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Suppression of hyperemia and DNA oxidation by indomethacin in cerebral ischemia

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Abstract

We investigated antioxidative activity and the effect of indomethacin, an agent that inhibits cyclooxygenase, on extracellular glutamate and cerebral blood flow in cerebral ischemia in gerbils. Pre-ischemic administration of indomethacin (5 mg/kg, i.p.) significantly rescued hippocampal CA1 neurons (9 ± 6 cells/mm in the ischemia, 87 ± 43 cells/mm in the indomethacin group, $P < 0.001$). DNA fragmentation induced by ischemia was also examined using the terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) method and indomethacin reduced TUNEL positive cells (140 ± 21 in the ischemia, 99 ± 31 in the indomethacin group, $P < 0.01$). In addition, indomethacin attenuated the increase in hippocampal blood flow during reperfusion, but not increased extracellular glutamate by ischemia. Eight-hydroxydeoxyguanosine (8-OH-dG), a highly sensitive marker of DNA oxidation, was measured 90 min following ischemia using high-pressure liquid chromatography. Indomethacin significantly decreased the level of ischemia-induced 8-OH-dG in the hippocampus ($P < 0.05$). These results suggest that indomethacin may protect neurons by attenuating oxidative stress and reperfusion injury in ischemic insult.

Keywords: Indomethacin; 8-OH-dG; Glutamate; Reperfusion injury; Cerebral blood flow; Reactive oxygen species

1. Introduction

Several researchers have reported that non-steroidal anti-inflammatory drugs (NSAIDs) like indomethacin, which are inhibitors of cyclooxygenase, have neuroprotective effects against both global (Kondo et al., 1998; Sasaki et al., 1988) and focal ischemia (Buccellati et al., 1998; Dempsey et al., 1985). Two isoforms of cyclooxygenase have been described: cyclooxygenase-1 and cyclooxygenase-2. Cyclooxygenase-1 is expressed constitutively in many organs and contributes to the synthesis of prostanoids involved in normal cellular functions (Seibert et al., 1997). Cyclooxygenase-2 is thought to be an inducible enzyme, the expression of which is up-regulated in inflammation (Seibert et al., 1997). In brain, cyclooxygenase-2 is present in selected neurons including the hippocampus (Breder et al., 1995; Yamagata et al., 1993), and its expression is up-regulated in several neurological diseases such as stroke and epilepsy (Miettinen et al., 1997; Yamagata et al., 1993). Activation of the cyclooxygenase pathway by cerebral ischemia, especially during reperfusion, accelerates conversion of arachidonic acid to prostaglandins and thromboxanes, producing reactive oxygen species such as hydroxyl radicals and superoxide radicals (Katsuki and Okuda, 1995; Miettinen et al., 1997). Reactive oxygen species are suggested to be one of the major causes of ischemic cell death (Lipton, 1999) and NSAIDs are considered to suppress reactive oxygen species production by inhibiting cyclooxygenase-2 (Miettinen et al., 1997).

Recent studies have suggested additional roles for cyclooxygenase in the brain. Prostaglandins produced in the

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cyclooxygenase pathway stimulated Ca^{2+}-dependent glutamate release in cultured astrocytes (Bezzi et al., 1998). Sanzgiri et al. (1999) also demonstrated that prostaglandin E_2-evoked Ca^{2+} elevation in astrocyte caused the release of glutamate that activates neuronal ionotropic receptors. In addition, cyclooxygenase may contribute to increased cerebral blood flow (Niwa et al., 2000; Niwa et al., 2001), although prostaglandins inhibit microcirculation due to induced edema (Siesjö, 1984). Niwa et al. (2000) demonstrated that cyclooxygenase-2 is involved in the increase in cerebral blood flow produced by synaptic activity in the somatosensory cortex. They also reported that cyclooxygenase-1 participates in maintaining resting vascular tone and in selected vasodilator responses of the cerebral microcirculation (Niwa et al., 2001). Therefore, the protective mechanism of NSAIDs on neurons in ischemia may involve extracellular glutamate and cerebral blood flow, which are considered to play important roles in ischemic brain damage (see Siesjö, 1992 for a review).

