Neuroprotective Activities of Saffron and Crocin

Shinji Soeda, Kosuke Aritake, Yoshihiro Urade, Hiroshi Sato, and Yukihiro Shoyama

Abstract We first considered that saffron is really safety food because it has a long-use history. The neuroprotective activities of saffron and its major constituent, crocin, are separately discussed in vitro and in vivo. We reviewed the inhibitory activities of crocin against PC-12 cell apoptosis. The oxidative stress decreased the cellular levels of glutathione (GSH) which is an inhibitor of neutral sphingomyelinase (N-SMase). Therefore, the level of GSH was assayed by the addition of crocin resulted in the activation of glutathione reductase (GR). It became evident that crocin treatment prevents the N-SMase activation resulting in the decrease of ceramide release. From these evidences we summarized the role of crocin for neuronal cell death. We used the ethanol-blocking assay system for learning and memory activities. The effect of saffron and crocin on improving ethanol-induced impairment of learning behaviors of mice in passive avoidance tasks has been clear. Further, we did make clear that saffron and crocin prevent the inhibitory effect of ethanol on long-term potentiation (LTP) in the dentate gyrus. Finally we found that 100 mg/kg of crocin gave non-rapid eye movement sleep (non-REM sleep) although mice were started to be active during night time.

Keywords Saffron • *Crocus sativus* • Crocin • Neuroprotective activity • Learning and memory • Non-REM sleep

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Abbreviation

DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FB1	Fumonisin B1
c-GCS	c-Glutamylcysteinyl synthase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
IL-6	Interleukin-6
JNK	c-Jun kinase
LTP	Long-term potentiation
MAb	Monoclonal antibody
NGF	Nerve growth factor
NMDA	*N*-Methyl-D-aspartate
Non-REM sleeping	Non-rapid eye movement sleep
N-Smase	Neutral sphingomyelinase
PS	Phosphatidylserine
SAPK	Stress-activated protein kinase
SD	Step-down
SM	Sphingomyelin
SOD	Superoxide dismutase
ST	Step through
TNF-a	Tumor necrosis factor
TCM	Traditional Chinese medicine

Introduction

Japan has been running into super aging society resulting in rapidly increasing lifestyle disease including dementia which is the most serious problem in recent Japan. Japanese patient survey by Ministry of Health, Labour, and Welfare reported 462,000 of dementia patients in 2012 and quickly increasing to 700,000 in 2025 in Japan meaning that the estimate of national medical care expenditure swells up, and therefore the Japanese health insurance system is considered to be confronted with the brink of collapse. This is the reason why the natural products having preventive activities for brain disease are particularly desirable in Japan. Considering such recent healthy circumstances in Japan, we select saffron as a neuroprotective natural product, and its function will be reviewed in this chapter.

Crocus sativus L. (Iridaceae) is a perennial herb that is widely cultivated mainly in Iran, where 90 % of saffron has been produced, and the other countries like Greece, Spain, and Morocco for its red stigmatic lobes that constitute saffron from 3500 years ago. This plant blooms only once a year, and the man-

ual harvest of stigmas should be performed within a very short time (Trease and Evans 2002).

The manual cultivation methods practiced with saffron crocus contribute greatly to its high price. About 100,000 flowers give about 1000 g of the dried saffron. Since weather conditions affect the quality of saffron, an indoor cultivation system was established in Japan from 100 or more years ago in Oita Prefecture in Japan (Fig. 1, upper). The stigmas can be collected from full blooming *C. sativus* in the room. This is the reason why the indoor cultivation method is advantageous for the achievement of a homogenous quality of saffron and for saving time (Morimoto et al. 1994). We confirmed that the concentration of crocin is increasing until full blooming and then decreased. Therefore, stig-

Fig. 1 Cultivation of *Crocus sativus* indoor and saffron corrected

mas can be collected in full blooming season in general in order to keep the higher concentration of crocin (Morimoto et al. 1994).

Saffron finds its use as folk medicines and traditional Chinese medicine (TCM) as well as a flavoring and a coloring agent. Saffron has three main chemical components, the bright yellow coloring carotenoids, a bitter taste, picrocrocin, and a spicy aroma, safranal. The carotenoid pigments consist of crocetin-diglucoside, crocin-2, crocin-3, crocin-4, and crocetin di-(β-D-digentiobiosyl)-ester (crocin) (Fig. 2). More recently we succeeded to isolate a novel crocetin glycoside, *trans*-crocetin-1-al 1-*O*-β-gentiobiosyl ester (Fig. 2) (Tung and Shoyama 2013). We confirmed that drying is important because an endogenous β-glucosidase is still active when moisture remains (Morimoto et al. 1994). Therefore, drying is completed in about 30–45 min, after which the drug is cooled and stored under dry condition (Morimoto et al. 1994).

