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Pesticides and impairment of mitochondrial function in relation with the parkinsonian syndrome

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1. ABSTRACT

The Parkinsonian syndrome induced by pesticides is associated with the impairment of mitochondrial function. Toxicants that inhibit selectively NADH-dehydrogenase activity, as rotenone or pyridaben, also show a selective inhibition of O_2 uptake and respiratory control in rat brain mitochondria in the presence of NAD-dependent substrates. The IC_{50} of rotenone and pyridaben for complex I inhibition were in the range 1.7-2.2 microM. The determination of NADH-cytochrome c reductase, succinate-cytochrome c reductase and cytochrome oxidase activities in rat brain submitochondrial showed again the selective inhibition of Complex I by rotenone and pyridaben, whereas paraquat produced a non-selective inhibition affecting all the respiratory chain complexes. In rat brain mitochondria, rotenone and pyridaben markedly decreased mtNOS functional activity with NAD-dependent substrates but not when the substrate was succinate. This observation suggest than mtNOS activity is regulated by the activity of complex I. This regulation and the role of mitochondrial NO diffusion as a signal for mitochondrial biogenesis could have a role in the etiopathology of Parkinson's disease.

2. INTRODUCTION

A pesticide is any substance or mixture of substances that prevent, destroy, repel, or mitigate any pest. This group includes insecticides, herbicides, fungicides, and various other substances used to control pests. Pesticides are used extensively throughout the world and evidences continue to accumulate showing that exposure to pesticides is associated with impaired health. The best-documented health effects involve the nervous system. The neurotoxic consequences of exposure to acute high-level of pesticides are well established, but the consequences of chronic exposure to more moderate levels remain controversial. Studies of chronic pesticide neurotoxicity have typically evaluated either the long-term sequelae of pesticide poisoning or the effects of occupational exposure. On the other hand, the wide-spread use of pesticides is a feature of modern times and there has been recognition of a temporal relationship between the prevalence of Parkinson's disease and industrialization (1-3).

An extensive literature suggests that rural living, farming as an occupation, drinking well water and pesticide exposure increase the risk of Parkinson's disease although

most of these studies have been unable to implicate specific substances (4). There are several explanations for this situation. First, chronic and sporadic low-level environmental exposures are difficult to detect. Second, acute environmental exposure produces delayed or slowly progressive degeneration and the appearance of symptoms can be remote in time from exposure. Third, individual genetic variation, such as polymorphism in disease-associated genes, xenobiotic metabolism, or even blood-brain-barrier function may influence the development of Parkinson's disease (5-7). Organophospates and organochlorinates insecticides, the carbamate fungicide Maneb, and the herbicide Paraquat are the pesticides most usually referred in these works (3,8-10). However, cohort studies of subjects submitted to pesticide exposure have not revealed an increased incidence of Parkinson's disease (11). It seems necessary to investigate thousands of exposed and non-exposed individuals to reach the statistical significance of results. The prevalence of Parkinson's disease in the general population over the age of 50 is about 1 %, with reports of up to 5-7% in rural areas (12).

The almost generally accepted role of mitochondria in Parkinson's disease pathogenesis led to the hypothesis that the mechanism of chronic toxicity of many pesticides involves the appearance of dysfunctional mitochondria. However, the acute toxicity of pesticides is not generally considered to be mediated by mitochondrial toxicity: *i.e.* pyrethroids and organochlorines alter membrane Na^+ channel function and organophosphates and carbamates inhibit acetylcholinesterase activity.

The systemic dysfunction in complex I, NADH-dehydrogenase, is associated with the occurrence of Parkinsonism, apparently due to a high intrinsic sensitivity to complex I defects of the *substantia nigra* dopaminergic neurons. Accordingly, the hypothesis of chronic environmental exposure to pesticides as the pathogenic cause of Parkinson's disease becomes biologically acceptable. Pesticides have been reported as able to interfere with mitochondrial function, either by inhibiting the respiratory chain or by uncoupling oxidative phosphorylation. The knowledge of pesticides mechanism of toxicity related to Parkinson's disease leads to a better understanding of the etiology of the disease and to suggest the ways of preventing the risks of the use of some pesticides.

3. MITOCHONDRIAL DYSFUNCTION AND PARKINSONIAN SYNDROME

The direct relation between Parkinson's disease and mitochondrial function was started to be evidenced when several drug addicts in the San Francisco bay area suffered a poisoning due to the injection of a pyridine contaminant, MPTP (1-methyl-4-1,2,3,6-tetrahydropyiridine) that caused an acute and permanent parkinsonian syndrome in these individuals (13,14). As result of the accident, the general hypothesis that Parkinson disease is secondary to exogenous environmental neurotoxins, particularly pesticides, was considered. The role of mitochondria in environmental Parkinson's disease was studied in relation with MPTP and it was found that its active metabolite MPP^+ (1-methyl-4-phenilpyiridinium ion) accumulated in mitochondria and exerted their toxicity by inhibiting complex I (15). Significantly, this finding supported the concept that mitochondrial dysfunction has a role in Parkinson's disease pathogenesis and that patients have a systemic complex I defect affecting both brain and peripheral tissues (16-18).

The mechanism by which complex I dysfunction leads to selective dopaminergic neurons degeneration in Parkinson's disease is not fully elucidated. Reduced complex I activity may predispose to excitotoxicity and oxidative damage, both implied in Parkinson's disease development (18). Brains from Parkinson's disease patients exhibit biomarkers of oxidative damage including lipid peroxidation, and DNA and protein oxidative modifications (19-21). It is considered that dopamine neurons support a constant oxidative stress because the enzymatic activity implied in the synthesis and catabolism of dopamine (tyrosine hydroxylase and monoamine oxidase, respectively) generate H_2O_2 as a normal product or by-product. Production of melanin from auto-oxidation of dopamine in the *substantia nigra*, also yields H_2O_2 as secondary product, which decomposes to $HO^{\cdot}$, the most dangerous oxygen free radical. The non-enzymatic homolysis of H_2O_2 is greatly accelerated in by the presence of free ferrous ion (Fe^{2+}), which is abundant physiologically in *substantia nigra*. Thus, mitochondrial oxidative damage is potentiated by dopamine (22) and dopamine renders dopaminergic cells more susceptible to the mitochondrial damaging effects of reactive oxygen species (ROS).

From the early stages of Parkinson's disease, NO production in the dopaminergic neurons increases until reaching a level that induces neuronal damage. Dopamine stored in dopaminergic cells may cause these cells susceptible to the deleterious effects of NO, which involve irreversible impairment of mitochondrial respiration (22). In addition, the inhibition of complex I activity markedly enhances superoxide radical ($O_2^{\cdot-}$) and H_2O_2 production (23), which by themselves, by the formation of $HO^{\cdot}$, and by the reaction of NO and $O_2^{\cdot-}$o to produce peroxinitrite ($ONOO^-$) that act synergistically to cause more complex I damage and mitochondrial dysfunction (24).

