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Anti-inflammatory and neuroprotective effects of auraptene, a citrus coumarin, following cerebral global ischemia in mice

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ABSTRACT

Cerebral ischemia causes delayed neuronal cell death in the hippocampus resulting in sequential cognitive impairments. Hyper-activated inflammation following ischemia is one of the etiologies for delayed neuronal cell death. In the present study, using a transient global ischemia mouse model, we showed that auraptene (AUR), a citrus coumarin, effectively inhibited microglia activation, cyclooxygenase-2 expression by astrocytes, and neuronal cell death in the hippocampus following ischemic insults. These results suggest that AUR acts as a neuroprotective agent in the ischemic brain, which may be mediated by suppression of the inflammatory response.

1. Introduction

Inflammation is a pathophysiological phenomenon that is involved in numerous diseases. One representative example is carcinogenesis. Stromal activation of inflammatory cells induces dormant tumor cells to grow and progress into malignant tumors (Murakami et al., 2005). Associated with this, nonsteroidal anti-inflammatory drugs such as aspirin have the ability to prevent the incidence of cancer in the human colon (Kune et al., 1988). A representative example of inflammation in the central nervous system is ischemia (Wang et al., 2007). After ischemic onset, inflammatory cells such as blood-derived leukocytes and microglia are activated and accumulate within brain tissue, subsequently leading to inflammatory injury. In the processes of ischemia/stroke, molecular cues generated by cerebral ischemia activate components of innate immunity, promote inflammatory signaling, and contribute to tissue damage (Iadecola and Anrather, 2012).

Auraptene (7-geranyloxycoumarin, AUR; Fig. 1), a coumarin derivative, occurs in a variety of citrus fruits. Numerous studies have indicated that AUR has valuable effects on various biological functions in peripheral tissues, such as anti-inflammation and anti-carcinogenesis. The role of AUR in inflammation-associated carcinogenesis has been well discussed Murakami et al. (2000). In contrast, there have been no reports describing the effect of AUR on cerebral ischemia. Although no data is available on whether or not AUR can penetrate into the brain, we decided to examine if AUR exerts a protective effect against ischemia-induced neuronal death.

Here, we successfully demonstrated that AUR suppressed the inflammatory response and, consequently, could suppress neuronal death.

2. Materials and methods

2.1. Animals and surgical procedures

All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation prepared by the Animal Care and Use Committee of Matsuyama University. Experiments were performed in nine-week-old male C57BL/6 strain mice obtained from Japan SLC (Hamamatsu, Japan). Procedures for bilateral common carotid artery occlusion (2-vessel occlusion: 2VO) have previously been described (Okuyama et al., 2012). Mice were anesthetized with 1.0–2.0% isoflurane and subjected to 2VO surgery. To induce global ischemia, micro-aneurysm clips (#14120, 30 g pressure; World Precision Instruments, Sarasota, FL) were applied for 12 min to occlude arteries. Sham control animals received the same surgical treatment without arterial occlusion. Body temperature during surgery was maintained with a heated blanket and mice were excluded

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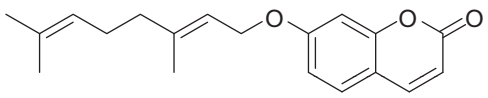

Fig. 1. Structure of auraptene (AUR).

if their brain temperatures (36.5 ± 0.2 °C) or core temperatures (37.0 ± 0.5 °C) were out of the range of criteria during ischemic surgery. After surgery, all mice were placed in a recovery cage under a heat lamp and had free access to drinking water.

2.2. AUR treatment

There were four experimental groups ($n=8$–10); sham operated group (Sham), 2VO-control group (2VO), AUR (25 mg/kg/day)-treated 2VO group (2VO+AUR25), and AUR (50 mg/kg/day)-treated 2VO group (2VO+AUR50). AUR (LKT Laboratories; St. Paul, MN) was dissolved in DMSO/Polyethylene glycol (PEG) 300 (1:1) solution. An osmotic pump (Alzet 1007D; DURECT Corporation, Cupertino, CA) was implanted in the back just after ischemic surgery. Mice were administered 0.5 μl/h of AUR or vehicle subcutaneously until the day of sacrifice.

2.3. Immunohistochemistry for optical microscopy

Eight days after surgery, mice were transcardially perfused with ice-cold PBS. Brains were removed and postfixed with 4% paraformaldehyde. Frozen brains were sagittally sectioned at 30 μm using a cryostat (CM3050S; Leica Microsystems, Heidelberger, Germany). Sections were incubated in 3% H_2O_2, followed by 5% normal goat serum blockade, and were immunostained at 4 °C overnight using a rabbit polyclonal antibody against ionized calcium binding adaptor molecule 1 (IBA1, dilution 1:1000; Wako, Osaka, Japan) to stain microglia. The secondary antibody was an EnVision-plus system-HRP labeled polymer (anti rabbit; Dako, Glostrup, Denmark). Immunoreactivity was developed and visualized by DAB substrate (SK-4100; Vector Laboratories, Burlingame, CA), and quantified using Image J software (NIH, Bethesda, MD) as previously described (Okuyama et al., 2012).

