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ACTIVATION OF TYROSINE HYDROXYLASE PREVENTS PNEUMONIA IN A RAT CHRONIC CEREBRAL HYPOPERFUSION MODEL

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Abstract—Pneumonia is a common complication with the highest attributable proportion of deaths in patients with stroke. Cilostazol is a potent type III phosphodiesterase inhibitor, approved as an anti-platelet aggregation agent. The present study was designed to determine the protective mechanism of cilostazol against post-stroke pneumonia using a rat chronic cerebral hypoperfusion model. Rats were subjected to bilateral common carotid artery ligation (LBCCA) and divided randomly into the vehicle group (n=72) and cilostazol group (n=72). Rats of each group were sacrificed at baseline and at days 14, 28 and 42 after LBCCA. Cilostazol significantly improved the swallowing reflex by shortening the latency to elicited swallowing and increasing the numbers of swallows (P<0.05) at 14 days of hypoperfusion. It also decreased the numbers of bacterial colonies grown in cultures from homogenized lungs. Cilostazol markedly upregulated cyclic AMP responsive element binding protein (CREB) phosphorylation, increased tyrosine hydroxylase (TH) expression in the substantial nigra, and maintained dopamine (84.7±2.3 vs. 79.2±4.1% control; P=0.0512) and substance P levels (86.6±7.9 vs. 73.9±6.5% control; P<0.05) in the striatum, compared with the vehicle group. Our results indicate that cilostazol improves the swallowing reflex by enhancing the expression of TH through the CREB phosphorylation signaling pathway, and suggest that cilostazol could be useful in preventing pneumonia in the chronic stage of stroke.
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Pneumonia occurs in about one-third of patients with stroke (Walker et al., 1981). It is the complication with the highest attributable proportion of deaths in the entire stroke population. Not only does it account for 31.2% to 35% of all in-hospital deaths after an acute stroke (Heuschmann et al., 2004; Hinchey et al., 2005), it is also the first major cause of death in patients with ischemic stroke and transient ischemic attack after hospital discharge (Kimura et al., 2005). Compared with post-stroke survivors whose recovery is not complicated by pneumonia, there is a threefold increased risk of dying when diagnosed with pneumonia after an acute stroke. Therefore, exploration of effective therapies is clinically desirable to reduce or prevent pneumonia-related mortality. Given the knowledge that dysphagia and aspiration as well as attenuated cough reflex caused by CNS disorders are the most important factors contributing to the risk of post-stroke pneumonia (Martino et al., 2005; Horner et al., 1988), post-stroke pneumonia could be thought as a disease of the brain, and should be a potentially preventable complication through improvement of swallowing reflex and coughing reflex.

Dopamine and substance P play critical roles in pneumonia based on their effects on the regulatory mechanisms that control the swallowing and coughing reflexes (Ujiie et al., 1993; Jin et al., 1994). Delayed triggering of the swallowing reflex is reported in patients with infarction in the basal ganglia, who also exhibit a parallel impairment of dopamine metabolism in the basal ganglia (Pinto et al., 1994; Itoh et al., 1994). Administration of levodopa was reported to improve the swallowing reflex in such patients who also had history of aspiration pneumonia (Kobayashi et al., 1996). On the other hand, patients with aspiration pneumonia were found to have low levels of substance P in the sputum (Nakagawa et al., 1995), and administration of inhibitors of angiotensin converting enzyme (ACE), which degrades substance P, significantly reduced the risk of pneumonia among hypertensive patients with stroke, and such effect was associated with elevation of serum substance P levels (Arai et al., 2003).

Recently, in a multi-center, randomized, placebo-controlled double-blinded clinical study, Shinohara and colleagues (Shinohara et al., 2006) reported that cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2 (1H)-quinolinone), a potent type III phosphodiesterase (PDE III) inhibitor approved as an anti-platelet aggregation agent, prevented pneumonia in patients with chronic-stage ischemic stroke, and suggested that the effect was mediated through improvement of the swallowing reflex. Furthermore, the neuroprotective mechanism of cilostazol also includes activation of phosphorylation of cyclic AMP responsive element binding protein (CREB) (Lee et al., 2005; Watanabe et al., 2006). However, to our knowledge, there is little information on the effect of cilostazol on the neurotransmitter system.

In the present study, a rat chronic cerebral hypoperfusion model was used to simulate the pathological condition of chronic cerebral ischemia. First, we established the model by permanent occlusion of both common carotid arteries, then

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Abbreviations: CBF, cerebral blood flow; CREB, cyclic AMP responsive element binding protein; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LBCCA, bilateral common carotid artery ligation; pCREB, phosphorylated cyclic AMP responsive element binding protein; PDE III, type III phosphodiesterase; SN, substantia nigra; TH, tyrosine hydroxylase.

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assessed the temporal changes in experimentally-elicited swallowing reflex and positive culture of bacteria from the lung, and evaluated the effect of cilostazol on tyrosine hydroxylase (TH) expression, dopamine synthesis and endogenous substance P content. We tested the hypothesis that cilostazol is useful in protecting against pneumonia in patients with chronic cerebral ischemia.

EXPERIMENTAL PROCEDURES

All animals used in the present study were acquired and cared for in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal protocols used in the present study were approved by the Animal Care Committee of Juntendo University, and efforts were made to minimize the number of animals used and their suffering. Adult male Wistar rats (8-week-old) weighing 250–270 g were obtained from Charles River Institute (Kanagawa, Japan) and maintained on a 12-h light/dark cycle with free access to food and water.

