Rotenone Model of Parkinson Disease

MULTIPLE BRAIN MITOCHONDRIA DYSFUNCTIONS AFTER SHORT TERM SYSTEMIC ROTTENONE INTOXICATION*

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Chronic infusion of rotenone (Rot) to Lewis rats reproduces many features of Parkinson disease. Rot (3 mg/kg/day) was infused subcutaneously to male Lewis rats for 6 days using Alzet minipumps. Control rats received the vehicle only. Presence of 0.1% bovine serum albumin during the isolation procedure completely removed rotenone bound to the mitochondria. Therefore all functional changes observed were aftereffects of rotenone toxicity in vivo. In Rot rat brain mitochondria (Rot-RBM) there was a 30–40% inhibition of respiration in State 3 and State 3U with Complex I (Co-I) substrates and succinate. Rot did not affect the State 4 ΔΨ of RBM and rat liver mitochondria (RLM). However, Rot-RBM required two times less Ca2+ to initiate permeability transition (mPT). There was a 2-fold increase in O2− or H2O2 generation in Rot-RBM oxidizing glutamate. Rot infusion affected RLM little. Our results show that in RBM, the major site of reactive oxygen species generation with glutamate or succinate is Co-I. We also found that Co-II generates substantial amounts of reactive oxygen species that increased 2-fold in the Rot-RBM. Our data suggest that the primary mechanism of the Rot toxic effect on RBM consists in a significant increase of O2− generation that causes damage to Co-I and Co-II, presumably at the level of 4Fe-4S clusters. Decreased respiratory activity diminishes resistance of RBM to Ca2+ and thus increases probability of mPT and apoptotic cell death. We suggest that the damage to Co-I and Co-II shifts O2− generation from the CoQ10 sites to more proximal sites, such as flavines, and makes it independent of the RBM functional state.

Currently, it is widely accepted that mitochondrial dysfunctions play an important role in pathogenesis of neurodegenerative diseases, such as Parkinson disease (PD)1, 2, Huntington disease (3), amyotrophic lateral sclerosis (4), Friedrich ataxia (5), and others. Various aspects of mitochondrial participation in neurodegeneration have been discussed in numerous recent reviews and papers (2, 6–9, 11–15). Large progress toward understanding the mechanisms of mitochondrial involvement in neurodegeneration occurred when genetic and toxic animal models of neurodegenerative diseases were developed. Inhibitors of mitochondrial respiratory chain are widely used in toxic models of neurodegenerative diseases. The inhibitors of Complex I (MPP+ and rotenone) are used to model Parkinson disease (1, 9, 17–19), whereas inhibitors of Complex II (3-nitropropionic acid and malonate) are used to model Huntington disease (20–22).

Among various animal models of PD, the rotenone model has recently drawn particular attention for two reasons: 1) unlike other models it reproduces most of the movement disorder symptoms and the histopathological features of PD including Lewy bodies (2, 19); and 2) rotenone and other pesticides are powerful inhibitors of mitochondrial respiration, and recent epidemiological studies suggest involvement of these toxic compounds in the higher incidence of sporadic Parkinsonism among the population of rural areas (23, 24). Since the first publication of Betarbet et al. (1), there have been many published papers describing various aspects of the in vivo rotenone model of Parkinsonism in rats (17, 18, 25, 26), and the results have been discussed in several recent reviews (2, 19). Most papers on in vivo rotenone intoxication address various aspects of behavioral and morphological signs of Parkinsonism (1, 17, 18, 26), but not specific mitochondrial functions, except for nitric oxide generation (25). Therefore, the complete picture of what happens to mitochondria is still absent.

There are three major mitochondrial functions that determine the performance and fate of the cell. These are: 1) oxidative phosphorylation that produces ATP for almost all cellular functions; 2) mitochondrial Ca2+−dependent permeability transition (mPT) that may initiate apoptotic or necrotic death of a cell; and 3) generation of reactive oxygen species (ROS), a byproduct of normal aerobic metabolism. Compelling evidence exist that increased generation of ROS is responsible for the dysfunction and sensitization of the cell to death signals. Thus systemic rotenone intoxication may cause damage to each of these major mitochondrial functions or all of them. However, it is unclear how increased ROS production relates to other events leading to the dopaminergic cells death (27). The important issue to be addressed is the time course of pathological events in the systemically poisoned animals.

In this paper we present data on relatively short term systemic rotenone intoxication (6 days). To find methods of therapeutic intervention it is important to understand the early primary mechanisms of rotenone toxicity. We expect that after 6 days of intoxication the organism will respond to the toxin but various adaptive mechanisms will not yet shadow the primary mechanisms of rotenone toxicity. We show that rotenone intoxication for 6 days results in multiple mitochondrial dysfunctions including increased ROS generation, inhibition of NAD-dependent substrates oxidation, and, surprisingly, succinate oxidation. Importantly, the inhibition of succinate oxidation was the most consist...
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ent fact that we observed both after short term and long term (4 weeks) rotenone intoxication. Also we show that in rat brain mitochondria (RBM), Complex II also generates ROS at a rate comparable with the rate in the presence of glutamate + malate. Although rotenone intoxication results in a 42% decrease of succinate-supported ROS generation at Complex I associated with the backward electron flow, there was a 2-fold increase of ROS generation by Complex II itself, which increased even further (3–4-fold) after 4 weeks of rotenone intoxication.3 We suggest that inhibition of electron flow at Complexes I and II results from the damages to 4Fe-4S clusters. As a result, the intraprotein sites of O2 generation shift to more proximal sites, presumably flavines, and thus generation of ROS in Rot-RBM becomes independent of the mitochondrial metabolic state. These changes lead to a higher probability of permeability transition of the brain mitochondria when challenged with Ca2+, and thus excitotoxic cell death. Unlike RBM, rotenone affected RLM little. Thus a complex interaction of several mitochondrial dysfunctions in the brain during systemic rotenone dysfunction is responsible for selective loss of neurons in this model of PD.

