Brain Research Bulletin 87 (2012) 397–405

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Probucol modulates oxidative stress and excitotoxicity in Huntington's disease models in vitro

Dirleise Colle[a,*], Juliana M. Hartwig[a], Félix A. Antunes Soares[b], Marcelo Farina[a,*]

[a] Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil
[b] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul, Brazil

ARTICLE INFO

Article history:
Received 21 October 2011
Received in revised form
27 December 2011
Accepted 1 January 2012
Available online 11 January 2012

Keywords:
Huntington's disease
3-Nitropropionic acid
Quinolinic acid
Probucol
Mitochondrial dysfunction
Oxidative stress

ABSTRACT

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disease characterized by symptoms attributable to the death of striatal and cortical neurons. The molecular mechanisms mediating neuronal death in HD seem to be related to oxidative stress, excitotoxicity and misbalance in energetic metabolism. In this study we evaluated the potential relationship between energetic impairment, excitotoxicity and oxidative stress in rat striatal slices exposed to quinolinic acid (QA; as an excitotoxic model), 3-nitropropionic acid (3-NP; as an inhibitor of mitochondrial succinate dehydrogenase), as well as a combined model produced by the co-administration of these two toxins at subtoxic concentrations. We took advantage of the direct antioxidant/scavenger properties of Probucol in order to investigate the role of reactive oxygen species (ROS) in mediating the toxicity of both compounds alone or in association. Experiments with MK-801 (a NMDA type glutamate receptor antagonist) and succinate (an energy precursor agent) were also performed in an attempt to better comprehend the mechanisms of damage and neuroprotection. QA (1 mM), 3-NP (1 mM) and QA plus 3-NP (0.1 mM of both) significantly induced mitochondrial dysfunction and produced an increase in ROS generation, as well as a significant increase in lipid peroxidation in striatal slices. Probucol (10 and 30 μM) prevented ROS formation and lipid peroxidation in all used models, but did not protect against the mitochondrial dysfunction induced by 3-NP (only by QA or QA plus 3-NP). Sodium succinate (1 mM) protected the striatal slices only against 3-NP-induced mitochondrial dysfunction. On the other hand, MK-801 protected against mitochondrial dysfunction in all used models. Our data suggest that the two studied toxic models (QA and 3-NP) or the combined model (QA plus 3-NP) can generate complex patterns of damage, which involve metabolic compromise, ROS formation, and oxidative stress. Moreover, a partial inhibition of SDH by subtoxic 3-NP and moderate excitotoxicty by subtoxic QA are potentiated when both agents are associated. The toxic action of QA plus 3-NP seems to be involved with Ca^{2+} metabolism and ROS formation, and can be prevented or attenuated by antioxidant/scavenger compounds and NMDAr antagonists.

1. Introduction

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disorder caused by an abnormal expansion of CAG repeat located in exon 1 of the gene encoding for the Huntingtin protein [8,58]. The CAG repeat expansion leads to an abnormal polyglutamine (polyQ) tract in mutant Htt (mHtt) N-terminal region, which triggers a variety of aberrant interactions leading to pathological gain of toxic functions as well as loss of normal functions [7,59,65]. Moreover, the polyQ expansion can cause conformational changes in the mutant protein leading to intranuclear and intracytoplasmic insoluble aggregates or inclusions, which seem to play important roles in HD pathogenesis [12,37].

HD symptoms consist of motor, cognitive and psychiatric disturbances [58], which are attributable to the death of medium spiny GABAergic striatal neurons and, to a lesser extent, cortical neurons

Abbreviations: DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; HD, Huntington's disease; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRB, Krebs–Ringer bicarbonate buffer; LP, lipid peroxidation; MDA, malonaldehyde-bis-dimethyl acetal; mHtt, mutant huntingtin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDAr, N-methyl D-aspartate receptors; 3-NP, 3-nitropropionic acid; QA, quinolinic acid; PB, Probucol; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TBA, thiobarbituric acid; TBA-RS, thiobarbituric acid-reactive substances.

* Corresponding authors at: Departamento de Bioquímica, Centro de Ciências Biológicas, Campus Universitário, Trindade, Universidade Federal de Santa Catarina, CEP 88040-900, Florianópolis, Santa Catarina, Brazil. Tel.: +55 4837219589; fax: +55 4837219672.
E-mail addresses: dirleise@yahoo.com.br (D. Colle), farina@ccb.ufsc.br (M. Farina).

0361-9230 © 2012 Elsevier Inc. Open access under the Elsevier OA license.
doi:10.1016/j.brainresbull.2012.01.003

[33,55]. Several lines of evidence have proposed that the striatal cell death observed in studies of HD pathogenesis in humans and animal models is mediated by a misbalance in energetic metabolism, as well as oxidative stress and excitotoxicity [8,10,42].

Different genetic and toxin-based protocols have been developed to induce HD-like symptoms in laboratory animals [55]. Of particular importance, the administration of either quinolinic acid (QA) or 3-nitropropionic acid (3-NP) to rodents and non-human primates represents useful experimental models of HD; both biochemical and behavioral characteristics observed in HD patients are reproduced in these models [20,56,60,70].

QA (2,3-pyridinedicarboxylic acid), a tryptophan metabolite at the kynurenine pathway in glial cells, is a well-known agonist of the N-methyl D-aspartate (NMDA) type glutamate receptors that typically produces excitotoxic damage [54,66]. Given its endogenous nature, QA itself has been directly implicated as a potential pathogenic factor in HD [71], since it has been recently demonstrated that neostriatal and cortical levels of this toxicant is significantly enhanced in postmortem brains from HD patients at early stages of the disease [22,77]. QA has been currently shown to exert selective striatal toxicity by means of excitotoxic, pro-inflammatory and oxidative mechanisms [26,29,57,61], and antioxidant compounds have been reported to protect against QA-induced damage [4]. In addition, recent in vivo and in vitro studies showed that QA also causes brain energy impairment, resulting in inhibition of the mitochondrial complexes I, II and IV, as well as oxidative stress [28,62].

3-Nitropropionic acid (3-NP) is a mitochondrial toxin that has been found to effectively produce HD-like symptoms in animals models [35,64,70]. The primary mechanism of 3-NP-induced neurotoxicity involves irreversible inhibition of succinate dehydrogenase (SDH), a key enzyme located at the inner mitochondrial membrane and responsible for succinate oxidation to fumarate [31,70]. SDH inhibition interferes with mitochondrial electron transport cascade and oxidative phosphorylation, which leads to cellular energy deficit [32]. 3-NP treatment causes depletion of ATP levels, alteration in calcium homeostasis, generation of reactive oxygen species (ROS) and neuronal death [34,36,44,50]. Interestingly, some studies have demonstrated that 3-NP-induced neuronal death may also occur as result of excitotoxic events [46,51], which likely represent a secondary response to a primary energetic deficit.

More recently, an emerging line of research has provided interesting models to study integrative toxic events occurring in neurodegenerative disorders, including HD [14,15]. These models comprehend the facilitation of excitotoxic events through the impairment of energy metabolism, and are produced by combination of toxic molecules in different biological systems and under different experimental conditions [70]. Recently, evidence showed that the energy impairment induced by 3-NP, added by a moderate toxic action of QA, produced synergic increase of striatal degeneration, in a mechanism involving intracellular calcium deregulation [24]. These evidences corroborate data from the studies of Pérez-De La Cruz and coworkers, who demonstrated that, in the combined model, both oxidative stress and energy deficit are likely synergically contributing to cell death in slices of striatum [53]. From a molecular point of view, it is noteworthy that impairment in energy metabolism and excitotoxicity, two common elements in HD, seem to affect each other and involve a significant increase in ROS generation and oxidative stress, which modulate pathways mediating neuronal death: the interesting integrative hypothesis for HD is proposed and discussed by Pérez-De La Cruz and Santamaría [51]. Furthermore, some lines of evidence indicate that antioxidants and energy precursor agents may reduce neuronal death in HD models [16,25,68]. Although these different events, namely (i) oxidative stress, (ii) excitotoxicity and/or (iii) energetic deficits affect each other and seem to mediate neuronal death in experimental models of HD [51], the relationship between them in either QA- or 3-NP-based models is not completely understood. In addition, to the best of our knowledge, the understanding about such relationship is significantly scarcer in combined models (e.g., QA plus 3-NP).