It is well known that saffron has anticancer activities against several cancer cell lines and in vivo investigations. We also investigated the anticancer activity of saffron and its constituent, crocin. In the first investigation, we tested the

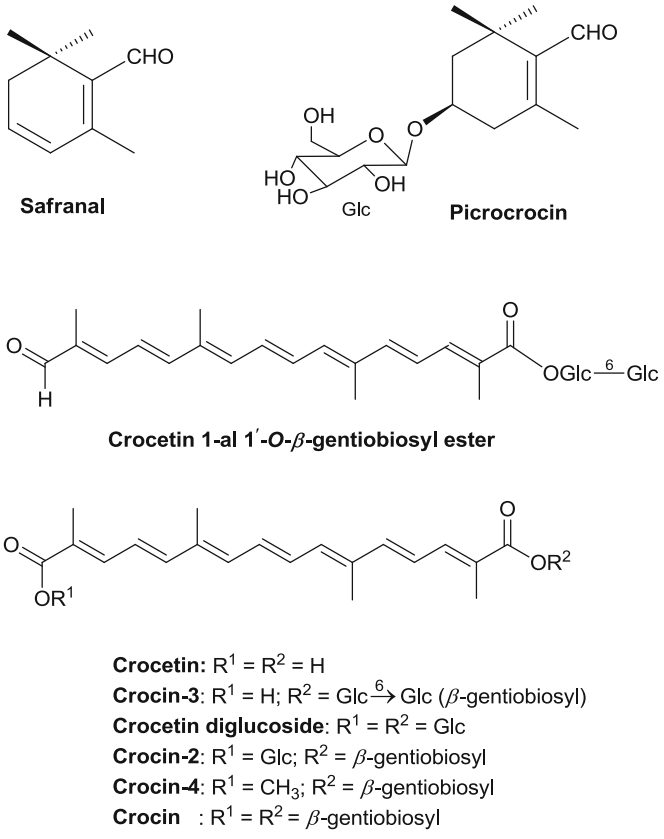

Fig. 2 Major constituent in saffron

inhibitory activity for skin tumor promoted by chemicals by using saffron and crocetin glucosides. When crocin was applied before each 12-*O*-tetradecanoylphorbol-13-acetate treatment, it delayed the formation of papillomas; only 10 % of mice bore papillomas at 9 weeks of promotion. The effect of crocin was not mimicked by gentiobiose or glucose alone (Konoshima et al. 1998).

Furthermore, we investigated in vitro anticancer-active evidences of saffron extract and its constituent, crocin, using several cancer cell lines like HTC-116, SW-480, and HT-29. Saffron extract and crocin significantly inhibited the growth of colorectal cancer cells while not affecting normal cell (Aung et al. 2007). From these data we started in vitro experiments using mice. The development of colonic adenocarcinomas in mice was induced by azoxymethane and dextran sodium sulfate. Crocin significantly inhibited the colonic adenocarcinomas depending on the inhibition of inflammation phenomenon resulting in the prevention of colitis and inflammation-associated colon carcinogenesis (Kawabata et al. 2012).

Saffron can be used as an antispasmodic, anticatarrhal, and nerve sedative ingredient and is reported to be useful in treating various human disorders such as heart and blood disorders (Konoshima et al. 1998; Aung et al. 2007; Kawabata et al. 2012; Lee et al. 2005). Crocin has a wide range of activities including antioxidant (Aung et al. 2007; Kawabata et al. 2012; Lee et al. 2005; Ochiai et al. 2004; Rigobello et al. 2002), anticancer (Konoshima et al. 1998; Ochiai et al. 2004; Chryssanthi et al. 2007; Abdullaev 2002), hypolipidemic (Aung et al. 2007; Rigobello et al. 2002; Sheng et al. 2006), anti-atherosclerotic (Chryssanthi et al. 2007; Abdullaev 2002; Sheng et al. 2006; Xu et al. 2005, 2006), and anti-inflammatory effects (Xu et al. 2005, 2006, 2009). The neuroprotective activities of crocin have also been demonstrated in various experimental animal models of brain disorders, such as cerebral ischemia (Xu et al. 2005; Ochiai et al. 2007), Alzheimer's disease (Xu et al. 2009; Papandreou et al. 2006), depression (Ochiai et al. 2007; Lechtenberg et al. 2008), and memory impairment (Papandreou et al. 2006; Lechtenberg et al. 2008; Abe et al. 1998; Sugiura et al. 1995a, b).

Neuronal cell death is required for the development of the nervous system. However, recent studies suggest that neurons die from programmed cell death (apoptosis) in the brains deprived of oxygen by stroke (Crowe et al. 1997; Zhang et al. 1994a) and trauma (Hill et al. 1995; Sugiura et al. 1995c) and in the brains of Alzheimer's patients (Pettmann and Henderson 1998; Xuan et al. 1999). Therefore, prevention of neuronal apoptosis has been considered to be a desirable therapeutic strategy for treating such neurodegenerative diseases, although the value of this approach is not yet evident. This review discusses the value of folk medicines in terms of learning and memory and also in modulating apoptotic cell death, together with our recent data of crocin's effect on neuronal cell death.

The development of natural products with properties for alleviating the symptoms of learning and memory impairments has been expected by clinicians and researchers in the field. In the brain, the hippocampus is a very important

region in the learning and memory processes, and the LTP induced from the brain tissue is closely related to learning and memory (Hill et al. 1995; Ishiyama et al. 1991). In earlier publications, we reported the effects of an ethanol extract of *C. sativus* and its purified components on the central nervous system in terms of learning behaviors in mice and LTP in the dentate gyrus of hippocampus in anesthetized rats and in the CA1 region of rat hippocampus slices (Pettmann and Henderson 1998; Ishiyama et al. 1991; Abe et al. 1991; Zhang et al. 1994a; Sugiura et al. 1995c).

