-3pSR cells transduced with empty plasmid without cDNA coding for a siRNA against DT-diaphorase both in the presence of 50 μm 6-OHDA (Fig. 1a). We evaluated the cell death under different concentrations of 6-OHDA and we found that the cell death significantly increased in RCSN-3NQ7 cells to 45 ± 1 % ($P < 0.001$) when the cells were incubated with 250 μm 6-OHDA in comparison to RCSN-3 cells (24 ± 1 % cell death) or RCSN-3pSR cells (21 ± 1 % cell death) (Fig. 1b). We tested the ability of 5-hydroxydopamine to induce cell death in RCSN-3NQ7 cells by incubating 150 μm during 24 h but no significant cell death was observed (5.7 ± 1 % cell death) contrasting with a significant cell death induced by 150 μm 6-OHDA (30 ± 1.3 % cell death; $P < 0.001$) in comparison with RCSN-3NQ7 cells incubated with cell medium (5.3 ± 1 % cell death, Fig. 1c).

The role of reactive oxygen species in the observed cell death when RCSN-3NQ7 cells were incubated with 6-OHDA was investigated by incubating 6-OHDA with the homogenate that contain all the flavoenzymes including DT-diaphorase in the presence of 500 μm NADH as electron donor. The incubation of RCSN-3NQ7 cell homogenate with oxidized 50 μm 6-OHDA resulted in a significant increase in oxygen consumption (3.8 ± 0.5 nmol O/mg protein; $P < 0.01$) in comparison RCSN-3 cells (1.8 ± 0.4) (Fig. 2). The presence of oxygen consumption when the cells were incubated with 6-OHDA should induce a decrease in reduced GSH. A significant decrease of reduced GSH was observed in both RCSN-3 cells and in RCSN-3NQ7 cells (17 ± 5, $P < 0.001$ and 0 ± 0 %, $P < 0.001$ of control, respectively)

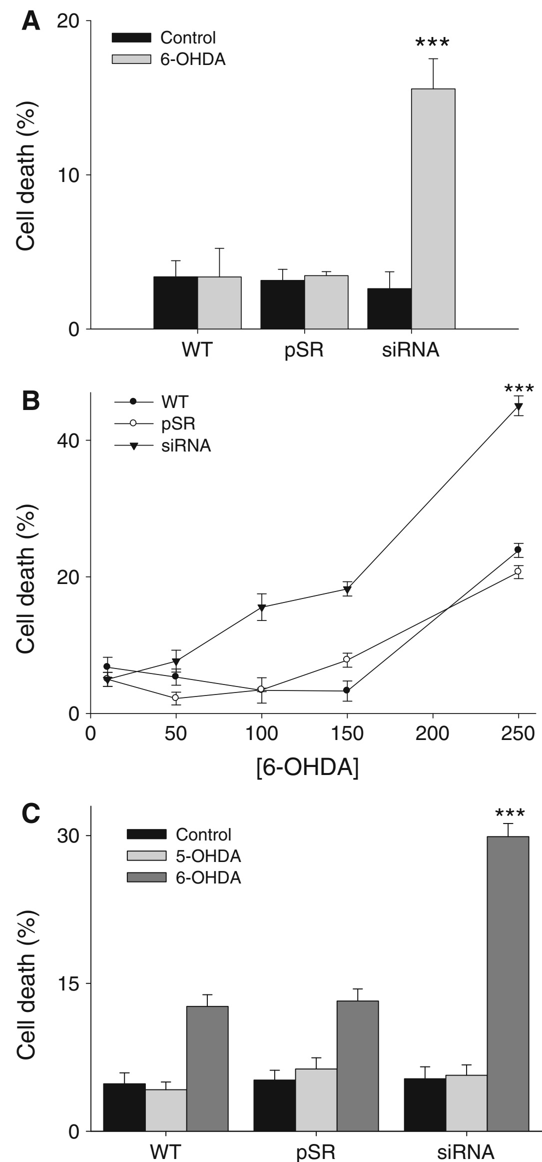

Fig. 1 The effect of 6- and 5-hydroxydopamine on RCSN-3 cells and RCSN-3NQ7 cells. **a** RCSN-3 cells (WT), RCSN-3pRS cells transduced with a empty pRetroSuper plasmid (pSR) and RCSN-3NQ7 cells expressing a siRNA that decrease in 70 % DT-diaphorase expression were incubated with cell culture medium (control) or 100 μm 6-OHDA (6-OHDA) during 24 h. A significant increase in cell death was observed in RCSN-3-siRNA-7 cells suggesting a protective role of DT-diaphorase. **b** The cell death at different concentrations of 6-OHDA (0, 10, 50, 100, 250 μm 6-OHDA was determined at 24 h incubation. **c** RCSN-3, pRetroS and RCSN-3NQ7 cells were incubated with 150 μm 5-hydroxydopamine (5-OHDA) during 24 h and compared with 6-OHDA toxicity. The values are expressed as the mean ± SD, $n = 3$. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's t test (***$P < 0.001$)

