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Caffeic acid phenethyl ester reduces spinal cord injury-evoked locomotor dysfunction

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ABSTRACT

Caffeic acid phenethyl ester (CAPE) is a component of propolis, which is a substance taken from the hives of honeybees, and is known to exhibit an anti-inflammatory activity. Such activity has been thought to be partly based on its potential and specific inhibitory activities toward nuclear factor-κB, a transcription factor. Therefore, in the present study, we evaluated the effect of CAPE on functional locomotor recovery after spinal cord injury (SCI) caused by hemi-transection, because inflammatory responses are a major cause of the secondary injury observed following SCI and play a pivotal role in regulating the pathogenesis of acute and chronic SCI. When CAPE was *i.p.*-administered at a dosage of 10 μmol/kg, it enhanced the recovery of locomotor function and reduced the lesion size while suppressing the expression of the mRNAs for a pro-inflammatory cytokine interleukin-1β and the inflammatory enzymes, inducible nitric oxide synthase and cyclooxygenase-2. These results suggest CAPE to be a promising therapeutic tool for reducing the secondary neuronal damage following primary physical injury to the spinal cord.

Traumatic spinal cord injury (SCI) causes a permanent and irreversible loss of motor function, which dramatically decreases the quality of life for patients with SCI. Nevertheless, no effective treatments have been established to date; therefore, therapeutic interventions for SCI are urgently needed.

Primary physical damage to the spinal cord is followed by delayed secondary neural degeneration that involves various cellular and biochemical events that lead to progressive tissue damage and cell death (6). In particular, inflammatory responses are a major component of this secondary injury and play a pivotal role in regulating the pathogenesis of acute and chronic SCI (4, 7). The nuclear factor (NF)-κB transcription factor is a key mediator in inflammatory responses and has been implicated in the progression of this secondary injury. NF-κB can be activated by a variety of stimuli, including inflammatory cytokines such as interleukin (IL)-1, which is released from spinal cord-resident cells and/or infiltrating monocytes (4, 25). In response to SCI, NF-κB induces the transcriptional activation of pro-inflammatory genes including inducible nitric oxide (NO) synthase (iNOS), which produces NO, and cyclooxygenase-2 (COX-2) (23). Both iNOS and COX-2 cause cellular damage by producing nitrogen radical species and oxygen radicals, respectively, which are potent mediators of cell death in the SCI (28, 31). Therefore, they are closely involved in the formation of the secondary injury.

Caffeic acid phenethyl ester (CAPE) is a component of propolis, which is a substance taken from the hives of honeybees; and it exhibits many biological and pharmacological activities including anti-oxidative, anti-inflammatory, anti-tumor, and anti-viral ones (2). These activities may be partly based on its potential and specific inhibitory actions toward NF-κB activation (20), lipid peroxidation (29) or lipoxygenase activity (16). It has been recently reported that the infarct volume and degree of neurological

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deficit induced by middle cerebral artery occlusion become smaller in CAPE-administered animals due to CAPE-mediated inhibition of the oxidative stress and inflammation (1, 14). These observations prompted us to examine the possible ameliorative effect of CAPE on traumatic SCI, because no reports on it have yet emerged.

Therefore, in the present study we evaluated the effect of CAPE on functional locomotor recovery after SCI and the anti-inflammatory effect of CAPE in the injury site. Conspicuously, CAPE enhanced the recovery of locomotor function and reduced the lesion size in the injured spinal cord while suppressing the expression of the mRNAs for a pro-inflammatory cytokine IL-1β and the inflammatory enzymes iNOS and COX-2. These results suggest CAPE to be a promising therapeutic tool for reducing the secondary neuronal damage following primary physical injury to the spinal cord.