Preparation of Monoclonal Antibody (MAb) Against Crocin and Confirmation for Incorporation of Crocin into PC-12 Cells by Immunostaining

In the first stage of neuronal investigations, we prepared monoclonal antibody (MAb) against crocin (Xuan et al. 1999). In the first step for preparation of MAb against crocin, the conjugate of crocin with carrier protein for immunization is necessary. Therefore, crocin was treated with $NaIO_4$ to cut sugar moiety releasing aldehyde in a molecule following addition of carrier protein. As the other way, the crocin hemisuccinate was prepared first and then conjugated with BSA to give crocin hemisuccinate-BSA conjugate as indicated in Fig. 3. The molecular weight of prepared schiff base was analyzed by MALDI-TOF mass spectrometry to determine the hapten number in the conjugate for suitability of immunization. Since the hapten number in crocin hemisuccinate-BSA conjugate was determined to be 8.6 which was suitably enough for immunization rather than that of crocin-BSA conjugate prepared by $NaIO_4$ treatment, the former was used as an antigen. Hybridoma-producing MAb reactive to crocin was obtained by general procedure and classified into IgG2a which had λ light chains. The reactivity of IgG-type MAb 12a was tested by varying antibody concentration and by performing a dilution curve, and then the antibody concentration was selected for competitive ELISA. The measuring range of this ELISA system extends from 10 to 200 ng/ml of crocin (Xuan et al. 1999).

In order to confirm the incorporation of crocin and the localization of crocin into PC-12 cells, we immunostained cells using the anti-crocin MAb prepared. Clear incorporation of crocin into PC-12 cells was confirmed after 30 min comparing with the control cells as indicated in Fig. 4 (Ochiai et al. 2004). The incorporation after addition of crocin in the medium was not enough after 15 min (Fig. 4b). From 30 min later, the clear staining occurred (Fig. 4c, d). From this evidence we confirmed that crocin can be incorporated into the cell and be functioned.