Moreover, delayed neuronal death is commonly observed after transient ischemia (Kirino, 2000) and is often reported as apoptosis associated with DNA fragmentation, although its mechanism is not fully understood (Hara et al., 1998; Nitatori et al., 1995). DNA is vulnerable against oxidative stress (Floyd et al., 1986) and ischemia-induced reactive oxygen species are responsible for apoptotic DNA fragmentation in neuronal cells (Morita-Fujimura et al., 2001). Kondo et al. (1998) demonstrated that indomethacin prevents delayed DNA fragmentation after transient ischemia, using the terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) method. They also suggested that this protective effect of indomethacin is due to inhibition of the cyclooxygenase pathway, with subsequent reductions in the level of reactive oxygen species. Thus, indomethacin has been postulated to protect neurons from delayed neuronal death after ischemia by suppression of DNA oxidation caused by reactive oxygen species, but this mechanism has been poorly examined to date.

The purpose of the present study was to examine the mechanism of the protective effects of indomethacin on neuronal death in ischemia. We investigated whether indomethacin influences extracellular glutamate and cerebral blood flow in ischemia. Furthermore, the direct prevention of oxidative DNA damage induced by ischemia was also examined.

2. Materials and methods

2.1. Animals

Male Mongolian gerbils weighing 60–70 g were obtained from Seiwa Experimental Animals (Fukuoka, Japan), and housed under a 12:12-h light–dark cycle at 24 °C with ad libitum access to food and water. Gerbils were divided into the following groups: sham control (Sham), ischemia with vehicle (0.5% methyl cellulose in saline; Ischemia), and ischemia with indomethacin (5 mg/kg; Ischemia–indomethacin). Indomethacin was supplied by Teikoku Seiyaku (Takamatsu, Japan) and administered intraperitoneally 30 min before or after ischemia (Ischemia–indomethacin-pre and Ischemia–indomethacin-post, respectively). The effects of indomethacin were evaluated at a dose of 5 mg/kg, since indomethacin prevented ischemic cell death in a dose-dependent manner until 5 mg/kg in our preliminary experiment (data not shown). All animal experiments were carried out in accordance with the guidelines described in the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan.

2.2. Ischemia

Transient global ischemia was induced for 5 min by occlusion of the bilateral common carotid arteries with micro-aneurysm clips (Sugita Clip, Mizuho, Nagoya, Japan) under sodium pentobarbital anesthesia (30 mg/kg, i.p.) as previously reported (Nakamura et al., 2001; Yamagami et al., 1998). Rectal and tympanic temperatures were maintained at around 37 °C using a feedback-controlled heating pad (CMA, Stockholm, Sweden) and overhead lamp during the operation. After restoration of blood flow, temperature was also maintained at 37 °C for 30 min. Sham-operated control animals were operated on in the same way except for occlusion of carotid arteries. Since the hippocampus is the most vulnerable part of the brain against oxidative stress (Hall et al., 1993) and hippocampal CA1 neurons selectively die in this gerbil ischemic model (Kirino, 2000), we investigated the protective mechanism of indomethacin with focusing on the hippocampus.

2.3. Histological examination

For histological examination, the animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.) 1 week after ischemia and transcardially perfused with 4% phosphate-buffered paraformaldehyde after a flush with 0.1 M phosphate-buffered saline. The brains were removed, post-fixed at 4 °C in the same fixative overnight and embedded in paraffin. Each brain was serially sectioned in the coronal plane at a thickness of 8 μm using a microtome, mounted on a silan-coated glass slide, then stained with hematoxylin and eosin (HE). A person with no prior knowledge of the groups examined neurons under a light microscope at ×400 magnification. The number of normal neurons in the bilateral CA1 subfield was counted on one histological section corresponding to roughly 1.7 mm posterior to the bregma from each animal, and is expressed as the number of cells per 1 mm of the pyramidal cell layer as neuronal density ($n=4$ in the Sham, $n=6$ in the Ischemia, $n=10$ in the Ischemia–indomethacin-pre, $n=6$ in the Ischemia–indomethacin-post group) (Nakamura et al., 2001; Yamagami et al.,

1998). To detect DNA fragmentation, gerbil brains in the Sham, the Ischemia and the Ischemia–indomethacin groups were perfusion-fixed 4 days after ischemia and 20-μm frozen sections at the dorsal hippocampus level were cut ($n=3$ for each group). TUNEL was performed using an in situ cell death detection kit according to the manufacturer's instructions (Intergen, New York, USA) on one section corresponding to roughly 1.7 mm posterior to the bregma from each animal. TUNEL-positive nuclei were visualized using 3,3-Diaminobenzidine-tetrahydrochloride and H_2O_2 with peroxidase-conjugated anti-digoxigenin. The number of TUNEL positive neurons was also counted and is expressed as the number of cells per 1 mm of the CA1 pyramidal cell layer.