MATERIALS AND METHODS

Animal surgery. Female Wistar rats (7 weeks of age, weighing 120–140 g; Japan SLC, Hamamatsu, Japan) were used in this study, and they were handled in accordance with the Guidelines of Experimental Animal Care issued by the Office of the Prime Minister of Japan. The surgery was performed according to the method reported previously (11, 13, 15). Briefly, the rats were anesthetized with sodium pentobarbital (40 mg/kg body weight), after which the spinal cord (left side) was hemi-transected or not (sham control) by use of microsurgical scissors after laminectomy at the level of the 10th thoracic vertebra. After arrest of hemorrhage, the muscle and skin were sutured. The rats were then placed in normal cages and given free access to food and water.

Administration of CAPE. CAPE was a generous gift from Api Co., Ltd., Gifu, Japan. It was diluted with sterile phosphate-buffered saline (PBS) containing 1% ethanol and intraperitoneally injected into the animals (2 or 10 μmol/kg) immediately after the injury and then once every 24 h for 28 days. The vehicle-treated animals received PBS containing ethanol whose final concentration was equal to that of the CAPE solution.

Assessment of locomotor function. Locomotor function of the left hindlimb was assessed by the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale in open-field as described earlier (3). Evaluation was performed 1 day after injury and subsequently once a week by observers blinded to the experimental treatments and was continued up to 28 days after the injury.

Tissue preparation for immunohistochemical analysis. The animals were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital and then cardio-perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The spinal cord tissues including the lesion site were dissected out and post-fixed in the same fixative overnight at 4°C. The tissues were then soaked in cold PBS containing 20% sucrose for 15 h, and frozen in embedding compound (Sakura Finetek, Tokyo, Japan). Frozen sections (25-μm thickness) were prepared with a cryostat (model CM 1800; Leica, Nussloch, Germany) and then put on adhesive-coated slides (Matsunami, Osaka, Japan).

Immunohistochemical analysis. Immunohistochemical analysis was performed by the method of Kasai *et al*. (12). Cryostat sections were fixed in 4% PFA solution for 10 min at room temperature and rinsed for 30 min at 37°C in 0.1 M Tris-HCl buffer (pH 7.4) containing 0.3% (v/v) Triton X-100. After having been washed with PBS, the sections were blocked for 30 min at room temperature in PBS containing 2% Block Ace and then reacted with the diluted primary antibody specific for glial fibrillary acidic protein (GFAP, 1 : 1000; Dakocytomation, Glostrup, Denmark) for appropriate times at 4°C. After another wash with PBS, the sections were incubated with a fluorescence-conjugated secondary antibody for 3 h at room temperature (Alexa 546-conjugated goat anti-rabbit IgG, 1 : 1000; purchased from Invitrogen, Carlsbad, CA). Finally, the slides were washed with PBS and coverslipped with PermaFluor Aqueous Mounting Medium (Thermoshandon, Waltham, CA). The images were observed with a confocal laser microscope (LSM 510; Zeiss).

Image analysis. For GFAP staining, horizontal sections were prepared. The lesion size of the injury site was measured by tracing the lesion area within the borders of the lesions as defined by GFAP labeling through 5 representative horizontal sections made 0.1 and 0.2 mm ventral to the midline, at the midline, and 0.1 and 0.2 mm dorsal to the midline in each rat. The mean value of the lesion size in the 5 sections was considered as the value for each animal.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of expression of inflammation-inducible genes. For the RT-PCR experiment, the spinal cords were dissected out immediately after the hemitransection or 1, 3, 7 or 14 days later; and the segments just rostral or caudal to the injury site (2-mm length each) were collected. Total RNA was isolated from the collected spinal cord segments by using TRIZOL Reagent (Invitrogen) according to the manufacturer's instruction. RT-PCR was performed as described previously (12) to assess the mRNA level of IL-1β, iNOS and COX-2. The sequences of primers used were as follow: forward primer, 5'-GCCGTCTTCCCCTCCATCGT-3', and reverse primer, 5'-CCCGTCTCCGGAGTCCATCA-3', for β-actin; IL-1β forward primer, 5'-CTTGGGCTGTCCAGATGAGAGCAT-3', and reverse primer, 5'-GAAGACACGGGTTCCATGGTGAAG-3' for IL-1β; forward primer, 5'-GCTGGAGGTGACCATGGAGCAT-3', and reverse primer, 5'-CCTGGCTAGCGCTTCCGACTTT-3', for iNOS; and forward primer, 5'-TGAAGCCCACCCCAAACACAGT-3', and reverse primer, 5'-TTGGGGATCCGGGATGAACTCT-3', for COX-2. The amplification was carried out with a thermal cycler at 94°C for 5 min, followed by 24 (for β-actin), 35 (for iNOS or COX-2) or 38 (for IL-1β) cycles consisting of 94°C for 30 s, 63°C for 1 min, and 72°C for 45 or 60 s. β-Actin was used as the internal control. After amplification, the PCR products were subjected to 2% agarose gel electrophoresis and then visualized by ethidium bromide staining. The images were captured with FLA-5100 (FUJIFILM, Tokyo, Japan). The optical density of each band was quantified by utilizing image-analysis software (NIH ImageJ).