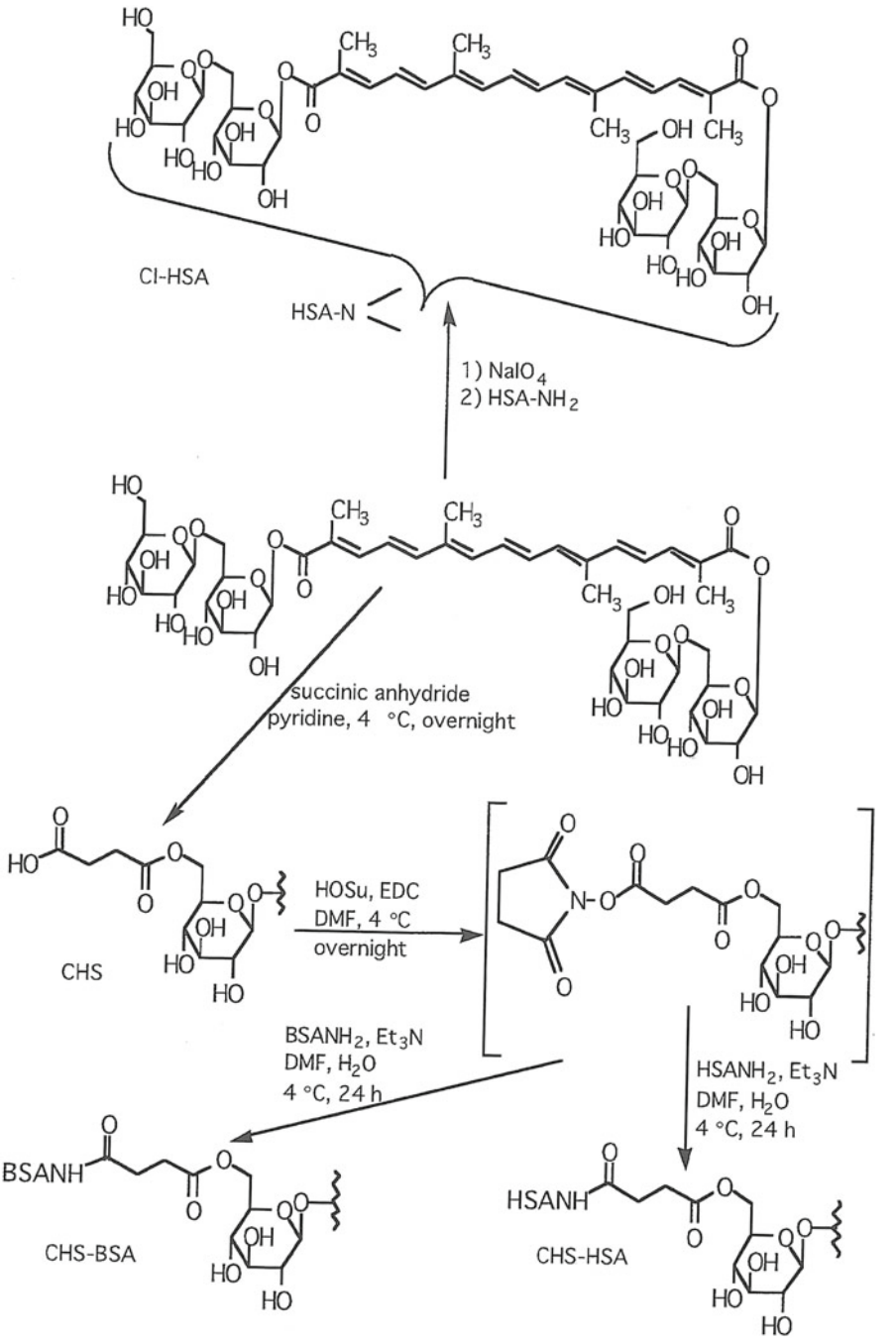

Fig. 3 Synthetic pathway for hapten-carrier protein conjugate of crocin

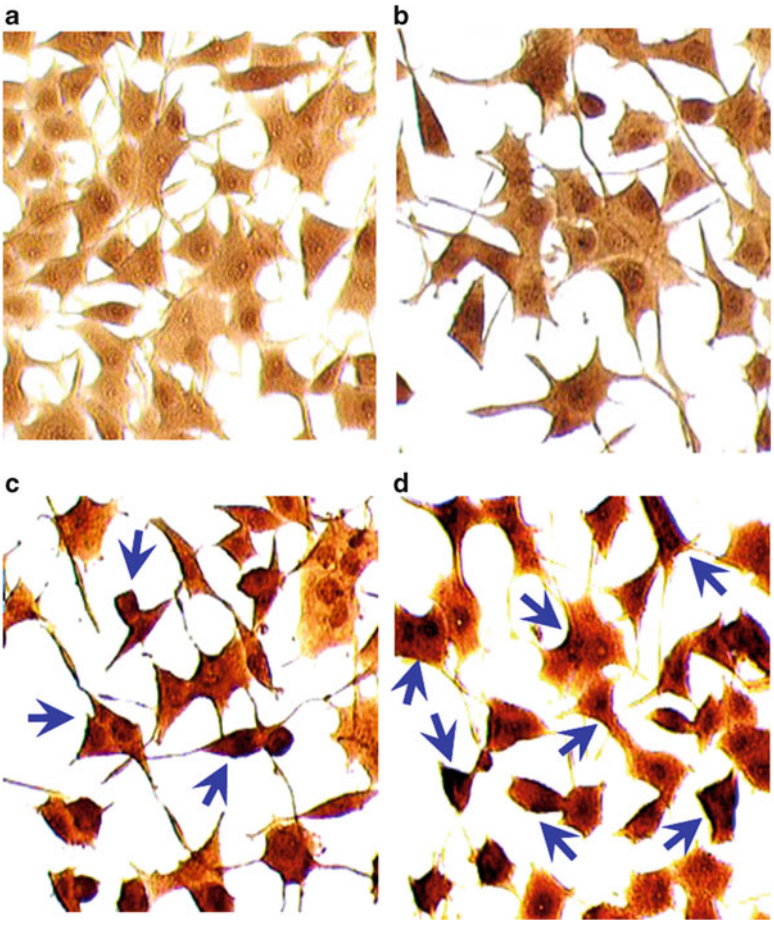

Fig. 4 Immunostaining of crocin using anti-crocin monoclonal antibody in PC-12 cells. (**a**) Stained after 0 min; (**b**) after 15 min; (**c**) after 30 min; (**d**) after 1 h. *Arrows* indicated stained cells by immunostaining using anti-crocin MAb

Neuroprotective Activity of Crocin In Vitro

Antioxidant Activity of Crocin in Preventing Neuronal Cell Death

First of all we examined the effects of crocin on PC-12 cells deprived of serum/glucose in comparison with those of α-tocopherol (Soeda et al. 2001). Depriving the PC-12 cells of serum/glucose caused changes in the morphology and peroxidation of their membrane lipids and decreased intracellular superoxide dismutase (SOD) activity. The oxidative stress transferred the phosphatidylserine (PS) residues into

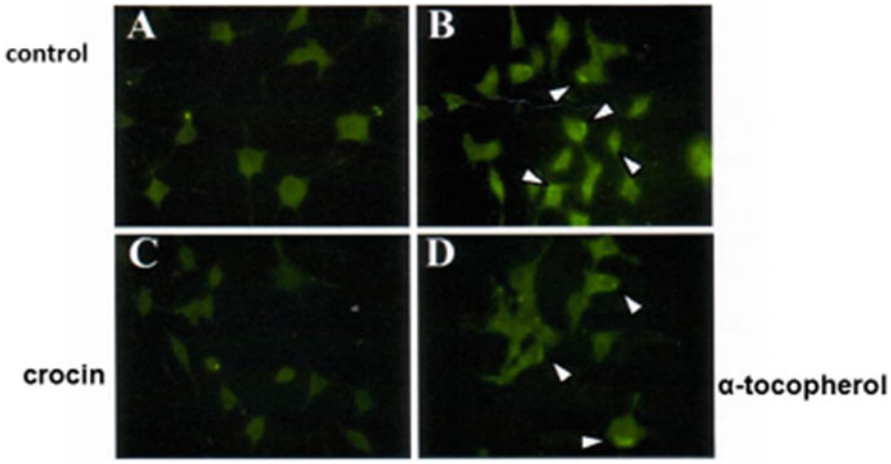

Fig. 5 Annexin V staining of PC-12 cells exposed for 3 h in serum/glucose-deprived medium. (**a**) Control cells in serum/glucose (+) DMEM; (**b**) cells in (−) DMEM alone; (**c**) cells in (−) DMEM plus 10 μM crocin; (**d**) cells in (−) DMEM plus 10 μM α-tocopherol. *Arrows* indicate ring-like stains

