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"STIMULATORY EFFECTS OF A PARTIAL RESPIRATORY COMPLEX I INHIBITION ON MITOCHONDRIAL H_2O_2 GENERATION"

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Orientador/Supervisor: Prof. Dr. Roger Frigério Castilho

"STIMULATORY EFFECTS OF A PARTIAL RESPIRATORY COMPLEX I INHIBITION ON MITOCHONDRIAL H_2O_2 GENERATION"

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Fisiopatologia Médica, da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para a obtenção do título de Mestre em Ciências.

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"Nosso medo mais profundo não é que sejamos inadequados. Nosso medo mais profundo é que sejamos poderosos demais. É nossa sabedoria, não nossa ignorância, o que mais nos apavora. Perguntamo-nos: ´Quem sou eu para ser brilhante, belo, talentoso, fabuloso?´ Na verdade, por que você não seria? Você é um filho de Deus. Seu medo não serve ao mundo. Não há nada de iluminado em se diminuir para que outras pessoas não se sintam inseguras perto de você. Nascemos para expressar a glória de Deus que há em nós. Ela não está apenas em alguns de nós; está em todas as pessoas. E quando deixamos que essa nossa luz brilhe, inconscientemente permitimos que outras pessoas façam o mesmo. Quando nos libertamos de nosso medo, nossa presença automaticamente liberta as outras pessoas."

Nelson Mandela

"O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis."

José de Alencar

"Paciência vale mais que valentia, dominar a si mesmo vale mais que conquistar cidades inteiras."

Provérbios 16:32

PÁG.

ADP	Adenosina difosfato
ATP	Adenosina trifosfato
BSA	Albumina soro bovina
CTE	Cadeia transportadora de elétrons
CuZnSOD	Superóxido dismutase dependente de cobre e zinco
$\Delta\psi_m$	Potencial elétrico de membrana mitocondrial
$\Delta\mu H^+$	Potencial eletroquímico de prótons
DNA	Ácido desoxirribonucléico
DNAmt	Ácido desoxirribonucléico mitocondrial
DP	Doença de Parkinson
DTNB	Ácido 5,5-ditiobis(2-nitrobenzóico)
DTT	Ditiotreitol
EDTA	Ácido etileno diamino tetra acético
EGTA	Ácido etileno glicol tetracético
EROs	Espécies reativas de oxigênio
FCCP	Carbonil cianido *p*-(trifluoromethoxi)fenil-hidrazona
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
H_2O_2	Peróxido de hidrogênio
HEPES	N-[2-hidroxietil] piperazina-N'-[ácido 2-etanosulfônico])
HRP	Peroxidase de raiz forte VIA
MAO	Monoamino oxidases
MCR	Mitocôndria de cérebro de rato
MDa	Megadalton
MnSOD	Superóxido dismutase dependente de manganês
MPTP	1-metil-4-fenil-1,2,3,6-tetrahidroperidina
MPP^+	Íon 1-metil-4-fenilpiridina
NAD^+	Nicotinamida adenina dinucleotídeo oxidada

NADH	Nicotinamida adenina dinucleotídeo reduzida
$NADP^+$	Nicotinamida adenina dinucleotídeo monofosfato oxidada
NADPH	Nicotinamida adenina dinucleotídeo monofosfato reduzida
O_2	Oxigênio molecular
$O_2^{\bullet-}$	Radical ânion superóxido
OH^-	Ânion hidroxila
$HO^\bullet$	Radical hidroxila
RNA	Ácido ribonucleico
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TRxSH	Tioredoxina reduzida
TRxS	Tioredoxina oxidada

Resumo

A inibição parcial do Complexo I da cadeia respiratória mitocondrial em ratos tratados cronicamente com rotenona está associada com o desenvolvimento de características neuroquímicas, comportamentais e neuropatológicas da doença de Parkinson. Os objetivos deste trabalho foram **(*i*)** caracterizar os efeitos de uma inibição parcial do Complexo I por rotenona na produção de peróxido de hidrogênio (H_2O_2) por mitocôndrias de cérebro de ratos (MCR) em diferentes estados respiratórios e **(*ii*)** avaliar a suscetibilidade de MCR velhos (24 meses) à inibição do consumo de oxigênio (O_2) e ao estímulo da produção de H_2O_2 por rotenona em comparação a MCR adultos (3-4 meses). A análise do potencial de membrana por citometria de fluxo em mitocôndrias isoladas indicou que a adição de rotenona promoveu uma inibição uniforme da respiração mitocondrial nestas organelas. Quando mitocôndrias foram incubadas na presença de uma baixa concentração de rotenona (10 nM) e de substratos geradores de NADH, o consumo de O_2 foi reduzido de 45,9±1,0 para 26,4±2,6 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ e de 7,8±0,3 para 6,3±0,3 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ nos estados respiratórios 3 (respiração estimulada por ADP) e 4 (respiração de repouso), respectivamente. Nessas condições, a produção mitocondrial de H_2O_2 foi estimulada de 12,2±1,1 para 21,0±1,2 pmol $H_2O_2 \cdot mg^{-1} \cdot min^{-1}$ e de 56,5±4,7 para 95,0±11,1 pmol $H_2O_2 \cdot mg^{-1} \cdot min^{-1}$ nos estados respiratórios 3 e 4, respectivamente. Resultados similares foram observados ao comparar preparações mitocondriais enriquecidas com organelas sinápticas e não-sinápticas ou quando o íon 1-metil-4-fenilpiridina (MPP^+) foi utilizado como inibidor de Complexo I mitocondrial. O estímulo da produção de H_2O_2 por rotenona nos estados respiratórios 3 e 4 foi associado a um aumento do estado reduzido de nucleotídeos de nicotinamida endógenos. Na respiração mitocondrial com succinato, onde a maior parte da produção de H_2O_2 se origina do fluxo reverso de elétrons do Complexo II para o I, baixas concentrações de rotenona inibiram a produção de H_2O_2. Rotenona não exerceu efeito sobre a eliminação mitocondrial de concentrações micromolares de H_2O_2. Em sinaptossomas intactos, observamos que rotenona 10 nM estimulou a liberação de

H_2O_2 em 20,2±3,3% no estado respiratório basal. Ao compararmos MCR adultos e velhos, verificamos que o consumo de O_2 no estado respiratório 3 e a atividade da citrato sintase foram 21,0±3,3% e 17,0±5,4% mais baixos em MCR velhos. Experimentos conduzidos na presença de diferentes concentrações de rotenona (5, 10 e 100 nM) demonstraram sensibilidade similar à inibição do consumo de O_2 por rotenona no estado respiratório 3, com IC_{50} de 7,8±0,4 e 6,5±0,5 nM para MCR adultos e velhos, respectivamente. De acordo com esses resultados, o estímulo da produção de H_2O_2 observado foi similar em MCR adultos e velhos, tratadas com diferentes concentrações de rotenona. Concluímos que, uma inibição parcial do Complexo I pode resultar em uma crise energética e/ou estresse oxidativo mitocondrial, enquanto o primeiro evento predominaria numa situação de alta demanda de fosforilação oxidativa, o segundo ocorreria em condições de respiração de repouso. Em adição, os experimentos com ratos velhos indicaram que rotenona exerce efeitos similares no consumo de O_2 e na produção de H_2O_2 em MCR adultos e velhos.

Palavras-chave: Mitocôndrias; Espécies de oxigênio reativas; Doença de Parkinson; Rotenona; Complexo I de transporte de elétrons.

Abstract

Partial inhibition of mitochondrial Complex I is associated with the development of neurochemical, behavioral, and neuropathological features of Parkinson´s disease in rats chronically and systemically treated with rotenone. The aims of this work were **(*i*)** to characterize the effects of partial inhibition of respiratory Complex I by rotenone on H_2O_2 production by rat brain mitochondria in different respiratory states and **(*ii*)** to evaluate the susceptibility of brain mitochondria from old rats (24 month-old) to rotenone-induced inhibition of oxygen consumption and increased generation of H_2O_2 when compared with organelles from adult rats (3-4 month-old). Flow cytometric analysis of membrane potential in isolated mitochondria indicated that rotenone leads to uniform respiratory inhibition when added to a suspension of these organelles. When mitochondria were incubated in the presence of a low concentration of rotenone (10 nM) and NADH-linked substrates, oxygen consumption was reduced from 45.9±1.0 to 26.4±2.6 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ and from 7.8±0.3 to 6.3±0.3 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ in respiratory states 3 (ADP-stimulated respiration) and 4 (resting respiration), respectively. Under these conditions, mitochondrial H_2O_2 production was stimulated from 12.2±1.1 to 21.0±1.2 pmol $H_2O_2 \cdot mg^{-1} \cdot min^{-1}$ and 56.5±4.7 to 95.0±11.1 pmol $H_2O_2 \cdot mg^{-1} \cdot min^{-1}$ in respiratory states 3 and 4, respectively. Similar results were observed when comparing mitochondrial preparations enriched with synaptic or nonsynaptic organelles or when 1-methyl-4-phenylpyridinium (MPP^+) ion was used as a respiratory Complex I inhibitor. Rotenone-stimulated H_2O_2 production in respiratory states 3 and 4 was associated with a high reduction state of endogenous nicotinamide nucleotides. In succinate-supported mitochondrial respiration, where most of the mitochondrial H_2O_2 production relies on electron backflow from Complex II to Complex I, low rotenone concentrations inhibited H_2O_2 production. Rotenone had no effect on mitochondrial elimination of micromolar concentrations of H_2O_2. In intact synaptosomes, we observed that 10 nM rotenone stimulated H_2O_2 release by 20.2 ± 3.3% under basal respiratory state. When

comparing isolated brain mitochondria from adult and old rats we observed that oxygen consumption under respiratory state 3 and citrate synthase activity were 21.0±3.3% and 17.0±5.4% lower in mitochondria from old rats. Experiments conducted in the presence of different rotenone concentrations (5, 10 and 100 nM) showed that brain mitochondria from adult and old rats have similar sensitive to rotenone-induced inhibition of oxygen consumption in respiratory state 3, with IC_{50} of 7.8±0.4 and 6.5±0.5 nM for adult and old rats, respectively. In line with these results, similar stimulations in H_2O_2 production were observed in mitochondria from adult and old rats treated with different concentrations of rotenone. We conclude that partial Complex I inhibition may result in mitochondrial energy crisis and oxidative stress, the former being predominant under oxidative phosphorylation and the latter under resting respiration conditions. Rotenone exerts similar effects on oxygen consumption and H_2O_2 production by isolated brain mitochondria from adult and old rats.

Keywords: Mitochondria; Reactive oxygen species; Parkinson disease; Rotenone; Electron transport Complex I.

1. Introdução

Doenças neurodegenerativas são caracterizadas pela morte de neurônios em diferentes regiões do cérebro, com o comprometimento de diferentes vias de neurotransmissão. Até o presente momento, inúmeras doenças neurodegenerativas foram reportadas, sendo as mais comuns, a doença de Alzheimer e a doença de Parkinson (DP) (NUSSBAUM e ELLIS, 2003).

A DP é considerada uma desordem com espectro universal, representando uma taxa de incidência bruta que varia de 4,5 a 19 em 100.000 pessoas por ano (WHO, 2006) e a prevalência corresponde a aproximadamente 0,3% da população total (SCHAPIRA, 2008). O envelhecimento possui um importante papel no aumento das taxas de incidência, demonstrada em indivíduos entre 50 a 59 anos onde a taxa de incidência é de 17,4 em 100.000 indivíduos, com aumento para 93,1 em 100.000 pessoas em indivíduos entre 70 a 79 anos (LEES, HARDY e REVESZ, 2009).

Em 2005, o número de portadores da DP acima de 50 anos ao redor do mundo era de aproximadamente 4,5 milhões e estima-se que este número duplique até 2030. A projeção para 2030 de pacientes portadores de DP no Brasil é de 340 mil indivíduos (DORSEY et al., 2007).

1.1. Doença de Parkinson

O nome da doença de Parkinson (DP), inicialmente chamada de Mal de Parkinson, foi proposto por Jean Martin Charcot, devido à descrição dessa síndrome ter sido realizada por James Parkinson em 1817, em sua monografia intitulada "*An Essay on the Shaking Palsy*" (LEES, HARDY e REVESZ, 2009).

A DP caracteriza-se pela degeneração predominante e progressiva de neurônios dopaminérgicos da substância negra *pars compacta* (NAVARRO e BOVERIS, 2009; VENDEROVA e PARK, 2012). Os indivíduos acometidos

apresentam, sobretudo, comprometimentos motores, incluindo tremor de repouso, rigidez, instabilidade postural, bradicinesia e hipocinesia (NUSSBAUM e ELLIS, 2003; LEES, HARDY e REVESZ, 2009).

Na maioria dos indivíduos com DP, em neurônios há a presença de inclusões citoplasmáticas ricas em α-sinucleína e filamentos de ubiquitina, denominados de corpos de Lewy e neuritos de Lewy (RUIPÉREZ *et al.*, 2010; BOUSSET *et al.*, 2013). Os corpos de Lewy possuem uma relação direta com a DP, sendo considerados um importante marcador anatomopatológico. Foram encontrados em autópsias realizadas em tecido cerebral de portadores da DP e estão presentes em neurônios do mesencéfalo e de outras regiões do sistema nervoso central (SPILLANTINI *et al.*, 1997).

A DP é considerada uma doença multifatorial, a qual existe uma relação direta entre fatores ambientais como, p.ex., exposição a pesticidas, herbicidas e metais como chumbo e manganês, e predisposição genética (ELBAZ e MOISAN, 2008).

Foram identificadas mutações em nove genes nucleares codificadores das proteínas α-sinucleína, parkina, ubiquitina hidroxilase carboxi-terminal L1, DJ-1, fosfatase homóloga tensina (PTEN)-induced cinase 1 (PINK1), cinase rica em repetições de leucina (LRRK2), receptor nuclear NURR1, HTRA2 e *tau*. Seis desses genes podem ter relação direta ou indireta com funções mitocondriais, a saber, os codificadores de α-sinucleína, parkina, DJ-1, PINK1, LRRK2 e HTRA2 (FILOSTO *et al.*, 2011). Mutações nestes genes estão relacionadas com a forma familiar da DP, que representa menos de 5% dos casos, sendo os demais classificados como esporádicos.

No início dos anos 90, Schapira e colaboradores realizaram estudos *postmortem* com tecido cerebral de pacientes com DP e reportaram uma diminuição da atividade do Complexo I (NADH:ubiquinona oxidoredutase) da cadeia transportadora de elétrons (CTE) nesses indivíduos, quando comparados com indivíduos normais (SCHAPIRA *et al.*, 1989; SCHAPIRA *et al.*, 1990).

Nos anos 80, a toxina 1-metil-4-fenil-1,2,3,6-tetrahidroperidina (MPTP) foi identificada como um contaminante presente em formulações de heroína sintética ("nova heroína"). Vários indivíduos expostos a esta toxina desenvolveram sinais clínicos e sintomas característicos da doença de Parkinson (LANGSTON *et al*., 1983; BOVÉ e PERIER, 2012; MARTINEZ e GREENAMYRE, 2012). Estudos posteriores mostraram que o 1-metil-4-fenilpiridina (MPP^+), metabólito ativo do MPTP, inibe a atividade do Complexo I mitocondrial e é capaz de causar morte seletiva de neurônios dopaminérgicos em animais de experimentação e humanos (BURNS et al., 1983; LANGSTON *et al*., 1983; NICKLAS, VYAS e HEIKKILA, 1985; RAMSAY *et al*., 1987). Posteriormente, foi caracterizado que o MPTP sofre metabolização pelas monoaminas oxidases (MAO-B) presentes em astrócitos (SINGER *et al*., 1988) e também por mitocôndrias de neurônios dopaminérgicos (BAJPAI *et al*., 2013), e que o MPP^+ é seletivamente captado por neurônios dopaminérgicos, através do transportador de dopamina (SINGER *et al*., 1988; BETARBET, SHERER e GREENAMYRE, 2002).

