Received: 8 March 2012 / Revised: 22 June 2012 / Accepted: 12 July 2012

Differential Toxicity of 6-Hydroxydopamine in SH-SY5Y Human Neuroblastoma Cells and Rat Brain Mitochondria: Protective Role of Catalase and Superoxide Dismutase

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Abstract

Oxidative stress and mitochondrial dysfunction are two pathophysiological factors often associated with the neurodegenerative process involved in Parkinson's disease (PD). Although, 6-hydroxydopamine (6-OHDA) is able to cause dopaminergic neurodegeneration in experimental models of PD by an oxidative stress-mediated process, the underlying molecular mechanism remains unclear. It has been established that some antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) are often altered in PD, which suggests a potential role of these enzymes in the onset and/or development of this multifactorial syndrome. In this study we have used high-resolution respirometry to evaluate the effect of 6-OHDA on mitochondrial respiration of isolated rat brain mitochondria and the lactate dehydrogenase cytotoxicity assay to assess the percentage of cell death induced by 6-OHDA in human neuroblastoma cell line SH-SY5Y. Our results show that 6-OHDA affects mitochondrial respiration by causing a reduction in both respiratory control ratio (IC_{50} = 200 ± 15 nM) and state 3 respiration (IC_{50} = 192 ± 17 nM), with no significant effects on state 4_o. An inhibition in the activity of both complex I and V was also observed. 6-OHDA also caused cellular death in human neuroblastoma SH-SY5Y cells (IC_{50} = 100 ± 9 μM). Both SOD and

CAT have been shown to protect against the toxic effects caused by 6-OHDA on mitochondrial respiration. However, whereas SOD protects against 6-OHDA-induced cellular death, CAT enhances its cytotoxicity. The here reported data suggest that both superoxide anion and hydroperoxyl radical could account for 6-OHDA toxicity. Furthermore, factors reducing the rate of 6-OHDA autoxidation to its p-quinone appear to enhance its cytotoxicity.

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Keywords

6-Hydroxydopamine · Superoxide dismutase · Catalase · Mitochondria · SH-SY5Y cells · High-resolution respirometry

Abbreviations

6-OHDA	6-Hydroxydopamine
PD	Parkinson's disease
ROS	Reactive oxygen species
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
RCR	Respiratory control ratio
LDH	Lactate dehydrogenase
E-MEM	Eagle's minimal essential medium
*p*Q	*p*-Quinone of 6-OHDA
$^{\cdot}$*s*QH	Semiquinone radical of 6-OHDA
$O^{\cdot -}_2$	Superoxide radical
H_2O_2	Hydrogen peroxide
$^{\cdot}OH$	Hydroxyl radical
$HO^{\cdot}_2$	Hydroperoxyl radical

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a progressive motor dysfunction as a result of the loss of nigrostriatal dopaminergic neurons. Although its etiology is still not fully understood, there is no doubt that animal models of PD provide useful mechanistic data for the understanding of this disorder and for the ongoing development of neuroprotective strategies for its treatment. Thus, 6-hydroxydopamine (6-OHDA) is a catecholaminergic neurotoxin widely used to generate experimental models of PD [1, 2]. Administration of 6-OHDA into certain areas of the rat brain (striatum, substantia nigra, and third ventricle) in combination with desipramine, to prevent noradrenergic neuron destruction, is able to cause a selective

loss of dopaminergic neurons [3–5]. It has been reported that in these models 6-OHDA promotes an apoptotic process in dopaminergic neurons [6–8], mediated by oxidative stress, which leads to cell death [9, 10]. In view of the well-known ability of 6-OHDA to react rapidly and non-enzymatically with molecular oxygen and form a series of reactive oxygen species (ROS) [11, 12], the molecular mechanism proposed to explain its neurotoxicity includes the action of these ROS on different cellular structures and metabolic systems [13–15]. However, the precise molecular mechanism involved in 6-OHDA-induced neurotoxicity remains to be clearly established [16, 17].

It is recognized that the mitochondrial electron transfer system in association with mitochondrial oxidative phosphorylation set up the primary source of high energy compounds for cells, and that a dysfunction of mitochondrial energy metabolism leads to both a reduction in ATP production and an increase in ROS formation [18, 19]. Under normal conditions a small amount of molecular oxygen is continuously reduced by a single electron transfer to superoxide radical ($O^{\cdot -}$) by complexes I and III [20–22], but an impairment in mitochondrial respiration may dramatically increase the production of $O^{\cdot -}$. This has been demonstrated in dopaminergic neurons where the inhibition of complex I activity causes an increase in the production of ROS and subsequent oxidative damage to proteins, lipids and DNA [23–25]. Evidently, this supports the opinion that the specific deficiency in the activity of complex I found in the substantia nigra of some PD patients is the etiological factor involved in the pathogenesis of these particular cases of PD [26, 27]. With regard to 6-OHDA, although some authors found no significant evidence of its effect on complex I activity [28], others have reported a clear inhibitory effect [29, 30].

However, to understand mitochondria behaviour it should be borne in mind that cells in a homeostatic state present an antioxidant defence, mainly composed of enzymatic systems, which help to protect mitochondria against oxidative stress. Evidently, the status of the cellular defence determines the viability of cells. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are the major enzyme systems in charge of modulating the mitochondrial redox environment, developing its activity mainly inside the mitochondria (Mn-SOD), in the cytosol (Cu, Zn-SOD and GPx), and inside the

peroxisome (CAT). For this reason, the use of cell cultures can provide valuable and additional information on the mitochondrial response to a particular insult and vice versa. SH-SY5Y is a human neuroblastoma cell line which expresses the dopamine uptake system and is sensitive to the toxicity of 6-OHDA [31–33]. Furthermore, it has been reported that both CAT and SOD protect against 6-OHDA-induced cytotoxicity [7, 34–36]. Nevertheless, several studies have failed to find a protective role for both SOD [1, 37] and CAT [38–40] against 6-OHDA-induced cytotoxicity.

In order to obtain fresh data on the molecular mechanisms involved in the toxicity of 6-OHDA we used two models: rat forebrain mitochondria and human neuroblastoma SH-SY5Y cells. To evaluate the effects of 6-OHDA on mitochondrial function we investigated its action on mitochondrial respiration and on the activity of each of the complexes of the electron transport system. The effects caused by the presence of CAT and SOD on this process were also investigated, together with the ability of 6-OHDA to induce cell death and the capacity of CAT and SOD to affect this process.

Experimental Procedure

All experimental protocols described in this article were formally approved by the Ethical Committee of the University of Santiago de Compostela and conformed to the European Community Council Directive of 24 November 1986 (86/609/EEC).