the outer membrane, although they are usually consisted in the inner membrane, and resulted that PS externalization can be used as an early marker of apoptotic induction. Annexin binds to the negatively charged PS, and the conjugated FITC shows a ring-like stain along the cellular boundary (Mukhopadhyay et al. 2007). The cells deprived of serum/glucose show strong ring-like stains compared to the control cells. Crocin kept the cell's morphology more intact than α-tocopherol. In PC-12 cells deprived of serum/glucose for 6 h, the level of peroxidized lipid membrane increased 1.8-fold in comparison to the control cells, and SOD activity decreased to 14 % of that in the control cells. However, crocin significantly decreased the formation of peroxidized membrane lipids and restored SOD activity compared to α-tocopherol activity. The restoration of SOD activity suggests that crocin has an important role in modulating antioxidative effects. Crocin also suppressed the activation of caspase-8 caused by serum/glucose deprivation; this activation was suppressed in a concentration-dependent manner (0.1–10 μM). Crocin did not inhibit caspase-8 activity in the cell lysates, and its inhibitory effect may be caused indirectly by the antioxidant activity (Fig. 5).

Inhibitory Activity of Crocin for PC-12 Cell Death Induced by Serum/Glucose Deprivation

Cells cultured in serum/glucose-containing Dulbecco's modified Eagle's medium [DMEM (+)] had a normal morphology at 24 h, while those cultured in the serum- and glucose-free medium [DMEM (−)] for 24 h were round in shape and showed

the characteristic properties of necrotic and/or apoptotic cells. We confirmed that approximately 60 % cell death had occurred in the latter culture c exclusion method. The addition of crocin (10 μM) significantly suppressed both the morphological changes and the PC-12 cell death induced by the DMEM (−) conditions as crocin inhibited TNF-a-induced PC-12 cell death (Oppenheim 1991), resulting in 85 % survival. It is well known that serum or nerve growth factor (NGF) (Batistatou and Green 1991; Rukenstein et al. 1991; Mesner et al. 1992; Pittman et al. 1993) deprivation induces apoptosis in PC-12 cells. Colombaioni et al. (2002) demonstrated that serum deprivation increased the intracellular ceramide levels in undifferentiated HN9.10e cells, resulting in apoptosis. These findings easily suggest a possibility that ceramide levels increase in PC-12 cells under DMEM (−) conditions. PC-12 cells cultured for 3 h in DMEM (−) showed a significant increase (3.5-fold increase) in the level of ceramide compared to the basal level in cells cultured in DMEM (+) conditions. The suppressive effect of crocin was dose dependent. We also tested the effect of fumonisin B1 (FB1), which inhibits de novo ceramide synthesis in cells at a concentration of 10–30 μM (Wang et al. 1991; Merrill et al. 1993). However, FB1 had no significant effect on ceramide levels, suggesting that the accumulation of ceramide through an enhancement of de novo synthesis following a 3-h culture in DMEM (−) was in itself not sufficient to explain the increase. It has been suggested that the sphingomyelin (SM) pathway and SAPK/JNK signaling systems may function together (Verheij et al. 1996) in stress-induced apoptosis of U937 cells and BAE cells. Since the environmental stress under DMEM (−) conditions may activate the stress-activated protein kinase (SAPK)/JNK cascade in PC-12 cells, we compared the amounts of phosphorylated JNK in the cells cultured in DMEM (+) and DMEM (−) for 6 h. The DMEM (−) conditions stimulated the phosphorylation of JNK in the cells by approximately 3.7-fold relative to the control cells.

Inhibitory Effect of Crocin on the Activation of N-SMase Induced in Serum/Glucose-Deprived PC-12 Cells

In order to confirm the resource of the accumulated ceramide, we measured the activity of magnesium-dependent N-SMase in the PC-12 cell homogenate. N-SMase activity in cells cultured in DMEM (−) reached a maximum at 1 h and decreased to around the level of the control cells at 3 h. However, there was no time-dependent change in the N-SMase activity during a 3-h culture in DMEM (−). This assay method can detect N-SMase activity in these supernatants by substitution of the reaction medium for 50 mM sodium acetate buffer (pH 5.6). The results demonstrated that the activity of N-SMase in PC-12 cells was unaffected by serum/glucose deprivation for at least for 3 h. The addition of crocin in the culture medium suppressed the enzyme activities at 1 and 2 h in a dose-dependent manner. To determine

whether or not the inhibition of N-SMase is a direct action of crocin on the enzyme, we added crocin to the reaction medium, whose cells had been cultured in DMEM (−) for 2 h. The addition of 1 or 10 μM crocin had no inhibitory effect on N-SMase activity in the reaction medium. However, the addition of GSH at concentrations of 1 and 10 mM inhibited the enzyme activity dose dependently. Earlier reports indicate that GSH is a physiological inhibitor of magnesium-dependent N-SMase in plasma membranes (Yoshimura et al. 1998, 1999; Liu and Hannun 1997). N-SMase is inactive in the presence of physiological concentrations (1–20 mM) of GSH. Therefore, these results suggest that the N-SMase activity in the reaction medium is derived from magnesium-dependent N-SMase contained in plasma membranes and that the observed N-SMase inhibition by crocin does not occur through its direct action on the enzyme.