2.4. Glutamate and cerebral blood flow in the hippocampus

Extracellular glutamate was measured as described (Yamagami et al., 1998). Briefly, a dialysis electrode (Microdialysis Biosensor, Sycopel International, London, UK) was filled with a perfusate of 10 mM phosphate-buffered saline (PBS, pH 7.4) containing glutamate oxidase (100 U/ml, Yamasa, Chiba, Japan). The perfusate entered the fluid inlet tube at a rate of 0.2 μl/min via a perfusion pump (IP-2, Bio Research Center, Nagoya, Japan). The dialysis electrode was connected to an ESP-800 potentiostat (Eicom, Kyoto, Japan) and a voltage clamp was controlled at +650 mV for 30 min to induce electropolymerization. After placing of the gerbils in a stereotaxic apparatus, the dialysis electrode was directed to the dorsal hippocampus (1.7 mm posterior to the bregma, 2 mm to the right of the midline and 1.5-mm depth from the dura surface). Indomethacin or vehicle was administered 30 min before ischemia, and glutamate was monitored in the Ischemia and the Ischemia–indomethacin groups ($n=5$ per each group) before, during and after ischemia. Body temperature was maintained at around 37 °C during measurement. After measuring glutamate, the brains were fixed and cut to confirm the position of the electrode. In each experiment, a regression line was obtained from the known glutamate concentration. Data from areas outside the dorsal hippocampus were excluded from the study.

Hippocampal blood flow was measured continuously throughout the experiment as previously described (Yamagami et al., 1998) with minor modifications. Briefly, blood flow in the dorsal hippocampus was monitored in the Ischemia and the Ischemia–indomethacin groups ($n=7$ in the Ischemia, $n=4$ in the Ischemia–indomethacin group) using a laser Doppler probe (Omega Flow, FLO-N1, Tokyo, Japan) with a 0.5-mm diameter. The fiberoptic probe was directed to the dorsal hippocampus as described for glutamate measurement. Hippocampal blood flow is expressed as a percentage of the average baseline because values displayed by the laser Doppler flowmeter are not absolute.

2.5. DNA oxidation in the hippocampus

Animals were sacrificed by decapitation 90 min after ischemic insult ($n=6$ in the Sham and the Ischemia groups, $n=3$ in the Ischemia–indomethacin group). Each brain was quickly removed from the skull, placed in ice-cold saline, and bisected. The hippocampus was dissected and stored at −70 °C until analysis. Oxidative DNA damage was evaluated by measurement of eight-hydroxydeoxyguanosine (8-OH-dG), which is one of the most abundant types of oxidative markers (Floyd et al., 1986; Kasai et al., 1986). Eight-OH-dG was analyzed as previously described using high-pressure liquid chromatography coupled with electro-