Com base nesses achados, diversos modelos animais utilizando-se toxinas foram propostos para estudar a fisiopatolgia da DP, assim como para avaliar abordagens terapêuticas. Neste trabalho, nos baseamos na toxina rotenona, um composto capaz de inibir a atividade do Complexo I da CTE (ESPOSTI, 1998). A rotenona é utilizada na piscicultura como agente para promover morte de peixes e na agricultura como pesticida (GONSÁLVEZ, 1983). A rotenona é utilizada no tratamento crônico de ratos Sprague-Dawley e ratos Lewis, sendo administrada por via intravenosa ou intraperitoneal (BERTABET *et al*., 2000; CANNON *et al*., 2009). Após tratamento crônico de ratos com rotenona, foi observada uma redução do número de neurônios dopaminérgicos na região da substância negra, com consequente acometimento da via nigroestriatal, presença de corpúsculos fibrilares ricos em ubiquitina e α-sinucleína nos neurônios dopaminérgicos remanescentes e aparecimento de sinais clínicos característicos da DP como bradicinesia e rigidez postural (BERTABET *et al*., 2000; CANNON *et al*., 2009).

Este modelo animal fornece certas vantagens em relação ao modelo com MPTP, pois reproduz um estado de inibição parcial do Complexo I em diferentes tecidos, além do tecido cerebral, assim como é observado em indivíduos com DP (BETARBET *et al.*, 2000; BETARBET, SHERER e GREENAMYRE, 2002).

1.2. Metabolismo Energético Oxidativo

A metabolização de carboidratos, lipídeos e aminoácidos, realizada em organismos aeróbios, resulta na transferência de elétrons desses nutrientes para o O_2, gerando H_2O e liberando energia que é armazenada na forma de um potencial eletroquímico de prótons ($\Delta\mu H^+$) através da membrana mitocondrial interna. O $\Delta\mu H^+$ será utilizado pela ATP sintase para promover a conversão de ADP e fosfato inorgânico à ATP. Este processo é denominado de fosforilação oxidativa (MITCHELL, 1966).

A atividade neuronal, incluindo a manutenção de gradientes iônicos através da membrana plasmática e a liberação de neurotransmissores, é dependente da energia livre gerada a partir da glicólise e da fosforilação oxidativa (MONGE *et al.*, 2008). Sendo que, o sistema nervoso central (SNC), representando apenas 2% da massa corporal, consome cerca de 20% do oxigênio provido da respiração em um indivíduo em repouso (KANN e KOVÁCS, 2007).

Mitocôndrias estão presentes no citoplasma da maioria das células eucariotas. A estrutura de uma mitocôndria é composta por uma membrana externa, que possui aspecto liso e envolve toda a organela, e uma membrana interna, que apresenta invaginações e envolve a matriz mitocondrial, originando as cristas mitocondriais (LEHNINGER, NELSON e COX, 2009).

Na membrana interna mitocondrial, localiza-se a CTE, que está acoplada ao sistema de fosforilação oxidativa. A CTE é composta por quatro complexos multiprotéicos, sendo a NADH:ubiquinona oxidoredutase (Complexo I), succinato:ubiquinona oxidoredutase (Complexo II), ubiquinona-citocromo c oxidase (Complexo III) e citocromo c oxidase (Complexo IV), além de outros componentes,

como a ubiquinona (coenzima Q) e o citocromo c (LEHNINGER, NELSON E COX, 2009). O sistema de fosforilação oxidativa é composto pela ATP sintase (também denominada por alguns autores como Complexo V), que possui duas subunidades; a subunidade F_o e a subunidade F_1 (ADAM-VIZI, 2005).

Pressupõe-se que, na DP a disfunção do Complexo I da CTE pode ocasionar déficit na produção de ATP e propensão ao aumento da produção de EROs, o que levaria à degeneração de neurônios (TRETTER, SIPOS e ADAM-VIZI, 2004).

A seguir, daremos ênfase na estrutura, atividade catalítica e produção de EROs pelo Complexo I mitocondrial.

1.3. Complexo I mitocondrial (NADH:Ubiquinona oxidoredutase)

O Complexo I, ou NADH:ubiquinona oxidoredutase (E.C. 1.6.5.3), é uma proteína multimérica com função enzimática, sendo o maior dos complexos proteicos presentes na CTE (HOEFS *et al.*, 2012).

Em mamíferos, foram identificadas 45 subunidades que compõe o Complexo I, sendo 7 subunidades codificadas pelo DNA mitocondrial e 38 subunidades codificadas pelo DNA nuclear, com massa molecular aproximada de 1 MDa (CARROLL *et al.*, 2006; MCKENZIE, LAZAROU e RYAN, 2009). Das 45 subunidades que compõe o Complexo I, apenas 14 possuem atividade catalítica. As outras subunidades são denominadas de subunidades acessórias, devido ao desconhecimento da função exata dessas subunidades até o momento (HOEFS *et al.*, 2012).

Devido à sua estrutura, o Complexo I mitocondrial pode ser dividido em três módulos, de acordo com sua funcionalidade (FASSONE e RAHMAN, 2012). O módulo N ou módulo desidrogenase é responsável pela oxidação do NADH; o módulo Q ou módulo hidrogenase é responsável pela transferência dos elétrons dos centros ferro-enxofre do módulo N para a coenzima Q; e o módulo P ou

módulo de translocação de prótons, que transloca prótons da matriz mitocondrial para o espaço intermembranas (BRANDT, 2006).

O Complexo I é a primeira enzima da CTE que promoverá a entrada dos elétrons presentes no equivalente redutor NADH, gerado pelas desidrogenases mitocondriais do ciclo do ácido cítrico e de uma pequena parte do NADH gerado diretamente pela β-oxidação de ácidos graxos, para a CTE (BRANDT, 2006; PETRUZZELLA *et al*., 2012).

A catálise de transferência do par de elétrons do NADH para a ubiquinona promoverá a translocação de quatro prótons da matriz mitocondrial, através da membrana interna mitocondrial (BRANDT, 2006; HIRST, 2009; FASSONE e RAHMAN, 2012). Esse processo gerará energia livre que será armazenada na forma de $\Delta\mu H^+$ no espaço intermembranas (WALKER, 1992). A reação geral realizada pelo complexo I é a seguinte:

$$NADH + H^+ + Q + 4\ H^+_{matriz} \rightarrow NAD^+ + QH_2 + 4\ H^+_{espaço\ intermembranas}$$

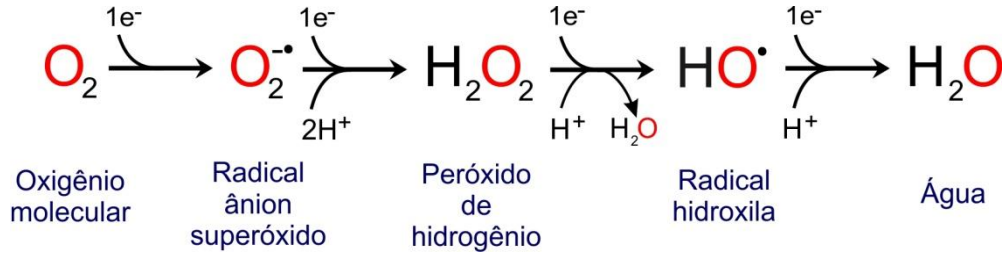
Cerca de 40% da geração do $\Delta\mu H^+$ é devida a atividade do Complexo I e essa energia gerada no espaço intermembranas é denominada de força próton motriz que, posteriormente, será utilizada pela ATP sintase para produção e liberação do ATP (PETRUZZELLA *et al*., 2012; FASSONE e RAHMAN, 2012; BOYER, 1977).

Diversas desordens neurológicas estão associadas com mutações em diferentes subunidades do Complexo I mitocondrial. Até o momento, foram identificadas algumas mutações em genes que codificam subunidades do Complexo I mitocondrial, ocasionando uma diminuição de sua atividade catalítica e aumentando a geração do radical ânion superóxido ($O_2^{\cdot -}$) em mitocôndrias (FASSONE e RAHMAN, 2012).

1.4. Produção Mitocondrial de Espécies Reativas de Oxigênio (EROs)

A produção de espécies reativas do oxigênio (EROs) pela CTE é uma inevitável consequência da produção de ATP pela fosforilação oxidativa, podendo levar à processos celulares deletérios e envelhecimento (BRAND *et al.*, 2004).

Sabe-se que mitocôndrias são importantes fontes produtoras de derivados parcialmente reduzidos do oxigênio molecular (O_2), que ao receber elétrons de fontes distintas, dará origem a espécies reativas como o radical ânion superóxido ($O_2^{\bullet-}$), peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($HO^{\bullet}$) (KOOPMAN *et al.*, 2010; VENDITTI, DI STEFANO e DI MEO, 2013). O ***Esquema 1*** ilustra a acepção de elétrons pela molécula de O_2 e a formação de radicais livres:



Esquema 1. Redução monoeletrônica sequencial do oxigênio molecular à água. A adição de um elétron ao oxigênio molecular (O_2) produz o radical ânion superóxido ($O_2^{\bullet-}$), que ao receber outro elétron dará origem a peróxido de hidrogênio (H_2O_2). Na presença de um metal bivalente, como o Fe^{2+}, o H_2O_2 pode aceitar outro elétron, originando o radical hidroxila ($HO^{\bullet}$) e ânion hidroxila (HO^-) (reação tipo Fenton). O $HO^{\bullet}$ pode receber um elétron e gerar uma molécula de água. Adaptado de Koop (2006).

O processo de formação do ânion $O_2^{\bullet-}$, inicia-se pela redução monoeletrônica do oxigênio molecular. O O_2 molecular possui dois elétrons desemparelhados em um orbital (***Figura 1***) e, ao receber um único elétron em um desses orbitais, dará origem ao ânion $O_2^{\bullet-}$, uma molécula doadora de elétron e altamente reativa, capaz de desencadear uma cascata de eventos oxidativos

deletérios (MURPHY, 2009; KOWALTOWSKI *et al.*, 2009; RIGOULET, YOBOUE e DEVIN, 2011).

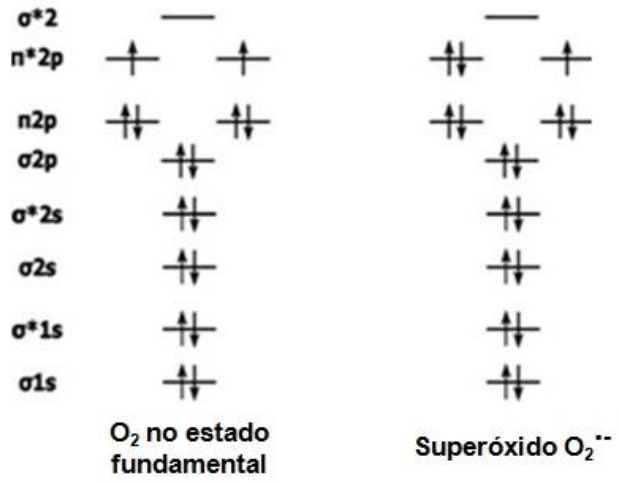


Figura 1: Acepção de um elétron no orbital n*2p do oxigênio molecular (O_2 no estado fundamental), dará origem ao radical ânion Superóxido ($O_2^{\bullet-}$). Adaptado de Rigoulet, Yoboue e Devin (2011).

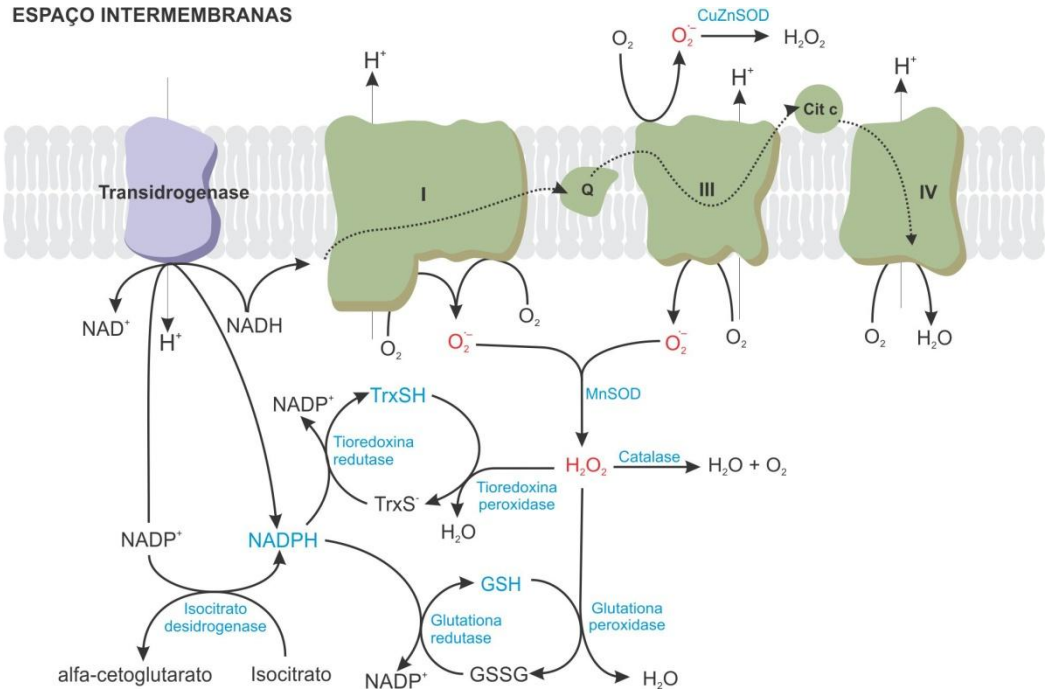
Até o presente momento, foram identificados oito locais de produção de $O_2^{\bullet-}$ em mitocôndrias, que são: piruvato desidrogenase, α-cetoglutarato desidrogenase, sítios I_F e I_Q presentes no Complexo I, flavoproteína transportadora de elétrons:ubiquinona oxidoredutase, glicerol 3-fosfato desidrogenase, sítio III_Q presente no Complexo III (BRAND, 2010) e, o mais recentemente identicado, Complexo II (QUINLAN *et al.*, 2012). Porém, as duas principais fontes de produção de $O_2^{\bullet-}$ em mitocôndrias são os Complexos I e III (HIRST, 2009; BRAND, 2010; DRÖSE e BRANDT; 2012).

Na matriz mitocondrial e no espaço intermembranas, há a presença de superóxido dismutases (Mn-SOD e Cu,Zn-SOD, respectivamente) que neutralizarão o ânion $O_2^{\bullet-}$ produzido, dando origem ao H_2O_2, uma ERO mais estável (NORDBERG E ARNER, 2001; KOWALTOWSKI *et al.*, 2009). O H_2O_2 possui vários destinos, podendo atuar como sinalizador celular (HAMANAKA e CHANDEL, 2010) ou ser metabolizado por enzimas do sistema antioxidante como

a catalase, tioredoxina peroxidase e glutationa peroxidase, presentes no citosol e na matriz mitocondrial (NORDBERG e ARNER, 2001; KOWALTOWSKI *et al.*, 2009; KOOPMAN *et al.*, 2010). Os níveis de tioredoxina reduzida (TRxSH) e glutationa reduzida (GSH) são mantidos pela atividade das enzimas tioredoxina e glutationa redutases, que utilizarão o NADPH como fonte doadora de elétrons, reduzindo as moléculas de tioredoxina (TRxS) e glutationa (GSSG) oxidadas (GHEZZI, 2005; HOLMGREN *et al.*, 2005).

Os níveis de NADPH na matriz mitocondrial são mantidos pela atividade da transidrogenase de nucleotídeos de nicotinamida (RYDSTRÖM, 2006) e também pela atividade da isocitrato desidrogenase (SAZANOV e JACKSON, 1994) (***Esquema 2***). A atividade da transidrogenase promoverá um transporte de prótons para a matriz mitocondrial, gerando uma ligação direta entre o potencial de membrana mitocondrial ($\Delta\Psi_m$) com a capacidade redox (KOWALTOWSKI *et al.*, 2009).

No entanto, a substância negra é rica em íons Fe^{2+}, o que contribui para a aceleração da reação não enzimática de degradação do H_2O_2 gerando o radical hidroxila ($H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\cdot}\mathbf{OH} + Fe^{3+}$), uma ERO altamente reativa (KOOPMAN *et al.*, 2010). Acredita-se que neurônios dopaminérgicos também estão expostos a um estado constante de desbalanço redox, devido à formação de $O_2^{\cdot -}/H_2O_2$ como produto final do catabolismo da dopamina (GREENAMYRE *et al.*, 2001).



