Neuroprotection by Corticotropin Releasing Factor During Hypoxia in Rat Brain

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Background and Purpose: Corticotropin releasing factor is an endogenous neuropeptide released by the hypothalamus that activates the pituitary-adrenocortical system in response to stressful stimuli. It has been demonstrated that corticotropin releasing factor increases the excitability of hippocampal neurons in both in vitro and in vivo studies, which may contribute to neurological injury during hypoxia. The purpose of this study was to determine the effects of corticotropin releasing factor and its synthetic competitive antagonist, α-CRF, on neuronal synaptic recovery after a hypoxic insult using the hippocampal slice.

Methods: Wistar rat hippocampal brain slices (n=120) were treated with various concentrations (10^{-6} to 10^{-11}) of corticotropin releasing factor or its synthetic antagonist during a 10-minute hypoxic episode. Extracellular recording of population spikes was used during and after the hypoxic insult to assess neuronal recovery.

Results: Corticotropin releasing factor provided dose-dependent neuronal protection with maximum recovery (37.95±8.71%) occurring at 10^{-7} concentrations. The competitive antagonist α-CRF provided a similar degree of recovery at 10^{-4} concentration, whereas 10^{-7} molar concentration of competitive antagonist resulted in 16.84±7.68% recovery.

Conclusions: Corticotropin releasing factor provides moderate protection to hypoxic hippocampal neurons in the brain slice preparation. The mechanism of action is unknown but appears to be a direct neuronal effect. These results support the hypothesis that corticotropin releasing factor may act as an endogenous neuroprotective hormone during hypoxia. (Stroke 1993;24:1072-1076)

KEY WORDS • hypoxia • neuropeptides • neuroprotection • rats

Corticotropin releasing factor (CRF) is a 41-amino acid polypeptide that regulates both endocrine and behavioral responses to stressful stimuli through activation of the hypothalamic-pituitary-adrenocortical system. Intraventricular administration of millimolar concentrations of CRF can directly stimulate central nervous system neurons, and these effects can be blocked by the synthetic CRF antagonist α-helical CRF 9-41 (α-CRF). CRF has been shown to increase the excitability and spontaneous discharge frequency of hippocampal neurons in vitro via hippocampal calcium-dependent CRF-specific receptor sites, which has been suggested though not proven to contribute to neurological injury during ischemic or hypoxic insults. It has been demonstrated by immunohistochemical studies that CRF gains access to the amygdalohippocampal complex through the medial forebrain bundle, the stria terminales, and the ventral amygdalofugal pathways. CRF effects appear to act through interneurons or cerebrospinal fluid pathways.

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It has recently been demonstrated that α-CRF may reduce both neuronal excitability and hippocampal ischemic neuronal injury in vivo. Based on this work, we hypothesized that the administration of CRF should augment hypoxic neuronal injury whereas the synthetic antagonist to CRF, α-CRF, could be neuroprotective under similar conditions. To test this hypothesis, we used rat hippocampal brain slices to expose neurons to various concentrations of CRF or α-CRF under hypoxic conditions. Neurophysiological methods were then used to measure synaptic function and determine neuronal recovery.

Materials and Methods

Slice Preparation

Overnight-fasted, unanesthetized male Wistar rats weighing 150-200 g were used for all experiments following approval by the Institutional Animal Welfare Committee. For each experiment, a rat was decapitated and its hippocampus rapidly dissected while being rinsed with cold (3-4°C) artificial cerebrospinal fluid (ACSF) solution containing (mol/L) NaCl 1.20×10^{-1}, KCl 4.00×10^{-3}, KH2PO4 1.57×10^{-3}, MgSO4 2.00×10^{-3}, CaCl2 2.00×10^{-3}, NaHCO3 2.60×10^{-2}, and glucose 1.00×10^{-2}. The hippocampus was sectioned transversely at 500 μM using a Starrett tissue chopper. Slices were
transferred to a room-temperature (16-18°C) prechamber filled with oxygenated ACSF. After 30 minutes, slices were transferred to a dual, linear-flow incubation chamber (Fine Science Tools, Foster City, Calif) perfused with oxygenated (95% O2-5% CO2) ACSF solution at 1.5 mL/min. Temperature was maintained at 34±0.5°C.15 Slices were supported on a nylon mesh at an ACSF/gas interface. The slices were equilibrated for a period of 45 minutes before the recording of baseline synaptic function.

