Neuroleptic-Induced Mitochondrial Enzyme Alterations in the Rat Brain

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ABSTRACT

For years, it has been known that neuroleptics have the capacity to interfere with the mitochondrial respiratory chain in vitro. We report that haloperidol and fluphenazine, classical neuroleptics, cause a generalized reduction in the activity of NADH:ubiquinone oxidoreductase (complex I) in the rat brain in vivo, an effect that was not observed with the atypical neuroleptic, clozapine. MPTP, which bears significant structural similarities with haloperidol, also demonstrated a significant reduction in complex I activity after low-dose, chronic administration. Interestingly, an increase in the activity of cytochrome-c oxidase (complex IV), probably reflecting enhanced functional neuronal activity, was observed in the frontal cortex of all chronically treated animals, an effect that is unlikely to result from compensation for the inhibition of complex I. Results suggest that previous findings, in which a reduction in the activity of cytochrome-c oxidase was observed in postmortem brain samples from schizophrenics, are not dependent on treatment with neuroleptics.

The finding that the activity of COX (complex IV of the mitochondrial respiratory chain) is significantly reduced in the caudate nucleus and frontal gyrus of schizophrenic patients (Cavalier et al., 1995) prompted us to address the hypothesis that neuroleptic treatment could underlie respiratory chain inhibition in vivo. A growing base of evidence provides support for the hypothesis that an imbalance in mitochondrial energy production may be important in the etiology of schizophrenia. Clinical studies have demonstrated that schizophrenic patients, in comparison with controls, have lower metabolic rates in wide areas of cortical and subcortical structures in the brain (Wiesel, 1992). Other metabolic changes, such as a decrease in creatine kinase levels, have also been found in the brains of schizophrenics, suggesting that local concentrations of ATP may be altered (Klushnik et al., 1991). However, whether or not these effects are a result of the disease or neuroleptic treatment is at present unknown. Previous studies, primarily involving chlorpromazine, have demonstrated a generalized reduction in oxygen consumption in isolated brain and liver mitochondria (Guth and Sprites, 1964), but despite the 40 yr that have transpired since the first results were obtained, no in vivo studies on specific mitochondrial enzyme systems have been performed. Our study was undertaken primarily to establish whether or not COX inhibition could be a direct effect of neuroleptic treatment or an adaptive change occurring as a response to the up-stream inhibition of the respiratory chain after chronic antipsychotic administration.

Recently, this line of research was revived based on findings that neuroleptics inhibit NADH:ubiquinone oxidoreductase (complex I) (Burkhardt et al., 1993) in vitro, with the implication that this could underlie the development of potentially irreversible extrapyramidal disturbances, of which tardive dyskinesia is the most important. Thus, in light of dissipating support for the involvement of D2 receptor supersensitivity in the etiology of tardive dyskinesia, it was suggested that neuroleptic treatments may produce the condition via a blockade of the mitochondrial respiratory chain, specifically complex I (Burkhardt et al., 1993).

Haloperidol, a prototype butyrophenone neuroleptic used commonly in the treatment of schizophrenia, possesses structural similarities with the dopaminergic neurotoxin MPTP (Subramanyam et al., 1990). Interestingly, it has been demonstrated that haloperidol can undergo oxidation to give the metabolite HPP⁺, resembling the active toxic metabolite of MPTP, MPP⁺ (Subramanyam et al., 1991) which is formed extraneuronally via oxidation by MAO-B (Chiba et al., 1984). MPP⁺ is thought to mediate its toxic effects via a selective inhibition of complex I resulting in bioenergetic compromise (Niklas et al., 1985). Recently, it has been demonstrated that HPP⁺ is also a potent inhibitor of complex I (Rollema et al., 1994). In further support of the concept that a complex I

ABBREVIATIONS: COX, cytochrome-c oxidase (complex IV); HPP⁺, haloperidol pyridinium derivative; MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP⁺, 1-methyl-4-phenylpyridinium; complex I, NADH:ubiquinone oxidoreductase; ATP, adenosine triphosphate.
deficiency may be involved in the development of extrapyramidal disturbances, are the findings that complex I abnormalities have been identified in platelet (Parker et al., 1989) and brain (Mizuno et al., 1989) mitochondria from individuals with idiopathic Parkinson's disease.