Esquema 2. Metabolismo mitocondrial de espécies reativas do oxigênio. A redução monoeletrônica do oxigênio molecular (O_2) dará origem ao ânion superóxido ($O_2^{\cdot-}$) pelos complexos I, II e III. O superóxido formado sofrerá dismutação pelas superóxido dismutases presentes no espaço intermembranas (CuZnSOD) e na matriz mitocondrial (MnSOD), gerando peróxido de hidrogênio (H_2O_2). O H_2O_2 poderá ser removido pela catalase, tioredoxina peroxidase ou glutationa peroxidase. A atividade da glutationa e tioredoxina peroxidase são dependentes de glutationa reduzida (GSH) e tioredoxina reduzida (TRxSH), respectivamente. As glutationa e a tioredoxina redutases são responsáveis pela redução da glutationa e tioredoxina oxidadas (GSSG e TrxS-, respectivamente), às custas de NADPH como fonte doadora de elétrons. Os níveis de NADPH são mantidos pela atividade da transidrogenase de nucleotídeos de nicotinamida. A isocitrato desidrogenase também é uma importante fonte de redução de NADP na matriz mitocondrial. Fonte: Adaptado de Kowaltowski *et al.*(2009).

Na DP há um aumento da agregação protéica, devido à diminuição da atividade do sistema proteassoma ubiquitina (SCHAPIRA, 2008). Esta alteração também é acompanhada de danos oxidativos a constituintes celulares como proteínas, lipídeos e DNA, levando a disfunções celulares e morte celular (TRETTER, SIPOS e ADAM-VIZI, 2004).

2. Objetivo

O objetivo geral dessa Dissertação foi avaliar e caracterizar o impacto de uma inibição parcial do Complexo I mitocondrial pela toxina rotenona no consumo de oxigênio (O_2) e na produção de peróxido de hidrogênio (H_2O_2), nos estados respiratórios de fosforilação oxidativa (estado 3) e no estado de repouso (estado 4). Como forma de abordar esta questão, utilizamos mitocôndrias isoladas de cérebro de ratos com diferentes idades (3-4 meses e 24 meses), preparações enriquecidas de mitocôndrias sinápticas e não-sinápticas e sinaptossomas intactos.

3. Capítulos

3.1. Capítulo I

"Effects of Partial Inhibition of Respiratory Complex I on Brain Mitochondrial H_2O_2 Production in Different Respiratory States"

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(Manuscrito submetido à publicação)

Effects of Partial Inhibition of Respiratory Complex I on Brain Mitochondrial H_2O_2 Production in Different Respiratory States

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Running Title: Inhibition of complex I and mitochondrial H_2O_2 production

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ABSTRACT

The aim of this work was to characterize the effects of partial inhibition of respiratory complex I by rotenone on H_2O_2 production by rat brain mitochondria in different respiratory states. Flow cytometric analysis of membrane potential in isolated mitochondria indicated that rotenone leads to uniform respiratory inhibition when added to a suspension of these organelles. When mitochondria were incubated in the presence of a low concentration of rotenone (10 nM) and NADH-linked substrates, oxygen consumption was reduced from 45.9 ± 1.0 to 26.4 ± 2.6 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ and from 7.8 ± 0.3 to 6.3 ± 0.3 nmol $O_2 \cdot mg^{-1} \cdot min^{-1}$ in respiratory states 3 (ADP-stimulated respiration) and 4 (resting respiration), respectively. Under these conditions, mitochondrial H_2O_2 production was stimulated from 12.2 ± 1.1 to 21.0 ± 1.2 pmol $H_2O_2 \cdot mg^{-1} \cdot min^{-1}$ and 56.5 ± 4.7 to 95.0 ± 11.1 pmol $H_2O_2 \cdot mg^{-1} \cdot min^{-1}$ in respiratory states 3 and 4, respectively. Similar results were observed when comparing mitochondrial preparations enriched with synaptic or nonsynaptic organelles or when 1-methyl-4-phenylpyridinium ion (MPP^+) was used as a respiratory complex I inhibitor. Rotenone-stimulated H_2O_2 production in respiratory states 3 and 4 was associated with a high reduction state of endogenous nicotinamide nucleotides. In succinate-supported mitochondrial respiration, where most of the mitochondrial H_2O_2 production relies on electron backflow from complex II to complex I, low rotenone concentrations inhibited H_2O_2 production. Rotenone had no effect on mitochondrial elimination of micromolar concentrations of H_2O_2. The present results support the conclusion that partial complex I inhibition may result in mitochondrial energy crisis and oxidative stress, the former being predominant under oxidative phosphorylation and the latter under resting respiration conditions.

Keywords: Brain mitochondria; Parkinson´s disease; reactive oxygen species; respiratory chain; rotenone.

INTRODUCTION

Parkinson's disease (PD) is characterized by a preferential and progressive loss of midbrain dopaminergic neurons in the substantia nigra pars compacta [1]. Postmortem studies of patients who died with apparently sporadic PD have provided evidence that this brain region displays a pronounced deficiency of mitochondrial respiratory chain complex I [2, 3]. Complex I deficiency has also been found in skeletal muscle, platelets and lymphoblasts of PD patients [4, 5]. This mitochondrial alteration may be the consequence of high levels of somatic mutations in mitochondrial DNA in PD patients [6, 7].

The importance of complex I deficiency in PD pathology is attested to by the fact that humans exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), whose metabolite 1-methyl-4-phenylpyridine (MPP^+) is taken up by dopaminergic neurons and inhibits complex I [8], develop parkinsonism [9]. Moreover, chronic and systemic treatment of rodents with rotenone, a classical complex I inhibitor, recapitulates many features of PD, including degeneration of nigral dopaminergic neurons, accumulation of Lewy bodies and Lewy neurites, activation of microglia, tissue oxidative damage and behavioral deficits [10, 11, 12, 13].

The mechanisms involved in dopaminergic neurodegeneration linked to complex I inhibition probably involve increased mitochondrial reactive oxygen species (ROS) generation and/or cellular energy crisis due to reduced respiratory capacity [10, 13, 14, 15, 16, 17, 18, 19]. Indeed, marked stimulation of H_2O_2 production by isolated brain mitochondria has been reported when complex I is extensively inhibited by rotenone [20, 21, 22, 23] through a mechanism linked to an increased NAD(P)H/$NAD(P)^+$ ratio [24]. Studies conducted in intact synaptosomes and cells also revealed increased production of ROS when complex I was partially inhibited by rotenone [16, 17, 25, 26]. Although the increased generation of ROS seems to be a determinant of rotenone-induced cellular fate under several experimental conditions, a limitation in respiratory capacity was shown to be the determining factor for rotenone toxicity in an acute glutamate excitotoxicity model using

cultures of rat cerebellar granule neurons [17]. This evidence indicates an important relationship between cellular energy status and rotenone-induced neurodegeneration.

The aim of the present study was to gain a better understanding of the impact of partial inhibition of complex I on mitochondrial oxidative phosphorylation and H_2O_2 production. Isolated rat brain mitochondria were energized either by NADH-linked substrates or succinate. The impact of partial inhibition of complex I by rotenone or MPP^+ on oxygen consumption and H_2O_2 production and elimination in respiratory states 3 (ADP-stimulated respiration) and 4 (resting respiration) was evaluated. We also compared mitochondrial preparations enriched with synaptic or nonsynaptic organelles.

MATERIAL AND METHODS

Materials

The majority of the chemicals used, including ADP, decylubiquinone, L-glutamic acid, malic acid, MPP^+ iodide, β-NADH dipotassium salt, oligomycin, rotenone, Percoll, succinic acid and tetraphenylboron sodium salt (TPB^-), were obtained from Sigma-Aldrich (St Louis, MO, USA). The enzyme horseradish peroxidase type VI-A was also obtained from Sigma-Aldrich. ADP, glutamate, malate and succinate solutions were prepared by dissolving the respective acids in water and adjusting the pH to 7.2 with KOH. The fluorescent probes Amplex Red and tetramethylrhodamine methyl ester (TMRM) were purchased from Invitrogen (Carlsbad, CA, USA).

Animals

Three- to four-month-old male Wistar rats (*Rattus novergicus albino*) were obtained from the State University of Campinas (UNICAMP) Animal Breeding Center. The animals were kept under standard laboratory conditions (20°C-22°C and 12 h/12 h light/dark cycle) with free access to a standard diet (Labina/Purina, Campinas, SP, Brazil) and tap water. Animal experiments followed UNICAMP guidelines for the use of animals in experimental studies and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication N^o 85-23, revised in 1996).

Isolation of rat brain mitochondria

Brain mitochondria were isolated from two rats, as described by Mirandola et al. [27], using digitonin to permeabilize the synaptosomal plasma membrane [28]. The cerebellum and the caudal part of brainstem were removed by a coronal cut at the level of mid pons and the remaining tissue was used for mitochondrial isolation. This preparation

results in a mixture of synaptic and nonsynaptic mitochondria. The mitochondria were stored on ice until the start of the experiments, which were conducted no more than five hours after the mitochondria had been isolated. Unless specified in the text or figure legends, this was the methodology used to obtain isolated brain mitochondria.

Isolation of synaptic and nonsynaptic rat brain mitochondria

Synaptic and nonsynaptic rat brain mitochondria were isolated using the Percoll gradient separation method and digitonin to permeabilize the synaptosomal plasma membrane [29]. Briefly, two rats were sacrificed by decapitation, and their brains were rapidly removed (within 1 minute) and placed into 10 mL ice-cold isolation buffer I containing 225 mM mannitol, 75 mM sucrose, 1 mM K^+-EDTA and 10 mM HEPES-K^+ (pH 7.4). The cerebellum and the caudal part of brainstem were removed by a coronal cut at the level of mid pons and the remaining tissue was used for mitochondrial isolation. The brains were cut into small pieces using surgical scissors and extensively washed in isolation buffer. The tissue was then manually homogenized in a glass Dounce homogenizer with both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 minutes at 1,300 x *g* in a Beckman Coulter JA-25.50 fixed angle rotor (Beckman, Palo Alto, CA). The supernatant (S1) was removed and centrifuged for 10 minutes at 20,000 x *g*. The resulting supernatant was discarded, and the pellet was resuspended in 15% Percoll and layered on a preformed Percoll gradient (40% and 23%) [30]. The Percoll solutions (pH 7.2) were prepared in isolation buffer I. After centrifugation at 30,000 x *g* for 10 minutes, the fractions enriched with nonsynaptic and synaptic mitochondria were located at the interfaces of the lower two and top two layers, respectively. These fractions were removed and separately resuspended in 10 mL of isolation buffer I and centrifuged for 10 minutes at 17,000 x *g*. The supernatants were discarded, and the loose pellets resuspended in 10 mL of isolation buffer II (225 mM mannitol, 75 mM sucrose, 200 µM K^+-EDTA and 10 mM HEPES-K^+, pH 7.4) containing 0.05% BSA and 10 µL of 10% digitonin. For standardization purposes, digitonin was used

in the isolation procedures of both synaptic and nonsynaptic mitochondria. The homogenates were then centrifuged for 10 minutes at 10,000 x *g*. The supernatants were discarded, and the pellets were resuspended in isolation buffer II and centrifuged again for 10 minutes at 10,000 x *g*. The resulting supernatants were discarded, and the final pellets were washed and resuspended in isolation buffer III (225 mM mannitol, 75 mM sucrose and 10 mM HEPES-K^+, pH 7.4) at an approximate protein concentration of 20-30 mg/mL. The entire procedure was performed at 4ºC.

Protein determination

The protein contents of the mitochondrial suspensions were determined using the Biuret assay in the presence of 0.2% deoxycholate [31]. Bovine serum albumin was used as a standard.

Incubation conditions

Measurements of mitochondrial membrane potential, oxygen consumption, H_2O_2 production and elimination and the redox state of NAD(P) were performed at 37°C with continuous magnetic stirring in a standard reaction medium containing 130 mM KCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM Hepes buffer (pH 7.2), 200 µM EGTA and 0.05% BSA. Other additions are described in the methodologies and/or figure legends. Except for the O_2 consumption measurements, which were performed in a 1.0 mL glass chamber, a 2 mL final volume was used in all the experiments performed in cuvettes.

Flow cytometric analysis of mitochondrial membrane potential

Flow cytometric analyses of mitochondrial membrane potential were performed as described by Mattiasson [32], with minor modifications. Nonsynaptic mitochondria (0.15 mg/mL) were incubated for 10 min in standard reaction medium containing 5 mM

glutamate, 5 mM malate and 10 nM TMRM. Measurements were taken immediately in a flow cytometer BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a 488 nm argon laser and Cell-Quest software (version 4.1). At least 10,000 events (mitochondria) were analyzed using the FL2-H channel (585/42 nm) [32].

Oxygen uptake measurement

Oxygen consumption was measured polarographically using a Clark-type electrode (Hansatech Instruments Limited, Norfolk, UK). Mitochondrial suspensions (0.5 mg/mL) were incubated in standard reaction medium in a sealed glass chamber equipped with a magnetic stirrer and kept at 37°C. The initial molecular oxygen (O_2) concentration in the reaction medium was considered to be 200 µmol/L [33].

Measurement of H_2O_2 production

Production of H_2O_2 by isolated brain mitochondria was monitored by measuring the conversion of Amplex Red to highly fluorescent resorufin in the presence of added horseradish peroxidase. Mitochondrial suspensions (0.5 mg/mL) were incubated in standard reaction medium containing 10 µM Amplex Red and 1 U/mL horseradish peroxidase. Fluorescence was monitored over time with a temperature-controlled spectrofluorometer (F-4010, Hitachi Ltd, Tokyo, Japan) using excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 3 nm. An external calibration curve was generated using different quantities of H_2O_2.

Measurement of H_2O_2 elimination

Elimination of H_2O_2 by brain mitochondria was measured as described by Tretter et al. [34], with minor modifications. Brain mitochondria (1.0 mg) were added to 2 mL of standard reaction medium containing 5 mM malate and 5 mM glutamate at 37 °C under

constant stirring. ADP (800 µM) or ADP plus 1 µg/mL oligomycin were added to obtain respiratory states 3 and 4, respectively. Partial inhibition of complex I was achieved with both 10 and 100 nM rotenone. After 2 min preincubation, 5 µM H_2O_2 was added and 50 µL samples were taken at different time periods (10, 60 and 150 sec) during incubation. The samples were added to cuvettes containing standard reaction medium (2 mL) and 10 µM Amplex Red plus 1 U/mL horseradish peroxidase (HRP). Resorufin fluorescence was measured after 1 min using excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 3 nm. For calibration, different concentrations of H_2O_2 were added to the standard reaction medium without mitochondria and samples were collected immediately.

Redox state of mitochondrial NAD(P)

Isolated rat brain mitochondria were suspended (0.5 mg/mL) in standard reaction medium, and the changes in the redox state of NAD(P) were monitored in a spectrofluorometer (Shimadzu RF-5301PC) using excitation and emission wavelengths of 366 and 450 nm, respectively, and slit widths of 15 and 20 nm [35]. Of note, only the reduced forms of NAD(P) exhibit a strong endogenous fluorescence signal. No attempt was made to discriminate between NADH and NADPH. To correct the continuous decrease in mitochondrial light scattering during the experimental time period, the fluorescence values from each experiment were subtracted from a blank consisting of mitochondria incubated in the presence of 0.5 µM FCCP. In the presence of FCCP, NAD(P)H is expected to be oxidized and the residual fluorescence can be considered background. As a reference, known amounts of NADH were added to the reaction medium in the absence of mitochondria.

Determination of complex I activity

Mitochondrial complex I activity was determined according to Long et al. [36]. After preincubation of the nonsynaptic and synaptic mitochondrial suspensions (0.15 mg/mL) in the presence of different rotenone concentrations for 12 min, samples (27 µL) were taken and added to 173 µL of the assay medium containing 25 mM K_2HPO_4 (pH 7.4), 3 mg/mL BSA, 60 µM decylubiquinone, 80 µM NADH and 2 µM antimycin A. Absorbance of NADH was followed at 340 nm for 6 min in a *PowerWave XS2* plate reader (BioTek Intruments, Winooski, VT, USA).