4. MITOCHONDRIA AS TARGET OF ENVIRONMENTAL TOXICS

Mitochondria are present in almost all types of eukaryotic cells being energy production their principal function; however it has been recognized that mitochondria have homeostatic functions in metabolic cell signaling, ion homeostasis, in the regulation of cell morphology, mobility and multiplication, and in triggering apoptosis.

Oxidative phosphorylation is the primary process by which the energy derived from the catabolism of carbohydrates, fats and proteins is used to synthesize ATP, the universal source of chemical energy in the cell. Therefore, any events that significantly alter ATP levels

Table 1. Mechanism of action and effects of pesticides on mammalian mitochondrial function

Compound / Chemical class / Main use	Active level	Assay site	Primary mechanism of action	Effect on mitochondrial function	Reference
DIELDRIN / Chlorinated hydrocarbon / Insecticide	IC_{50} = 30-100 microM	Dopaminergic PC12 cell culture	ROS generation in a dose-dependent manner	ROS generation. Mitochondrial membrane depolarization	96
	IC_{50} = 143 microM			Initiation of apoptotic cascade in dopaminergic cells	
		Rat liver mitochondria	Inhibition of Complex III, ATP-synthase and phosphate transporter	Decreased respiratory control and ADP/O ratios. Decreases mitochondrial membrane potential and prevents complete repolarizarion	49
PARATHION / Organophosphate/ Insecticide		Rat liver mitochondria	Inhibition of Complex II, ATP-synthase and phosphate transporter	Decreased respiratory control and ADP/O ratios. Decreases mitochondrial membrane potential	50
PERMETHRYN / Type I non-alpha-cyano-substituted pyrethroid/ Insecticide	IC_{50} = 0.73 microM	Rat liver mitochondria	Inhibition of Complex I sigmoidal dependence	Inhibition of state 3 respiration. State 4 respiration and ADP/O ratio are not affected	108
CYHALOTHRIN / Type II alpha-cyano-substituted pyrethroid/ Insecticide	IC_{50} = 0.57 microM	Rat liver mitochondria	Inhibition of Complex I sigmoidal dependence	Inhibition of state 3 respiration. State 4 respiration and ADP/O ratio are not affected	108
DELTAMETHRIN / Type II alpha-cyano-substituted pyrethroid / Insecticide	IC_{50} = 10-200 nmol / mg protein	Rat liver mitochondria	Inhibition of Complex II and Complex III	Inhibition of mitochondrial respiration in states 3 and 4	127
ROTENONE / Rotenoid / Insecticide	IC_{50} = 0.1-0.2 nmol / mg protein	Bovine heart mitochondria	Selective inhibition of complex I		57,58,128
FENPYROXIMATE / Pyrazole / Acaricide	IC_{50} = 4.6 nM	Bovine heart mitochondria	Selective inhibition of complex I		43
	IC_{50} = 0.4 microM	Rat liver mitochondria	Competitive inhibition of ubiquinone-2 reduction		103
FENAZAQUIN / Quinazolin / Acaricide	IC_{50} = 25-50 nM	Rat liver and bovine heart mitochondria	Selective inhibition of complex I		129
TERBUFENPYRAD / Pyrazole./ Acaricide	IC_{50} = 6 nM	Bovine heart mitochondria	Selective inhibition of complex I		43
PYRIDABEN / Pyridazinone / Acaricide	IC_{50} = 2.4 nM	Rat liver and bovine heart mitochondria	Selective inhibition of complex I		42,43
PARAQUAT / Bipirydilium / Herbicide	IC_{50} = 1.3 – 15 mM	Rat liver mitochondria	ROS generation Inhibition of complex I, partial inhibition of complex III and IV Inhibition of ATP-synthase activity Uncoupling effect	Decreased respiration in state 3. Increased respiration in state 4	47
		Rat brain mitochondria			82
CHLORPROPHAM / N-aryl carbamate / Herbicide	IC_{50} = 0.1-0.5 mM	Rat liver mitochondria	Uncoupling effect. Inhibition of electron transfer	Increased respiration in state 4. Decreased respiratory control. Decreased respiration in state 3	130
TTFB / 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazol / Insecticide and herbicide	IC_{50} = 0.08 microM	rat isolated liver mitochondria	Inhibition of electron transfer. Uncoupling effect	Decreased respiration in state 3. Decreased CCCP-stimulated respiration. Decreased respiratory control	55
2,4-D / Phenoxy acid /.Herbicide	IC_{50} = 150 microM	Rat liver mitochondria	Inhibition of Complex II and Complex III. Uncoupling effect at high concentrations	Decreased state 3 respiration. Decreased CCCP-stimulated respiration	44
DINOSEB / Dinitrophenol / Herbicide	IC_{50} = 500 nM	Rat liver mitochondria	Uncoupling effect. Inhibition of complex III	Concentration-dependent increase of state 4 respiration. Moderate decrease of state 3 respiration	44
MANEB / Manganese ethylene-bis-dithiocarbamate / Fungicide	IC_{50} = 4 microM	Rat brain mitochondria	Inhibition of complex III	Decreased state 3 respiration	88

will have physiological consequences. Several insecticides and acaricides exert their primary action on oxidative phosphorylation. Many others, *i.e.* the neurotoxic pesticides, have mitochondria as a secondary target. This second action of impairment of oxidative phosphorylation is sometimes a consequence of a primary action, *i.e.* an inhibition of a biosynthetic pathway essential for mitochondrial function or an extramitochondrial generation of ROS and reactive nitrogen species (RNS) (Table 1).

Chemical toxicants exhibit both species and tissue specificity. The specificity between species can be explained by the effects of toxicants over mitochondrial complex I. This complex is present in all eukaryotic organisms possessing mitochondria and in many bacteria, but their structure and sensitivity to inhibitors differ between species. In general, insect and fish complex I are the most sensitive to inhibition and in mammalians, neuronal mitochondria tend to be the most sensitive (25). The different tissue sensitivity may be also approached from the analysis of metabolic demand and bioenergy reserve. Cells requiring large quantities of energy, such as brain and cardiac cells, have large metabolic demands and, after energy depletion can survive for not more that three minutes before they cease functioning. Despite of toxicokinetic and toxicodinamics considerations, number and size, enzyme profiles, and membrane transporters systems of mitochondria determine organ-specific differences (26).

Some pesticides affect directly the mitochondrial electron transfer chain (Table 1), which in almost all cases lead to a further increased formation of damaging oxygen and nitrogen free radicals, because mitochondria are both a source and a target of free radicals (24).

