NMDA receptor mediated dendritic plasticity in cortical cultures after oxygen-glucose deprivation

Zhigang Lei, Yiwen Ruan, Angela N. Yang, Zao C. Xu *

Department of Anatomy and Cell Biology, Indiana University School of Medicine, 635 Barnhill Drive, MS 507, Indianapolis, IN 46202, USA

Received 6 March 2006; received in revised form 25 May 2006; accepted 5 June 2006

Abstract

Dendrites and spines undergo dynamic changes in physiological and pathological conditions. Dendritic outgrowth has been observed in surviving neurons months after ischemia, which is associated with the functional compensation. It remains unclear how dendrites in surviving neurons are altered shortly after ischemia, which might reveal the mechanisms underlying neuronal survival. Using primary cortical cultures, we monitored the dendritic changes in individual neurons after oxygen-glucose deprivation (OGD). Two to four hours of OGD induced approximately 30–50% cell death in 24 h. However, the total dendritic length in surviving neurons was significantly increased after OGD with a peak at 6 h after re-oxygenation. The increase of dendritic length after OGD was mainly due to the sprouting rather than the extension of the dendrites. The dendritic outgrowth after 2 h of OGD was greater than that after 4 h of OGD. Application of NMDA receptor blocker MK-801 abolished OGD-induced dendritic outgrowth, whereas application of AMPA receptor antagonist CNQX had no significant effects. These results demonstrate a NMDA receptor-dependent dendritic plasticity shortly after OGD, which provides insights into the early response of surviving neurons after ischemia.

© 2006 Elsevier Ireland Ltd. All rights reserved.

Keywords: Cerebral ischemia; Dendritic outgrowth; NMDA receptor; Neuronal survival

Dendrites and spines are the basic structures of a neuron and contribute to its integrative properties [8,34]. Plasticity changes of dendrites and spines have been observed in physiological conditions, such as learning and memory in the form of long-term potentiation (LTP) [18]. Alterations of dendrites have also been reported in pathological conditions including ischemia [1,4]. Dendritic changes after ischemia, such as the formation of focal varicosities along the dendrites and loss of spines, are the signs of degeneration [23]. On the other hand, it has been shown that the dendritic and axonal arborization significantly increased several months after focal ischemia [1,4] or prolonged hypoxia [25]. The increase of dendritic number and dendritic length in surviving neurons several months after ischemia/hypoxia is probably one of the mechanisms underlying the functional compensation. Despite the extensive studies on neuronal plasticity after ischemia, little is known about the dendritic changes in surviving neurons shortly after ischemia. The behavior of surviving neurons shortly after the insult might reveal the mechanisms associated with ischemic resistance. Using in vitro preparation, the present study examined the dendritic arborization of cortical neurons in primary culture after oxygen-glucose deprivation (OGD). We found a significant increase of dendritic outgrowth in surviving neurons within 24 h after OGD.

Cortical tissues were collected from embryonic day 18 Wistar rat embryos. After digestion with 0.125% trypsin (Invitrogen), cells were re-suspended in Neurobasal media (Invitrogen) containing 2% B27 (Invitrogen), 2 mM glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 25 \( \mu \)M glutamate (Sigma), 100 U/ml penicillin and 100 \( \mu \)g/ml streptomycin (Invitrogen). Then cells were seeded in 24-well plates (Falcon) coated with 0.01% (w/v) poly-L-lysine (Sigma) at a density of 10^5 cells/cm^2 and put into a standard incubator (Taibai Espec) maintained at 37°C in 95% air, 5% CO2. A half medium was changed once a week.

To determine the percentage of neurons in the culture, immunocytochemistry was conducted using MAP2 and GFAP.
antibodies. Cells were plated on coverslips coated with 0.01% poly-L-lysine. On days in vitro (DIV) 10, cultures were fixed with 4% paraformaldehyde for 10 min at room temperature, and were treated with 2% horse serum in 0.05% Triton X-100 for 30 min. Neurons were labeled with mouse monoclonal anti-MAP2 (1:1000, Sigma) for 2 h. Then, cultures were incubated in a solution containing Texas red-conjugated secondary antibody (1:100; Vector) for 1 h. After repeated washes in PBS and blocked with 2% coat serum, astrocytes were labeled with rabbit anti-GFAP (1:1000, Chemicon) for 2 h, followed by fluorescein-conjugated secondary antibody (1:100; Vector) for 1 h. After washing in PBS, cultures were mounted with Vectashield media (Vector) and visualized with a fluorescence microscope (Nikon) at 20× objective.

