Correlation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity with blood–brain barrier monoamine oxidase activity (par-}gyline/brain capillary endothelium/Parkinson disease)

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ABSTRACT Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes parkinsonism in humans and subhuman primates, but not in rats and many other laboratory animals; mice are intermediate in their susceptibility. Since MPTP causes selective dopaminergic neurotoxicity when infused directly into rat substantia nigra, we hypothesized that systemic MPTP may be metabolized by monoamine oxidase and/or other enzymes in rat brain capillaries and possibly other peripheral organs and thus prevented from reaching its neuronal sites of toxicity. We tested this hypothesis by assessing monoamine oxidase in isolated cerebral microvessels of humans; rats, and mice by measuring the specific binding of [3H]paragline, an irreversible monoamine oxidase inhibitor, and by estimating the rates of MPTP and benzylamine oxidation. [3H]Paragline binding to rat cerebral microvessels was about 10-fold higher than to human or mouse microvessels. Also, MPTP oxidation by rat brain microvessels was about 30-fold greater than by human microvessels; mouse microvessels yielded intermediate values. These results may explain, at least in part, the marked species differences in susceptibility to systemic MPTP. They also suggest the potential importance of “enzyme barriers” at the blood–brain interface that can metabolize toxins not excluded by structural barriers, and may provide biological bases for developing therapeutic strategies for the prevention of MPTP-induced neurotoxicity and other neurotoxic conditions including, possibly, Parkinson disease.

Systemic administration of small quantities of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to humans and other primates destroys nigrostriatal dopaminergic neurons and causes an acute and irreversible parkinsonian syndrome (1–5). Neurotoxicity probably results from MPTP metabolism by brain monoamine oxidase [MAO; amine:oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4] to 1-methyl-4-phenyl-2,3-dihydropyridinium cation (2,3-MPDP⁺), which is further oxidized to 1-methyl-4-phenylpyridinium (MPP⁺) (Fig. 1) (6–10). Although selective uptake of MPP⁺ by dopaminergic neurons may explain the relative specificity of MPTP toxicity (11), many of the mechanisms that underlie the toxicity remain unknown (see ref. 12 for review). A most intriguing observation is that many common laboratory animals, with the exception of the mouse (13), are quite resistant to systemic MPTP neurotoxicity. This observation has focused our attention on the biological bases for the marked species differences in systemic MPTP neurotoxicity, which may have important heuristic and practical implications concerning not only the mechanisms of the neurotoxicity but also the pathogenesis of Parkinson disease and other human neurodegenerative disorders.

Although systemic MPTP does not cause nigrostriatal abnormalities in the rat, direct infusion of MPTP into rat substantia nigra selectively destroys zona compacta dopaminergic neurons and depletes striatal dopamine and its metabolites (14, 15). This suggests that the rat’s resistance to systemic MPTP may be a property of the blood–brain barrier and other organs that can metabolize MPTP and prevent it from reaching its brain targets in sufficient concentrations. Because MPTP is a known substrate for enzymatic oxidation by MAO-B (6, 16), and since brain capillaries possess MAO activity (17–22), we reasoned that MAO activity in brain microvessels from different species may correlate inversely with their susceptibility to systemic MPTP neurotoxicity.

We examined isolated brain microvessels of humans, mice, and rats (examples of high susceptibility, partial sensitivity, and resistance to MPTP toxicity) for their MAO content and activity. We also studied mitochondria-enriched preparations from the cerebral cortex and liver of these three species because of the importance of brain MAO in converting MPTP to the neurotoxic MPP⁺, and the importance of the liver in the systemic metabolism of MPTP. We assessed MAO in these tissue preparations by assaying specific [3H]paragline binding to MAO molecules, which is irreversible and stoichiometric (23, 24), and by measuring the enzymatic oxidation of MPTP and benzylamine.

METHODS

Preparation of Tissues. Human, rat, and mouse tissues were used. Human brain and liver were sampled at autopsy from six subjects (four men and two women), aged 30 to 71

Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 2,3-MPDP⁺, 1-methyl-4-phenyl-2,3-dihydropyridinium; MPP⁺, 1-methyl-4-phenylpyridinium; MAO, monoamine oxidase; P₂, crude mitochondrial fraction.