Probucol (PB) is a phenolic lipid-lowering agent with antioxidant properties that had been clinically used during the past few decades for the treatment and prevention of cardiovascular diseases [11,74,75]. Of particular importance, previous experimental studies have reported that Probucol plays protective effects in experimental models of neurotoxicity/neuropathology [18,49]. Although the beneficial effects of Probucol under in vivo conditions are mediated by its hypocholesterolemic and anti-inflammatory properties [74], its beneficial roles under short-term incubations in in vitro models are likely related to its direct antioxidant (scavenger) properties [73].

Taking into account that (i) the combined model of HD (QA plus 3-NP) represents an useful tool in studding events mediating HD pathogenesis and that (ii) the potential relationship between energetic impairment, excitotoxicity and oxidative stress in the QA plus 3-NP-based model is not completely understood, we took advantage of the direct antioxidant/scavenger properties of Probucol to comprehend the role of ROS in the neurotoxic effects of QA plus 3-NP, as well as in the synergistic relationship between both challenges. Because of the relevant contribution of astrocytes in the combined model [51], striatal slices were used in this study since neuronal-glial interactions are preserved, thereby resembling the physiological conditions of the brain in a more integrative manner. Markers of energetic metabolism and oxidative stress were evaluated in the slices exposed to 3-NP, QA and/or Probucol in order to investigate the role of ROS in mediating the toxicity of both compounds alone or in association. Additional studies using the NMDA type glutamate receptor antagonist MK-801 and the energy precursor agent succinate were also performed in an attempt to better comprehend the mechanisms of damage and neuroprotection.

2. Methods

2.1. Chemicals

3-Nitropropionic acid, quinolinic acid, Probucol, MK-801, sodium succinate, thiobarbituric acid (TBA), malonaldehyde-bis-dimethyl acetal (MDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). All other reagents were obtained from local suppliers.

2.2. Animals

Adult male Wistar rats (200–250 g) ($n = 30$) from our own breeding colony were kept in cages with continuous access to food in a room with controlled temperature ($22 \pm 3\,^{\circ}C$) and a 12 h light/dark cycle, with lights on at 7:00 am. All experiments were conducted in accordance with the Guiding Principles of the Animal Care and Wellness Committee of the Universidade Federal de Santa Catarina (CEUA/UFSC PP00424; 23080.008706/2010-52).

2.3. Preparation and incubation of striatal slices

Rats were killed by decapitation and the striatum was rapidly removed and placed in ice-cold Krebs–Ringer bicarbonate buffer (KRB) (pH 7.4) containing (122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose). The striatum was removed and slices (0.4 mm) were rapidly prepared using a McIlwain Tissue Chopper, separated in KRB at 4 °C and allowed to recover for 30 min in KRB at 37 °C [47].

2.4. Slice treatment

QA and 3-NP were dissolved in PBS buffer and neutralized to pH 7.4 with NaOH, and they were freshly prepared each time before treatment. Probucol was dissolved

in dimethylsulfoxide (DMSO), which was used as control/vehicle and whose concentration did not exceed 0.1%. Sodium succinate and MK-801 were dissolved in PBS buffer.

After the preincubation time striatal slices were incubated with vehicle, QA (0.1, 0.5 and 1 mM), 3-NP (0.1, 0.5 and 1 mM), or with the combination of subtoxic concentrations of both agents (0.1 mM for both) at 37 °C for 2 h in KRB. After this period, the medium was removed, the slices were washed with KRB and the medium was replaced by a nutritive culture medium composed of 50% of KRB, 50% of Dulbecco's modified Eagle's medium (DMEM, Gibco), 20 mM of HEPES and 100 μg/mL of gentamicine in a humidified 5% CO_2/95% air atmosphere at 37 °C [43], and slices were maintained for additional 4 h to evaluate mitochondrial viability, lipid peroxidation and ROS formation.

Some experiments were performed in the presence of Probucol (10 and 30 μM), sodium succinate (1 mM), MK-801 (50 μM) or their respective vehicles. These compounds were co-incubated with the toxins (QA and/or 3-NP) and re-add in the slice medium during the second incubation. The analytical procedures were performed immediately after the last incubation.

2.5. MTT reduction assay

MTT reduction assay was evaluated as an index of mitochondrial function, according to previous reports [16,52]. This method is based in the ability of cells to reduce MTT to a dark violet formazan product by mitochondrial dehydrogenases in viable cells [45].

Striatal mitochondrial viability was evaluated after the second incubation. The slices (one per probe) were added with 15 μL of MTT (5 mg/mL), and re-incubated at 37 °C for 60 min in KRB (750 μL). Then, the medium was removed and the slices were washed for 30 min in 1 mL of dimethylsulfoxide (DMSO) to remove the formazan. Quantification of formazan was estimated by measuring optical density at 540 nm. The slices were solubilized (1% SDS; 0.1 N NaOH) and an aliquot was used for protein determination. Results were expressed as the percentage of MTT reduction with respect to control values. Preliminary experiments showed that 0.1% DMSO (Probucol's vehicle) did not interfere with the analyzed biochemical parameters per se. Data from five experiments per group were collected and analyzed.

2.6. Lipid peroxidation assay

Lipid peroxidation (LP) was assessed in homogenates obtained from the striatal slices (four slices per probe) by the assay of thiobarbituric acid-reactive substances (TBA-RS) formation, according to previous reports [57].

Immediately, after the last incubation, the slices were homogenized in 500 μL of ultra-purified water, and an aliquot of 20 μL of the homogenate was separated for protein determination. The homogenates remaining were mixed with 1 mL of the TBA reagent (containing 15% of trichloroacetic acid, 0.375% of thiobarbituric acid and 2.5%, v/v of HCl) to be re-incubated in a boiling water bath (95 °C) for 30 min. Samples were then centrifuged at 3000 × *g*, 15 min. The optical density of supernatants was estimated in 540 nm. The concentrations of MDA (expressed as nmol of MDA per mg protein) were calculated by interpolation in a standard curve of MDA (constructed in parallel), corrected by the content of protein per sample and expressed as percent of MDA formed vs. the control values. Data from five experiments per group were collected and analyzed.

2.7. Estimation of reactive oxygen species (ROS) formation

Formation of ROS was estimated with the fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as described by [2]. After cellular uptake, DCFH-DA is enzymatically hydrolyzed by intracellular esterase to form non fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. At the end of incubation, striatal slices were homogenized in order to read the ROS production. An aliquot of 20 μL of the homogenate was separated for protein determination. DCFH-DA (5 μM) was added to supernatants and fluorescence was read after 30 min using excitation and emission wavelengths of 480 and 525 nm, respectively. ROS levels (expressed as nmol of oxidized DCF per mg protein) were calculated by interpolation in a standard curve of oxidized DCF (constructed in parallel), corrected by the content of protein per sample expressed as percent of DCF oxidized formed vs. the control values. Data from five experiments per group were collected and analyzed.

2.8. Protein determination

The protein measurements content of the homogenized slice were assessed according to Lowry method [39].

2.9. Statistical analysis

Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Differences among the groups were analyzed by one-way ANOVA followed by the Tukey's post hoc test. Results are expressed as mean ± SEM. The differences were considered significant when $p < 0.05$.

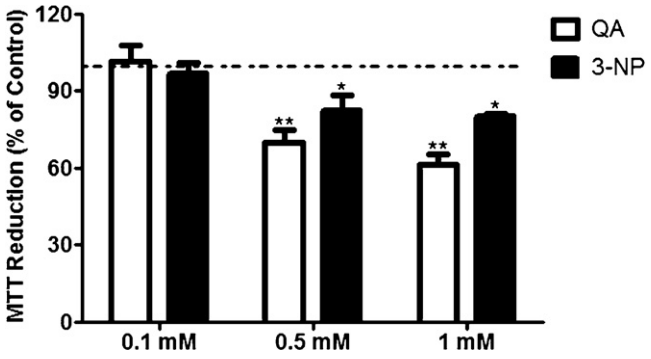

Fig. 1. Mitochondrial dysfunction induced by QA and 3-NP. Striatal slices were incubated with QA (0.1, 0.5 and 1 mM), 3-NP (0.1, 0.5 and 1 mM) or vehicle at 37 °C for 2 h in KRB. After this period, the medium was replaced for fresh culture medium without QA or 3-NP and the slices were maintained for additional 4 h. Mitochondrial viability was evaluated by the MTT reduction method. Results are expressed as the percentage of MTT reduction with respect to control values (dotted line). Data are represented as mean ± SEM ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical difference from control by one-way ANOVA, following by Tukey's post hoc test.