Statistical analysis. All numerical data were presented as group mean values with standard deviation (SD). Statistical analyses of gene expressions in the injury site and locomotor function assessed by using the BBB scale were performed by two-way analysis of variance (ANOVA) followed by the Bonferroni post-test. A statistically significant difference in the size of the lesion site was determined by performing one-way ANOVA with Tukey's *post hoc* test.

RESULTS

CAPE improves locomotor function after SCI

We evaluated the effect of 2 doses of CAPE on the locomotor activity of the left hindlimb for over 28 days after the SCI by using the BBB locomotor rating scale. This scale was originally developed for assessment of bilaterally impaired hindlimb function following contusive SCI, but it has been also used for lateral hemi-transection SCI (11, 13, 15). Hemi-transection of the spinal cord resulted in complete paralysis of the hindlimb ipsilateral to the transection side (left) for several days in all groups. Although the locomotor function of the rats of all groups was improved by 28 days after the surgery, the rats treated with CAPE at 10 μmol/kg maintained significantly better hindlimb locomotor performance than the vehicle-treated rats from 14–28 days (Fig. 1). The CAPE-treated (10 μmol/kg) rats attained over 11 points of BBB score, which reflects consistent weight-supported hindlimb movements; whereas the vehicle-treated rats demonstrated only limited hindlimb weight bearing (nearly 9 points of BBB score). No improvement in locomotor activity was observed in the rats treated with the low dose of CAPE (2 μmol/kg) throughout the experimental period (Fig. 1).

CAPE reduces the lesion size in the injured spinal cord

To assess histologically the anti-inflammatory effect of CAPE on the injured spinal cord, we measured the lesion size of the injured spinal cord by outlining the lesion area defined as GFAP labeling on experimental day 28. Compared with the vehicle-treated rats, the rats that had received daily administration

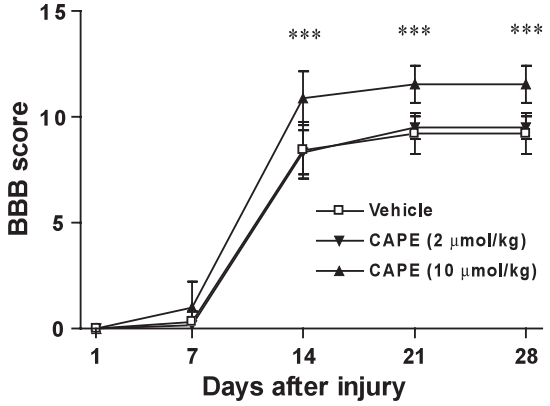

Fig. 1 CAPE improves locomotor function of the animals with SCI. The changes in locomotor function of the vehicle- (n = 9) or CAPE-treated animals (2 μmol/kg; n = 6, 10 μmol/kg; n = 9) were assessed for 28 days after SCI. Only the group treated with the high-dose CAPE (10 μmol/kg) showed a significant behavioral improvement compared with that for the vehicle-treated group. The values of BBB score are expressed as the mean ± SD. Significant differences from the vehicle-treated group were determined by using repeated measures of two-way ANOVA followed by Bonferroni post-test. Significance: $^{***}P < 0.001$.

