Crocetin reduces the oxidative stress induced reactive oxygen species in the stroke-prone spontaneously hypertensive rats (SHRSPs) brain

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Crocetin is a natural carotenoid compound of gardenia fruits and saffron, which has various effects in biological systems. In this study, we investigated the antioxidant effects of crocetin on reactive oxygen species such as hydroxyl radical using in vitro X-band electron spin resonance and spin trapping. Crocetin significantly inhibited hydroxyl radical generation compared with the control. Moreover, we performed electron spin resonance computed tomography ex vivo with the L-band electron spin resonance imaging system and determined the electron spin resonance signal decay rate in the isolated brain of stroke-prone spontaneously hypertensive rats, a high-oxidative stress model. Crocetin significantly reduced oxidative stress in the isolated brain by acting as a scavenger of reactive oxygen species, especially hydroxyl radical, as demonstrated by in vitro and ex vivo electron spin resonance analysis. The distribution of crocetin was also determined in the plasma and the brain of stroke-prone spontaneously hypertensive rats using high-performance liquid chromatography. After oral administration, crocetin was detected at high levels in the plasma and the brain. Our results suggest that crocetin may participate in the prevention of reactive oxygen species-induced disease due to a reduction of oxidative stress induced by reactive oxygen species in the brain.

Key Words: crocetin, antioxidant, oxidative stress, brain, electron spin resonance (ESR)

Reactive oxygen species (ROS) such as the superoxide (O$_{2}^•$-) and/or hydroxyl radical (HO$^•$) have been implicated in the pathogenesis of various types of brain dysfunction including ischemia-reperfusion injury,(1) Alzheimer’s disease,(2) aging,(3) and other neurodegenerative disease.(4) Among the organs that can be affected by ROS-induced diseases, the brain is particularly susceptible to the effects of aging and oxidative stress.(5) The brain protective properties of several carotenoids are well known.(6–9) It has recently been reported that antioxidant carotenoids such as β-carotene and lycopene reduce ischemia-reperfusion injury of the brain via their antioxidant properties.(10,11)

Crocetin is a natural carotenoid compound found in the stigmas of saffron (Crocus sativus L.) and the fruits of Gardenia jasminoides Ellis. This yellow compound has been used as an important spice and natural food colorant in various parts of the world.(12,13) In addition, saffron and gardenia fruits have been used as traditional medicine and crocetin is one of the major active compounds of these herbal medicines. Crocetin is an amphiphilic low-molecular weight carotenoid compound, as shown in Fig. 1. Extensive research on crocetin has indicated that it inhibits tumor promotion,(14) is hepatoprotective,(15) has neuroprotective potential,(16) exerts anti-inflammatory effects,(17) and is beneficial in cardiac diseases.(18) In a recent clinical studies, crocetin showed positive effects on asthenopia(19) and attenuating effects on physical fatigue.(20) Antioxidant potential of crocetin may be contributing to these pharmacological actions. However, there are almost no reports on a direct ROS scavenging effect of crocetin.

We previously reported on the use of an electron spin resonance (ESR)-based technique for the detection of free radical reactions in biological systems.(21–26) Nitroxyl radicals are very useful as spin probes for measuring ROS distribution, oxygen concentration, and redox metabolism by ex vivo ESR in biological systems.(21–25) It has been reported that the nitroxyl radical, referred to as a ‘nitroxyl spin probe’, loses its ESR signal by rapidly reacting with HO$^•$ (k$>$10$^9$ M$^{-1}$ s$^{-1}$),(27,28) O$_2^•$ (k$=10^5$–10$^4$ M$^{-1}$ s$^{-1}$) in the presence of thiols or NAD(P)H,(29) and other radicals such as alkyl (k$=10^7$–10$^4$ M$^{-1}$ s$^{-1}$) and lipid peroxy radicals.(30) The signal decay rate of the nitroxyl spin probe provides evidence of ROS generation and changes in the redox status of biological systems.(31,32)

The stroke-prone spontaneously hypertensive rat (SHRSP) is a genetic model of spontaneous hypertension, stroke, and endothelial dysfunction.(33–35) It has several characteristics of increased oxidative stress(21,35–38) and other features such as blood pressure elevation, high blood pressure, and increased vascular wall thickness.(39) The blood brain barrier-permeable nitroxyl spin probe 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (MC-PROXYL) has been used for ESR assessment of oxidative stress in the rodent brain.(31,37,38) In the present study, we used the ESR technique to investigate the ROS scavenging effect of crocetin and the decay rate constant of MC-PROXYL in the isolated brain of the SHRSPs. In addition, we investigated the absorption and distribution of crocetin in the plasma and the brain following oral administration in SHRSPs. The results showed that oral administration of crocetin to SHRSPs was capable of reducing ROS-mediated oxidative stress in the brain due to a direct ROS-scavenging effect.