EXPERIMENTAL PROCEDURES

Animals—Male Lewis rats weighing 300–350 g were used in this study. All animal use complied with National Institutes of Health guidelines and was approved by the Emory University Institutional Animal Care and Use Committee.

Subcutaneous Implantation of Osmotic Pumps—Alzet osmotic minipumps (Alzet Corp., Palo Alto, CA) were filled with rotenone (Sigma) dissolved in equal volumes of dimethyl sulfoxide (Me2SO) and polyethylene glycol (polyethylene glycol-300, Sigma) or solvent alone as described in Ref. 17. Ketamine (75 mg/kg) and xylazine (10 mg/kg) were injected intramuscularly for anesthetic. Alzet osmotic minipumps were implanted under the skin on the back of each animal (17). Control rats received Me2SO:polyethylene glycol (1:1) only. Treated rats received 3.0 mg of rotenone/kg/day (based on the weight at the time of surgery). The animals were sacrificed on the 6th day after surgery.

Isolation of Rat Liver and Brain Mitochondria—Both liver and brain mitochondria were isolated in medium containing the following: (in mM) 225 mannitol, 75 sucrose, 20 MOPS (pH 7.2), 1 EGTA, and 0.1% bovine serum albumin. Liver mitochondria (RLM) were isolated by conventional differential centrifugation with a final spin at 8600 × g (28). Brain mitochondria were isolated from the pooled forebrains of three rats. We used the modified method of Sims (30) to isolate and purify brain mitochondria (RBM) in a Percoll gradient. The modifications were as follows: brain tissue was homogenized with 15 strokes of a loose pestle in a Dounce homogenizer, and 5-ml volumes per tube of 15, 23, and 40% (v/v) of Percoll solutions were used to purify the brain mitochondria. After the final sedimentation of mitochondria at 8600 × g, the mitochondria were suspended in 250 mM sucrose and 10 mM MOPS (pH 7.2). Mitochondrial protein was determined using the Pierce Coomassie Protein Assay Reagent Kit.

Preparation of the Submitochondrial Particles (SMP)—The inverted nonphosphorylating SMP were prepared according to Ref. 29. Fresh RLM or RBM mitochondria were suspended in 0.25 M sucrose with 2 mM EDTA to contain about 20–30 mg/ml. 0.5 ml of the suspension was saturated with N2, placed into a beaker filled with a mixture of ice, water, and KCl for effective cooling, and subjected to sonic oscillations (5 times for 5 s). The sonicated mitochondria were diluted with a double volume of 0.25 M sucrose buffered with 10 mM MOPS, and centrifuged in a Beckman ultracentrifuge at 16,000 × g for 10 min. The supernatant was centrifuged at 150,000 × g (rotor SW41) for 45 min. The sediment of SMP was collected and homogenized in a small glass homogenizer in a volume of 0.25 ml sucrose to give a final concentration of SMP of 10–20 mg/ml. The usual recovery of the protein as SMP was about 20–30% of the original concentration of the mitochondria.

Simultaneous Registration of Mitochondrial Respiration and Membrane Potential—Respiratory activities of the mitochondria were measured using a custom made plastic minichamber of 765 μl volume equipped with a standard YSI (Yellow Spring Instrument Co., Inc.) oxygen minielectrode connected to a YSI model 5300 Biological Oxygen Monitor, a custom made tetraphenylphosphonium-sensitive minielectrode, and a KCl bridge to a Ag/AgCl reference electrode connected to a pH meter. All instruments were connected to a Keep and Zonnen paper chart recorder and the data acquisition system. To obtain maximum oxidative phosphorylation activity (see Ref. 31) the following incubation medium was used (Medium A): (in mM) 125 KCl, 10 MOPS (pH 7.2), 2 MgCl2, 2 KH2PO4, 10 NaCl, 1 EGTA, 0.7 CaCl2. At the Ca2+/EGTA ratio of 0.7 the free [Ca2+] is close to 1 μM as determined with the Fura-2 method (31). The substrate concentrations were as follows: (in mM) 5 succinate without rotenone, 20 glutamate + 2 malate, 5 pyruvate + 2 malate, 10 α-ketoglutarate + 2 malate. Oxidative phosphorylation (State 3) was initiated by addition of 150 μM ADP. The uncoupled respiration (State 3U) was stimulated by titration with CCCP (0.05 μM aliquots) until the maximum rate of oxygen consumption was obtained.

Membrane potential was measured with a tetraphenylphosphonium-sensitive electrode as described in Refs. 28 and 32. Because the volume of the matrix space and binding constants for tetraphenylphosphonium in brain mitochondria are unknown, the calculated ΔΨ values were approximate.

Registration of Permeability Transition and Estimation of Calcium Retention Capacity (CRC)—We have introduced a quantitative parameter CRC that allows a meaningful comparison of the sensitivity to Ca2+ of mitochondria from different organs and species (33). CRC is the amount of calcium that can be accumulated and retained by mitochondria until the mPT occurs. It is expressed as nanomole of Ca2+ per mg of mitochondrial protein. For our work we used two different methods to estimate CRC and register permeability transition: 1) potentiometric measurements of pH changes of the incubation medium during Ca2+ accumulation and release by the mitochondria as described in Ref. 33, and 2) depolarization of the mitochondria using a tetraphenylphosphonium-sensitive electrode as described elsewhere (33). The pH measurements were performed using Corning pH meter model 440 equipped with a mono pH microelectrode from Lazar Co., and Ag/AgCl reference electrode connected to the incubation chamber by a KCl bridge.

The mitochondrial CRC values were estimated in medium (Medium B) containing: (in mM) 125 KCl, 10 NaCl, 0.5 MgCl2, 3 glycyl-glycin (pH 7.2), 1 KH2PO4, and 20 glutamate + 5 malate as substrates for brain and liver mitochondria. Mitochondrial protein was 0.5 mg/ml. Measurements were done in a final volume of 1.0 ml. Ca2+ was added to mitochondria in 5-μl aliquots of 5, 10, or 20 μl stock solutions of CaCl2 of very high purity, 99.99% (Sigma).