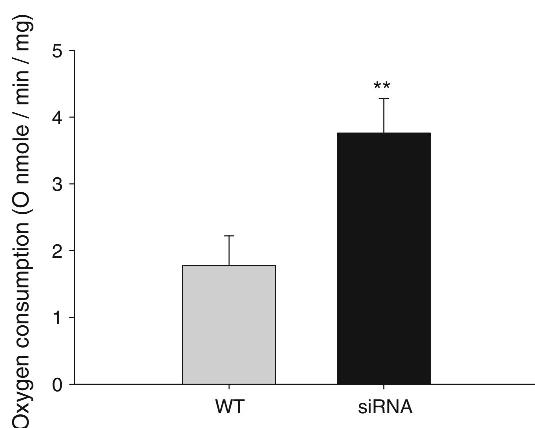

Fig. 2 Oxygen consumption in RCSN-3 and RCSN-3NQ7 cell homogenates in the presence of 6-OHDA. The oxygen consumption in cell homogenates were measured polarographically with a Clark electrode as described under "Material and Methods." The incubation included 50-mm Tris–HCl pH 7.5 containing 0.08 % Triton X100, 150 µm 6-OHDA, 500 µm NADH. The oxygen consumption rates were expressed as nmol O/min/mg protein. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's *t* test (**$P < 0.01$)

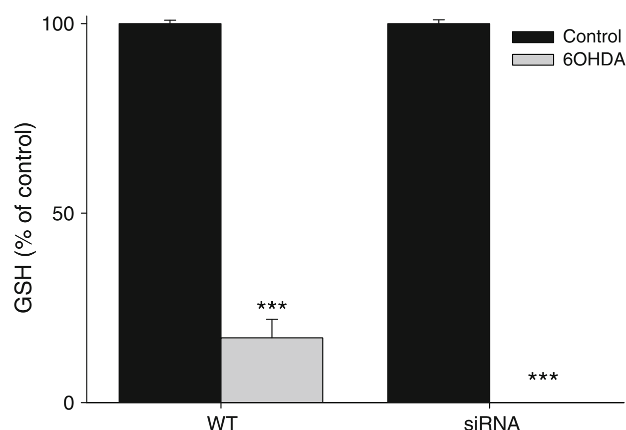

Fig. 3 The level of reduced glutathione in cells incubated with 6-OHDA. RCSN-3 and RCSN-3NQ7 cells were incubated 100-µm 6-OHDA during 24 h and the level of reduced GSH was measured as described under "Material and Methods." The results were expressed as percentage of control cells incubated in the absence of 6-OHDA. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's *t* test (**$P < 0.01$)

when they were incubated with 100-µm 6-OHDA. However, the decrease of reduced GSH in RCSN-3NQ7 was extensive resulting in a complete depletion of reduced GSH in comparison to RCSN-3 cells ($P < 0.01$; Fig. 3).

The oxidation of 6-OHDA to topaminequinone (Fig. 5) and its reduction by one-electron when DT-diaphorase is silenced by the expression of siRNA in RCSN-3NQ7 suggest that NADH can be depleted resulting in a decrease in ATP level. Incubation of RCSN-3 and RCSN-3NQ7 cells with 10 µm 6-OHDA induced a significant decrease of ATP level ($P < 0.001$) in comparison with cells incubated with cell culture medium. Interestingly, the ATP level in RCSN-3NQ7 cells was significantly lower ($P < 0.05$) than in RCSN-3 cells in the presence of 10 µm 6-OHDA (Fig. 4).

