

## Pharmacological activities of crocin in saffron

Shinji Soeda · Takashi Ochiai · Hiroshi Shimeno ·  
Hiroshi Saito · Kazuho Abe · Hiroyuki Tanaka ·  
Yukihiro Shoyama

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**Abstract** The effect of crocin on improving ethanol-induced impairment of learning behaviors of mice in passive avoidance tasks is reported. Based on these results, it became evident that crocin prevents the inhibitory effect of ethanol on long-term potentiation (LTP) in the dentate gyrus in vivo. We confirmed that crocin inhibits tumor necrosis factor (TNF)- $\alpha$ -induced apoptosis of PC-12 cells. PC-12 cells showed a rapid increase in cellular ceramide levels, followed by an increase in the phosphorylation of c-Jun kinase (JNK), leading to apoptosis by serum/glucose deprivation in the medium. The production of ceramide was dependent on the activation of magnesium-dependent neutral sphingomyelinase (N-SMase), but not on de novo synthesis. The oxidative stress also decreased the cellular levels of glutathione (GSH), which is the potent inhibitor of N-SMase. Crocin treatment resulted in the prevention of N-SMase activation, ceramide production and JNK phosphorylation. Exploration of the crocin's preventive mechanism in oxidative stress-in-

duced cell death revealed that the activities of GSH reductase and  $\gamma$ -glutamylcysteinyl synthase ( $\gamma$ -GCS) in the  $\gamma$ -glutamyl cycle affected the stable GSH supply that blocks the activation of N-SMase. These results strongly support the importance of the proposed GSH-dependent inhibitory mechanism in oxidative stress-mediated cell death.

**Keywords** Antioxidant effect · Crocin · *Crocus sativus* L. · Neuronal apoptosis · Neuroprotective effect

### Abbreviation

A-Smase	Acidic Smase
BSO	Buthionine sulfoximine
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FB1	Fumonisin B1
$\gamma$ -GCS	$\gamma$ -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
N-Smase	Neutral sphingomyelinase
PS	Phosphatidylserine
SAPK	Stress-activated protein kinase
SD	Step down
SM	Sphingomyelin
SOD	Superoxide dismutase
ST	Step through
TNF- $\alpha$	Tumor necrosis factor

S. Soeda · T. Ochiai · H. Shimeno  
Department of Biochemistry,  
Faculty of Pharmaceutical Sciences,  
Fukuoka University, Fukuoka 814-0180, Japan

H. Saito · K. Abe  
Faculty of Pharmacy, Musashino University,  
Nishi-tokyoshi, Tokyo 202-8585, Japan

H. Tanaka · Y. Shoyama (✉)  
Department of Pharmacognosy,  
Graduate School of Pharmaceutical Sciences,  
Kyushu University, Fukuoka 812-8582, Japan  
e-mail: shoyama@phar.kyushu-u.ac.jp

## Introduction

The saffron crocus *Crocus sativus* L. (family: Iridaceae), from which saffron is obtained, was first cultivated in Greece more than 3000 years ago. It is now extensively grown in Spain, Greece, France, Macedonia, Iran and, more recently, in China. Saffron is derived from the stigmas of the saffron flowers, and about 90,000–100,000 flowers are required to yield 5000 g of fresh stigmas or about 1000 g of the dried saffron. On a per-weight basis, saffron is the currently the most expensive spice commercially available, but in addition to its use as a flavoring and coloring agent, it has various therapeutic properties and has been used as a natural medicine for many thousands of years. Three main chemical compounds have been identified in saffron: the bright red coloring carotenoids; picrocrocin, which gives the spice its characteristic bitter taste; safranal, which provides the spicy aroma. The carotenoid pigments consist of crocetin di-( $\beta$ -D-glucose)-ester, crocetin-( $\beta$ -D-gentiobiosyl)-( $\beta$ -D-glucosyl)-ester and crocetin-di-( $\beta$ -D-digentiobiosyl)-ester (crocin), as shown in Fig. 1. We have found that saffron cultivated indoors in Taketa City, Oita prefecture contains approximately 15% more crocin (dry weight) than saffron cultivated elsewhere. In an earlier investigation, we found that the level of crocetin glucose esters increase from the period prior to blooming, reaching a maximum in the full blooming period [1], and that they are sensitive to oxygen, light irradiation and to an indigenous  $\beta$ -glucosidase, which hydrolyzes crocin to crocetin di-( $\beta$ -D-glucose)-ester [1]. The storage of saffron at  $-20^{\circ}\text{C}$ , however, facilitates maintenance of a constant supply of saffron with a homogeneous pharmacological activity [1]. In

