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Short communication

Neuroprotective effects of lipoxygenase inhibitors against ischemic injury in rat hippocampal slice cultures

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Abstract

Using organotypic cultures of rat hippocampal slices, we investigated the possible involvement of arachidonate cascades in neuronal death following ischemic insult. Oxygen/glucose deprivation-induced neuronal damage was efficiently attenuated by various inhibitors of lipoxygenase, whereas cyclooxygenase inhibitors were less effective. Interestingly, 5- and 12-lipoxygenases are likely to separately mediate ischemic injury in the hippocampus. The present study will provide novel therapeutic targets for the development of neuroprotective agents.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: Ischemia; Hippocampus; Phospholipase A_2; Arachidonic acid; Lipoxygenase

The usual cause of stroke is an acute interruption of cerebral blood flow that compromises the stores of tissue energy, i.e. glucose and oxygen [16,19,20]. The ultimate consequence of this metabolic deprivation is neuronal cell death. The hippocampus, particularly the CA1 subfield, is highly vulnerable to ischemic injury [3,17]. However, mechanisms by which the energy deficiency results in neuronal degeneration remain to be determined.

Phospholipase A_2 is an enzyme that hydrolyses the *sn*-2 fatty acyl ester bond of glycerophospholipids, and is abundantly expressed in the hippocampus [7,11]. The release of arachidonic acid from membrane phospholipids is a well-documented observation following transient global ischemia [1,4,16]. The released arachidonic acid rapidly decreases during the postischemic period, as compared with other products of phospholipase A_2 including free fatty acids and lysophospholipids [22], which suggests that the ischemic stimulus simultaneously facilitates subsequent arachidonate metabolism, probably through lipoxygenase (LOX) and cyclooxygenase (COX) [18]. Indeed, LOX [9,12] and COX [2] are highly expressed in the hippocampus. However, it remains controversial how the arachidonate metabolites, e.g. prostaglandins, leukotrienes, and other eicosanoids, contribute to ischemic injury. Therefore, we focused the present study on the role of the arachidonate cascade in ischemia-induced neuronal death. Using organotypic cultures of hippocampal slices, this work shows that the ischemic injury is attenuated by various LOX inhibitors.

Hippocampal slice cultures were prepared from 9-day-old Wistar/ST rats (SLC, Shizuoka, Japan), essentially as described [5]. Animals were deeply anesthetized by hypothermia, and their brains were aseptically removed and cut into transverse slices (300 μm thick) in aerated, ice-cold Gey's balanced salt solution supplemented with 6.5 mg/ml glucose using a vibratome (DTK-1500; Dosaka EM, Kyoto, Japan). The entorhino-hippocampi were dissected out under stereomicroscopic control. They were fed with 1 ml of culture medium consisting of 50% minimal essential medium, 25% horse serum (Cell Culture Lab, Cleveland, OH, USA), and 25% Hanks' balanced salt solution (HBSS), and were maintained in a humidified incubator at 37°C in 5% CO_2. The medium was changed every 3 days. Experiments were performed after 12–13 days in vitro.

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Combined oxygen/glucose deprivation (OGD) experiments were conducted in a chamber containing an anaerobic gas mixture (95% N_2 and 5% CO_2). The culture medium was replaced with deoxygenated, glucose-free HBSS. During the deprivation procedure, cells were placed in a humidified, 37°C incubator. Deprivation was terminated by replacing the exposure medium with normal medium containing 0.5 μg/ml propidium iodide (PI) (Molecular Probes, Eugene, OR, USA). The cultures were then incubated in a CO_2 incubator at 37°C for 24 h. PI fluorescence images were obtained with the BioRad MRC-600 confocal imaging system (BioRad Microscience Division, Cambridge, MA, USA). Pixel fluorescence intensity of 8-bit resolution (F_t) was measured at three different areas of the slice, i.e. the CA1 and CA3 stratum pyramidale and the stratum granulosum of the dentate gyrus (DG). Background intensity (F_0) was obtained outside the slices. Forty-eight hours after OGD insult, total cells were killed by 24-h incubation at a low temperature (4°C), and then the final PI fluorescence (F_{fin}) was measured. PI uptake was determined from