Statistical analysis

The experimental data were analyzed by repeated-measures one-way ANOVA followed by a post hoc Bonferroni test. H_2O_2 production rates were transformed to $\log_{10}$ values before statistical analysis. The data are presented as mean ± standard error (SEM) of at least four independent experiments. The level of significance was set at $p < 0.05$. All data were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

RESULTS

Effects of rotenone on brain mitochondrial respiration and H_2O_2 production in the presence of NADH-linked substrates

Initially, experiments were conducted to determine whether the addition of a low concentration of rotenone to the mitochondrial suspension would result in similar inhibition of respiratory chain complex I in all the mitochondria. For this purpose, mitochondria were incubated for 10 min in the presence of rotenone, and membrane potential was estimated by flow cytometry. Percoll-purified nonsynaptic mitochondria were employed to yield a suspension of organelles of uniform size. The results shown in **Figure 1** indicate that a low concentration of rotenone (20 nM) results in a small decrease in membrane potential in the entire mitochondrial population. A sub-population of depolarized mitochondria, indicative of extensive complex I inhibition, was not observed under this condition. When the mitochondria were incubated in the presence of a high concentration of rotenone (2000 nM), the whole population of organelles was depolarized, as occurred with the mitochondria incubated in the presence of the protonophore FCCP. These results indicate that treatment of a mitochondrial suspension with rotenone at nanomolar concentrations results in uniform inhibition of respiratory chain complex I in the whole mitochondrial population.

Next, the effect of different concentrations of rotenone (6, 10 and 50 nM) on oxygen consumption and H_2O_2 production by mitochondria during respiratory states 3 (ADP-stimulated respiration) and 4 (resting respiration) was tested using NADH-linked substrates. In both respiratory states, increasing concentrations of rotenone result in progressive inhibition of mitochondrial oxygen consumption and increased production of H_2O_2 (**Figure 2**). For example, when mitochondria were incubated in the presence of 10 nM rotenone (20 pmol rotenone · mg protein^{-1}), oxygen consumption was reduced from 45.9 ± 1.0 to 26.4 ± 2.6 nmol O_2 · mg^{-1} · min^{-1} and from 7.8 ± 0.3 to 6.3 ± 0.3 nmol O_2 · mg^{-1} · min^{-1} in respiratory states 3 and 4, respectively. Under these conditions, mitochondrial

H_2O_2 production was stimulated from 12.2 ± 1.1 to 21.0 ± 1.2 pmol $H_2O_2 \cdot mg^{-1} \cdot min^{-1}$ and 56.5 ± 4.7 to 95.0 ± 11.1 pmol $H_2O_2 \cdot mg^{-1} \cdot min^{-1}$ in respiratory states 3 and 4, respectively.

Following this, the redox status of nicotinamide nucleotides (NAD(P)) was evaluated by monitoring changes in their endogenous fluorescence in response to different concentrations of rotenone. **Figure 3** shows the effect of two rotenone concentrations (10 and 100 nM) on the redox status of mitochondrial nicotinamide nucleotides in respiratory states 3 and 4 (panels **A** and **B**, respectively). Panel **A** shows that the addition of ADP to brain mitochondria results in a drop in fluorescence, indicating nicotinamide nucleotide oxidation due to the faster flow of electrons through the respiratory chain during oxidative phosphorylation. Under these conditions the addition of rotenone elicited a large increase in fluorescence, indicating NAD(P) reduction. When rotenone was added to mitochondria under resting respiration (in the presence of oligomycin; **panel B**), a slight but significant increase in reduction of nicotinamide nucleotides was observed. These results indicate a relationship between nicotinamide nucleotide redox status and rotenone-stimulated mitochondrial H_2O_2 production under our experimental conditions.

Effects of MPP^+ on brain mitochondrial respiration and H_2O_2 production

Figure 4 shows the effect of complex I inhibitor MPP^+ on brain mitochondrial oxygen consumption (**panel A**) and H_2O_2 production (**panel B**) in respiratory states 3 and 4. A concentration of 25 µM of MPP^+ was used to achieve partial complex I inhibition. TPB^- (10 µM) was also present to potentiate the effect of MPP^+ on complex I inhibition [37]. In the absence of TPB^-, a nearly 25-fold higher concentration of MPP^+ is required to achieve a similar degree of respiratory inhibition in brain mitochondria [38]. In addition, in the presence of TPB^- a faster and more uniform pattern of respiratory inhibition by MPP^+ was obtained (data not shown). As with rotenone, partial complex I inhibition by MPP^+ resulted in slower mitochondrial respiration rates (**panel A**) and increased H_2O_2 production (**panel B**) in respiratory states 3 and 4.

Comparative study of the effects of rotenone on synaptic and nonsynaptic mitochondrial respiration and H_2O_2 production

The results in **Figure 5A** show that synaptic mitochondria were more sensitive than nonsynaptic mitochondria to rotenone-induced inhibition of oxygen consumption. While 10 nM rotenone inhibited oxygen consumption by 51.2 ± 1.4% in state 3 in synaptic mitochondria, only 17.0 ± 1.0% inhibition was observed in nonsynaptic organelles. In state 4, 10 nM rotenone significantly inhibited oxygen consumption only in synaptic mitochondria. These results correlate with measurements of complex I activity in both mitochondrial preparations; complex I activity was inhibited to a greater degree by rotenone in synaptic mitochondria than in nonsynaptic organelles (**Figure 5B**). However, both mitochondrial preparations showed a similar pattern of rotenone-stimulated H_2O_2 production (**Figure 5C**). These results show that although synaptic mitochondria were more sensitive to rotenone-induced complex I inhibition, H_2O_2 production levels were not significantly higher in these mitochondria than in nonsynaptic organelles. Control experiments (N = 3) conducted with synaptic mitochondria in the presence and absence of exogenous cytochrome *c* (5 µM) did not indicate that their lower respiratory rate when compared with the nonsynaptic mitochondria is due to the loss of cytochrome *c*.

Inhibitory effect of rotenone on H_2O_2 production by succinate-supported mitochondria

It is well known that isolated brain mitochondria incubated in the presence of succinate and absence of rotenone produce high levels of H_2O_2 because of electron backflow from complex II to complex I [20, 21, 39]. **Figure 6** depicts the results of mitochondrial H_2O_2 production when succinate-supported mitochondria were incubated in the presence of low concentrations of rotenone (6, 10 and 50 nM) to partially inhibit complex I. Under these experimental conditions, increasing rotenone concentrations

progressively inhibited H_2O_2 production in respiratory state 4, while no significant effect of rotenone was observed on this parameter in respiratory state 3.

Lack of inhibitory effect of rotenone on mitochondrial elimination of H_2O_2

Brain mitochondria rapidly degrade H_2O_2 at micromolar concentrations [34, 40]. To determine whether the higher production of H_2O_2 caused by rotenone could be at least partially explained by a reduced mitochondrial capacity to remove H_2O_2, the next experiment measured H_2O_2 elimination by brain mitochondria in respiratory states 3 and 4. The results in **Figure 7** show that rotenone does not significantly impair mitochondrial capacity to remove H_2O_2 under the conditions tested.

DISCUSSION

The findings reported here make a valuable contribution to the understanding of the impact of partial inhibition of complex I on brain mitochondrial bioenergetics and redox balance. Uniform and partial respiratory inhibition was achieved by adding rotenone at nanomolar concentrations to suspensions of isolated brain mitochondria supported by NADH-linked substrates (**Figure 1**). Our results indicate that partial complex I inhibition results in a similar percentage increase in H_2O_2 production by mitochondria in respiratory states 3 and 4 (**Figure 2**), e.g., 10 nM rotenone stimulated H_2O_2 production by 74.7 ± 11.2 % and 67.4 ± 11.4 % in respiratory states 3 and 4, respectively. Considering that basal production of H_2O_2 in respiratory state 3 is four- to five fold lower than in state 4, the impact of partial complex I inhibition on the amount of H_2O_2 produced is much more marked in respiratory state 4. Importantly, no alterations in the mitochondrial property of H_2O_2 elimination were detected under our experimental conditions (**Figure 7**), indicating that the differences in mitochondrial H_2O_2 release under these conditions (respiratory states 3 and 4 and the presence of rotenone at nanomolar concentrations) are due to changes in H_2O_2 production rather than impairment of antioxidant systems.

Regarding mitochondrial oxygen consumption, the effect of rotenone at 10 nM was more pronounced in respiratory state 3, resulting in 42.5 ± 5.1 % inhibition of oxygen consumption, while in state 4 only 20.0 ± 1.8 % inhibition was detected. The minor effect of rotenone in respiratory state 4 was expected, as there is greater spare respiratory capacity in this state.

Under our experimental conditions, using NADH-linked substrates, rotenone-stimulated mitochondrial superoxide production originates from the flavin mononucleotide (FMN)-containing NADH binding site of complex I (site I_F) and from upstream dehydrogenases [41]. Starkov and Fiskum [24] showed that H_2O_2 production by brain mitochondria supported by NADH-linked substrates is closely related to membrane potential and levels of endogenous reduced NAD(P). Our results also show a correlation

between H_2O_2 production and the reduced levels of endogenous NAD(P) when the respiratory chain was partially inhibited by rotenone (**Figure 3**). In addition, in state 4 respiration, when most NAD(P) was already reduced, a small increase in the reducing state of this nucleotide by the addition of rotenone resulted in a large increase in H_2O_2 production, probably reflecting the reduction of site I_F under this condition.

Comparison of partial inhibition of mitochondrial respiration by rotenone and by MPP^+ shows that stimulation of H_2O_2 production was similar for both compounds (**Figure 4**). This result may be explained by the fact that MPP^+ inhibits complex I by interacting at or near the same site as rotenone [42]. Although these toxins show similar effects when applied to isolated brain mitochondria, differences may be expected when they are used in intact cells. While rotenone is highly cell-permeable, MPP^+ is selectively taken up by dopaminergic neurons via the dopamine transporter [43, 44]. A recent study also presented evidence that MPP^+ may be produced inside mitochondria from dopaminergic neurons incubated with MPTP [45].

Parkinson´s disease has been correlated with mitochondrial respiratory chain deficiency in neurons and/or glial cells [46, 47, 48]. In order to determine whether rotenone could have a different impact on organelles from these cells, we conducted experiments comparing the effect of rotenone on nonsynaptic mitochondria, which originate mainly from the neuronal cell body and glial cells, and on synaptic mitochondria, which are predominantly neuronal and presynaptic in origin [29, 49]. The results indicate that synaptic mitochondria are more sensitive than nonsynaptic organelles to respiratory inhibition by rotenone (**Figure 5**). This observation is correlated with higher inhibition of complex I activity by rotenone in synaptic mitochondria. Interestingly, while synaptic mitochondria were more sensitive than nonsynaptic organelles to rotenone-induced respiratory inhibition, similar stimulation by rotenone of H_2O_2 production was observed in both mitochondrial preparations. Although we have no definite explanation for this greater sensitivity of synaptic mitochondria to rotenone, these results could reflect differences in either mitochondrial constituents or bioenergetics [50]. The higher sensitivity of synaptic

mitochondria to rotenone may contribute to the predominantly neuronal cell death [10, 11] when rodents are treated with this toxin. In addition, Davey et al. [51, 52], by conducting titrations of complex I activity and oxygen consumption in brain mitochondria incubated with rotenone, showed that complex I from synaptic mitochondria has lower spare respiratory capacity than nonsynaptic mitochondria.

In succinate-supported mitochondria under non-phosphorylating conditions, most of the H_2O_2 production relies on electron backflow from complex II to complex I [20, 21, 53, 54, 55], with superoxide production at a putative ubiquinone reduction site of complex I (I_Q site) [55, 56]. Under these conditions, extensive inhibition of complex I by rotenone strongly inhibits H_2O_2 production by brain mitochondria [20, 21, 53]. Here we show that an inhibitory effect on H_2O_2 production by succinate-supported mitochondria also occurs when complex I is partially inhibited by rotenone (**Figure 6**). Our results suggest that partial complex I inhibition is enough to decrease superoxide production at site I_Q, probably by decreasing electron backflow from complex II to complex I. In aggrement with these results, Chinta et al [38] showed that H_2O_2 production caused by reverse electron transfer to complex I during succinate-suported respiration was 50% lower in brain mitochondria from a *pcd8*-mutated mouse that exhibit a 40-50% reduction in complex I level. The relevance of electron backflow for mitochondrial ROS production under physiological conditions remains unclear [41]. A recent publication indicates that site I_Q is not a significant source of superoxide/H_2O_2 in isolated muscle mitochondria supported by glutamate plus malate under non-phosphorylating conditions [56]. The rotenone effect in in situ brain mitochondrial H_2O_2 production also seems to occur predominantly at site I_F, since several reports show that rotenone increases, rather than decreases, H_2O_2 production when applied to intact cells and synaptosomes [16, 17, 25, 26, 57, 58].

In conclusion, our results support the proposal that partial inhibition of respiratory chain complex I can contribute to neurodegeneration by compromising energy metabolism and/or promoting oxidative stress [10, 13, 14, 15, 16, 17, 18, 19]. An energy crisis can be expected to predominate under conditions with high metabolic demands such as in acute

excitotoxicity [17] and excessive opening of L-type calcium channels [59]. However, during basal metabolic conditions spare respiratory capacity can be expected in neurons with partial respiratory complex I inhibition. Under these circumstances, oxidative stress will be favored as a cause of neurodegeneration. In fact, oxidative damage has been demonstrated to be associated with the dopaminergic neurodegeneration observed in PD [13, 60, 61, 62, 63]. Factors not investigated in the present study, including the availability of substrates and impairment of the activity of Krebs cycle enzymes, such as α-ketoglutarate dehydrogenase [64], may also contribute to determine whether spare respiratory capacity and/or oxidative stress are determinants of the neurodegeneration associated with partial inhibition of respiratory complex I.

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FIGURES AND LEGENDS

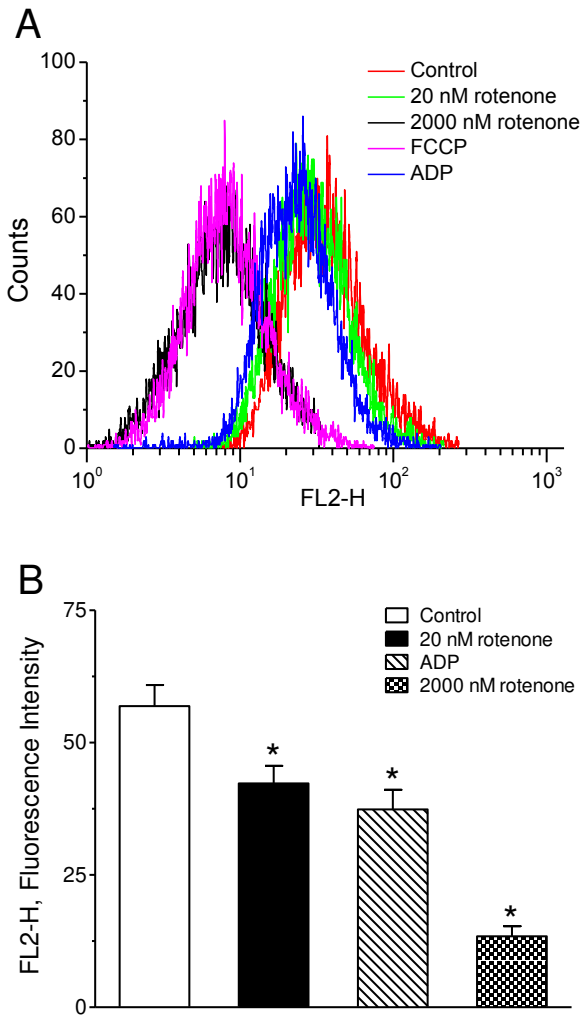

Figure 1 - Flow cytometric analysis of brain mitochondrial membrane potential: effect of rotenone. Nonsynaptic mitochondria (0.15 mg/mL) were preincubated for 10 min at 37°C in standard reaction medium containing 10 nM TMRM, 5 mM glutamate and 5 mM malate without other additions (Control) or in the presence of either 20 nM rotenone, 2000 nM rotenone, 200 nM FCCP or 1 mM ADP, as indicated in the figure. **Panel A:** Representative fluorescence histograms of TMRM-loaded mitochondria. **Panel B:** Mean fluorescence intensity determined using the FL2-H channel. Bar values represent the mean ± SEM of at least six independent experiments. *P<0.01 vs. Control.