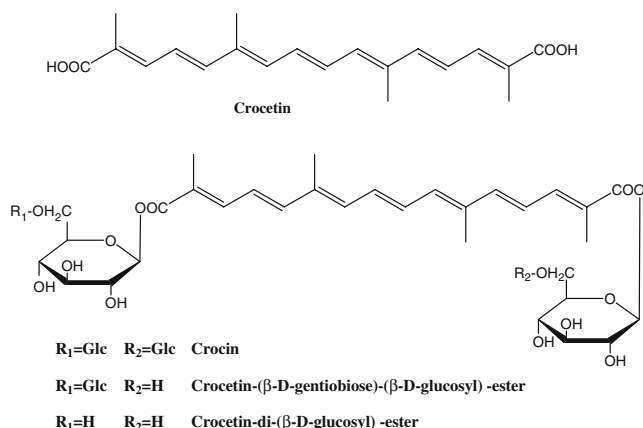
order to evaluate the quantity of saffron, we have already prepared a monoclonal antibody (MAb) against crocin and established a competitive enzyme-linked immunosorbent assay (ELISA) using the anti-crocin MAb [2].

The anti-tumor activity of saffron has been observed in mice transplanted with several types of tumor cell lines, including sarcoma 180, Ehrlich ascites carcinoma and Dalton's lymphoma ascites [3]. Saffron shows an inhibitory effect on chemical carcinogenesis in mice [4], and the effect of crocetin on skin papilloma and Rous sarcoma has been reported [5]. Escribano et al. [6] have recently demonstrated that crocin inhibits the growth of HeLa cells and suggested that the compound has pro-apoptotic properties. More recently, we have reported that an orally administered ethanol extract of saffron and crocin exhibit inhibitory effects on the two-stage carcinogenesis of mouse skin papillomas [7]. These results suggest that crocetin and/or the crocetin glucose esters contribute to the anti-tumor activities of saffron. Furthermore, in the peripheral blood system, crocetin derivatives prevent an elevation in bilirubin levels [8] and also reduce elevated levels of serum cholesterol and triglyceride [9].

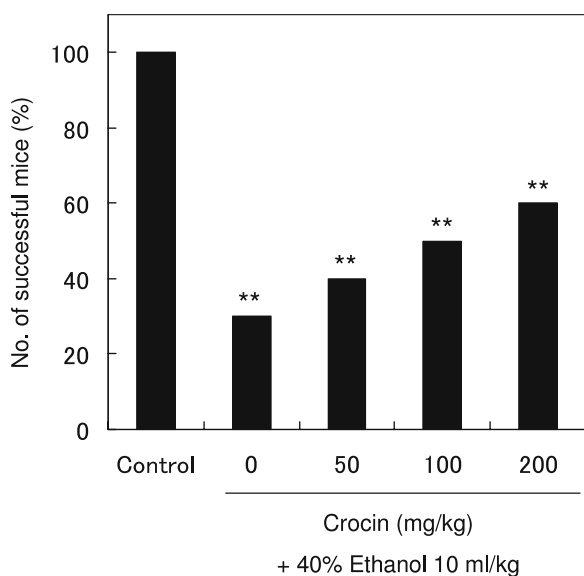
The development of natural products with properties for alleviating the symptoms of learning and memory impairments has been expected by clinicians and researchers in the field. In the brain, the hippocampus is a very important region in the learning and memory processes, and the LTP induced from the brain tissue is closely related to learning and memory [10]. 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 [11–13].

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 brains deprived of oxygen by stroke [14] and trauma [15] and in the brains of Alzheimer's patients [16]. 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.



**Fig. 1** Structure of crocetin and its glycosides



**Fig. 2** Effect of crocin on 30% ethanol-induced impairment of memory acquisition in step through (ST) and step down (SD) tests

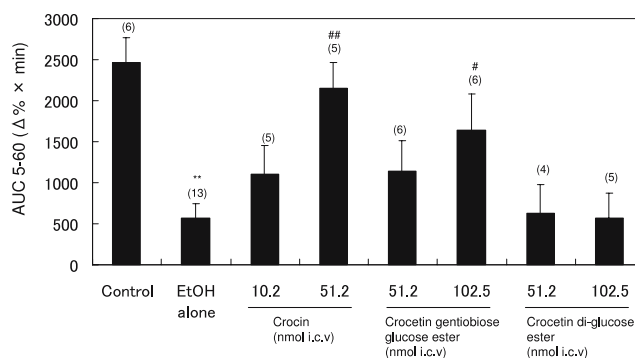
### Crocine can improve ethanol-induced impairments of learning behavior

The crude extract of saffron prevents the ethanol-induced impairment of memory acquisition in step through (ST) and step down (SD) tests [12]. On the basis of these results it is relatively easy to suggest that some components of saffron are capable of antagonizing the blocking effect of ethanol for memory acquisition. Fractionation of the crude extract based on activity revealed that crocine is actual active component in saffron.

