Ca^{2+}-induced oxidative stress in brain mitochondria treated with the respiratory chain inhibitor rotenone

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Received 28 February 2003; revised 4 April 2003; accepted 14 April 2003

First published online 29 April 2003

Edited by Vladimir Skulachev

Abstract In this study we show that micromolar Ca^{2+} concentrations (>10 μM) strongly stimulate the release of reactive oxygen species (ROS) in rotenone-treated isolated rat forebrain mitochondria. Ca^{2+}-stimulated mitochondrial ROS release was associated with membrane lipid peroxidation and was directly correlated with the degree of complex I inhibition by rotenone. On the other hand, Ca^{2+} did not increase mitochondrial ROS release in the presence of the complex I inhibitor 1-methyl-4-phenylpyridinium. Cyclosporin A had no effect on Ca^{2+}-stimulated mitochondrial ROS release in the presence of rotenone, indicating that mitochondrial permeability transition is not involved in this process. We hypothesized that Ca^{2+}-induced mitochondrial oxidative stress associated with partial inhibition of complex I may be an important factor in neuronal cell death observed in the neurodegenerative disorder Parkinson's disease.

Key words: Brain mitochondrion; Calcium; Free radical; Parkinson's disease; Rotenone

1. Introduction

Post mortem studies have shown evidence that oxidative stress and mitochondrial dysfunction are involved in the pathogenesis of idiopathic Parkinson's disease (PD). Indications of oxidative stress include depletion of reduced glutathione, iron accumulation and the presence of lipid, protein and DNA oxidation products [1–3]. Mitochondrial dysfunction is mainly suggested by a partial inhibition (20–40%) of respiratory chain complex I activity [4,5], present also in peripheral tissues [6,7].

Recently Betarbet and collaborators [8] showed that chronic treatment of rats with the mitochondrial complex I inhibitor rotenone results in selective nigrostriatal dopaminergic degeneration, including formation of Lewy bodies and cell loss. However, the concentration of rotenone found in the rat brains was insufficient to substantially inhibit mitochondrial respiration [8], indicating that a bioenergetic defect with ATP depletion could not explain the reported neurodegeneration. Under these conditions, an increased mitochondrial production of reactive oxygen species (ROS) secondary to partial inhibition of complex I could contribute to rotenone-induced nigrostriatal dopaminergic degeneration [9,10]. This experimental model for PD resembles the classical selective degeneration of dopaminergic neurons obtained by systemical treatment of primates and rodents with *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (for a review see [11]). MPTP toxicity for dopaminergic neurons occurs when it is converted to the active neurotoxin, 1-methyl-4-phenylpyridiniun ion (MPP^+), an inhibitor of mitochondrial respiratory chain complex I [11,12].

Glutamate receptor-mediated neuronal cell death – i.e. excitotoxicity – has been hypothesized to be involved in the pathogenesis of PD [13,14]. In excitotoxicity, Ca^{2+} influx to the cytosol is correlated with cellular toxicity [15,16]. In this paper, we study the effect of Ca^{2+} on rotenone-treated isolated rat forebrain mitochondria. The results indicate that micromolar Ca^{2+} concentrations induce mitochondrial oxidative stress in the presence of rotenone. The possible implications of this observation for the progressive neurodegeneration observed in PD are discussed.

2. Materials and methods

2.1. Mitochondrial isolation

Mitochondria were isolated by conventional differential centrifugation from forebrains of adult female Wistar strain rats as described by Rosenthal et al. [17]. Digitonin was used to disrupt synaptosomal membranes and release any mitochondria trapped within. The final pellet was resuspended in medium containing 225 mM mannitol, 75 mM sucrose, 5 mM K^+-HEPES pH 7.2 and 1 mg/ml bovine serum albumin, at an approximate protein concentration of 30–40 mg/ml. Rat liver and gastrocnemius muscle mitochondria were isolated by the same procedure described above for forebrain mitochondria, including the addition of digitonin to the second pellet.