$$(F_t - F_0)/(F_{fin} - F_0) \times 100\%$$

Ibuprofen, indomethacin, and nordihydroguaiaretic acid (NDGA) were purchased from Wako Pure Chemicals (Osaka, Japan). AA8861, baicalein, and cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) were purchased from Sigma (St. Louis, MO, USA). These drugs were added to culture medium from 40 min before until 40 min after the OGD. 5(*S*)-Hydroperoxyeicosatetraenoic acid (HpETE), 12-HpETE 5(*S*)-hydroxyeicosatetraenoic acid (HETE), and 12-HETE were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Cell morphology was assessed by using a fluorescent membrane dye DiO. The slices were fixed with 4% paraformaldehyde for 30 min, and then the DiO crystal was carefully placed on the stratum pyramidale. Following incubation in the fixative at room temperature for 2 days, DiO-labeled cells were observed using a confocal microscope.

Although apparent neuronal cell death was not detected in hippocampal slice cultures 24 h after OGD exposure of $<$30 min, a significant increase in PI uptake was observed in the pyramidal cell layer 24 h after OGD exposure of $>$32.5 min (Fig. 1A and B). Neurons and glial cells in the slices exposed to the 35-min OGD were individually labeled with DiO to visualize their morphology. Confocal microscopy observation revealed that dying neurons showed severe damage of the cytomembrane (Fig. 1C). Such characteristic injures were not found in glial cells following the OGD (data not shown). These results indicate that the increase in PI uptake essentially reflects the neuronal cell death. In the following experiments, therefore, the neuronal death was assessed by the PI fluorescent intensity. When cultures were exposed to OGD for 32.5 or 35 min, massive neuronal death was evident mainly in the hippocampal CA1 subregion (Fig. 1B). When they were exposed to OGD for 40 to 50 min, cell death was found in the CA1 and CA3 regions. Neurons in the DG were relatively resistant to the OGD insult. Even when the OGD was applied for up to 120 min, the DG neurons underwent no apparent injury ($N = 7$, data not shown).

We investigated the contribution of LOX and COX to the ischemic injury. The neuronal death induced by OGD of either 35 or 40 min was efficiently attenuated by NDGA, a broad-spectrum inhibitor of LOX, in a concentration-dependent manner, while NDGA per se had no effect on the cell survival in intact slices (Fig. 2A). On the other hand, ibuprofen, a broad-spectrum inhibitor of COX, did not block 35-min OGD-induced neuronal death (Fig. 2B), although it partially reduced the CA3 neuronal death following 40-min OGD without affecting the CA1 neuron survival (Fig. 2B). Similar results were obtained for indomethacin, another inhibitor of COX ($N = 8$, data not shown). These results suggest that the LOX pathway predominantly mediates the ischemic injury in the hippocampus.

LOX is an enzyme that incorporates molecular oxygen into specific positions of polyunsaturated fatty acids, and generally classified as 5-, 12-, and 15-LOXs based on the site of insertion of the oxygen [18]. We therefore determined which subtype of LOX is involved in the OGD-induced neuronal death. Pharmacological blockade of 12-LOX by baicalein significantly inhibited the neuronal death induced by 35-min OGD, but not by 40-min OGD (Fig. 3A). Another 12-LOX inhibitor, CDC, led to similar results (Fig. 3B). These data suggest that other isozymes of LOXs mediate neurodegeneration following 40-min exposure to OGD. Actually, AA861, a 5-LOX inhibitor, efficiently reduced the 40-min OGD-induced neuronal death, while it did not prevent the 35-min OGD-induced neuronal death (Fig. 3C). The partial effect of this inhibitor may indicate the possible involvement of another isozyme, 15-LOX. However, no selective 15-LOX inhibitor is available at present, and thus it is not possible to definitively distinguish which enzyme is responsible for the cell death in 40-min OGD insult. Consistent with these observations, esculetin, a dual inhibitor of 5- and 12-LOXs, attenuated the neuronal death induced by either 35- or 40-min OGD ($N = 8$, data not shown). Incidentally, none of these inhibitors alone affected the cell survival in intact slices (Fig. 3).

