1. Effects of nitric oxide in the simulated retinal ischemia induced by deprivation of glucose and oxygen

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Abstract

Purpose. To examine the neuroprotective effects of neuronal nitric oxide synthetase (NOS) inhibitor in the development of ischemic retinal degeneration.

Methods. In the rat ex vivo retinal preparations, simulated ischemia was induced by deprivation of oxygen or glucose from the incubation medium. GYKI (non-NMDA receptor antagonist) were applied to the retinal samples in combination with 7-NIA (neuronal NOS inhibitor). The neuroprotective effects of these agents were evaluated by light microscopically and lactate dehydrogenase enzyme assay.

Results. Oxygen deprivation induced axonal swelling of the retinal ganglion cells remaining other retinal layer intact. Deprivation of oxygen and glucose induced retinal excitotoxic damages. This neurodegeneration was prevented by the combination of GYKI and 7-NIA.

Conclusions. The present study revealed that the neuronal NOS inhibitor seems to be partially neuroprotective against the ischemic retinal degeneration.

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Introduction

Ischemia is a pathological condition with a restriction in blood flow, causing an inadequate supply of oxygen and glucose needed for cellular metabolism. Excitotoxicity is considered to play an important role in the ischemic degeneration of the retina.\textsuperscript{1} Excitotoxicity is predominantly mediated by the overstimulation of NMDA receptors due to their extreme permeability to calcium ions.\textsuperscript{2} The NMDA receptors are activated followed by production of nitric oxide (NO), and NO affords hazardous effects in the retinal ischemia.\textsuperscript{8} It has been reported the possibility that neuronal-type NO synthetase (NOS) inhibitors may ameliorate the ischemic changes in the retina.\textsuperscript{3-8} There is also a possibility that glutamate acts via non-NMDA receptor to initiate neurotoxicity.\textsuperscript{9-13} Therefore, co-administration of NOS inhibitor and non-NMDA receptor antagonists may be effective to protect the retina from ischemia.\textsuperscript{14}

In the present study, we induced the simulated ischemia in the rat retinal ex vivo preparation by deprivation of glucose or oxygen, and examined the neuroprotective effects of neuronal NOS inhibitor in combination with non-NMDA receptor antagonists.

Materials and methods

Animals

The present experiments were performed in accordance with the guidelines of the ARVO. Male Sprague-Dawley (SD) rats were obtained from Charles River Laboratories International Inc. (Wilmington, Mass) at postnatal date 30.

Chemicals

A non-NMDA type glutamate receptor antagonist GYKI52446 (GYKI), and neuronal types of NOS inhibitors, 7-Nitroindazole (7-NIA) were obtained from Sigma Chemical Co (St. Louis, MO).

Retinal preparation

Retinal segments were prepared from 30-day-old SD rats, using previously described methods.\textsuperscript{14} Briefly, albino rats were anesthetized with
halothane and decapitated. The eyes were carefully enucleated and placed in chilled artificial cerebrospinal fluid (aCSF). After removal of the lens, iris, and vitreous, the empty eyecup was sliced into retinal segments.

Simulated ischemia was induced by replacement of oxygen to N₂. In some experiments, glucose was deprived from aCSF. The ex vivo retinal preparations were incubated in the condition of simulated ischemia for 60 min.

GYKI was used at the concentration of 10 μM, and applied to the incubation medium for 60 min of simulated ischemia. 7-NIA (200 μM) was also applied separately or in combination with glutamate receptor antagonists to the incubation medium for 60 min.

**LDH assays**

LDH levels were determined from whole retina following gentle sonication using methods described previously. LDH activity was measured with a Sigma LDH assay kit (Sigma Chemical Company, St. Louis, MO). Based on our prior studies, there is a good correlation between LDH levels and histological measures of neuronal damage.

**Results**

Compared with the control retina (Fig. 1a), axonal swelling of the retinal ganglion cell was induced by deprivation of oxygen (Fig. 1b). Simulated ischemia induced by deprivation of oxygen and glucose for 60 min was characterized by spongy appearance of the inner plexiform layer and the bull’s eye formation of the inner nuclear layer (Fig. 1c).

