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 in 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 (O₂⁻) 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 asthenopia19 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 in vivo ESR in biological systems.21–26 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>10¹⁰ M⁻¹ s⁻¹),27,28 O₂− (k = 10⁻⁸ – 10⁻⁹ M⁻¹ s⁻¹) in the presence of thiols or NAD(P)H,29 and other radicals such as alkyl (k = 10⁻⁷ – 10⁻⁸ M⁻¹ s⁻¹)30 and lipid peroxyl radicals.31 The signal decay rate of the nitroxyl spin probe provides evidence of ROS generation and changes in the redox status of biological systems.32,33

The stroke-prone spontaneously hypertensive rat (SHRSP) is a genetic model of spontaneous hypertension, stroke, and endothelial dysfunction.34–36 It has several characteristics of increased oxidative stress.21,35–38 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.21,37,38 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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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-propanediol-
phosphoryl)-1-pyrroline-N-oxide (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.(25,37)
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.25-29,40 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.41 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 ± 7.5 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
suspected in 0.5% (w/v) carboxymethylcellose-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.25,42

Ex vivo ESR-CT imaging system constructed in our laboratory
and JEOL ESR application laboratory software were used.26,37,38
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 Lauterburb’s method,43 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 trans-
fomation to the spatial domain. The 2D imaging pictures of
212 ± 512 points were obtained from 18 projections per gradient step at 10 f 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: 1.44 ± 0.07 μg 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, Nikoh 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.43 In recovery experiments, the recovery
percentage of crocetin extracted from plasma and brain homo-
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,41 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 SHRSsPs-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 and ex vivo ESR
detection.42,43 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

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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
Diseases for centuries.

Various antioxidants have been investigated for the prevention of oxidative stress caused by the generation of ROS and H₂O² in ischemia-reperfusion injury, stroke, and atherosclerosis. It is possible to generate HO² via the Fenton reaction and/or Harber-Weiss reaction in biological systems. These free radicals play an important role in brain damage after stroke. In addition to oxidizing macromolecules, leading to cell injury, oxidants are also involved in cell death/survival signaling pathways and cause mitochondrial dysfunction. Various antioxidants have been investigated for the treatment of stroke, and 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.

Crocetin, a kind of carotenoid originally found in the dried stigma of saffron, has been used in the treatment of diversiform disease has been suggested.

ROS in the pathogenesis of many diseases including brain dysfunction has been proposed. 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 disease has been suggested. In this in vitro X-band ESR study, crocetin reduced the generation of HO² from irradiation of H₂O² in a dose-dependent manner (Fig. 2). This study represents the first report of the scavenging effect of crocetin on HO².

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 possible 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. Regarding as the concentration of ROS generation on in vitro experiments, they would be much higher than in vivo situation.

Thus, in order to prevent oxidative stress-induced neurologic disorder induced brain disease.

The involvement of O₂⁻ in ischemia-reperfusion injury, stroke, and atherosclerosis is well known. It is possible to generate HO² from O₂⁻ via the Fenton reaction and/or Harber-Weiss reaction in biological systems. These free radicals play an important role in brain damage after stroke. In addition to oxidizing macromolecules, leading to cell injury, oxidants are also involved in cell death/survival signaling pathways and cause mitochondrial dysfunction. Various antioxidants have been investigated for the treatment of stroke, and 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.

Crocetin is known to exhibit increased levels of oxidative stress in the brain of SHRSP (Fig. 3 and 4). Other study revealed that crocetin would be helpful in preventing oxidative stress-induced neurologic disorder. Our present study has shown that the high concentration of crocetin treatment resulted in the recovery of normal levels of oxidative stress in the brain of SHRSP (Fig. 3 and 4). Other study revealed that crocetin would be helpful in preventing oxidative stress-induced neurologic disorder. These results suggest the possibility that crocetin may show useful antioxidant activity for in vivo rodent model for human application. However, further studies will be required to examine the human application of crocetin upon oxidative stress-induced brain disease.

The SHRSP is a well-known model for atherosclerosis and is useful for the study of oxidative stress caused by the generation of O₂⁻ and HO² in the brain. 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. Spontaneous ROS generation has been demonstrated in association with ischemia-reperfusion injury accompanying atherosclerosis 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. We assessed the metabolic fate and the bioavailability of crocetin in the plasma and the brain of SHRSPs and found that the compound was detected in both within 90 min after oral administration (Table 1). This result suggested that orally administrated crocetin 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...
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-propoxycyclophosphoryl)-5-methyl-1-pyrroline-N-oxide  
ESR    electron spin resonance  
GPx    glutathione peroxidase  
H₂O₂  hydrogen peroxide  
HO·  hydroxyl radical  
HPLC  high performance liquid chromatography  
MC-PROXYL  3-methoxy carbonyl-2,2,5,5-tetramethylpyrroldine-1-oxyl  
O₂⁻  superoxide  
ROS  reactive oxygen species  
SHRSP  stroke-prone spontaneously hypertensive rat  
SOD  superoxide dismutase  
UV    ultraviolet

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