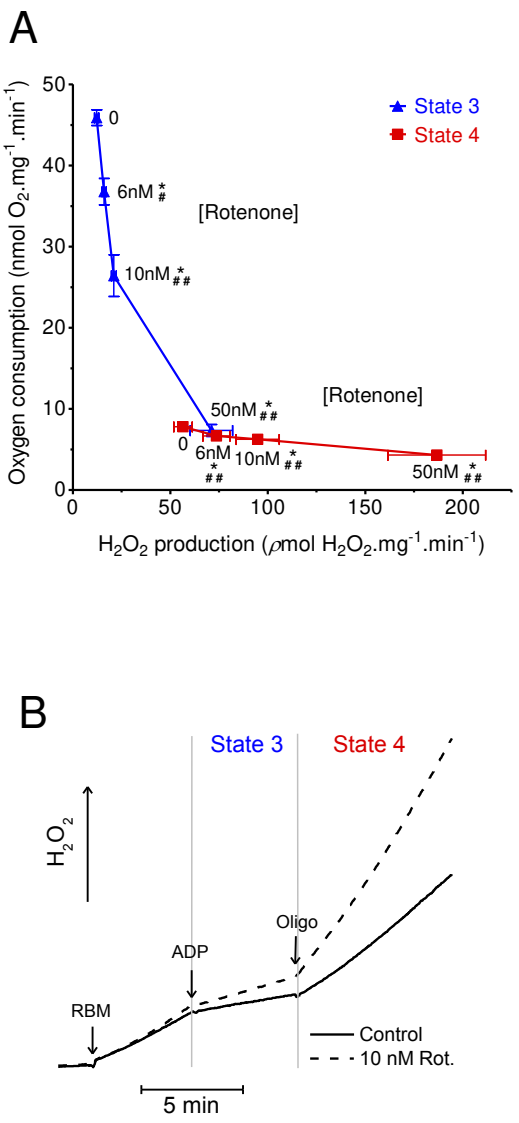

Figure 2 - Effects of rotenone on brain mitochondrial oxygen consumption and H_2O_2 production in the presence of NADH-linked substrates in different respiratory states. Isolated rat brain mitochondria (0.5 mg/mL) were incubated at 37°C in standard reaction medium containing 5 mM glutamate and 5 mM malate in the absence or presence of different concentrations of rotenone. ADP (800 µM) and 1 µg/mL oligomycin were added to the reaction medium to obtain respiratory states 3 and 4, respectively. **Panel A:** Effects of different concentrations of rotenone (6, 10 and 50 nM) on oxygen consumption and H_2O_2 production by mitochondria in respiratory states 3 and 4. **Panel B:** Representative traces of mitochondrial H_2O_2 production under control conditions (*full line*) and in the presence of 10 nM rotenone (Rot.; *dashed line*). Where indicated by the arrows, 0.5 mg/mL brain mitochondria (RBM), 800 µM ADP and 1 µg/mL oligomycin (Oligo) were added. The values in Panel A represent the mean ± SEM of at least four independent experiments performed in duplicate. $^{*}P<0.01$ vs. control for oxygen consumption. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. control for H_2O_2 production.

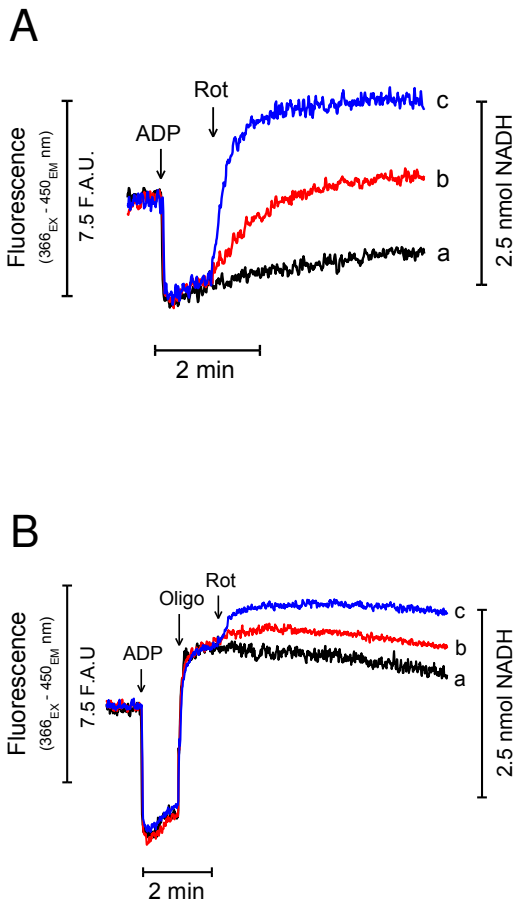

Figure 3 - Rotenone reduces endogenous NAD(P) in brain mitochondria in respiratory states 3 (Panel A) and 4 (Panel B). Brain mitochondria (0.5 mg/mL) were incubated at 37°C in standard reaction medium containing 5 mM glutamate and 5 mM malate. Where indicated by the arrows, ADP (800 µM), 1 µg/mL oligomycin (Oligo) and rotenone (Rot) were added to the experiments. *Traces b* and *c* represent experiments with the addition of 10 and 100 nM rotenone, respectively. *Traces a* represent control experiments without rotenone. Traces are representative of at least four independent experiments. F.A.U.: Fluorescence arbitrary units.

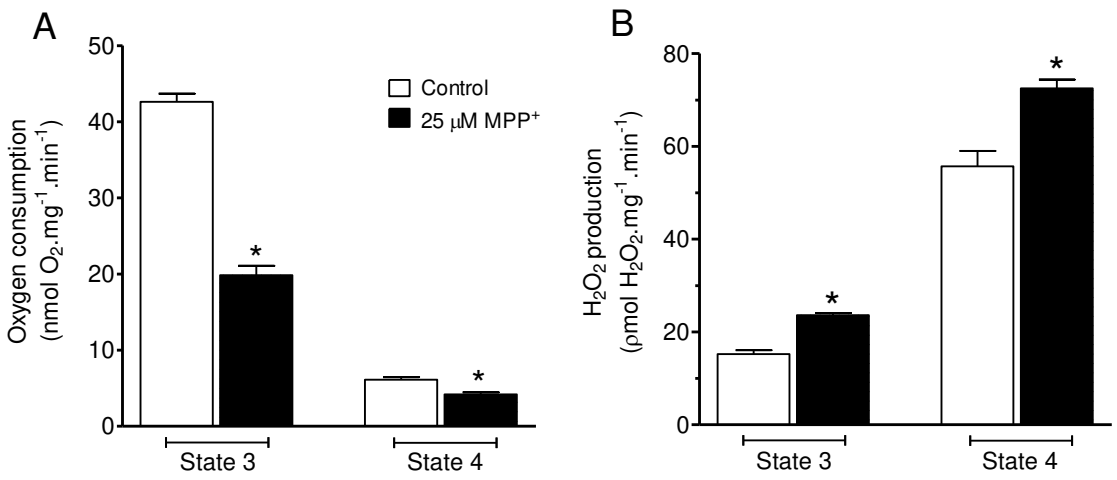

Figure 4 - Partial inhibition of mitochondrial oxygen consumption by MPP^+ stimulates H_2O_2 production. Isolated brain mitochondria (0.5 mg/mL) were incubated at 37°C in standard reaction medium containing 5 mM glutamate and 5 mM malate under control conditions or in the presence of 25 μM MPP^+ plus 10 μM TPB^-. ADP (800 μM) and 1 μg/mL oligomycin were added to the reaction medium to obtain respiratory states 3 and 4, respectively. **Panel A:** Inhibitory effect of MPP^+ on mitochondrial oxygen consumption. **Panel B:** Stimulatory effect of MPP^+ on mitochondrial H_2O_2 production. The values in Panels A and B represent the mean ± SEM of at least four independent experiments performed in duplicate. **P*<0.01 vs. respective control.

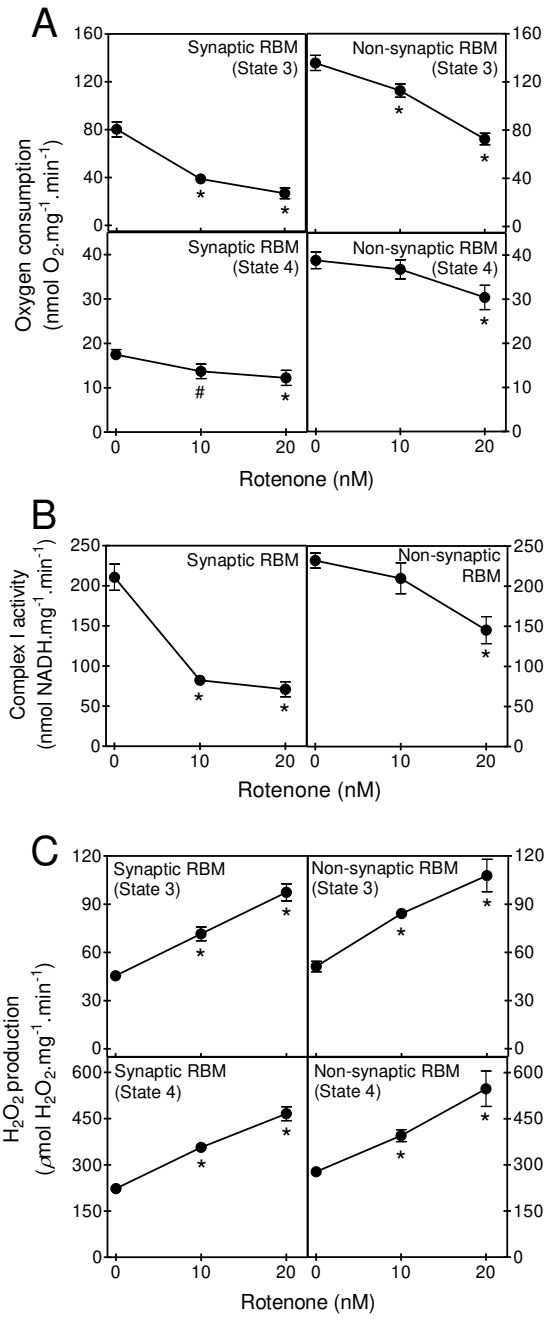

Figure 5 - **Effects of rotenone on oxygen consumption (Panel A), complex I activity (Panel B) and H_2O_2 production (Panel C) by synaptic and nonsynaptic mitochondria**. Fractions enriched with synaptic or nonsynaptic mitochondria (0.15 mg/mL) were incubated at 37°C in standard reaction medium containing 5 mM glutamate and 5 mM malate in the absence or presence of different concentrations of rotenone (10 and 20 nM). ADP (800 µM) and 1 µg/mL oligomycin were added to the reaction medium to obtain respiratory states 3 and 4, respectively. To determine complex I activity, samples of mitochondrial suspension were taken after oxygen consumption measurements. The values in Panels A-C represent the mean ± SEM of at least four independent experiments performed in duplicate. $^{\#}P<0.05$, $^{*}P<0.01$ vs. respective control.

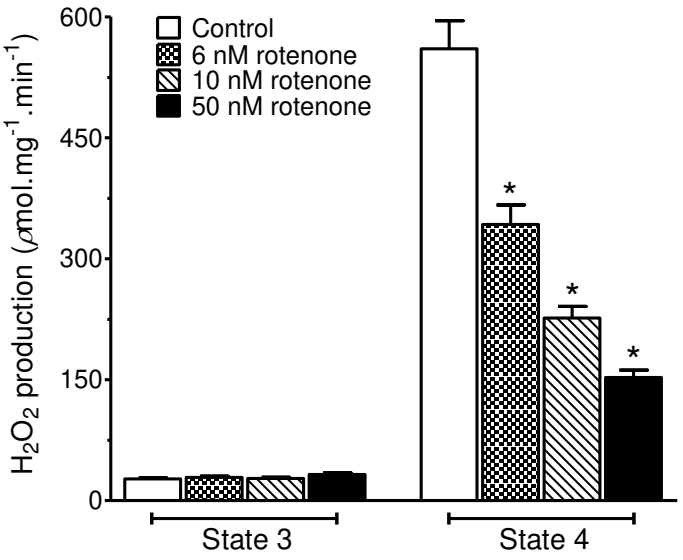

Figure 6 - Partial inhibition of mitochondrial complex I by rotenone inhibits H_2O_2 production by succinate-supported brain mitochondria. Brain mitochondria (0.5 mg/mL) were incubated at 37°C in standard reaction medium containing 5 mM succinate. ADP (800 µM) and 1 µg/mL oligomycin were added to the reaction medium to obtain respiratory states 3 and 4, respectively. H_2O_2 production was measured under control conditions and in the presence of different concentrations of rotenone (6, 10 and 50 nM). The values represent the mean ± SEM of at least four independent experiments performed in duplicate. *P<0.01 vs. respective control.

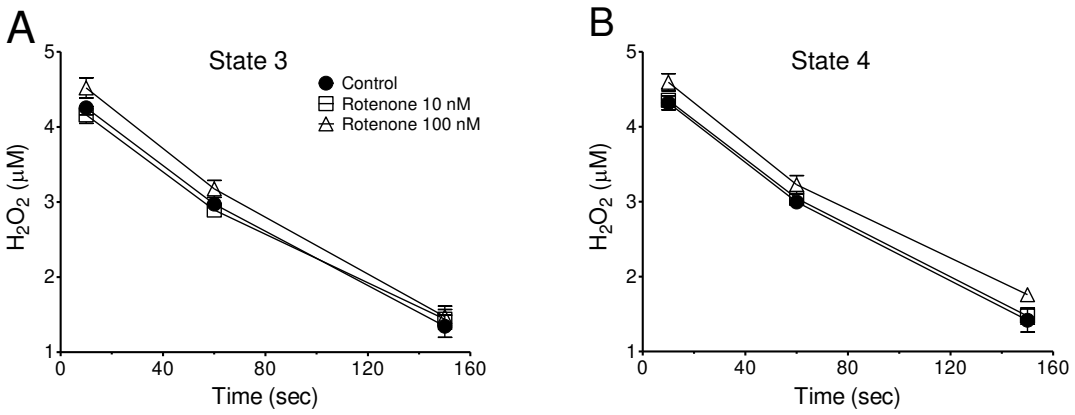

Figure 7 - H_2O_2 elimination by brain mitochondria. Brain mitochondria (0.5 mg/mL) were preincubated for 2 min at 37°C in standard reaction medium containing 5 mM glutamate and 5 mM malate under control conditions or in the presence of 10 and 100 nM rotenone. ADP (800 µM) or ADP plus 1 µg/mL oligomycin were also present in the reaction medium to obtain respiratory states 3 and 4, respectively. H_2O_2 (5 µM) was added and 50 µL samples were taken at 10, 60 and 150 s to measure H_2O_2 elimination. The values represent the mean ± SEM of at least four independent experiments performed in duplicate.