Earlier investigations on the effects of neuroleptics on MAO have failed to demonstrate any significant alterations (Van Der Krogt et al., 1982). In light of the fact that MPTP, itself a substrate and inhibitor of MAO (Fuller and Hemrick-Luecke, 1985), bears structural homology with haloperidol, MAO activity was analyzed after chronic neuroleptic treatment as well as after a low dose, chronic treatment with MPTP. Our interest in the analysis of MAO was also based on its outer mitochondrial membrane localization, lack of involvement in energy metabolism, and thus its use as a marker for mitochondrial number.

Materials and Methods

Materials. Haloperidol (Haldol) was obtained from Janssen Pharmaceutica (Beerse, Belgium). Clozapine was a gift from Sandoz Pharma Ltd. (Basel, Switzerland). Fluphenazine was purchased from Sigma Chemical Co. (St. Louis, MO). MPTP-HCl was purchased from Research Biochemicals Inc. (Natick, MA). Ubiquinone-1 was purchased noncommercially through F. Hoffman-La Roche AG (Basel, Switzerland). MPTP-HCl was purchased from Research Biochemicals Inc. (Natick, MA). Ubiquinone-1 was purchased noncommercially through F. Hoffman-La Roche AG (Basel, Switzerland). β-[ethyl-14C]phenylethylamine and 5-[2-14C]hydroxytryptamine were from Amersham (London, UK). All other compounds were obtained from Sigma.

Animal treatments. Eight-wk-old Male Sprague-Dawley albino rats initially weighing 250 to 300 g were housed in groups of three or five animals per cage with a 12-hr light/dark cycle, at a constant temperature of 21°C. For investigations with haloperidol, animals were divided into six groups: saline; 2 days (N = 6), 14 days (N = 6), and 28 days (N = 10), and Haldol; 2 days (N = 6), 14 days (N = 6), and 28 days (N = 10). Clozapine (N = 10), fluphenazine (N = 10) and MPTP (N = 10) were administered to animals for 28 days. Animals received injections i.p. with either saline (control) or Haldol (10 mg/kg/day), clozapine (20 mg/kg/day), fluphenazine (1.0 mg/kg/day) or MPTP (1.0 mg/kg/day). All compounds with the exception of Haldol (commercial solution) were dissolved in 0.02 M lactic acid and saline. At the times indicated in the text, animals were killed by decapitation. Brains were rapidly removed and the striatum, frontal cortex, hippocampus and cerebellum were dissected from both the left and right hemispheres (excluding cerebellum) and frozen immediately on dry ice. The total amount of protein in the samples was determined according to the Markwell modification of the Lowry procedure (Markwell et al., 1978; Lowry et al., 1951).

MAO assay. MAO activity was determined by a radiometric method similar to that described by Hallman et al. (1987) using β-[ethyl-14C]phenylethylamine and 5-[2,14C]hydroxytryptamine as substrates. Dissected brain regions were resuspended in 0.01 M phosphate buffer, 1 mM EDTA, 0.25 M sucrose (pH 7.7), homogenized by 10 strokes with an Ultra-Turrax and frozen at −70°C until assayed. For MAO-A, sonicated samples containing approximately 100 μg protein were incubated at 37°C for 20 min in a final volume of 100 μl 0.05 M phosphate buffer (pH 7.4) in the presence of 0.05 μCi C14-labeled 5-hydroxytryptamine (50 μM). For MAO-B, samples were performed identically except that samples were incubated for 10 min in the presence of 0.025 μCi C14-labeled β-phenylethylamine (25 μM). The reaction was terminated by the addition of 50 μl HCl (1 M). The acid oxidation products were extracted with the addition of 750 μl toluene-ethylecetate (1:1, v/v). Aliquots (500 μl) of the organic extracts were transferred to vials containing 8 ml ReadySafe counting fluid and analyzed by liquid scintillation spectrometry using a Packard Tri-carb Liquid Scintillation Analyser model 1900 CA.

