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Neuroprotective effects of clarithromycin against neuronal damage in cerebral ischemia and in cultured neuronal cells after oxygen-glucose deprivation

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ABSTRACT

Aims: Rats subjected to transient focal ischemia and cultured neuronal cells subjected to oxygen-glucose deprivation (OGD) were treated with clarithromycin (CAM) to evaluate the effects of CAM in protecting against neuronal damage.
Main methods: Sprague-Dawley rats were subjected to middle cerebral artery occlusion (MCAO) for 90 min and then reperfused. Each animal was given an oral dose clarithromycin (CAM, 100 mg/kg) or vehicle alone just after the ischemia was commenced. The infarct volume, edema index and neurological performance were assessed after 24 and 72 h of reperfusion. The cerebral blood flow (CBF) was measured with an MRI system at 90 min after MCAO. After 24 and 72 h, oxidative stress (4-HNE, 8-OHdG) and inflammation (Iba-1, TNF-α) were assessed by immunohistochemical analyses and degenerative cells were assessed in the cortex by Fluoro-Jade C (FJC) labeling. The cultured neuronal cells were also used to examine the effects of CAM exposure on the viability of the cells after OGD.
Key findings: CBF was unchanged between the two groups. Significant reductions of the infarct volume and edema index, an improved neurological deficit score, a significant suppression of 4-HNE and 8-OHdG expression, marked reductions of Iba-1 and TNF-α expression, and a significant reduction of FJC-positive cells were also observed in the CAM-treated animals at both time points. Treatment with 10 μM and 100 μM CAM in vitro significantly reduced cell death after OGD.
Significance: CAM appears to provide antioxidant and anti-inflammatory effects and protect against neuronal damage after cerebral ischemia and OGD.

1. Introduction

Ischemic stroke often kills or permanently incapacitates its victims. The thrombolytic agent recombinant tissue plasminogen activator (rtPA) is sometimes given to restore blood flow to the ischemic tissue after acute ischemic stroke. Yet strict eligibility criteria have limited the patients who receive the agent to only 2–5% [11]. The remainder of patients who suffer acute cerebral ischemia therefore require neuroprotective agents to salvage the ischemic penumbra.

Investigators have developed neuroprotective compounds such as glutamate antagonists, calcium channel blockers, anti-inflammatories, antioxidant, GABA agonist, and growth factors over the last few decades. Regretfully, few of the neuroprotective agents used to treat ischemic insults bring about desired effects after acute stroke [25]. The free radical scavenger NXY-059, for example, showed no efficacy for acute stroke in SAINTII (Stroke-Acute Ischemic NXY Treatment II) [32].

Erythromycin (EM), clarithromycin (CAM, 6-0-methylerythromycin) and other macrolide antibiotics have an extended history of clinical success and generally elicit few side effects. Pretreatment with erythromycin mediates the transcriptional response to ischemia by bolstering tolerance against hypoxia in vitro and cerebral ischemia in vivo [6,14,20]. Macrolide antibiotics have also been found to mediate inflammatory mechanisms in vitro in both animal and clinical studies [3,21,22,26].

In our own studies with a transient focal ischemic model and oxygen-glucose deprivation (OGD) in cultured neuronal cells, EM treatment brought about significant neuroprotective effects against cerebral ischemia by reducing inflammation, oxidant stress, and OGD [17].

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Pretreatment with various macrolide antibiotics confers neuroprotective effects against cerebral ischemia in rat [16]. The anti-ischemic effects of CAM are among the strongest elicited by the numerous antibiotics tested [16].

CAM is an EM (6-*O*-methylerythromycin) derivative with more potent antibacterial effects and higher acid-stability than its precursor [30].

In this study we examined the neuroprotective effects of CAM against neuronal damage in a rat model of transient focal ischemia by post-ischemic treatment and in cultured neuronal cells subjected to OGD.

2. Materials and methods

All experimental protocols were approved by our Institutional Committee on Animal Research (Graduate School of Medicine, Nippon Medical School) and carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.1. Effect of CAM in transient focal ischemia

2.1.1. Experimental protocol and drug administration

Male Sprague-Dawley rats (8-week-old, 250–300 g) purchased from Sankyo Labo Service (Tokyo, Japan) were used in the present study. The animals were randomly divided into CAM-treated (n = 20) and vehicle-treated groups (n = 20) and then subdivided into 24-hour reperfusion (n = 10) and 72-hour reperfusion groups (n = 10). Each group was given 100 mg/kg of CAM or saline by gavage just after the cerebral ischemia was induced. Clarithromycin (CAM, LKT Laboratories, Inc., MN, USA) was dissolved to a 30 mg/ml concentration with physiological saline. Blood samples were collected from the tail artery before and during MCAO (45 and 90 min after MCAO was commenced) for measurement of pH, PO_2, and PCO_2. The infarct volume and edema index were measured and neurological symptoms were assessed in the same animals after 24 and 72 h of reperfusion. Oxidative stress (4-HNE, 8-OHdG) and inflammatory markers (Iba-1, TNF-α) were evaluated by immunohistochemical analyses. The number of degenerative cells labeled by FJC staining in the cortex was counted in other animal groups after 24 and 72 h of reperfusion.