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years, who had no evidence of brain or liver diseases. The time interval between death and autopsy ranged between 3 and 12 hr (mean = 8 hr). Male Wistar rats (250 g) and male COBS CF-1 mice (25 g) were purchased from Hilltop Lab Animals (Scottdale, PA) and were killed by decapitation. Brain and liver samples were immersed in ice-cold physiological buffers. The cerebral cortical mantles were dissected free of meninges and choroid plexus before microvessel isolation. All procedures were performed at 0–4°C unless otherwise stated.

Microvessels were obtained from cerebral cortical mantles by bulk isolation (25). Each human cerebral microvessel preparation was obtained from the cerebral cortex of one individual, whereas each rat and mouse microvessel preparation was obtained from cerebral cortical mantles pooled from 5–14 animals. Microvessel purity was checked by microscopy and occasionally by biochemical assays of their enrichment with γ-glutamyl transpeptidase and alkaline phosphatase (26, 27). Mitochondria-enriched P2 fractions were prepared from the cerebral cortex and liver samples by homogenization in 0.3 M sucrose containing 10 mM sodium phosphate buffer (pH 7.4). The homogenates were centrifuged at 1000 × g for 10 min and the supernatants were recentrifuged at 50,000 × g for 20 min. The pellets were washed twice in 50 mM Tris/HCl buffer (pH 7.4). Tissue pellets were often stored at −80°C for no longer than 1 month. Such storage had no appreciable effect on MAO activity or pargyline binding. On the day of the assay, the pellets were resuspended in 50 mM Tris buffer (to 1–4 mg of tissue protein per ml) and thoroughly mixed by a 10-s burst of a Brinkmann Polytron at a setting of 6. Tissue protein concentrations were assayed according to Lowry et al. (28), using bovine serum albumin as standard.

**Pargyline Binding.** Binding of [3H]pargyline to tissue preparations was assayed at 37°C for 1 hr. The reaction mixture (0.2 ml) contained: 50–150 μg of tissue protein and ~300 nM [3H]pargyline (specific activity 13 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq), with or without excess (20 μM) unlabeled pargyline. The reaction was stopped by rapid filtration under reduced pressure over Whatman GF/B filters, and the filters were washed three times with 4 ml of ice-cold 50 mM Tris buffer. The 3H content of filters was assayed in a scintillation spectrometer at an efficiency of ~45%. Specific binding was calculated by subtracting nonspecific binding (in the presence of 20 μM unlabeled pargyline) from total binding. The ratio of nonspecific to total binding varied with the tissue preparation, from a high of ~60% in tissues that had meager amounts of pargyline binding activity to a low of ~10% in tissues with high pargyline binding. [3H]Pargyline binding was expressed in pmol/mg of tissue protein.

In preliminary experiments, we found that specific [3H]pargyline binding was linear with respect to the amount of protein used, and that binding was maximal at 1 hr of incubation. [3H]Pargyline binding was also linear with increasing concentrations of [3H]pargyline, up to ~300 nM (Fig. 2). Pargyline binding was irreversible, and tissue-bound radioactivity was not decreased by repeated washings. The relative contribution of the two forms of MAO, MAO-A and MAO-B, to [3H]pargyline binding was estimated in parallel incubations with 0.6 μM unlabeled deprenyl or 0.1 μM unlabeled clorgyline, respectively. These concentrations of deprenyl and clorgyline were determined from preliminary competition–displacement experiments. When unlabeled pargyline, deprenyl, or clorgyline was used, tissues were preincubated with the unlabeled antagonist for 15 min at 37°C before [3H]pargyline was added.

In some experiments, [3H]pargyline binding was followed by extensive washing of the tissue pellet, which was then dissolved in 5% NaDodSO4 and processed for PAGE (29). The gels were either sliced into 2-mm segments, and each segment was dissolved and assayed for its 3H content, or were processed for autoradiography.