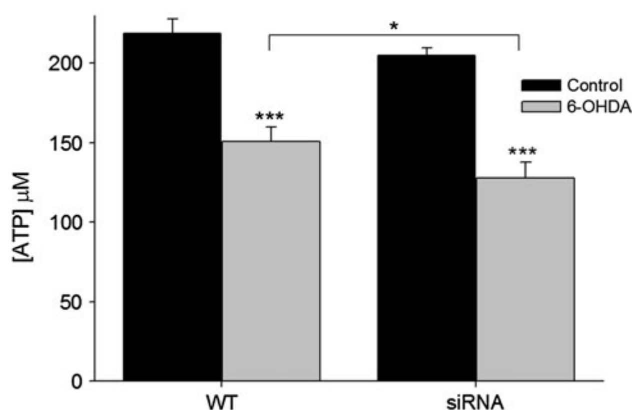

Fig. 4 The effect of 6-OHDA on ATP level in RCSN-3 cells. RCSN-3 cells and RCSN-3-NQ7 cells were incubated with 10 µm 6-OHDA (6-OHDA) or with cell culture medium during 2 h and the level of ATP was determined as described under "Material and Methods." A significant decrease in ATP was observed both in RCSN-3 and RCSN-3-NQ7 cells when the cells were incubated with 6-OHDA. However, or when RCSN-3-NQ7 cells compared RCSN-3 cells with in the presence of 6-OHDA showed a significant statistical decrease. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's *t* test (*$P < 0.05$; ***$P < 0.001$)

Discussion

It has been postulated that 6-OHDA neurotoxicity is depending on its ability to form free radicals depending on 6-OHDA auto-oxidation in the presence of oxygen (Glinka et al. 1997). However, our results strongly support our hypothesis that 6-OHDA neurotoxicity is depending on 6-OHDA quinone metabolism since 6-OHDA induced a significant sixfold increase in cell death in RCSN-3NQ7 cells expressing a siRNA against DT-diaphorase, contrasting with the lack of significant cell death observed in RCSN-3 cells wild type or RCSN-3pSR cells when they were incubated with 6-OHDA. The cell death observed in wild type RCSN-3 cells at 250 µm is probably due to that DT-diaphorase neuroprotection capacity was surpassed at this high concentration. DT-diaphorase is a unique flavoenzyme which catalyzes the two-electron reduction of quinones (Segura-Aguilar et al. 1992). Therefore, the silencing of DT-diaphorase expression by siRNA in RCSN-3NQ7 cells suggests that one-electron reduction of topaminequinone mediated by flavoenzymes that use

