Rapid Hypoxia Preconditioning Protects Cortical Neurons From Glutamate Toxicity Through δ-Opioid Receptor

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Background and Purpose—Hypoxia preconditioning (HPC), rapid or delayed, has been reported to induce neuroprotection against subsequent severe stress. Because δ-opioid receptor (DOR) plays an important role in delayed HPC-induced neuroprotection against severe hypoxic injury, we asked whether DOR is also involved in the rapid HPC-induced neuroprotection.

Methods—Cultured rat cortical neurons at culture days 8 to 9 were exposed to a short-term hypoxia (1% O₂ for 30 minutes) to induce HPC followed by 30-minute normoxia before exposing to glutamate toxicity (100 μmol/L; 4 hours). Neuronal viability was assessed by lactate dehydrogenase leakage and morphological assessment. Protein and mRNA levels of DOR were detected by receptor binding and RT-PCR, respectively. Naltrindole was used to block DOR. Developmental changes in NMDA receptor expression was measured by Western blots.

Results—HPC significantly reduced the glutamate-induced neuronal injury. Receptor binding showed that HPC increased DADLE (a DOR ligand) binding density in the cultured cortical neurons by 90% over control level (P<0.05), although RT-PCR did not detect any appreciable change in DOR mRNA. DOR inhibition with naltrindole had no effect on neuronal injury and completely abolished the HPC-induced neuroprotection. In contrast to HPC-induced increase in DADLE binding density, prolonged hypoxia caused severe neuronal injury with a significant decrease in DADLE binding density and DOR mRNA level.

Conclusions—DOR is involved in neuroprotection induced by rapid HPC in cortical neurons. (Stroke. 2006;37:1094-1099.)

Key Words: cerebral cortex ■ hypoxia ■ neurons ■ neuroprotection ■ receptors, opioid

Ischemia preconditioning/hypoxia preconditioning (IPC/HPC) is a phenomenon whereby brief ischemia/hypoxia “preconditions” cells and increases cellular resistance against subsequent lethal ischemia/hypoxia injury. It has been documented in various preparations including the brain, in vitro brain slice, and cultured neurons.1–4 However, most studies have been focused on the delayed IPC/HPC neuroprotection (ie, 24 hours [or more] of recovery are required between the “preconditioning” and subsequent insults). Only several studies that performed on the whole brain,8,10–13 spinal cord,14 and hippocampal slice7,12,15,16 showed a rapid IPC/HPC-induced protection (ie, the interval between “preconditioning” and lethal insult was between 30 and 60 minutes), in which the protection develops within 60 minutes after the preconditioning. We observed that delayed HPC protects cultured cortical neurons from subsequent insults.17 However, it is unknown whether rapid HPC could produce protection in cortical neurons.

Moreover, the mechanism of rapid HPC-induced protection has not been well understood, although several signal pathways have been suggested for delayed IPC/HPC protection.3,18,19 Our work has shown that δ-opioid receptor (DOR), which is neuroprotective against excitotoxic/hypoxic stress,20,21 is involved in delayed HPC protection in cortical neurons.17 We hypothesized that rapid HPC, like delayed HPC, may render cortical neurons more tolerant to subsequent severe stress. However, there are no data available as to whether DOR plays a role in rapid HPC. So far, three fundamental questions remain unknown regarding this issue. First, does rapid HPC protect cortical neurons from neuronal excitotoxicity? Second, does rapid HPC influence DOR density in cortical neurons? Third, does blockade of DOR attenuate/abolish rapid HPC effect?

As a first step toward better understanding of neuronal response to rapid HPC, this work is performed to answer the above 3 questions. Specifically, we investigated: (1) whether rapid HPC can trigger neuroprotection in cortical neurons; (2) whether DOR density is altered by rapid HPC at protein and mRNA levels; and (3) whether DOR antagonists affect the rapid HPC neuroprotection.

Materials and Methods

This study was performed in accordance with the guidelines of the animal care committee of Yale University School of Medicine.