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Fig. 1. Chemical structure of crocetin.
Materials and Methods

Reagents. Crocetin was provided by Riken Vitamin Corpora-
tion Limited (Tokyo, Japan). Hydrogen peroxide (H₂O₂) was purchased from Wako Pure Chem. Ind. (Osaka, Japan). The ESR spin trapping studies, using 5-(2,2-dimethyl-1,3-propoxycyclo-
ephenoxy)-1-pyrroline-N-oxyl (CYPMO), Radical Research, Tokyo, Japan), indicated production of HO·. Pento-
barbital sodium was purchased from Kyoritsu Seiyaku Co. (Tokyo, Japan). MC-PROXYL was synthesized from 3-carboxy-
2,2,5,5-tetramethyl-pyrroline-1-oxyl (carboxy-PROXYL, Tokyo Kasei, Tokyo, Japan) by a method described previously. All other reagents were analytical grades.

In vitro ESR measurement. HO· was generated by ultra-
violet (UV, emission: 310–400 nm, 5 sec; 40 mW; SUPERCURE-
203S, RU-360, Radical Research, Tokyo, Japan) irradiation of H₂O₂ as described previously. Crocetin was prepared in 10% alkaline buffer (50 mM Na₂B₄O₇–50 mM Na₂CO₃, pH 10.0). Other solutions were prepared in ultra-pure water. ESR spin-trapping was conducted with an ROS-generating system containing CYPMO. ESR observations were performed with a JES-RE 3X, X-band spectrometer (JEOL, Tokyo, Japan) connected to a WIN-RAD ESR Data Analyzer (Radical Research, Tokyo, Japan) at the following instrument settings: microwave power, 8.00 mW; magnetic field, 335.6 ± 0.07 mT; field modulation width, 0.079 mT; receiver gain, 200; sweep time, 1 min; and time constant, 0.03 sec. All experiments were repeated a minimum of 3 times. For each experiment, the effects of the compounds were calculated and presented as the percentage of the mean control value (designated as 100%).

Animal and ex vivo ESR-CT imaging measurements. The procedures used in this study were in accordance with the guide-
lines of the US National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication NO. 85–23, revised 1985) and the protocols were approved by the Animal Care Committees (Yokosuka, Japan). Male SHRSPs (6-weeks old) were purchased from Japan SLC (Shizuoka, Japan). Animals were housed in a light-controlled room with a 12-h light/dark cycle and were allowed ad libitum access to food and water. Crocetin was suspended in 0.5% (w/v) carboxymethylcellulose-sodium (CMC-
Na) solution at a concentration of 10 mg/ml (crocetin/CMC-Na). We have previously confirmed that crocetin arrived at the maximum blood concentrations 90 min after oral administration (data not shown). Crocetin (100 mg/kg) or 0.5% CMC-Na solution was administered orally 90 min prior to measurement by ex vivo ESR. ESR-computed tomography (CT) imaging of the isolated rat brain was performed as follows. The rats were anesthetized with 50 mg/kg (i.p.) pentobarbital and injected with 140 mmol/l MC-PROXYL solution (10 mg/kg) i.v. via the tail vein. The brain was isolated 30 sec after the treatment and subsequently analyzed using ex vivo L-band ESR imaging, as described previously.

Ex vivo ESR-CT imaging system constructed in our laboratory and JEOL ESR application laboratory software were used. This system consists of a commercially available electromagnet (modified JES-RE 3X, JEOL, Tokyo, Japan), a pair of field scan coils, power supplies, a personal computer, and a 1-GHz micro-
wave unit containing a 4-window loop-gap resonator (28 ×
φ43 mm, the measurement position centered on bregma). The system is provided with 4 different coil sets; 3 for the gradients (0.9 mT/cm, max) and 1 for rapid scanning. The gradient field was controlled by a current stabilizer linked with a personal computer (Dell Precision PWS 380).

The ESR-CT images were constructed on the basis of Lauterbuch’s method, known as a 3D zeugmatography. We applied linear magnetic field gradients along the x-, y-, and z-axes produced by the magnetic field gradient coils. For the 2D imaging, 36 projections alternating between gradient and non-gradient were acquired in 55 s. Each projection required 1.024 points of acquisition data for imaging. The ESR absorption spectra were obtained by integrating the derivative spectrum with the recorded gradient. The mid-field hyperfine line in the spectrum was separated from the triplet signal of the nitroxy radicals. Each signal data set was convoluted with Shepp’s filter function into the Fourier domain before performing the inverse Fourier transform to the spatial domain. The 2D imaging pictures of 512 × 512 points were obtained from 18 projections per gradient step at 10⁷ in the spatial domain. Instrument settings for ESR detection of MC-PROXYL were as follows: microwave power, 20 mW; magnetic field, 31.0–34.0 ± 1.0 mT; field modulation width, 0.1 mT; receiver gain, 63–125; time constant, 0.01 sec; field intensity, 0.7 mT/cm.