2.1.2. Transient focal cerebral ischemia

The animals were fasted overnight with free access to tap water before the surgery. The anesthesia was initially induced with 5% halothane and then maintained with 1% halothane in a mixture of 70% N_2O and 30% O_2 under spontaneous breathing. A polyethylene catheter (PE-50) was inserted into the tail artery to monitor the mean arterial blood pressure and sample blood. Blood gases and blood glucose levels were measured just before and during ischemia. The animals were subjected to focal cerebral ischemia for 90 min followed by 24 or 72 h of reperfusion using a modified intraluminal suture technique described in detail previously [2,29]. Briefly, the left common, internal, and external carotid arteries were carefully exposed through a midline cervical incision, and the common and external carotid arteries were doubly ligated using 4-0 silk sutures. The left middle cerebral artery (MCA) was occluded by inserting a silicone-rubber-coated 4-0 nylon thread through the left internal carotid artery for 90 min. The vessel was reperfused by withdrawing the thread.

2.1.3. Evaluation of cerebral blood flow

The MCAO and reduced cerebral blood flow (CBF) were confirmed by magnetic resonance imaging (MRI) experiments using a 7T/18-cm horizontal magnet (Magnex Scientific, Abingdon, UK) with a Varian Unity-INOVA-300 (Varian Inc., Palo Alto, CA, USA) system equipped with an actively shielded gradient and 6-cm-internal-diameter quadrature birdcage coil. Cerebral perfusion images at the level of the bregma were obtained at 90 min after MCAO by a continuous arterial spin labeling (CASL) method [36] modified with a centrally encoded variable-tip-angle gradient echo (VTE-GE) technique [13]. The conditions were described in detail previously [16,17]. The images were manipulated using MR-Vision software (MR Vision Co., Menlo Park, CA, USA) on a Sun Blade 1000 workstation (Sun Microsystems, Milpitas, CA, USA).

2.1.4. Measurement of infarct volume and edema index

The animals were anesthetized and decapitated after reperfusion for 24 or 72 h. Two-mm-thick coronal sections were cut and stained by immersion in 2% TTC (2,3,5-triphenyltetrazolium chloride) at 37 °C and fixed in 10% buffered formalin overnight. The infarct volume was determined by measuring the area of the ischemic lesion in each section and multiplying the area by the interval thickness using an image analysis system (National Institute of Health image software) [2,29].

The edema index (%) after 24 and 72 h of reperfusion was calculated as follows: (ipsilateral hemispheric volume − contralateral hemispheric volume) × 100/contralateral hemispheric volume [1,17].

2.1.5. Examination of neurological symptoms

After 24 and 72 h of reperfusion, neurological symptoms in several experimental animals were assessed by an observer blinded to the study protocol using a scoring system based on the detection of hemiplegia and abnormal posture [37]. The flexor response of the right hindlimb was scored as follows while extending the limb with round-tipped forceps: 0, normal; 1, slight deficit; 2, moderate deficit; 3, severe deficit. To assess posture, forelimb flexion and body twisting were scored while suspending the animal by the tail: 0, normal; 1, slight twisting; 2, marked twisting; or 3, marked twisting and forelimb flexion.

2.1.6. Immunohistochemistry and Fluoro-Jade C staining

Lipid peroxidation, DNA oxidative damage, microglial activation, and proinflammatory cytokine induction were immunohistochemically assessed in the boundary area around the ischemia after 24 and 72 h of reperfusion.

The rats in the 24-hour and 72-hour reperfusion groups (n = 5, each) were transcardially perfused with heparinized saline, followed by 4% paraformaldehyde. The brains were removed, postfixed in the same solution overnight at 4 °C, cryoprotected, and rapidly frozen. The frozen coronal sections were sliced (20 μm), collected on glass slides, and incubated in 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxide activity. After washing in Tris-buffered saline, nonspecific binding was blocked with 5% normal bovine serum in Tris-buffered saline. The sections were incubated overnight at 4 °C with the following antibodies: a mouse monoclonal antibody against 4-hydroxy-2-nonenal (4-HNE) (4-HNE; 50:1; Japan Institute for the Control of Aging [JIFCA], Shizuoka, Japan) to assess lipid peroxidation; a mouse monoclonal antibody against 8-hydroxy-deoxyguanosine (8-OHdG) (8-OHdG; 50:1; JIFCA) to detect oxidative DNA damage; a rabbit polyclonal antibody against ionized calcium-binding adapter molecule (Iba-1) (Iba-1; 500:1; Wako Pure Chemical Industries, Osaka, Japan) as a marker of active microglia; and a goat polyclonal antibody against tumor necrosis factor-alpha (TNF-α) (TNF-α; 10:1; Invitrogen, Carlsbad, CA, USA). The sections were then incubated with secondary antibodies, followed by avidin-biotin-peroxide complex (Vector Laboratories, Burlingame, CA, USA) for 30 min. To analyze the effect of the treatment on neuronal degeneration, Fluoro-Jade C (FJC) staining was conducted using a commercially available kit according to the manufacturer's protocols (Fluoro-Jade C Ready-to-Dilute Staining Kit for Identifying Degenerating Neurons; Biosensis, Temecula, CA, USA) [31]. Positively stained cells in a 0.56 mm^2 region of the ischemic boundary zone next to the ischemic core were counted in four randomly selected microscopic fields by an investigator blinded to the experimental groups (Eclipse E600W; Nikon, Tokyo, Japan).