2.2. Standard incubation procedure

The experiments were carried out at 28°C, with continuous magnetic stirring, in a standard reaction medium containing 100 mM sucrose, 65 mM KCl, 10 mM K^+-HEPES buffer (pH 7.2), 50 μM EGTA, 1 mM P_i, 2 mM Mg^{2+}, 5 mM malate, 10 mM pyruvate, 200 μM ATP and 1 μg/ml oligomycin. Other additions are indicated in the figure legends. The results shown are representative of a series of at least four experiments, using different mitochondrial preparations. The results were reproduced within 10% of variation.

2.3. Oxygen uptake measurements

Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, OH, USA) in 1.3 ml of standard reac-

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Abbreviations: DCF, dichlorofluorescein; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone; H_2-DCFDA, dichlorodihydrofluorescein diacetate; MPP^+, 1-methyl-4-phenylpyridinium; MPT, mitochondrial permeability transition; PD, Parkinson's disease; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00421-6

tion medium, in a sealed glass cuvette equipped with a magnetic stirrer.

2.4. Estimation of mitochondrial ROS release

Mitochondrial release of ROS (H_2O_2) was determined spectrofluorometrically, using the membrane-permeable fluorescent dye dichlorodihydrofluorescein diacetate (H_2-DCFDA; 1 μM) [18,19]. Fluorescence was determined at 488 nm for excitation and 525 nm for emission. Calibration was performed by adding known concentrations of dichlorofluorescein (DCF), the product of H_2-DCF oxidation. Alternatively ROS were measured using either 200 nM MitoTracker® Red CM-H_2XRos, at 579 nm for excitation and 599 nm for emission; 1 μM scopoletin plus 1 μM horseradish peroxidase, at 365 nm for excitation and 450 nm for emission; or 50 μM Amplex Red plus 0.025 μM horseradish peroxidase [20], at 563 nm for excitation and 587 nm for emission. The rate of mitochondrial H_2O_2 release detected under control conditions when using the horseradish peroxidase substrates scopoletin and Amplex Red was 42 ± 6 and 245 ± 26 pmol H_2O_2/min/mg, respectively.

2.5. Determination of thiobarbituric acid reactive substances (TBARS)

TBARS production in mitochondria was measured as described by Maciel et al. [19].

2.6. Materials

Most chemicals, including ATP, antimycin A, cyclosporin A, digitonin, HEPES, malic acid, MPP^+, myxothiazol, pyruvic acid, rotenone (R-8875, minimum 95%), scopoletin and thiobarbituric acid were obtained from Sigma Chemical Company (St. Louis, MO, USA). Amplex Red, H_2-DCFDA and MitoTracker Red CM-H_2XRos were purchased from Molecular Probes (Eugene, OR, USA). Rotenone was prepared at a stock concentration of 1 mM in 100% ethanol.

3. Results

Mitochondrial ROS release was measured using the membrane-permeable probe H_2-DCFDA, which is oxidized mainly by H_2O_2 and peroxynitrite [18,21] generating highly fluorescent DCF. The results in Fig. 1A show that the addition of Ca^{2+} to isolated rat brain mitochondria treated with rotenone strongly stimulates ROS detection (line d). A fast increase in the rate of DCF production was observed 3–5 min after Ca^{2+} addition. Rotenone alone did not increase the rate of DCF production (line b) while only Ca^{2+} addition in the absence of rotenone slightly stimulates ROS detection (line c). Similar results were obtained using the mitochondrial respiratory substrates glutamate/malate instead of pyruvate/malate (results not shown). The increased release of ROS promoted by rotenone and Ca^{2+} was completely blocked by Ca^{2+} removal by EGTA (Fig. 1B, line b) or by the H_2O_2-removing system ebselen plus glutathione (line c). In Fig. 1C, we tested the effect of Ca^{2+} on brain mitochondria treated with the complex I inhibitor MPP^+, a classical dopaminergic neurotoxin [11]. Interestingly, MPP^+ alone increased the rate of DCF production (line b) without any further stimulatory effect of Ca^{2+} addition (line c). The results of Fig. 1D show the effect of different concentrations of Ca^{2+} (rotenone at 100 nM) and different concentrations of rotenone (free Ca^{2+} at 50 μM) on H_2-DCF oxidation.