Measurements of Hydrogen Peroxide Generation—H2O2 was measured using the Amplex Red (Molecular Probes). In the presence of horseradish peroxidase there is a reaction: Amplex Red + H2O2 → resorufin + O2. Resorufin is a stable and highly fluorescent compound whose wavelength spectra excitation/emission are 570/585 nm. The fluorescence of resorufin was determined in 1 ml incubations in Medium A, 0.2 mg/ml mitochondrial protein, 5 μM Amplex Red, and 3 units of horseradish peroxidase. Calibration was made using standard

3 A. Panov, S. Dikalov, N. Shalbueva, G. Taylor, T. Sherer, J. T. Greenamyre, unpublished data.
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FIGURE 1. Measurements of O$_2^-$ release by mitochondria using ESR and spin probe PPH. PPH reacts with O$_2^-$ producing stable PP-nitroxide detected with ESR spectroscopy (inset). Superoxide radicals were measured by superoxide dismutase-inhibited ESR signal of the PP-nitroxide in the sample containing mitochondria, substrate, and PPH as described under “Experimental Procedures.”

solutions of H$_2$O$_2$ (Fluka) and resorufin (Sigma). Fluorimetric measurements were made using fluorometer from C&L Co., Middletown, PA. Resorufin was measured using an excitation filter of 570 nm, and the emission filter was 585 nm. The stock solutions of the mitochondrial inhibitors used for inhibitor analysis of H$_2$O$_2$ generation sites were made in ethanol.

Measurements of O$_2^-$ Release by Mitochondria—The cyclic hydroxylamine PPH (Alexis Biochemicals, San Diego, CA) was used for quantitative measurements of O$_2^-$ release by mitochondria. PPH allows extracellular and intramitochondrial detection of O$_2^-$ (34). It reacts with O$_2^-$ producing stable PP-nitroxide detected with ESR spectroscopy (Fig. 1, inset). Briefly, 10 mM PPH was dissolved in deoxygenated media with 50 μM deferoxamine. Mitochondria preparations and PPH stock solutions were kept on ice (50 μg of protein mixed with 1 mM PPH and mitochondrial substrates in 100 μl of Medium A). Detection of O$_2^-$ radical was confirmed by inhibition of the ESR signal with 50 units/ml of superoxide dismutase (Fig. 1). Accumulation of PP-nitroxide was measured using a Bruker EMX ESR spectrometer and a super-high Q microwave cavity. The concentration of PP-nitroxide was determined by calibration with standards of H$_2$O$_2$ (Fluka) and resorufin (Sigma). Fluorimetric measurements were made using fluorometer from C&L Co., Middletown, PA. Resorufin was measured using an excitation filter of 570 nm, and the emission filter was 585 nm. The stock solutions of the mitochondrial inhibitors used for inhibitor analysis of H$_2$O$_2$ generation sites were made in ethanol.

Mitochondrial Yields—Using our standard procedure for isolation of brain and liver mitochondria with a final sedimentation at 8,600 × g, we have found that the yield of brain mitochondria (normalized per 1 g of wet tissue) was on average 20% higher for rotenone-treated animals (1.74 ± 0.14 mg of RBM/mg of wet tissue) as compared with the sham operated rats (1.44 ± 0.1 mg of RBM/mg of wet tissue). There was no change in mitochondrial yields from the livers of rotenone rats. Interestingly, Koopman et al. (35) recently reported that in cells chronically treated with rotenone there is a significant increase in mitochondrial length and branching without changing the number of mitochondria per cell. Evidently, the increased yield of RBM from Rot rats was not associated with glia because at this early stage of rotenone intoxication there was no significant increase in the number of glial cells in the brain (1).

Effects of Systemic Rotenone Infusion on Respiratory Activities and Membrane Potential of the Rat Liver and Brain Mitochondria—Mitochondrial oxidative phosphorylation is evaluated by measuring the respiratory activity in different metabolic states. When mitochondria oxidize a substrate but do not perform useful work such as ATP generation or cation transport, the membrane potential is at maximum and the rate of oxygen consumption is controlled by the intrinsic proton conductiv-

TABLE ONE shows that after 6 days of rotenone infusion there is a significant inhibition of the brain mitochondria (Rot-RBM) respiration with all substrates in metabolic States 3 and 3U as compared with control rats receiving Me$_2$SO + polyethylene glycol only. We did not find any difference between the control untreated rats and the rats exposed for 6 days to Me$_2$SO + polyethylene glycol. The State 4 respiratory rates with Rot-RBM were also inhibited but not significantly. Remarkably, oxidation of succinate in States 3 and 3U, which is a Complex II substrate, was inhibited to the same degree (∼40%) as oxidation of Com-

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Effects of systemic rotenone infusion on oxidation by the rat brain and liver mitochondria of various substrates in different metabolic states

Incubation conditions are described under "Experimental Procedures."