Experimental protocol

Hypoperfusion was induced by ligating both common carotid arteries, as described previously (Watanabe et al., 2006). Briefly, animals were initially anesthetized with 4% isoflurane and maintained with 1.5% isoflurane mixed in 70% N_2O and 30% O_2. Through a midline incision, the bilateral common carotid arteries were carefully separated from the cervical sympathetic and vagal nerves, and ligatured permanently. During this procedure, the body temperature was maintained at 37.0±0.5 °C. Cerebral blood flow (CBF) was measured in a window over the left temporal region using laser Doppler flowmetry (Laser Tissue Blood Flow Meter FLO-C1; Omega Wave, Inc., Tokyo, Japan). After ligature of both common carotid arteries (LBCCA), the rats were divided at random into the following three groups: 1 the vehicle group: rats of this group (n=72) were provided normal animal food; 2 the cilostazol group: rats of this group (n=72) were provided laboratory food mixed with 0.1% cilostazol (Otsuka Pharmaceutical, Tokyo, Japan) at 50 mg/kg/day. The selected dose was based on the pharmacokinetic profile of cilostazol supplied by the manufacturer, our own preliminary experiments, and relevance to long-term administration clinically; 3 the control sham-operated vehicle- and cilostazol-treated groups. These rats (n=36 for each subgroup) underwent the same aforementioned protocol except for LBCCA. Rats of each group were sacrificed at baseline (before LBCCA) or 14, 28 and 42 days after LBCCA, and the brain was dissected out immediately and sectioned for subsequent analyses (*see below*).

Swallowing reflex

At the day before LBCCA, 14, 28 and 42 days after LBCCA, swallowing reflex was elicited experimentally by intra-pharyngeal injection of distilled water and evaluated in vehicle-treated and cilostazol-treated groups (Kajii et al., 2002). Rats (total 64) were anesthetized with i.p. injection of 40 mg/kg pentobarbital sodium (the dosage maintained the anesthesia at stage 3 with preservation of the blink and swallowing reflexes (Fosburgh, 1997)), and then fixed in the supine position on a heated pad to maintain body temperature at 37 °C. A catheter was inserted through the mouth, with its tip placed into the pharynx. About 50 μl of distilled water was applied to the pharyngolaryngeal region three times at intervals of 3 min. A midline incision was made in the ventral surface of the neck. Swallowing movement was identified by visual inspection of the laryngeal movement. The duration from the start of instillation to the first swallow was recorded as latency to swallowing, and the number of swallows elicited by the maneuver in 1 min was counted.

Bacteria culture

At 14, 28 and 42 days after BLCCA, rats (total 54) of each group were anesthetized with i.p. injection of 100 mg/kg pentobarbital and washed with ethanol under sterile conditions. The lungs were removed after thoracotomy and homogenized. For determination of colony-forming units, tissue homogenate was diluted and plated onto 5% sheep blood agar plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After incubation at 37 °C for 72 h, the numbers of bacterial colonies were counted (Meisel et al., 2004).

Immunohistochemical staining

Immunohistochemistry was performed on 20 μm-thick free-floating coronal sections of the substantia nigra (SN) and striatum. After incubation in 3% H_2O_2 followed by 10% Block Ace in 0.1% PBS(−), the sections (total 64 rats) were immunostained overnight at 4 °C with a mouse monoclonal antibody against TH (2000:1, EMD Chemicals, Inc., San Diego, CA, USA), and a goat polyclonal antibody against substance P (100:1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The sections were then treated with secondary antibodies (300:1, Vector Laboratories, Burlingame, CA, USA). Immunoreactivity was visualized subsequently by the avidin–biotin complex method (Vector Laboratories). The bound complex was visualized with 3,3′-diaminobenzidine and urea–H_2O_2 tablets from Sigma (St. Louis, MO, USA) dissolved in water. As a negative control, sections were immunohistochemically stained without the primary antibodies. The images were analyzed with an Olympus CX40 microscope (Tokyo) connected to a digital camera (DXM1200, Nikon, Tokyo) and analyzed with the ACT-1 image system (version 2.20, Nikon) (Komine-Kobayashi et al., 2006).

Immunofluorescence

Double immunofluorescence staining and confocal laser scanning microscopy (Axiovert 100M, Carl Zeiss, Jena, Germany) were performed (in total of 64 rats) to determine co-localization of phosphorylated cyclic AMP responsive element binding protein (pCREB) and TH, as well as co-localization of dopamine Da1 receptor in substance P-positive cells. The primary antibodies used were rabbit polyclonal antibody against pCREB (1:100, Upstate Biotechnology, Lake Placid, NY, USA) with mouse monoclonal antibody against TH (1:2000, EMD Bioscience, Inc., San Diego, CA, USA), or rabbit polyclonal antibody against dopamine Da1 receptor (1:200, Chemicon International, Inc., Temecula, CA, USA) with goat polyclonal antibody against substance P (1:100, Santa Cruz Biotechnology). Alexa Fluor 594–conjugated IgG (1:300, Molecular Probes, Inc., Eugene, OR, USA) was used to demonstrate pCREB or dopamine D1a receptor in red fluorescence. On the other hand, fluorescein isothiocyanate (FITC)–conjugated IgG (1:300, Jackson Immunoresearch Laboratories, West Grove, PA, USA) was used to detect TH or substance P in green fluorescence (Komine-Kobayashi et al., 2006).

Western blotting

Proteins (from total of 64 rats) were extracted from the striatum or SN (n=5 each group), and measured as described previously (Komine-Kobayashi et al., 2006). Briefly, aliquots containing 50 μg of protein were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were transferred onto polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA) and probed for TH by incubation with the primary antibody (3000:1, EMD Bioscience), followed by incubation with horseradish peroxidase–conjugated secondary antibody (10,000:1, Santa Cruz Biotechnology). Blots were visualized using the ECL system (Amersham Biosciences, Arlington Heights, IL,

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