3. Results

3.1. Probucol protects against mitochondrial dysfunction induced by QA, 3-NP or QA plus 3-NP

In order to investigate the potential deleterious effects of QA and 3-NP on energy metabolism, MTT reduction was assessed as an index of the mitochondrial reductive capacity of striatal slices. Fig. 1 depicts a concentration-response study where slices of striatum were exposed to different concentrations of QA or 3-NP (0–1 mM). QA and 3-NP (at 0.5 and 1 mM, but not 0.1 mM) caused a significant decline in mitochondrial function ($p < 0.05$), as indicated by a decrease in the mitochondrial MTT reductive capacity in striatal slices (Fig. 1). Sub-toxic concentrations of QA (0.1 mM) and 3-NP (0.1 mM), which did not affect mitochondrial function when individually presented in the incubation medium, caused significant

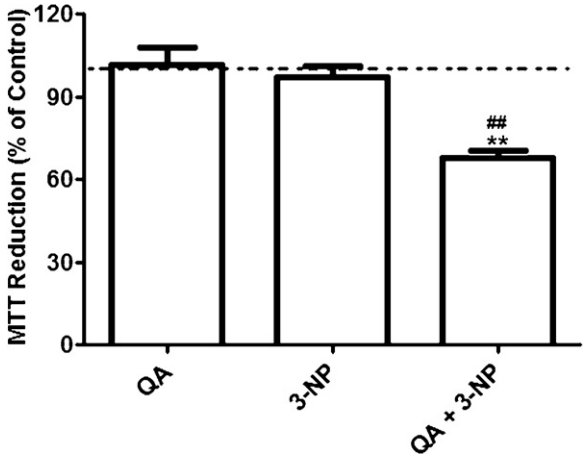

Fig. 2. Mitochondrial dysfunction induced by QA plus 3-NP. Striatal slices were incubated with 0.1 mM QA, 0.1 mM 3-NP, 0.1 mM QA plus 0.1 mM 3-NP or vehicle at 37 °C for 2 h in KRB. After this period, the medium was replaced for fresh culture medium without QA and/or 3-NP and the slices were maintained for additional 4 h. Mitochondrial viability was evaluated by the MTT reduction method. Results are expressed as the percentage of MTT reduction with respect to control values (dotted line). Data are represented as mean ± SEM ($n = 3$). $^{**}p < 0.01$ indicates statistical difference from control. $^{\#\#}p < 0.01$ indicates statistical difference from QA or 3NP by one-way ANOVA, following by Tukey's post hoc test.

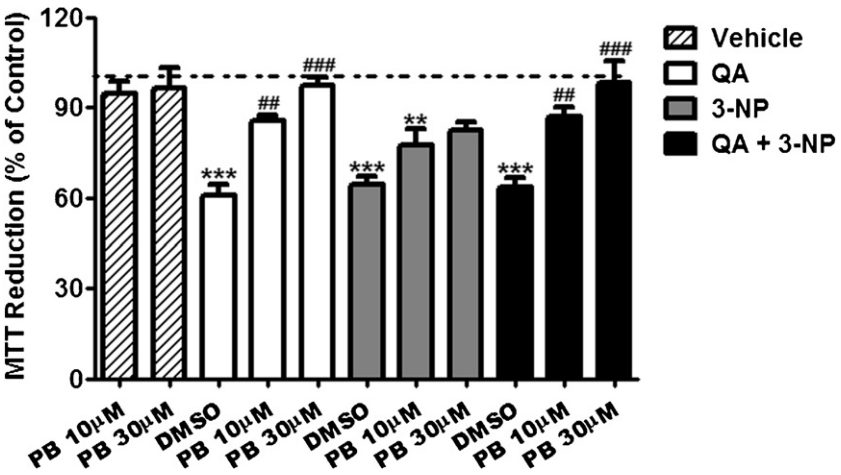

Fig. 3. Protective effect of Probucol against QA, 3-NP or QA plus 3-NP-induced mitochondrial dysfunction. Striatal slices were incubated with 1 mM QA, 1 mM 3-NP, 0.1 mM QA plus 0.1 mM 3-NP or vehicle at 37 °C for 2 h in KRB. After this period, the medium was replaced for fresh culture medium without QA and/or 3-NP and the slices were maintained for additional 4 h. PB (10 and 30 μM) or vehicle were co-incubated with the toxins and re-added in the culture medium during the second incubation. Mitochondrial viability was evaluated by the MTT reduction method. Results are expressed as the percentage of MTT reduction with respect to control values (dotted line). Data are represented as mean ± SEM ($n = 5$). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical difference from control. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate statistical difference from QA, 3NP or QA plus 3NP by one-way ANOVA, following by Tukey's post hoc test.

mitochondrial dysfunction in striatal slices when presented simultaneously (Fig. 2). Because 0.2 mM QA or 3-NP alone also did not affected mitochondrial function (data not shown), it is possible to state that the incubation with QA plus 3-NP induced a synergistic toxicity.

In order to investigate the molecular mechanisms mediating the toxicity induced by QA and 3-NP (alone or in association), the potential protective effect of Probucol (an antioxidant and scavenger compound) was evaluated in the presence of 1 mM of each toxin (alone) or 0.1 mM of both toxins (simultaneously). Fig. 3 show that the three toxic treatments (1 mM QA; 1 mM 3-NP; or 0.1 mM of both) significantly induced mitochondrial dysfunction in striatal slices (Fig. 3). Probucol was effective in protecting the insult elicited by QA in both concentrations ($p < 0.01$ and $p < 0.001$ for 10 and 30 μM, respectively) (Fig. 3). On the other hand, Probucol had no effect in striatal slices against the insult elicited by 3-NP (Fig. 3). However, in QA plus 3-NP model, Probucol (10 and 30 μM) completely preserved the mitochondrial function showed a potent neuroprotective activity, managing to restore the mitochondrial function (Fig. 3).

3.2. ROS production and lipid peroxidation elicited by QA, 3-NP or QA + 3-NP and protective effect of Probucol

Considering that mitochondrial dysfunction and ROS generation are closely related phenomena, which also can contribute to increased lipid peroxidation, ROS levels were investigated in QA- and/or 3-NP-exposed slices. ROS generation was significantly increased in the striatal slices exposed to 1 mM QA, 1 mM 3-NP or 0.1 mM of both compounds (Fig. 4A). As expected, Probucol, which presents scavenger activity, completely prevented the QA, 3-NP and QA plus 3-NP-induced ROS formation in the slices analyzed (Fig. 4A).

Lipid peroxidation, which represents a consequence of increased ROS formation, was assessed as an index of oxidative damage to lipids. The statistical analysis revealed a significant increased of lipid peroxidation by all toxic conditions (1 mM QA, 1 mM 3-NP, or 0.1 mM QA plus 0.1 mM 3-NP) in striatal slices (Fig. 4B). The lipoperoxidative effects induced by either QA, 3-NP or QA plus 3-NP were completely blocked by Probucol 10 and 30 μM (Fig. 4B).

3.3. Protective effect of sodium succinate and MK-801 against QA, 3-NP and QA plus 3-NP-induced mitochondrial dysfunction

Several studies have demonstrated that antioxidant compounds are able to protect against the neurotoxicity elicited by QA, 3-NP and QA plus 3-NP models. However, recently, the particular interest in characterizing the protective properties of energy precursor agents against the toxic insult with 3-NP has been increased [3,30,40,76]. In this study, we used sodium succinate as an energy precursor and its capacity to restore the mitochondrial function was investigate in an attempt to understand the mechanism of damage induced by the toxins and the potential contribution of the energetic metabolism disruption in either QA, 3-NP or QA plus 3-NP-induced toxicity. Sodium succinate (1 mM) was unable to recover the mitochondrial dysfunction induced by QA and QA plus 3-NP treatments (Fig. 5A). On the other hand, sodium succinate effectively protected striatal slices against 3-NP-induced mitochondrial dysfunction ($p < 0.05$, Fig. 5A).

In addition, mitochondrial dysfunction and ROS generation can trigger excitotoxicity and induce massive entry of calcium ions (Ca^{2+}) from the extracellular environment, prompting the activation of cell death pathways [17,72]. Furthermore, QA stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes [69], which could lead to excitotoxic events. MK-801, a well-known NMDA antagonist, has protective action against QA insults [27]. However, studies on the potential protective effect of NMDA receptor antagonists in the combined model (QA plus 3-NP) are lacking in the literature. To further determine the contribution of the excitotoxic events linked with NMDA receptor activation in QA, 3-NP and QA plus 3-NP-induced damage, slices were incubated with the toxins in the absence or presence of MK-801, a non-competitive antagonist of NMDA receptor.