Increase of Intracellular GSH Levels in Serum/Glucose-Deprived PC-12 Cells Through Activation of GR and c-GCS by Crocin

In an investigation aimed at testing the above-mentioned hypothesis, we examined the effect of crocin on intracellular GSH levels in serum/glucose-deprived PC-12 cells. The GSH levels in PC-12 cells exposed for 3 h to serum/glucose-free DMEM decreased to half than that found in the control cells and thereafter remained constant. However, the addition of crocin to the medium increased the intracellular GSH level dose dependently, maintaining it at the 3-h time point at a higher level. The most significant effect of crocin occurred at a concentration of 10 μM. The concentration of GSH was high enough to inactivate N-SMase. We then investigated the mechanism by which crocin increased the GSH levels. The GR activities in serum/glucose-deprived PC-12 cells decreased in a time-dependent fashion, whereas the copresence of 10 μM crocin enhanced GR activity each hour (approximately fourfold elevation at 6 h). This result indicates that crocin has no significant effect on the GPx activity in the cells. GSH synthesis is regulated by the rate-limiting enzyme *c*-GCS. This enzyme is thought to be regulated by several mechanisms. In mouse endothelial cells, the TNF-a- or IL-1b-induced increase in *c*-GCS activity is associated with an increase in mRNA expression (Urata et al. 1996). IL-6 also stimulates the expression of *c*-GCS mRNA and increases the activity of this enzyme, which leads to increased GSH levels in PC-12 cells. In contrast, Nakajima et al. (2002) reported that NGF had an ability to increase the activity of *c*-GCS at the transcription level by extending the half-life of *c*-GCS mRNA. The addition of crocin (10 μM) doubled *c*-GCS mRNA expression in PC-12 cells in serum/glucose-free DMEM, while it had no effect on the mRNA levels of the control PC-12 cells. The crocin-induced increase in *c*-GCS mRNA expression is reflected in an increase in the activity of this enzyme in the cells. These results suggest that crocin can increase GSH levels by increasing the 1 h DMEM.

Neuroprotective Activity of Crocin In Vivo

Inhibitory Activity of Crocin for Infarcted Areas Caused by Occlusion of the Middle Cerebral Artery in Mice Brain

We investigated the effect of crocin on an infarcted area caused by occlusion of the middle cerebral artery (MCA) in mice. Crocin was administrated immediately before and 3 h after the MCA occlusion. The administration of crocin (10 mg/kg) reduced the infarct volume significantly resulting in half compared to vehicle as indicated in Fig. 3. Recently Vakili et al. reported the protective effect of crocin against cerebral ischemia in a dose-dependent manner using a rat model (Vakili et al. 2014). These data had a good correlation with our data although the model animals were different. Furthermore, it has been reported that a ginseng saponin, ginsenoside Rb1, also has the protective effects against ischemic hippocampal neurons (Lim et al. 1997) although these structures are completely different from each other having strong antioxidant activities. These evidences suggest that it may be possible to search and find out new active compounds against infarct induced by occlusion of the MCA from natural products reaching to preventive medicine because the infarct patients are 260,000 in Japan and quickly increasing (Fig. 6).