Animals and Reagents

Male Sprague–Dawley rats weighing 200–250 g were used. Animals were housed in an animal room under controlled temperature (22 ± 1 °C) and 12-h light–dark cycle (light on from 8:00 to 20:00), with free access to tap water and standard laboratory chow. Animals were allowed to acclimatize for at least 4 days before the experiment. Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Isolation of Rat Brain Mitochondria

Mitochondria were isolated according to a previously described procedure [41] with slight modifications to obtain much better functional characteristics. Briefly, each rat was stunned with carbon dioxide, killed by decapitation, and the rostral forebrain quickly dissected with a transaction at the level of the two colliculli. Meninges and cerebellum were removed immediately, and the resultant forebrain minced prior to subsequent manual homogenization in a Dounce-type glass homogenizer using an icecold isolation medium (1/10, w/v) containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 3 mM EDTA2K and 1 mg/ml fatty acid free BSA (pH 7.4). The resulting brain homogenate was brought to 15 ml, distributed into three tubes, and then centrifuged at 600*g* for 10 min (JA-20 rotor, Beckman Instruments, Palo Alto, CA, USA). The supernatant was preserved while the pellet was resuspended in isolation medium to 10 ml and centrifuged as earlier to recover fast sedimented mitochondria. The resulting supernatants were then pooled and centrifuged in four tubes at 12,000*g* for 8 min. The pellets, including the fluffy synaptosomal layer, were resuspended in isolation medium, distributed into two tubes each containing a final volume of 10 ml and centrifuged at 12,000*g* for 10 min. The brown mitochondrial pellets obtained after discarding the synaptosomal layer were resuspended again in 10 ml of isolation medium and recentrifuged in two tubes at 12,000*g* for 10 min. The resulting mitochondrial pellets were combined and stocked in 1 ml of isolation medium until respiratory assay. All the procedures described were carried out at 4 °C. Protein concentration was determined by the method of Markwell et al. [42], using bovine serum albumin as standard. This procedure yielded 8–12 mg of protein per rat brain. Mitochondria prepared in this way were active for 3–4 h, as determined by the value obtained for respiratory control ratio (RCR).

Measurement of Mitochondria Respiration

Stocked mitochondria were diluted to 0.2 mg/ml in 2 ml of a standard respiration medium containing 125 mM KCl, 5 mM HEPES, 3 mM $MgCl_2$, 2 mM KH_2PO_4, and 0.5 mM EGTA (pH 7.4). The organelles were fuelled by a NADH-linked substrate (2.5 mM L-glutamate and 1.25 mM L-malate) under oxygen saturation conditions to evaluate the respiratory activity related to complex I. Oxygen consumption was measured at 30 °C using an Oroboros® high resolution respirometer, model Oxygraph-2k (Innsbruck, Austria) stirred at 500 rpm. Oxygen consumption under active respiration (state 3) was monitored after the addition of ADP (200 µM) and oxygen consumption under resting conditions was measured after the addition of 4 lg/ml of the mitochondrial ATP synthase inhibitor oligomycin (state 4_o). The rate of oxygen consumption before addition of mitochondria was subtracted from all measurements. RCR values (state 3/state 4_o) were calculated as the ratio of oxygen consumption in the presence of ADP divided by that obtained in the presence of oligomycin [43].

Measurement of Activities of Respiratory Chain Complex

The activities of respiratory chain complex, including complex I, II, IV and V, were measured in isolated mitochondria using the rapid ELISA kit for rodents (MitoSciences, Eugene, OR, USA), according to manufacturer's instructions. Complex III activity was determined by monitoring the reduction of cytochrome c in the presence of coenzyme Q_{10}, according to a slight modification introduced in a previously reported procedure [44]. Briefly, cytochrome c (15 µM) and coenzyme Q_{10} (100 µM) were added to an assay medium containing 25 mM KH_2PO_4, 2 mM KCN, 5 mM $MgCl_2$, 2 lg/ml rotenone at pH 7.2. Mitochondria (0.25 mg/ml) were added to the mixture and the increase in the absorbance recorded at 550 nm for 5 min. In some experiments, 6-OHDA (200 µM) was preincubated with mitochondria for 3 min. Data are expressed as percentage of activity compared to control.

Cell Culture and Treatment

Human neuroblastoma SH-SY5Y cells were grown at 37 °C in Eagle's minimal essential medium (E-MEM) with Earle's BSS:Ham's F12 (1:1, v/v), 2 mM L-glutamine, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1.5 g/l sodium bicarbonate, 10 % foetal bovine serum, 100 units/ml penicillin, and 100 lg/ml streptomycin in the presence of 5 % CO_2. Cells were grown to 80–90 % confluence and then seeded into different culture plates. Several experimental groups were treated for 24 h with different concentrations of 6-OHDA (from 1 µM to 1 mM) to induce cell death. 6-OHDA was dissolved in a 0.9 % sterile saline containing 0.02 % ascorbic acid. Another group was treated with vehicle alone and used as control. Other experimental groups were treated with different concentrations of CAT (250–2,000 IU/ml) or SOD (100–1,000 IU/ml) 24 h prior tothe addition of 6-OHDA (100 µM).

LHD Cytotoxicity Assay

Cellular death was fluorimetrically quantified by monitoring the activity of the cytoplasmic enzyme LDH released by cells with damaged plasma membranes and comparing this data with that obtained after a complete lysis of cells [45]. SHSY-5Y cells grown onto 96-well plates were treated as described above. LDH activity was determined using the CytoTox-ONE™ assay kit (Promega, Southampton, UK), according to manufacturer's instructions. Briefly, CytoTox-ON reagent was added to the culture medium (1:1, v/v) up to 100 ll in each well, following by plate shaken for 30 s. After 20 min of plate incubation at 37 °C, stop solution supplied with the kit was added and fluorescence recorded with a microplate spectrofluorometer (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA, USA), using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. LDH release was quantified by comparison with 100 % LDH release and was obtained using the lysis solution supplied with the kit.

Monitoring of *p*-Quinone Formation During 6-OHDAAutoxidation

The formation of *p*-quinone during 6-OHDA autoxidation was monitored spectrophotometrically at 490 nm [46]. The incubation medium was a buffer solution (Na_2PO_4/ KH_2PO_4 isotonized with KCl, pH 7.4) and *p*-quinone formation was followed after the addition of 20 µl of a stock of 6-OHDA (20 µM) dissolved in 1 mM KCl (pH 2.0) to prevent its autoxidation. In some experiments, 10 µl of CAT (1,000 IU/ml) or SOD (1,000 IU/ml) were incorporated prior to the addition of 6-OHDA. Final volume of the incubation was 400 µl and all concentrations are final concentrations in the incubation.

Statistical Analysis

Data were expressed as the mean ± SD or mean ± SE. Statistical differences between means were tested using one-way ANOVA followed by Duncan's test for multiple comparisons. IC_{50} values were obtained by non-linear regression analysis of the sigmoidal inhibition-concentration curves, using the program Sigmaplot v. 11.0 (Systat Software Inc., Chicago, IL, USA). The statistical significance was set at $p < 0.05$.