As already demonstrated, MK-801 protected against QA toxicity in striatal slices ($p < 0.001$, Fig. 5B). Interestingly, MK-801 also was effective in protecting the mitochondrial function disrupted by 3-NP and QA plus 3-NP in striatal slices ($p < 0.001$ and $p < 0.01$, Fig. 5B).

4. Discussion

The present study showed that QA (but not 3-NP)-induced mitochondrial dysfunction in striatal slices was prevented by Probucol, an antioxidant compound with scavenger properties in vitro. When

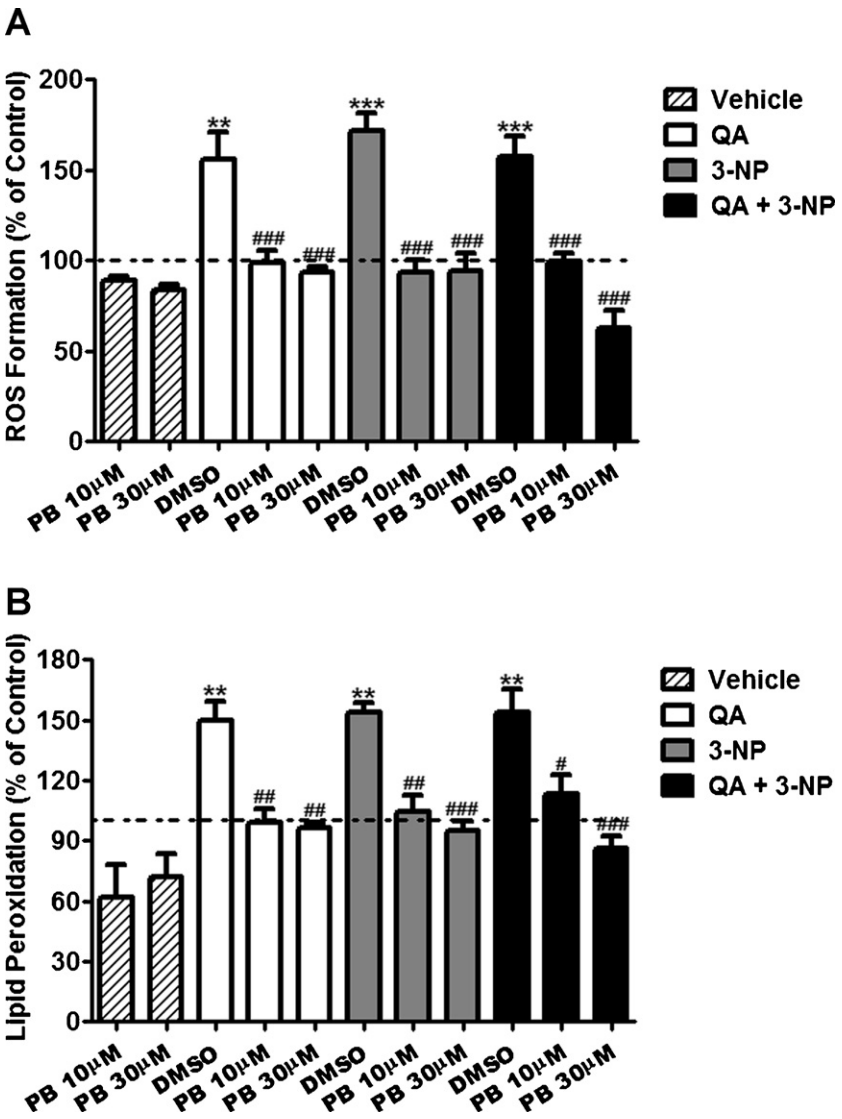

Fig. 4. Protective effect of Probucol against QA, 3-NP or QA plus 3-NP induced ROS formation and lipid peroxidation. Striatal slices were incubated with 1 mM QA, 1 mM 3-NP, 0.1 mM QA plus 0.1 mM 3-NP or vehicle at 37 °C for 2 h in KRB. After this period, the medium was replaced for fresh culture medium without QA and/or 3-NP and the slices were maintained for additional 4 h. PB (10 and 30 μM) or vehicle were co-incubated with the toxins and re-add in the culture medium during the second incubation. Formation of ROS was estimated with the fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) using excitation and emission wavelengths of 480 and 525 nm, respectively. ROS levels (expressed as nmol of oxidized DCF per mg protein) are expressed as percent of control (dotted line) (A). Lipid peroxidation (LP) was assessed in homogenates obtained from the slices by TBARS formation and expressed as nmol of MDA per mg of protein. Results are expressed as percent of MDA formed vs. the control values (dotted line) (B). Data are represented as mean ± SEM ($n = 5$). $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistical difference from control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate statistical difference from 3NP by one-way ANOVA, following by Tukey's post hoc test.

QA and 3-NP were simultaneously present at concentrations that are sub-toxic when incubated individually (0.1 mM), a synergistic mitochondrial toxicity was observed, which was totally protected by Probucol. These results contribute to the understanding on molecular mechanisms mediating QA- and/or 3-NP-induced mitochondrial dysfunction, corroborating previous data pointing to excitotoxicity, oxidative stress and energetic deficit as important events mediating the toxicity [24,53]. In addition, the presented results add new insights on the involvement of ROS in the toxic effects of QA and 3-NP to mitochondria, indicating that the blockade of QA-induced ROS is enough to significantly blunt the decreased mitochondrial dysfunction, although this event was not observed for 3-NP.

Probucol prevented ROS formation and lipid peroxidation in all used models, but did not protect against the mitochondrial dysfunction induced by 3-NP (only by QA or QA plus 3-NP). This data indicates that QA and 3-NP might share similar damage mechanisms (ROS formation), but also may have different mechanisms. Although the predominant hypothesis on QA toxicity is oriented to sustained NMDAr overactivation and excitotoxicity [66], further leading to cell damage produced by enhanced levels of Ca^{2+} [41,63], evidence showed that QA is also able to produce damage via ROS production [33,67] and/or alterations in energy metabolism [28,62]. In this study, we demonstrated that QA was able to induced mitochondrial dysfunction in striatal slices, but this effect may be secondary to excitotoxicity, calcium influx and ROS production. Interestingly, Probucol prevented mitochondrial dysfunction probably by avoiding the secondary effect (energetic deficit) due to the blockade of the primary event (ROS production). Thus, when QA-induced ROS production was prevented by Probucol, there was protection against mitochondrial dysfunction. These results indicate that ROS production and oxidative stress played an important role of QA mediating deleterious effects in striatal slices (mitochondrial dysfunction) and that the antioxidant and