Crocetin analysis in plasma and brain. Blood and brain was collected after crocetin administration orally 90 min later. After the collection of blood from common carotid artery, it was centrifuged at 1,500 g for 5 min at 4°C and plasma was separated. Brain was isolated after phosphate buffer saline perfused from the heart atrium. The samples were stored at ~80°C. Plasma (100 μl) was mixed with 2.0 ml of methanol and centrifuged (3,000 rpm, 10 min). The supernatant was evaporated under nitrogen gas. We used the whole brain to analysis the crocetin distribution (control group: 1.46 ± 0.07 g wet weight, crocetin group: 1.67 ± 0.04 g wet weight) was homogenized in 2.0 ml of alkaline buffer and the homogenate was mixed with 6.0 ml of methanol/chloroform (1:1). The mixture was centrifuged (3,000 rpm, 10 min) and the supernatant was evaporated under nitrogen gas. The residue of plasma or brain was dissolved in 2.0 ml of alkaline buffer and loaded onto a solid-phase extraction cartridge (Oasis HLB Extraction Cartridge, Nihon Waters, Tokyo, Japan) pre-conditioned with methanol (2.0 ml) and alkaline buffer (2.0 ml). The cartridge was washed with water (2.0 ml) and hexane (2.0 ml). The analysis was eluted with methanol (2.0 ml) and the eluate was concentrated to dryness under nitrogen gas. The residue was reconstituted in 200 μl of methanol and filtered with a 0.45-μm Millipore filter for reversed-phase high performance liquid chromatography (HPLC) analysis. Crocetin was quantified by the HPLC method as described previously. In recovery experiments, the recovery percentage of crocetin extracted from plasma and brain homoge-
genate was determined to be 99% and 92%, respectively.

Statistical analysis. Results are expressed as mean ± SD. Student’s t test was used for comparisons between pairs of groups and Dunnet’s test was used for comparisons among 3 or more groups. Data were analyzed for statistical significance, and the significance level was set at p<0.05.

Results

Effects of crocetin on HO· generation by H₂O₂ with UV irradiation. We investigated the effects of crocetin on HO·, which had been generated from H₂O₂ by UV irradiation, and by ESR spin trapping with CYPMO. In agreement with our previous report, we observed that H₂O₂ generated by UV irradiation in the presence of CYPMO led to the formation of a characteristic CYPMO-OH spin adduct spectrum with hyperfine splitting giving rise to 14 resolved peaks (Fig. 2A). The generation of HO· was not influenced by the 10% alkaline buffer (data not shown). As shown in Fig. 2B, CYPMO-OH adduct formation was reduced in a dose-dependent manner by crocetin dissolved in 10% alkaline buffer (p<0.05). These data indicate that crocetin might be an effective HO· scavenger.

Effects of crocetin on SHRSPs-induced oxidative stress in the brain. MC-PROXYL is a suitable spin probe for the study of free radical reactions in the brain by in vivo ESR detection. The effect of crocetin on SHRSPs-induced oxida-
tive stress in the brain was investigated using MC-PROXYL and the resulting spectra were analyzed with the ESR-CT imaging system. Administration of crocetin to SHRSPs significantly
decreased the decay rate of the 2D ESR-CT image of MC-PROXYL in the isolated brain (Fig. 3). The signal decay rate of MC-PROXYL in this study was confirmed with preliminary data from a previous study using ESR-CT imaging with L-band ESR analysis. The signal decay rate constant of MC-PROXYL in brain of SHRSPs brain was significantly lower than that of the control ($p<0.05$) (Fig. 4).

**Crocetin analysis in plasma and brain.** Crocetin was given to SHRSPs ($n=6$) by oral administration at the same dose (100 mg/kg) used in the ESR experiments (Fig. 3 and 4). Plasma
and brain were both collected 90 min after the administration of crocetin. The crocetin concentrations in plasma and brain measured by HPLC are shown in Table 1. These concentrations (about 0.14 mM in plasma and 2.43 nmol/g in brain) were significantly higher in the group that received crocetin as compared with the control group that did not receive crocetin.

### Discussion

Various ROS may be generated by essential metabolic processes or by environmental stress such as UV exposure. Although the ROS plays important roles to cell signaling, it also has a potential to cause significant cellular damage. The participation of ROS in the pathogenesis of many diseases including brain dysfunction has been suggested. Thus, in order to prevent ROS-induced disease, supplementation of antioxidants such as vitamin C, vitamin E, and carotenoids has been proposed.