Sections on slides were immersed in 0.1% Cresyl violet solution for neuronal cell Nissl staining. After coverslipping slides, the number of viable-looking neurons with defined cell membranes and nuclei in the hippocampal pyramidal cell layer were counted in a microscopic field (CX21; Olympus, Tokyo, Japan) at $\times 1000$ magnification.

2.4. Immunohistochemistry for confocal fluorescence microscopy

Sagittal sections at 30 μm were incubated with the primary antibodies used in immunofluorescence studies, goat anti-cyclooxygenase-2 (COX-2, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-glial fibrillary acidic protein (GFAP, 1:200; Sigma-Aldrich). Secondary antibodies were Alexa Fluor 488 donkey anti-goat IgG (H+L) (1:300; Invitrogen, Carlsbad, CA) and Alexa Fluor 568 goat anti-mouse IgG (H+L) (1:300). A mounting medium was used (Vectashield; Vector Laboratories), and images in the hippocampus were captured with a confocal fluorescence microscopy system (LSM510; Zeiss, Oberkochen, Germany).

2.5. Statistical analysis

Data for individual groups are expressed as means ± S.E.M. Data were analyzed by a one-factor ANOVA followed by Dunnett's Multiple Comparison Test (Prism 5; GraphPad Software, La Jolla, CA). A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of AUR on neuronal cell loss in the hippocampus of ischemic model mice

As shown in Fig. 2, the survival rate of the Sham group was 100% (open circle) at the end of the experiment (Day 8). On the other hand, mice under 2VO surgery started to die from Day 2 to 3, and the mortality at Day 5 of the 2VO group, 2VO+AUR25 group, and 2VO+AUR50 group was 50% (open square), 89% (closed triangle), and 78% (closed diamond), respectively.

To evaluate the neuroprotective effect of AUR on ischemia-induced delayed neuronal death, histological analysis (by Nissl staining) was done in CA1, CA2, and CA3 regions of the hippocampus (Fig. 3A) of mice 8 days after 2VO surgery. Fig. 3B contains representative photomicrographs of Nissl staining in hippocampal regions for analysis. We used mice of the C57BL/6 strain in our study as a global ischemia mouse model. In most rodent global ischemia models, the CA1 region is the most fragile site in the hippocampus (Bobyn et al., 2005; Olsson et al., 2003); however, remarkable neuronal cell death in the C57BL/6 ischemia model also occurred in the CA2 region (Cho et al., 2007; Olsson et al., 2003). Fig. 3B and C shows that neuronal cell death in the CA2 region was the most severe among those in the three hippocampal regions in the present ischemic model mice (2VO group) as previously reported (Cho et al., 2007); the percentage of viable cells was lower than 50% in the CA2 region ($^{***}P < 0.001$), 76.5% in the CA1 region ($^{*}P < 0.05$) of 2VO group, and there was no significant difference in the CA3 region. However, administration of a lower dose (25 mg/kg/day) of AUR could markedly suppress neuronal loss (at CA1, $^{\#}P < 0.05$; CA2, $^{\#\#\#}P < 0.001$; and CA3, $^{\#}P < 0.05$) and the percentage of vital cells returned to the level of the Sham group. In the 2VO+AUR50 group, neuronal loss was not effectively suppressed in all three regions, contrary to our expectations.

3.2. Effect of AUR on microglial activation

Cerebral ischemia induces an inflammatory response in the injured brain (Wang et al., 2007). After ischemic onset, inflammatory cells such as blood-derived leukocytes and microglia are activated and accumulate within brain tissue, subsequently leading to inflammatory injury. Whether microglia are necessarily damaging or not following brain ischemia is unclear, but inhibition of microglial activation has been shown to significantly reduce infarct volume and improve neurological deficit scores for ischemic mice by reducing microglial activation (Suk, 2004; Fox et al., 2005; Zhang et al., 2005). We thus investigated whether or not AUR had the ability to suppress microglial activation 8 days

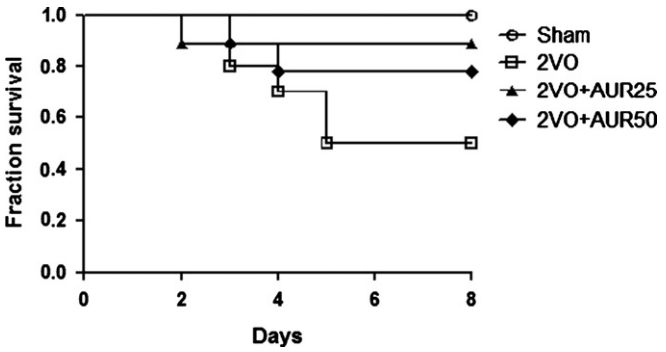

Fig. 2. Survival rate of AUR-treated mice after ischemic surgery. Administration of test samples with controlled-release osmotic pumps were started immediately after bilateral common carotid artery occlusion ischemic surgery (2VO) and lasted for 8 days.