Neuroprotective Action of Halogenated Derivatives of L-Phenylalanine

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Background and Purpose—The aromatic amino acid L-Phenylalanine (L-Phe) significantly and reversibly depresses excitatory glutamatergic synaptic transmission (GST) via a unique set of presynaptic and postsynaptic mechanisms. Therefore, we hypothesized that endogenous derivatives of L-Phe, which display potent antiglutamatergic activity, may safely and efficaciously protect the brain during conditions characterized by overactivation of glutamate receptors.

Methods—We tested this hypothesis in vitro with a combination of patch-clamp and lactate dehydrogenase (LDH) analyses in rat cultured neurons exposed to simulated ischemia, and in vivo using a rat model of experimental stroke caused by transient middle cerebral artery occlusion (MCAO).

Results—3,5-diiodo-L-tyrosine (DIT) and 3,5-dibromo-L-tyrosine (DBrT), endogenous halogenated derivatives of L-Phe, attenuated GST by similar mechanisms as L-Phe, but with greater potency. For example, the IC<sub>50</sub> s for DIT and DBrT to depress the frequency of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptor-mediated mEPSCs were 104.6±14.1 μmol/L and 127.5±13.3 μmol/L, respectively. Depression of GST by DIT and DBrT persisted during energy deprivation. Furthermore, DBrT significantly reduced LDH release in neuronal cultures exposed to oxygen glucose deprivation. In rats subjected to transient MCAO, DBrT decreased the brain infarct volume and neurological deficit score to 52.7±14.1% and 57.1±12.0% of control values, respectively. DBrT neither altered atrioventricular nodal and intraventricular conduction in isolated heart, nor heart rate and blood pressure in vivo.

Conclusion—DBrT, an endogenous halogenated derivative of L-Phe, shows promise as a representative of a novel class of neuroprotective agents by exerting significant neuroprotection in both in vitro and in vivo models of brain ischemia. (Stroke. 2004;35:1192-1196.)

Key Words: excitotoxicity ■ neuroprotection ■ receptors, glutamate ■ stroke

During cerebral ischemia, glutamate is released in supraphysiologi­cal quantities that cause neurotoxicity within the brain. Thus, the search for effective neuro­protective agents has focused on compounds that block glutamate receptors or inhibit glutamate release. However, many of these agents cause significant side effects or lack efficacy in clinical trials. One reason for the failure to achieve neuroprotection may be that most antiglutama­tergic agents only target one subtype of glutamate recep­tors, or excitatory neurotransmitter release, leaving other pathways of glutamatergic excitotoxicity intact. Therefore, drugs which are capable of inhibiting multiple pathways that are involved in excitotoxicity may exhibit superior neuroprotective efficacy.

Our recent results indicate that an endogenous substance, the aromatic amino acid L-Phenylalanine (L-Phe), may fit this profile. We demonstrated that L-Phe produces a selective, significant, and reversible depression of ionotropic glutamate receptor function at excitatory synapses in hippocampal and cerebrocortical neuronal cultures prepared from rats or mice. This action of L-Phe is unique in that 3 distinct mechanisms combine to mediate depression of glutamatergic synaptic transmission (GST): (1) competition for the glycine-binding site of N-methyl-D-aspartate receptors (NMDARs); (2) competition for the glutamate-binding site of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate receptors (non-NMDARs); and (3) attenuation of glutamate release. Furthermore, as observed in patients with phenylketonuria or in experiments with neuronal cultures, temporary increases in the concentration of L-Phe are not likely to cause permanent changes in neuronal and brain function. The objectives of the present study were 2-fold. First, to identify endogenous derivatives of L-Phe that exhibit a similar pattern of antiglutamatergic activity, but are more potent. Second, to determine whether these compounds cause neuroprotection.
Materials and Methods

All experiments were approved by the University of Florida Animal Care and Use Committee.

Neuronal Cultures

Hippocampi or cerebral cortices were dissected from newborn rats (Charles River Laboratories, Wilmington, Mass.), treated with 0.25% trypsin to dissociate the cells, and plated in poly-L-lysine coated 35 mm Nunc plastic tissue culture dishes using procedures previously described.7,8 Spectrophotometry.10

Electrophysiological Recordings

Neurons were used for electrophysiological recordings between 12 and 27 days in vitro. Patch-clamp recordings and analysis of spontaneous and miniature postsynaptic currents (s/mEPSCs) were performed as previously described.7,8 Spontaneous EPSCs were recorded in the presence of 1 mmol/L Mg2+ in extracellular solution at a holding potential of −30 mV. To record mEPSCs, action potential propagation was blocked by tetrodotoxin (TTX). Strychnine (1 mmol/L) and bicuculline (20 mmol/L) or picrotoxin (100 mmol/L) were added to the extracellular solution to block glycine and gamma-aminobutyric acid (GABA) receptors, respectively. The NMDAR-mediated EPSCs were recorded in the presence of 1.2,3,4-Tetrahydro-6-nitro-2,3-dioxobenzo(f)quinoxaline-7-sulfamomide (NBQX) (10 mmol/L). The non-NMDAR–mediated EPSCs were recorded in the presence of MK-801 (5 mmol/L). To record GABAR-mediated miniature inhibitory postsynaptic currents (mIPSCs), bicuculline (20 mmol/L) in the extracellular solution and Cs gluconate (135 mmol/L) in the intrapipette solution were replaced with NBQX (5 mmol/L) and with KCl (135 mmol/L), respectively. mEPSCs and IPSCs were recorded at a holding potential of −60 mV.

