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 IC₅₀ 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 ionotopic 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 receptor (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
Hippocampal 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

Electrophysiological Recordings
Neurons were used for electrophysiological recordings between 12 and 27 days in vitro. Patch-clamp recordings and analysis of spontaneous and miniature excitatory postsynaptic currents (s/mEPSCs) were performed as previously described.9,10 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). Stryclbine (1 μmol/L) and bicineuline (20 μmol/L) or picrotoxin (100 μmol/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 μmol/L). The non-NMDAR–mediated EPSCs were recorded in the presence of MK-801 (5 μmol/L). To record GABAR-mediated miniature inhibitory postsynaptic currents (mIPSCs), bicuculline (20 μmol/L) in the extracellular solution and Cs gluconate (135 mmol/L) in the intrapipette solution were replaced with NBQX (5 μmol/L) and with KCl (135 mmol/L), respectively. All 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 μmol/L) and L-tyrosine (L-Tyr) (50 μmol/L). Cultures were then exposed to oxygen glucose deprivation (OGD) as follows: Culture media were removed from each dish and reserved under sterile conditions. Media were replaced with DMEM (1 mL/dish) devoid of glucose, with or without 3,5-dibromo-L-tyrosine (DBrT) (400 μmol/L). Cultures were then placed into an airight chamber (Billups-Rothenberg Inc) and flushed with 95% N2/5% CO2 until the oxygen concentration was less than 1%. These conditions were maintained for 15 minutes at 37°C. Following this period of OGD, the glucose-free media were removed and replaced with the original media either with or without DBrT (400 μmol/L). The neuronal death was evaluated by measuring the amount of lactate dehydrogenase (LDH) released from the cells into media using spectrophotometry.10

Measurements of Cardiovascular Parameters in Conscious Rats
Rats with catheters implanted in the common carotid artery and internal jugular vein were anesthetized with halothane for placement of electrocardiogram (ECG) electrodes and access of the catheters. ECG and blood pressure data were recorded continuously. A minimum of 30 minutes was allowed after anesthesia before drug administration.

Isolated Perfused Hearts
Rat hearts were isolated and perfused with the Langendorff method as previously described.11 ECGs were recorded with the use of unipolar electrodes placed on the surface of the left atrium and in the His bundle position.

Transient Middle Cerebral Artery Occlusion (MCAO)
Male Sprague-Dawley (Charles River Laboratories) rats weighing 280 to 400 g were housed on a 12-hour day–night cycle with free access to food and water. Anesthesia was induced with 4% isoflurane and 1L/min oxygen in a chamber and maintained with 2% isoflurane and 1L/min oxygen by a face mask for the surgical procedures. During the operation, the body temperature was maintained at 37°C using a heating pad. Prior to the MCAO induction, the jugular vein was canulated for drug infusion. MCAO for 2 hours with subsequent reperfusion was induced by an intraluminal method modified from Koizumi et al.12 The rats received 2 bolus injections of 12 mmol/L DBrT (10 minutes before the MCAO procedure and 10 minutes before the reperfusion) at a volume of 5 mL/kg body weight (BW). The second bolus was followed by a continuous infusion, which began immediately after the reperfusion and continued for 4 hours. The rate of the infusion was 5 mL/kg BW/h of the same solution. The 12 mmol/L solution of DBrT was prepared in 0.9% NaCl in the presence of NaOH (12 μmol/L). The control rats received 0.9% NaCl under the same protocol. Bupivacaine was infiltrated to the wound as analgesia. Neurological evaluation was performed 3 days after the MCAO procedure using a score scale modified from Longa et al.13:0=no neurological deficit, 1=failure to extend left paw fully, 2=decreased resistance to lateral push, 3=circling to the left, 4=failing to the left. Animals that died prematurely or had subarachnoid hemorrhage at postmortem examination were excluded and replaced.