Since a partial inhibition (20–40%) of mitochondrial complex I activity has been detected in PD patients [4,5,7], we tested if a partial inhibition of mitochondrial respiration by rotenone would increase the rate of ROS release in the presence of Ca^{2+}. The results of Fig. 2A show that, under our experimental conditions, 5 nM rotenone results in 30–40% inhibition of uncoupled mitochondrial respiration supported by NADH-linked substrates. Using this rotenone concentra-

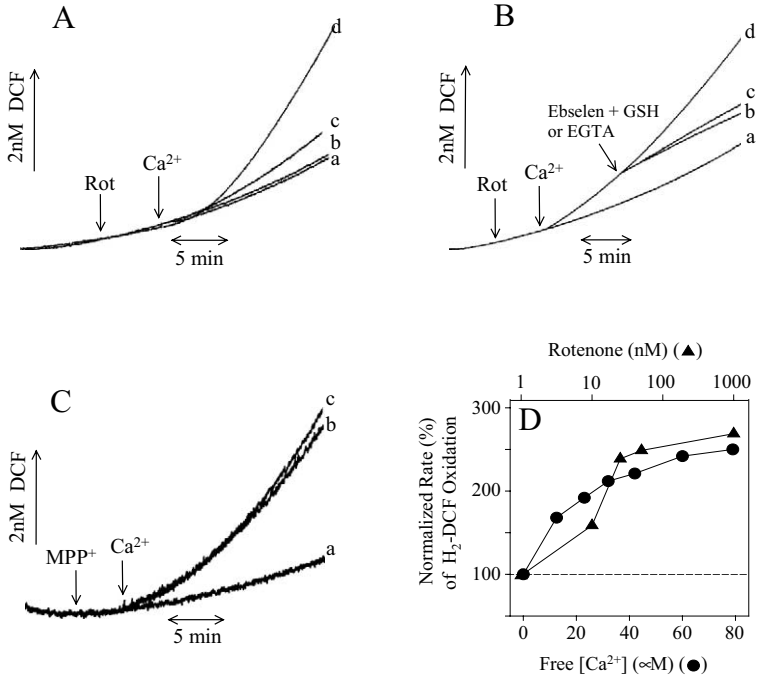

Fig. 1. Ca^{2+}-induced increase in ROS release in isolated brain mitochondria treated with rotenone: effect of rotenone and Ca^{2+} concentrations. Isolated forebrain mitochondria (0.5 mg/ml) were added to standard reaction medium containing 1 μM H_2-DCFDA. In A, 50 nM rotenone (lines b and d) and 120 μM Ca^{2+} (lines c and d) were added to the experiments where indicated by the arrows. In B, 50 nM rotenone (lines b, c and d), 120 μM Ca^{2+} (lines b, c and d), 10 μM ebselen plus 200 μM glutathione (line b) and 500 μM EGTA (line c) were added to the experiments where indicated by the arrows. In C, 1 mM MPP^+ (lines b and c) and 120 μM Ca^{2+} (line c) were added where indicated by the arrows. Panel D shows the effect of different concentrations of rotenone (free Ca^{2+} at 50 μM) (▲) and different concentrations of Ca^{2+} (rotenone at 100 nM) (●) on H_2-DCF oxidation. Lines a represent control experiments without the addition of rotenone, MPP^+ and Ca^{2+}.

