Erythropoietin Receptor-mediated Inhibition of Exocytotic Glutamate Release Confers Neuroprotection during Chemical Ischemia*

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Erythropoietin (EPO) reduced Ca\(^{2+}\)-induced glutamate (Glu) release from cultured cerebellar granule neurons. Inhibition was also produced by EPO mimetic peptide 1 (EMP1), a small synthetic peptide agonist of EPO receptor (EPO-R), but not by iEMP1, an inactive analogue of EMP1. EPO and EMP1 induced autophosphorylation of Janus kinase 2 (JAK2), a tyrosine kinase that associates with EPO-R. Furthermore, genistein, but not genistin, antagonized both the phosphorylation of JAK2 and the suppression of Glu release induced by EPO and EMP1. During chemical ischemia, substantial amounts of Glu were released from cultured cerebellar and hippocampal neurons by at least two distinct mechanisms. In the early phase, Glu release occurred by exocytosis of synaptic vesicle contents, because it was abolished by botulinum type B neurotoxin (BoNT/B). In contrast, the later phase of Glu release mainly involved a BoNT/B-insensitive non-exocytotic pathway. EMP1 inhibited Glu release only during the early exocytotic phase. A 20-min exposure of hippocampal slices to chemical ischemia induced neuronal cell death, especially in the CA1 region and the dentate gyrus, which was suppressed by EMP1 but not iEMP1. However, EMP1 did not attenuate neuronal cell death induced by exogenously applied Glu. These results suggest that activation of EPO-R suppresses ischemic cell death by inhibiting the exocytosis of Glu.

Brain ischemia induces delayed neuronal cell death, especially in the CA1 region of the hippocampus (1). Glutamate (Glu)\(^3\) and related excitatory amino acids are well-known neurotoxins, and their extracellular levels increase dramatically in the course of brain ischemia. The excitotoxicity hypothesis suggests that, in neuronal hypoxia/ischemia, neurodegeneration can be triggered by cytoplasmic Ca\(^{2+}\) overload, which occurs when N-methyl-D-aspartate receptors are overstimulated by excessive Glu (2). Numerous pharmacological approaches have been explored to prevent or attenuate neuronal cell death in ischemia, however, no satisfactory methods have been developed (3–5).

The hematopoietic growth factor erythropoietin (EPO) is a primary regulator of mammalian erythropoiesis and is produced by kidney and liver in an oxygen-dependent manner (6, 7). The EPO receptor (EPO-R) is a member of the type 1 superfamily of single-transmembrane cytokine receptors. EPO binding to EPO-R induces receptor oligomerization and subsequent activation by autophosphorylation of Janus kinase 2 (JAK2), a protein-tyrosine kinase that associates with EPO-R. JAK2 phosphorylates a cytoplasmic transcription factor called signal transducer and activator of transcription (STAT), leading to translocation of STAT into the nucleus and regulation of transcription (8). EPO and EPO-R are also expressed in mammalian brain, where hypoxia strongly stimulates EPO production (9, 10). Although activation of EPO-R has been suggested to play a neuroprotective role (10, 11), little is known about the action of EPO in the nervous system. Recently, we showed that EPO inhibits Ca\(^{2+}\)-induced dopamine release from clonal rat pheochromocytoma PC12 cells (12); however, it has not yet been deduced whether EPO also suppresses neurotransmitter release from central neurons. In the present study, we investigated the effect of EPO on Glu release from cultured cerebellar granule cells and hippocampal neurons. We found that the activation of EPO-R attenuates Ca\(^{2+}\)-induced Glu release from these neurons possibly through activation of JAK2 tyrosine kinase. We also found that EPO-R activation protects hippocampal neurons from ischemic neuronal damage through the inhibition of Glu release via exocytosis.

EXPERIMENTAL PROCEDURES

Materials—EPO mimetic peptide 1 (EMP1) was synthesized by the solid-phase methodology of Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. After trifluoroacetic acid cleavage, crude peptide was purified by preparative HPLC with an ODS column by applying isocratic EPO-R, EPO receptor; EMP1, EPO mimetic peptide 1; iEMP1, inactive analogue of EMP1; DMEM, Dulbecco’s modified Eagle’s medium; TBS, Tris-buffered saline; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PI, propidium iodide; JAK2, Janus kinase 2; BoNT/B, botulinum type B neurotoxin; Fmoc, N-(9-fluorenyl)methoxycarbonyl; STAT, signal transducer and activator of transcription; HPLC, high performance liquid chromatography.