3.2. Capítulo II

"Rotenone exerts similar effects on brain mitochondrial oxygen consumption and H_2O_2 production in adult and old rats"

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(Manuscrito a ser submetido à publicação)

Rotenone exerts similar effects on brain mitochondrial oxygen consumption and H_2O_2 production in adult and old rats

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Running Title: Effects of rotenone on brain mitochondria from old rats

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ABSTRACT

Aging is the main risk factor associated with Parkinson´s disease. Chronic and systemic treatment of rodents with rotenone, a classical inhibitor of mitochondrial respiratory complex I, results in neurochemical, behavioral and neuropathological features of Parkinson´s disease. The aim of the present study was to evaluate whether brain mitochondria from old rats (24 months old) would be more susceptible to rotenone-induced inhibition of oxygen consumption and increased generation of H_2O_2 than organelles from adult rats (3-4 months old). Isolated brain mitochondria were incubated in the presence of different rotenone concentrations (5, 10 and 100 nM), and oxygen consumption and H_2O_2 production were measured during respiratory states 3 (ADP-stimulated respiration) and 4 (resting respiration). Respiratory state 3 and citrate synthase activity were, respectively, 21.0 ± 3.3% and 17.0 ± 5.4 % lower in mitochondria from old rats. Both types of mitochondria showed similar sensitivity to rotenone-induced inhibition of oxygen consumption in respiratory state 3, with an IC_{50} of 7.8 ± 0.4 and 6.5 ± 0.5 nM for adult and old rats, respectively. Similarly, H_2O_2 production rates by mitochondria from adult and old rats were dose-dependently stimulated to the same extent by increasing concentrations of rotenone. We conclude that rotenone exerts similar effects on oxygen consumption and H_2O_2 production by isolated brain mitochondria from adult and old rats. Therefore, aging does not increase the mitochondrial H_2O_2 generation in response to complex I inhibition.

Keywords: Aging; Brain mitochondria; Hydrogen peroxide; Oxygen consumption; Parkinson´s disease; Rotenone.

INTRODUCTION

Parkinson´s disease (PD) is a chronic progressive neurodegenerative disorder characterized by a preferential loss of dopaminergic neurons in the substantia nigra pars compacta (Braak et al., 2004). The majority of people who develop this neurodegenerative disorder are 60 years of age or older. In addition to altered protein handling and inflammation, mitochondrial dysfunction and oxidative stress have been implicated as major factors in the pathogenesis of PD (for recent review, see Dexter and Jenner, 2013). The most marked mitochondrial alteration in PD is a partial loss of respiratory complex I activity (for review see Schapira, 2011), probably associated with age-dependent accumulation of somatic mutations in mtDNA (Bender et al., 2006; Schon et al., 2012).

Worthy of note is the fact that humans exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), whose metabolite 1-methyl-4-phenylpyridine (MPP^+) is taken up by dopaminergic neurons and inhibits complex I, develop parkinsonism (Langston et al., 1983). Similarly, there is an increased risk for PD with exposure to organic pesticides such as rotenone, a classical inhibitor of respiratory complex I (Dhillon et al., 2008). In light of these evidences, many studies have employed MPTP or rotenone to generate experimental animal models with neuropathological characteristics and behavior deficits that resemble those of PD (Przedborski et al., 2004; Martinez and Greenamyre, 2012). Interestingly, aging has been associated with enhanced dopaminergic neurodegeneration induced by these toxins (Irwin et al., 1992; Phinney et al., 2006). Neurodegeneration mediated by partial respiratory complex I inhibition most likely arises from increased production of reactive oxygen species in the mitochondrial respiratory chain and/or impaired cellular energy state (Barrientos and Moraes, 1999; Yadava and Nicholls, 2007; Sanders and Timothy Greenamyre, 2013).

Bearing in mind the association of PD with aging and impaired respiratory complex I function, the aim of the present study was to test the hypothesis that isolated mitochondria from old rat brains are more susceptible to rotenone-induced inhibition of oxygen consumption and/or increased generation of H_2O_2.

MATERIALS AND METHODS

Materials

Most of the chemicals used, including acetyl coenzyme A sodium salt, ADP, bovine serum albumin, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), L-glutamic acid, malic acid, oligomycin, oxaloacetic acid and rotenone, were obtained from Sigma-Aldrich (St Louis, MO, USA). Type VI-A enzyme horseradish peroxidase was also obtained from Sigma-Aldrich. ADP, glutamate and malate solutions were prepared by dissolving the respective acids in water and adjusting the pH to 7.2 with KOH. The fluorescent probe Amplex Red was purchased from Invitrogen (Carlsbad, CA, USA).

Animals

Male Wistar rats (*Rattus norvegicus albino*) were obtained from the State University of Campinas (UNICAMP) Animal Breeding Center. To age the rats for the old rats group, animals were kept under standard laboratory conditions (20-22°C and 12 h/12 h light/dark cycle) with a standard diet (Labina/Purina, Campinas, SP, Brazil) and tap water *ad libitum* until they reached 24 months. Six apparently healthy old rats were then used for the experiments, which followed UNICAMP guidelines for the use of animals in experimental studies and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication N^{o} 85-23, revised in 1996).

Isolation of Rat Forebrain Mitochondria

Rat brain mitochondria were isolated as described by Mirandola et al. (2008), using digitonin to permeabilize synaptosomal plasma membrane (Rosenthal et al., 1987). This was done simultaneously for adult and old rats. Briefly, one adult rat (3-4 months old) and one old Wistar rat were killed by decapitation and their brains rapidly removed (within 1 min) and put into 15 mL of ice-cold "isolation buffer I" (225 mmol/L mannitol, 75 mmol/L sucrose, 1 mmol/L K^+-EGTA, 0.1% BSA and 10 mmol/L K^+-Hepes, pH 7.4). The cerebellum and the caudal part of brainstem were removed by a coronal cut at the level of mid pons and the remaining tissue was used for mitochondrial isolation. This preparation results in a mixture of synaptic and nonsynaptic mitochondria. The tissues were cut into small pieces using surgical scissors and extensively washed. The small pieces were then manually homogenized in a *Dounce* homogenizer with a loose-fitting glass pestle followed by a tight-fitting pestle. The homogenates were centrifuged for 3 min at 2000 x *g* in a Beckman JA-25.50 rotor (Beckman, Palo Alto, CA, USA), and the supernatants were recentrifuged for 8 min at 12000 x *g*. The pellets were resuspended in 10 mL "isolation buffer I" containing 20 µL of 10% digitonin and recentrifuged for 10 min at 12000 x *g*. The supernatants and the upper layers of the pellets were discarded, and the dark pellets were resuspended in "isolation buffer II" (225 mmol/L mannitol, 75 mmol/L sucrose, 0.1% BSA and 10 mmol/L K^+-Hepes, pH 7.4). These homogenates were then centrifuged for 10 min at 12000 x *g*. The supernatants were discarded and the final pellets were gently washed and resuspended in "isolation buffer II" at a protein concentration of 40-50 mg/mL and kept on ice. The mitochondrial protein yield per gram of brain tissue was slightly higher for the old rats (12.1 ± 0.9 and 15.0 ± 1.1 mg of protein/g of brain tissue for the adult rats and old rats, respectively; $p<0.05$).

Protein determination

The protein contents of the mitochondrial suspensions were determined using the Biuret assay in the presence of 0.2% deoxycholate (Kaplan and Pedersen, 1983) and fatty acid-free bovine serum albumin (BSA) as a standard.

Oxygen Uptake Measurement

Oxygen consumption was measured polarographically using a Clark-type electrode in a 1.4 mL sealed glass cuvette equipped with a magnetic stirrer and kept at 37°C. The initial molecular oxygen (O_2) concentration in the reaction medium was considered to be 200 µmol/L. The experiments to measure oxygen consumption by isolated mitochondria (0.5 mg/mL) were carried out at 37°C in standard reaction medium containing 130 mM KCl, 10 mM K^+-HEPES (pH 7.2), 2 mM KH_2PO_4, 1 mM $MgCl_2$, 0.05% BSA and 200 µM EGTA. NADH-linked substrates (5 mM malate and 5 mM glutamate) were added to the reaction medium prior to the addition of mitochondrial suspension. In all the experiments, 800 µM ADP was used to stimulate respiration by oxidative phosphorylation (respiratory state 3) and 1 µg/mL oligomycin was used to inhibit respiration and obtain the resting state (respiratory state 4).

Hydrogen Peroxide Production Measurement

H_2O_2 production by isolated brain mitochondria was monitored by measuring the conversion of Amplex Red to highly fluorescent resorufin in the presence of added horseradish peroxidase. Mitochondrial suspensions (0.5 mg/mL) were incubated in standard reaction medium containing 5 mM malate, 5 mM glutamate, 10 µM Amplex Red

and 1 U/mL horseradish peroxidase. Fluorescence was monitored over time with a temperature-controlled spectrofluorometer (F-4010, Hitachi Ltd, Tokyo, Japan) using excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 3 nm. An external calibration curve prepared with different quantities of H_2O_2 was used.

Citrate Synthase Activity

Conversion of oxaloacetate and acetyl-CoA to citrate and CoA-SH catalyzed by citrate synthase was monitored by measuring the colorimetric product thionitrobenzoic acid (Shepherd and Garland, 1969). Mitochondrial suspensions (20 µg) were incubated at 37°C in 2 mL of the reaction medium containing 50 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 250 µM oxaloacetate, 50 µM acetyl-CoA and 100 µM DTNB. The increase in absorbance at 412 nm was followed for 5 min in a UV-1800 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Statistical Analysis

The experimental data were analyzed by repeated-measures one-way ANOVA, followed by the post hoc Bonferroni test. H_2O_2 production rates were transformed to $\log_{10}$ values before statistical analysis. The data are presented as mean ± standard error (SEM) of six independent experiments. The level of significance was set at $P<0.05$. All data were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

RESULTS

Effects of rotenone on oxygen consumption and H_2O_2 production by mitochondria from adult and old rat brains

To evaluate the effect of rotenone on oxygen consumption and H_2O_2 production by mitochondria from adult and old rat brains, isolated organelles were incubated in the presence of different concentrations of this toxin (5, 10 or 100 nM) and the NADH-linked substrates malate and glutamate. Under these experimental conditions, rotenone-stimulated mitochondrial superoxide ($O_2^{\cdot-}$) production originates in the flavin mononucleotide (FMN)-containing NADH binding site of complex I (site I_F) and from upstream dehydrogenases (Brand, 2010; Figueira et al., 2013).

Figure 1A depicts the results of representative experiments to measure oxygen consumption by mitochondria isolated from adult and old rat brains in the absence of rotenone. Oxygen consumption rates were determined in respiratory states 3 (ADP-stimulated respiration) and 4 (resting respiration induced by the ATP synthase inhibitor oligomycin). **Figure 1C** shows the oxygen consumption rates for mitochondria from adult and old rat brains in the presence of different concentrations of rotenone in respiratory states 3 and 4. Under control conditions (i.e. absence of rotenone), oxygen consumption in respiratory state 3 was 21.0 ± 3.3% lower in brain mitochondria from old rats than in mitochondria from adult rats. Mitochondria from adult and old rats showed a similar pattern of rotenone-induced inhibition of oxygen consumption in respiratory state 3, with an IC_{50} of 7.8 ± 0.4 and 6.5 ± 0.5 nM, respectively. Oxygen consumption did not differ between adult and old rats in respiratory state 4 under control conditions. Only the highest tested concentration of rotenone (i.e. 100 nM) led to significant inhibition of oxygen consumption in respiratory state 4 in mitochondria from both groups.

Representative measurements of H_2O_2 production by isolated mitochondria from adult and old rat brains in the absence of rotenone are shown in **Figure 1B**. In respiratory state 3, mitochondrial H_2O_2 production was significantly lower than in respiratory state 4. **Figure 1D** shows H_2O_2 production rates for mitochondria from adult and old rat brains in the presence of different rotenone concentrations in respiratory states 3 and 4. Under control conditions (absence of rotenone), H_2O_2 production by mitochondria from old rats was 15.6 ± 4.0% and 15.0 ± 4.3% lower in respiratory states 3 and 4, respectively, than the corresponding figures for mitochondria from adult rats. A similar pattern of stimulation of H_2O_2 production by rotenone was observed in mitochondria from adult and old rats.

Effects of rotenone on citrate synthase-normalized oxygen consumption and H_2O_2 production by brain mitochondria from adult and old rats

Mitochondria from old rats exhibited lower activity of the mitochondrial biomarker citrate synthase (Larsen et al., 2012) than those from adult rats (0.93 ± 0.03 and 1.12 ± 0.04 U/mg protein, respectively; $P<0.05$). **Figure 2** shows oxygen consumption and H_2O_2 production rates normalized to citrate synthase activity (CS). When the data were normalized to CS, the differences in oxygen consumption and H_2O_2 production rates between mitochondria from adult and old rats were smaller than when the rates were normalized to total sample protein. In the absence of rotenone, oxygen consumption in respiratory state 3 was only 4.8 ± 1.8 % lower for old rats than for adult rats. In respiratory state 3, mitochondria from old rats showed slower oxygen consumption rates (9.2 ± 1.9 % lower) in the presence of 5 nM rotenone than mitochondria from adult rats. Rotenone concentrations of 10 and 100 nM inhibited mitochondrial oxygen consumption to the same extent in adult and old rats. No differences between adult and old rats were observed for H_2O_2 production under control conditions and in the presence of different concentrations of rotenone.

DISCUSSION

The results reported here show that nanomolar concentrations of rotenone stimulate H_2O_2 production to the same extent in brain mitochondria from adult and old rats (**Figure 1D**). Dose-dependent rotenone-stimulated mitochondrial H_2O_2 production and dose-dependent inhibition of oxygen consumption were observed under these conditions (**Figure 1C**). Similar IC_{50} values were observed for the effect of rotenone on oxygen consumption by brain mitochondria from adult and old rats. In apparent contrast with this last result, Genova et al. (1997) showed a higher IC_{50} for rotenone inhibition of respiratory complex I activity in nonsynaptic, but not in synaptic, mitochondria from old rats than in mitochondria from adult controls. Similarities between the inhibitory effects of rotenone on oxygen consumption by brain mitochondria from adult and old rats under our conditions may be explained by the presence of both synaptic and nonsynaptic mitochondria in our preparation of isolated organelles. Moreover, nonsynaptic mitochondria exhibit respiratory complex I activity that considerably exceeds the electron-flow demand of this complex during oxidative phosphorylation (Davey et al., 1997), suggesting that partial inhibition of complex I may not be directly reflected in inhibition of nonsynaptic mitochondrial respiration.

Under control conditions (i.e., in the absence of rotenone), brain mitochondria from old rats showed a 21.0 ± 3.3% decrease in oxygen consumption rate in respiratory state 3 compared with the corresponding figure for adult rats (**Figure 1C**). Interestingly, this difference was reduced to 4.8 ± 1.8% when the respiratory data were normalized to citrate synthase activity (**Figure 2A**). This finding agrees with data in the literature indicating that brain mitochondrial bioenergetic functions decay with aging (for a review see Boveris and Navarro, 2008; Paradies et al., 2011). Also worthy of note is that under control conditions similar H_2O_2 production rates by brain mitochondria from adult and old rats were observed when the data were normalized to citrate synthase activity (**Figure 2B**) or to oxygen

consumption rates (results not shown). Previous reports have shown increased H_2O_2 production by brain mitochondria from old rats (Sohal et al., 1994; Petrosillo et al., 2013), but these authors' experimental conditions were quite different from ours. Instead of using succinate as the electron donor for respiratory complex II, we used NADH-linked substrates, providing the entry of electrons at complex I level; also, we used digitonin to permeabilize synaptosomes, while other authors used intact synaptosomes (Kilbride et al., 2008; Lores-Arnaiz and Bustamante, 2011). These are fundamental experimental differences that may affect ROS measurement because (***1***) brain mitochondrial H_2O_2 production is approximately sevenfold higher with succinate than with NADH-linked substrates (Fornazari et al., 2008); (***2***) the contribution of each superoxide production site to reactive oxygen species generation may vary considerably in the respiratory chain depending on whether NADH-linked substrates or succinate are used (Brand, 2010; Figueira et al., 2013); and (***3***) H_2O_2 measurements in synaptosomes may be affected by non-mitochondrial $O_2^{\cdot-}/H_2O_2$ generation sites and antioxidant systems. Finally, differences in animal strains, incubation procedures and methods used to detect $O_2^{\cdot-}/H_2O_2$ are all potential factors that may explain divergent results between this and previous studies.

Our findings show that the effects exerted by rotenone on oxygen consumption and H_2O_2 production by isolated brain mitochondria are similar for adult and old rats. Our results do not support the notion that the association of aging, complex I deficiency and dopaminergic neurodegeneration in Parkinson´s disease is due to greater mitochondrial $O_2^{\cdot-}/H_2O_2$ release following complex I inhibition by toxins in the elderly. Indeed, dopaminergic neurodegeneration in PD is a complex process that may involve cumulative complex I defects with aging (Bender et al., 2006; Schon et al., 2012) and greater vulnerability of older neurons to energy impairment and/or reactive oxygen species generated by partial complex I inhibition.