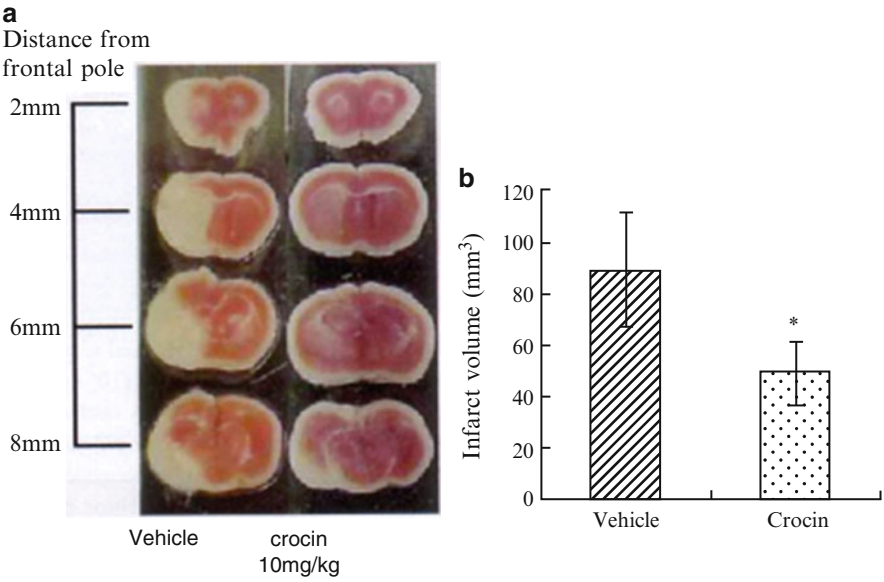

Fig. 6 The effects of crocin on the infarct volume induced by the middle cerebral artery occlusion in mice. Crocin was injected i.v. immediately before and 3 h after the middle cerebral artery occlusion. Slices were immediately stained with 2 % 2,3,5-triphenyltetrazolium chloride. (**a**) Representative infarcted area caused in each group of mice. (**b**) Each bar represents the mean ± S.E. of five to seven mouse brains

Learning and Memory by Saffron Extract and Crocin

Although the saffron extract had no effect on memory registration in normal mice, the crude extract of saffron prevents the ethanol-induced impairment of memory acquisition in step-through (ST) and step-down (SD) tests (Sugiura et al. 1995d). On the basis of these results, it is suggested that some components of saffron are capable of antagonizing the blocking effect of ethanol for memory acquisition. Activity-guided fractionation of the crude extract revealed that crocin is actual active component in saffron.

Single oral administration of crocin had no effect on mice in passive avoidance tasks. Oral administration of 30 % ethanol induced an impairment in memory acquisition in ST and SD tests. However, the subsequent oral administration of crocin (50 mg/kg) improved the impairment of memory acquisition in both tests in a dose-dependent manner (Sugiura et al. 1996; Zhang et al. 1994b).

Effect of Saffron and Crocin on LTP

We already discussed that the saffron crude extract and crocin can improve the blocking effect of ethanol on learning and memory dose dependently. In order to continue the story, the saffron extract was injected intracerebroventricularly, and the blocking effect of ethanol on the LTP decreased dose dependently. These results led us to hypothesize that crocin might antagonize the blocking effect of ethanol on the induction of LTP. Following the activity-guided separation from the crude extract, we confirmed that crocin is the actual active component in saffron. When a 50 mg/kg dose of crocin was injected 5 min before the administration of ethanol, LTP was induced at 84 % than that of control, suggesting that the LTP-blocking effect of ethanol was improved dose dependently with the administration of crocin (Sugiura et al. 1995a, b, c). The activities of the crocetin gentiobiose glucose ester and crocetin di-glucose ester, which are analogs of crocin on the LTP-blocking effect of ethanol, were investigated at the same dose scale. These activities were found to be distinctly lower than that of crocin. The active improvement effect against blocking was clearly proportional to the number of glucoses because crocin, which possesses four glucoses in a molecule, showed the highest improvement effect, while the activity of crocetin di-glucose ester was almost the same as the control (Sugiura et al. 1995b).

Sleep-Promoting Effect of Crocin

It is known that saffron with traditional Chinese medicines (TCM) and/or Japanese Kampo medicine promotes the sleep activity in the field of therapy of mental disorders and is used for sleep promotion as a folk medicine in Japan. From these evidences we

started to search the activity of saffron and its component, crocin. We examined the sleep-promoting effect of crocin on mice after an intraperitoneal administration at 20:00 during the wake period. Figure 7a shows time courses of the hourly amounts of non-REM sleep after the administration of vehicle or crocin (100 mg/kg). During the period of 20:00 to 01:00, mice with vehicle treatment spent more time in wake than in sleep. When 100 mg/kg of crocin was injected on the experimental day, the amount of non-REM sleep was increased immediately after the injection, and the effect was statistically significant from 2 to 4 h after the administration. Crocin did not change the REM sleep after the administration (Masaki et al. 2012). This augmentation effect on non-REM sleep time was accompanied by reduction in wakefulness. The increase in non-REM sleep and decrease in wake lasted more than 4 h after the injection. There was no further disruption of the sleep architecture during the subsequent period (8:00 to 20:00). These data indicated that crocin induces non-REM sleep without occurrence of adverse effects, such as rebound insomnia after the sleep induction. Similar time-course profiles were observed with a low dose of 10 mg/kg, but the effect on sleep was small and lasted only about 1–2 h after the injection.

We calculated the total time spent in non-REM and REM sleep and wakefulness for 4 h after the crocin injection (Fig. 7b). Crocin at 10 mg/kg did not affect the cumulative amounts of non-REM and REM sleep and wakefulness for 4 h after injection. Crocin given at 30 and 100 mg/kg statistically significantly increased the total amount of non-REM sleep by 160 % and 270 %, respectively, and