Finally, we examined whether LOX products per se elicit neuronal injury. 5-HpETE, 12-HpETE, 5-HETE, or 12-HETE (1, 10, or 100 nM) was applied continuously for 24 h, but no apparent cell death was detected in any subregion of the hippocampus (each $N = 4$, data not shown).

Although the release of arachidonic acid from membrane phospholipids has been documented following transient global ischemia [1,4,16], it has not been fully

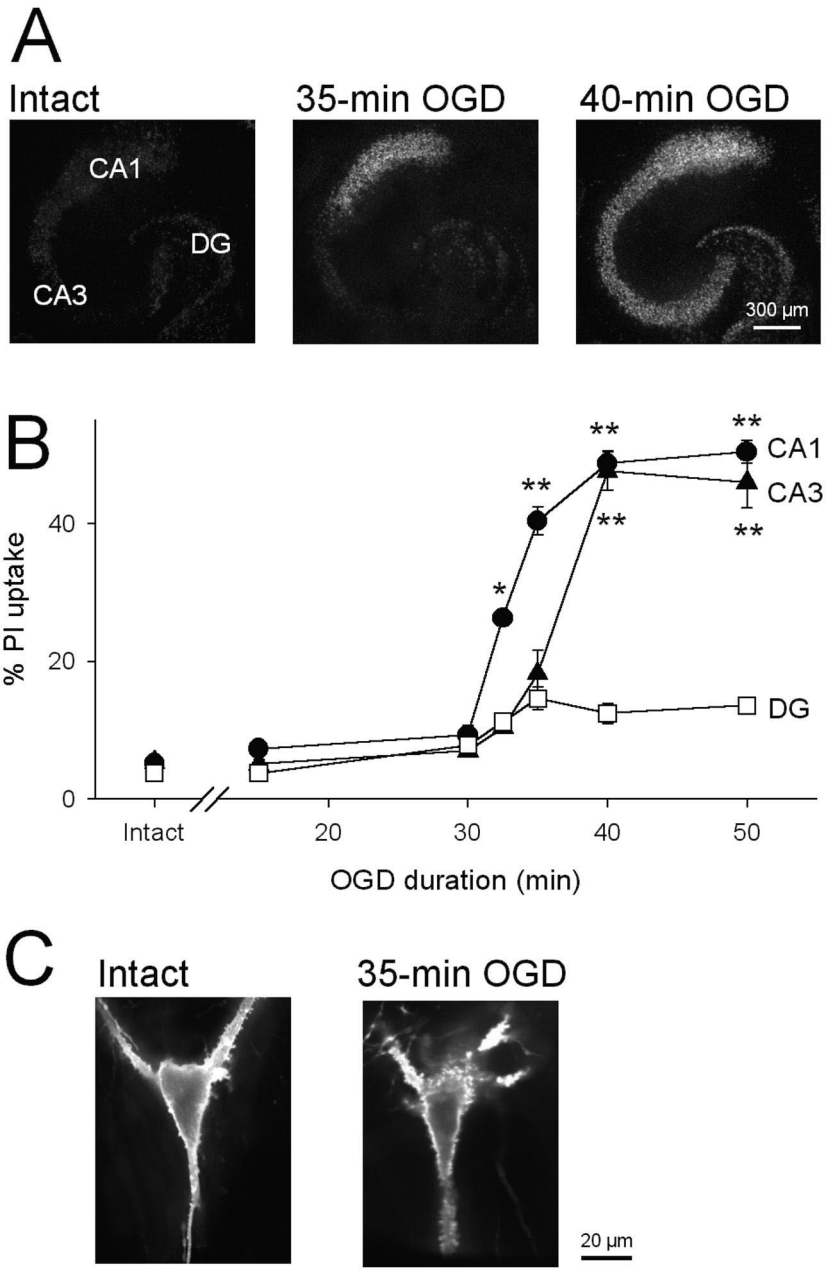

Fig. 1. OGD-induced neuronal death in rat hippocampal slice cultures. (A) Representative confocal images of PI fluorescence show an intact slice (left panel), slices that received OGD exposure of 35 min (middle panel) and 40 min (right panel). (B) Relationship between the duration of OGD exposure and the neuronal damages. PI fluorescence was quantified in the pyramidal cell layer of the CA1 region (●), the CA3 region (▲), and the granule cell layer of the DG (□) 24 h after the OGD exposure. Vertical bars on data points are S.E.M. of eight slices; when not indicated, they fell within the data symbols. $*P<0.05$, $**P<0.01$ versus corresponding subregions in intact slices: Tukey's multiple range test following two-way ANOVA. (C) DiO-labeled CA1 pyramidal cells in control cultures or the slices exposed to 35-min OGD.