Results

Effect of 6-OHDA on Both Cellular Viability and Mitochondrial Functionality

6-OHDA was described as a dopaminergic neurotoxin whose effects are mediated by oxidative stress. Human SH-SY5Y dopaminergic neuroblastoma cells were exposed for 24 h (peak-time for LDH release [47]) to different concentrations of the neurotoxin, as described in Experimental Procedure. To evaluate the toxicity of 6-OHDA, the percentage of cellular death was estimated by using the LDH assay. The IC_{50} value was calculated by non-linear regression analysis of the toxicity-concentration curve,

obtaining a value of 100 ± 9 µM after 24 h of exposition (Fig. 1a).

Under control conditions, the mean active respiration rate (state 3) was 468 ± 60 pmol O_2/min mg protein and the mean resting respiration rate (state 4o) was 86 ± 19 pmol O_2/min mg protein, which results in a mean RCR of 5.45 ± 0.02. As illustrated in Fig. 1b, c, the treatment of forebrain mitochondria with different concentrations of 6-OHDA caused a significant reduction in both RCR and rate of oxygen consumption at state 3, with no significant change in the rate of oxygen consumption at state 4_o. The non-linear regression of the inhibition-concentration graph obtained for RCR inhibition revealed the existence of a decrease in the degree of coupling that was dependent on 6-OHDA concentration and gave an IC50 of 200 ± 15 nM. As shown in Fig. 1c, the inhibition of state 3 respiration by 6-OHDA gave an IC_{50} value (192 ± 17 nM) very close to that obtained for RCR inhibition. In addition, our results also show that concentrations of 6-OHDA higher than 10 µM shift the active oxygen consumption rate (state 3) into the value obtained for resting respiration (state 4_o) (data not shown). To understand the point of electron transport system altered by the presence of 6-OHDA (200 nM), the activity of complexes I–V was analyzed. As shown in Fig. 2, only complex I and V showed a significant reduction in their activity of 12 and 27 %, respectively, when compared with control,while complexes II–IV remained unaltered.

Neuroprotection of SH-SY5Y Cells and Isolated Mitochondria by CAT and SOD

To assess the potential neuroprotective activity of the antioxidant enzymes SOD and CAT on the neurotoxicity induced by 6-OHDA, SH-SY5Y neuroblastoma cells were pre-treated with each of the antioxidant enzymes 24 h prior to the addition of 6-OHDA. At this point, it is interesting to note that the hydrophilicity and molecular size of SOD and CAT restrict their action to the outside of cells. As shown in Fig. 3a, SOD protects significantly SH-SY5Y cells against the cellular death induced by 6-OHDA (100 µM). Furthermore, this effect resulted dependent on enzyme concentration. Nevertheless, CAT failed to prevent the damage induced by 6-OHDA (100 µM), even showing a high ability to enhance the cellular death caused by 6-OHDA (Fig. 3b). This enhancement

decreased in a concentration-dependent manner for concentrations over250 IU/ml. Mitochondria were also pre-treated with increasing concentrations of both SOD and CAT prior to their exposition to 6-OHDA (200 nM). In this study we observed that both antioxidant enzymes caused an increase in active respiration (state 3) with no significant effects on resting respiration (state 4_o). As illustrated in Fig. 3c, d, our results clearly show how the presence of both SOD and CAT cause a significant increase in the value of the RCR (state 3/ state 4_o) when compared with the value obtained in the absence of the assayed enzymes. In both cases, the protective action of both SOD and CAT against 6-OHDAtoxicity was dependent on the enzyme concentration.

Effect CAT and SOD on p-Quinone Formation During 6-OHDA Autoxidation

To determine whether the presence of both CAT and SOD affects the rate of 6-OHDA autoxidation, the formation of the corresponding *p*-quinone (*p*Q) was followed by direct recording of the increase in the absorbance at 490 nm. As illustrated in Fig. 4, the presence of both CAT (1,000 IU/ml) and SOD (1,000 IU/ml) caused a visible reduction in the rate of *p*Q formation during the autoxidation of 20 µM 6-OHDA under physiological conditions of pH. As can be seen, the slowing down observed in *p*Q formation washigher with CAT than with SOD.

Discussion

Oxidative stress has been cited as one of the main factors involved in the pathogenesis of PD [10, 48]. The use of different neurotoxins (6-OHDA, MPTP, and rotenone) to develop experimental models of PD has become an essential tool to gain insight into the molecular mechanisms involved in the etiology of this disorder. Although there is consensus that 6-OHDA neurotoxicity is due to the oxidative stress triggered by the production of ROS [49], the precise mechanism of its cytotoxicity remains uncertain. In this study we report the results obtained from a combined study carried out on the

toxicity of 6-OHDA using two models: rat forebrain mitochondria and human neuroblastoma SH-SY5Y cells. The aim of this study is to shed some new light on the molecular mechanisms involved in the toxicity of 6-OHDA from the effects caused by this toxin on mitochondrial function and cell viability, and particularly from the response of both models to the addition of SOD and CAT.

It is well-known that under physiological conditions 6-OHDA is rapidly oxidized in the presence of molecular oxygen to form the corresponding *p*Q and a series of ROS, which include the corresponding semiquinone radical ($^{\cdot}$*s*QH), superoxide radical ($O^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), and hydroperoxyl radical ($HO^{\cdot}_2$) [11, 12, 14, 15]. To illustrate our arguments, Fig. 5 shows the reactions involved in the formation of the above mentioned substances, together with the potential involvement of SOD and CAT in such reactions.

Assuming the reported involvement of mitochondria in the generation of ROS and the subsequent implication of these reactive compounds in neurodegeneration, our first goal was to quantify the effects caused by the presence of different concentrations of 6-OHDA on mitochondrial respiration. In our opinion, high-resolution respirometry appeared to be the most appropriate technique to perform this study because, as previously reported by Gnaiger [50], it requires only small amounts of mitochondria, thus allowing the use of low respiratory fluxes without the problems derived from the low signal–noise ratio reported by using Clark type oxygen electrodes. Thus, the accuracy of this technique to measure oxygen concentration at the picomolar level gave us the opportunity to investigate events occurring at very low concentrations of 6-OHDA and in the presence of antioxidants altering the concentration of oxygen into the chamber. However, highresolution respirometry requires the use of metabolically well-preserved mitochondria with good degree of integrity in order to detect slight variations in the rate of oxygen consumption. The RCR obtained under our experimental conditions with untreated brain mitochondria confirms the usefulness of the here reported procedure to obtain mitochondria with an appropriate degree of integrity and functionality for high-resolution respirometry.