For OGD, cultures of DIV 10 were placed in an anaerobic chamber (ThermoForma) containing 5% CO₂, 10% H₂, 85% N₂. The culture medium was replaced with deoxygenated, glucose-free BSS consisting of (in mM) 116 NaCl, 5.4 KCl, 0.8 MgSO₄, 1.0 NaH₂PO₄, 26.2 NaHCO₃, 0.8 CaCl₂, 20 sucrose. The chamber was humidified and maintained at 37 °C, and cells were exposed to the OGD condition for a designated period. OGD was terminated by returning the cultures to the normal medium and standard incubator. Control cultures were exposed to BSS that contained 20 mM D-glucose and maintained in the standard incubator. In some experiments, 10 μM MK-801 or 10 μM CNQX (Sigma) was applied during and after OGD. Twenty-four hours after OGD, neuronal damage was examined with lactate dehydrogenase (LDH) and neuronal survival was determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [32]. For the LDH assay, background LDH concentrations were determined in control cultures. The background values were subtracted from experimental values to yield the LDH concentration specific to OGD. Results were pooled and expressed as a percentage of the maximal LDH concentration corresponding to cultures exposed to 10 h OGD. For the MTT assay, the background concentration was determined in the cultures exposed to 10 h OGD and the results of different OGD duration were normalized to the values of cultures in the control ones.

To examine the dendritic morphology, cells at DIV 2 were transfected with pEGFP-N1 (Clontech) using FuGENE 6 (Roche). At DIV 10, GFP positive (+) neurons were randomly selected from different areas of the well using an inverted fluorescence microscope (Nikon) and the co-ordinates were recorded with image acquisition software IPLab (Scanalytics). The same neuron could be identified at different time points by the computer based on the co-ordinates. The fluorescence images of the selected neurons were acquired and stored as TIF-images. A 10× objective lens was chosen to make sure that all the processes of the neuron were included in the image. Fluorescence images of the same GFP (+) neuron were captured before OGD, 6 and 24 h after OGD, respectively, using the same aperture and exposure time. To eliminate individual bias, no attempts were made to normalize the fluorescent intensity between the images recorded at different time points. The fluorescence images of control neurons were also captured at matched time points under the same condition. Dendritic arborizations were traced based on the images using Neurolucida (MicroBrightField) by two researchers blinded to the experimental conditions and the data were pooled for further analysis. The dendritic morphometry was analyzed using Neuroexplorer (MicroBrightField).

All results were expressed as mean ± S.E.M. The effects of OGD on dendritic morphometry were analyzed with paired Student’s t-test and the other data were analyzed with one-way ANOVA followed by post hoc analysis with Fisher’s PLSD using StatView (SAS). The difference was considered statistically significant when P < 0.05.

In our preparation, most of the cells in culture were MAP2 positive neurons with a few GFAP positive astrocytes (Fig. 1A). Cell counting from 10 visual fields revealed that more than 95% of the cells in culture were neurons (635 neurons in 662 total cells). Neurons in culture were vulnerable to OGD. The percentage of cell death increased with prolonged duration of OGD accordingly (Fig. 1B). Exposure of cultures to OGD for 2 or 4 h resulted in 32.3 ± 2.9% or 52.6 ± 3.7% cell death, respectively (n = 18, Fig. 1B).

The morphology of neurons was examined on GFP (+) neurons. These neurons had round somata with extensively branching dendrites and axons (Fig. 2A1, B1, C1). The GFP (+) neurons were healthy and continuously expressed GFP for more than 2 weeks. In both control and OGD neurons, dynamic changes in dendrites were observed during the 24 h monitoring period with some dendrites displayed retraction while others exhibited...
Fig. 2. Dendritic changes of cortical neurons after OGD. (A–C) Fluorescence images of individual neurons taken at 0 and 24 h under different experimental conditions. The dendrites of control neurons remained about the same in 24 h (A). However, after 2 h of OGD, some neurons had degenerated, leaving fragments in their original locations (B), whereas other neurons remained intact with increasing dendritic length (C). Scale bar, 20 μm. (D) Superimposed dendritic arborization reconstructed from the images of a neuron before and at 6 h after OGD. As shown in this picture, OGD induced dendritic retraction (arrow), extension (arrow head) and sprouting (asterisk) in cultured cortical neurons. (E) Increase of total dendritic length after OGD. Quantitative analysis indicated that the total dendritic length was significantly increased after 2 h OGD with a peak at 6 h after re-oxygenation. Four-hour OGD also induced dendritic outgrowth but was less dramatic as compared with that after 2 h OGD. Data at 6 and 24 h were compared with values at 0 h of the same group with paired t-test. *P < 0.01.