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Neuronal Culture
Primary neuronal culture was prepared from the cortex of embryonic day 16 to 17 rats as described previously. In brief, the cortical neurons were suspended in neuron-defined culture medium and plated onto poly-D-lysine-coated 35-mm dishes (1×10^6 cells per dish). Except for the plating medium, no glutamate existed in normal culture medium. Half of the medium was replaced every 3 to 4 days. Under this condition, >99% of the total population is neurons up to the day when all experiments were finished.

Cell Treatment
Culture dishes were randomly divided into normoxia (control) and HPC groups at 8 days in vitro (DIV 8). Control dishes were kept in normoxia, whereas HPC dishes were exposed to 1% O₂ for 30 minutes, followed by 30 minutes of normoxia. Four-hour exposure to 100 μmol/L glutamate, which has been shown to induce neuronal injury, was used as the subsequent insult. DOR antagonist naltrindole (10 μmol/L) was administered right before the glutamate exposure. To compare effects of HPC and prolonged hypoxia on DOR expression, some dishes were exposed to hypoxia for up to 120 hours.

Lactate Dehydrogenase Assay
Lactate dehydrogenase (LDH) release, a reliable index of cellular injury, was measured using LDH kit (procedure No. 228-UV; Sigma). A total of 100 μL of medium was mixed with 1 mL of prewarmed LDH reagent (50 mmol/L lactate, 7 mmol/L NAD⁺ in 0.05% sodium azide buffer, pH 8.9, 30°C). After stabilization for 30 s, absorbance at 340 nm was recorded at 30-s intervals for 2.5 minutes with spectrophotometer (Beckman DU-640). The LDH activity in the medium (U/L) was then calculated and converted to percentage of control levels.

Morphological Studies
The “same field” assessment of viable and injured neurons was performed using a computer-based image analysis system as described in our previous work. The number of injured cells was expressed as a percentage of the number of viable cells before treatment.

Receptor Binding
The procedures were similar to those described in our previous work. In brief, culture dishes were incubated at room temperature for 60 minutes with 4 mmol/L [3H]-DADLE in 50 mmol/L Tris-HCl buffer, pH 7.4, containing 100 mmol/L NaCl, 40 mg/L bacitracin, and 1 μmol/L PL017, a μ-ligand, to prevent [3H]-DADLE from binding to μ-receptor. After incubation, culture dishes were rinsed and cells were dislodged from the dish with a 400-g force and 300 μL of medium was mixed with 1 mL of scintillation liquid (Opti-Fluor; Packard), and radioactivity (counts per minute) was counted by a Packard beta counter. The specifically bound [3H]-DADLE was determined by subtracting the nonspecific binding from the total.

Western Blot
The neurons were collected by cell scrapers and homogenized in lysis buffer containing protease inhibitor cocktail. Protein samples (30 μg) were subjected to electrophoresis in 10% sodium dodecyl sulfate–containing polyacrylamide gels and then transferred to a polyvinylidene fluoride membrane (Amersham). The membrane was blocked with 5% nonfat milk in Tris-buffered saline and then incubated at 4°C with anti-NMDAR1 affinity purified polyclonal antibody, Chemicon) or anti-actin (Santa Cruz Biotechnology) antibodies. The protein was visualized with chemiluminescence ECL (Amersham).

Data Analysis
Data were expressed as mean±SE and subjected to Student t test using Graphpad Prism 3.0 software. P<0.05 was considered statistically significant.

Results
Differential NMDA Expression and Neuronal Response to Glutamate Toxicity Between Ages
First, we compared morphological and LDH measurements in the neuronal culture in terms of cellular viability after glutamate exposure. Before and after the glutamate exposure, the cultured media were sampled for LDH measurement. Immediately after the sampling, neuronal images were taken from the same culture dishes for “same field” morphological assessment. As shown in Figure 1, LDH assay was very consistent with morphological measurements. A 200% increase in LDH leakage was approximately equivalent to 70% of injured neurons detected by the “same filed” morphology. Both of them showed proportional changes in response to glutamate toxicity or DOR-induced protection.