Single oral administration of crocine 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 crocine (50 mg/kg) improved the impairment of memory acquisition in both tests in a dose-dependent manner, as indicated in Fig. 2.

### Effect of crocine on LTP [17]

We previously confirmed that the saffron crude extract can improve the blocking effect of ethanol on the LTP in a dose-dependent manner. However, since the active component had not yet been isolated at that stage, we discuss our search for the active compound in this review.



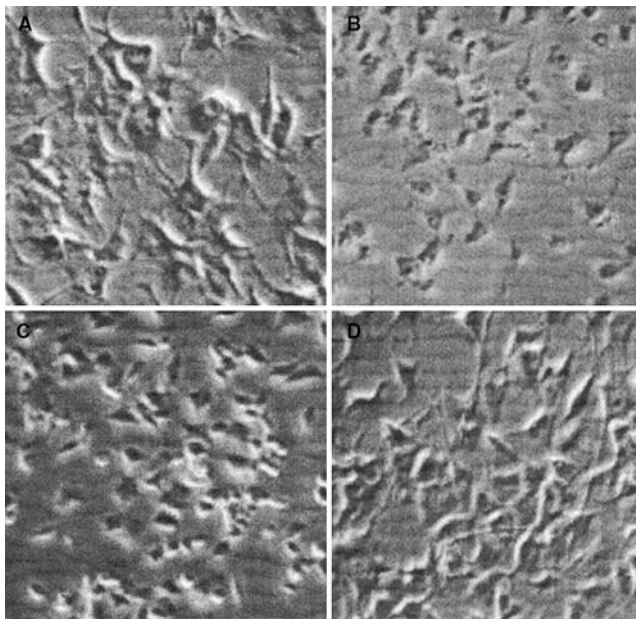
**Fig. 3** Dose-dependent effects of crocine and its analogs on the LTP-blocking effect of ethanol

The saffron extract were injected intracerebroventricularly, and the blocking effect of ethanol on the LTP decreased dose-dependently [13]. These results led us to hypothesize that crocine might antagonize the blocking effect of ethanol on the induction of LTP, as already discussed in the context of the improvement of impairments in memory acquisition. Following the activity-guided separation from the crude extract, we confirmed that crocine is the actual active component in saffron.

When a 50 mg/kg dose of crocine was injected 5 min before the administration of ethanol, LTP was induced at 84% of that that of control, suggesting that the LTP blocking effect of ethanol was improved dose dependently with the administration of crocine, as indicated in Fig. 3. The activities of the crocetin gentiobiose glucose ester and crocetin di-glucose ester, which are analogs of crocine, on the LTP blocking effect of ethanol was investigated at the same dose scale. These activities were found to be distinctly lower than that of crocine (Fig. 3). The active improvement effect against blocking was clearly proportional to the number of glucoses because crocine, 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 (Fig. 3). From this result we concluded that crocine is the actual active component in saffron related to learning and memory phenomenon.

### Inhibitory activity of crocine for PC-12 cell death induced by serum/glucose deprivation [18]

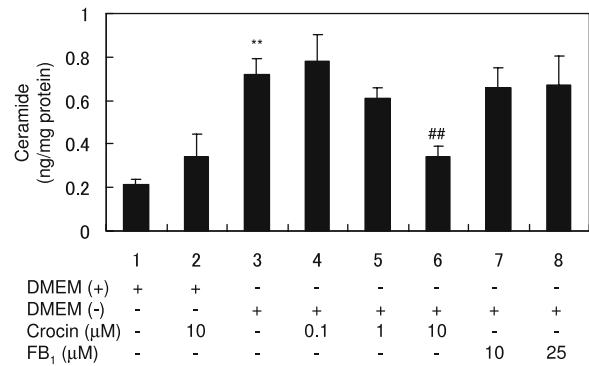
Figure 4a–d shows the effect of crocine on the morphological changes and PC-12 cell death induced by serum/glucose deprivation. Cells cultured in serum/glucose-containing Dulbecco's modified Eagle's med-