Extracellular recordings of evoked postsynaptic population spikes from the stratum pyramidale of the CA1 region were used as a measurement of synaptic function15,16 using a Paralene-C tungsten microelectrode (Diamond General, Ann Arbor, Mich) with a 1-μm tip diameter and 2-MΩ resistance. Orthodromic stimulation of hippocampal CA3 Schaffer collaterals was performed with a bipolar stimulating electrode (Rhodes Medical Instruments, Woodland Hills, Calif) with stimulating pulses 0.1 milliseconds in duration. The stimulus voltage used was 20 V. Hippocampal slices with population spike amplitudes of 2.0 mV or greater were used for all experiments. Baseline synaptic function was recorded for 1 hour to ensure neuronal viability.

Protocol

Each experiment (n=120) was initiated by rapidly changing the perfusing medium to an ACSF solution saturated with 95% N-5% CO2 and changing the atmosphere within the recording chamber to 95% N-5% CO2. A 10-minute hypoxic episode was used for each experiment, during which various concentrations of CRF, α-CRF, or ACSF vehicle were added directly to the recording chamber at 0, 2.5, 5.0, 7.5, and 10.0 minutes. A volume of 0.15 mL was used for each timed addition. At the end of 10 minutes, oxygenated ACSF was reestablished to the slice chamber. Evoked population spikes were then recorded for 60 minutes, at which time maximal synaptic recovery had been achieved by all slices. The measurement of the population spike amplitude was from the A1 segment of the waveform displayed on the oscilloscope (Fig 1).

Ten experiments were performed as normoxic controls without changing the perfusing medium or humidity in the recording chamber. Similarly, 10 slices were subjected to a hypoxic insult but had only ACSF vehicle added to the recording chamber (hypoxic controls). Finally, 10 experiments were performed at each concentration of CRF or α-CRF tested. Concentrations ranging from 10⁻¹¹ to 10⁻⁶ mol/L were used.

To determine the agonist-antagonist relation between CRF and α-CRF, additional experiments were performed by concurrent addition of CRF and α-CRF to the slice media during the hypoxic episode. CRF (10⁻⁹ mol/L)/α-CRF (10⁻⁷ mol/L) (n=5) and CRF (10⁻⁹ mol/L)/α-CRF (10⁻¹⁰ mol/L) (n=5) were compared.

Statistical analysis of results was determined using analysis of variance with Tukey’s post hoc test for multiple comparison, with P<.05 considered significant. Data were expressed as mean±SE.

Results

Normoxic controls maintained stable synaptic function throughout the 130-minute experimental period. Hypoxic controls (slices exposed to 10 minutes of hypoxia with addition of ACSF vehicle only at 0, 2.5, 5.0, 7.5, and 10.0 minutes) had an average 1.85±1.67% recovery of population spike amplitude 60 minutes after the end of the hypoxic episode. The addition of neither CRF nor α-CRF to the recording chamber housing the hypoxic neurons prevented the loss of synaptic function, but both caused a dose-dependent recovery of synaptic function following resumption of normal conditions (Fig 2, part A). Maximal synaptic recovery (37.95±8.71%, P<.05) was obtained at CRF concentrations of 10⁻⁵ mol/L, whereas the same concentrations of CRF antagonist resulted in a recovery of 16.84±7.68%, not statistically different from controls. Concentrations of both CRF (10⁻⁶ mol/L) or α-CRF (10⁻⁶ mol/L) resulted in baseline synaptic activity recoveries of 33.9±9.11% and 43.27±10.39% (P<.05), respectively (Fig 2, part B). Thus, hypoxic hippocampi exposed to CRF or α-CRF demonstrated increasing survivability as the concentrations were increased, with plateaus for both drugs at concentrations of 10⁻⁶ mol/L.

Concurrent addition of CRF/α-CRF demonstrated that α-CRF antagonized the effects of CRF alone (10⁻¹⁰ mol/L) in a dose-dependent manner (Fig 2, part C).

Discussion

This study supports previous findings that demonstrate a neuroprotective effect of the synthetic antagonist to CRF, α-helical CRF 9-41, during hypoxic-ischemic insults.10 Contrary to what had been previously postulated, however, CRF was also neuroprotective. These findings support the hypothesis that CRF, which is released during stressful episodes (including ischemic or hypoxic insults), could serve as an endogenous neuroprotective hormone. The experiment using CRF and α-CRF concurrently suggests that both may act at the same receptor site and are likely to be competitive, but CRF has a higher affinity for the receptor.

The protective effects of CRF at nanomolar concentrations and those of its antagonist, α-CRF, may be explained by a number of different mechanisms, including alteration of cerebral vascular perfusion, increased glucose availability, or alteration of intracellular adenylyl cyclase activity. The results reported here support a possible neuronal effect since both agents provided in vitro neuronal protection. The finding that both an agonist and its competitive antagonist (α-CRF) offer
neuronal protection could be explained by their similar polypeptide homologies and stereo configurations acting at the same membrane receptor.