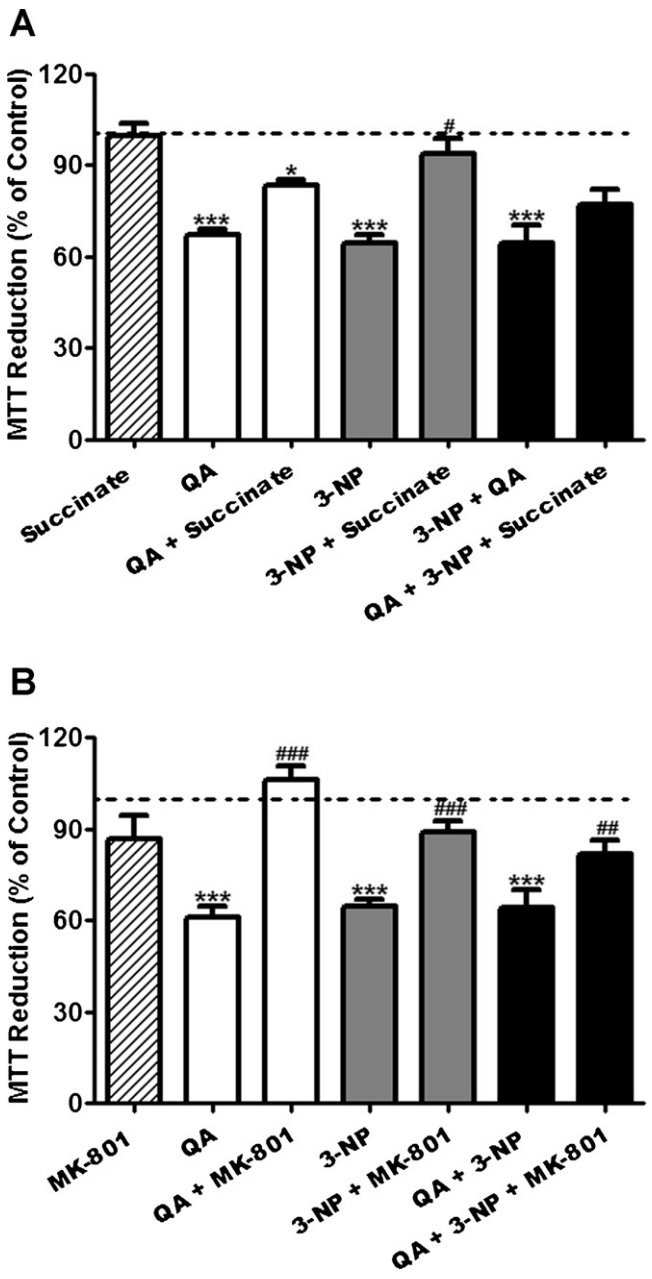

Fig. 5. Protective effect of sodium succinate and MK-801 against QA, 3-NP and QA plus 3-NP-induced mitochondrial dysfunction. Striatal slices were incubated with 1 mM QA, 1 mM 3-NP, 0.1 mM QA plus 0.1 mM 3-NP or vehicle at 37 °C for 2 h in KRB. After this period, the medium was replaced for fresh culture medium without QA and/or 3-NP and the slices were maintained for additional 4 h. Sodium succinate (1 mM) (A) or MK-801 (50 μM) (B) were co-incubated with the toxins and re-add in the slices medium during the second incubation. Mitochondrial viability was evaluated by the MTT reduction method. Results are expressed as the percentage of MTT reduction with respect to control values (dotted line). Data are represented as mean ± SEM ($n = 5$). $^{*}p < 0.05$ and $^{***}p < 0.001$ indicate statistical difference from control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate statistical difference from QA, 3NP or QA plus 3NP by one-way ANOVA, following by Tukey's post hoc test.

scavengers properties of Probucol were important in counteracting these effects. This idea is supported by other studies reporting the involvement of oxidative stress induced by QA [33]. In this regard, the toxicity induced by QA in striatal slices could be linked not just to NMDAr overactivation and excitotoxicity, but also to ROS production-inducing mitochondrial dysfunction.

On the other hand, Probucol, which diminished ROS production and lipid peroxidation induced by 3-NP, did not protect against the mitochondrial dysfunction induced by this toxin. The scavenger property of Probucol protected only against QA probably because it was not able to modulate a direct energetic deficit induced by 3-NP. 3-NP is a suicide inactivator of the mitochondrial Complex II, directly leading to mitochondrial dysfunction [23], decreased ATP levels, membrane depolarization and ROS formation [34,36,44]. Thus, 3-NP-induced ROS formation is a consequence of mitochondrial dysfunction and despite Probucol antioxidant effects, mitochondrial function was not re-established. Thus, this work demonstrated a crucial effect of ROS in mitochondrial dysfunction induced by QA, but also demonstrated that 3-NP-induced ROS production is not the only responsible by mitochondrial dysfunction in this model. Noteworthy, the novelty of this study does not contradict previous data from literature, which show that ROS, excitotoxicity and energetic deficit are mechanisms modulating the toxicity in all the models [24,53].

However, the temporal profile of primary and secondary events seems to be important in these models. This idea was better understood by using MK-801 (an NMDAr antagonist) and succinate (an energy precursor agent). MK-801, which prevents excitotoxicity, also prevented QA, 3-NP and QA plus 3-NP-induced mitochondrial dysfunction. These findings indicate that 3-NP also can induce excitotoxic events. However, the incubation of striatal slices with succinate, an energetic precursor, was able to prevent against 3-NP-induced mitochondrial dysfunction, but did not protect against QA effects. Interesting, succinate also did not protect against QA plus 3-NP. These findings demonstrate the involvement of primary and secondary events in these models. Although oxidative stress, energetic deficit and excitotoxicity represent important events in the models (QA, 3-NP and QA plus 3-NP), their sequences are likely different depending upon the specific model. On the other hand, the prevention of excitotoxicity-induced by 3-NP (by using MK-801) was able to prevent mitochondrial dysfunction. This data prove a critical role of excitotoxicity in mitochondrial dysfunction induced by 3-NP model alone or in association with QA. Studies demonstrated that 3-NP can induce excitotoxicity [46,51]. The hypothesis of an indirect or "secondary" excitotoxicity suggests that 3-NP-induced striatal degeneration is due in first place to depletion in ATP levels produced by a deficit in energy metabolism, further leading to membrane depolarization and sustained voltage-gated NMDAr activation by primary alteration of membrane Na^+, K^+-ATPases [1,5,46,51]. Under these conditions, 3-NP is able to cause excitotoxicity by making neurons vulnerable to endogenous basal levels of glutamate [51], producing neuronal necrotic death [6,48].

Liot et al. [38] showed that 3-NP induced the activation of NMDAr in neuronal cells, leading to ROS formation, as well as a significant mitochondrial fragmentation and cell death [38]. Remarkably, pretreatment with AP5, a glutamate receptor antagonist blocked the 3-NP-induced ROS formation, mitochondrial fragmentation, and neuronal cell death [38]. This study provides evidence that secondary excitotoxicity (caused by primary complex II inhibition) may play an important role in 3-NP-induced cell death. This is in accordance with our study, which indicates that MK-801 was able to protect striatal slices from 3-NP-induced mitochondrial toxicity. The protective effect of MK-801 against 3-NP toxicity may indicate a secondary excitotoxicity with involvement of NMDAr activation. This is in accordance with other studies indicating the involvement of glutamate receptor activation in 3-NP-induced cell death [5,9,13,19]. The results also suggest that 3-NP-induced damage may be partially glutamate receptor-mediated because the energy deficiency induced by this toxin might lead to increases in glutamate release, cellular depolarization, activation of NMDA receptors, and increases in damaging calcium cascades [15,21,38].

Our results also showed that the simultaneous exposure to subtoxic concentrations of QA plus 3-NP (which cannot induce