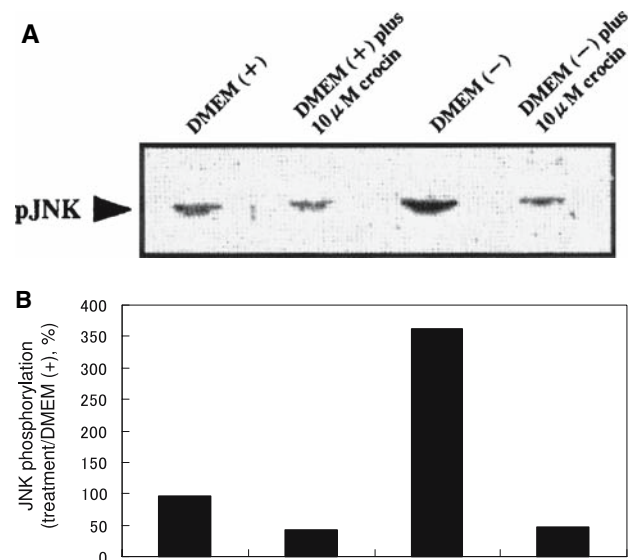
**Fig. 4** The effect of crocin on morphological changes and PC-12 cell death induced by serum/glucose deprivation. **a** Control cells in serum/glucose-containing DMEM [DMEM (+)], **b** cells in DMEM alone [DMEM (-)], **c** cells in DMEM (-) plus 0.1  $\mu$ M crocin, **d** cells in DMEM (-) plus 10  $\mu$ M crocin

ium [DMEM (+)] had a normal morphology at 24 h (Fig. 4a), 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 (Fig. 4b). We confirmed that approximately 60% cell death had occurred in the latter culture using the Trypan blue dye exclusion method. The addition of crocin (10  $\mu$ M) significantly suppressed both the morphological changes and the PC-12 cell death induced by the DMEM (-) conditions as crocin inhibited TNF- $\alpha$ -induced PC-12 cell death [19], resulting in 85% survival (Fig. 4c). It is well known that serum [20–22] or nerve growth factor (NGF) [23, 24] deprivation induces apoptosis in PC-12 cells. Colombaroni et al. [25] 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, as indicated in Fig. 5. 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  $\mu$ M [26, 27]. However, FB1 had no



**Fig. 5** Effect of crocin or FB1 on ceramide accumulation in serum/glucose-deprived PC-12 cells



**Fig. 6** Effect of crocin on phosphorylation of JNK in serum/glucose-deprived PC-12 cells

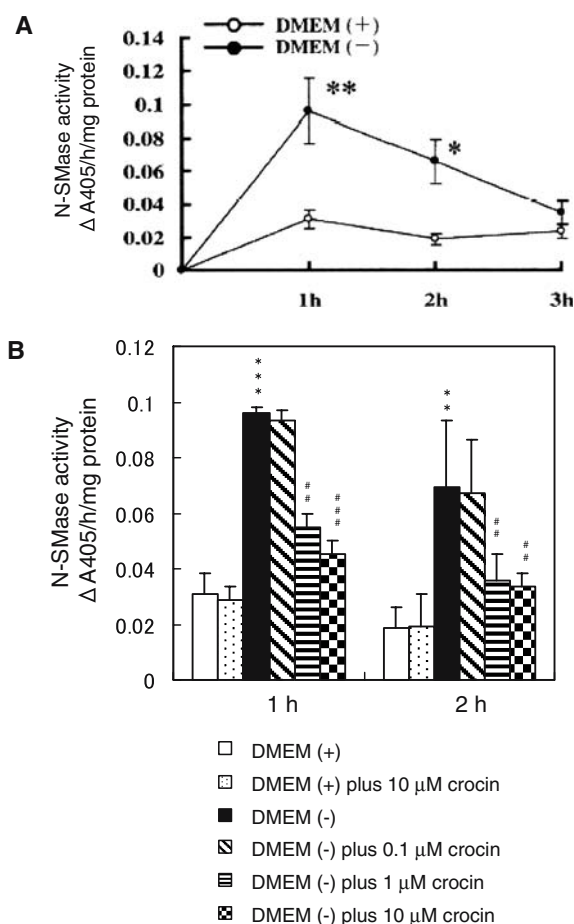
significant effect on ceramide levels, suggesting that the accumulation of ceramide through an enhancement of de novo synthesis following a 3-h culture in DMEM (-) was in itself not sufficient to explain the increase. It has been suggested that the sphingomyelin (SM) pathway and SAPK/JNK signaling systems may function together [28] 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 (Fig. 6a, b). The DMEM (-) conditions stimulated the phosphorylation of JNK in the cells by approximately 3.7-fold relative to the control cells

cultured in DMEM (+). The addition of 10  $\mu\text{M}$  crocin, however, prevented the increase in phosphorylated JNK. These results suggest that crocin inhibits ceramide accumulation in PC-12 cells induced by serum/glucose deprivation and thereby contributes to the inhibition of the ceramide-induced activation of JNK that leads to cell survival. The ceramide formed at the early stage (3 h) may be the product of SM hydrolysis by magnesium-dependent N-SMase, as observed during hypoxic PC-12 cell death [29, 30].