CRF is widely distributed throughout the central nervous system. It is synthesized and released from the hypothalamus to stimulate the release of adrenocorticotropic hormone from the anterior pituitary. Intracerebroventricular administration of CRF in the rat produces prolonged elevations of epinephrine, norepinephrine, and glucose, and increases mean arterial pressure and heart rate. In vivo, this may result in an increased cerebral vascular perfusion that may reduce ischemic or hypoxic neuronal injury. In this in vitro brain slice model, however, there is an absence of an intact vasculature, which may account for the paradoxical effects of CRF and its antagonist.

CRF and glucose levels are intimately related, with hypoglycemia resulting in CRF release and hyperglycemia resulting in an inhibition of CRF release. This effect occurs rapidly and is reversible. Additionally, activation of the hypothalamic-pituitary-adrenocortical axis during stressful periods causes increased glucocorticoid and glucose levels. Glucocorticoids have been shown to directly damage hippocampal neurons by impairing energy metabolism due to an inhibition of glucose utilization, leaving neurons less resistant to ischemic or hypoxic insults. Hyperglycemia during ischemia-hypoxia has been shown to be detrimental due to increased lactic acid formation by anaerobic metabolism. However, recent work has shown that mild acidosis may in fact be mildly neuroprotective during ischemia-hypoxia by the inhibition of N-methyl-D-aspartate receptors. In this situation, CRF-induced elevated glucose levels and the resulting mild acidosis may serve as a protective pathway for hippocampal neurons in this study. Although this mechanism is attractive in vivo, there is no increase in glucocorticoid, glucose, or acid levels during this in vitro study.

Finally, CRF acts on different central neuronal populations by receptor-mediated and calcium-dependent increases in intracellular cyclic adenosine monophosphate (cAMP). Intracerebroventricular millimolar injections of CRF in the rat result in a rapid increase in intracellular adenylate cyclase activity, whereas α-CRF partially reduces this response. Because the effects of CRF and its antagonist have opposite effects on cAMP yet were both effective neuroprotectors in this preparation, it is difficult to attribute these results to a simplistic mechanism involving cAMP. Discrepancies between the effects of the two peptides could reflect stability of proteolytic enzymes or penetration/affinity (K) constant to the receptors.

Interestingly, CRF, adrenocorticotropic hormone, and corticosteroids have been used successfully to treat infantile and childhood seizure disorders. Unfortunately, the mechanism of action of these neuropeptides is unclear, although some neuroprotective action on epileptogenic cortex is suggested. In conclusion, the results of the present study demonstrate that CRF may act as an endogenous neuroprotective hormone during hypoxia. Further investigation of the effects of CRF and α-CRF on hypoxic neurons is necessary to better define their mechanisms of action and determine their potential clinical roles in the treatment of cerebral ischemia.

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**References**

Corticotropin releasing factor (CRF) is a potent hypothalamic stimulator of the synthesis and secretion of adrenocorticotropic hormone (ACTH; i.e., pro-opiomelanocortin) from pituitary corticotrophs. The mature hormone, a 41–amino acid peptide, is synthesized as a precursor of 196–amino acid polypeptide, which is broadly expressed in a variety of tissues besides the hypothalamus. Although the primary action of CRF is still believed to be the release of ACTH (and possibly endorphins), a few recent studies revealed additional pharmacological properties of CRF, such as modulation of neuronal excitability and, most prominently, its ability to decrease postburst hyperpolarization in hippocampal slices. Because increased neuronal excitability has been argued by some to render neurons vulnerable to excitotoxic conditions, the property of CRF (vide supra) calls for a role in neuronal death. This hypothesis needs validation of significant research and data, including the following: demonstration that CRF is neurotoxic in vivo (on its own or in certain conditions); that CRF is present at neurotoxic concentrations at vulnerable neurons; that functional CRF receptors are present on vulnerable neurons in vivo; and that the timing of CRF synthesis, release, and actions concurs with the neuropathologic events at vulnerable neurons.

In the study by Fox et al., an in vitro system to monitor the activity of hippocampal circuits has been used to study the neurotoxic effects of CRF and its peptide analog, α-CRF, a competitive antagonist of CRF. The premise of this study was that CRF would exacerbate hypoxia-induced disruption of hippocampal population spikes. Contrary to the authors' hypothesis, both CRF and its antagonists enhanced recovery of the posthypoxic activity. The data, however, must be interpreted with great caution, as the following caveats are apparent: (1) The
Neuroprotection by corticotropin releasing factor during hypoxia in rat brain.
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