extension (Fig. 2D). These observations indicated that OGD treatment did not increase or decrease the fluorescence level that in turn affected the outcome of the present study. At 6 h after OGD, some neurons displayed dendritic varicosity, a sign of degeneration as described by previous investigators [23]. By 24 h, most of these neurons had died and only GFP positive fragments were observed in the sites where the neurons were previously located (Fig. 2B1 and 2). On the other hand, some neurons did not exhibit obvious degenerating features after OGD (Fig. 2C1 and 2). Quantitative analysis indicated a significant increase of dendritic length in these neurons after OGD. In control neurons, the total dendritic length remained about the same in 24 h (Fig. 2E). However, after 2 h OGD, the total dendrite length was significantly increased from 722.9 ± 35.56 μm before OGD to 890.4 ± 41.99 μm and 814.7 ± 44.72 μm at 6 and 24 h after re-oxygenation, respectively (paired t-test, P < 0.01, n = 80, Fig. 2E). The increase of dendritic length could be due to the increase of dendritic branching singularly or in combination with the extension of the individual dendrites. To reveal which component was the major contributing factor to the increase of dendritic length after OGD, the number of dendritic endings and primary dendrites were used to evaluate the dendritic branching, and the internode interval (the length between the points where dendrites bifurcate) and terminal length (the length of last branches) were used to evaluate the dendritic extension. As shown in Table 1, the number of dendritic endings was significantly increased from 23.4 ± 1.11 of before OGD to 27.8 ± 1.25 (P < 0.01) and 24.9 ± 1.24 (paired t-test, P < 0.05, n = 80) at 6 and 24 h after re-oxygenation, respectively. The number of primary dendrites was also increased from 8.2 ± 0.34 of control values to 9.3 ± 0.42 and 9.4 ± 0.4 at 6 and 24 h after OGD (P < 0.01, n = 80). On the other hand, the internode interval was slightly decreased at 6 h after OGD while no significant changes in terminal length were observed after OGD. These results suggested that the increase of dendritic length after OGD was mainly due to the dendritic sprouting rather than the extension of the dendrites. The pattern of dendritic changes in surviving neurons

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Dendritic ending</th>
<th>Primary dendrites</th>
<th>Internode interval (μm)</th>
<th>Terminal length (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>23.4 ± 1.11</td>
<td>8.2 ± 0.34</td>
<td>11.8 ± 0.70</td>
<td>19.5 ± 0.96</td>
</tr>
<tr>
<td>6 h after</td>
<td>27.8 ± 1.25*</td>
<td>9.3 ± 0.42*</td>
<td>10.3 ± 0.59†</td>
<td>20.5 ± 1.02†</td>
</tr>
<tr>
<td>24 h after</td>
<td>24.9 ± 1.24†</td>
<td>9.4 ± 0.44*</td>
<td>12.0 ± 0.69</td>
<td>21.3 ± 1.24</td>
</tr>
</tbody>
</table>

*P < 0.01, †P < 0.05, paired t-test.
after 4 h OGD was similar to that after 2 h OGD but the extent of dendritic outgrowth was smaller (Fig. 2E).

Activation of glutamate receptors plays important roles in dendritic development and plasticity [15,19,21]. To investigate the involvement of glutamate receptors in OGD-induced dendritic plasticity, the dendritic morphometry of neurons after 2 h OGD was compared between cultures with or without application of glutamate receptor antagonists. The total dendritic length and number of dendritic endings were significantly increased 6 h after OGD. However, in cultures incubated with NMDA receptor blocker MK-801, no significant change in dendritic length and dendritic endings was observed after OGD (n = 23, Fig. 3). On the other hand, in cultures with AMPA receptor antagonist CNQX application, the dendritic length increased to 126.3 ± 5.19% and the number of dendritic endings increased to 127.3 ± 1.4% after OGD (ANOVA, *P < 0.01, n = 17, Fig. 3). These results indicated that the NMDA receptors, but not AMPA receptors, were involved in the dendritic outgrowth after OGD.