5. MITOCHONDRIAL PRODUCTION OF FREE RADICALS

Mitochondria are organelles with a strongly reducing chemical environment with most of the steps in respiratory chain function involve single-electron reactions. The intermediates with unpaired number of electrons favor auto-oxidation with the univalent reduction of O_2. The respiratory chain generates O_2^- almost exclusively at complex I and at ubisemiquinone (23,27-29). In Complex I, the principal site of O_2^- generation seems to be one of the iron-sulfur cluster (either N-1 alfa or N2), since inhibitors as rotenone increase O_2^- generation by the full reduction of those carriers upstream from the site of inhibition (28-30). In complex III, the main mechanism of O_2^- generation is the autoxidation of ubisemiquinone, the Boveris-Cadenas reaction (28).

Spontaneous and enzymatic dismutation of O_2^- produce H_2O_2 in the mitochondrial matrix (23). The steady state concentration of O_2^- and H_2O_2 have been calculated and measures as 10^{-10} M and as 0.5-1.5 x 10^{-7} M, respectively (31). Moreover, the two species of the partial reduction of O_2, O_2^- and H_2O_2, are able to sustain the biochemical free-radical chain-reaction with formation of other free radicals such as $HO^{\cdot}$, alkyl, peroxyl, and alcoxyl. Non-radical, but also reactive species are also formed in the biochemical free-radical chain-reaction, such as alkyl hydroperoxides, organic peroxides and singlet oxygen ($^1O_2$) (24).

Nitric oxide (NO) is generated in the mitochondrial matrix by mitochondrial nitric oxide synthase (mtNOS), from the breakdown of arginine to citrulline with $NADPH_2$ and O_2 as substrates (32,33). Nitric oxide inhibit cytochrome oxidase activity and stimulates O_2^- production which, in turn, reacts with NO and generate the dangerous peroxinitrite ($ONOO^-$) (34,35). Peroxynitrite is produced by the rapid reaction of NO and O_2^-, and is considered to be an important factor in causing cellular damage mediated by mitochondrial dysfunction (36,37). The steady state levels of NO and peroxynitrite in the liver are in the ranges of 30-50 nM and 2-10 nM respectively (29). Many drugs and foreign agents can exert their toxicity mediated by production of toxic levels of peroxynitrite, i.e. by stimulation of the endogenous production of O_2^- or NO or by a decrease in antioxidant defenses, which is the case for drugs which are metabolized by conjugation with GSH or interfere with the biosynthesis of GSH (38). Nitric oxide and RNS have been related to the pathogenesis of several neurodegenerative disorders, among them Parkinson's disease, associated to the damage of the mitochondrial electron transfer chain (39-41).

6. TOXICANTS AFFECTING DIRECTLY MITOCHONDRIAL FUNCTION

Toxicants affect the mitochondrial function in several forms and the effect is variable in different tissues. Mitochondria vary widely in number, form and activity in different tissues and the differences are likely to determine which tissue are injured and which are resistant after exposure to mitochondrial poisons. Human synthesizes, and utilizes, approximately 40 kg/day of ATP. Brain, with a 2% to the body weight, is an important user of ATP, because it produces and uses 20% of the ATP. This high necessity of energy leads to a permanent brain damage if ATP is interrupted for more than a few minutes (42).

The maintenance of the selective permeability of inner membrane is the key to energy conservation during oxidative phosphorylation. The inner mitochondrial membrane is anisotropic, the matrix side being negatively charged and slightly alkaline; mitochondrial membrane potential is in the range of -170 to -220 mV with a pH variation of 0.4-0.6 (42). Because this feature, mitochondria can accumulate large amounts of positively charged lipophilic compounds such as MPP^+ and some acids in concentration that exceed that of the cytosol by orders of magnitude (25).

The toxicants can affect mitochondrial bioenergetics, either by interfering with the generation of the electrochemical gradient of H^+ or by causing its dissipation. The acute inhibition of electron transfer is associated to inability to utilize O_2 and cytotoxic hypoxia is associated with acidosis and hyperpnea, despite the normal PO_2 and absence of cyanosis. Inhibitors of the supply of reducing substrates for the respiratory chain cause a similar metabolic syndrome that is difficult to distinguish from inhibition of the electron transfer. The toxicants that dissipate the H^+ electrochemical gradient are uncouplers of oxidative phosphorylation and inducers of the permeability transition of the mitochondrial membrane, which likewise cause ATP deficits and metabolic acidosis and hyperpnea, but also induce excessive O_2 consumption, as reflected the lower PO_2 and cyanosis (25).

In summary, we can group the pesticides in relation to their mechanism of action on mitochondrial bioenergetics: a) pesticides that are inhibitors of the respiratory chain; and b) pesticides that are uncouplers of oxidative phosphorylation.

6.1 Pesticide inhibitors of the respiratory chain

Most of pesticides that are inhibitors of the respiratory chain are inhibitors of complex I, mitochondrial NADH: ubiquinone oxidorreductase or NADH-dehydrogenase (EC 1.6.5.3) that is the first in the series of membrane H^+ pumps of the mitochondrial respiratory chain and the most vulnerable to chemical induced malfunction within the respiratory chain (26). A large number of compounds with structural diversity have been recognized as complex I inhibitors, noticing a relationship between potency of their activity and a modular similarity with ubiquinone, with a cyclic "head" and a hydrophobic "tail". Compounds that seem to be specific and potent inhibitors act at concentrations low enough to have no effects on other components of the respiratory chain (43) (Table 1, see below point 7).

Pesticides with a selective mechanism of action by inhibition of Complex II, succinate: ubiquinone

oxidorreductase or succinate-dehydrogenase (EC 1.3.5.1) have not been clearly described.

Complex III, ubiquinol: cytocrome c oxidorreductase (EC 1.10.2.2) is the thirth membrane-spannning H^+-translocating component of the electron transfer chain. The variability in its structure from diverse species explains the different response and allows a relatively safe application of those chemicals as pesticides and drugs (25). Some pesticides as the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) act at the center Qi inhibing electron transfer from heme b_H to ubiquinone or ubisemiquinone bound at the center (44). In presence of the inhibitor, ubisemiquinone is not normally reduced by cytochrome b_H and not normally oxidized by heme b_H due to blockage of the electron transfer from b_L to center Qo, two processes that greatly increase the probability of partial reduction of O_2 and the generation of O_2^- (45,46).

In addition, many phenolic and substituted nitrophenolic compounds that act as uncouplers also partially inhibit complex III in isolated mitochondria, but the conclusions about the structure-activity relationship remain unclear (25).

The herbicide paraquat (47), several pyrethroid insecticides, DDT (48,49), and parathion have been reported as able to inhibit F_1-ATPase (complex V, ATP-synthase, EC 3.6.1.34) (50) (Table 1). Complex V is the unique molecular pathway implied in the mitochondrial synthesis of ATP from ADP and phosphate, constituting the major source of ATP in aerobic cells.