Three days after the MCAO procedure, rats were deeply anesthetized and perfused transcardially with heparinized saline. The brain was removed and sectioned coronally into seven slices of 2 mm thickness starting from the frontal pole. Slices were stained with 2% 2,3,5-triphylthiazolium chloride (TTC) for 30 minutes at room temperature. Areas ipsilateral to the occlusion, which were not stained, were recorded as infarcted. After fixation with 10% formaldehyde, infarct volume was measured using an imaging system (AIS, Imaging Research Inc). To compensate for the effect of brain edema, the corrected infarct volume was calculated using an indirect method.14

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).8 In contrast, DIT and DBrT, which differ from L-Phe by modifications to the aromatic ring of the molecule, de-
pressed GST in hippocampal cultured neurons to a much greater extent than L-Phe (Figure 1A). In addition, DIT and DBrT (1 mmol/L) decreased the current activated by NMDA (INMDA) to 64.3 ± 3.7% and 79.1 ± 2.1% of control values, respectively (n = 4 to 7, P < 0.01). DIT and DBrT also depressed spontaneous action potentials, but did not alter elicited action potentials (Figure 1B). DIT and DBrT at this concentration did not affect GABA receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) (Figure 1C).

DIT and DBrT depressed excitatory postsynaptic currents in a concentration-dependent manner (Figure 2). The IC50 values for DIT and DBrT to depress the frequency of non-NMDAR-mediated mEPSCs were 104.6 ± 14.1 μmol/L and 127.5 ± 13.3 μmol/L, respectively.

**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% 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

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**Figure 3.** L-Phe, DIT, and DBrT depress EPSCs enhanced by energy deprivation (ED) in rat hippocampal and cerebrocortical cultured neurons. A, Representative traces of non-NMDAR mEPSCs recorded from a hippocampal neuron under the following conditions: control; during ED; during ED plus DBrT (1 mmol/L); after washout of DBrT. B, Non-NMDAR mEPSCs recorded from a cerebrocortical neuron under the same treatment conditions as in A. Summary data for the effects of L-Phe (1 mmol/L), DIT (1 mmol/L), and DBrT (1 mmol/L) on the frequency of mEPSCs enhanced by ED in hippocampal neurons and for the effect of DBrT in cerebrocortical neurons. Data were normalized to mEPSC frequency values during ED. Data are plotted as mean ± SEM, n=5 to 9, Drug versus Drug-free during ED; * $P<0.01$.

**Figure 4.** DBrT markedly decreases neuronal death in vitro. Cerebrocortical neuronal cultures were exposed to OGD for 15 minutes as detailed under Methods. DBrT (400 μmol/L) was added at the start of the OGD procedure, and also following OGD. Cell cultures submitted to OGD but not treated with DBrT were called control cells. At the indicated times the amount of LDH was assayed. Data for LDH activity was normalized to values of LDH measured in cultures before exposure to OGD (presented as 100%) and plotted as mean ± SEM, n=6, against the time. *$P<0.05$ compared with control (no DBrT). The levels of LDH measured in cultures before induction of OGD were 55.9 ± 7.69 Berger-Broida units/mL.

**Figure 5.** Cardiovascular effects of DBrT in rats. A, The effects of DBrT on heart rate (A,a) and blood pressure (A,b) in conscious rats. Data are plotted as mean ± SEM, n=4, Drug versus Control: *$P<0.05$. The protocol of DBrT administration was the same as that for MCAO-treated rats (see Methods). B,a, Representative tracings acquired from a rat isolated heart instrumented for electrogram recordings during perfusion of the heart with control solution or after addition of DBrT (3 mmol/L). B,b, Summary data for the effect of DBrT (3 mmol/L) on the stimulus-to-atrium (SA) interval, atrium-to-ventricle (AV) interval, QRS interval, and duration of MAPD90. Data are plotted as mean ± SEM, n=4, Drug versus Control: *$P<0.08$.

**Figure 6.** DBrT decreases infarct volume and neurological deficits in rat brain following middle cerebral artery occlusion (MCAO) for 2 hours. A and B, Bar graphs show infarct volumes (% of contralateral hemisphere) in saline- and DBrT-treated rats in cortex and subcortex, respectively. C, The total neurological scores for the same control and DBrT-treated rats as shown in A and B. Values are mean ± SEM, n=8, DBrT versus Saline: *$P<0.05$. The rats received either 0.9% NaCl (control) or DBrT (DBrT-treated). For details on drug administration and neurological score evaluation see Methods.
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 H₂O₂, physiological concentrations of Br⁻ and protein tyrosine residues. On the other hand, reactive oxygen species such as H₂O₂ 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 H₂O₂ during ischemia by acting as an end product of the oxidation reaction that involves H₂O₂.

**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 exogenous DBrT may diminish the neurotoxic effects of H₂O₂ during ischemia by acting as an end product of the oxidation reaction that involves H₂O₂.

**References**

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