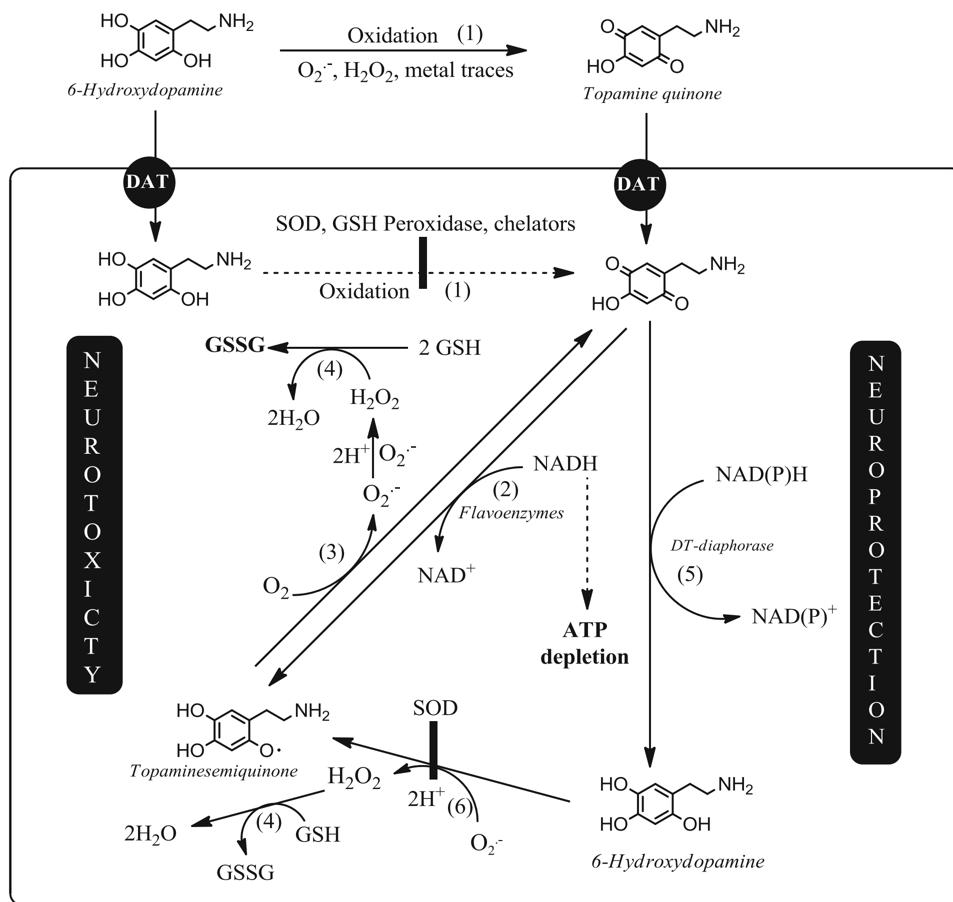

Fig. 5 Possible mechanism of 6-hydroxydomine neurotoxicity in RCSN-3NQ7 cells. The oxidation of extra- and intracellular 6-OHDA (*reaction 1*) result in the formation of topaminequinone that can be one-electron reduced to topamine-o-semiquinone radical (*reaction 2*) that auto-oxidizes to topaminequinone by reducing dioxygen to superoxide radicals (*reaction 3*) that dismutate spontaneously to hydrogen peroxide that oxidize GSH to GSSG (*reaction 4*). When the expression of DT-diaphorase is silenced by a siRNA expressed in RCSN-3NQ7 cells GSH is depleted due to one-electron reduction of topaminequinone induces a redox cycling that also deplete NADH required for ATP production in the mitochondria, inducing neurotoxicity. Interestingly, DT-diaphorase prevents 6-OHDA neurotoxicity by reducing topaminequinone with two-electrons to form again 6-OHDA (*reaction 5*) that is able to auto-oxidize in the presence of superoxide radicals, hydrogen peroxide and metal traces. However, in the cytosol superoxide dismutase, glutathione peroxidase, and proteins that chelate metals prevent 6-OHDA auto-oxidation (*reaction 6*)

NADH or NADPH is the major mechanism responsible for 6-OHDA neurotoxicity.

It has been reported that pyruvate provided cytoprotection in rat cerebellar granular cell cultures treated with 6-OHDA by preventing lipid peroxidation and up-regulating glutathione peroxidase mRNA levels (Fernandez-Gomez et al. 2006). It seems plausible that this protective action of pyruvate in 6-OHDA neurotoxicity was dependent on the use of pyruvate as substrate to form of Acetyl CoA catalyzed by pyruvate dehydrogenase complex, preventing that this enzyme complex catalyzes topaminequionone reduction. The oxidized flavin of a flavoenzyme is always able to be reduced by electrons donated from NADH or NADPH and transfer one-electron to an electron acceptor such as topaminequinone. The induction of DT-diaphorase in SH-SY5Y cells mediated by tert-butylhydroquinone (Hara et al. 2003) and 3*H*-1,2-dithiole-3-thione (Jia et al. 2008) associated with a decrease in 6-OHDA-induced cell death, support the protective role of DT-diaphorase against one-electron reduction of topaminequinone. The neuroprotective action of DT-diaphorase against topaminequinone in RCSN-3 cells support the neuroprotective role proposed for DT-diaphorase in dopaminergic neurons against other quinones such as aminochrome formed during dopamine oxidation (Paris et al. 2001, 2005, 2008, 2009a, b, 2010, 2011; Arriagada et al. 2004; Fuentes et al. 2007).

The 6-OHDA is very reactive with oxygen since it immediately autoxidizes in the presence of oxygen to topaminequinone (Napolitano et al. 1995) with concomitant formation of superoxide (Heikkila and Cohen 1971). However, 6-OHDA instability in the presence of oxygen is dependent on the presence of metal traces, hydrogen peroxide and superoxide radicals since the presence of the chelator diethylenetriaminepentaacetic acid, catalase, and

superoxide dismutase completely prevent 6-OHDA oxidation during 12 h (Gee and Davison 1984; Heikkila and Cohen 1973). The auto-oxidation of 6-OHDA to topaminequinone is one step event resulting in the production of reactive oxygen species while it seems to be plausible that one-electron reduction of topaminequinone to topaminesemiquinone (Fig. 5, reaction 2) induces a redox cycling as a consequence of topaminesemiquinone auto-oxidation in the presence of oxygen. This redox cycling generates reactive oxygen species that will deplete reduced intracellular glutathione as it was observed in RCSN-3NQ7 cells incubated with 6-OHDA. The depletion of reduced glutathione potentiates the neurotoxicity induced by topaminequinone one-electron reduction in RCSN-3NQ7. The depletion of reduced glutathione (Fig. 5, reaction 4) will prevent the protective activity of glutathione peroxidase that requires reduced glutathione to remove hydrogen peroxide. Interestingly, 5-hydroxydopamine did not induce cell death in RCSN-3NQ7 or RCSN-3 cell suggesting the importance of hydroxyl in the position 6 to form a *p*-quinone during 6-OHDA oxidation. 5-Hydroxydopamine is able to form two possible *o*-quinones that are not neurotoxic probably due to the presence a hydroxyl group in the next carbon.