| Table One |
|-----------|
| Effects of systemic rotenone infusion on oxidation by the rat brain and liver mitochondria of various substrates in different metabolic states |

| Succinate | Glutamate |
|-----------|-----------|
| RLM       | Rot-RLM   | % Changes | RLM       | Rot-RLM   | % Changes |
| State 4   | 120 ± 4   | Rot-RBM   | 106 ± 2  | -12, NS   | 54 ± 4    | 11 ± 0   | -6, NS   |
| State 3   | 498 ± 14  |           | 306 ± 19 | -39, p < 0.001 | 325 ± 17  | 242 ± 4  | -26, < 0.05 |
| State 3U  | 523 ± 32  |           | 304 ± 17 | -38, p < 0.001 | 403 ± 25  | 305 ± 6  | -29, p < 0.05 |

| Pyruvate  | α-Ketoglutarate |
|-----------|-----------------|
| Succinate |                 |
| RLM       | Rot-RLM         | % Changes | RLM       | Rot-RLM   | % Changes |
| State 4   | 66 ± 4          | Rot-RBM   | 49 ± 10  | -24, NS   | 37 ± 5    | 21 ± 5   | -17, NS   |
| State 3   | 366 ± 17        |           | 218 ± 17 | -40, p < 0.05 | 292 ± 5  | 175 ± 6  | -40, p < 0.001 |
| State 3U  | 511 ± 29        |           | 318 ± 26 | -38, p < 0.05 | 318 ± 12  | 213 ± 15 | -33, p < 0.01 |

| Glutamate |                 |
|-----------|-----------------|
| RLM       | Rot-RLM         | % Changes | RLM       | Rot-RLM   | % Changes |
| State 4   | 38 ± 4          | Rot-RBM   | 39 ± 2   | NS        | 25 ± 9    | 19 ± 2   | NS        |
| State 3   | 163 ± 16        |           | 139 ± 5  | NS        | 127 ± 9   | 62 ± 2   | -52, p < 0.01 |
| State 3U  | 267 ± 21        |           | 192 ± 4  | NS        | 201 ± 14  | 87 ± 5   | -57, p < 0.001 |

| Pyruvate  | α-Ketoglutarate |
|-----------|-----------------|
| Glutamate |                 |
| RLM       | Rot-RLM         | % Changes | RLM       | Rot-RLM   | % Changes |
| State 4   | 22 ± 2          | Rot-RBM   | 22 ± 10  | NS        | 26 ± 2    | 23 ± 2   | NS        |
| State 3   | 55 ± 2          |           | 38 ± 3   | -31, p < 0.05 | 58 ± 3   | 24 ± 2   | -59, p < 0.05 |
| State 3U  | 63 ± 2          |           | 48 ± 3   | NS        | 50 ± 3    | 42 ± 3   | NS        |

*NS, not significant.

plex I substrates pyruvate and α-ketoglutarate, and more than glutamate (TABLE ONE). Inhibition of respiration with these substrates was not associated with diminished activity of Complex IV because with 5 mM ascorbate + 0.3 mM TMPD the rates of oxygen consumption in State 3U were (in nanogram atom O/min/mg of protein) 2368 ± 180 for control RBM and 1988 ± 236 for Rot-RBM. Interestingly, after 4 weeks of rotenone intoxication the rate of ascorbate + TMPD oxidation in State 3U was inhibited by 62%.

In Rot-RBM oxidation of succinate was practically unaffected. There was only a 50% inhibition of the State 3 glutamate oxidation. Other substrates of Complex I, pyruvate and α-ketoglutarate, were also inhibited. However, the State 3 and State 3U respiratory rates with these substrates were very slow as compared with glutamate. Evidently, livers of Lewis rats do not utilize glucose as a source of energy for mitochondria.

Because of a more profound inhibition of the State 3 oxidation of the substrates as compared with the State 4 respiration, there was a significant decline in the respiratory control ratios in RBM and RLM with all substrates as shown in Fig. 2, A and B, with the exception of RLM oxidizing α-ketoglutarate + malate (see Fig. 2B).

The first question that arises regarding the observed inhibitions of the Complex I substrates oxidation in rat brain and liver mitochondria of the rotenone-treated rats (TABLE ONE) is whether the inhibitions were associated with a damage of Complex I or by rotenone that may remain bound to the mitochondrial Complex I. To answer this question we isolated normal rat brain mitochondria and added in vitro 400 nm/mg of protein rotenone. This concentration of rotenone inhibits the State 3 oxidation of glutamate + malate by 80–90% (1). The rotenone-loaded and control RBM were then washed twice in 30 ml of the isolation medium (see ’Experimental Procedures’) that contained 0.1% defatted bovine serum albumin. The resulting mitochondrial sediments were resuspended and the State 3 and State 3U respiratory rates with glutamate were compared. We found that washing of the rotenone-loaded RBM with the bovine serum albumin-containing medium completely removed rotenone from the mitochondria (data not shown). Therefore, we suggest that the rotenone was removed from the mitochondria isolated from rotenone-treated animals because both RLM and RBM were isolated in the presence of 0.1% bovine serum albumin. Thus inhibitions of Complex I substrate oxidation observed in Rot-RBM and Rot-RLM were indeed associated with damage of Complex I. A 40% inhibition of the State 3 and State 3U succinate oxidation in RBM also speaks in favor of damage of Complex II because rotenone in vitro has no direct effect on succinate oxidation.

In line with insignificant inhibitions of the State 4 respiration in the Rot-RBM, rotenone at this stage of intoxication had no effect on the resting membrane potentials in both Rot-RBM and Rot-RLM (data not shown). This suggests that after 6 days of rotenone intoxication the inner mitochondrial membrane was not yet damaged by lipid peroxidation or other mechanisms (such as oxidative damage of transmembrane proteins) that would result in increased proton conductivity of the inner mitochondrial membrane. However, when a functional load was imposed on the mitochondria (such as phosphorylation of ADP) with Rot-RBM, the drop in membrane potential was larger and it took more time to phosphorylate the same amount of ADP as compared with the control RBM oxidizing glutamate + malate or succinate (not shown). Thus Rot-RBM have diminished ability to maintain energization during increased energy demands because of diminished activities of Co-I and Co-II. This assumption was further studied using Ca2+ consumption as an energy-dependent function.