6.2 Pesticide uncouplers of the oxidative phosphorilation

A wide variety of pesticides are uncouplers of mitochondrial oxidative phosphorylation resulting in a decreased efficiency of ATP production. For a molecule to be an uncoupler it must be liphophilic, able to transverse mitochondrial membranes, and weakly acidic, able to dissociate protons (42). Certainly, one group of these chemicals possess protonophoric activity and their structural requirements include the presence of an acid-dissociable group, a bulky lipophilic groups and a strong electron withdrawing moiety, with a pKa in the range of 5-7. 2,4-Dinitrophenol (DNP) and other substituted phenols are the best studied class of mitochondrial poisons and their widespread use as herbicides and insecticides has sustained the interest in this mode of toxicity (51).

2-Trifluoromethylbenzimidazole (TBF) and derivatives were introduced in the 60s as a new class of potent herbicides and insecticides but early showed a high toxicity to animals. Many of these compounds act similar to DNP but their activity is significantly higher. The principal manifestation of toxicity of injected TBF is dyspnea, occasional salivation, weakness, and death in an extended position with immediate *rigor mortis*, which is typical for other mitochondrial uncouples as well. The most active uncoupler of this class is 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), which produces 50% uncoupling of oxidative phosphorylation, measured as ATP synthesis, at 8 x 10^{-8} M in isolated liver mitochondria. In a study comparing the toxicity of substituted TBFs in mitochondria isolated from mice, houseflies, and honeybees it was found that mice brain mitochondria were much more sensitive than liver mitochondria to TBF derivatives (52-56).

7. PESTICIDES RELATED WITH PARKINSONIAN SYNDROME, THEIR EFFECT OVER MITOCHONDRIAL FUNCTION

7.1. Rotenoids

The classic compound is the rotenone (2R,6aS,12aS) - 1,2,6,6ª,12,12ª - hexahidro - 2 - isoprenil - 8,9 - dimetoxicromeno [3,4 - *b*]furo [2,3-*h*] cromen - 6 - one), a natural isoflavonoid produced by *Leguminosae* plants. Rotenone, commonly used as pesticide in vegetable gardens, as to kill nuisance fish in lakes and reservoirs, is a potent inhibitor of complex I (57,58) (Table 1). In isolated beef heart or liver mitochondria, the median inhibitory concentration (IC_{50}) is 0.07 nmol/mg of protein with a *Ki* of 4 nM (43). It is not widely used in commercial agriculture but organic farmers are also permitted to use of rotenone joint to ryania, sabadilla and pyrethrum. This agriculture modality is practiced in about 111 countries in the world. Currently almost 23 million hectares (Mha) are managed organically with 11.6 Mha in Australia/Oceania, 5.1 Mha in Europe, 4.7 Mha in Latin America, 1.5 Mha in North America, 0.6 Mha in Asia, and 0.2 Mha in Africa (59).

The enzymatic activities of submitochondrial particles, obtained from rat brain mitochondria were determined after supplementation with rotenone in 0-10 microM range (Figure 1A). Results show the selective inhibition of complex I by rotenone, which is seen as a marked decrease of NADH-cytochrome c reductase activity (complex I-III activity) without modification of succinate-cytochrome c reductase (complex II-III activity. Rotenone had no effect on complex IV, *i.e.* cytochrome oxidase, activity.

Coupled mitochondria isolated from rat brain incubated with rotenone at concentrations in the 0-10 microM range, also showed the selective inhibition of complex I. Rotenone was able to show a dose-dependent and marked decrease in the respiratory control when the substrate used was malate-glutamate (IC_{50} 2.2 microM), whereas respiratory control was not modified when the substrate was succinate (Figure 2A).

Rotenone is considered a model toxic of environmental Parkinson (60-63). After weeks of rotenone administration, a selective nigrostriatal dopaminergic degeneration develops that is similar to that observed in Parkinson disease. Electron microscopy revealed cytoplasmic inclusions in nigral neurons containing alpha-synuclein and ubiquitin that are reminiscent of Lewy bodies (64). A brain rotenone concentration of 20–30 nM was reported enough to partially inhibit complex I, but too low to significantly impair respiration of brain mitochondria, produce ATP depletion and explain neurodegeneration (64).

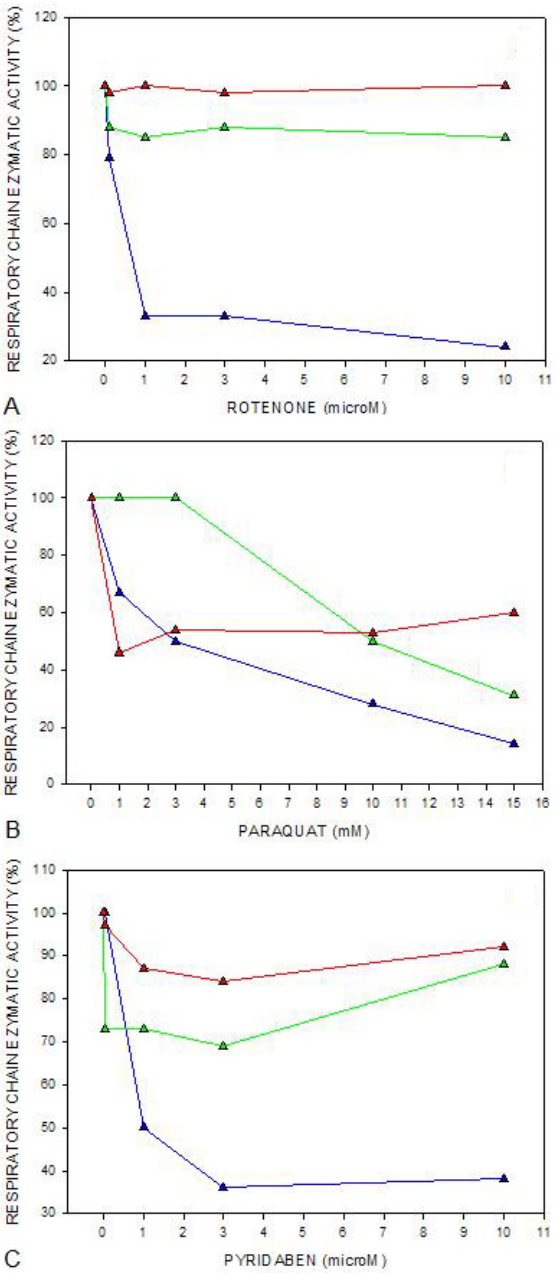

Figure 1. Effects of rotenone (A), paraquat (B) and pyridaben (C) on the mitochondrial electron transfer activities of rat brain submitochondrial particles. Blue line and symbols: complexes I-III activity; green line and symbols: complexes II-III activities; and red line and symbols: complex IV activity. Mitochondrial samples, isolated as described, were twice frozen and thawed and homogenized each time by passage through a tuberculin needle (131-133). Submitochondrial particles were incubated 3 min with the pesticides. The enzyme activities of Complexes I-III, II-III, and IV were determined spectrophotometrically at 30°C with the submitochondrial particles suspended in 100 mM phosphate buffer (pH 7.4) added with the corresponding substrates (121,131,132).