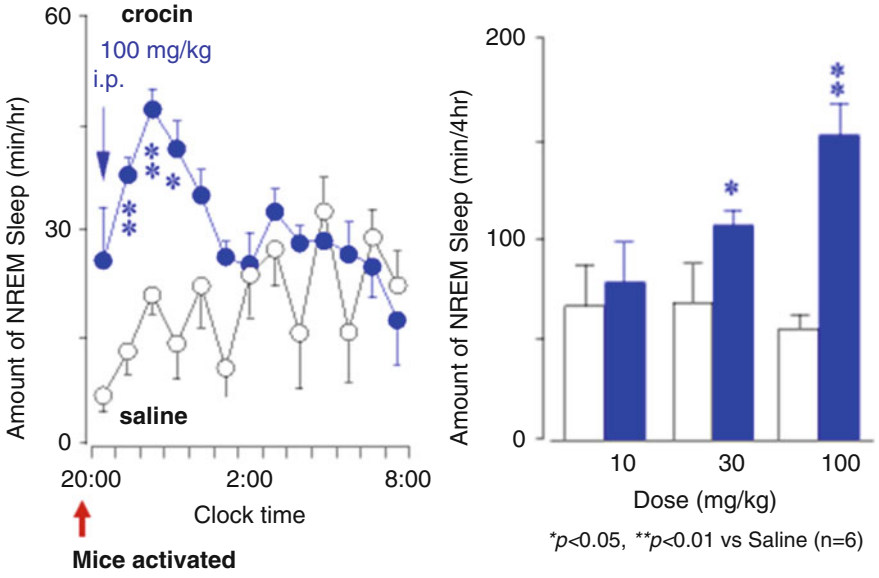

Fig. 7 Increase in non-REM sleep by crocin. (**a**) Time courses of non-REM sleep after an intraperitoneal administration of vehicle (*open circle*) or crocin (*filled circle*) at a dose of 100 mg/kg. (**b**) Total time spent in non-REM sleep for 4 h after the administration of vehicle (*open column*) or crocin (*closed column*) at a dose of 10, 30, or 100 mg/kg, respectively

decreased the total amount of wakefulness by 20 % and 50 %, respectively, without changing the amount of REM sleep during a 4-h period as compared with the vehicle control.

Conclusion

Since we do not have preventive medicine in Japan, some preventive natural products are strongly required instead of medicines. There are thousands of health food in Japan; however safeties against many of them are not clear enough. From these circumstances Consumer Affair Agency, Government of Japan, started a new system, Functional Labeling System on health foods, from April 2015. This system is basically focusing to confirm the safety and to make sure the real function on health food. Regarding safety of functional food, the most important point is how much longer the eating experiences and nothing of toxicity of major constituents contained in the functional food. From these viewpoints we selected saffron because saffron has been used from approximately 3000 years ago as a spice and coloring and a medicine as listed in materia medica 2000 years ago. In fact saffron is known well as safety food because oral administration of saffron extract at concentrations of up to 5 g/kg is still nontoxic in mice (Abdullaev 2002). Therefore, we have been searching the neuroprotective activities in saffron in this chapter.

The main constituent in saffron, crocin, increases the intracellular glutathione level and prevents PC-12 cell death in serum deprived by its antioxidant property. The antioxidant capacities of crocin have been reported in association with a variety of neuroprotective potentials. In PC-12 cells, a cell culture model for brain ischemia, the generation of ROS activates neutral SMase to generate ceramide, which induces cell death. Glutathione directly inhibits the activation of the SMase. Therefore, we hypothesized that crocin might prevent the activation of N-SMase in serum/glucose-deprived PC-12 cells by a GSH-dependent inhibition mechanism. Ceramide releases and activates the caspase family as discussed already. More recently we reported the inhibitory effects of crocin on Bcl-2, Bax, and caspase-3 expression of PC-12 cells injured by H_2O_2 (Cui et al. 2015). Moreover, we found the protective effect of crocin on an infarcted area induced by MCA occlusion of the middle cerebral artery in mice. This evidence also helps the brain health care by saffron and/or crocin.

We reported that crocin is the actual active component involved both in the improvement of learning and memory and with the preventive effect of LTP blocked by ethanol in vivo although oral administrations of saffron and/or crocin had no effect on memory acquisition in normal mice. Recently Naghibi et al. investigated the effect of saffron extract on morphine-induced memory impairment and concluded that the saffron extract attenuated morphine-induced memory impairment (Naghibi et al. 2012). We further demonstrated for the first time that crocin selectively antagonizes the inhibitory effect of ethanol on *N*-methyl-D-aspartate (NMDA)-receptor-mediated responses in hippocampal neurons (Abe et al. 1998). The sugar numbers in crocetin glycosides reflected the LTP-blocking activity by ethanol like that of crocin having four glucoses in a molecule attenuated

strongest compared to the other smaller molecules. This tendency is a good agreement with the previous reports that the sugar residues are important for the activities of some drugs like cardiac steroids (Shimada et al. 1986), streptozotocin (Gunnarsson et al. 1974), ginsenosides (Takemoto et al. 1984), and so on.

Twenty-five percent of population have some sleeping problems in Japan resulting in brain diseases. Saffron has been widely prescribed with Japanese Kampo medicine and/or TCM for mental disorder patient having sleeping problem in Japan. Therefore, crocin was tested for sleep promotion, and we found crocin increased the total time of non-REM sleeping. This finding promotes the clinical use of saffron since saffron has been used as safety food from thousands ago. Moreover, crocin was approved by the State Food and Drug Administration for the clinical trial in 2006 and now a drug for angina in China. From various kinds of phenomena described in this review, saffron and crocin have a multifunctional natural product in the brain.

Compliance with Ethics Requirements The authors declare that they have no conflicts of interest.

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