determined whether arachidonic acid and/or its metabolites are actually involved in the ischemic injury. Using organotypic cultures, we have shown for the first time that blockade of LOX activity during OGD exposure improves survival of hippocampal neurons. The effects of COX inhibitors were substantially limited. However, recent reports have shown that some specific inhibitors of the inducible isozyme COX-2 prevent neuronal death following global ischemia [10,13]. The non-selective COX inhibitors used in this study may mask their effects that could be detected by selective inhibition of each enzyme. Very recently, however, the neuroprotective effect of piroxicam, which has been believed to be a selective COX-2 inhibitor against hypoxia injury, seems to be independent of COX inhibition but may rather depend on COX-independent activation of extracellular signal-regulated kinase [21]. Therefore, although our finding does not exclude the possibility that the COX pathway is

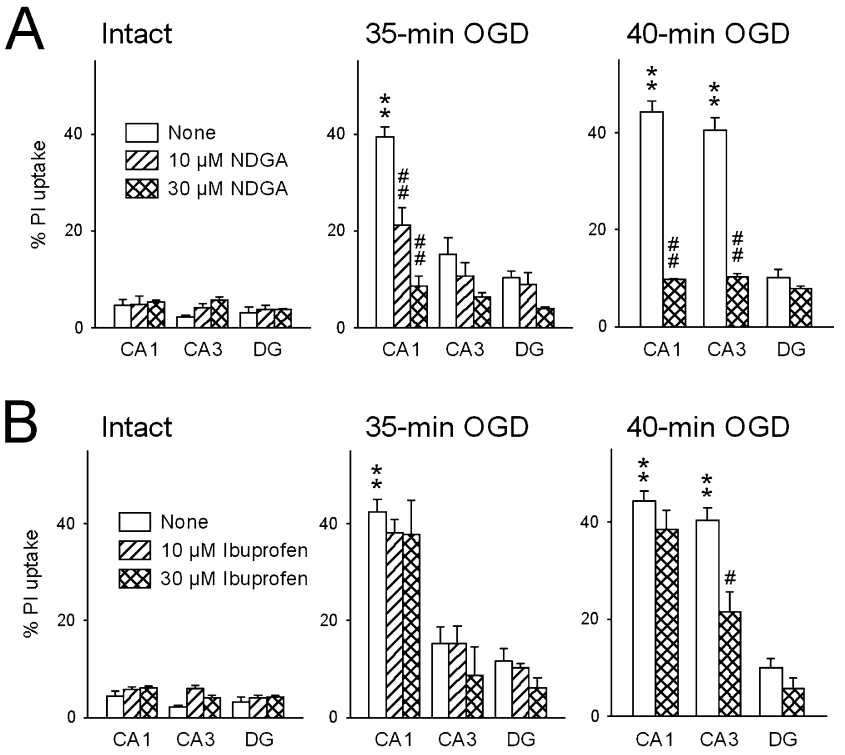

Fig. 2. LOX is involved in OGD-induced neuronal death. NDGA (A) or ibuprofen (B) was added to culture medium from 40 min before until 40 min after 35-min OGD (middle panels) or 40-min OGD (right panels). Left panels show PI uptake in intact slices that received treatment with each drug. PI uptake was assessed in the CA1 region, CA3 region, and the DG 24 h after the OGD insult. Data represent mean±S.E.M. of eight slices. $^{**}P < 0.01$ versus corresponding subregions in intact slices, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus none: Tukey's test following one-way ANOVA.