Under our experimental conditions, 6-OHDA clearly showed a high capacity to reduce

mitochondrial respiration in rat forebrain mitochondria and to cause cellular death in human neuroblastoma SH-SY5Y cells. Both effects were dependent on 6-OHDA concentration and gave IC_{50} values of 200 nM for mitochondrial respiration (expressed in terms of RCR inhibition) and of 100 μM for cellular death in human neuroblastoma SH-SY5Y cells. Our results also show that 6-OHDA causes a noteworthy decrease in NADH-linked state 3 respiration, with no significant effects on state 4_o. Curiously, similar behavior was previously observed using dopamine at high concentrations [51]. Taking into account that under our experimental conditions 6-OHDA reduces the activity of complex I, a fact which agrees with previously reported data [29, 30], our findings may be interpreted as a consequence of a reduction in the flow of electrons through the mitochondrial electron transfer system. At this point, it should be emphasized that under our experimental conditions state 4_o mitochondrial oxygen consumption is limited by the rate of protons influx across the inner membrane, and consequently the resulting absence of change in state 4_o respiration by 6-OHDA shows that the permeability of mitochondria membranes to protons is not affected by the presence and autoxidation of 6-OHDA. To assess the potential ability of 6-OHDA to affect proton leak through the inner mitochondrial membrane and taking into consideration the results reported by Masini et al. [52], we made some additions to the incubation medium. These were: EGTA (to prevent the cycling of Ca^{2+} across the inner membrane of mitochondria), fatty acid free BSA (to prevent the oxidation of fatty acids), and oligomycin (to prevent the action of broken mitochondria with uncoupled ATPase activity). The inhibition observed in the activity of ATP synthase by 6-OHDA may also contribute to the reduction found in active respiration. Evidently, the uncoupling found in respiration-phosphorylation may help to explain the neurotoxicity of 6-OHDA through the consequent reduction in neuronal ATP production. Assuming the known ability of 6-OHDA to undergo autoxidation and generate ROS (Fig. 5), in both models (human neuroblastoma SH-SY5Y cells and rat forebrain mitochondria) it seems understandable to attribute the toxicity of 6-OHDA to the formation of ROS and their ability to cause both lipid peroxidation and protein oxidation [31]. The particular toxicity here reported of 6-OHDA on mitochondrial function could also be linked to the

presence of cytochromes and Fe-Cu clusters involved in mitochondrial respiration and the well-known ability of iron and copper to promote the reduction of H_2O_2 through the participation of the Fenton reaction to give $^{\cdot}OH$, which is considered the most damaging free radical for living cells [53]. With regard to dopaminergic neurons, this effect could be enhanced by: (a) the presence of neuromelanin in these cells and its ability to accumulate iron [54], and (b) the reported ability of 6-OHDA to release iron from strongly coordinating ligands [55]. Evidently, when cells are the model, the toxicity of 6-OHDA for mitochondrial function may be diminished by the antioxidant machinery within the cell which protects the mitochondrial function [56]. Both SOD and CAT have been shown to protect against the toxic effects caused by 6-OHDA on mitochondrial respiration. Although, the neuroprotective role of CAT and SOD against the neurotoxicity induced by 6-OHDA has been previously reported under different experimental conditions [7, 31, 34–36], some reports have failed to show the protective role of SOD [1, 37] and CAT [38, 39] against 6-OHDA-induced cytotoxicity. The protective effect here reported for both SOD and CAT on mitochondrial respiration appears to corroborate the involvement of ROS in early stages of 6-OHDA toxicity and the ability of these highly reactive substances to affect state 3 respiration and consequently to uncouple respiration to phosphorylation. As can be seen in Fig. 5, the activity of SOD may boost the formation of H_2O_2, and consequently the participation of the Fenton reaction in the formation of $^{\cdot}OH$. However, the dismutation of superoxide radical caused by the activity of SOD decreases the formation of $HO^{\cdot}_2$, with a slight alteration in the rate of *p*Q formation. Assuming the previously suggested role of $HO^{\cdot}_2$ in the initiation of both lipid peroxidation and in particular protein oxidation [57–59], our hypothesis explains the protective effects of SOD on mitochondrial respiration. The protective effect of CAT on the toxicity induced by 6-OHDA on mitochondrial respiration appears to be caused by an efficient elimination of H_2O_2, which must be associated to a significant decrease in the rate of 6-OHDA autoxidation, together with a reduction in the production of $^{\cdot}OH$ by the Fenton reaction. It is evident that CAT activity diverts the 6-OHDA autoxidation to the direct reaction between 6-OHDA and molecular oxygen without the involvement of H_2O_2. The possibility that this effect

could increase the production of $O^{\cdot -}$, and consequently the formation of $HO^{\cdot}_2$ (Fig. 5), is precluded by the notable decrease observed in the rate of 6-OHDA autoxidation. Furthermore, our results show that SOD is more efficient than CAT in reducing the 6-OHDA-induced toxicity, which underlines the particular contribution of $O^{\cdot}_2$ to the toxicity of 6-OHDA. In this way, our findings appear to corroborate the view that the actual free radical acting on the components of electron transport system and uncoupling mitochondrial respiration is the $HO^{\cdot}_2$ generated from $O^{\cdot -}_2$. As we previously reported [60], it is reasonable to think that an uncharged free radical with a relatively non short half-life such as $HO^{\cdot}_2$ could be involved in this process because of the following features: (a) its ability to pass through biological membranes to reach the inner mitochondrial membrane, (b) its easy access to the internal hydrophobic environment of proteins, and (c) its reported ability to oxidize proteins [58, 59]. Thus, we suggest $HO^{\cdot}_2$ as the main chemical species involved in mitochondrial impairment.

Finally, our results revealed a difference in behaviour concerning the effects of SOD and CAT on the cytotoxicity induced by 6-OHDA in human dopaminergic neuroblastoma cells. Thus, SOD showed a highly protective effect against 6-OHDA-induced toxicity, while CAT surprisingly enhanced the potential of 6-OHDA to cause cellular death, at least, when relatively low concentrations of CAT were used. Once again, these observations suggest the involvement of ROS in 6-OHDA-induced cellular death. Nevertheless, this lethal effect diminished as CAT concentration increased, but without achieving a protective effect, even at the highest concentrations used in this study. A putative explanation for these data may be the reduction in the rate of 6-OHDA autoxidation caused by the elimination of hydrogen peroxide by CAT, which causes an increase in the half-life of 6-OHDA and its derivates in the environment of the dopaminergic SH-SY5Y cells. In this way, the toxic potential of 6-OHDA is increased, particularly by the increased accessibility of 6-OHDA to the inside of cells by active transport [31]. It is probably that CAT at concentrations over 1,000 IU/ml displaces completely the reaction of $O_2^{\cdot -}$ dismutation to the formation of H_2O_2 and consequently, the instantaneous consumption of H_2O_2 by CAT prevents the formation of $HO^{\cdot}_2$ during 6-OHDA autoxidation, thus contributing to the protective effect observed under these

circumstances.

In summary, this study demonstrated that low concentrations of 6-OHDA (at nanomolar levels) are able to affect mitochondrial function by causing both a reduction of active respiration (state 3) and an inhibition of complex I and V activities. At higher concentrations (at micromolar levels) this neurotoxin is also able to cause cellular death in human dopaminergic neuroblastoma SH-SY5Y cells. Whereas both SOD and CAT have been shown to protect efficiently against the toxicity caused by 6-OHDA on mitochondrial respiration, only SOD has a protective effect on 6-OHDA-induced cellular death. Evidently, these data contribute to corroborate the involvement of oxidative stress in 6-OHDA toxicity. Furthermore, our data appear to suggest that a low rate of 6-OHDA autoxidation, the amount of superoxide anion formed and a high production of hydroperoxyl radical are the major causes of the toxic effects induced by 6-OHDA. This indicates that only specific ROS derived from 6-OHDA autoxidation are involved in its neurotoxicity and that more work is required to confirm the chemical nature of the species involved inmitochondrial and cellular injury.