Cox assay. COX was assayed according to a modification of the spectrophotometric method of Yonetani and Ray (1965). Reduced cytochrome c was prepared by the addition of 30 mg Na2S2O4/100 mg cytochrome c in 0.01 M phosphate buffer, 1 mM EDTA, pH 7.4 and separated on a G-25 Sephadex column. Brain samples were prepared as in the MAO assay, but with the addition of 0.05% deoxycholate as a solubilizing agent after sonication for 30 sec. Incubation mixtures consisted of 10 mM phosphate buffer, 2 mM EDTA and 25 μM reduced cytochrome c. Upon the addition of approximately 100 μg protein, the change in absorbance at 548 nm was determined on an Hitachi model U-2000 spectrophotometer for 5 min at which point K4Fe(CN)6 was added and the final absorbance read. Initial rates were determined by the differential method in which d[ferrocytochrome c]/dt is determined from polynomial plots solved at zero time (Yonetani and Ray, 1965).

NADH:ubiquinone oxidoreductase assay. NADH:ubiquinone oxidoreductase (complex I) was assayed according to a modification of the method described by Whitfield et al. (1981) in the presence and absence of 5 μM rotenone. Approximately 500 μg of brain homogenate were added to a reaction mixture containing 0.042 M DPPS, pH 7.4, 0.2 mM EDTA, 80 μM coenzyme-Q, and 2 mM NaN3. Reaction mixtures were equilibrated to 25°C for 3 min before the addition of 0.1 M NADH to initiate the reaction. The decrease in absorbance at 340 nm was monitored for 2 min and activity calculated from linear plots using a combined extinction coefficient for Q1 and NADH of 6.81 mM−1 cm−1.

Statistics. Statistically significant differences in biochemical data were established using Student’s t test. Significance was assumed for all values where P < .05.

Results

Classical neuroleptics and MPTP cause generalized reduction in NADH:ubiquinone oxidoreductase in rat brain. Consistent with the results obtained by Burkhardt et al. (1993) on isolated brain mitochondria, we found a generalized reduction in NADH:ubiquinone oxidoreductase (complex I) in the rat brain after both acute (2 days) and chronic (14 and 28 days) haloperidol treatment (fig. 1). Fluphenazine, a phenothiazine more widely used clinically than its predecessor, chlorpromazine, also inhibited complex I significantly after chronic treatment. Clozapine, an atypical neuroleptic compound, failed to induce any significant reduction in complex I activity after 28 days of treatment. Interestingly, MPTP at a subtoxic dose, administered chronically, also induced a significant reduction in complex I activity. In terms of regional variation, this effect was absent in the hippocampus (fig. 1).

Chronic treatment with neuroleptics and MPTP causes increase in activity of cytochrome-c oxidase. In contrast to expectations, all treatments resulted in a significant increase in the activity of COX (complex IV) in the rat brain (fig. 2). This effect was localized to the frontal cortex in case of haloperidol, but was also seen to a significant degree in the hippocampus after treatment with fluphenazine and clozapine. Interestingly, MPTP also produced a regionally specific increase in COX activity, in which a significant elevation in the frontal cortex, and a nonsignificant increase in the hippocampus were observed. A moderate, significant reduction in COX activity in the frontal cortex was observed 14 days after the initiation of haloperidol treatment, implying that increases after 28 days may be due to compensatory mechanisms.

Chronic treatment with neuroleptics and MPTP induce changes in activities of monoamine oxidase-A and -B. A moderate, generalized increase in the activities of
Both MAO-B and MAO-A was found after chronic treatments with all of the compounds under investigation (fig. 3). However, although statistical significance was not reached in all cases, a general trend, suggesting that effects were localized to the striatum and hippocampus, was observed. Interestingly, an increase in MAO-A in the hippocampus, correlates well with the observed trend found with COX, in which fluphenazine, clozapine and MPTP all produced an increase. An absolute increase in the activity of MAO-A was observed in the cerebellum and striatum of both controls and treated animals in the acute and chronic haloperidol experiments (fig. 1, A–C). This may reflect late occurring changes in the postnatal development of these brain areas that have been demonstrated previously (Tsang et al., 1986).