The analysis of oxygen consumption in a cell homogenates that contain both one-electron transfer flavoenzymes and DT-diaphorase support this idea since the oxygen consumption increased twofold in the homogenate of RCSN-3NQ7 in comparison with RCSN-3 cells. We have to remember that DT-diaphorase is responsible for 94 % of the total quinone reductase activity in RCSN-3 cells (Paris et al. 2008). The observed oxygen consumption in RCSN-3 homogenate it is probably dependent on 6-OHDA autooxidation since DT-diaphorase catalyzes the two-electron reduction of topaminequinone to 6-OHDA (Fig. 5 reaction 5) which is very reactive with superoxide radicals, hydrogen peroxide and metal traces and immediately autoxidizes to topaminequinone (Napolitano et al. 1995). However, 6-OHDA instability in the presence of oxygen is dependent on the presence of metal traces, hydrogen peroxide and superoxide radicals since the presence of the chelator diethylenetriaminepentaacetic acid, catalase, and superoxide dismutase prevent 6-OHDA oxidation during 12 h (Gee and Davison 1984; Heikkila and Cohen 1973). In the RCSN-3 cell, homogenate 6-OHDA autoxidation is probably dependent on the presence of metal traces present in our buffer since the presence of superoxide dismutase and peroxidases in the homogenates will prevent that superoxide and hydrogen peroxide play a role in 6-OHDA.

The other mechanism proposed for 6-OHDA neurotoxicity is its ability to inhibit mitochondrial respiratory chain complexes I and IV (Glinka et al. 1997). Our results showed a significant decrease in ATP level both in RCSN-3 and RCSN-3-siRNA-7 supporting the idea that 6-OHDA in part exerts its neurotoxicity effects by interfering electron transport chain in mitochondria. However, the level of ATP was significantly lower in RCSN-3-siRNA-7 compared to RCSN-3 after 6-OHDA treatment although this decrease was not too strong as we expected since one-electron reduction of topaminequinone to dopaminesemiquinone radical should generate a redox cycling (Fig. 5, reactions 2 and 3) consuming both dioxygen and NADH required for ATP production in electron transport chain in the mitochondria. A possible explanation for this low decrease of ATP is the existence of a compensatory effect similar to the observed under anaerobic condition where the glycolysis rate is increased to compensate the inhibition of mitochondrial ATP (Hardie 2000). In RCSN-3NQ7 cells treated with 6-OHDA, we have similar conditions such as under anaerobic glycolysis since oxygen is depleted and the production of mitochondrial ATP is inhibited, due to the lack of NADH that it is oxidized to NAD^+ and 6-OHDA also inhibit mitochondrial respiratory chain complexes I and IV (Glinka et al 1997), increasing the glycolysis rate and compensating the loss of mitochondrial ATP.

Our results suggest a protective role of DT-diaphorase since the expression decrease (70 %) in RCSN-3NQ7 cells is essential for 6-OHDA neurotoxicity. DT-diaphorase is a unique flavoenzyme that catalyzes the two-electron reduction of quinones to hydroquinones and it has been proposed that this enzyme play a neuroprotective role in dopaminergic neurons by preventing aminochrome to participate in neurotoxic reactions such as the formation of adducts of proteins such as parkin, alpha synuclein, actin, α- and β-tubulin, complex I and III of mitochondrial electron transport, DJ1, UCH-L1 or be one-electron reduced generating reactive oxygen species a redox cycling that deplete NADH and oxygen (Van Laar et al. 2009; LaVoie et al. 2005; Norris et al. 2005; Paris et al 2009c, 2010, 2011; Lozano et al. 2010; Muñoz et al. 2012a, b, c).

In conclusion, our results suggest that 6-OHDA neurotoxicity is dependent on 6-OHDA oxidation to topaminequinone and subsequent one-electron reduction when DT-diaphorase is inhibited. DT-diaphorase prevents 6-OHDA neurotoxicity supporting the proposed neuroprotective role in dopaminergic neurons.

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