In cultured cortical neurons after day 8, 4-hour exposure to glutamate caused 70±5% of neurons injured. However, cortical neurons in the first 3 days of culture were extremely resistant to glutamate toxicity. No appreciable injury could be found in these neurons even after exposure to 10 mmol/L level of glutamate. These observations are consistent with our previous work. Because neuronal excitotoxicity is depen-
We asked whether there was a major difference in NMDA receptor expression between neuronal ages in the cultured cortical neurons. Western blots showed that NMDA receptor density was very low in the first 1 to 2 days in culture and reached mature level after culture day 8 (Figure 2). Therefore, we performed all the subsequent studies in neurons at culture day 8 to 9.

Rapid HPC Attenuated Glutamate-Induced Neurotoxicity

LDH leakage from neurons was used as an index of neuronal injury. We compared LDH leakage to morphological studies and found that LDH leakage is very reliable for determining neuronal viability. As shown in Figure 3, exposure to 100 μmol/L glutamate for 4 hours could elevate LDH release in DIV 8 neurons by nearly 1-fold higher than that in the control, which was consistent with our previous report. If the neurons were preconditioned with 30 minutes of 1% O₂ followed by 30 minutes of reoxygenation (rapid HPC), the same glutamate exposure increased LDH release by only 0.5-fold higher, indicating that rapid HPC could increase neuronal resistance to subsequent excitotoxic insult. In the HPC-only group, there was no significant change in the LDH release compared with control, suggesting that HPC we used here is a sublethal insult to the neurons at DIV 8.

Rapid HPC Increased DADLE Binding Density but Did Not Change DOR mRNA Level

To elucidate the role of DOR in the rapid HPC-induced neuroprotection, we asked whether rapid HPC could affect DOR expression at protein and mRNA levels. Receptor binding showed that DADLE binding density was low at DIV 4 (about one tenth of the mature level) and increased with cultured day, with peaking around DIV 11. As shown in Figure 4A, HPC increased DADLE binding density by >1-fold higher than that of the control (n=15; P<0.01). Semiquantitative RT-PCR revealed no appreciable changes in DOR mRNA level (Figure 4B).

DOR Antagonist Abolished the Rapid HPC-Induced Neuroprotection

Because DOR protein level was upregulated by rapid HPC, we hypothesized that the rapid HPC-induced neuroprotection might rely on the function of DOR. Therefore, we applied DOR antagonist naltrindole (10 μmol/L) with glutamate in the cortical neurons treated with rapid HPC. The application of naltrindole itself exerted no significant effect on LDH leakage (Figure 3) in the HPC-treated neurons. However, naltrindole remarkably affected the rapid HPC-induced neuroprotection in the cortical neurons. As shown in the last bar in Figure 3, the increase of LDH leakage in...
mRNA had no appreciable change in the group of 2-hour exposure. After 24 hours of hypoxic exposure, DADLE binding density returned to the control level (P>0.05). After 72 hours, DADLE binding density and DOR mRNA level significantly decreased by >30% (Figures 5 and 6), with serious neuronal injury as described in our previous work.20 A 4-day hypoxia further reduced DADLE binding density (~50% compared with the control; P<0.05).

Discussion

This study presents the first data to show that rapid HPC renders cortical neurons more tolerant to subsequent severe neuroexcitotoxicity via DOR system. Our work provides a simple model for exploring the mechanisms of rapid HPC neuroprotection in cortical neurons.