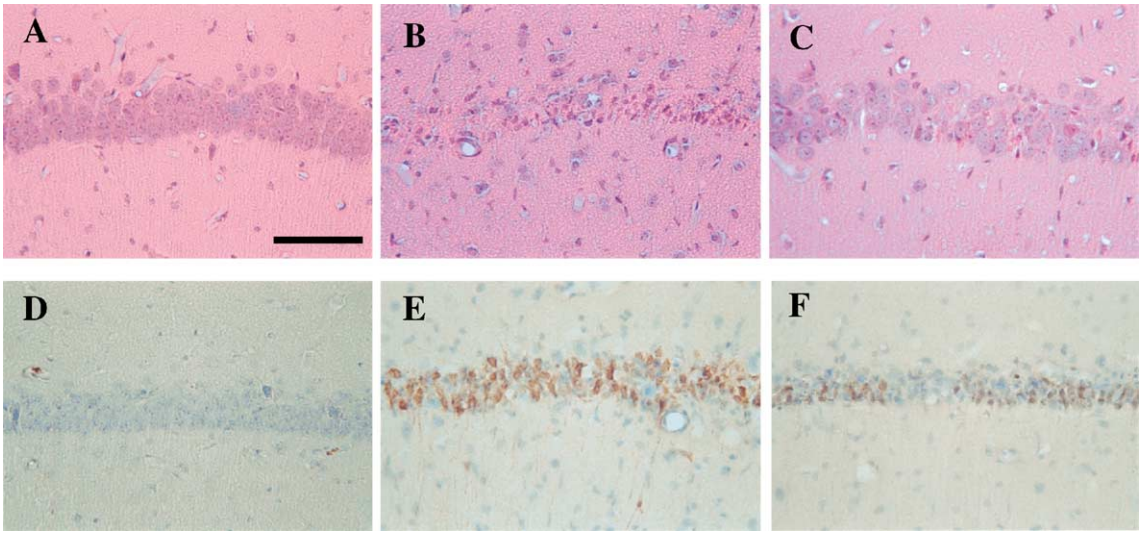


Fig. 1. Representative hematoxylin–eosin stained hippocampus (HE, upper panels) and terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL, lower panels) in the hippocampal CA1 subfield following transient ischemia. Indomethacin (5 mg/kg, i.p.) was administered 30 min before ischemia in the Ischemia–indomethacin group, and gerbil brains were fixed 1 week or 4 days after ischemia for HE (A,B,C) and TUNEL (D,E,F), respectively. A,D, Sham; B,E, Ischemia; C,F, Ischemia–indomethacin. Viable neurons are numerous in the Ischemia–indomethacin group. Scale bar = 100 μm.

chemical detection (HPLC–ECD) (Asami et al., 1996). Briefly, the samples were homogenized in lysis buffer, and the nuclear DNA in the homogenate extracted using the DNA Extractor WB Kit. The extracted nuclear DNA was digested with nuclease P1 and alkaline phospatase in a 10 mM sodium acetate solution at 37 °C for 30 min. The mixture was then treated with the ion exchange resin Muromac (Muromachi kagaku, Tokyo, Japan) and centrifuged at 15000 × *g* for 5 min. The supernatant was transferred to a filter tube (Milipore; Sampep C; 0.2 μm), centrifuged at 5000 × *g* for 5 min, and injected onto a high-performance liquid chromatography column (Beckman; Ultrasphere-ODS; 5 μm, 4.6 × 250 nm) equipped with an electrochemical detector (ESA Coulochem II: detector 1, 0.15 V; detector 2, 0.30 V). As standard samples, 20 μl each