**Assay of MAO Activity.** MAO activity was assayed by a modification of the procedure of Szutowicz et al. (30), which is based on the colorimetric determination of the H2O2 formed. The reaction mixture (0.7 ml) contained 100 mM sodium phosphate buffer (pH 7.4), 3 mM sodium azide, 100–250 μg of tissue protein, and one of two substrates, MPTP or benzylamine (a preferred MAO-B substrate), at 1 mM. To determine the maximal velocity of MPTP oxidation (Vmax) and the concentration of MAO at half-maximal velocity (Km), MPTP concentration was varied from 30 μM to 3 mM. Blanks consisted of parallel incubations either without substrate or following preincubation with 0.1 mM pargyline. Incubation took place for 30 min at 37°C, and the reaction was stopped by the addition of 0.5 ml of the H2O2-measuring solution, which contained 0.5 M phosphate/citrate buffer (pH 4.0), 1.8 mM 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), and 5 units of horseradish peroxidase. After 15 sec, 0.25 ml of 0.75 M hydrochloric acid containing 5% NaDodSO4 was added, and the colored product was measured spectrophotometrically at 414 nm. Readings were compared to a H2O2 standard curve. Oxidase activity was expressed as nmol of H2O2 formed per mg of tissue protein per hr. In preliminary experiments, we confirmed that H2O2 production was linear with incubation time and with amount of tissue used. The Vmax and Km values for MPTP oxidation were calculated from Lineweaver–Burk plots where the linear regression lines were computer-generated by the least-squares method.

**Data Analysis.** Statistical analyses of species differences were computed by the two-sample Student’s t test (two-tailed). Bonferroni’s method was used to correct significance levels for multiple comparisons (31). Significance is considered at P < 0.05.

## RESULTS

As previously reported (25–27), microvessel preparations consisted of segments of capillaries, arterioles, and venules that ranged from 4 to 30 μm in diameter. The vast majority were less than 10 μm in diameter. Larger microvessels often had smooth muscles, but we estimate their contribution at <15%. γ-Glutamyl transpeptidase and alkaline phosphatase activities were >20-fold higher in microvessels than in the cerebral cortex from which microvessels were obtained. Based on the microscopic and biochemical assessment of microvessel purity, we estimate that contaminants do not exceed 5% of the preparation.

Fig. 2 depicts total, nonspecific, and specific [3H]pargyline binding to rat cerebral microvessel and cerebral P2 preparations as a function of increasing [3H]pargyline concentrations in the incubation medium. Tissues with high pargyline binding, such as rat brain microvessels, have a low ratio of nonspecific to total binding. A single concentration of [3H]pargyline (~300 nM) was used in most experiments. Specific [3H]pargyline binding to the various tissues is presented in Table 1. Pargyline binding to rat cerebral microvessels was about 10-fold higher than that to human or mouse cerebral microvessels (P < 0.001). There were no significant differences in pargyline binding between human and mouse microvessels. Pargyline binding to human cerebral cortex was higher than that of the mouse (P < 0.05), but no significant difference was noted between human and rat cortex. Pargyline binding to liver preparations from all three species was similar. All tissues with high specific [3H]pargyline binding had a high MAO-B/MAO-A ratio (Table 1).

Similar results were also obtained by PAGE of solubilized tissues after [3H]pargyline binding. Figs. 3 and 4 depict the
findings in human, rat, and mouse cerebral microvessels. Rat microvessels (and human, rat, and mouse livers; results not shown) exhibited two distinct radioactive bands, corresponding to the apparent molecular weights of the two types of MAO (29). No other radioactive bands were noted either in autoradiograms of these gels or in segments of gels that were subjected to scintillation counting. Human and mouse microvessels showed minimal radioactivity in the same region of the gel, but no definite bands (Fig. 3). In the case of rat microvessels, the smaller peak closer to the origin (Mr = 65,000) was abolished in the presence of clorgyline, which inhibits MAO-A at low concentrations; whereas the larger peak towards the front (Mr = 58,000) was completely abolished by deprenyl, which inhibits MAO-B at low concentrations (results not shown). Both peaks were abolished when rat microvessels were preincubated with excess unlabeled pargyline. These results indicate that rat cerebral microvessels, but not human cerebral microvessels, contain a high concentration of MAO, mostly type B.

To corroborate the pargyline-binding results, we assayed tissue MAO activity using MPTP and benzylamine as substrates. The latter is a standard preferred substrate for MAO-B. The results show that rat brain microvessels have the highest, and human brain microvessels have the lowest, capacity to oxidize MPTP and benzylamine (Table 2). The mouse cerebral cortex has a significantly lower ability to oxidize MPTP than either human or rat cerebral cortices, which were not significantly different from each other. Benzylamine was a better substrate than MPTP, particularly in human tissues where the capacity to oxidize MPTP was limited. In general, there was good correlation between [3H]pargyline binding and MPTP and benzylamine oxidation, with the exception of the rat and mouse liver, both of which had high pargyline binding but limited ability to oxidize benzylamine (Tables 1 and 2).