Discussion

The primary findings of our study indicate that a reduction in the activity of COX, which is evident in the schizophrenic brain (Cavalier et al., 1995), is not a result of drug treatment. Instead, we demonstrate that chronic neuroleptic treatment leads to a significant increase in COX enzyme activity in specific regions of the rat brain, which may reflect the therapeutic value of these agents.

Disturbances in energy metabolism in cortical and subcortical structures of the brain have been implicated in the etiology of schizophrenia (Wiesel, 1992). In relation to this, the concept of "hypofrontality" as a predominant neurobiological characteristic of schizophrenia was postulated in connection with the findings that blood flow in the dorsolateral prefrontal cortex is abnormally low after challenge to specific tasks in the schizophrenic brain (Weinberger et al., 1992). After the discovery that elderly schizophrenic patients display a marked reduction in brain COX activity, it became relevant to investigate whether or not this effect could be mediated by neuroleptic agents (Cavalier et al., 1995). The inhibitory effects of neuroleptics, particularly chlorpromazine, on oxygen consumption have been exhaustively documented in vitro (Guth and Sprites, 1964), but previous studies have failed to demonstrate a direct effect of neuroleptics upon COX activity (Moraczewski and Anderson, 1965; Burkhardt et al., 1993). Nonetheless, despite the fact that more than 40 yr have passed since the original experiments were performed (Grenell et al., 1955), no in vivo tests on specific mitochondrial enzymes after chronic neuroleptic treatment have been performed.

Our focus on the activity of COX is based primarily on a...
gradually expanding body of work pointing to an association between energy use in brain tissue and neuronal functional activity (reviewed by Wong-Riley, 1989). Neurons are highly dependent on oxidative phosphorylation as the primary pathway for the generation of ATP, of which 40 to 60% is used in the maintenance of ion gradients by ATPases. Indeed, a strong correlation exists between the regulation of COX and Na⁺,K⁺ ATPase in brain tissue (Hevner et al., 1992). In addition, changes in COX activity can be induced by experimental interventions that alter neuronal functional activity. In this regard, studies have shown that monocular retinal impulse inhibition with tetrodotoxin results in a decrease in COX activity in specific regions of the monkey visual thalamus and cortex (Wong-Riley and Carrol, 1984). Thus, an increase in the activity of COX (fig. 2) most likely points to an enhancement of functional neuronal activity in specific brain regions, particularly the frontal cortex and hippocampus of neuroleptic-treated animals. This may indicate that the effects of these compounds are specific for predominantly glutamatergic brain regions. The latency of this effect, as demonstrated with haloperidol in which an early decrease in the activity of COX was noted followed by an increase after longer treatment, is also in agreement with the late occurring therapeutic effects of neuroleptic agents, which are generally evident after a period of weeks.

One of the difficulties in the interpretation of our results with COX is that we also observed a significant reduction in the activity of complex I in neuroleptic-treated animals (fig. 1). This finding appears paradoxical, but several factors point to it being an unrelated effect. First, the inhibition of complex I is evident in animals treated acutely with haloperidol suggesting that the effect is a result of a direct inhibition of the respiratory chain. As well, this effect appears to subside somewhat after 28 days, implying that some compensatory recovery occurs. Second, clozapine induced an increase in COX activity in the absence of any inhibitory effect on complex I. Third, to date, no evidence exists demonstrating a stoichiometric relationship between respiratory complexes. In correlating the activities of complex I and IV, we found an extremely low regression coefficient ($r^2 = 0.039$), indicating that these systems are, in principle, independent of one another. Finally, the lack of an increase in COX activity in brain regions where a decrease in complex I occurs (i.e., striatum), demonstrates that a COX induction is most likely not a compensatory effect after up-stream inhibition of the respiratory chain.

Recent evidence involving the effects of neuroleptics on mitochondrial function have led to the hypothesis that this effect may underlie the development of extrapyramidal disturbances (Burkhardt et al., 1993). Early evidence supported
the hypothesis that an impairment of energy metabolism could underlie neurodegenerative processes (reviewed by Beal et al., 1993), primarily on the basis that several neurological and muscle disorders are the result of mitochondrial DNA mutations and deletions (Wallace, 1994). Thus, neurons with depleted energy reserves and thus abnormal ion gradients could be subject to sustained depolarization or hyperpolarization, perhaps resulting in excitotoxicity, seizures or a dysregulation of neurotransmitter metabolism (Hevner and Wong-Riley, 1993).