Acknowledgments

This study was supported by grants SAF200766114 (to R. S.-O.) from the Ministerio de Ciencia e Innovación (Madrid, Spain) with the contribution of the European Regional Development Fund and 09CSA005298PR (to E. M.-A.) from the Xunta de Galicia (Santiago de Compostela, Spain). J. I.-G. was supported by a scholarship from the Fundación Obra Social La Caixa (Barcelona, Spain).

References

1. Blum D, Torch S, Nissou MF, Benabid AL, Verna JM (2000) Extracellular toxicity of 6-hydroxydopamine on PC12 cells. Neurosci Lett 283:193–196. doi:10.1016/S0304-3940(00)00948-4

2. Bove´ J, Prou D, Perier C, Przedborski S (2005) Toxin-induced models of

Parkinson's disease. NeuroRx 2:484–494. doi: 10.1602/neurorx.2.3.484

3. Sauer H, Oertel WH (1994) Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. Neuroscience 59:401–415. doi:10.1016/0306-4522(94)90605-X

4. Rodríguez M, Barroso-Chinea P, Abdala P, Obeso J, González Hernández T (2001) Dopamine cell degeneration induced by intraventricular administration of 6-hydroxydopamine in the rat: similarities with cell loss in Parkinson's disease. Exp Neurol169:163–181. doi:10.1006/exnr.2000.7624

5. Soto-Otero R, Méndez-Álvarez E, Hermida-Ameijeiras A, López-Real AM, Labandeira-García JL (2002) Effects of (-)-nicotine and (-)-cotinine on 6-hydroxydopamine-induced oxidative stress and neurotoxicity: relevance for Parkinson's disease. Biochem Pharmacol 64:125–135. doi:10.1016/S0006-2952(02) 01070-5

6. Biswas SC, Ryu E, Park C, Malagelada C, Greene LA (2005) PUMA and p53 play required roles in death evoked in a cellular model of Parkinson disease. Neurochem Res 30:839–845. doi: 10.1007/s11064-005-6877-5

7. Kulich SM, Horbinsky C, Patel M, Chu CT (2007) 6-Hydroxydopamine induces mitochondrial ERK activation. Free Radic Biol Med 43:372–383. doi:10.1016/j.freeradbiomed.2007.04.028

8. Gomez-Lazaro M, Galindo MF, Concannon CG, Segura MF, Fernandez-Gomez FJ, Llecha N, Comella JX, Prehn JHM, Jordan J (2008) 6-Hydroxydopamine activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA. J Neurochem 104:1599–1612. doi: 10.1111/j.1471-4159.2007.05115.x

9. Marti MJ, James CJ, Oo TF, Kelly WJ, Burke RE (1997) Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of the substantia nigra.J Neurosci 17:2030–2039

10. Jenner P (2003) Oxidative stress in Parkinson's disease. Ann Neurol 53:S26–S36. doi:10.1002/ana.10483

11. Graham DG, Tiffany SM, Bell WR, Gutknecht WF (1978) Autoxidation versus

covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. Mol Pharmacol 14:644–653

12. Gee P, Davison AJ (1989) Intermediates in the aerobic autoxidation of 6-hydroxydopamine: relative importance under different reaction conditions. Free Radic Biol Med 6:271–284. doi: 10.1016/0891-5849(89)90054-3

13. Halliwell B, Gutteridge JM, Cross CE (1992) Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med 119:598–620

14. Soto-Otero R, Méndez-Á lvarez E, Hermida-Ameijeiras A, Muñoz-Patiño AM, Labandeira-Garcia JL (2000) Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J Neurochem 74:1605–1612. doi: 10.1046/j.1471-4159.2000.0741605.x

15. Méndez-Álvarez E, Soto-Otero R, Hermida-Ameijeiras A, López-Martín ME, Labandeira-García JL (2001) Effect of iron and manganese on hydroxyl radical production by 6-hydroxydopamine: mediation of antioxidants. Free Radic Biol Med 31:986–998. doi:10.1016/S0891-5849(01)00679-7

16. Blum D, Torch S, Lambeng N, Nissou MF, Benabid A-L, Sadoul R, Verna JM (2001) Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Progress Neurobiol 65:135–172. doi: 10.1016/S0301-0082(01)00003-X

17. Simola N, Morelli M, Carta AR (2007) The 6-hydroxydopamine model of Parkinson's disease. Neurotox Res 11:151–167

18. Vercesi AE, Kowaltowski AJ, Grijalba MT, Meinicke AR, Castilho RF (1997) The role of reactive oxygen species in mitochondrial permeability transition. Biosci Rep 17:43–52. doi: 10.1023/A:1027335217774

19. Lenaz G, Bovina C, D'Aurelio M, Fato R, Formiggini G, Genova ML, Giuliano G, Merlo Pich M, Paolucci U, Parenti Castelli G, Ventura B (2002) Role of mitochondria in oxidative stress and aging. Ann NY Acad Sci 959:199–213

20. Boveris A, Cadenas E, Stoppani AO (1976) Role of ubiquinone in the mitochondrial

generation of hydrogen peroxide. Biochem J 156:435–444

21. Cadenas E, Boveris A, Ragan CI, Stopani AO (1977) Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. Arch Biochem Biophys 180:248–257. doi:10.1016/0003-9861(77)90035-2

22. Turrens JF, Boveris A (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem J 191:421–427

23. Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889–909. doi:10.1016/S0896-6273(03)00568-3

24. Vila M, Przedborski S (2003) Targeting programmed cell death in neurodegenerative diseases. Nat Rev Neurosci 4:365–375. doi:10.1038/nrn1100

25. Liss B, Haeckel O, Wildmann J, Miki T, Seino S, Roeper J (2005) K-ATP channels promote the differential degeneration of dopaminergic midbrain neurons. Nat Neurosci 8:1742–1751. doi: 10.1038/nn1570

26. Parker WD Jr, Swerdlow RH (1998) Mitochondrial dysfunction in idiopathic Parkinson disease. Am J Hum Genet 62:758–762. doi:10.1086/301812

27. Schapira AHV, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD (1990) Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 54:823–827. doi:10.1111/j.1471- 4159.1990.tb02325.x

28. Mazzio EA, Reams RR, Slimaqn KFA (2004) The role of oxidative stress, impaired glycolysis and mitochondrial respiratory redox failure in the cytotoxic effects of 6-hydroxydopamine in vitro. Brain Res 1004:29–44. doi:10.1016/j.brainres.2003.12.034

29. Glinka Y, Tipton K, Youdim M (1996) Nature of inhibition of mitochondrial respiratory complex I by 6-hydroxydopamine.J Neurochem 66:2004–2010