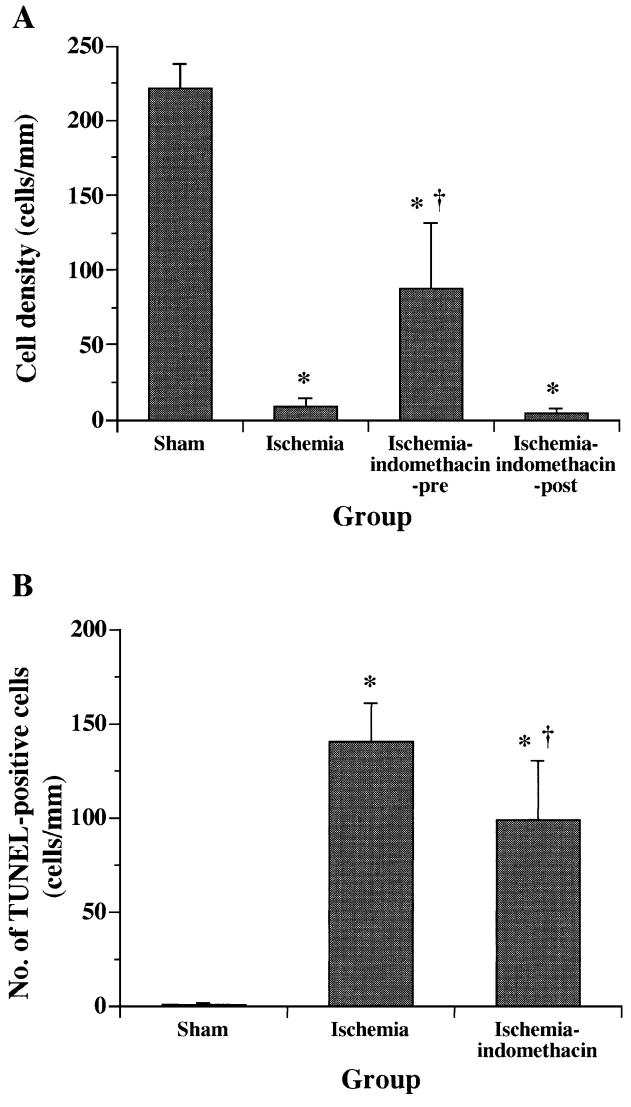


Fig. 2. (A) Bar graph of neuronal density and of the CA1 subfield 1 week after ischemia. Indomethacin (5 mg/kg, i.p.) was administered 30 min before (Ischemia–indomethacin-pre) or after (Ischemia–indomethacin-post) ischemia. Pre-ischemic injection of indomethacin significantly rescued neurons from ischemic insult but not post-ischemic injection. (B) Bar graph of TUNEL positive cells of the CA1 subfield 4 days after ischemia. Indomethacin (5 mg/kg, i.p.) was administered 30 min before ischemia. TUNEL positive cells were significantly decreased by indomethacin. Data represent the mean ± S.D. *$P<0.0001$ compared with the Sham group, †$P<0.01$ compared with the Ischemia group.

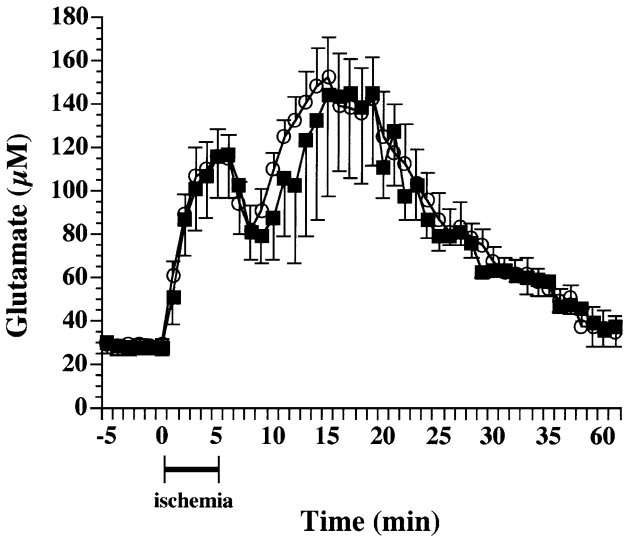


Fig. 3. Effect of indomethacin on increased extracellular glutamate by ischemia in the hippocampus. ■, Ischemia; ○, Ischemia–indomethacin. Ischemia was produced by 5-min occlusion of bilateral common carotid arteries. Indomethacin (5 mg/kg, i.p.) was administered 30 min before ischemia in the Ischemia–indomethacin group and glutamate was measured by an electro-enzymatic method of microdialysis. Ischemia was induced from 0 to 5 min. Glutamate was significantly increased by ischemia and no significant difference was detected between the Ischemia and Ischemia–indomethacin groups. Data represent the mean ± S.D.

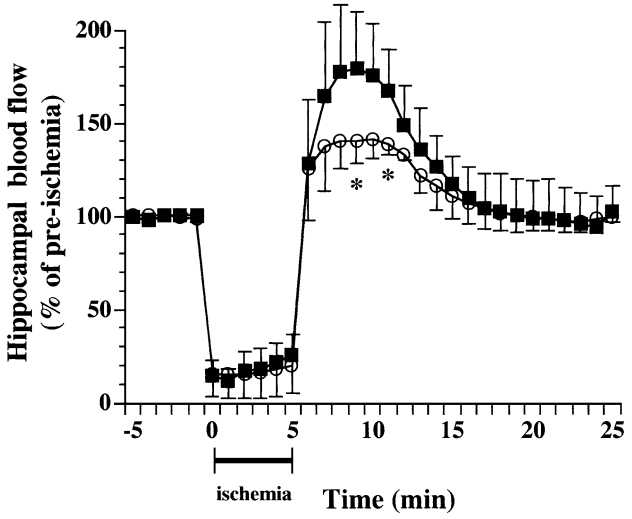
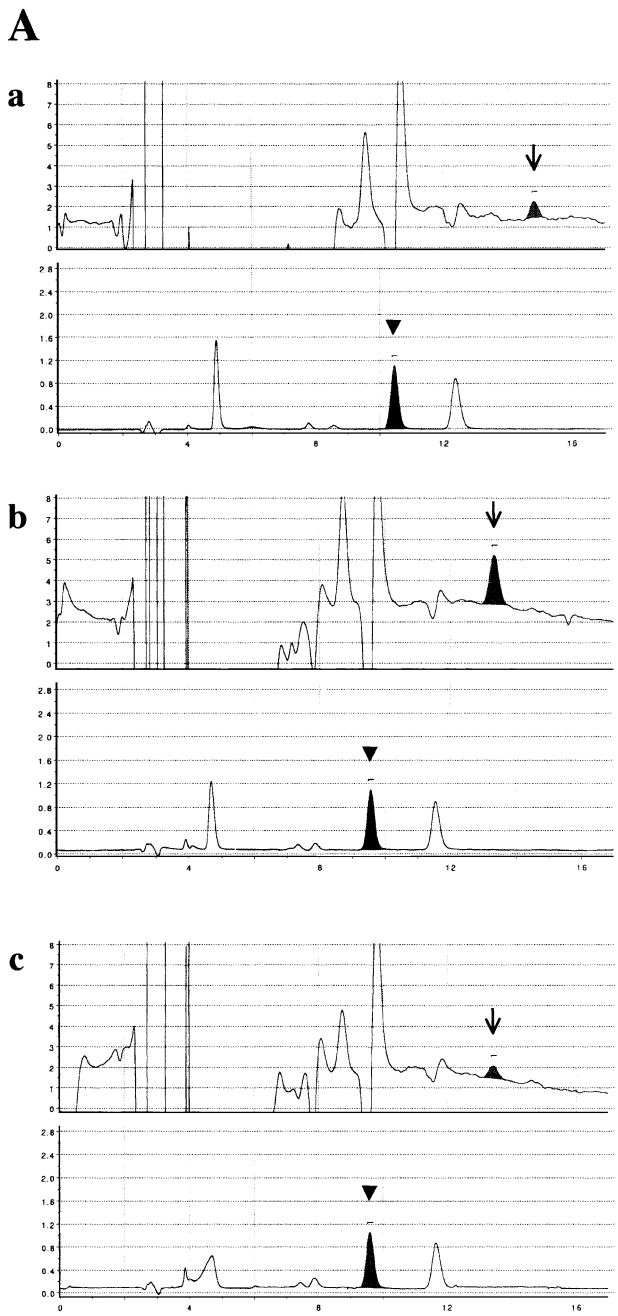