Our results, demonstrating that complex I is reduced in vivo after chronic neuroleptic treatment, are in agreement with the in vitro findings of Burkhardt (1993). In addition, clozapine, an atypical neuroleptic that lacks the extrapyramidal side-effects associated with the classical neuroleptics, also failed to induce an inhibition of complex I. Recent evidence, involving the use of 3-nitropropionic acid, a mitochondrial toxin, in the induction of vacuous chewing movements in rats, also lends support to the concept that mitochondrial inhibition can underlie tardive dyskinesias (Andreassen and Jorgensen, 1995). However, the immediate question posed is how much inhibition is necessary to result in neuronal damage or dysfunction. Despite our findings, in which only a relatively mild reduction in complex I is found, synonymous effects by haloperidol, fluphenazine and MPTP imply that their mechanisms of action may be similar. This is indeed supported by recent work demonstrating that HPP⁺, a metabolite of haloperidol which is similar in structure to MPP⁺, is a dopaminergic toxin and an potent inhibitor of complex I (Rollema et al., 1994). It has been established that rats, in comparison with humans and other primates, are extremely resistant to its toxic effects of MPTP (Riachi et al., 1990). Thus, the possibility that localized, significant reductions in complex I activity in humans could be caused by haloperidol is supported by our results.

Species variation in the pharmacokinetic profiles of the compounds under investigation is a critical point in terms of applying the data to humans. Indeed, many examples exist showing significant species differences in metabolic pathways and clearance for neuroleptics (Korpi et al., 1984; Zetler and Baumann, 1985; Volavka and Cooper, 1987). However, as an illustration of the problems associated with extrapolat-
ing animal results, haloperidol is metabolized much more slowly in humans than rats (Ereshevsky et al., 1986; Hubbard et al., 1987), whereas fluphenazine is eliminated equally well (within 2–3 days) in humans and rats (Cohen et al., 1992; Midha et al., 1988). Nonetheless, although a linear relationship exists in terms of neuroleptic concentration and degree of complex I inhibition (Burkhardt et al., 1993), dose effects on COX activity in vivo are at present unknown.

Significant increases were found in the activities of MAO-A and MAO-B in the rat brain, primarily in the striatum and hippocampus after chronic neuroleptic and MPTP treatment. The pharmacological implications of an enhancement of MAO-A and MAO-B activity is presently unknown. Early studies demonstrated a reduction in platelet MAO activity in chronic schizophrenic patients undergoing neuroleptic therapy (Wyatt and Murphy, 1976). In support of this being a result of neuroleptic treatment, recent work has shown that metabolites of haloperidol are potent inhibitors of MAO-B (Fang et al., 1995). One previous study has failed to demonstrate alterations in MAO activity in the rat brain (Van Der Krogt et al., 1982). However, in their study, a 9-day washout period was applied, which may have been sufficient for enzyme recovery to have occurred.

Nonetheless, the lack of an effect on MAO-A and -B in the frontal cortex indicates that the increase we observed with COX was most likely not dependent on changes in mitochondrial number. The independent nature of the increase in COX activity is further supported by the finding that no correlation exists between complex I and complex IV.

In conclusion, postmortem findings in schizophrenics are complicated by the extreme difficulties that exist in obtaining brain material from untreated individuals. Our results suggest that a reduction in COX activity in the schizophrenic brain (Cavalier et al., 1995) is independent of neuroleptic treatment. Interestingly, results also imply that part of the therapeutic effect of neuroleptics may result from a stimulation of glutamatergic transmission as reflected by an enhancement in COX activity in predominantly glutamatergic brain regions. Future studies involving the analysis COX by enhancement in COX activity in predominantly glutamatergic brain regions and liver. Further studies involving the analysis of the effect of neuroleptics and neurotoxic metabolites derived from haloperidol: In vivo microdialysis and in vitro mitochondrial studies. Psychopharmacology 1995. 119: 189–192, 1992.


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