30. Glinka Y, Tipton KF, Youdim MBH (1998) Mechanism of inhibition of mitochondrial respiratory complex I by 6-hydroxydopamine and its prevention by desferroxamine. Eur J Pharmacol 351:121–129. doi:10.1016/S0014-2999(98)00279-9

31. Tiffany-Castiglioni E, Saneto RP, Proctor PH, Perez-Polo R (1982) Participation of active oxygen species in 6-hydroxydopamine toxicity to a human neuroblastoma cell line. Biochem Pharmacol 31:181–188. doi:10.1016/0006-2952(82)90208-8

32. Simantov R, Blinder E, Ratovitski T, Tauber M, Gabbay M, Porat S (1996) Dopamine-induced apoptosis in human neuronal cells: inhibition by nucleic acids antisense to the dopamine transporter. Neuroscience 74:39–50. doi:10.1016/0306-4522(96)00102-9

33. Storch A, Kaftan A, Burkhardt K, Schwarz J (2000) 6Hydroxydopamine toxicity towards human SH-SY5Y dopaminergic neuroblastoma cells: independent of mitochondrial energy metabolism. J Neural Transm 107:281–293. doi:10.1007/s007020050023

34. Asanuma M, Hirata H, Cadet JL (1998) Attenuation of 6-hydroxydopamine-induced dopaminergic nigrostriatal lesions in superoxide dismutase transgenic mice. Neuroscience 85:907–917. doi:10.1016/S0306-4522(97)00665-9

35. Kabuto H, Yokoi I, Iwata-Ichikawa E, Ogawa N (1999) EPC-K1, A hydroxyl radical scavenger, prevents 6-hydroxydopamineinduced dopamine depletion in the mouse striatum by up-regulation of catalase activity. Neurochem Res 24:1543–1548. doi: 10.1023/A:1021152115752

36. Barkats M, Millecamps S, Bilang-Bleuel A, Mallet J (2002) Neuronal transfer of the human Cu/Zn superoxide dismutase gene increases the resistance of dopaminergic neurons to 6-hydroxydopamine. J Neurochem 82:101–109. doi:10.1046/ j.1471-4159.2002.00952.x

37. Hanrott K, Gudmunsen L, O'Neill MJ, Wonnacott S (2006) 6-Hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase C delta. J Biol Chem 281:5373–5382. doi:10.1074/ jbc.M511560200

38. Choi W-S, Yoon S-Y, Oh TH, Choi E-J, O'Malley KL, Oh YJ (1999) Two distinct mechanisms are involved in 6-hydroxydopamineand MPP^{+}-induced dopaminergic neuronal cell death: role of caspases, ROS, and JNK. J Neurosci Res 57:86–94. doi:10.1002/ (SICI)1097-4547(19990701)57:1↘86::AID-JNR9▯3.0.CO;2-E

39. Saito Y, Nishio K, Ogawa Y, Kinumi T, Yoshida Y, Masuo Y, Niki E (2007) Molecular mechanisms of 6-hydroxydopamineinduced cytotoxicity in PC12 cells: involvement of hydrogen peroxide-dependent and -independent action. Free Radic Biol

Med 42:675–685. doi:10.1016/j.freeradbiomed.2006.12.004

40. Izumi Y, Sawada H, Sakka N, Yamamoto N, Kume T, Katsuki H, Shimohama S, Akaike A (2005) p-Quinone mediates 6-hydroxydopamne-induced dopaminergic neuronal death and ferrous iron accelerates the conversion of p-quinone into melanin extracellularly. J Neurosci Res 79:849–860. doi:10.1002/jnr.20382

41. Rosenthal RE, Hamud F, Fiskum G, Varghese PJ, Sharpe S (1987) Cerebral schemia and perfusion: prevention of brain mitochondrial injury by lidofalzine. J Cereb Blood Flow Metab 7:752–758

42. Markwell MAK, Haas SM, Bieber LL, Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87:206–210. doi:10.1016/0003-2697(78)90586-9

43. Chance B, Williams GR (1956) Respiratory enzymes in oxidative phosphorylation. VI. The effects of adenosine diphosphate on azide-treated mitochondria. J Biol Chem 221:477–489

44. Zhang S, Fu J, Zhou Z (2004) In vitro effect of manganese chloride exposure on reactive oxygen species generation and respiratory chain complexes activities of mitochondria isolated from rat brain. Toxicol Vitro 18:71–77. doi:10.1016/j.tiv.2003.09.002

45. Brzozowski MJ, Alcantara SL, Iravani MM, Rose S, Jenner P (2011) The effect of nNOS inhibitors on toxin-induced cell death in dopaminergic cell lines depends on the extent of enzyme expression. Brain Res 1404:21–30. doi:10.1016/j.brainres.2011.05.063

46. Heikkila RE, Cohen G (1973) 6-Hydroxydopamine: evidence for superoxide radical as an oxidative intermediate. Science 181:456–457. doi:10.1126/science.181.4098.456

47. Ossola B, Kääräinen TM, Raasmaja A, Männistö PT (2008) Time-dependent protective and harmful effects of quercetin on 6-OHDA-induced toxicity in neuronal SH-SY5Y cells. Toxicology 250:1–8. doi:10.1016/j.tox.2008.04.001

48. Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97:1634–1658. doi:10.1111/j. 1471-4159.2006.03907.x

49. Bove´ J, Perier C (2012) Neurotoxin-based models of Parkinson’s

disease. Neuroscience 211:51–76. doi:10.1016/j.neuroscience. 2011.10.057

50. Gnaiger E (2008) Polarographic oxygen sensors, the oxygraph, and high-resolution respirometry to assess mitochondrial function. In: Dyens J, Will Y (eds) Drug-induced mitochondrial dysfunction. Wiley, Hoboken, pp 327–352

51. Berman SB, Hastings TG (1999) Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria: implications for Parkinson's disease. J Neurochem 73:1127–1137. doi:10.1046/j.1471-4159.1999.0731127.x

52. Masini A, Ceccarelli-Stanzani D, Muscatello U (1983) The effect of oligomycin on rat liver mitochondria respiring in state 4. FEBS Lett 160:137–140. doi:10.1016/0014-5793(83)80953-3

53. Pryor WA (1986) Oxy-radicals and related species: their formation, lifetimes, and reactions. Ann Rev Physiol 48:657–667. doi: 10.1146/annurev.physiol.48.1.657

54. Enochs WS, Sarna T, Zecca L, Riley PA, Swartz HM (1994) The roles of neuromelanin, binding of metal ions, and oxidative cytotoxicity in the pathogenesis of Parkinson's disease: a hypothesis. J Neural Transm 7:83–100. doi:10.1007/BF02260963

55. Linert W, Herlinger E, Jameson RF, Kienzl E, Jellinger K, Youdim MBH (1996) Dopamine, 6-hydroxydopamine, iron, and dioxygen-their mutual interactions and possible implication in the development of Parkinson's disease. Biochim Biophys Acta 1316:160–168. doi:10.1016/0925-4439(96)00020-8