Crocetin, a kind of carotenoid originally found in the dried stigma of saffron, has been used in the treatment of diversiform diseases for centuries. It is well known that various carotenoids scavenge ROS such as HO and O. However, the antioxidant activity of crocetin appeared to involve the activation of the endogenous antioxidant enzymatic activities such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), rather than a direct ROS scavenging effect. There are few reports of the scavenging activity for ROS of the crocetin. 

In recent years, it has been suggested that crocetin might be an effective antioxidant to counter oxidative stress in a hemi-parkinsonian rat model. However, the antioxidant activity of crocetin appeared to involve the activation of the endogenous antioxidant enzymatic activities such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), rather than a direct ROS scavenging effect. There are few reports of the scavenging activity for ROS of the crocetin. 

**Table 1. Distribution of crocetin in plasma and brain**

| Group   | Concentration of crocetin |
|---------|---------------------------|
| Plasma  |                           |
| Control | nd                        |
| Crocetin| 0.14 ± 0.05*              |

| Brain   |                           |
|---------|---------------------------|
| Control | nd                        |
| Crocetin| 2.43 ± 0.40*              |

Plasma and brain crocetin concentrations (mean ± SD) after the administration of 100 mg/kg crocetin suspended in 0.5% CMC-Na solution (crocetin group) or 0.5% CMC-Na solution alone (control group). Crocetin was not detected (nd) in plasma and brain of the control group.

Crocetin, like other carotenoids, has the potential to be an effective treatment for diseases related to ROS, such as stroke, ischemia-reperfusion injury, and atherosclerosis. The SHRSP is a well-known model for atherosclerosis and is useful for the study of oxidative stress caused by the generation of O and HO. Our research group previously reported the utility of quantitative ESR analysis with MC-PROXYL for the assessment of redox status under conditions of oxidative stress in SHRSPs brain. In this study, we used ex vivo ESR-CT imaging to demonstrate the ability of crocetin (at a dose of 100 mg/kg) to reduce ROS generation and decrease the decay rate constant of MC-PROXYL in SHRSPs brain. This result suggested that orally administrated crocetin may cross the blood-brain barrier and distribute to the brain. Taken together, these results indicate that crocetin attenuates oxidative stress in the isolated brain of SHRSPs.

In conclusion, the present study demonstrated that crocetin exhibits antioxidant properties by scavenging ROS, and that it may reduce oxidative stress induced by ROS generation in the brain. If we turn our attention to how our results may relate to the brain in vivo, it is critical to consider the relative concentration of the crocetin used in the present study. The concentration in rat brain of absorption of crocetin was about 2.43 nmol/g (Table 1), compared to the concentration of crocetin of 250 μM used in our in vitro experiments (Fig. 2). Indeed, it would be possibility that the scavenging effects of crocetin, much used in studies, may reach the brain. The redox potential of those unchanged crocetin and crocetin metabolites that reach the brain enables them to scavenge damaging radicals, but the endogenous brain antioxidants, especially ascorbate, would be less effective at scavenging radicals in the brain. The redox potential of those unchanged crocetin and crocetin metabolites that reach the brain enables them to scavenge damaging radicals, but the endogenous brain antioxidants, especially ascorbate, would be less effective at scavenging radicals in the brain.

Regarding as the concentration of ROS of the crocetine. Tseng et al. reported the scavenging effect of crocetine on ROS scavenging effect. There are few reports of the scavenging effect of crocetin on ROS scavenging effect. We assessed the metabolic fate and the distribution of crocetin (at a dose of 100 mg/kg) to reduce ROS generation and decrease the decay rate constant of MC-PROXYL in SHRSPs brain.

ESR-CT imaging to demonstrate the ability of crocetin to reduce ROS generation and decrease the decay rate constant of MC-PROXYL in SHRSPs brain. This result suggested that orally administrated crocetin may cross the blood-brain barrier and distribute to the brain. Taken together, these results indicate that crocetin attenuates oxidative stress in the isolated brain of SHRSPs.

In conclusion, the present study demonstrated that crocetin exhibits antioxidant properties by scavenging ROS, and that it may reduce oxidative stress induced by ROS generation in the brain.
isolated brain of SHRSPs. By extension, crocetin might be able to prevent ROS-related brain diseases such as stroke.

**Abbreviations**

- CMC-Na: carboxymethylcellulose-sodium
- CT: computed tomography
- CYPMPO: 5-(2,2-dimethyl-1,3-propanoylcyclophosphoryl)-5-methyl-1-pyrrrole-N-oxide
- ESR: electron spin resonance
- GPx: glutathione peroxidase

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