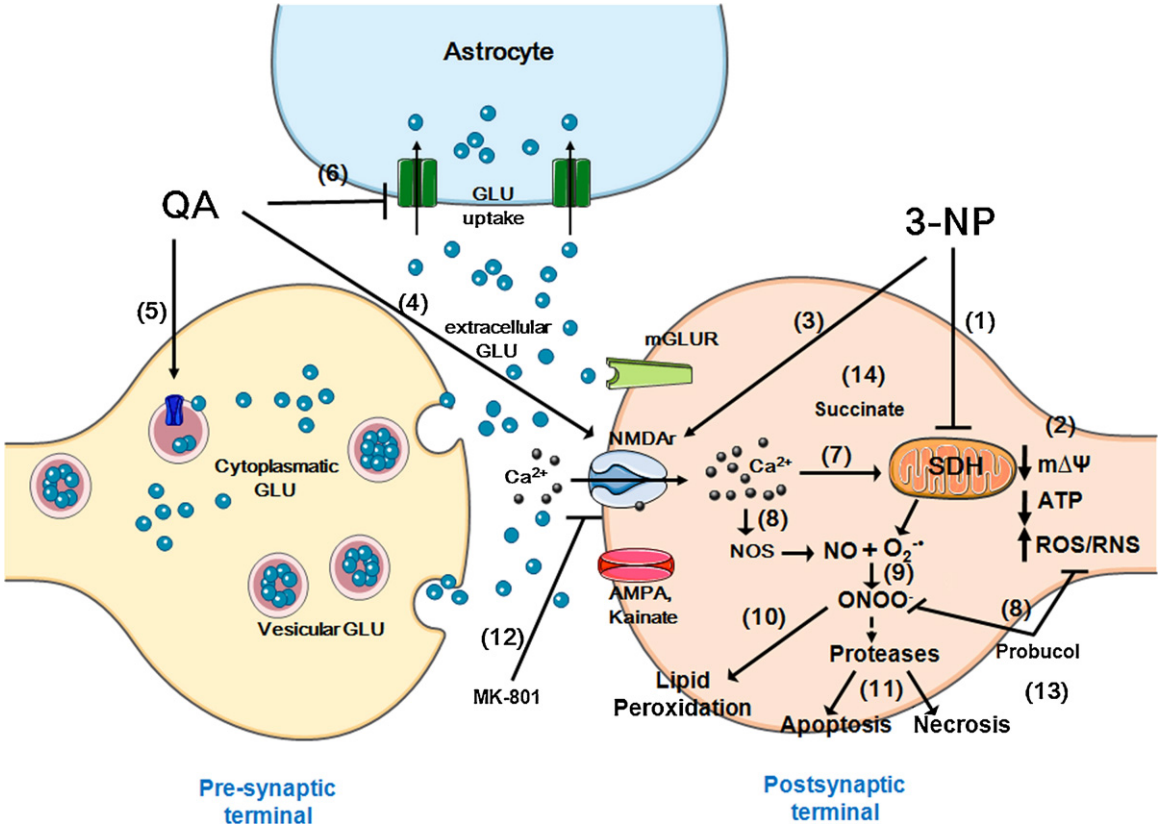

Fig. 6. Schematic representation of the mechanisms of QA plus 3NP-induced toxicity. Low concentrations of 3-NP primarily induce a moderate mitochondrial respiratory complex II inhibition (SDH) (event 1), which in turn triggers ATP drop, decrease in mitochondrial membrane potential ($m\Delta\Psi$) and massive production of ROS/RNS (event 2). The energy deficit makes neurons more vulnerable to endogenous basal levels of glutamate ("secondary" excitotoxicity). This scenario leads to plasma membrane depolarization, which may release the Mg^{2+} blockade of voltage-gated NMDAr (event 3). Opening of NMDAr causes intracellular Ca^{2+} influx. On the other hand, QA induce a moderate activation NMDAr (event 4), thus causing increased intracellular Ca^{2+} concentrations. In addition, QA also stimulates synaptosomal glutamate release (event 5) and affects glutamate re-uptake into astrocytes (event 6), and so increasing extracellular concentrations of glutamate. Increased levels of intracellular calcium, which can directly lead to mitochondrial dysfunction (event 7), might activate nitric oxide synthase (NOS) (event 8) thus increasing nitric oxide (NO) formation. NO can combine with O^{2-} to form $ONOO^-$ (event 9). In addition, ROS/RNS generation might induce lipid peroxidation (event 10). Altogether, these events lead to proteases activation, thus inducing cell death by necrosis and/or apoptosis (event 11). The blockade of NMDAr with MK-801 (event 12) and the scavenging activity of Probucol toward ROS/RNS (event 13) can block mitochondrial dysfunction and neuronal cell death. Succinate, as an energetic precursor, may mitigate mitochondrial dysfunction induced by 3-NP (event 14).

mitochondrial damage alone) induced mitochondrial dysfunction and oxidative stress in a synergistic manner. In fact, when subtoxic concentrations of QA (0.1 mM) and 3-NP (0.1 mM) were combined, there was a significant decrease in the mitochondrial viability in striatal slices, as well as increased in ROS levels and lipid peroxidation, showing the involvement of oxidative stress in the impaired of mitochondrial function induced by this mixed exposure. Interestingly, Probucol (by scavenging ROS) prevented mitochondrial toxicity in the mixed model. Probucol was able to protect striatal slices against QA plus 3-NP-inducing mitochondrial dysfunction, suggesting that oxidative stress played an important role in mediating the deleterious effects of QA plus 3-NP. In addition, MK-801 also provides protection against QA plus 3NP. This is in accordance with Pérez-De La Cruz and coworkers, who reported a reduction of lipid peroxidation by MK-801 in QA plus 3-NP-treated synaptosomal membranes [52]. On the other hand, succinate did not protect striatal slices against mitochondrial dysfunction induced by QA plus 3-NP. Conversely, MK-801 effectively blunted the mitochondrial toxicity induced by the association of both toxins. Altogether, these findings suggest that a cascade of toxic events related with NMDAr overactivation may play a relevant role for cell damage following the toxic insult that involves deficit in energy metabolism and excitotoxicity when QA and 3-NP are associated.

As already mentioned, we found a synergistic toxicity of QA and 3-NP, which produced oxidative damage to striatal slices. Based on literature data [24,51,53,70] and on our current findings, Fig. 6 depicts the main molecular mechanisms of QA plus 3NP-induced toxicity. The primary mechanism mediating such damage may involve a moderate energy metabolism deficit induced by SDH inhibition by 3-NP. The energy metabolism dysfunction makes neuronal cells more vulnerable to be damage by physiologic glutamate levels ("secondary" excitotoxicity) [51]. In addition, QA induces a moderate activation NMDAr, thus likely causing increased intracellular Ca^{2+} concentrations, which in turn can lead to major alterations in synaptic and mitochondrial functions, generation of ROS and RNS and activation of cell death pathways [51]. 3-NP also induces ROS and RNS formation, as well as increases in intracellular Ca^{2+} levels and further activation of proteases [70]. Altogether, these events might cause neuronal cell death (either necrotic or apoptotic) (Fig. 6).

In summary, the findings of this study show that the two studied toxic models (QA and 3-NP) or the combined model (QA plus 3-NP) can generate complex patterns of damage, which involve metabolic compromise, ROS formation, and oxidative stress. These neurotoxic models share common mechanisms of cell damage, despite each model recruits these processes in a differential manner: QA by NMDAr activation and 3-NP by SDH inhibition. These events were counteracted by Probucol, an antioxidant compound with scavenger properties under in vitro conditions. Moreover, a partial inhibition of SDH by subtoxic 3-NP and moderate excitotoxicty by subtoxic QA are potentiated when both agents are associated.

The toxic action of QA plus 3-NP seems to involve changes in Ca^{2+} metabolism and ROS/RNS formation, and can be prevented or attenuated by antioxidant/scavenger compounds and NMDAr antagonists. Therefore, oxidative stress remains as a major expression in these toxic models, as well as a potential key target to ameliorate neuronal damage in HD patients.

Disclosures

The authors report no declarations of interest.

Acknowledgements

The financial supports by (i) FINEP Research Grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00, (ii) Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), (iii) Fundação de Apoio à Pesquisa do Estado de Santa Catarina (FAPESC - TO 1346/2010-3), (iv) Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and (v) INCT-CNPq-Excitotoxicity and Neuroprotection are gratefully acknowledged. M.F. and F.A.A.S. recipient of CNPq fellowship.