Oxygen Glucose Deprivation and Assessment of Neuronal Death in Vitro

For these experiments, cerebral cortical neurons were cultured for 10 to 12 days in DMEM containing physiological concentrations of L-Phe (50 mmol/L) and L-tyrosine (L-Tyr) (50 mmol/L). Cultures were then exposed to oxygen glucose deprivation (OGD) as follows: Culture media were replaced from each dish and reserved under sterile conditions. Media were replaced with DMEM (1 mL/dish) and maintained for 15 minutes at 37 °C. Oxygen concentration was less than 1%. These conditions were considered to be significant. All statistical calculations were carried out using raw data.

Data Analysis

All measurements are reported as mean±SEM. Statistical analyses were carried out using SigmaStat version 2.03 (SPSS Inc). One-way, repeated measures ANOVA followed by Student-Newman-Keuls testing were used to analyze multiple comparisons among control and interventions. When appropriate, paired t testing (2-tailed) was used for single comparisons. A probability value less than 0.05 was considered to be significant. All statistical calculations were carried out using raw data.

Results

Screening of Endogenous Derivatives of L-Phe for Antiglutamatergic Effects

Screening experiments for possible antiglutamatergic potency were carried out for (1) L-Phe derivatives whose concentrations are elevated in phenylketonuria (PKU),15 and (2) endogenous halogenated derivatives of aromatic amino acids, including DBrT and DIT.16,17 The derivatives of L-Phe, which occur in PKU as a result of transamination of L-Phe, were significantly less potent than L-Phe. Phenylpyruvic, phenylacetic, and phenyllactic acids (1 mmol/L) reduced the frequency of non-NMDAR–mediated mEPSCs by 12.6±1.9%, 25.6±4.2%, and 23.9±2.7%, respectively (n=6, P<0.01, compared to a reduction of 48.7±2.0% caused by 1 mmol/L L-Phe).6 In contrast, DIT and DBrT, which differ from L-Phe by modifications to the aromatic ring of the molecule, de-
DIT and DBrT selectively depress glutamatergic transmission in rat hippocampal cultured neurons. A, Representative non-NMDAR–mediated sEPSCs before, during, and after application of DIT and DBrT. Horizontal bars denote the application of DIT (1 mmol/L) or DBrT (1 mmol/L). Histograms summarize the effects of DIT and DBrT on the amplitude and frequency of NMDAR and non-NMDAR–mediated sEPSCs. Summary data are expressed as mean±SEM of 8 to 9 cells. Intervention versus Control: *P<0.01. The small number of sEPSCs in the presence of DIT made it impossible to adequately determine the average amplitude of non-NMDAR–mediated sEPSCs. B, DIT and DBrT depress spontaneous action potentials (left and center panels) but do not influence elicited action potentials (right panel). Action potentials were elicited by depolarizing the membrane with inward current pulses of 2 ms duration and 2 nA amplitude. The concentration of DBrT and DIT was 1 mmol/L. C, DBrT and DIT do not affect GABA receptor-mediated mIPSCs. Representative GABA-mediated mIPSCs recordings (center panel) from the same neuron before (control), during (1 mmol/L), and after (wash) application of DBrT. Histograms summarizing the effects of DIT (1 mmol/L) and DBrT (1 mmol/L) on the amplitude and frequency of GABA–mediated mIPSCs (right panel). Summary data are expressed as mean±SEM of 4 to 5 cells.

Effect of L-Phe and Its Derivatives on GST in Simulated Ischemia

In order to determine the ability of L-Phe and its derivatives to depress GST under conditions characteristic of brain ischemia, we studied their effects during energy deprivation (ED), a surrogate of ischemia at the single cell level. To induce ED, glucose was replaced with 2 mmol/L 2-deoxyglucose, and 5 mmol/L sodium cyanide was added to the extracellular solution. ED markedly enhanced glutamate release, as indicated by an increase in mEPSC frequency from 9.3±1.5 Hz to 50.7±8.5 Hz and from 2.7±0.6 Hz to 31.8±5.7 Hz in rat hippocampal and cerebrocortical neurons, respectively. L-Phe, DIT, and DBrT significantly attenuated this effect of ED by depressing the frequency of mEPSCs (Figure 3).

Because DBrT is less likely to cause side effects in vivo, it was chosen for further experiments to investigate whether this class of compounds may produce neuroprotection in vitro and in vivo models of brain ischemia.