Fig. 4. Effect of indomethacin on hippocampal blood flow during and after ischemia. ■, Ischemia; ○, Ischemia–indomethacin. Ischemia was produced by 5-min occlusion of bilateral common carotid arteries. Indomethacin (5 mg/kg, i.p.) was administered 30 min before ischemia in the Ischemia–indomethacin group and hippocampal blood flow was measured by laser Doppler flowmetry. Ischemia was induced from 0 to 5 min. Hyperemia during reperfusion was suppressed by indomethacin. Data represent the mean ± S.D. *$P<0.05$ compared with the Ischemia group.



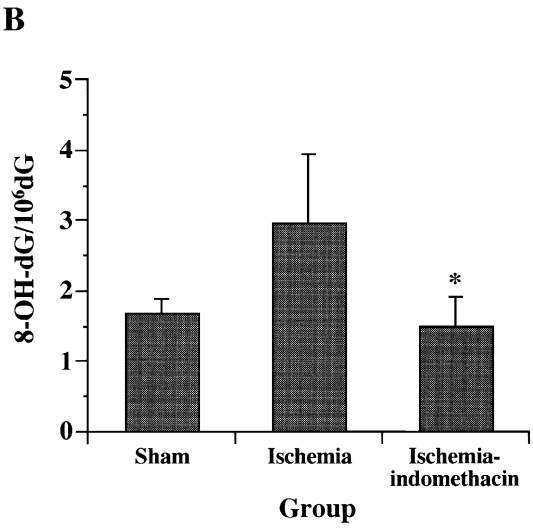


Fig. 5. 8-Hydroxydeoxyguanosine (8-OH-dG) level in gerbil hippocampus after ischemia. Indomethacin (5 mg/kg, i.p.) was administered 30 min before ischemia. Hippocampus was removed and analyzed 90 min after ischemia by high-pressure liquid chromatography coupled with electrochemical detection (HPLC–ECD). (A) Typical HPLC–ECD patterns for each group. The *x*-axis shows retention time (min) and the *y*-axis shows EC detector response (mV). (a) Sham; (b) Ischemia; (c) Ischemia–indomethacin group. The level of 8-OH-dG was calculated by the ratio of 8-OH-dG (arrows) to dG (arrowheads). (B) Bar graph of the level of 8-OH-dG. Indomethacin significantly prevented the 8-OH-dG increase in the Ischemia–indomethacin group. Data represent the mean ± S.D. *$P<0.05$ compared with the Ischemia group.