56. Tatsuta T, Langer T (2008) Quality control of mitochondria: protection against neurodegeneration and ageing. EMBO J 27:306–314. doi:10.1038/sj.emboj.7601972

57. Aikens J, Dix JA (1991) Perhydroxyl radical ($HOO^{\cdot}$) initiated lipid peroxidation. The role of fatty acid hydroperoxides. J Biol Chem 266:15091–15098

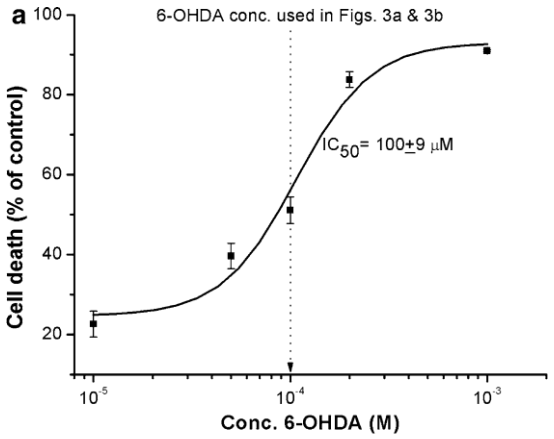
58. Gebicki S, Gebicki JM (1993) Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. Biochem J 289:743–749

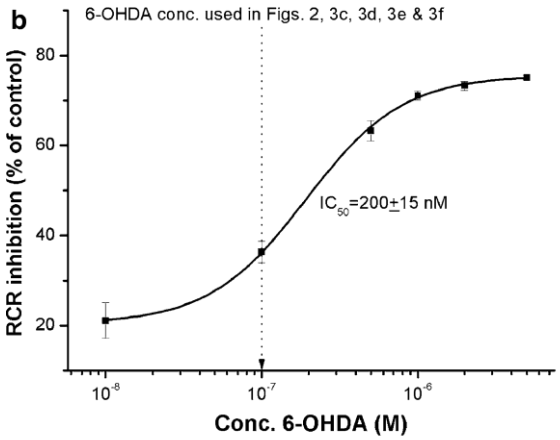
59. Fu S, Gebicki S, Jessup W, Gebicki JM, Dean RT (1995) Biological fate of amino acid, peptide and protein hydroperoxides. Biochem J 311:821–827

60. Hermida-Ameijeiras A, Méndez-Á lvarez E, Sánchez-Iglesias S, Sanmartín-Suárez C, Soto-Otero R (2004) Autoxidation and MAO-mediated metabolism of dopamine as a

potential cause of oxidative stress: role of ferrous and ferric ions. Neurochem Int 45:103–116. doi:10.1016/j.neuint.2003.11.018

Fig. 1 a Cellular death induced by 6-OHDA in human neuroblastoma SH-SY5Y cells. Cells were incubated with 6-OHDA during 24 h. Cell death was assayed by LDH assay and represented as the percentage of untreated control (in the absence of 6-OHDA). b Reduction of mitochondrial respiratory control ratio (RCR) caused by 6-OHDA after incubation of rat brain mitochondria in the presence of L-glutamate (2.5 mM) plus L-malate (1.25 mM). RCR was obtained as the quotient of active respiratory rate (state 3; 200 μM ADP) and resting rate (state 4_o; 4 lg/ml oligomycin), and represented as the percentage of inhibition in relation to untreated control. c Effects of 6-OHDA on the rate of oxygen consumption in state 3 and state 4_o. The active respiratory state 3 suffered a significant decrease and the percentage of inhibition is represented. Data are expressed as mean ± SD from three different experiments with three replicas per experiment ($n = 3$). IC_{50} values were obtained by non-linear regression analysis (Sigmaplot v. 11.0)


a
6-OHDA conc. used in Figs. 3a & 3b
Cell death (% of control)
IC50= 100±9 μM
Conc. 6-OHDA (M)


b
6-OHDA conc. used in Figs. 2, 3c, 3d, 3e & 3f
RCR inhibition (% of control)
IC50=200±15 nM
Conc. 6-OHDA (M)

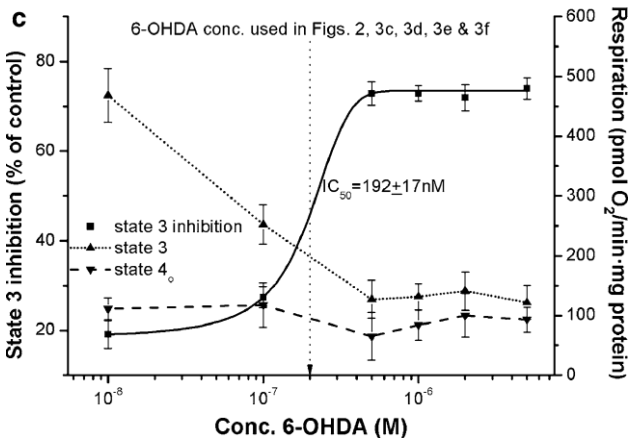

c
6-OHDA conc. used in Figs. 2, 3c, 3d, 3e & 3f
State 3 inhibition (% of control)
Respiration (pmol O2/min·mg protein)
IC50=192±17nM
state 3 inhibition
state 3
state 4o
Conc. 6-OHDA (M)

Fig. 2 Effects of 6-OHDA (200 µM) on the activity of complexes from the mitochondrial electron transport system. Mitochondria were isolated from forebrains as detailed in the ‘‘Experimental Procedure’’ section. Complex activities were determined using an antibody for each complex (*complex I*, *II*, *IV* and *V*) to perform an ELISA and a spectrophotometric method for *complex III*. Values are mean ± SE ($n = 3$) from independent experiments and represent the percentage of activity compared with controls. *Statistical significance (one-way ANOVA followed by Duncan’s test) was $p < 0.05$

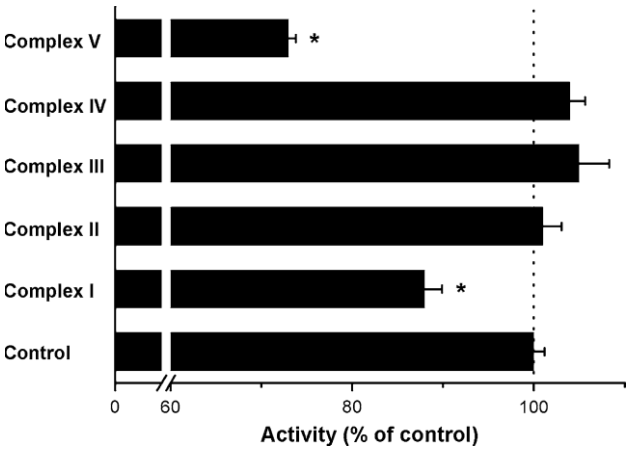


Fig. 3 Effect of SOD (a) and CAT (b) on SH-SY5Y cell death induced by 6-OHDA (100 μM) after 24 h of incubation. Cell death was assayed by LDH assay and represented as the percentage of untreated control (in the absence of 6-OHDA). Effect of SOD (c) and CAT (d) on the ability of 6-OHDA (200 nM) to reduce mitochondrial coupled respiration of rat brain mitochondria in the presence of L-glutamate (2.5 mM) plus L-malate (1.25 mM). RCR was obtained as the quotient of active respiratory rate (state 3; 200 μM ADP) and resting rate (state 4_o; 4 lg/ml oligomycin), and represented as the percentage of untreated control (in the absence of 6-OHDA). Effects of SOD (e) and CAT (f) on the effects caused by 6-OHDA (200 nM) on the rates of oxygen consumption in state 3 and state 4_o. Data are presented as the mean ± SE from three different experiments with three replicas per experiment. *Significant difference ($p < 0.05$) when compared with control exposed to 6-OHDA but in the absence of the antioxidant enzyme (one-way ANOVA followed by Duncan's test)