Effects of Systemic Rotenone Infusion on Permeability Transition and the CRC of RBM and RLM—The amount of calcium phosphate salts (CaP) that mitochondria can sequester and retain determines whether mitochondria will undergo mPT or not when a cell is challenged with calcium. The data published in the literature suggest that Ca2+-induced mPT may be responsible for the excitotoxic death of glutamatergic neurons (37). Fig. 3A shows CRC for RBM and RLM when the mitochondria...
were not protected by ADP or cyclosporin A (CsA). Rot-RBM required 50% less Ca\(^{2+}\) to open the permeability transition pore than control RBM. ADP (in the presence of oligomycin to prevent phosphorylation of ADP) significantly increases CRC, particularly in brain mitochondria (33). This is a more physiological situation because ADP is always present in the cell (38). Fig. 3B shows that although in the presence of ADP/Ca\(^{2+}\)/oligomycin CRC increased severalfold, in Rot-RBM CRC was 27% lower as compared with control RBM. However, when the mitochondria were protected by CsA, a specific inhibitor of the mitochondrial permeability transition pore opening (Fig. 3C), the CRC of Rot-RBM was the same as that of control RBM. We used CsA together with ADP because ADP is always present in the cells, and ADP substantially increases CsA binding to mitochondria (39). We have also shown recently that the protective effect of CsA alone is small with brain mitochondria (33). Thus rotenone affects only the energy-dependent Ca\(^{2+}\) sequestration process without direct influence on mPT pore.

Effects of Systemic Rotenone Infusion on Generation of Superoxide Radical and H\(_2\)O\(_2\) by RBM and RLM—Superoxide radical (O\(_2\)\(^{\bullet-}\)) and H\(_2\)O\(_2\) are natural products of mitochondrial aerobic metabolism when electrons spontaneously reduce molecular oxygen (40). Fig. 4 compares the rates of O\(_2\)\(^{\bullet-}\) production by the RBM and RLM. The superoxide dismutase-inhibitable PP-nitroxide formation reflects the amount of O\(_2\)\(^{\bullet-}\) released from the mitochondria. Note the protein differences in the experiments. When normalized for 1 mg of protein in the presence of antimycin A and with succinate as a substrate, RBM generate six times more O\(_2\)\(^{\bullet-}\) than RLM. These data demonstrate a much higher potential for O\(_2\)\(^{\bullet-}\) production by RBM than RLM. Therefore, there are three factors that make RBM the primary target for rotenone-induced oxidative damage: 1) under the State 4 conditions RBM produce more O\(_2\)\(^{\bullet-}\) than RLM (Fig. 4, 14.2 \pm 2.3 versus 7.7 \pm 1.6 pmol/min/mg); 2) mitochondrial dysfunction may lead to a 6-fold higher O\(_2\)\(^{\bullet-}\) production by RBM than RLM (Fig. 4); and 3) RBM have less antioxidant protection than RLM (41).

Indeed, after 6 days of systemic rotenone infusion the isolated RBM oxidizing glutamate + malate generated O\(_2\)\(^{\bullet-}\) at a rate two times faster.
than RBM from the control animals (Fig. 5, A and B) (30 ± 3.2 versus 14.2 ± 2.3 pmol/min/mg). At the same time O$_2^-$ production by Rot-RLM oxidizing succinate was not significantly different from the control RLM (Fig. 5, 11.0 ± 1.8 versus 7.7 ± 1.6 pmol/min/mg).

Interestingly, rotenone added in vitro to control RBM oxidizing glutamate + malate leads to a noticeable decrease in O$_2^-$ determined outside the mitochondria (Fig. 6A). Experiments conducted with SMP from RBM showed that rotenone inhibits the O$_2^-$ release from the mitochondria, but increases its production. Fig. 6B shows that rotenone added to SMP caused a 4-fold increase in O$_2^-$ generation. Note also that the scale of the Fig. 6B is 1 order larger than that of the Fig. 6A. Fig. 6C shows that rotenone added in vitro to intact RBM doubles the release of H$_2$O$_2$. Of interest, the amount of H$_2$O$_2$ released was close to that for O$_2^-$ generated by SMP. Addition of superoxide dismutase 1 to the mitochondrial suspension did not further increase generation of H$_2$O$_2$ (not shown). Two important conclusions can be drawn from the data presented in Fig. 6 (A--C): 1) only a small portion of O$_2^-$ generated by the mitochondria can be determined outside the brain mitochondria, and 2) the release of O$_2^-$ depends on mitochondrial energization. The latter indicates that with the respiratory inhibitors, which are usually used for localization of the sites responsible for ROS generation, the observed O$_2^-$ does not reflect actual O$_2^-$ production by the intact mitochondria. Therefore, generation of H$_2$O$_2$ is a more accurate marker of the mitochondrial ROS production under these conditions.

After 6 days of rotenone infusion there was a 2-fold increase in H$_2$O$_2$ generation with glutamate + malate (Fig. 7A) and a 42% decrease with succinate as substrate. The latter occurred possibly because of a 40% inactivation of Complex II (Fig. 7B). The data presented in Figs. 7--9, A and B, show that measuring H$_2$O$_2$ is probably the best choice to study ROS generation by intact brain mitochondria. Fig. 7, A and B, show that
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**FIGURE 8. Effects of respiratory inhibitors on H\textsubscript{2}O\textsubscript{2} generation by control RBM and Rot-RBM oxidizing glutamate + malate.** Incubation conditions are as described in the legend to Fig. 7. A, responses of control RBM to additions of respiratory inhibitors; B, generation of H\textsubscript{2}O\textsubscript{2} by RBM from control and rotenone-treated animals. Additions: catalase (Roche Diagnostics), 400 \mu g; antimycin A, 5 \mu M; rotenone, 5 \mu M; myxothiazol, 5 \mu M.

**FIGURE 9. Effects of respiratory inhibitors on H\textsubscript{2}O\textsubscript{2} generation by control RBM and Rot-RBM oxidizing succinate.** Incubation conditions and additions are as described in the legend to Fig. 8. A, responses of control RBM oxidizing succinate to additions of respiratory inhibitors; B, generation of H\textsubscript{2}O\textsubscript{2} by RBM from the control and rotenone-treated animals. AntA, antimycin A; Stig, stigmatellin.

RBM from control animals generate 4–5 times more H\textsubscript{2}O\textsubscript{2} with succinate as a substrate than with glutamate + malate.