Fig. 5 (*continued*).

of deoxyguanosine (0.5 mg/ml) and 8-OH-dG (5 ng/ml) solutions were injected. The value of 8-OH-dG was calculated as the number per 10^6 deoxyguanosine (dG).

2.6. Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.). The data of neuronal density, TUNEL positive cells and DNA oxidation were analyzed by one-way analysis of variance (ANOVA) followed by Scheffé's test. The data of glutamate and cerebral blood flow were analyzed by two-way ANOVA, and Student's *t*-test was used to assess significance between the Ischemia and the Ischemia–indomethacin group. A probability level of <0.05 was considered to be statistically significant.

3. Results

3.1. Neuronal density and TUNEL positive cells (Figs. 1 and 2)

Neuronal density of the CA1 subfield 1 week after ischemia in the Sham group was 222 ± 17 cells/mm, and almost all neurons died as a result of 5-min ischemic insult (9 ± 6 cells/mm in the Ischemia group) (Figs. 1 and 2A). A significant and marked difference was seen among groups by ANOVA ($P<0.0001$) and pre-ischemic administration of indomethacin showed a significant neuroprotective effect (87 ± 43 cells/mm) compared with the Ischemia group ($P=0.0006$). However, the administration of indomethacin 30 min after ischemia did not provide any neuroprotective effect (5 ± 3 cells/mm). Numerous TUNEL positive cells in the CA1 subfield were apparent 4 days after ischemia (Figs. 1E and 2B, 140 ± 21 cells/mm) and

pre-ischemic administration of indomethacin clearly decreased the number of TUNEL positive cells (Figs. 1F and 2B, 99 ± 31 cells/mm, $P = 0.0055$ compared with the Ischemia group).

3.2. Glutamate and hippocampal blood flow (Figs. 3 and 4)

The time course of glutamate efflux in the CA1 subfield during the 5-min ischemia and reperfusion in the Ischemia and the Ischemia–indomethacin groups is shown in Fig. 3. A remarkable increase in glutamate was observed during ischemia. After recirculation, glutamate levels were increased again and reached a maximum in about 10 min. The increased levels of glutamate had almost returned to pre-ischemic levels after 60 min of reperfusion, and thereafter tended to be slightly higher than the pre-ischemic levels. Indomethacin did not affect increased extracellular glutamate by ischemia. The effect of indomethacin on hippocampal blood flow intra- and post-ischemia is illustrated in Fig. 4. During ischemia, blood flow decreased to less than 20% of the pre-ischemic level in both the Ischemia and the Ischemia–indomethacin groups. An increase in blood flow occurred starting just after reperfusion (hyperemia), and lasted for around 15 min after reperfusion. The increase in blood flow during reperfusion tended to be suppressed by the administration of indomethacin ($P = 0.039$, 0.050, and 0.033 at 9, 10, and 11 min after reperfusion, respectively).

3.3. DNA oxidation (Fig. 5)

The level of 8-OH-dG in the hippocampus 90 min after ischemia is shown in Fig. 5. A significant increase in 8-OH-dG was observed following ischemia ($P = 0.022$, compared with Sham). The levels of 8-OH-dG per 10^6 dG in the Sham, Ischemia, and Ischemia–indomethacin groups were 1.69 ± 0.20, 2.96 ± 0.99, and 1.50 ± 0.41, respectively. Indomethacin significantly prevented the ischemia-induced 8-OH-dG increase ($P = 0.031$, compared with the Ischemia group), while no significant difference was observed between the Sham and Ischemia–indomethacin groups ($P = 0.926$).