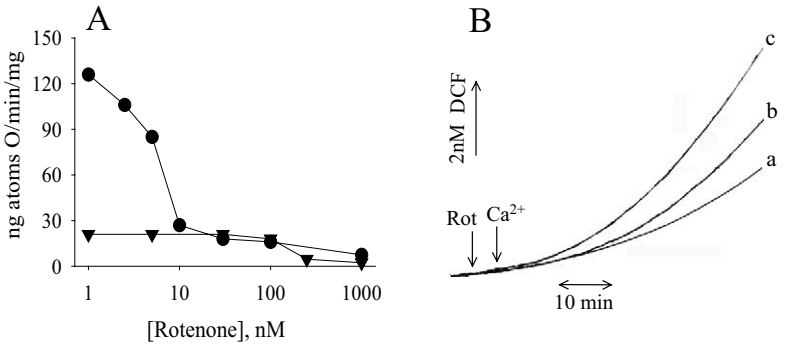

Fig. 2. A partial respiratory chain inhibition by rotenone results in mitochondrial oxidative stress in the presence of Ca^{2+}. In A, forebrain mitochondria (0.5 mg/ml) were incubated in standard reaction medium, without ATP and oligomycin, in the absence (▼) or presence or 1 mM ADP (●). In B, forebrain mitochondria (0.5 mg/ml) were incubated in standard reaction medium, 5 nM rotenone (line c) and 120 μM Ca^{2+} (lines b and c) were added where indicated by the arrows. Line a represents a control experiment without the addition of rotenone and Ca^{2+}.

tion, in the presence of Ca^{2+}, we observed a nearly 200% increase in the rate of the detection of mitochondrially generated ROS (Fig. 2B).

The effect of cyclosporin A, an inhibitor of mitochondrial permeability transition (MPT; for a review see [22]), was tested on mitochondrial ROS release (Fig. 3). Cyclosporin A had no inhibitory effect on rotenone plus Ca^{2+}-induced increased release of ROS (line c), indicating that MPT is not involved in this process. In addition, the results of Fig. 3 show that the protonophore carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP; line d) does not inhibit Ca^{2+} plus rotenone-induced increased release of mitochondrial ROS, and the mitochondrial respiratory chain inhibitors antimycin A (line e) and myxothiazol (line f) do not substitute rotenone in stimulating mitochondrial ROS release in the presence of Ca^{2+}. Interestingly Ca^{2+} slightly inhibited antimycin A-stimulated mitochondrial ROS release (result not shown).

In Fig. 4, mitochondrial ROS release was measured using the membrane-permeable fluorescent dye Mito-Tracker Red CM-H_2XRos (Fig. 4A), or the fluorescent peroxidase substrates scopoletin (Fig. 4B) and Amplex Red (Fig. 4C), in order to guarantee that the results obtained with H_2-DCFDA were accurate. The presence of rotenone plus Ca^{2+} strongly stimulated ROS detection using any of these methods, while rotenone in the absence of Ca^{2+} resulted in a smaller stimulatory effect on ROS detection that was more pronounced when using Amplex Red. Ca^{2+} alone did not increase the rate of ROS detection. The results obtained with Mito-Tracker Red and Amplex Red (Fig. 4A,C) are in accordance with recent reports showing a stimulatory effect of rotenone on mitochondrial ROS release [10,23].

Mitochondrial lipid peroxidation was quantified in order to study a possible correlation between Ca^{2+} plus rotenone-induced increased release of ROS and membrane damage (Fig. 5). Incubation of brain mitochondria in the presence of Ca^{2+} plus rotenone increased the basal content of TBARS three- to four-fold, while either Ca^{2+} or rotenone had no significant effect on the basal content of TBARS.

4. Discussion

In the present work, we report that micromolar Ca^{2+} concentrations in the presence of rotenone strongly increase the detection of ROS production in isolated rat forebrain mitochondria. This Ca^{2+} effect was dose dependent, while the effect of rotenone was correlated with the degree of inhibition of uncoupled (respiratory state 3) mitochondrial respiration (Figs. 1 and 2). Mitochondrial oxidative damage was evidenced by increased membrane lipid peroxidation in the presence of Ca^{2+} plus rotenone (Fig. 5). The effect of Ca^{2+} and rotenone on mitochondrial ROS release was only observed for isolated forebrain mitochondria, and not for organelles isolated from two other tissues, liver and muscle (results not shown). Although we have no plausible explanation for the selective effect of Ca^{2+} plus rotenone to brain mitochondria, this effect correlates with the selective pathology of PD to the nervous system [1,24].