7-NIA alone did not block the retinal degeneration induced by simulated ischemia induced by deprivation of oxygen and glucose for 60 min (Fig. 2a) or deprivation of oxygen alone (data not shown). Combination of 7-NIA and GYKI offered substantial protection against neuronal damage induced by deprivation of oxygen alone (Fig. 2b) or deprivation of oxygen and glucose (Fig. 2c).

To qualify retinal degeneration under ischemic conditions, we assayed LDH release into the media using previously described methods. The results of each experiment were summarized in Table 1.
Figure 1a. Control retina incubated for 3 h in drug-free solution. b. Sixty min of incubation in aCSF with 95% N₂ and 5% CO₂ induces axonal swelling of the ganglion cell (double arrows). c. Retinas exposed to 60-min simulated ischemia induced by deprivation of glucose and oxygen shows a irregular appearance in the ONL, along with neuronal and dendritic swelling in the INL and IPL. The nerve fiber layer shows severe damage (long arrows).

Figure 2a. Similarly, 200 μM 7-NIA alone does not inhibit excitotoxic damage in a retina exposed to 60-min simulated ischemia. The nerve fiber layer shows severe damage (long arrows). b. Combination of GYKI and 7-NIA provides the substantial neuroprotection, although the axonal swelling of the ganglion cells (arrow) still remains in the retinas incubated in aCSF with 95% N₂ and 5% CO₂. c. In the retinas exposed to 60-min simulated ischemia induced by deprivation of glucose and oxygen, combination of GYKI and 7-NIA diminishes the neuronal changes. However, the damages in the GCL, INL, IPL and the endfeet of the Müller cells are remained. Scale bar = 30 μm
Nitric oxide & retinal ischemia

Table 1. LDH release from the retinal preparation. In four sets of experiments, LDH levels were determined after 60 min incubation of whole retina using gentle sonication as described in the Methods. Retinas were exposed to 60 min simulated ischemia (IS) in the presence of GYKI or GYKI plus 7-NIA. Data are mean ± S.E.

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>LDH U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>5 ± 4</td>
</tr>
<tr>
<td>Deprivation of oxygen</td>
<td></td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Deprivation of oxygen and glucose (IS)</td>
<td>6</td>
<td>206 ± 59</td>
</tr>
<tr>
<td>IS + 7-NIA</td>
<td>5</td>
<td>192 ± 60</td>
</tr>
<tr>
<td>IS + GYKI + 7-NIA</td>
<td>5</td>
<td>*35 ± 25</td>
</tr>
<tr>
<td>Deprivation of oxygen + GYKI + 7-NIA</td>
<td>4</td>
<td>10 ± 3</td>
</tr>
</tbody>
</table>

Asterisks mean the significant reduction of LDH (p<0.001) compared with simulated ischemia.

Discussion

In the present study, the simulated ischemia induced by deprivation of oxygen and glucose demonstrated the inner retinal degeneration, which is characterized the bull’s eye formation in the inner nuclear layer and the spongy appearance of the inner plexiform layer. Such histological appearance seems corresponding to the excitotoxicity in the adult rat.\(^{14,17}\) By contrast, the retina is well tolerated with deprivation of oxygen alone, when glucose is supplied. It is because glycolytic cycle still works in such an anaerobic condition, and produce adenosine triphosphate to metabolize the extracellular glutamate by glutamine synthetase. The vulnerability of the ganglion cell axon in such ischemic condition may be caused by the fact that the ganglion cell is rich in NMDA receptors.

It has been revealed that the activation of both types of glutamate receptors, NMDA and non-NMDA receptors, were involved in the development of retinal ischemic degeneration, because anti-NMDA and anti-non-NMDA receptor antagonists are needed to block the ischemic degeneration.\(^{18}\) Because NO release occurred downstream of activation of NOS and NMDA receptor, we attempted to examine the neuroprotective effects of NOS inhibitor (7-NIA) combined with non-NMDA receptor antagonist (GYKI) in simulated ischemia.

NO formation is considered to be a downstream of the activation of glutamate receptors. NO is generated by three types of NOSs; inducible NOS, endothelial NOS, and neuronal NOS (nNOS). Recently, the immediate
activation of nNOS under the ischemia has been reported. Our present results that nNOS inhibitor, 7NIA, demonstrated the neuroprotective effect against the simulated ischemia under the presence of GYKI, indicate the involvement of nNOS to the development of retinal ischemia. These results were corresponding to the previous reports of the ischemia in the central nervous system.

References