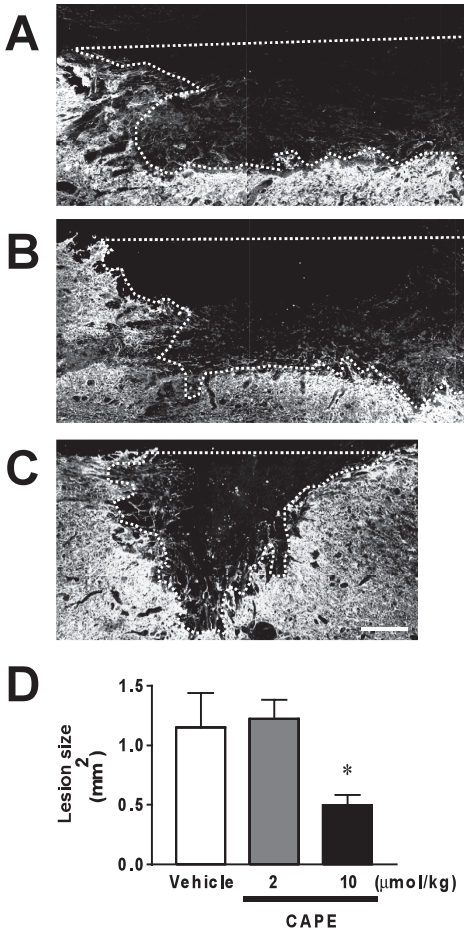

Fig. 2 CAPE reduces the lesion size. **A**–**C**, Representative sections of the injured spinal cord including the lesion site were prepared from the rats (n = 3 for all groups) administered vehicle (**A**), 2 μmol/kg (**B**) or 10 μmol/kg of CAPE (**C**) for 28 days after SCI, and stained for GFAP. The area surrounded by GFAP staining was compared between CAPE-treated and the vehicle-treated groups. **D**, Quantitative analysis confirmed that the lesion size in the CAPE-treated group (10 μmol/kg) was significantly smaller than that in the vehicle-treated group. The left is rostral, and the right, caudal, in panels "**A**", "**B**", and "**C**". The values are expressed as the mean ± SD. Significant differences from the vehicle-treated group were determined by using one-way ANOVA with Tukey's *post hoc* test. Significance: $^{*}P < 0.05$. Scale bar: 200 μm.

of 10 μmol/kg of CAPE showed a significantly smaller lesion size (Fig. 2). There was no significant difference in the lesion size between the vehicle-treated and the low-dose (2 μmol/kg) CAPE-treated rats (Fig. 2).

CAPE attenuates the expression of inflammation-inducible genes in the SCI site

Finally, we examined the mRNA expression level of genes of pro-inflammatory cytokine IL-1β and inflammatory mediators iNOS and COX-2 in the injury site by using the RT-PCR method. The expression of IL-1β mRNA markedly increased and attained its maximal level 1 day after SCI, and gradually decreased to the original level irrespective of CAPE administration. It was significantly lower when the animals were treated with the high-dose (10 μmol/kg) CAPE than when they were given the vehicle at times of 1, 3, 7 or 14 days after the SCI (Fig. 3A). Similar results were observed for the iNOS mRNA expression; *i.e.*, the high-dose CAPE (10 μmol/kg) significantly suppressed the lesion-induced remarkable elevation of the level of iNOS mRNA in the lesion site during 14 days after injury (Fig. 3B). Likewise, the injury-induced COX-2 expression level was significantly lower at 1, 3 or 7 days after the injury when the high-dose CAPE was administered than when the vehicle was used (Fig. 3C). The low-dose CAPE (2 μmol/kg) was ineffective throughout the experimental period (Fig. 3A–C).