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FIGURES AND LEGENDS

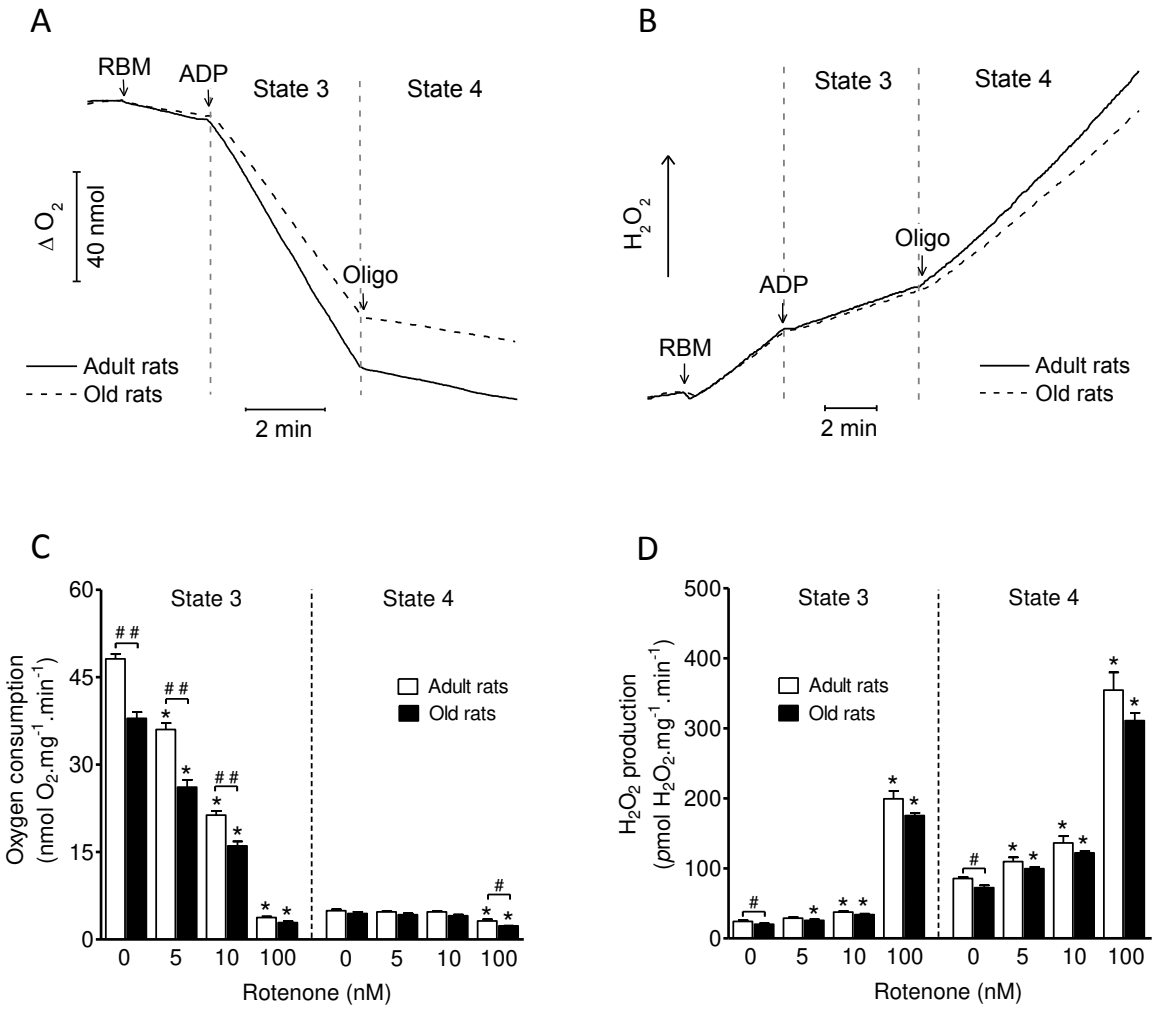

Figure 1 - Effects of rotenone on oxygen consumption and H_2O_2 production by brain mitochondria from adult and old rats in different respiratory states. Isolated brain mitochondria (0.5 mg/mL) from adult and old rats were incubated at 37°C in standard reaction medium containing 5 mM glutamate and 5 mM malate in the absence or presence of different concentrations of rotenone. ADP (800 µM) and 1 µg/mL oligomycin were added to the reaction medium to obtain respiratory states 3 and 4, respectively. **Panels A** and **B:** Representative traces of oxygen consumption (**Panel A**) and H_2O_2 production (**Panel B**) by brain mitochondria from adult (*solid lines*) and old rats (*dashed lines*) under control conditions. Where indicated by the arrows, 0.5 mg/mL brain mitochondria (RBM), 800 µM ADP and 1 µg/mL oligomycin (Oligo) were added. **Panels C** and **D:** Effects of different concentrations of rotenone (5, 10, and 100 nM) on oxygen consumption (**Panel C**) and H_2O_2 production (**Panel D**) by brain mitochondria from adult and old rats in respiratory states 3 and 4. The values in Panels C and D represent the mean ± SEM of six independent experiments. $^{*}P<0.01$ vs. respective control (adult or old rats in the absence of rotenone) for oxygen consumption and H_2O_2 production. $^{\#}P<0.05$, $^{\#\#}P<0.01$, difference between adult and old rats at the same rotenone concentration.

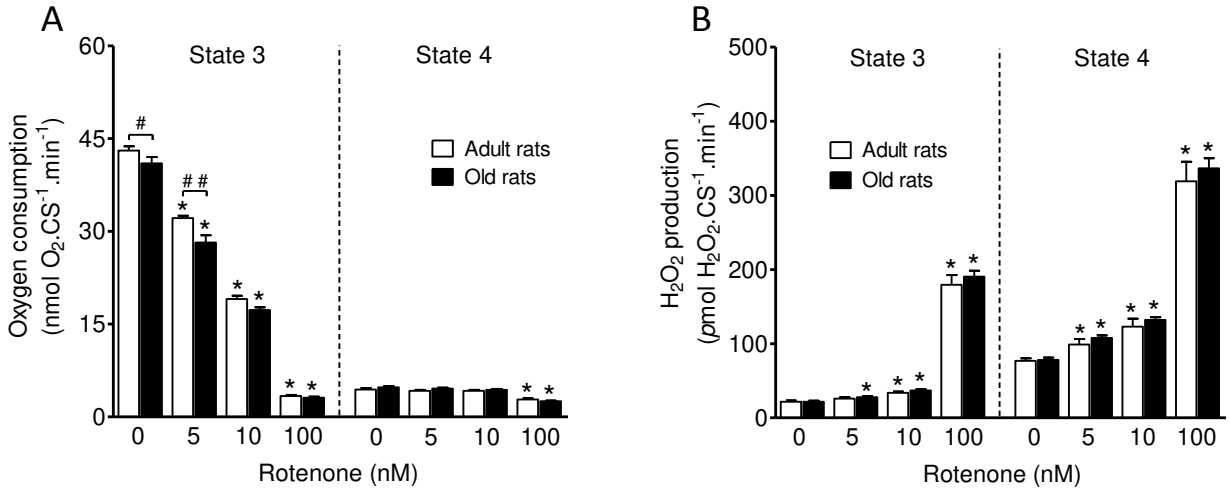

Figure 2 - Effects of rotenone on citrate synthase-normalized oxygen consumption and H_2O_2 production by brain mitochondria from adult and old rats in different respiratory states. All rates were normalized to citrate synthase activity (CS) measured in duplicate in each mitochondrial suspension. Oxygen consumption and H_2O_2 production data were derived from Figure 1. The mean ± SEM citrate synthase activity in mitochondrial suspensions from adult and old rats was 1.12 ± 0.04 and 0.93 ± 0.03 U/mg, respectively. **P*<0.01 vs. respective control (adult or old rats in the absence of rotenone) for oxygen consumption and H_2O_2 production. $^{\#}P$<0.05, $^{\#\#}P$<0.01, difference between adult and old rats at the same rotenone concentration.

3.3. Capítulo III

"Inibição parcial do consumo de O_2 e estímulo da liberação de H_2O_2 por rotenona em sinaptossomas intactos."

MATERIAIS E MÉTODOS DO CAPÍTULO III

Animais de experimentação – Ratos machos *Wistar*, com 3-4 meses de idade, foram utilizados nos experimentos. Os animais foram fornecidos pelo biotério central da UNICAMP (Centro Multidisciplinar para Investigação Biológica - CEMIB) e foram alojados em gaiolas mantidas em sala climatizada a 22 ± 2°C, com ciclo 12 horas claro e escuro, com livre acesso à água e ração Nuvilab CR-1 irradiada da Nuvital (Dois Vizinhos, Paraná, Brasil). Os procedimentos experimentais utilizados neste estudo foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas (CEUA/UNICAMP), por estarem de acordo com os Princípios Éticos na Experimentação Animal.

Preparo de sinaptossomas intactos – O preparo de sinaptossomas intactos foi realizado seguindo o método de gradiente de Percoll proposto por Dunkley, Jarvie e Robinson (2008) com algumas modificações. O cérebro de um rato foi removido e extensivamente lavado em meio de homogeneização contendo sacarose 320 mM, EDTA (K^+) 1 mM, ditiotreitol (DTT) 0,25 mM e Tris 10 mM (pH 7,4), seguido por homogeneização em homogeneizador do tipo *Dounce*. O homogenato foi centrifugado a 1.000 x *g* em rotor *Beckman 25.50 JA* (Beckman, Palo Alto, CA, USA). O sobrenadante foi distribuído em seis tubos sobre gradiente de Percoll nas concentrações de 3, 10 e 23%, com auxílio de bomba peristáltica. Após serem centrifugados por 5 minutos a 31.000 x *g*, houve formação de três bandas entre os gradientes de Percoll, onde a fração enriquecida em sinaptossomas intactos encontrava-se sobre o gradiente de Percoll 23% (Fração 3). A Fração 3, contendo sinaptossomas, foi ressuspensa em meio contendo sacarose 320 mM, EDTA (K^+) 4 mM e Tris 10 mM (pH 7,4) e centrifugada a 20.000 x *g* por 30 minutos. O *pellet* formado foi ressuspenso em meio iônico contendo NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1 mM, $NaHCO_3$ 5 mM, HEPES(K^+) 20 mM (pH 7,4), Na_2HPO_4 1,2 mM e glicose 10 mM, e novamente centrifugado a 18.000 x *g* por 10 minutos. O *pellet* formado foi ressuspenso em meio iônico, obtendo-se uma concentração de proteínas entre 35 e 55 mg/mL e mantido em banho de gelo.

Medida do consumo de O_2 - O consumo de O_2 por suspensões de sinaptossomas isolados foi monitorado utilizando-se um oxígrafo de alta resolução OROBOROS *Oxygraph-2K* equipado com agitador magnético, no qual os dados reproduzidos, foram calculados pelo software específico do aparelho, *DatLab 4* (Oroboros Instruments, Innsbruck, Áustria). Sinaptossomas intactos (0,5 mg/mL) foram incubados em meio de reação contendo NaCl 120 mM, KCl 3,5 mM, K_2HPO_4 0,4 mM, $MgSO_4$ 2 mM, Na_2SO_4 1,2 mM, $CaCl_2$ 1,3 mM, HEPES(K^+) 20 mM (pH 7,4) e BSA 4 mg/mL e foram mantidos em câmaras fechadas (2 mL), sob agitação constante de 750 r.p.m. e termostatizada a 37°C. A solubilidade do oxigênio molecular (O_2) no meio de reação foi considerada de 200 µmol x L^{-1}, a 37°C e 1 atm (Robinson e Cooper, 1970). Glicose 15 mM foi utilizada como substrato metabólico nas suspensões de sinaptossomas intactos e FCCP 4 µM foi adicionado para se obter o estado de alta fosforilação oxidativa e oligomicina 1 µg/mL foi adicionada para se obter o estado de repouso.

Medida da liberação de H_2O_2 – A liberação de H_2O_2 foi mensurada fluorimetricamente através da conversão de Amplex Red (2,5 µM), na presença de peroxidase raiz forte (HRP, 1 U/mL), à um composto altamente fluorescente, resorufina (Zhou *et al.,* 1997). A fluorescência foi monitorada ao longo do tempo em espectrofluorímetro F-4010 (Hitachi Ltda, Tókio, Japão) para suspensão de sinaptossomas, usando comprimentos de onda de excitação e emissão de 563 e 587 nm, respectivamente, com largura das fendas de 3 nm. Sob essas condições, um aumento linear na fluorescência indicou um aumento da taxa de liberação de H_2O_2 sinaptossomal. Os dados foram calculados através de um experimento controle na presença de catalase 2.125 U/mL, obtendo-se uma taxa de H_2O_2 sinaptossomal sensível à catalase. Para a conversão de unidades arbitrárias de fluorescência (U.A.F.) em valores absolutos de H_2O_2, foram realizadas curvas de calibração, através da adição de concentrações conhecidas de H_2O_2.

Análise estatística – Os dados experimentais foram analisados utilizando-se o software *GraphPad Prism 5* (GraphPad Software, La Jolla, CA), pelo teste de análise de variância one-way ANOVA, seguido pelo teste *post-hoc* de *Bonferroni* com múltiplas comparações. Os dados de produção de H_2O_2 foram transformados para valores de $\log_{10}$ antes da análise estatística. Os dados estão representados como a média ± erro padrão (± S.E.M.) de oito experimentos independentes. O nível de significância foi considerado $P<0,05$.

RESULTADOS DO CAPÍTULO III

Utilizamos sinaptossomas, para que pudéssemos avaliar o impacto de uma inibição parcial do consumo de O_2 por rotenona na liberação de H_2O_2 em um modelo *in situ* de mitocôndrias de cérebro (**Figura 1 do Capítulo III)**.

Realizamos ensaios de consumo de O_2 em oxígrafo de alta resolução (Oroboros-2k), no qual incubamos os sinaptossomas intactos (0,5 mg/mL) em meio de reação para sinaptossomas (pH 7,4) e glicose 15 mM foi utilizada como substrato metabólico. Os experimentos foram conduzidos sob agitação constante, a 37°C.

Na **Figura 1A**, avaliamos o estado respiratório em que os sinaptossomas intactos se encontravam. Para isso, utilizamos o FCCP (4 µM), um desacoplador da cadeia transportadora de elétrons, para estimular a respiração mitocondrial e oligomicina (1 µg/mL) para simular o estado respiratório de repouso (estado 4). Observamos que, o consumo de O_2 basal dos sinaptossomas (3,6 ± 0,28 nmol.mg^{-1}.min^{-1}) encontrava-se próximo ao estado respiratório 4 (linha azul, 2,7 ± 0,21 nmol.mg^{-1}.min^{-1}). A adição de rotenona 10 nM (linha vermelha) resultou em uma diminuição do consumo de O_2 no estado respiratório 3 (linha preta) de 6,8 ± 0,44 nmol.mg^{-1}.min^{-1} para 5,7 ± 0,49 nmol.mg^{-1}.min^{-1} de O_2, o que equivale a uma inibição de 17% no consumo de O_2.

Utilizamos o sistema Amplex Red e HRP para mensurar o estímulo de liberação de H_2O_2 nos sinaptossomas intactos, porém, devido ao pequeno estímulo de liberação de H_2O_2 obtido, foi necessário reajustar a concentração de Amplex Red. Testamos diferentes concentrações de Amplex Red (dados não mostrados) e obtivemos melhores resultados com uma concentração de 2,5 µM.

O estímulo da produção de H_2O_2 em sinaptossomas foi avaliado no estado respiratório basal, visto que, não observamos diferenças na liberação de H_2O_2 no estado 3 e no estado 4 (dados não mostrados). Na **Figura 1B**, podemos observar que rotenona 10 nM foi capaz de estimular a liberação de H_2O_2 no estado respiratório basal de 28,8 ± 1,79 para 34,67 ± 2,44 *p*mol.mg^{-1}.min^{-1}.