Fig. 5 depicts the results of a typical experiment where the velocity of the reaction catalyzed by MAO in human and rat microvessels and cerebral cortex preparations is plotted against increasing MPTP concentrations. V_max and K_m values were estimated from Lineweaver–Burk plots (Fig. 5 Inset). The K_m values were close (48–63 μM), whereas V_max values varied markedly, from a high of 191 nmol per mg of protein per hr in rat microvessels to a negligible low value in human microvessels. The V_max for mouse microvessels was intermediate between human and rat microvessels, at 24 nmol per mg of protein per hr (see Fig. 5 legend).

**DISCUSSION**

Our most important findings are the paucity of MAO in human cerebral microvessels and the rich endowment of rat cerebral microvessels with MAO-B. These contrasting results are evident from experiments using [3H]pargyline binding and MPTP and benzylamine oxidation. These findings support our hypothesis that a possible reason for the species differences in susceptibility to systemic MPTP is the capacity

**Table 1.** [3H]Pargyline binding to cerebral microvessels and cerebral cortex and liver preparations

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific pargyline binding (mean ± SEM)</th>
<th>MAO-A/B ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral microvessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human (n = 5)</td>
<td>1.54 ± 0.47 (P &lt; 0.001)</td>
<td>1:2</td>
</tr>
<tr>
<td>Rat (n = 6)</td>
<td>11.40 ± 1.47</td>
<td>1:8</td>
</tr>
<tr>
<td>Mouse (n = 3)</td>
<td>0.63 ± 0.07 (NS)</td>
<td>1:1</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human (n = 4)</td>
<td>5.12 ± 0.75</td>
<td>1:14</td>
</tr>
<tr>
<td>Rat (n = 5)</td>
<td>3.05 ± 0.51 (NS)</td>
<td>1:2</td>
</tr>
<tr>
<td>Mouse (n = 3)</td>
<td>1.76 ± 0.12 (P &lt; 0.05)</td>
<td>1:5</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human (n = 4)</td>
<td>12.70 ± 3.72</td>
<td>1:9</td>
</tr>
<tr>
<td>Rat (n = 6)</td>
<td>11.80 ± 2.59 (NS)</td>
<td>1:12</td>
</tr>
<tr>
<td>Mouse (n = 3)</td>
<td>16.24 ± 0.68 (NS)</td>
<td>1:36</td>
</tr>
</tbody>
</table>

n is the number of observations. NS means no statistically significant differences. The concentration of [3H]pargyline in the incubation medium was about 300 nM. MAO-A/MAO-B ratio was estimated from [3H]pargyline binding in the presence of deprenyl or clorgyline, as described in Methods. Statistical differences between humans and the two other species were computed by the two-sample Student's t test (two-tailed), with Bonferroni's modification.
neurobiology: Kalaria et al.

Fig. 4. PAGE analysis of [3H]pargyline binding. Tissue preparations (110–118 μg of protein) were incubated with [3H]pargyline, washed, and dissolved in 5% NaDodSO₄. After electrophoresis, the gel was stained with Coomassie blue (Left), dried, and then subjected to autoradiography (Right). Lanes A, B, and C: human brain microvessels, rat brain microvessels, and human brain P₂ preparations, respectively. No radioactive bands are seen in human brain microvessels, while two distinct bands are seen in rat brain microvessels. Molecular weight markers are phosphorylase b (M₉, 92,000), bovine serum albumin (M₉, 66,000), and ovalbumin (M₉, 45,000).

for MPTP metabolism by the “enzyme barrier” at the blood–brain interface, which may prevent access of MPTP and its metabolites to their targets. The findings of low [3H]pargyline binding and of intermediate MPTP and benzylamine oxidation by brain microvessels of the mouse, a species that is partially sensitive to systemic MPTP (13), further support our hypothesis. In fact, of the three indices of MAO activity that were tested in this study (pargyline binding and MPTP and benzylamine oxidation), MPTP oxidation by brain microvessels from the three species correlated best with their known susceptibility to systemic MPTP neurotoxicity. However, it should be emphasized that a larger number of animal species need to be tested before this hypothesis can be accepted. Even then, it is our belief that MPTP neurotoxicity is a multifaceted and complex process, and therefore we must caution that our observations may explain only in part the differences among species in their susceptibility to systemic MPTP. The results also support the concept of a “chemical” blood–brain barrier (19) that complements the “physical” blood–brain barrier with its tight endothelial cell junctions and few pinocytotic vesicles, by metabolizing substances that are not excluded by the physical barrier.