DISCUSSION

In the present study, we found that CAPE attenuated the mRNA expression for the pro-inflammatory cytokine IL-1β and inflammatory enzymes iNOS and COX-2 in the injured spinal cord, which was followed by significant improvement of hindlimb locomotor activity after SCI. The decreased lesion size was thought to be a consequence of these actions of CAPE. To our knowledge, this is the first report to identify the active component of propolis to repair traumatic SCI. Propolis has been used in folk medicine in many regions of the world for centuries (8). In light of this finding, it may be greatly advantageous for patients with SCI to ingest this natural compound, *i.e.*, CAPE, as a supplement or whole propolis for treatment, which intake would be both safe and easy.

Inflammation that occurs after central nervous system injury causes secondary injury facilitating neuronal dysfunction (4, 7). Harmful actions are at least partly due to the production of NO, reactive oxygen species, and inflammatory cytokines (17, 32). Transcripts of pro-inflammatory cytokines are rapidly up-regulated after traumatic SCI. IL-1β is a major pro-inflammatory cytokine that activates the transcription factor NF-κB, which stimulates gene transcription of inflammation enzymes such as iNOS and COX-2 in the injured spinal cord, resulting in

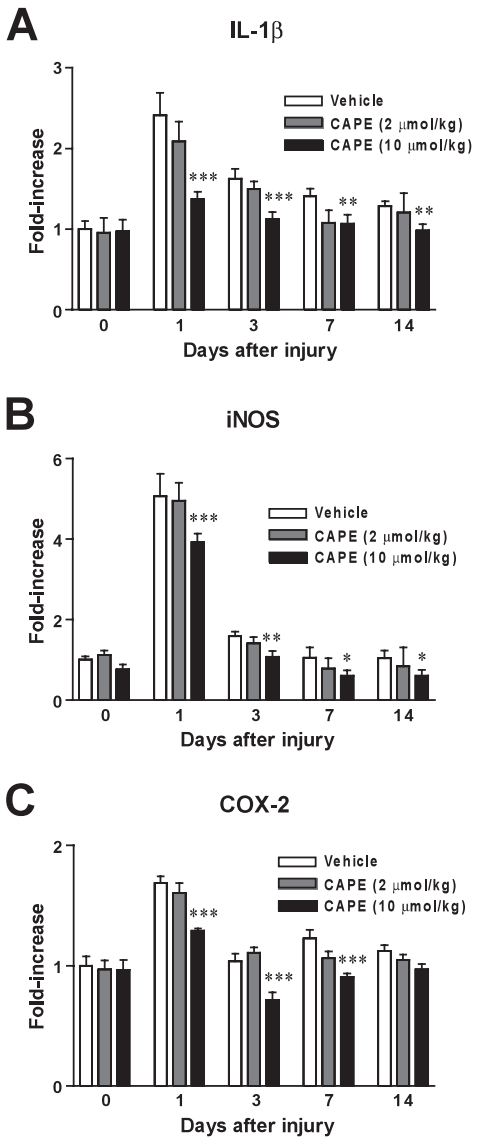

Fig. 3 CAPE attenuates the expression of inflammation-inducible genes in the SCI site. Time-courses of injury-induced expression of IL-1β (**A**), iNOS (**B**), and COX-2 (**C**) mRNAs in the spinal cords after intraperitoneal administration of vehicle (n = 3) or CAPE (2 μmol/kg; n = 3, 10 μmol/kg; n = 5). The mRNA expression level of IL-1β in the injury site was significantly lower in the high-dose (10 μmol/kg) CAPE-treated group than in the vehicle-treated group on experimental days 1, 3, 7, and 14 (**A**). The expression of iNOS mRNA was significantly inhibited in the high-dose CAPE-treated group compared with that in the vehicle-treated group during 14 days after SCI (**B**). The expression level of COX-2 mRNA was significantly decreased in the high-dose CAPE-treated group when compared with that for the vehicle-treated group at 1, 3, and 7 days following SCI (**C**). The low-dose (2 μmol/kg) CAPE did not affect the expression levels of these mRNAs throughout the entire experimental period (**A**–**C**). The values are expressed as the mean±SD. Significant differences from the vehicle-treated group were determined by using two-way ANOVA followed by Bonferroni post-test. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