Rotenone impairs complex I causing dopaminergic neurodegeneration via oxidative stress, rather than by bioenergetic defect, and lipid peroxidation may play a critical role in mitochondrial damage (65). It has been reported that brain mitochondria from rotenone treated animals show enhanced O_2^- production. Also, *in vivo* experiment with rotenone infusion to rats elevates soluble and insoluble protein carbonyls mainly in dopaminergic areas, the midbrain and the olfactory bulb. Pre-treatment with alpha-tocopherol *in vitro* markely attenuated protein carbonyl elevation chronically induced in organotypic slice culture including *substantia nigra* of rat brain (63).

It is noteworthy that rotenone induces apoptosis in human dopaminergic cells and that may contribute to the etiology of Parkinson disease. Rotenone-induced reduction in dopamine uptake was attributed not to an alteration in dopamine transporter but rather to dopaminergic neuronal death. Assays with HL-60 (promyelocitic leukemia) and BJAB (B-cell lymphoma) culture human cells at 5 microM rotenone induce apoptosis with changes in mitochondrial membrane potential, caspase-3 activation, and DNA ladder formation, and this apoptotic events are secondary to H_2O_2 production and mitochondrial dysfunction (66). On the other hand, the presence of microglia increased rotenone neurotoxicity in mouse neuron-glia cultures and this observation can be attributed to the release of NADPH oxidase-derived O_2^- from activated microglia (67).

Although rotenone represent an useful model of Parkinson disease it is unlikely that rotenone is a major cause of Parkinson disease. Rotenone has poor oral bioavailability and it is rapidly biodegraded in the environment. It is highly lipophilic and easily crosses the blood-brain barrier and it does not depend on the dopamine transporter for access to cell, causing an uniform complex I inhibition throughout the brain (68).

7.2. Paraquat

Several studies have correlated Parkinson disease and exposure to the herbicide paraquat, which it is known to produce selective degeneration of dopaminergic neurons (69-72).

Paraquat (1,1-dimethyl-4,4'-bipyridilium chloride) is the most potent of bipyridyl herbicides and is widely used in agriculture in more than 100 countries. Acute intoxication with paraquat has provoked a large number of human fatalities and the situation attracted much attention and interest about the biochemical mechanisms involved in the toxicity. Pathologic changes observed at autopsy in all of these human poisonings showed evidences of liver, heart, kidney and lung damage. Lung exhibited the most striking injury, a wide-spread cellular proliferation and fibrosis, closely related with the marked respiratory distress previous to patient death. The mechanism of action implied in lethal respiratory failure has been accepted to be mediated by O_2^- anions generated by the reduction of paraquat by NAD(P)H in mitochondria and microsomes from type I and II alveolar epithelial cells. A further generation of H_2O_2 and HO^-, this latter by the Fenton

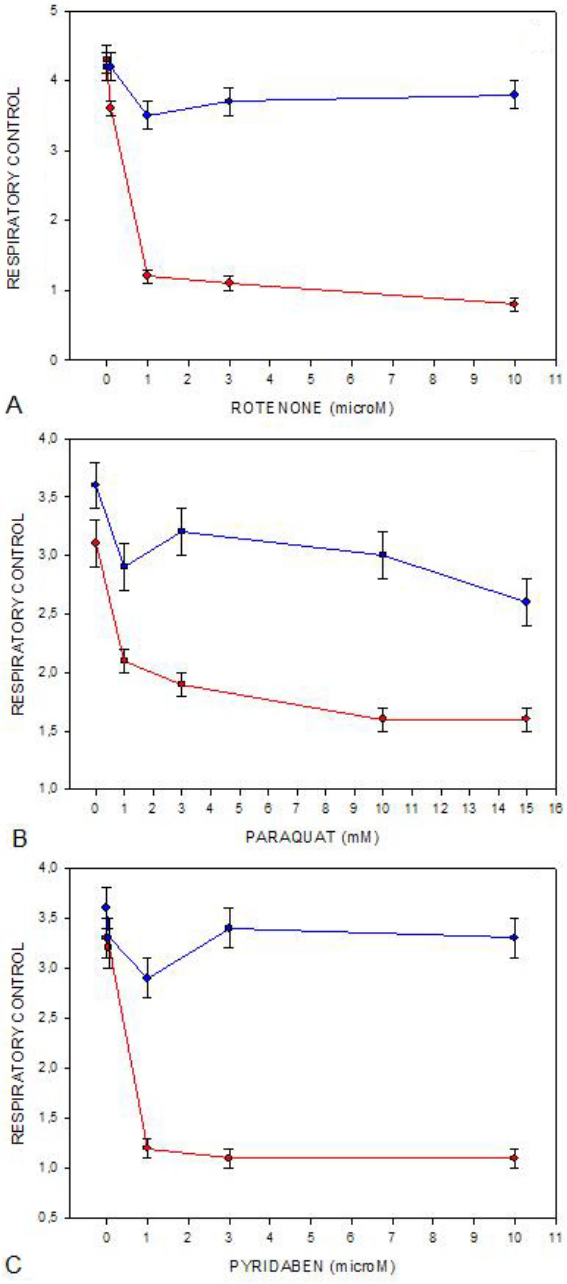

Figure 2. Effects of rotenone (A), paraquat (B) and pyridaben (C) on the respiratory control of rat brain mitochondria. Respiratory rates were determined with 5 mM malate-5 mM glutamate (red line and symbols) and with 10 mM succinate (blue line and symbols) as substrates. State 3 active respiration was established by addition of 0.5 mM ADP. Mitochondrial O_2 uptake was determined polarographically with a Clark-type electrode (Hansatech Instrumens Ltd.) as described in Navarro *et al.* (121) and in Boveris *et al.* (80).

reaction in the presence of Fe^{2+}, promotes lipid peroxidation by a redox cycling enhanced by high concentration of O_2 in the lung (45,73-76).

Paraquat was reported to damage mitochondria *in vivo* and *in vitro*, resulting in cell death. It was postulated that tissue-selective toxicity may be caused by an accumulation of compound in the target organ (77,78), however paraquat is not transported into the mitochondrial (79). Paraquat induces a Ca^{2+}-dependent permeability increase of the inner mitochondrial membrane leading to membrane depolarization, uncoupling and matrix swelling. The Ca^{2+}-dependent permeability increase is due to inappropriate opening of the endogenous permeability transition pore (MTP), a regulated, voltage-dependent channel of the inner mitochondrial membrane. The pore is primarily affected by paraquat through a shift of the gating potential to more negative values, allowing pore opening at physiological membrane potential. This effect apparently involves oxidation of a critical dithiol in the pore voltage sensor, while other regulatory aspects of the MTP (matrix pH and Ca^{2+}) are unaffected by paraquat (79).