involved in cell death, we consider that the LOX pathway is of particular importance for ischemic injury in the hippocampus.

Following the OGD exposure, hippocampal slices showed a massive cell loss in the pyramidale cell layer but not in the granule cell layer of the DG. The rank of hippocampal vulnerability was the CA1 region>the CA3 region>the DG, which is closely reminiscent of the in vivo ischemic situation. This OGD system is, therefore, assumed to reproduce in vivo ischemic injury. Survival of hippocampal neurons following the OGD insult was strictly dependent on the duration of OGD exposure. OGD with a duration of >32.5 min severely produced neuronal degeneration, while OGD of <30 min evoked no apparent neuronal death. This remarkable difference between the results of 30- and 35-min OGD implies that the period required for depriving cellular energy is around 30 min.

In the case of 35-min OGD, neuron loss was observed only in the CA1 region. Therefore, the CA1 neurons may be most vulnerable to energy depletion. However, this regional difference in vulnerability cannot be attributed merely to a difference in the amount of energy store in neurons in each subregion, because different isozymes of LOXs appear to be involved in 35- and 40-min OGD. The neuronal death evoked by 35-min OGD was efficiently attenuated by 12-LOX inhibitors. In contrast, 40-min OGD-induced cell damage was rather reduced by 5-LOX inhibitor. At an early stage of the OGD, therefore, 12-LOX is selectively activated and then converts arachidonic acid to 12-HpETE, which may exert its aversive effect on neuron survival. With a slight delay, 5-LOX is also activated by OGD and produces 5-HpETE. At this stage, 12-LOX inhibitors were virtually ineffective, which suggests that 5-HpETE is a major participant. On the other hand, the present study revealed that treatment with 5-HpETE, 12-HpETE, 5-HETE, or 12-HETE alone did not mimic the ischemic injury. Therefore, we consider that, in spite of a critical role of LOX, the LOX cascade cannot solely account for the OGD-induced neuronal death.

Considering that the 35-min OGD caused selective damage in the CA1 region, ischemia-induced 12-LOX activation is likely to be a specific event for the CA1 neurons. Because clinical studies on transient forebrain ischemia revealed that the CA1 subfield is particularly vulnerable to ischemic injury [3,17], the 35-min OGD may represent pathological conditions in humans. Therefore, it is our impression that 12-LOX plays a main role in pathogenic processes of cell damage. On the other hand, Normandin et al. [14] indicated that the 12-LOX pathway is involved in long-term depression of synaptic transmission in the CA1 region. Therefore, 12-LOX activity is required for normal, not pathophysiological, functions of the hippocampus, but excessive 12-LOX activation may cause a collapse of neuronal functions and thereby trigger