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1 The abbreviations used are: Glu, glutamate; EPO, erythropoietin;
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**RESULTS**

**Fig. 1**. EPO inhibits Ca\(^{2+}\)-induced Glu release from cerebellar granule cells. A, cultured cerebellar granule cells were preincubated in low K\(^+\) solution containing the indicated concentrations of EPO for 10 min. The cells were further incubated in the low K\(^+\) solution with or without EPO for 2 min, and the release of Glu during this period represented basal release (open squares). Then the cells were incubated for 2 min in low K\(^+\) solution containing 5 \(\mu\)M ionomycin, and Glu release during this period represented Ca\(^{2+}\)-induced Glu release (closed squares). B, the cells were preincubated in low K\(^+\) solution containing 50 ng/ml EPO for various periods as indicated. Basal (open squares) and Ca\(^{2+}\)-induced Glu release (closed squares) were measured as above. Glu was quantified using HPLC as described under “Experimental Procedures.” The results are means ± S.E. (n = 4). * significantly different (p < 0.05) from the control (t test).

**EPO Inhibits Ca\(^{2+}\)-induced Glu Release from Cerebellar Granule Cells**—Cerebellar granule cells release substantial amounts of Glu in response to 5 \(\mu\)M ionomycin treatment in the presence of extracellular Ca\(^{2+}\). As shown in Fig. 1A, EPO reduced Ca\(^{2+}\)-dependent Glu release in a concentration-dependent manner. The inhibition appeared at concentrations above 10 ng/ml (0.28 nM) and about 30% inhibition was achieved at 50 ng/ml (Fig. 1A). The inhibition of Glu release by...
EPO was induced rapidly and attained a maximal level within 20 min after at 50 ng/ml (Fig. 1B). No significant change was observed in basal Glu release in low K (+) solution.

**EMP1, a Synthetic EPO-R Agonist, Inhibits Ca2+ -induced Glu Release in an Activity-dependent Manner**—The inhibition of Ca2+ -induced Glu release was also induced by another EPO-R agonist, EMP1. EMP1 is a synthetic 20-mer peptide having no sequence similarity to EPO (22). EMP1 binds to an extracellular region of EPO-R distinct from the EPO binding site and induces receptor dimerization and activation (23). As shown in Fig. 2, EMP1 also inhibited Ca2+ -induced Glu release from cerebellar granule cells in a concentration-dependent manner. Inhibition by EMP1 was specific, because no significant decrease in Glu release was observed with iEMP1, an inactive analogue of EMP1 with two Cys residues mutated to Ser (22). These results indicate that Ca2+ -induced Glu release from cerebellar granule cells was reduced by the activation of EPO-R.

**Activation of JAK2 by EPO and EMP1**—JAK2 is a tyrosine kinase associated with EPO-R, and the activation of JAK2 is essential for signal transduction via the EPO-R. As shown in Fig. 3, genistein, a tyrosine kinase inhibitor, but not genistin, an inactive analogue of genistein, abolished EPO and EMP1-induced inhibition of Glu release from cultured cerebellar granule cells. Activation of JAK2 was further investigated by immunoblotting of cellular homogenates using antibodies specific for tyrosine-phosphorylated JAK2 and for EPO-R. As shown in Fig. 4, the intensity of the immunoreactive band increased markedly after a treatment with EPO. Pretreating the cells with genistin but not with genistin blocked the tyrosine phosphorylation of JAK2. Tyrosine phosphorylation of JAK2 was also induced by EMP1 but not by iEMP1. The amounts of EPO-R estimated by immunoblotting did not change significantly in these conditions. These results suggest that the activation of JAK2 is involved in EPO-R-mediated suppression of Glu release from cerebellar granule cells.

**Activation of EPO-R Suppresses Exocytotic Glu Release from Cerebellar Granule Cells Induced by Chemical Ischemia**—Next we examined in cultured cerebellar granule cells whether Glu release induced by ischemia could be suppressed by activation of EPO-R (24). The extracellular solution was replaced with low K (+) solution without glucose and supplemented with 1 mM KCN to induce chemical ischemia. The extracellular solution was changed every 5 min, and the amount of Glu released into the solution was determined. As shown in Fig. 5A, chemical ische-
30 min after treatment. To determine whether chemical ischemia-induced Glu release occurred via exocytosis, or another mechanism, we examined the effect of BoNT/B. BoNT/B blocks neurotransmitter release by specifically cleaving vesicle-associated membrane protein-2, a synaptic vesicle protein essential for exocytosis (25). We found that there was a difference in the BoNT/B sensitivity of Glu release in the presence of 10 μM EMP1. The amount of Glu was quantified using HPLC as described under “Experimental Procedures.” The values represent the means ± S.E. (n = 3), *, significantly different (p < 0.05) from the level in chemical ischemia (t test).