Interestingly, Ca^{2+} did not stimulate MPP^+-induced increase in mitochondrial ROS release (Fig. 1), and Ca^{2+} did not stimulate ROS release in brain mitochondria treated with antimycin A or myxothiazol, inhibitors of respiratory chain complex III (Fig. 3). These results suggest that the effect of Ca^{2+} on increasing ROS release under a condition of partial inhibition of the mitochondrial respiratory chain may be specific for the rotenone-sensitive site of complex I. The ability of Ca^{2+} to stimulate mitochondrial ROS generation may be at-

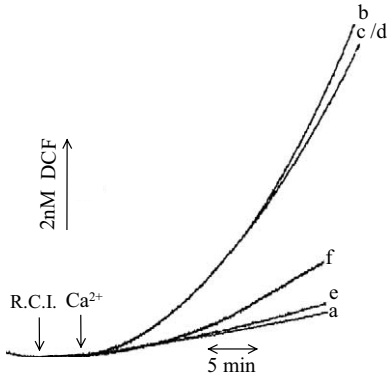

Fig. 3. Effect of cyclosporin A, FCCP and respiratory chain inhibitors on mitochondrial ROS release in the presence of Ca^{2+}. Forebrain mitochondria (0.5 mg/ml) were added to standard reaction medium containing 1 μM cyclosporin A (line c), 1 μM FCCP (line d), or no other additions (lines a, b, e and f). Mitochondrial respiratory chain inhibitors (R.C.I.), 50 nM rotenone (lines b–d), 0.5 μM antimycin A (line e) or 0.5 μM myxothiazol (line f) were added where indicated by the arrow. Ca^{2+} was added to the experiments represented by lines b–f where indicated by the arrow. Line a represents a control experiment without the addition of respiratory chain inhibitors and Ca^{2+}.

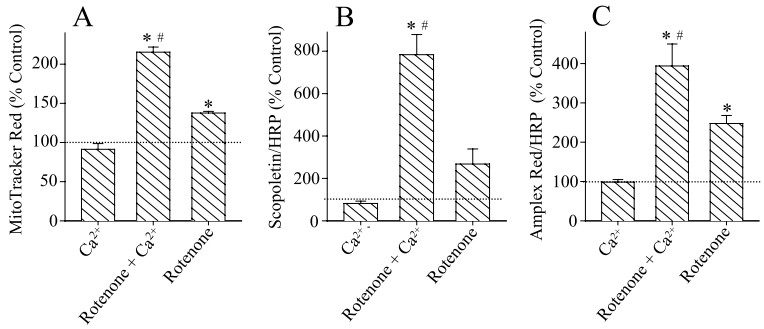

Fig. 4. Rotenone plus Ca^{2+}-induced increase in detection of ROS in isolated brain mitochondria: measurement of ROS using different methods. Forebrain mitochondria (0.5 mg/ml) were incubated in standard reaction medium containing 200 nM MitoTracker Red CM-H_2XRos (A), 1 μM scopoletin plus 1 μM horseradish peroxidase (B) or 50 μM Amplex Red plus 0.025 μM horseradish peroxidase (C). Rotenone (50 nM) and/or 120 μM Ca^{2+} were added to the experiments as indicated in the figure. Values represent averages of three to four experiments (±S.E.M.), using different mitochondrial preparations. $^{*}P<0.01$, post hoc Bonferroni/Dunn's test compared with control. $^{\#}P<0.01$, post hoc Bonferroni/Dunn's test compared with rotenone.

tributed to alterations promoted by this cation on the mitochondrial membrane structure, possibly through binding to cardiolipins of the inner mitochondrial membrane [25]. These alterations are characterized by an increased lipid packing and lipid domain formation that may contribute toward electron leakage at the respiratory chain complex I [25].