References

[1] R.L. Albin, J.T. Greenamyre, Alternative excitotoxic hypotheses, Neurology 42 (4) (1992) 733–738.
[2] S.F. Ali, C.P. LeBel, S.C. Bondy, Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity, Neurotoxicology 13 (1992) 637–648.
[3] O.A. Andreassen, A. Dedeojlu, R.J. Ferrante, B.G. Jenkins, M. Thomas, Creatine increases survival and delays motor symptoms in a transgenic animal model of Huntington's disease, Neurobiol. Dis. 8 (2001) 479–491.
[4] D.S. Avila, P. Gubert, A. Palma, D. Colle, D. Alves, C.W. Nogueira, J.B. Rocha, F.A. Soares, An organotellurium compound with antioxidant activity against excitotoxic agents without neurotoxic effects in brain of rats, Brain Res. Bull. 76 (2008) 114–123.
[5] M.F. Beal, B.T. Hyman, W. Koroshetz, Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases, Trends Neurosci. 16 (1993) 125–131.
[6] M.I. Behrens, J. Koh, L.M. Canzoniero, S.L. Sensi, C.A. Csernansky, D.W. Choi, 3-Nitropropionic acid induces apoptosis in cultured striatal and cortical neurons, Neuroreport 6 (1995) 545–548.
[7] P.F. Behrens, P. Franz, B. Woodman, K.S. Lindenberg, G.B. Landwehrmeyer, Impaired glutamate transport and glutamate-glutamine cycling: downstream effects of the Huntington mutation, Brain 125 (2002) 1908–1922.
[8] A. Benchoua, Y.I. Trioulier, D. Zala, M.C. Gaillard, N. Lefort, N. Dufour, F. Saudou, J.M. Elalouf, E. Hirsch, P. Hantraye, N. Déglon, E. Brouillet, Involvement of mitochondrial complex II defects in neuronal death produced by N-terminus fragment of mutated huntingtin, Mol. Biol. Cell 17 (2006) 1652–1663.
[9] E. Brouillet, F. Conde, M.F. Beal, P. Hantraye, Replicating Huntington's disease phenotype in experimental animals, Prog. Neurobiol. 59 (1999) 427–468.
[10] S.E. Browne, M.F. Beal, Oxidative damage in Huntington's disease pathogenesis, Antioxid. Redox Signal. 8 (2006) 2061–2073.
[11] M.M. Buckley, K.L. Goa, A.H. Price, R.N. Brogden, Probucol. A reappraisal of its pharmacological properties and therapeutic use in hypercholesterolaemia, Drugs 37 (1989) 761–800.
[12] C. Chen, Mitochondrial dysfunction, metabolic deficits, and increased oxidative stress in Huntington's disease, Chang Gung Med. J. 34 (2011) 135–152.
[13] D.W. Choi, Cerebral hypoxia: some new approaches and unanswered questions, J. Neurosci. 10 (1990) 2493–2501.
[14] P. Del Río, L. Massieu, Mild mitochondrial inhibition in vivo enhances glutamate-induced neuronal damage through calpain but not caspase activation: role of ionotropic glutamate receptors, Exp. Neurol. 212 (2008) 179–188.
[15] P. Del Río, T. Montinel, V. Chagoya, L. Massieu, Exacebation of excitotoxic neuronal death induced during mitochondrial inhibition in vivo: relation to energy imbalance or ATP depletion? Neuroscience 146 (2007) 1561–1570.
[16] D. Elinos-Calderón, Y. Robledo-Arratia, V. Pérez-De La Cruz, P.D. Maldonado, S. Galván-Arzate, J. Pedraza-Chaverrí, A. Santamaría, Antioxidant strategy to rescue synaptosomes from oxidative damage and energy failure in neurotoxic models in rats: protective role of S-allylcysteine, J. Neural Transm. 117 (1) (2010) 35–44.
[17] G. Ermak, K.J.A. Davies, Calcium and oxidative stress: from cell signaling to cell death, Mol. Immunol. 38 (2001) 713–721.
[18] M. Farina, F. Campos, I. Vendrell, J. Berenguer, M. Barzi, S. Pons, C. Suñol, Probucol increases glutathione peroxidase-1 activity and displays long-lasting protection against methylmercury toxicity in cerebellar granule cells, Toxicol. Sci. 112 (2) (2009) 416–426.
[19] S.L. Fink, D.Y. HO, R.M. Sapolsky, Energy and glutamate dependency of 3-nitropropionic acid neurotoxicity in culture, Exp. Neurol. 138 (1996) 298–304.
[20] C. Giampà, S. Patassini, A. Borreca, D. Laurenti, F. Marullo, G. Bernardi, F.S. Menniti, F.R. Fusco, Phosphodiesterase 10 inhibition reduces striatal excitotoxicity in the quinolinic acid model of Huntington's disease, Neurobiol. Dis. 34 (2009) 450–456.
[21] J.G. Greene, S.S. Sheu, R.A. Gross, J.T. Greenamyre, 3-Nitropropionic acid exacerbates N-methyl-D-aspartate toxicity in striatal culture by multiple mechanisms, Neuroscience 84 (1998) 503–510.
[22] P. Guidetti, R.E. Luthi-Carter, S.J. Augood, R. Schwarcza, Neostriatal and cortical quinolinate levels are increased in early grade Huntington's disease, Neurobiol. Dis. 17 (2004) 455–461.
[23] L. Huang, G. Sun, D. Cobessi, A. Wang, J.T. Shen, E.Y. Tung, V.E. Anderson, E.A. Berry, 3-Nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme, J. Biol. Chem. 281 (9) (2006) 5965–5972.
[24] C. Jacquard, Y. Trioulier, F. Cosker, C. Escartin, N. Bizat, P. Hantraye, J.M. Cancela, G. Bonvento, E. Brouillet, Brain mitochondrial defects amplify intracellular [Ca^{2+}] rise and neurodegeneration but not Ca^{2+} entry during NMDA receptor activation, FASEB J. 20 (2006) 1021–1023.
[25] S. Jang, H.S. Jeong, J.S. Parka, Y.S. Kim, C.Y. Jin, M.B. Seol, B.C. Kim, M.C. Lee, Neuroprotective effects of (−)-epigallocatechin-3-gallate against quinolinic acid-induced excitotoxicity via PI3K pathway and NO inhibition, Brain Res. 1313 (2010) 25–33.
[26] H. Kalonia, A. Kumar, Suppressing inflammatory cascade by cyclo-oxygenase inhibitors attenuates quinolinic acid induced Huntington's disease-like alterations in rats, Life Sci. 88 (2011) 784–791.
[27] H. Kalonia, P. Kumar, B. Nehru, A. Kumar, Neuroprotective effect of MK-801 against intra-striatal quinolinic acid induced behavioral, oxidative stress and cellular alterations in rats, Indian J. Exp. Biol. 47 (11) (2009) 880–892.
[28] H. Kalonia, P. Kumar, A. Kumar, B. Nehru, Protective effect of rofecoxib and nimesulide against intra-striatal quinolinic acid-induced behavioral, oxidative stress and mitochondrial dysfunctions in rats, Neurotoxicology 31 (2010) 195–203.
[29] H. Kalonia, P. Kumar, A. Kumar, Attenuation of proinflammatory cytokines and apoptotic process by verapamil and diltiazem against quinolinic acid induced Huntington like alterations in rats, Brain Res. 1372 (2011) 115–126.
[30] S. Kasparová, Z. Sumbalová, P. Bystrický, J. Kucharská, T. Liptaj, V. Mlynárik, A. Gvozdjáková, Effect of coenzyme Q10 and vitamin E on brain energy metabolism in the animal model of Huntington's disease, Neurochem. Int. 48 (2) (2005) 93–99.
[31] P. Kumar, A. Kumar, Possible role of sertraline against 3-nitropropionic acid induced behavioral, oxidative stress and mitochondrial dysfunction in rat brain, Prog. Neuropsycopharmacol. Prog. Neuropsychopharmacol. Biol. Psych. 33 (1) (2009) 100–108.
[32] P. Kumar, S.S.V. Padis, P.S. Naidu, A. Kumar, Cyclooxygenase inhibition attenuates 3-nitropropionic acid-induced neurotoxicity in rats: possible antioxidant mechanisms, Fundam. Clin. Pharmacol. 21 (2007) 297–306.
[33] P. Kumar, H. Kalonia, A. Kumar, Huntington's disease: pathogenesis to animal models, Pharmacol. Rep. 62 (2010) 1–14.
[34] P. Kumar, H. Kalonia, A. Kumar, Role of LOX/COX pathways in 3-nitropropionic acid-induced Huntington's disease-like symptoms in rats: protective effect of licofenole, Br. J. Pharmacol. 162 (2b) (2011) 644–654.
[35] W.T. Lee, C. Chang, Magnetic resonance imaging and spectroscopy in assessing 3-nitropropionic acid-induced brain lesions: an animal model of Huntington's disease, Prog. Neurobiol. 72 (2004) 87–110.
[36] W.T. Lee, H.S. Yin, Y.Z. Shen, The mechanisms of neuronal death produced by mitochondrial toxin 3-nitropropionic acid: the roles of N-methyl-D-aspartate glutamate receptors and mitochondrial calcium overload, Neuroscience 112 (2002) 707–716.
[37] X.J. Li, S. Li, Proteasomal dysfunction in aging and Huntington disease, Neurobiol. Dis. 43 (2011) 4–8.
[38] G. Liot, B. Bossy, S. Lubitz, Y. Kushnareva, N. Sejbuk, E. Bossy-Wetzel, Complex II inhibition by 3-NP causes mitochondrial fragmentation and neuronal cell death via an NMDA- and ROS-dependent pathway, Cell Death Differ. 16 (2009) 899–909.
[39] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[40] L. Massieu, P. Del Río, T. Montiel, Neurotoxicity of glutamate uptake inhibition *in vivo*: correlation with succinate dehydrogenase activity and prevention by energy substrates, Neuroscience 106 (4) (2001) 669–677.
[41] E. Matyja, Intracellular calcium overload in a model of quinolinic acid neurotoxicity in organotypic culture of rat hippocampus; inhibited by nimodipine, Folia Neuropathol. 35 (1997) 8–17.
[42] F. Mochel, R.G. Haller, Energy deficit in Huntington disease: why it matters, J. Clin. Invest. 121 (2) (2011) 493–499.
[43] S. Molz, H. Decker, T. Dal-Cim, C. Cremonez, F.M. Cordova, R.B. Leal, C.I. Tasca, Glutamate-induced toxicity in hippocampal slices involves apoptotic features and $p38^{MAPK}$ signaling, Neurochem. Res. 33 (2008) 27–36.
[44] P. Montilla, I. Túnez, M.C. Muñoz, M. Salcedo, M. Feijóo, J.R. Muñoz-Castañeda, I. Bujalance, Effect of glucocorticoids on 3-nitropropionic acid-induced oxidative stressing synaptosomes, Eur. J. Pharmacol. 488 (2004) 19–25.
[45] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1-2) (1983) 55–63.