secondary injury (19, 22). The administration of an IL-1 receptor antagonist reduces IL-1β production in the spinal cord, diminishes apoptosis, and enhances locomotor functional recovery after SCI (21). Therefore, it is plausible that the SCI induces an excessive inflammatory reaction via facilitated production of IL-1β and initiates apoptosis to suppress locomotor recovery. Alternatively, CAPE may directly inhibit transcriptional activity of NF-κB irrespective of down-regulation of IL-1β gene expression, because it has been reported that CAPE prevents the binding of NF-κB to DNA (20). In fact, an NF-κB decoy ameliorates behavior outcomes in SCI animal models (25), supporting the involvement of NF-κB in the CAPE-dependent amelioration of locomotor function.

In the CNS, NO is involved in a variety of biological processes to maintain homeostasis; however, it also plays a detrimental role by acting as a potent oxidant during pathophysiological processes occurring in neural tissues including those caused by SCI (5). In fact, a high amount of NO is closely involved in the development of pathological processes *in vivo*, such as post-traumatic spinal cord cavitation (18). After traumatic SCI, iNOS is predominantly expressed in inflammatory cells infiltrating into the damaged tissue (5, 27), and suppression of SCI-evoked iNOS expression is thought to be a strategy to ameliorate abnormal locomotor activity (11, 24). Given the potent ability of CAPE to inhibit iNOS expression, as previously reported (14) and as shown in the present study (Fig. 3), CAPE becomes a promising candidate molecule for the treatment of cord injuries.

COX, a prostaglandin H/G synthase, is the rate-limiting enzyme in the metabolism of free arachidonic acid leading to the formation of prostaglandins (PGs). The inducible isoform, COX-2, is expressed primarily in the CNS and in activated immune cells such as macrophages (10) and causes the inflammatory response after SCI. PGs themselves have a pathogenic effect in SCI, but COX-2 also produces hydroxyl radicals that cause lipid peroxidation (26). Pharmacologic down-regulation of COX-2 reduces the lesion volume and enhances locomotor recovery (9, 26), suggesting that COX-2 causes deterioration of neural function after SCI. CAPE inhibited SCI-induced COX-2 mRNA expression in the lesion site, which may have contributed to amelioration of the impaired locomotion.

In addition to CAPE, we have found that ethanol extract of Chinese propolis (EECP) (13) and low-molecular-weight substances such as pyrroloquino-

line quinone (PQQ), which is a natural redox cofactor that acts as a nutrient, antioxidant, and redox modulator (11), improve functional recovery of locomotor activity. Particularly, EECP, PQQ, and CAPE are similar in that they effectively reduce iNOS gene expression in the injury site and ameliorate the locomotor recovery of rats with lateral hemi-transection of the spinal cord. However, it should be noted that EECP and PQQ only accelerated the rate of functional recovery after the injury, but did not change the maximal point of BBB scale finally attained. In the case of CAPE, significant improvement of the recovery from the motor paralysis lasted over 28 days. Considering this superiority of CAPE, some unknown action mechanism(s) such as modulation of intracellular signal transduction in neurons to facilitate axonal growth might be involved in the improvement of locomotor activity. In fact, we have preliminary results (Kasai *et al.*, unpublished results) showing that CAPE activates the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) 1/2 signaling pathway (ERK1/2) in neurons cultured from embryonic rat cerebral cortex. The ERK1/2 signaling is involved in regulatory mechanisms of neuritegenesis (30). Further investigations are needed to elucidate this issue.

Although there are several problems for the clinical use of CAPE such as clarification of the critical action mechanism(s) and pharmacokinetics to decide the validity of administering CAPE by oral administration, CAPE may be expected as a promising therapeutic tool for the treatment of traumatic SCI.

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