Palmeira *et al.* (47) reported that paraquat impairs mitochondrial function in rat liver mitochondria by uncoupling oxidative phosphorylation, by inhibiting electron transfer in complex III y IV, and by a direct inhibitory effect on ATPase complex. The assays were carried out in the range 0-10 mM, that is about similar to the level reached in serum after subcutaneous administration of a lethal dose of 340 mg/l. In our laboratory, mitochondria isolated from rat brain rat showed an impairment of electron transfer activities (Figure 1B), that can be summarized as similar to the data from Palmeira in liver mitochondria (47). The incubation of submitochondrial particles with paraquat at 0-15 mM showed a dose-dependent inhibition of NADH-cytochrome c reductase, succinate-cytochrome c reductase, and cytochrome oxidase activities. These results suggest a direct oxidative damage of paraquat over the electron transfer chain complexes. It is worth noting that NO has been proposed as endogenous mediator of the oxidant damage induced by paraquat in the lung (80)

A study involving paraquat acute poisoning in rats disclosed a decreased complex I activity in brain, lung and liver mitochondria prior to the appearance of respiratory dysfunction (81,82). Dopamine in the rat striatum nucleus was significantly lower than in controls. In addition, high levels of lipid peroxides were detected in brain, which brings up the hypothesis of mitochondrial dysfunction secondary to oxidative damage (82). The effect of paraquat on MTP opening has been referred to inhibition of electron transfer at NADH dehydrogenase or to respiratory chain inhibition by NO (80).

Rat brain mitochondria incubated with 0-15 mM paraquat show a decrease in respiratory control when malate-glutamate was used as substrate (IC_{50} = 1.6 mM), whereas respiratory control was not modified in the presence of succinate as substrate (Figure 2B). This result indicates that complex I is selectively inhibited by paraquat, an action that is likely due to an oxidative damage mediated by the pesticide. Although some authors have described that Paraquat was a weak inhibitor ((IC_{50} = 10 mM) of brain mitochondria complex I (68).

However, it remains unclear how paraquat, a charged hydrophilic compound, can cause selective dopaminergic neurodegeneration in treated animals. Due to the structural similarity with MPP^+ (identical to the herbicide Cyperquat) it has been speculated that paraquat may be actively transported into dopaminergic neurons. A study comparing the mechanism of action of MPTP, rotenone and paraquat showed that paraquat is neither a substrate for neuronal dopamine transporter nor it inhibits *in vivo* dihydrorotenone binding site in brain mitochondria complex I.

7.3. Ethylene-bis-dithiocarbamate (EBDC)

Ethylene-bis-dithiocarbamate (EBDC) fungicides (Maneb and Mancozeb) are used heavily in the United States. Their use has been restricted due to controversial implication in human thyroid cancer. These substances metabolize to ethylene thiourea, a carcinogen. Ethylene thiourea also decreases thyroxine (T4) and increases thyroid-stimulating (TSH) plasma levels in rodents (83,84). Rarely EBDCs were involved in acute toxicity, although some cases with neurological reversible symptoms, as headache, dizziness, confusion and seizures, have been reported. Long-term exposure has been associated with neurodegenerative disorders and increased risk of neurocognitive impairment (85).

Mn-EBDC is the main component of Maneb, (2-[(dithiocarboxy) amino]ethyl] carbamodithioato]](2-kappaS,kappaS′)]manganese), that has been associated to parkinsonism in chronically exposed agricultural workers (86), with the effect correlated with selective damage of dopaminergic neurons of the striatal system (87). Maneb induced selective dopaminergic striatal degeneration the brain of rats chronically injected in the lateral ventricle (20 nM, for 14 days). Also, extracellular striatal dopamine accumulation after infusion (500 microM) was comparable with that induced by MPP^+ (88). The possible mechanisms implicated in dopamine cell damage have been investigated. Some researchers have speculated about the role of oxidative stress and damage in Maneb mediated toxicity (89,90). A study reported that Mn-dithiocarbamates and Zn-dithiocarbamates (Mn-DTC and Zn-DTC), the major ingredients in Maneb and Zineb, respectively, had the potential to provoke oxidative damage in the catecholaminergic regions of brain. *In vitro*, these compounds catalyzed the auto-oxidation of dopamine and norepinefrine to the semiquinone form with generation of O_2^- (91). Moreover, the role of mitochondria was indicated when brain mitochondria exposed to Maneb showed a selective damage, dose-dependent, of complex III (IC_{50} = 6.8 microM). Neither $MnCl_2$ nor EBDC individual components separately caused any of those effects (88).

7.4. Dieldrin

Dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*endo,exo*-1,4,:5,8-dimethanonaphtalene (HEOD) is a chlorinated cyclodiene high lipophilic compound widely used as insecticide since 1970s in agriculture and industry. It is classified as one of the 12 more persistent and bioaccumulative toxics by the US Environmental Protection Agency (US-EPA) with a half-life of 300 days in humans and up to 25 years in the environment. General population is exposed primary via daily intake of contaminated food. Acute human poisoning as well as a biocide effect have been accepted to be mediated by an increase in the permeability of neuronal membrane ion channels and the inhibition of GABA receptor in sensory and motor nerve fibers and the motor cortex. The resulting neuronal hyperexcitation is correlated with the clinical symptoms: paresthesia, irritability, dizziness, disturbed equilibrium, tremor, and mainly tonic and clonic convulsions (92).

In the middle 80s, US-EPA banned the use of dieldrin for almost all application due their carcinogenic and genotoxic properties. Dieldrin was recognized as inductor of liver tumors specifically in mice, probably due to a different metabolization pathway, while the carcinogenic effect in human has not been demonstrated. This pesticide integrate group 3 (not classifiable as carcinogenic to humans) of the International Agency for Research on Cancer (93). Unlikely in mice in human, central nervous system nor the liver appears to be a critical target organ.

Kanthasamy *et al.* (94) reported dieldrin as an inductor of Parkinson disease. The cause-effect relationship remains unclear. Dieldrin appears as a relatively selective dopaminergic neurotoxin in mesencephalic cultures at low levels (EC_{50} = 8.0 microM) (95). Other experimental studies reported that subchronical and chronical administration of dieldrin to rats depleted the brain levels of dopamine (59%) and norepinefrine (38%). The effects are related to an enhanced reuptake in pre-synaptic terminals and to an inhibition of vesicular monoamine transport in striatum, associated both to an increase in free dopamine. These alterations in dopamine turnover seem to correlate with an increase in ROS production. In fact, pre-treatment with a monoamine oxidase (MAO) inhibitor or with the SOD reduces intracellular dose- and time-dependent ROS increase in dopaminergic cell culture PC12 after acute exposition to 143 microM dieldrin (96). Higher concentrations of dieldrin have been detected in *post-mortem* brain of Parkinson and Alzheimer's disease patients in comparison with other neurological control cases (97-99).