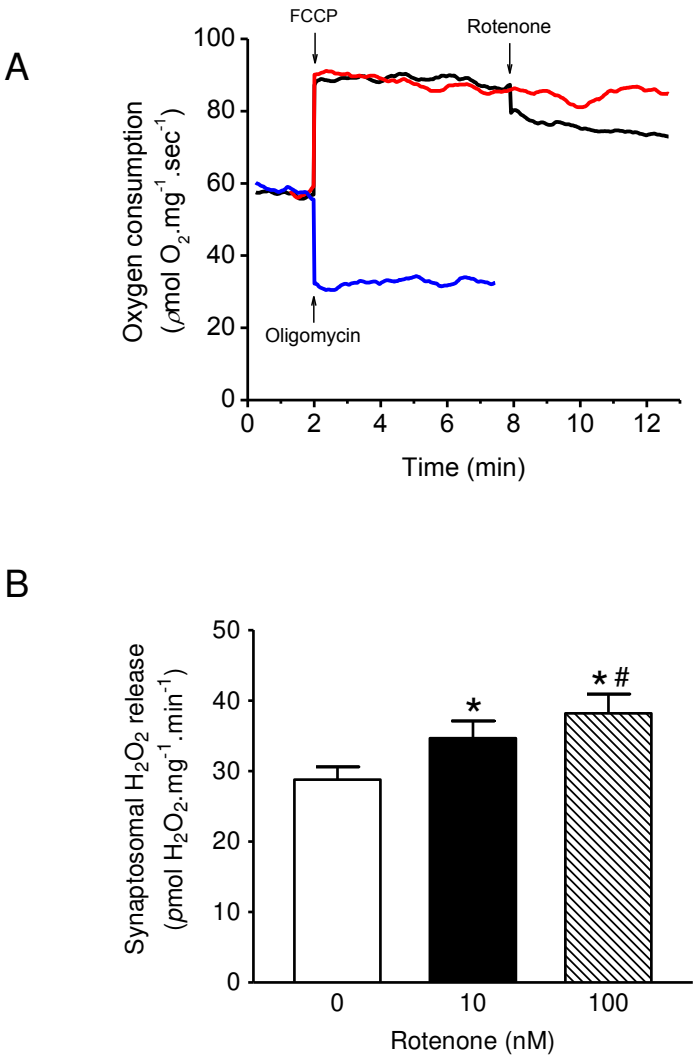

FIGURA 1 DO CAPÍTULO III – Inibição parcial do consumo de oxigênio por rotenona em sinaptossomas intactos estimula a liberação de H_2O_2. Sinaptossomas intactos (0,5 mg/mL) foram incubados em meio de reação contendo NaCl 120 mM, KCl 3,5 mM, K_2HPO_4 0,4 mM, $MgSO_4$ 2 mM, Na_2SO_4 1,2 mM, $CaCl_2$ 1,3 mM, HEPES(K^+) 20 mM (pH 7,4) e BSA 4 mg/mL, a 37°C. Glicose 15 mM foi utilizada como substrato metabólico. Em **A**, o consumo de oxigênio foi monitorado em oxígrafo de alta resolução e FCCP 4 µM foi adicionado para estimular a respiração mitocondrial (em **vermelho**) e oligomicina 1 µg/mL foi adicionada para obtenção do consumo de oxigênio no estado de repouso (em **azul**). Rotenona 10 nM foi adicionada para avaliar a inibição no consumo de oxigênio no estado respiratório 3 (em **preto**). **Painel B**: Na presença de Amplex Red 2,5 µM e HRP 1 U/mL, diferentes concentrações de rotenona (10 e 100 nM) estimularam a liberação de H_2O_2 no estado respiratório basal. Os dados representam a média ± e.p.m. de oito experimentos independentes. $^*P<0,01$ vs. ausência de rotenona (controle) e $^{\#}P<0,01$ vs. rotenona 10 nM.

4. Discussão Geral

Os dados obtidos ao longo dessa Dissertação contribuem para o entendimento do impacto de uma inibição parcial do Complexo I da cadeia respiratória na bioenergética mitocondrial e no balanço redox em cérebro.

Os dados apresentados no ***Capítulo I*** mostraram que, a adição de rotenona em concentrações nanomolares a suspensões de mitocôndrias isoladas de cérebro de ratos resulta em uma inibição respiratória parcial e uniforme nas mitocôndrias (***Figura 1***, ***Capítulo I***). Nossos resultados indicaram que, uma inibição parcial do Complexo I resulta em um percentual similar de estímulo de produção de H_2O_2 por mitocôndrias nos estados respiratórios 3 e 4 (***Figura 2***, ***Capítulo I***), isto é, rotenona 10 nM estimulou a produção de H_2O_2 em 74,7 ± 11,2 % e 67,4 ± 11,4 % nos estados reapiratórios 3 e 4, respectivamente. Considerando que a produção basal de H_2O_2 no estado respiratório 3 é quatro a cinco vezes menor que no estado respiratório 4, uma inibição parcial do Complexo I resulta em uma maior quantidade de H_2O_2 produzida neste último estado respiratório. Outro dado importante observado, foi que não detectamos alterações na propriedade de detoxificação de H_2O_2 mitocondrial (***Figura 7***, ***Capítulo I***), indicando que as diferenças de liberação de H_2O_2 mitocondrial nas condições experimentais estudadas (estados respiratórios 3 e 4 e na presença de rotenona em concentrações nanomolares) são resultantes de alterações na produção de H_2O_2, e não nos sistemas antioxidantes mitocondriais.

Sob nossas condições experimentais, com a utilização de substratos geradores de NADH, o estímulo de produção de superóxido mitocondrial por rotenona origina-se na porção contendo flavina mononucleotídeo (FMN) do complexo respiratório I (sítio I_F) e em desidrogenases presentes no ciclo do ácido cítrico (BRAND, 2010). Starkov e Fiskum (2003) demonstraram que, a produção de H_2O_2 por mitocôndrias de cérebro energizadas com substratos geradores de NADH, está intimamente relacionada com o potencial de membrana mitocondrial e

níveis de NAD(P) endógenos reduzidos. Nossos resultados também demonstraram uma correlação entre a produção de H_2O_2 e os níveis de NAD(P) endógeno reduzidos, quando o Complexo I estava parcialmente inibido por rotenona (***Figura 3***, ***Capítulo I***). No estado respiratório 4, a maior parte do NAD(P) encontrava-se reduzida e ao se adicionar rotenona, pode-se notar a ocorrência de um pequeno aumento no estado reduzido deste nucleotídeo. O grande aumento da produção de H_2O_2 por rotenona nesta condição está provavelmente relacionado à redução de componentes do sítio I_F do Complexo I.

O estudo comparativo do efeito de uma inibição parcial da respiração mitocondrial por rotenona e por MPP^+ demonstrou que o estímulo da produção de H_2O_2 foi similar para ambos os compostos (***Figura 4***, ***Capítulo I***). Este resultado pode ser explicado pelo fato do MPP^+ inibir o Complexo I pela interação no mesmo sítio da rotenona ou em um sítio próximo (RAMSAY *et al.*, 1991).

A DP tem sido correlacionada com uma deficiência da cadeia respiratória mitocondrial em neurônios e/ou células da glia (HATTORI *et al.*, 1991; MCNAUGHT e JANNER, 1999; SCHAPIRA, 1999). A fim de determinar se a rotenona poderia ter um impacto diferente em organelas provenientes dessas células, realizamos experimentos comparando os efeitos de rotenona em mitocôndrias não-sinápticas, que são originadas principalmente de corpos celulares de neurônios e de células gliais e, em mitocôndrias sinápticas, que são originadas predominantemente de neurônios e de terminais pré-sinápticos (BROWN, SULLIVAN e GEDDES, 2006; SIMS e ANDERSON, 2008). Nossos resultados indicaram que, mitocôndrias sinápticas são mais sensíveis à inibição do consumo de O_2 por rotenona em relação às organelas não-sinápticas (***Figura 5***, ***Capítulo I***). Esta observação se correlacionou com uma extensa inibição da atividade do Complexo I por rotenona em mitocôndrias sinápticas. Notadamente, enquanto mitocôndrias sinápticas foram mais sensíveis à inibição da respiração por rotenona em relação às organelas não-sinápticas, um estímulo semelhante da produção de H_2O_2 por rotenona foi observado em ambas as preparações. Embora não tenhamos uma explicação para a maior sensibilidade de mitocôndrias

sinápticas à rotenona quanto comparadas às não-sinápticas, este resultado pode ser o reflexo de diferenças constitutivas e bioenergéticas entre estas mitocôndrias (NAGA, SULLIVAN e GEDDES, 2007). A alta sensibilidade de mitocôndrias sinápticas à rotenona pode contribuir para a morte de neurônios em roedores submetidos a tratamentos sistêmicos com esta toxina (BETARBET *et al*., 2000; GREENAMYRE *et al*., 2010).

Mitocôndrias isoladas de cérebro mantidas com o substrato succinato e em respiração de repouso (i.e., na ausência de fosforilação oxidativa) apresentam a maior parte da produção de H_2O_2 depende do fluxo reverso de elétrons do Complexo II para o Complexo I (CINO e DEL MAESTRO, 1989; VOTYAKOVA e REYNOLDS, 2001; LIU, FISKUM e SCHUBERT, 2002; PRYDE e HIRST, 2011; TREBERG, QUINLAN e BRAND, 2011). Nesta condição, é proposto que a produção de superóxido na cadeia repiratória ocorra, sobretudo, em um sítio de redução da ubiquinona no Complexo I (sítio I_Q) (TREBERG, QUINLAN e BRAND, 2011; ORR *et al*., 2013). Sob essas condições, uma inibição completa do Complexo I por rotenona inibe fortemente a produção de H_2O_2 em mitocôndrias de cérebro (CINO e DEL MAESTRO, 1989; VOTYAKOVA e REYNOLDS, 2001; LIU, FISKUM e SCHUBERT, 2002). Os resultados da ***Figura 6*** (***Capítulo I***) mostraram que um efeito inibitório na produção de H_2O_2 por mitocôndrias energizadas com succinato, também ocorre quando o Complexo I é parcialmente inibido por rotenona.

Os dados descritos no ***Capítulo I*** suportam a proposta de que, uma condição de inibição parcial do Complexo respiratório I pode contribuir para neurodegeneração por comprometimento do metabolismo energético e/ou promovendo estresse oxidativo (BARRIENTOS e MORAES, 1999; BETARBET *et al*., 2000; CHINOPOLOS e ADAM-VIZI, 2001; TESTA, SHERER e GREENAMYRE, 2005; YADAVA e NICHOLLS, 2007; PÖLTL *et al*., 2012; SANDERS e GREENAMYRE, 2013; SURMEIER e SCHUMACKER, 2013). Uma inibição parcial do Complexo I pode levar a uma crise energética em determinadas situações que requerem alta demanda energética, como em condições de

excitotoxicidade (YADAVA e NICHOLLS, 2007) e na abertura excessiva de canais de cálcio do tipo L em neurônios dopaminérgicos (CHAN *et al.*, 2007). No entanto, em neurônios em condições metabólicas basais, a capacidade respiratória pode ser suficiente para atender a demanda energética, mesmo com uma inibição parcial do Complexo I. Sob estas condições, o estresse oxidativo será favorecido e poderá ser o principal fator que levará à neurodegeneração. De fato, lesões oxidativas têm sido associadas à ocorrência da neurodegeneração dopaminérgica na DP (FAHN e COHEN, 1992; JENNER *et al.*, 1992; CAMARA, LESNEFSKY e STOWE, 2010; PERFEITO, CUNHA-OLIVEIRA e REGO, 2013; SANDERS e GREENAMYRE, 2013).

Em sinaptossomas intactos incubados na presença de rotenona 10 nM notou-se um estímulo de 20,2 ± 3,3 % da liberação H_2O_2 (***Figura 1B***, ***Capítulo III***). O estímulo de liberação de H_2O_2 foi de 32,2 ± 3,1 % na presença de rotenona 100 nM. Os dados descritos no ***Capítulo III*** suportam a ideia de que, uma inibição parcial da respiração em sinaptossomas intactos por rotenona, resulta em aumento da liberação de H_2O_2. Esta observação está de acordo com resultados presentes na literatura, que mostram um aumento da liberação de H_2O_2 por sinaptossomas na presença de concentrações nanomolares de rotenona (SIPOS, TRETTER e ADAM-VIZI, 2003).

Os resultados apresentados na ***Figura 1D*** do ***Capítulo II*** mostraram que concentrações nanomolares de rotenona estimulam na mesma extensão a produção de H_2O_2 por mitocôndrias isoladas de cérebro de ratos adultos e velhos. O estímulo da produção de H_2O_2 mitocondrial por rotenona foi dose-dependente, assim como, a inibição do consumo de O_2 nessas condições experimentais (***Figura 1C***, ***Capítulo II***). Valores similares de IC_{50} para rotenona foram obtidos para a inibição do consumo de O_2 por mitocôndrias isoladas de cérebro de ratos adultos e velhos.

Sob condições controle (na ausência de rotenona), mitocôndrias de cérebro de ratos velhos mostraram uma redução na velocidade de consumo de O_2 de 21,0 ± 3,3 % no estado respiratório 3, quando comparadas às organelas de ratos

adultos (***Figura 1C***, ***Capítulo II***). Esta diferença foi reduzida para 4,8 ± 1,8 % quando os dados de consumo de O_2 foram normalizados pelos valores da atividade da enzima citrato sintase (***Figura 2A***, ***Capítulo II***). Este achado está de acordo com dados na literatura, indicando que funções bioenergéticas mitocondriais em cérebro decaem com o envelhecimento (para revisão, ver BOVERIS E NAVARRO, 2008; PARADIES *et al.*, 2011). Sob condições controle, quando os dados de produção de H_2O_2 foram normalizados pela atividade da citrato sintase (***Figura 2B***, ***Capítulo II***) ou pelas taxas de consumo de O_2 (resultados não mostrados), foi observado que os níveis de produção de H_2O_2 foram similares entre mitocôndrias de cérebro de ratos adultos e velhos.

Nossos dados mostraram que, os efeitos exercidos pela rotenona no consumo de O_2 e produção de H_2O_2 por mitocôndrias de cérebro são similares entre ratos adultos e ratos velhos. Desta forma, estes resultados não suportam a idéia de que a associação entre envelhecimento, deficiência de Complexo I e neurodegeneração doparminérgica na DP, seria devido a maior liberação de $O_2^{\cdot-}$ /H_2O_2 mitocondrial por uma inibição do Complexo I por toxinas em animais com idade avançada. De fato, a neurodegeneração dopaminérgica na DP é um processo complexo que pode envolver o acúmulo de deficiências do Complexo I devido ao envelhecimento (BENDER *et al.*, 2006; SCHON *et al.*, 2012) e uma maior vulnerabilidade de neurônios envelhecidos a um déficit energético e/ou geração de EROs por inibição parcial do Complexo I.

5. Conclusões

Concluímos que uma inibição parcial do Complexo I da cadeia respiratória mitocondrial por rotenona:

- Pode contribuir para neurodegeneração por comprometimento do metabolismo energético e/ou promovendo estresse oxidativo, enquanto que, o primeiro evento predominaria numa situação de alta demanda de fosforilação oxidativa, o segundo ocorreria em condições de respiração de repouso;
- Exerce efeitos similares no consumo de O_2 e na produção de H_2O_2 em mitocôndrias isoladas de cérebro de ratos adultos e ratos velhos; e
- Aumenta a liberação de H_2O_2 por mitocôndrias em condições *in situ*, em sinaptossomas.

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7. Anexo

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
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C E R T I F I C A D O,

Certificamos que o projeto **"Produção mitocondrial de espécies reativas de oxigênio na presença de uma inibição parcial do complexo I: Efeito de diferentes estados metabólicos"** (protocolo nº **2553-1**), sob a responsabilidade de **Prof. Dr. Roger Frigério Castilho / Luiz Guilherme Michelini Bueno**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **07 de novembro de 2011**.

Campinas, 07 de novembro de 2011.

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