The present study provides direct evidence of dendritic outgrowth in surviving neurons shortly after ischemia/hypoxia. The total dendritic length of cultured neurons is significantly increased after OGD with a peak at 6 h after re-oxygenation. The OGD-induced dendritic outgrowth mainly results from the dendritic sprouting rather than extension. Activation of NMDA receptors, but not AMPA receptors, contributes to the OGD-induced dendritic outgrowth. It is worthwhile to point out that despite the increase of total dendritic length, dendritic retraction has also been observed after OGD (Fig. 2D). These results not only demonstrate the dynamic changes of dendrites after ischemic insult but also exclude the possibility that the increase of dendritic length after OGD might result from the alteration of GFP fluorescence levels.

The neuronal dendrite is a specialized structure to integrate excitatory and inhibitory signals from synapses on its complex arbor. Plasticity changes in dendrites and spines have been implicated in the generation and maintenance of LTP [19,20]. Most of the reports on dendritic plasticity in neurological disorders such as epilepsy and cerebral ischemia are the long-term changes of surviving neurons [1,4,31]. Studies on short-term morphological changes after ischemia are mainly focused on the vulnerable neurons and degenerating process [3,29,35]. Investigations of morphological changes in surviving neurons shortly after ischemia are less extensive and controversial. It has been reported that the immunostaining of dendritic marker MAP2 is enhanced in the boundary zone of penumbra 48 h after focal ischemia [16], suggesting the dendritic outgrowth shortly after ischemia. In contrast, the reduction of dendritic number has been shown in hippocampal neurons 1 day after hypoxia [26]. Using *in vitro* preparations, studies have shown that brief period of hypoxia/hypoglycemia (sublethal OGD) promotes the sprouting of filopodia and elongation of existing spines in cultured neurons [10,24], but causes reversible dendritic varicosities [23]. The present study provides direct evidence, for the first time, demonstrating the dendritic outgrowth in surviving neurons after severe ischemia *in vitro*. Coincidently, using 3-D reconstruction of intracellularly stained neurons, an *in vivo* study also reports a significant dendritic outgrowth in CA1 pyramidal neurons at 24 h after transient cerebral ischemia [28].

Many intracellular and extracellular signals are involved in dendritid development and plasticity [8,22]. Glutamate, mediated by NMDA receptors, has been implicated as an important mediator for spine remodeling after ischemia/hypoxia [7,10]. The present study indicates that OGD-induced dendritic outgrowth is also mediated by NMDA receptors. Intracellular signals that mediate glutamate-induced dendrite plasticity have not been fully established. Accumulating evidence indicates that the intracellular calcium ([Ca2+]i) might be involved in this process [12]. Studies have shown that the concentration of [Ca2+]i is directly correlated with the amount of extracellular glutamate [13]. A transient increase of [Ca2+]i promotes dendrite and spine outgrowth, probably through calmodulin-CaM kinase-CREB pathway, while a sustained increase in [Ca2+]i causes dendritic retraction via calpain activation [5,13,27,33]. It has been indicated that calcium regulates the expression of brain-deprived neurotrophic factors (BDNF) [30]. BDNF levels are significantly increased after ischemia [11,17]. Through activation of PI3K-Akt pathway, BDNF promotes dendritic outgrowth and arborization [9,14]. PI3K-Akt pathway also plays dominant roles in cell survival [2,6]. It is conceivable that the BDNF activated PI3K-Akt pathway might be responsible for the
OGD-induced dendritic outgrowth in surviving neurons. Despite that NMDA receptors have been implicated in dendritic plasticity after ischemia/hypoxia, the present study could not exclude the involvement of other factors. For example, the dendritic plasticity might result from the consequences to the lysis of adjacent cells that undergo necrosis. Two lines of evidence suggest that this probably is not the case. First, the dendritic outgrowth after 4 h OGD with more cell death and lysis (~50%) was less dramatic as compared to that after 2 h OGD (~30% cell death). Secondly, NMDA receptor antagonist MK801 completely blocked the dendritic outgrowth induced by OGD.

The functional significance of dendritic outgrowth in surviving neurons shortly after ischemia/OGD remains unclear. One possibility is that the increase in dendritic arbors, possibly also spines and axons, is the response of these neurons to some survival signals, such as BDNF and PI3K-Akt pathway that also promote neurite outgrowth [9,14]. Another possibility is that the dendritic outgrowth shortly after the insult is an initial compensation of surviving neurons to reorganize neuronal circuits for the functional recovery after ischemic brain injury [1,4]. Further experiments are necessary to determine the causal relationships between structural changes and functional recovery of surviving neurons after ischemic injury.

Acknowledgement

This work is supported by grants NS38053 to Z.C.X., AHA056007Z to Z.G.L.

References