We also found that [3H]pargyline binding and MPTP oxidation in the rat cerebral cortex are not significantly different from that in the human cortex (Tables 1 and 2). This further supports previous findings (14, 15) that MPTP, when directly injected into the rat brain, can induce toxicity via its eventual conversion to MPP⁺ by MAO (Fig. 1). However, the finding of low MAO in the mouse cerebral cortex (Tables 1

![Graph](image)

**Fig. 5.** Rate of MPTP oxidation as a function of MPTP concentration in the incubation medium. Results of cerebral microvessel (circles) and cerebral cortex P₂ (triangles) preparations from human (open symbols) and rat (solid symbols) are shown. The reaction is saturable, and the Vₘₐₓ and Kₐₖ results are estimated from Lineweaver-Burk plots (Inset). Human cerebral microvessels had very low activity and are not represented in the Lineweaver-Burk plots. Vₘₐₓ and Kₐₖ values for the other three tissues are as follows: human cerebral cortex, 14 nmol per mg of protein per hr and 58 μM; rat microvessels, 191 nmol per mg of protein per hr and 63 μM; rat cerebral cortex, 25 nmol per mg of protein per hr and 48 μM; Mouse cerebral microvessels and cerebral cortex P₂ preparations yielded Vₘₐₓ values of 24 and 15 nmol per mg of protein per hr and Kₐₖ values of 26 and 34 μM (data not shown).
Several methodological questions brought up by our results should be addressed. The first concerns the purity of microvessels, and the possibility that any of the results could be due to contamination of microvessels with neuronal or glial elements. This is unlikely because human cerebral cortex has higher MAO than human microvessels, and contamination would result in higher MAO in human microvessels. On the other hand, MAO in rat cerebral cortex is much lower than in rat microvessels, and contamination would result in underestimation of the high MAO activity in rat microvessels. Another concern is the cellular heterogeneity of microvessels, which in addition to endothelium, contain pericytes and a minor component of smooth muscle cells (26). Since endothelial cells constitute the major component of microvessels, we believe that most of the MAO in rat brain microvessels is located in these cells. A third concern is the possible postmortem instability of MAO in human tissues. This was ruled out as an explanation for the low MAO in human tissues, especially microvessels, because pargyline binding and MPTP oxidation were not decreased in experiments where rats were killed and kept at room temperature for 6–8 hr before sampling of their tissues and subsequent isolation of cerebral microvessels and crude mitochondrial preparations. This is consistent with the known resistance of MAO to postmortem changes (32).

As MPTP seems to be vigorously oxidized by rat brain microvessels, the fate of the eventual product, MPP*, and the reason for its inability to reach its sites of action in the nigrostriatal system should be addressed. When MPTP and MPP* were infused directly into the rat substantia nigra, MPTP selectively destroyed dopaminergic neurons of the pars compacta, without affecting other neurons or glia at the injection site (15). In contradistinction, MPP* caused indiscriminate tissue destruction at the infusion site, suggesting that it is a potent nonspecific cytotoxin (14, 15). We suspect that, in the rat, systemic MPTP is actively metabolized to MPP* in brain microvessels, and the MPP* thus formed is either dispersed into the blood or interacts with endothelial cell membranes. Such interaction may have adverse effects on endothelial cells, but in view of their short life span (33) they, unlike neurons, are better suited to withstand the cumulative toxic effects of MPTP.

Recent evidence indicates that the two forms of MAO are separate molecular entities that are independently regulated (34). Levels of MAO-A and MAO-B vary widely in tissues of the same animal and also in the same tissue obtained from different animal species. The two forms of MAO also differ in their half-life (35) and in their response to the action of hormones and other enzyme-inducing factors (34). Differences in MAO among the various tissues of the three species (Tables 1 and 2) probably reflect differences in the number of MAO molecules. The important question is whether MAO of human brain microvessels could be induced to increase. Such induction may have practical implications for preventing systemic MPTP-induced neurotoxicity, and possibly for other human neurotoxic conditions, perhaps including Parkinson disease. Our results should be considered by physicians who use deprenyl and other MAO inhibitors in the treatment of patients, whether or not they have Parkinson disease. In particular, care should be exercised when using MAO inhibitors that primarily affect MAO in brain microvessels and peripheral tissues but which have only minor effects on neuronal and glial MAO. Such agents could possibly enhance the toxicity of MPTP and other neurotoxins.

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