Under our experimental conditions, Ca^{2+}-induced oxidative stress in rotenone-treated brain mitochondria does not seem to depend on mitochondrial Ca^{2+} accumulation. The Ca^{2+} effect was observed even in the presence of high rotenone concentrations and FCCP (Fig. 3), situations in which mitochondrial Ca^{2+} uptake is limited due to dissipation of the transmembrane electrical potential ($\Delta\Psi$). These results indicate that Ca^{2+} could be acting at an external site of the inner mitochondrial membrane facing the intermembrane space to stimulate ROS release in the presence of rotenone. High Ca^{2+} concentrations could also induce MPT, a phenomenon characterized by a non-specific inner membrane permeabilization (for reviews see [22,26]) that results in increased detection of mitochondrial ROS production [19]. In our experiments, we had no indications of the participation of permeability transition (Fig. 3), probably because the experiments were conducted in the presence of ATP and Mg^{2+}, effective inhibitors of this phenomenon in isolated brain mitochondria [27]. Recently, Starkov and collaborators [28] reported that Ca^{2+} approximately doubles brain mitochondrial ROS release in the presence of rotenone. However, different from the present study, the results reported by Starkov and collaborators [28] were obtained using succinate as respiratory substrate, a situation in which mitochondrial Ca^{2+} uptake and retention are maintained even in the presence of high concentrations of rotenone. Moreover, using succinate as substrate rotenone decreases by 70–80% mitochondrial ROS release and the presence of Ca^{2+} seems only to decrease this inhibitory effect of rotenone. In fact, the rate of H_2O_2 detection in the presence of rotenone and Ca^{2+} was lower than in their absence [28].

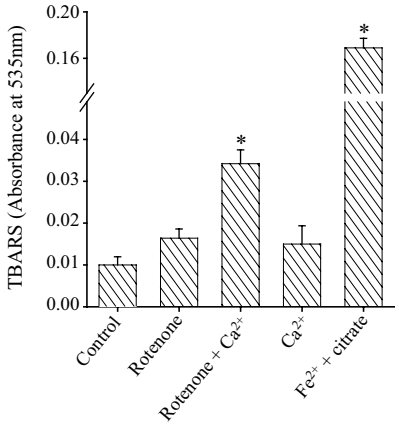

Fig. 5. Rotenone plus Ca^{2+}-induced TBARS formation. Forebrain mitochondria (0.5 mg/ml) were incubated in standard reaction medium for 30 min in the presence of 50 nM rotenone and/or 120 μM Ca^{2+} as indicated in the figure. Fe^{2+} (20 μM) plus 2 mM citrate were used to induce lipid peroxidation independent of mitochondrially generated ROS [19]. Values represent averages of five experiments (±S.E.M.), using different mitochondrial preparations. $^{*}P<0.01$, post hoc Bonferroni/Dunn's test compared with control.

Recently, Barrientos and Moraes [9] showed that a partial inhibition of mitochondrial complex I activity is not enough to explain neuronal cell death primarily by energy deprivation. Moreover, partial inhibition of complex I, obtained either by genetic modification of cell lines or rotenone treatment, promotes cell death in a manner quantitatively associated with increased free radical production, and not with a decrease in respiratory chain function [9]. This is in accordance with several reports showing an increased production of mitochondrial ROS after brain mitochondrial complex I inhibition [9,10,23,29]. A partial inhibition of respiratory chain complex I may also cause a subliminal energy deprivation that could increase the susceptibility of nigral neurons to glutamate receptor-mediated cell death, i.e. excitotoxicity [15,16].

In conclusion, our results show that micromolar Ca^{2+} concentrations induce oxidative stress in isolated brain mitochondria when complex I is partially inhibited by rotenone. A low grade excitotoxicity, promoted predominantly by hyperstimulation of *N*-methyl-D-aspartate receptors, which mediated Na^{+} and Ca^{2+} influx to the cytosol, has been proposed to play an important role in the neuropathology of PD [13,14]. Under these conditions, Ca^{2+}-stimulated mitochondrial ROS release may participate as an important link between the partial mitochondrial complex I inhibition and oxidative damage observed in PD.

Acknowledgements: We thank Dr. Alicia J. Kowaltowski for critical reading of the manuscript and Mrs. Elisangela J. Silva for the prep-

aration of rat forebrain mitochondria. This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundo de Apoio ao Ensino e Pesquisa (FAEP-UNICAMP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). S.C.S. and E.N.M. are supported by FAPESP fellowships.

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