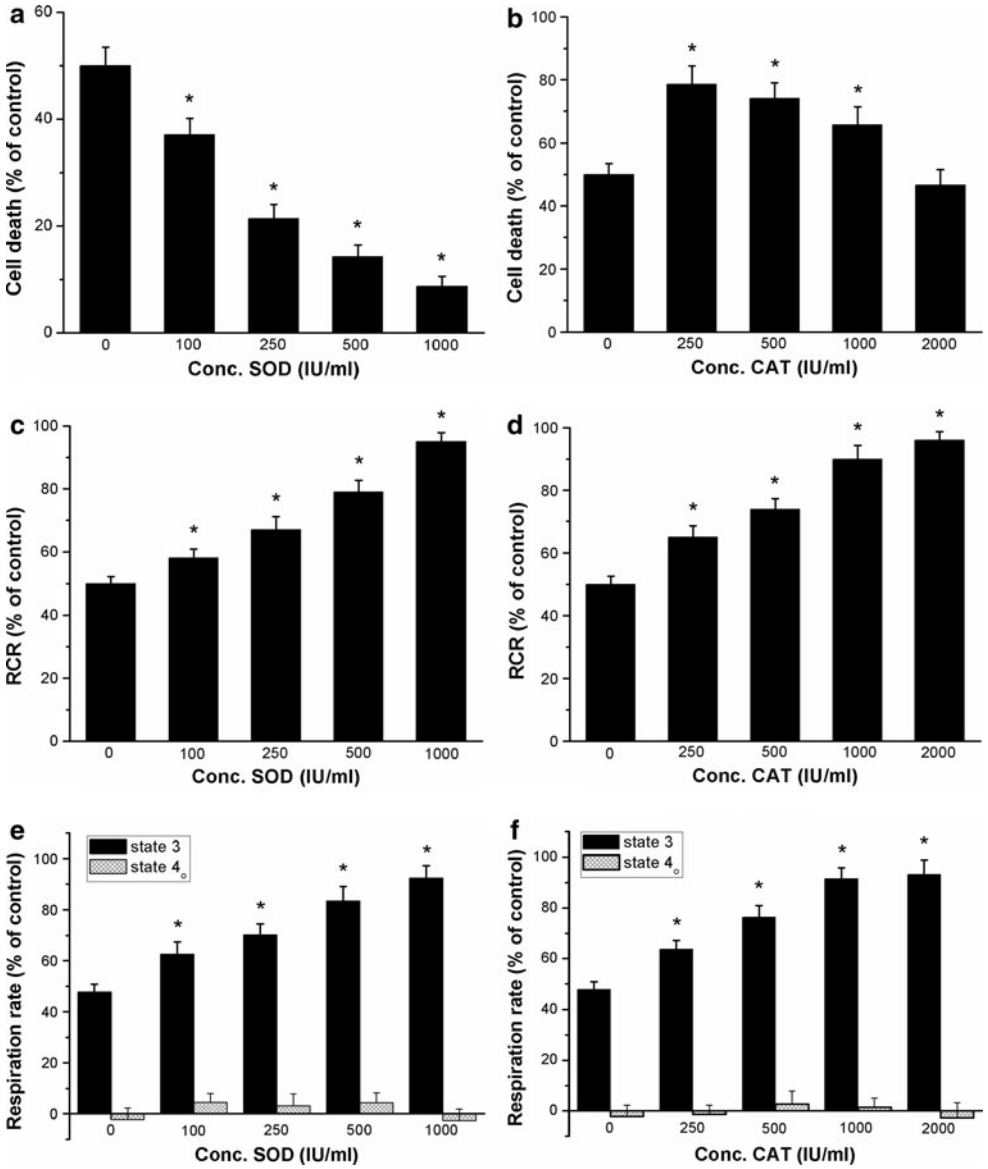

a
Cell death (% of control)
Conc. SOD (IU/ml)
b
Cell death (% of control)
Conc. CAT (IU/ml)
c
RCR (% of control)
Conc. SOD (IU/ml)
d
RCR (% of control)
Conc. CAT (IU/ml)
e
state 3
state 4o
Respiration rate (% of control)
Conc. SOD (IU/ml)
f
state 3
state 4o
Respiration rate (% of control)
Conc. CAT (IU/ml)

Fig. 4 Representative recording of the formation of *p*Q during 6-OHDA autoxidation in the absence (control) and presence of either CAT or SOD. The formation of *p*Q was followed spectrophotometrically after the addition of 6-OHDA (20 µM) to a phosphate buffer (isotonic with KCl, pH 7.4) containing none of the antioxidant enzymes, CAT (1,000 IU/ml) or SOD (1,000 IU/ml). All concentrations are final concentrations in the incubation

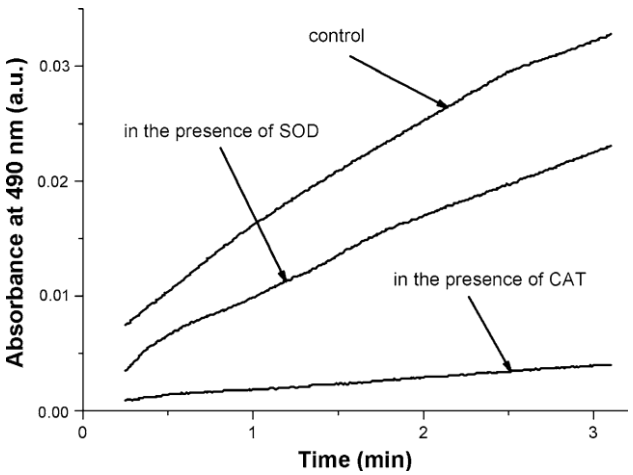


Fig. 5 Schematic representation of 6-OHDA autoxidation showing its ability to generate different reactive oxygen species (ROS) and the potential involvement of SOD and CAT in this process. As illustrated, ferrous iron (Fe^{2+}) can convert H_2O_2 to $^{\cdot}OH$ through what is called the Fenton reaction, but note that in the absence of Fe^{2+}, H_2O_2 can also generate $^{\cdot}OH$ by reacting with 6-OHDA (QH_2) or the semiquinone radical ($^{\cdot}sQH$) [15]