4. Discussion

Indomethacin did not affect increased extracellular glutamate by ischemia in the present experiment. Prostaglandin E_2 produced in the cyclooxygenase pathway stimulates Ca^{2+}-dependent glutamate release in astrocytes (Bezzi et al., 1998; Sanzgiri et al., 1999); however, ischemia-induced efflux of glutamate in the hippocampus in 5-min ischemia may originate mainly from neurons not astrocytes (Mitani et al., 1994). On the other hand, indomethacin attenuated hyperemia during reperfusion. Prostaglandin E_2 and prostacyclin, metabolites of the cyclooxygenase-2 reaction product prostaglandin H_2 (Brock et al., 1999), or superoxide, the other product of cyclooxygenase-2, are potent cerebrovasodilaters (Ellis et al., 1979; Wei et al., 1996). Niwa et al. (2000) demonstrated that cyclooxygenase-2 contributes to cerebral blood flow increase coupling synaptic activity, and the inhibition of cyclooxygenase-2 attenuates the increase in cerebral blood flow produced by somatosensory activation. Cyclooxygenase-1 is also involved in maintaining resting cerebral blood flow and in the increase in cerebral blood flow produced by hypercapnia and bradykinin (Niwa et al., 2001). Indomethacin inhibits both cyclooxygenase-1 and cyclooxygenase-2, and reduces the elevation in cerebral blood flow produced by endothelium-dependent vasodilators or hypercapnia (Sakabe and Siesjö, 1979; Rosenblum, 1987; Leffler et al., 1985). Bakalova et al. (2002) also reported that indomethacin reduced cerebral blood flow evoked by somatosensory stimulation, although it did not affect the baseline level of cerebral blood flow. In our previous study, a transient increase in cerebral blood flow was commonly observed during reperfusion just after ischemia (hyperemia) and continued for about 30 min in the gerbil hippocampus (Yamagami et al., 1998). A considerable amount of reactive oxygen species is produced in ischemia, especially during reperfusion, due to the increase in oxygen supply and metabolism, and this exacerbates neuronal cell damage (reperfusion injury) (Aronowski et al., 1997). Indomethacin may suppress hyperemia during reperfusion after ischemia due to a decrease in the production of reactive oxygen species and prostaglandinds, consequently preventing reperfusion injury. Compared with pre-ischemic injection, post-ischemic injection of indomethacin failed to provide a neuroprotective effect for ischemic neurons; a finding supports the notion that indomethacin rescues neurons from ischemic brain damage by influencing acute events in ischemia, such as reperfusion injury.

To gain further insight into the role of antioxidative activity in the prevention of delayed neuronal death after ischemia, we also evaluated the antioxidative effect of indomethacin. A marked increase in 8-OH-dG, which is a sensitive marker of DNA oxidation (Floyd et al., 1986; Kasai et al., 1986), was seen at 90 min following ischemia, after which hippocampal CA1 neurons died with DNA fragmentation in less than a week. These results suggest that transient ischemia produces a large amount of reactive oxygen species, which directly damages DNA, and results in apoptotic cell death (Chan et al., 1998; Peng et al., 1998). Park et al. (2000) also demonstrated that 8-OH-dG increases and peaks within 30 min after 10-min ischemia in the hippocampus of gerbils, and that the level of 8-OH-dG is maintained for at least 90 min after ischemia. DNA in neuronal cells may be quickly damaged by reactive oxygen species after ischemia and destined to die.

During ischemia and reperfusion, prostaglandins and thromboxanes are produced in the arachidonate cascade, which is one of the major sources of reactive oxygen species (Hall, 1995; Lipton, 1999). DNA damage by reactive oxygen species contributes to apoptotic neuronal injury in

ischemia (Morita-Fujimura et al., 2001). In our previous experiment, TUNEL positive cells started to appear within 3 days after ischemia, and co-localization of TUNEL staining with low affinity NGF receptor, which is closely associated with apoptosis, was observed in CA1 cells (Bagum et al., 2001). Thus, oxidative stress may in part induce apoptosis due to DNA damage in delayed neuronal death (Morita-Fujimura et al., 2001). In the present study, indomethacin significantly inhibited DNA oxidation and reduced TUNEL positive cells, consequently protecting neurons against ischemic insult. Indomethacin has been shown to abolish the ischemia-induced prostaglandin release in the rat brain due to inhibition of cyclooxygenase (Buccellati et al., 1998). Kondo et al. (1998) reported that indomethacin decreases the number of TUNEL positive CA1 neurons and delays the appearance of TUNEL positive neurons without changing the morphological features of cell death. Results from such studies, taken together with the present results, suggest that indomethacin protects neurons from delayed neuronal death by suppression of ischemia-induced reactive oxygen species production.

In conclusion, pre-ischemic administration of indomethacin (5 mg/kg) suppressed hyperemia in reperfusion after ischemia. Furthermore, indomethacin inhibited an increase in 8-OH-dG in gerbil hippocampus. Consequently, indomethacin decreased TUNEL positive cells in the CA1 subfield and rescued about 40% of CA1 cells after ischemia. These results indicate that elevation of cyclooxygenase products is responsible, at least in part, for delayed neuronal death with DNA fragmentation following transient ischemia in gerbils. The inhibition of cyclooxygenase by indomethacin may contribute to the attenuation of reactive oxygen species and hyperemia, leading to neuroproteciton from reperfusion injury.

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