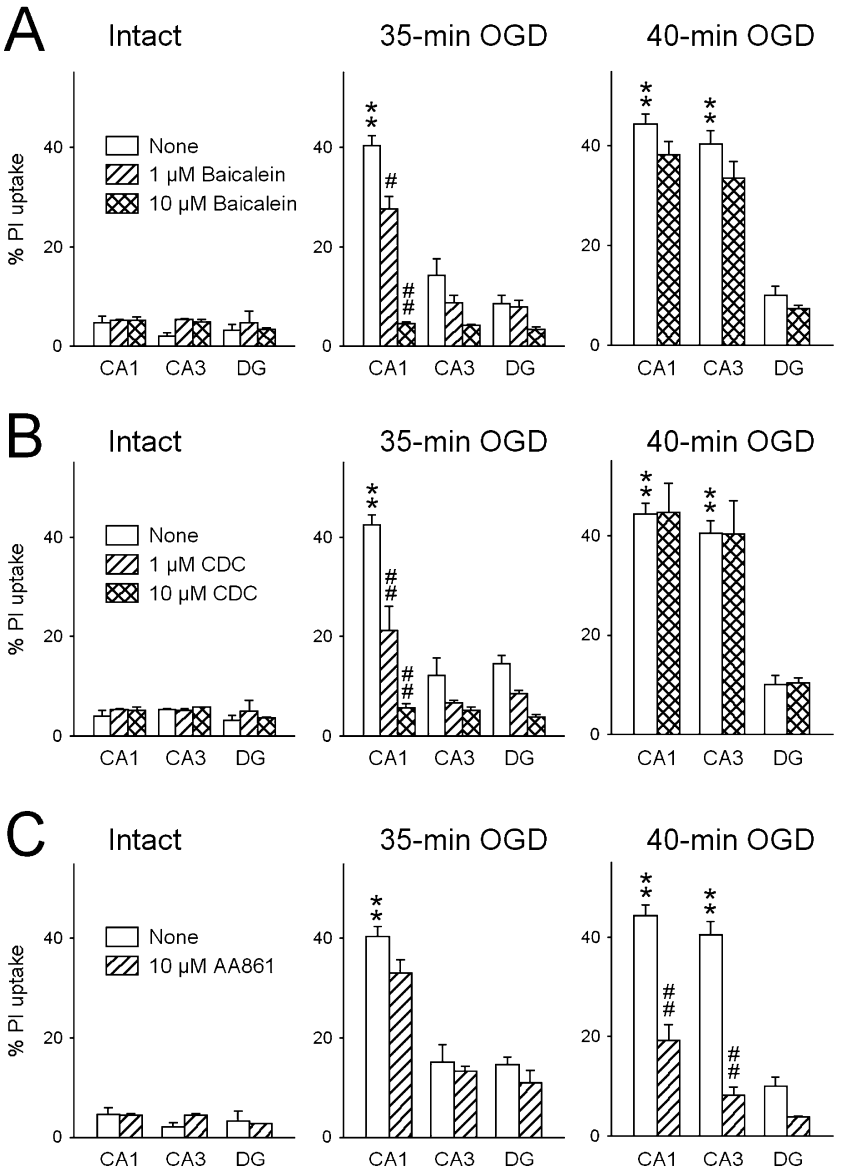

Fig. 3. Effects of diverse LOX inhibitors on OGD-induced neuronal death. Baicalein (A), CDC (B) or AA861 (C) was added to culture medium from 40 min before until 40 min after 35- or 40-min OGD. PI uptake was assessed in the CA1 region, CA3 region, and the DG 24 h after the OGD insult. Data represent mean±S.E.M. of eight slices. $^{**}P < 0.01$ versus corresponding subregions in intact slices, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus none; Tukey's test following one-way ANOVA.

eventual neurodegeneration. Although the mechanisms by which 12-LOX downstream causes neuronal death are unclear, 12-HETE receptors have recently been shown to interact with steroid receptor coactivator-1 [8], which may in turn be recruited by several nuclear receptors, e.g. the estrogen, glucocorticoid, progesterone, thyroid hormone, and the 9-*cis*-retinoic acid receptor [15]. Importantly, all these steroid hormones are potent regulators of apoptosis in various types of cells [6]. Therefore, the 12-LOX pathway may activate intrinsic death signals via steroid receptor recruitment.

In conclusion, we have shown that pathological activation of arachidonate cascades, especially the LOX pathway, contributes to the ischemic damage in the hippocampus. Interestingly, it appears that 5- and 12-LOXs mediate the process of ischemic injury independently. Further investigations on the LOX pathway in the hippocampus may give new insights into the cellular mechanisms underlying ischemic neurodegeneration, and may therefore provide novel therapeutic targets for the development of neuroprotective agents.

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