With RLM oxidizing succinate or glutamate + malate there was almost no difference in the rates of H\textsubscript{2}O\textsubscript{2} generation (correspondingly 92.1 ± 2 and 80 ± 2 pmol/min/mg of protein), which is in stark contrast to the production of O\textsubscript{2\textsuperscript{-}} by RBM shown in Fig. 7. A and B. We have also found that with the exception of antimycin A, addition of respiratory inhibitors rotenone, stigmatellin (or myxothiazol), and their combinations to RLM had no effect on the observed rates of H\textsubscript{2}O\textsubscript{2} production (not shown). Thus measurements of H\textsubscript{2}O\textsubscript{2} cannot be used for analysis of the sites of ROS generation by the liver mitochondria.

**Inhibitor Analysis of the ROS Generation Sites in RBM from the Control and Rotenone-treated Rats—**Fig. 8, A and B, show the effects of various respiratory chain inhibitors on generation of H\textsubscript{2}O\textsubscript{2} by the control RBM and Rot-RBM oxidizing glutamate + malate. These data show that regardless of the mechanism, inhibition of the electron transport caused a severalfold increase in generation of H\textsubscript{2}O\textsubscript{2} by RBM. With glutamate + malate the increase in H\textsubscript{2}O\textsubscript{2} upon addition of antimycin A was relatively modest. This might be interpreted as an indication that with Complex I substrates the rate of electron input from Complex I limits the rate of O\textsubscript{2\textsuperscript{-}} generation on Complex III in the presence of antimycin A.

It should be mentioned that the rates of H\textsubscript{2}O\textsubscript{2} production by RBM oxidizing 5 mM pyruvate + 2 mM malate were very similar to those shown in Figs. 7A and 8A for glutamate + malate (pyruvate data are not shown).

The data presented in Fig. 8, A and B, show that with glutamate + malate as substrates, the major source of H\textsubscript{2}O\textsubscript{2}, that reflect generation of O\textsubscript{2\textsuperscript{-}} is Complex I. Fig. 8B summarizes quantitatively the results of several experiments with the control RBM and the Rot-RBM. One can see that after 6 days of rotenone intoxication there was a 2-fold increase of ROS generation.

Fig. 9, A and B, show the effects of respiratory inhibitors on H\textsubscript{2}O\textsubscript{2} generation by RBM oxidizing 5 mM succinate. In general, control RBM oxidizing succinate in the metabolic State 4 generate 6–8 times more H\textsubscript{2}O\textsubscript{2} than RBM oxidizing glutamate or pyruvate (see Fig. 7, A and B). Fig. 9A shows that with the exception of antimycin A, addition of rotenone, myxothiazol (stigmatellin), or a combination of these inhibitors caused a significant inhibition of H\textsubscript{2}O\textsubscript{2} generation. This suggests that most, but not all, of the succinate supported O\textsubscript{2\textsuperscript{-}} generation occurs on the Complex I reduced by the energy-dependent backward electron flow. This conclusion agrees with earlier publications (42, 43). Addition of antimycin A caused a 3-fold increase in H\textsubscript{2}O\textsubscript{2} generation that was abolished by a simultaneous addition of myxothiazol or stigmatellin (Fig. 9A). We have found no difference between myxothiazol and stigmatellin in their effects on succinate-supported generation of H\textsubscript{2}O\textsubscript{2} by RBM. Fig. 9A shows that in the presence of myxothiazol + rotenone there was a substantial rate of H\textsubscript{2}O\textsubscript{2} production (53 ± 10 pmol/min/mg of protein) comparable with the State 4 rate of ROS generation with glutamate + malate (65 ± 5 pmol/min/mg of protein). These are significantly higher rates for ROS generation reported recently for the rat heart mitochondria (42). However, the authors used incubation conditions that were not optimal for the heart mitochondria (sucrose medium, no Ca\textsuperscript{2+}, see Ref. 31).

Fig. 9B summarizes quantitatively the results of several experiments. Although systemic rotenone poisoning resulted in a reduced generation of ROS by RBM with succinate as a substrate (see Figs. 7B and 9B) in metabolic State 4, there was a significant increase in ROS generation by Rot-RBM in the presence of myxothiazol and myxothiazol + rotenone (Fig. 9B). In the presence of both myxothiazol and rotenone the electrons cannot go upstream or downstream of the respiratory chain, and therefore the only source of ROS under these conditions may be Complex II. After 6 days of rotenone infusion H\textsubscript{2}O\textsubscript{2} generation becomes two times higher in the Rot-RBM (109 ± 6 pmol/min/mg of protein) as compared with the control RBM (53 ± 10 pmol/min/mg of protein).
Thus the data presented in Fig. 9, A and B, argue that at least in RBM, Complex II may also generate ROS that can be increased by rotenone-induced damage to Complex II.

**DISCUSSION**

Previous studies have shown that the subcutaneous infusion of rotenone for 28 or 56 days reproduces many features of PD in rats including selective nigrostriatal dopaminergic degeneration (2, 17). Despite numerous recent publications on rotenone toxicity in vitro (44, 45) and in vivo (1, 2, 17, 18, 25, 26, 47), the exact mechanisms of the specific rotenone-induced neurotoxicity remain obscure. Evidently during a long-term systemic rotenone infusion there is complex interplay of the deleterious effects of the toxin and defensive and adaptive mechanisms that in some neuronal cells fail to prevent the lethal outcome. It is known that toxic models of neurodegenerative diseases are subject to a large variability between various species as well as within one species (1, 26). In this study we exposed animals to rotenone infusion for 6 days to find the primary mechanisms of rotenone neurotoxicity that may be later disguised by adaptive reactions. It should be noted that unlike the long-term intoxication experiments, after 6 days of systemic rotenone infusion there was little variability between animals. For the first time we present data on the in vivo effects of rotenone on the three major mitochondrial functions, and define a cascade of early events that later may result in specific neurodegeneration.