An important observation of this work is that rapid HPC increased DOR binding density in cortical neurons. Although the underlying mechanism is still unclear, there is evidence suggesting that Ca\(^{2+}\) entry may be involved in DOR upregulation as an early response of neurons to hypoxia.21 Because L-type Ca\(^{2+}\) channel blockers were reported to reduce the induction of DOR expression, it is possible that the increase in DOR binding density is partially attributable to an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\_i\) through L-type Ca\(^{2+}\) channels activated by depolarization.22 The rapid HPC-induced increase in DOR binding density seen in this work is, at least partially, associated with the similar mechanism because hypoxia leads to an increase in [Ca\(^{2+}\)]\_i\). In addition, the mechanisms may vary in different cell types. For example, the Ca\(^{2+}\) entry leads to an increase in DOR mRNA levels in NG108-15 cells.23 However, the present work shows that in cultured neurons, short-term hypoxia, which may temporally increase [Ca\(^{2+}\)]\_i\), increased DOR binding density but not DOR mRNA level. Such results suggest that the cultured cortical neurons, rapid HPC triggers some post-transcriptional events to increase the density of the DOR protein. However, we are cautious about the increase in DOR binding density in our work. This may not necessarily indicate an increase in general amount of DOR in a whole cell. Instead, it could be simply related to DOR translocation from cytoplasma to neuronal membrane. From the energy saving point of view, this cellular procedure (ie, post-transcriptional regulation/translocation instead of both transcriptional and post-transcriptional expression) costs less ATP in the neurons with energy shortage under hypoxia. A longer period of HPC or interval (ie, delayed HPC) may induce an upregulation of DOR mRNA level, which may serve as 1 of the pathways of neuroprotection of delayed HPC. Indeed, our recent work17 has shown that preconditioning with mild hypoxia for 6 to 9 hours followed by 24-hour normoxia (ie, delayed HPC) increases both mRNA and protein levels of DOR in cortical neurons.

Past work has shown that DOR is protective against neuronal stress. For example, we observed that DOR activation protects cortical neurons from hypoxia-20 or glutamate-induced injury,21 whereas DOR antagonist blocks such protection. Other investigators also noticed that preincubation of cerebellar slice with morphine (0.1 to 10 µmol/L) increased
the survival rate of Purkinje cells under oxygen-glucose deprivation, which was blocked by DOR antagonist. Therefore, it is reasonable to predict that the DOR system is associated with rapid HPC-induced neuroprotection. Because the rapid HPC effect was completely blocked by DOR antagonist as shown in this work, we suggest that the function of DOR plays a role in neuroprotection induced by rapid HPC. Under hypoxia, increase in extracellular excitatory neurotransmitters and \([\text{Ca}^{2+}]\), causes excitotoxicity. The DOR system may play a role in feedback regulation because activation of DOR leads to hyperpolarization and lower neuronal excitability, which may partially form the base for HPC neuroprotection. Because DOR also participates in the ischemic preconditioning-induced myocardial protection, we believe that DOR may serve as a general protector against ischemic/hypoxic stress in oxygen/energy-sensitive organs like the brain and heart.

It is important to point out that the ability of neuronal adaptation to stress is within limitation in terms of DOR regulation. As shown in this work, cortical neurons lost the ability to increase DOR binding density after prolonged (>24 hours) hypoxia. With increase in hypoxic duration, both DOR mRNA and protein levels decreased in a major way with severe neuronal injury. Our data suggest that a short-term hypoxia (eg, HPC) may upregulate DOR system in the cortical neurons and render neurons more tolerant to subsequent stress, whereas a prolonged hypoxia may impair DOR system and thus cause neuronal injury. Indeed, several reports showed that after severe hypoxia or ischemia, DOR density decreased in the brain.

DOR-mediated IPC/HPC protection has significant impacts on clinical aspects. Certain neurosurgical procedures may require, or cause, brief periods of brain hypoxia/ischemia. Sleep apnea is another common condition. In acute stroke patients who have previous transient ischemic attack, for example, a less severe clinical picture at admission and a more favorable outcome has been documented when compared with those without the experience of transient ischemic attack, suggesting a role for IPC/HPC in the neuroprotection of human brain. Therefore, neuronal preconditioning is a promising strategy against ischemia/hypoxia.
Acknowledgments

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References

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