Effect of DBrT on Cell Death in Cerebrocortical Cultures Subjected to OGD

Figure 4 illustrates that exposure of cerebrocortical neuronal cultures to 15 minutes of OGD produces a significant increase in cell death, as indicated by a time-dependent elevation of LDH levels in the culture media. DBrT (400 μmol/L) significantly decreased LDH release in these cultures.

Neuroprotective Effects of DBrT on Ischemic Brain Injury in Vivo

DBrT did not cause clinically significant changes in blood pressure or heart rate in conscious rats (Figure 5A and 5B).
Both heart rate and blood pressure stayed within about 15% of their baseline values, a range within normal diurnal variations. Likewise, in isolated rat hearts, DBrT (3 mmol/L) did not significantly affect the stimulus-to-atrium, atrium-to-ventricle, and QRS intervals as well as the duration of MAPD90 (Figure 5B). In contrast, DBrT significantly decreased brain infarct volume and neurological deficits following MCAO. Three days after MCAO surgery, control rats exhibited visible intracerebral damage (infarct volume, 317.0±24.7 mm³) and major neurological deficits. In rats treated with DBrT, the infarct volume was significantly reduced to 170.1±39.7 mm³. The reduction in infarct size caused by DBrT was significant in both cortex and subcortex (Figure 6A and 6B). It was accompanied by a 57.1±12.0% improvement in the neurological test score data (P<0.038) (Figure 6C). Similar effects were caused by DIT administered using the same protocol (preliminary observations).

Discussion

The results of the current study indicate for the first time that aromatic amino acids, namely halogenated derivatives of L-Phe, can exert major neuroprotective actions. Findings in 2 complementary experimental models support this contention: (1) DBrT markedly improves the viability of neuronal cultures exposed to OGD, and (2) significantly decreases infarct
volume and neurological deficits in a rat stroke model of transient MCAO.

Possible Mechanisms of Neuroprotective Effects

The multisite antiglutamatergic actions of aromatic amino acids may provide a molecular basis for the neuroprotective action of DBrT observed in cell cultures and in vivo in rats subjected to MCAO. Each of the 3 models of ischemia that were employed in this study initiates a broad spectrum of processes, which simulate with different degrees of precision the complex neurotoxicity of brain ischemia in vivo. An important common property of ED, OGD, and MCAO is that all 3 are characterized by abnormal release of glutamate. The correlation of the inhibitory effects of DBrT on GST during ED, and of its neuroprotective actions in OGD and MCAO, presents indirect evidence that antiglutamatergic properties of DBrT may contribute to its neuroprotective actions. However, the mechanisms that mediate these neuroprotective effects of DBrT in cell cultures and, especially, in vivo are likely to be more complex and require further investigation. For example, DBrT is a stable and specific oxidation product that is formed by the action of eosinophil peroxidase, in the presence of H$_2$O$_2$, physiological concentrations of Br$^-$ and protein tyrosine residues. On the other hand, reactive oxygen species such as H$_2$O$_2$ have been shown to modulate GST and to mediate neurotoxicity. It is plausible that high concentrations of exogenous DBrT may diminish the neurotoxic effects of H$_2$O$_2$ during ischemia by acting as an end product of the oxidation reaction that involves H$_2$O$_2$.

Potential Clinical Implications

An important consideration for the success of neuroprotective compounds is attainment of sufficiently high concentrations, which produce neuroprotection but do not adversely affect cardiovascular or nervous systems. In this regard, it is important to emphasize that high blood concentrations of L-Phe (>1200 μmol/L versus 55 to 60 μmol/L in healthy subjects) cause the neurological disease PKU. The measurements of brain Phe concentrations in PKU patients indicate that following an oral load of L-Phe, its concentration varies and may rise up to 0.8 mmol/L. Such increases in the brain concentration of L-Phe for 1 week in treated PKU patients and healthy volunteers only caused reversible impairment of brain activity. However, the effects of L-Phe, and especially its derivatives, on brain function are far from completely understood. For example, some researchers advocate pathogenetic roles of transamination products of L-Phe in PKU.

Likewise, high concentrations of DIT, a precursor of thyroid hormone, may potentially modulate multiple physiological processes by modifying synthesis of thyroid hormone.

Based on our experimental results and the fact that L-Phe and its derivatives are endogenous substances, these compounds, or at least some of them, may represent a new therapeutic approach to safely, efficaciously, and inexpensively mitigate the consequences of ischemic stroke. It is plausible to speculate that these neuroprotective effects will not be limited to brain ischemia, but may extend to other neurological disorders where overactivity of glutamate receptors plays an etiological role (eg, traumatic brain injury, epileptic seizures). Future investigation and correlation of structure–activity relationships of L-Phe, DBrT, and DIT to their ability to depress GST may serve as a guide for the rational development of a newer and more effective class of neuroprotective agents.

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References

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