**Effects of the 6-Day Systemic Rotenone Infusion on Mitochondrial Respiration**—Rotenone, when introduced in vivo, binds specifically to the mitochondrial Complex I (NADH:ubiquinone oxidoreductase) all over the brain, as revealed by [3H]dihydrorotenone autoradiography (48), and we presume to Complex I in other tissues as well. With the rate of rotenone infusion by the Alzet osmotic pump at 3 mg/kg/24 h, it has been approximated that the concentration of "free" rotenone in brain should be about 20–30 nM (1). At this concentration rotenone had no inhibitory effect on State 3 oxidation of glutamate + malate by isolated RBM, whereas with RLM it was inhibited by 50% (1). Although rotenone is a highly specific inhibitor of mitochondrial Complex I, it also binds nonspecifically to proteins and hydrophobic compounds. Therefore, the actual concentration of rotenone in the tissues that are accessible to Complex I may be much lower than 20–30 nM. On the other hand, the amount of rotenone bound to Complex I can increase with time and thus gradually enhance inhibition of mitochondrial respiration. The steady-state degree of inhibition of RBM in vivo evidently depends on the rates of rotenone incoming from the Alzet pump and its removal by metabolism and excretion.

In control experiments we have found that isolation of mitochondria in the presence of 0.1% bovine serum albumin completely removes rotenone bound to mitochondria (see "Results"). Therefore, the observed inhibitions of mitochondrial respiration with NAD-dependent substrates and succinate shown in TABLE ONE are associated with structural damages to Complex I and Complex II. After 6 days of rotenone infusion, oxidations of the NAD-dependent substrates and succinate in metabolic States 3 and 3U were inhibited by 30–40%. However, we can presume that because in vivo rotenone is bound to Complex I, the inhibition of oxidation of the NAD-dependent substrates may be more than 40%. The unexpected fact that in vivo rotenone severely damages activity of Complex II argues that the inhibition was caused indirectly by a secondary mechanism that involves ROS because in vitro rotenone has no direct effect on activity of Complex II.

**Effects of Systemic Rotenone Toxicity on Ca2+-dependent Permeability Transition**—Although the control and Rot-RBM had the same State 4 membrane potential with glutamate + malate or succinate, our data show that in Rot-RBM, diminished activities of Complex I or Complex II result in a decreased ability of the mitochondria to restore membrane potential during increased energy consumption. In agreement with this presumption, Rot-RBM have a lower ability to sequester and retain CaPi even in the presence of ADP + oligomycin. However, Rot-RBM protected by CsA + ADP had the same CRC as the control RBM.

Recently we (33) have shown that the Ca2+-dependent mPT depends on the two events: 1) energy-dependent sequestration of Ca2+ and P, and 2) interactions of Ca2+ and ADP with the pore-forming protein, which do not depend on mitochondrial energization. Evidently the reduced ability of Rot-RBM to maintain energization during Ca2+ and P, sequestration was responsible for diminished CRC, whereas the CsA-dependent mechanism of mPT was not affected. Because Rot-RBM underwent mPT at significantly lower Ca2+ loads than the control RBM, this would make neuronal cells (particularly glutamatergic neurons) more vulnerable to the Ca2+-induced excitotoxic cell death.

Relatively small changes in the respiratory activity of the Rot-RLM and practically absence of the effects of systemic rotenone infusion on mPT may be explained by several mechanisms. First, hepatocytes have a powerful enzymatic machinery to metabolize rotenone. Second, liver has a very high regenerative capacity and thus, the damaged cells would be eliminated and replaced by the "new" ones.

**Effects of Systemic Rotenone Toxicity on ROS Generation by RBM**—Under normal conditions mitochondria in the actively functioning neuronal cells do not generate ROS because oxidative phosphorylation and other mitochondrial activities diminish the steady-state membrane potential and thus inhibit generation of ROS (38, 43). Therefore, investigations on the mitochondrial ROS generation under conditions of metabolic State 4 (which is a highly artificial situation) and the effects of various respiratory inhibitors reveal only a potential capacity of the given mitochondria to generate ROS under certain pathological conditions. The pathological situations that may increase generation of ROS by brain mitochondria include ischemia-reperfusion and hypoxia (reviewed in Ref. 49), Ca2+-induced permeability transition (excitotoxicity) (43), drugs that inhibit respiratory activity (for example, barbiturates), and poisoning with inhibitors of the respiratory chain, such as rotenone (50) and MPT (51). Each of these pathological situations has evidently different impact on neuronal cells and mitochondria and a different sequence of metabolic events that lead to cell death.

In this paper we studied the effect of short-term systemic rotenone poisoning on mitochondrial functions. Recently it was shown that agricultural and household toxins may contribute to a rise of sporadic PD cases and probably other pathologies as well (23, 24). Thus, to find corresponding prophylaxis measures and treatment methods it is important to understand the sequence of pathological events during systemic poisoning with mitochondrial toxins.

The data presented in this paper shows that the primary mechanism of systemic rotenone toxicity is an increase in generation of superoxide radical because of partial inhibition and damage of Complex I. As we have shown earlier (1), the initial small doses of rotenone are too small to affect oxidative phosphorylation. Addition of rotenone in vitro to mitochondria increases generation of ROS at Complex I (see Fig. 7). Thus, it is not the primary inhibitory effect of rotenone on the electron transport activity of Complex I, but the resulting secondary mechanism, increased superoxide radical production that is responsible for development of further structural and functional damages to mitochondria. We have previously discussed that rotenone toxicity results in a substantial loss of activity of Complex I and Complex II (see TABLE ONE).