Mitochondria seem centrally involved in dieldrin toxicity as triggers of apoptosis. Intracellular ROS elevation, altered mitochondrial membrane potential, formation of the transition pore, and cytochrome *c* release were reported in PC12 cells supplemented with dieldrin (94). In the same preparation, attenuation of citochrome c release was observed by treatments with free radical scavengers and the over-expression of the antiapoptotic Bcl-2 protein completely prevented the cell death caused by dieldrin. In human leukemic T-cells and in mouse nigral dopaminergic cells, dieldrin activated caspase-3 and caspase-9 whereas did not activate caspase-8, indicating that dieldrin-cytotoxic death is mediated by mitochondria and not by death receptors. In the same way, dieldrin promoted the proteolytic cleavage of polyADP-ribose polymerase and protein kinase C-delta mediated by

caspase-3 in dopaminergic cells. DNA fragmentation, a hallmark of the terminal phase of the apoptotic process present in neurodegenerative disorders, and stimulation of alpha-synuclein fibril formation has been after dieldrin supplementation (100,101).

7.5. Synthetic insectide/acaricide inhibitors of Complex I

Due to a wide use of insecticides, insect resistance against almost all of chemical pesticides is arising. Many of these insecticides are neurotoxins directed to a few receptor and enzyme targets: acetylcholinesterase in organopophosphates and carbamates; the voltage-dependent Na^+ channel in DDT, pyrethroids and dihydropirazoles; the GABA receptor and Cl^- channel in cyclodienes and phenilpyrazoles; and the nicotinic receptor of acetylcholine in nitromethylenes (102).

A new class of insectides/acaricides has been developed with the synthesis in the last twenty years of a series of compounds with a mechanism of toxic action based on the inhibition of the activity of complex I (NADH: ubiquinone oxidoreductase) following the classic example of the naturally occurring rotenone. These numerous synthetic inhibitors of mitochondrial electron transfer are included in some pesticide list as "unclassified acaricides" and have been classified into two main groups: a) pyrazoles, and b) substituted pyrimidines, pyridines, and quinazolines (103). The most used are: a) the pyrazole fenpyroximate (tert-butyl (E)-alpha-(1,3-dimethyl-5-phenoxypyrazol-4-yl-methyleneamino-oxy)-p-toluate; Dow AgroScience); b) the pyridazinone pyridaben (2-*tert*-butyl-5-(4-*tert*-butylbenzylthio)-4-chloropyridazin-3(2*H*)-one; Basf)); and c) the quinazoline fenazaquin (4-[2-[4-(1,1-dimethylethyl)-phenyl]ethoxy]quinazoline; Dow AgroScience) (104).

It has been speculated about the similarity of these compounds with ubiquinone with a cyclic "head", the N-heterocyclic ring, substituted with a lipophilic side chain via nitrogen or oxygen atom. These compounds resemble the classical inhibitor rotenone, that specifically blocks electron transfer between iron-sulfur cluster N2 y ubiquinone (Q) in complex I. With respect to the inhibition mechanism the proposed models implied several Q binding sites. Enzyme kinetic studies revealed two inhibitor classes: pyrazoles bloqued ubiquinone-2 reduction in a partially competitive manner; rotenone showed a non-competitive behavior under the same experimental conditions, but the two inhibitors specifically inhibited the ND1 subunit of complex I. Moreover, a close competition between the insecticide and hydrorotenone for a binding site at the ND1 subunit has been reported. Other studies placed the inhibition binding in the PSST site, a 20-kDa subunit of complex I with highly conserved cysteine motifs in their primary structure, probably located at the final component involved in electron transfer from the N2 cluster to ubiquinone (105,106).

Although it seems possible that this group of insectides/acaricides could produce a parkinsonian syndrome, this has not been described yet.

One of the compounds more used of this group is pyridaben, a pyridazinone acaricide which act by contact with a high acute effect in different insects and a moderate persistence (6-8 weeks; soil half-life 14-30 days) (104). It is described as a inhibitor of mammalian complex I stronger than rotenone with a twice higher inhibitory relative potency and with IC_{50} values = 0.8-4.0 nM in different mammalian tissues (42,43).

The effects of 0-10 microM pyridaben on respiratory chain enzymatic activities were assayed in rat brain submitochondrial particles (Figure 1C). The pattern of inhibition resulted similar to the one of rotenone pattern, with a selective inhibition of marked decrease of NADH-cytochrome reductase activity (complex I-III) and without modification of succinate-cytochrome c reductase activity (complex II-III) and of cytochrome oxidase activity (complex IV).

Rat brain mitochondria incubated with 0-10 microM pyridaben, showed a marked and dose-dependent marked decrease in respiratory control in the presence of malate-glutamate as substrate (IC_{50} = 1.7 microM), but respiratory control was not affected when the substrate was succinate (Figure 2C). These changes in respiratory control corroborate the selective inhibition of complex I by pyridaben.

7.6. Pyrethroids

Pyrethroids are becoming the major class of pesticides for agricultural and public health applications. Interestingly, mammals are about 3 orders of magnitude less sensitive to pyrethroids than insects. The widespread use of these insecticides opened an active interest in the human repercussions of the highly increased exposure to these compounds. Recent reports suggested that pyrethroid toxicity has significant aspects that were not considered in the original evaluation. Acute functional neurotoxicity is the major effect seen in mammals. *In vitro* studies provided evidence of a series of complex pyrethroid effects: interaction with the voltage-dependent gate of the Na^+ channel (the classical insecticide mechanism), impairment of nicotinic cholinergic transmission, enhancement of noradrenaline release, direct action on Ca^{2+} and Cl^- ion channels, and inhibition of GABA-mediated neurotrasmission (107). The inhibition of complex I has been again considered as the mechanism of the toxic action in both type of Pyrethroids, type I (non-alpha-cyano-substituted; permethrin) and type II (alpha-cyano-substituted; cyhalothrin). This inhibition of complex I would be the mechanism of the chronic motor and sensory disorders induced by Pyrethroids. Interestingly, and as mentioned before, the concentrations to produce a significant *in vitro* effect in mammalian cells are higher (10^{-7} M) than those needed to alter insect Na^+ channel (10^{-10} M) (108).