Activation of EPO-R Protects Hippocampal Neurons from Ischemic Neuronal Damage through the Inhibition of Glu Release by Exocytosis—Because an excessive release of Glu in ischemia induces neuronal cell death in the hippocampus, we next examined whether neuronal cell death could be attenuated by activation of EPO-R. Hippocampal slices in organotypic culture were maintained in chemical ischemic conditions for 20 min to induce Glu release by exocytosis. After washing with normal low K+ solution, slices were cultured for an additional 24 h in normal culture conditions. Neuronal cell death was evaluated before and 24 h after ischemic treatment by incorporation of PI, a fluorescent marker. PI incorporation was

FIG. 5. EMP1 suppresses exocytotic Glu release induced by chemical ischemia in cerebellar granule cells. A, cultured cerebellar granule cells were incubated in either the low K+/H11001 solution (closed squares) or the chemical ischemia solution (glucose-free + 1 mM RCN) (closed circles). The solution was changed every 5 min, and the amount of Glu released in the solution was quantified using HPLC as described under “Experimental Procedures.” The values represent the means ± S.E. (n = 3). B, cultured cerebellar granule cells were incubated in various conditions, and Glu release either from 15 to 20 min (Early phase) or 45 to 50 min (Late phase) after switching to the ischemic condition was measured. The values represent the means ± S.E. (n = 3), *, significantly different (p < 0.05) from the level in chemical ischemia (t test).

FIG. 6. EMP1 suppresses exocytotic Glu release induced by chemical ischemia in cultured hippocampal neurons. A, cultured hippocampal neurons were incubated in either the low K+ solution (closed squares) or the chemical ischemia solution (glucose-free + 1 mM RCN) (closed circles). The solution was changed every 5 min, and the amount of Glu released in the solution was quantified using HPLC as described under “Experimental Procedures.” The values represent the means ± S.E. (n = 3). B, cultured hippocampal neurons were incubated in various conditions, and Glu release either from 15 to 20 min (Early phase) or 45 to 50 min (Late phase) after switching to the ischemic condition was measured. The values represent the means ± S.E. (n = 3), *, significantly different (p < 0.05) from the level in chemical ischemia (t test).
dramatically increased 24 h after chemical ischemia, especially in the CA1 region and the dentate gyrus, suggesting that exocytotic Glu release induced neuronal cell death in these regions (Fig. 7, A and B). Neuronal cell death was markedly suppressed by treating the slice with EMP1 during chemical ischemia, whereas its inactive analogue iEMP1 showed no suppressive effect. These results indicate that EPO-R activation could protect neurons from ischemic damage.

Because it was not clear whether the neuronal cell death was prevented by EPO-R activation via a presynaptic or postsynaptic mechanism, we next examined the effects of EPO-R activation on neuronal cell death induced by exogenously applied Glu (Fig. 8). If EMP1 suppressed cell death through a postsynaptic mechanism, protection against exogenous Glu would be expected. As shown Fig. 8, exogenously applied Glu induced neuronal cell death not only in the CA1 region and the dentate gyrus but also in the CA2 and CA3 regions. EMP1 did not provide significant protection from Glu-induced cell death in any of these regions even reducing Glu concentration down to 0.5 mM (PI fluorescent ratios were 1.01 ± 0.22 and 1.04 ± 0.10 in control slices; 3.19 ± 0.52 and 2.84 ± 0.60 in slices treated with 0.5 mM Glu; 3.05 ± 1.52 and 1.52 ± 0.39 in slices treated with the chemical ischemia, in the absence or presence of 10 mM EMP1, respectively). These results suggest that EPO-R activation attenuates neuronal cell death by inhibiting Glu release via exocytosis.
ulating a step subsequent to Ca\(^{2+}\) entry. The onset of inhibition by EPO/EMP occurred very rapidly after application, suggesting that regulation is likely to involve post-translational modulation of a pre-existing protein. Previously, we have shown that phosphorylation of GAP-43 at Ser41 was decreased after EMP1 treatment in PC12 cells (12). However, we have not detected any dephosphorylation of GAP-43 in cultured cerebellar granule cells, suggesting that this process is not necessary for EPO/EMP1-induced inhibition of neurotransmitter release (data not shown). The identity of the protein substrate(s) of tyrosine kinase essential for this modulation has yet to be elucidated. Various protein kinases, including protein kinase C, CAM-dependent protein kinase, mitogen-activated protein kinase, and TrkA receptor protein-tyrosine kinase, are known to stimulate neurotransmitter release from a variety of neural preparations (27, 28). However, reports on protein kinases involved in negative regulation of neurotransmitter release are extremely scarce to date. We have shown for the first time that EPO-R activation inhibits Ca\(^{2+}\)-induced catecholamine release from PC12 cells (12) and Ca\(^{2+}\)-induced Glu release from neurons of hippocampus and cerebellum. Thus, EPO-R-mediated suppression of release seems not to be restricted to any particular type of neurotransmitter, and it will be important to dissect the underlying mechanisms to improve our understanding of the molecular basis of synaptic plasticity.