**Mechanism of Damages to Complex I and Complex II**—The most likely mechanisms of the decreased activities of the two complexes is a
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damage of the 4Fe-4S clusters in these complexes caused by superoxide radical \(O_2^\cdot\) and/or peroxynitrite \((ONOO^\cdot)\). Previous work revealed that enzymes containing 4Fe-4S clusters are particularly vulnerable to damage by these two radicals (52–54). Bashkatova et al. (25) have shown that after 10 days of chronic rotenone intoxication there was no increase in the levels of nitric oxide, or lipid peroxidation-like products that would result from increased generation of \(ONOO^\cdot\). Thus, it is plausible that after the short term poisoning with rotenone the inhibitions of Complexes I and II were primarily associated with increased generation of \(O_2^\cdot\) and \(ONOO^\cdot\). Even if \(ONOO^\cdot\) does participate in the damage to the respiratory enzymes its generation depends on increased levels of both nitric oxide and \(O_2^\cdot\).

The most evident proof of a particular vulnerability of the 4Fe-4S clusters was obtained in experiments with aconitase and some other enzymes of the aconitase family (52–54). Complex II has one and Complex I has six 4Fe-4S clusters (see Fig. 10). It is thus logical to suggest that these structures are also highly sensitive to the damaging effects of \(O_2^\cdot\) and \(ONOO^\cdot\). Because Complex I has more 4Fe-4S clusters than Complex II, one would expect that chronic systemic rotenone intoxication would cause more damage to Complex I than Complex II. However, we have found that both Complex I and Complex II were damaged to the same degree. The explanation of this "controversy" arises from recent work by Ohnishi et al. (42). These authors suggested that cluster N2 and bound semiquinones play key roles in the function of Complex I, such as electron transfer, proton transport, and superoxide generation. Five other 4Fe-4S clusters of Complex I are protected from reacting with oxygen (54). The only exception may be cluster N2 (42). It has been hypothesized that this cluster may serve as the coupling site between electron transfer and proton transport in Complex I (46). This requires that the region of cluster N2 and protein-bound semiubiquinones must be accessible to \(H^+\) and water. Hence, this region is also accessible to oxygen and the possibility of reducing oxygen to superoxide would also occur (42).

**Complex II as a Site of ROS Production**—When discussing the generation of ROS by mitochondria, only Complex I and Complex III are usually considered as the major sites for production of the superoxide radical by normal *in situ* and isolated mitochondria. After years of controversy recent x-ray studies have shown that the [4Fe-4S] cluster is part of a linear electron transport chain located between FAD cofactor and the quinone or heme (reviewed in Ref. 16). Thus, the [4Fe-4S] cluster of Complex II can also be a target for the damaging effects of \(O_2^\cdot\) and \(ONOO^\cdot\) radicals. There are known human diseases associated with mutations of genes for Complex II and interestingly many of these mutations cause various tumors (16).

Studies of bacterial or yeast mitochondria (reviewed in Ref. 16) have shown that Complex II (succinate-quinone oxidoreductase) is capable of generating only \(O_2^\cdot\), which occurs presumably at the FAD cofactor (10). In our experiments we used a highly sensitive fluorometer to find that in the presence of myxothiazol or myxothiazol + rotenone, RBM oxidizing succinate does generate ROS (measured as \(H_2O_2\)) at a rate \((53 \pm 10\) pmol/min/mg of protein) comparable with that in the presence of Complex I substrates (65.5 \pm 8 pmol/min/mg of protein). Although systemic treatment of rats with rotenone caused a 42% decrease of ROS generation on Complex I because of a succinate-driven backward electron flow (see Figs. 7B and 9B), generation of ROS on Complex II in the presence of rotenone + myxothiazol increased two times \((109 \pm 5\) pmol/min/mg of protein, see Fig. 9B) after 6 days, and three times \((156 \pm 15\) pmol/min/mg of protein) after 4 weeks of rotenone intoxication.

We presume that an important consequence of the damaging of [4Fe-4S] clusters is a shift of the major sites of SO generation. Fig. 10 shows the sequence of electron transport chain components in mammalian mitochondria. The tentative damaged [4Fe-4S] clusters in Complex I and Complex II are shown in *bold*. One can see that because of the imposed limit on the electron transport by the damage to [4Fe-4S] clusters in both complexes, there must be a higher reduction of components located before the damage. As a result, flavin components of the Complex I and Complex II will be in a more reduced state and thus may produce more \(O_2^\cdot\) (10). We have already mentioned that in active mitochondria under normal conditions membrane potential is significantly

![FIGURE 10. Respiratory components in mammalian mitochondria. The figure was adapted from Ref. 42.](image-url)
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lower that observed in metabolic State 4. Therefore, normal mitochon-
dria generate very little ROS and it is strongly dependent on the
functional state of the mitochondria. However, in the rotenone-treated
animals the limits imposed on electron transport by the damages to
Complex I and Complex II make generation of ROS not dependent any
more on the functional state of the mitochondria. Evidently, even a slow
but constant generation of mitochondrial ROS may be very harmful for
the neuronal cells, particularly when glial generation of nitric oxide
increases.

Our data show that a relatively brief (6 days) intoxication with rote-
none does not result in a significant amount of damage of lipid mem-
branes. Our data support the idea that inactivation of Complex I and
Complex II was mediated by an increase in formation of O$_2^-$
and ONOO$^-$ because they are chemically active and can reach their specific
targets, one of which are 4Fe-4S clusters in various enzymes.

We show that systemic intoxication of animals with a highly specific
inhibitor of Complex I also results in damage of Complex II. This fact
indicates that the concepts that associate damage of Complex I with
Parkinson disease, and damage of Complex II with Huntington disease
are at least too simplistic. Our data show that even a brief systemic
exposure of an animal to rotenone caused multiple mitochondrial dys-
functions, which are interconnected and each of them may probably
cause the demise of the cell by different death pathways.

Altogether our data shows that to understand the role of mitochon-
dria in the pathogenesis of neurodegenerative diseases various mito-
chondrial functions have to be studied. The multiple natures of mito-
chondrial dysfunctions have to be considered when designing methods
of cure or prophylaxis of neurodegenerative diseases.

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