8. EFFECT OF ROTENONE, PARAQUAT AND PYRIDABEN ON MITOCHONDRIAL NITRIC OXIDE PRODUCTION

A relation between NO production and the mitochondrial damage induced by rotenone and paraquat has been proposed (62,109,110). The role of mitochondrial

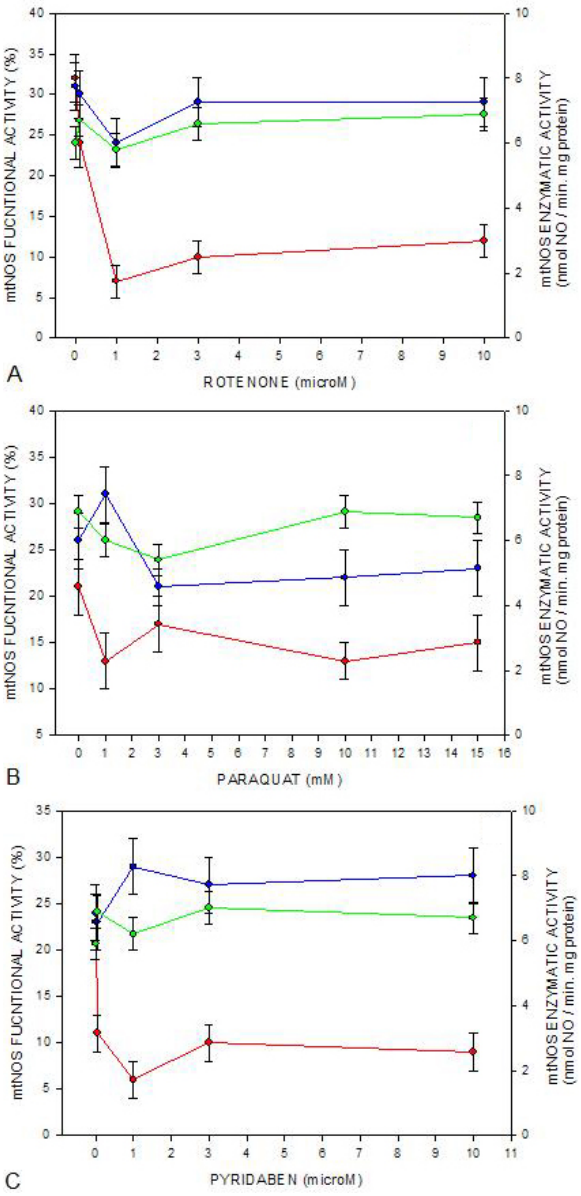

Figure 3. Effects of rotenone (A), paraquat (B) and pyridaben (C) on the mtNOS functional activity of rat brain mitochondria and on the mtNOS biochemical activity of rat brain submitochondrial particles. The mtNOS functional activity was determined by the difference between the rates of mitochondrial state 3 respiration at maximal and minimal intramitochondrial NO levels (134,135). The first condition was achieved by supplementation with 0.2 mM L-arginine and 1 microM Cu,Zn-superoxide dismutase, and the second one by addition of 1 mM L-NAME (the NOS inhibitor). Respiratory rates were determined as indicated in Figure 2. Malate-glutamate (red line and symbols); succinate (blue line and symbols). Spectrophotometric determination of mtNOS biochemical activity (green line and symbols) by the oxyhemoglobin oxidation assay at 30°C, as described (122).

NO production in relation with the mechanism of action of these toxicants is an open question. Nitric oxide is an O_2-competitive inhibitor of mitochondrial cytochrome oxidase (111-115). It has been calculated that endogenous mitochondrial nitric oxide synthase (mtNOS) activity inhibits mitochondrial respiration in the tissues by 18-25 % (116). On the other hand, it is important to consider the possibility that $ONOO^-$, the product of the termination reaction between O_2^- and NO, acting as irreversible inhibitor of complex I (114,117) and of complex III (118,119).

In preliminary assays we determined the effects of rotenone, paraquat, and pyridaben on the mtNOS functional activity of rat brain mitochondria. The assay is performed by a simple measurement of mitochondrial O_2 uptake. Mitochondria are placed in active state 3, and the mtNOS functional activity is the difference of the rates of O_2 uptake between (a) the state 3 supplemented with L-arginine (mtNOS substrate), and (b) the state 3 supplemented with a selective (L-arginine-competitive) inhibitor of mtNOS (and NOS in general) (120,121). Rotenone (Figure 3A) and pyridaben (Figure 3C) produced a marked decrease in mtNOS functional activity, when malate-glutamate was used as substrate, but not when the substrate was succinate. The effect is then specific of complex I inhibitors in conditions of an effective activity of complex I in the oxidation of NAD-dependent substrates. At variance, paraquat did not produce significant effects on mtNOS functional activity, neither using malate-glutamate nor succinate as substrates (Figure 3B). Paraquat is not able to cross the mitochondrial membrane and has a weak effect on O_2 uptake.

The effects of rotenone, paraquat and pyribaden on the enzymatic activity of mtNOS were assayed, at the previously used range of concentrations, in rat brain submitochondrial particles (122). Interestingly, there was no effect of the pesticides on mtNOS biochemical activity (Figure 3). The results suggest that the effects of rotenone and pyridaben on mtNOS functional activity depend of the establishment of a NAD-dependent O_2 uptake.

9. PERSPECTIVES

The selective inhibition of mitochondrial NADH-dehydrogensae activity by pesticides as rotenone and pyridaben may constitute part of the pathogenic mechanism of Parkinson's disease. Rotenone and pyridaben, selective inhibitors of complex I, were able to inhibit the mtNOS functional activity, *i.e.* the effect of endogenous mitochondrial NO on cytochrome oxidase, of rat brain mitochondria only in conditions of an active oxidation of NAD-dependent substrates. This suggests than the activity of the mtNOS inserted in the inner mitochondrial membrane depends on complex I activity. Since the players, complex I, NO and complex I inhibitors, have already being implicated in the etiopathology of Parkinson's disease, a role of the mtNOS regulated by complex I seems interesting to investigate.

Recently Boveris *et al.* (123) reported that the NO release from rat liver mitochondria exhibit an

exponential dependence on membrane potential. The phenomenon is interpreted as that membrane potential determines the protein conformation and enzyme activity of mtNOS, a constituent of the inner mitochondrial membrane. The almost parallel decreases in respiratory control, O_2 uptake and mtNOS functional activity produced by the selective complex I inhibitors, rotenone and pyridaben, could de interpreted as a close regulation of mtNOS by NADH-dehydrogenase by protein-protein interaction. In other words, the physiological activity of mtNOS woul be regulated by the electron transfer through NADH-dehydrogenase. In such a way, complex I and mtNOS would be at the center of the stage in the molecular mechanism underlying parkinsonism. Mitochondrial NO is necessary in controlled concentrations for cell proliferation or apoptosis (121,124). Recently, NO has been implicated in mitochondrial biogenesis through stimulation of guanylate cyclase, generation of cGMP and activation of PGC1-alpha (125,126). The decreased activity of complex I in Parkinson's disease would imply a decreased mtNOS activity and lower NO diffusion to the cytosol with the consequent diminished mitochondrial biogenesis in the dopaminergic neurons.

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Abbreviations: EBDC: ethylene-bis-dithiocarbamate; MPTP:1-methyl-4-1,2,3,6-tetrahydropyiridine; MPP^+ : 1-methyl-4-phenilpyiridinium ion; O_2^-: superoxide radical; H_2O_2: hydrogen peroxide; NO: nitric oxide; $ONOO^-$: peroxinitrite, MTP: permeability transition pore; US EPA: US Environmental Protection Agency; mtNOS: mitochondrial nitric oxide synthase; CCCP: carbamyl cianyde 3-chloro phenyl hydrazone

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