There are a number of reports showing that there are two phases of Glu release during ischemia (29). We found in the present study that Glu release in chemical ischemia was induced by at least two different mechanisms in cultured cerebellar granule cells and hippocampal neurons. In the early phase (up to 20-min treatment), Glu release was almost abolished by BoNT/B, indicating that it occurs via exocytosis of the contents of synaptic vesicles. In contrast, Glu release in the later phase was only partially inhibited by BoNT/B, suggesting that a non-exocytotic mechanism is involved, possibly by reversal of neuronal Glu transporters (30). EMP1 almost abolished Glu release in the early phase, but scarcely affected the release in the late phase of ischemia. These results indicate that during chemical ischemia the activation of EPO-R only suppressed exocytotic Glu release. The excessive release of Glu in brain ischemia is known to trigger neuronal cell death. In the present study, we found that a 20-min exposure of cultured hippocampal slices to chemical ischemia induced neuronal cell death selectively in the CA1 region and the dentate gyrus, which suggests that Glu released by exocytosis was enough to induce the neuronal cell death in ischemia. EMP1 suppressed cell death induced by the chemical ischemia for 20 min. Activation of EPO-R was involved in this process, because iEMP1, the inactive analogue of EMP1, did not produce any protective effect. Neuronal cell death induced by exogenously applied Glu was not suppressed by EMP1. Thus, it is reasonable to conclude that EMP1 attenuated neuronal cell death postnatally by inhibiting exocytotic Glu release, rather than through modification of a post-synaptic step downstream of Glu receptor activation.

Previously, EPO has been reported to protect neurons against ischemia-induced cell death in living gerbils. Infusion of EPO into the lateral ventricles of gerbils prevented ischemia-induced learning disability and rescued hippocampal CA1 neurons from lethal ischemic damage (11, 31). Because EPO rescued cultured neurons from NO-induced death, it was concluded that EPO exerts its neuroprotective effect by postsynaptic mechanisms, possibly by reducing the NO-mediated formation of free radicals or antagonizing their toxicity (11, 32). EPO also protected cultured hippocampal and cortical cerebral neurons from glutamate neurotoxicity (10). However, treatment of neurons with EPO (~8 h prior to exposure to glutamate was required, and RNA and protein synthesis were also necessary, to demonstrate a neuroprotective effect. The action of the EPO-R revealed in the present study is clearly different, because EPO and EMP1 confer protection very quickly by inhibiting presynaptic functions. Thus, the activation of EPO-R seems to protect neurons from ischemic damage through multiple mechanisms, and presynaptic and postsynaptic mechanisms are likely to be involved in inducing protection in the early and the late phases of ischemia, respectively.

Adenosine A\(_1\) receptor agonists had been used to prevent neuronal cell death in ischemia, because they depress Glu release from cortical slice preparations, however, cardiovascular side effects have constituted major obstacles to clinical implementation (3). EPO has proven to be a safe therapeutic agent with minimal adverse effects (33, 34). However, it is thought that direct delivery of EPO into the brain is not a practical approach in clinical contexts, because the blood-brain barrier effectively excludes large glycosylated molecules (35, 36). Recently, EPO was found to cross the blood-brain barrier and intraperitoneal administration of recombinant EPO before or up to 6 h after focal brain ischemia reduced injury (37). These observations raise the possibility that EPO and EMP1 could be used therapeutically to attenuate neuronal damage in the early phase of ischemia. Further studies, including elucidation of the molecular mechanisms of JAK2-mediated inhibition of neurotransmitter release, are required with a view to developing novel strategies for neuroprotection in ischemia.

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