[46] A. Novelli, J.A. Reilly, P.G. Lysko, R.C. Henneberry, Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced, Brain Res. 451 (1988) 205–212.
[47] I.J.L. Oliveira, S. Molz, D.O. Souza, C.I. Tasca, Neuroprotective effect of GMP in hippocampal slices submitted to an *in vitro* model of ischemia, Cell. Mol. Neurobiol. 22 (3) (2002) 335–344.
[48] Z. Pang, J.W. Geddes, Mechanisms of cell death induced by the mitochondrial toxin 3-nitropropionic acid: acute excitotoxic necrosis and delayed apoptosis, J. Neurosci. 7 (1997) 3064–3073.
[49] S.Y. Park, J.H. Lee, C.D. Kim, B.Y. Rhim, K.W. Hong, W.S. Lee, Beneficial synergistic effects of concurrent treatment with cilostazol and probucol against focal cerebral ischemic injury in rats, Brain Res. 1157 (2007) 112–120.
[50] R.P. Pereira, R. Fachinetto, A. de Souza Prestes, R.L. Puntel, G.N. Santos da Silva, B.M. Heinzmann, T.K. Boschetti, M.L. Athayde, M.E. Bürger, A.F. Morel, V.M. Morsch, J.B. Rocha, Antioxidant effects of different extracts from *Melissa officinalis*, *Matricaria recutita* and *Cymbopogon citratus*, Neurochem. Res. 34 (5) (2009) 973–983.
[51] V. Pérez-De La Cruz, A. Santamaria, Integrative hypothesis for Huntington's Disease: a brief review of experimental evidence, Physiol. Res. 56 (2007) 513–526.
[52] V. Pérez-De La Cruz, M. Konigsberg, J. Pedraza-Chaverri, N. Herrera-Mundo, M. Díaz-Muñoz, J. Morán, T. Fortoul-van der Goes, A. Rondán-Zárate, P.D. Maldonado, S.F. Ali, A. Santamaría, Cytoplasmic calcium mediates oxidative damage in an excitotoxic/energetic deficit synergic model in rats, Eur. J. Neurosci. 27 (2008) 1075–1085.
[53] V. Pérez-De La Cruz, D. Elinos-Calderón, P. Carrillo-Mora, D. Silva-Adaya, M. Konigsberg, J. Morán, S.F. Ali, M.E. Chánez-Cárdenas, G. Pérez-De La Cruz, A. Santamaría, Time-course correlation of early toxic events in three models of striatal damage: modulation by proteases inhibition, Neurochem. Int. 56 (2010) 834–842.
[54] R.L. Puntel, C.W. Nogueira, J.B. Rocha, N-methyl-D-aspartate receptors are involved in the quinolinic acid, but not in the malonate pro-oxidative activity in vitro, Neurochem. Res. 30 (3) (2005) 417–424.
[55] S. Ramaswamy, J.L. McBride, J.H. Kordower, Animal models of Huntington's disease, ILAR J. 48 (4) (2007) 356–373.
[56] L. Rekik, V. Daguin-Nerrière, J. Petit, P. Brachet, 3γ-Aminobutyric acid type B receptor changes in the rat striatum and substantia nigra following intrastriatal quinolinic acid lesions, J. Neurosci. Res. 89 (2011) 524–535.
[57] C. Ríos, A. Santamaría, Quinolinic acid is a potent lipid peroxidant in rat brain homogenates, Neurochem. Res. 16 (1991) 1139–1143.
[58] R.A.C. Roos, Huntington's disease: a clinical review, Orphanet J. Rare Dis. 5 (40) (2010) 1–8.
[59] C.A. Ross, S.J. Tabrizi, Huntington's disease: from molecular pathogenesis to clinical treatment, Lancet Neurol. 10 (2011) 83–98.
[60] J. Rossignol, C. Boyer, X. Lévèque, K.D. Fink, R. Thinard, F. Blanchard, G.L. Dunbar, L. Lescaudron, Mesenchymal stem cell transplantation and DMEM administration in a 3NP rat model of Huntington's disease: morphological and behavioral outcomes, Behav. Brain Res. 217 (2011) 369–378.
[61] A. Santamaría, S. Galván-Arzate, V. Lisy, S.F. Ali, H.M. Duhart, L. Osorio-Rico, C. Rios, F. St'Astny, Quinolinic acid induces oxidative stress in rat brain synaptosomes, Neuroreport 12 (2001) 871–874.
[62] P.F. Schuck, A. Tonin, G.C. Ferreira, R.B. Rosa, A. Latini, F. Balestro, M.L.S. Perry, C.M.D. Wannmacher, A.T.S. Wyse, M. Wajner, In vitro effect of quinolinic acid on energy metabolism in brain of young rats, Neurosci. Res. 57 (2007) 277–288.
[63] R. Schwarcz, W.O.J.R. Whetsell, R.M. Mangano, Quinolinic acid: an endogenous metabolite that produces axon-sparing lesions in rat brain, Science 219 (1983) 316–319.
[64] D. Silva-Adaya, V. Pérez-De La Cruz, M.N. Herrera-Mundo, K. Mendoza-Macedo, J. Villeda-Hernández, Z. Binienda, S.F. Ali, A. Santamaría, Excitotoxic damage, disrupted energy metabolism and oxidative stress in the rat brain: antioxidant and neuroprotective effects of L-carnitine, J. Neurochem. 105 (2008) 677–689.
[65] A.L. Southwell, P.H. Patterson, Gene therapy in mouse models of Huntington disease, Neuroscientist 17 (2) (2011) 153–162.
[66] T.W. Stone, M.N. Perkins, Quinolinic acid: a potent endogenous excitant at amino acid receptors in CNS, Eur. J. Pharmacol. 72 (1981) 411–412.
[67] T.W. Stone, W.M. Behan, M. MacDonald, L.G. Darlington, Possible mediation of quinolinic acid-induced hippocampal damage by reactive oxygen species, Amino Acids 19 (1) (2000) 275–281.
[68] I. Tasset, V. Pérez-De La Cruz, D. Elinos-Calderón, P. Carrillo-Mora, I.G. González-Herrera, A. Luna-López, M. Konigsberg, J. Pedraza-Chaverrí, P. Maldonado, S.F. Ali, I. Túnez, A. Santamaría, Protective effect of tert-butylhydroquinone on the quinolinic-acid-induced toxicity in rat striatal slices: role of the Nrf2-antioxidant response element pathway, Neurosignals 18 (1) (2010) 24–31.
[69] R.G. Tavares, C.I. Tasca, C.E.S. Santos, L.B. Alves, L.O. Porciúncula, T. Emanuelli, D.O. Souza, Quinolinic acid stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes, Neurochem. Int. 40 (2002) 621–627.
[70] I. Túnez, I. Tasset, V. Pérez-De La Cruz, A. Santamaría, 3-Nitropropionic acid as a tool to study the mechanisms involved in Huntington's disease: past, present and future, Molecules 15 (2010) 878–916.
[71] E. Vamos, A. Pardutz, P. Klivenyi, J. Toldi, L. Vecsei, The role of kynurenines in disorders of the central nervous system: possibilities for neuroprotection, J. Neurol. Sci. 283 (2009) 21–27.
[72] S. Wang, E.K. Michaelis, Selective neuronal vulnerability to oxidative stress in the brain, Front. Aging Neurosci. 2 (2010) 1–13.
[73] P.K. Witting, B.J. Wu, M. Raftery, P. Southwell-Keely, R. Stocker, Probucol protects against hypochlorite-induced endothelial dysfunction: indentification of a novel pathway of probucol oxidation to a biologically active intermediate, J. Biol. Chem. 280 (16) (2005) 15612–15618.
[74] S. Yamashita, Y. Matsuzawa, Where are we with probucol: a new life for an old drug? Atherosclerosis 207 (2009) 16–23.
[75] S. Yamashita, H. Hbujo, H. Arai, M. Harada-Shiba, S. Matsui, M. Fukushima, Y. Saito, T. Kita, Y. Matsuzawa, Long-term probucol treatment prevents secondary cardiovascular events: a cohort study of patients with heterozygous familial hypercholesterolemia in Japan, J. Atheroscler. Thromb. 15 (6) (2008) 292–303.
[76] L. Yang, N.Y. Calingasan, E.J. Wille, K. Cormier, K. Smith, R.J. Ferrante, M.F. Beal, Combination therapy with coenzyme Q10 and creatine produces additive neuroprotective effects in models of Parkinson's and Huntington's diseases, J. Neurochem. 109 (5) (2009) 1427–1439.
[77] D. Zadóri, P. Klivényi, E. Vámos, F. Fulo, J. Toldi, L. Vécsei, Kynurenines in chronic neurodegenerative disorders: future therapeutic strategies, J. Neural. Transm. 116 (2009) 1403–1409.