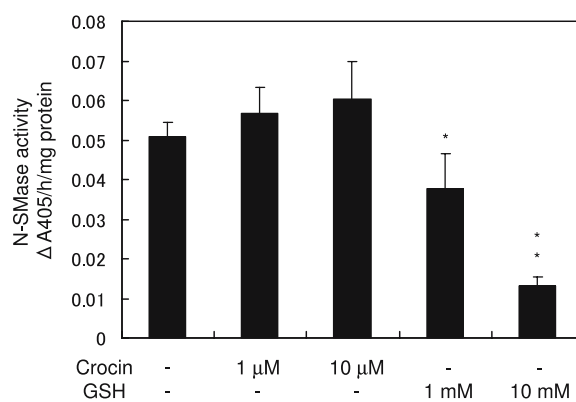
### Inhibitory effect of crocin on the activation of N-SMase induced in serum/glucose-deprived PC-12 cells [18]

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, as indicated in Fig. 7a. Under the same assay conditions, the supernatants obtained following centrifugation at 2000  $g$  exhibited considerable N-SMase activity. However, there was no time-dependent change in the N-SMase activity during a 3-h culture in DMEM (–). This assay method can detect A-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 A-SMase in PC-12 cells was unaffected by serum/glucose deprivation for at least for 3 h. Figure 7b shows the effect of crocin on the N-SMase activity was enhanced in cells cultured in DMEM (–). 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 containing a 2000  $g$  sediment of the PC-12 cell homogenate, whose cells had been cultured in DMEM (–) for 2 h.

The addition of 1 or 10  $\mu\text{M}$  crocin had no inhibitory effect on N-SMase activity in the reaction medium, as indicated in Fig. 5. However, the addition of GSH at concentrations of 1 and 10 mM inhibited the enzyme activity in a dose-dependent manner (Fig. 8). Earlier reports indicate that GSH is a physiological inhibitor of magnesium-dependent N-SMase in plasma membranes [29, 31, 32] and that N-SMase is inactive in the presence of physiological concentrations (1–20 mM) of GSH [31]. Therefore, these results suggest that the SMase activity in the reaction medium is derived from magnesium-dependent N-SMase contained in plasma



**Fig. 7** Effect of crocin on the activity of magnesium-dependent N-SMase enhancement in serum/glucose-deprived PC-12 Cells. **a** Time-dependent N-SMase activity of PC-12 cells cultured in DMEM (–), **b** dose-dependent effect of crocin on N-SMase activity in PC-12 cells cultured in DMEM (–)



**Fig. 8** Direct effects of crocin and GSH on the activity of magnesium-dependent N-SMase

membranes and that the observed N-SMase inhibition by crocin does not occur through its direct action on the enzyme. We hypothesized that crocin might

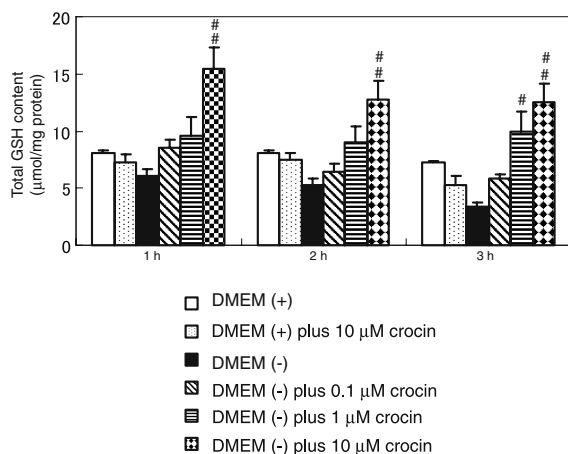


prevent the activation of N-SMase in serum/glucose-deprived PC-12 cells by a GSH-dependent inhibition mechanism.

**Increase of intracellular GSH levels in serum/glucose-deprived PC-12 cells through an increase in the activities of GR and  $\gamma$ -GCS by crocin [18]**

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. As shown in Fig. 9, the GSH levels in PC-12 cells exposed for 3 h to serum/glucose-free DMEM decreased to half 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  $\mu$ M. The concentration of GSH was high enough to inactivate N-SMase.

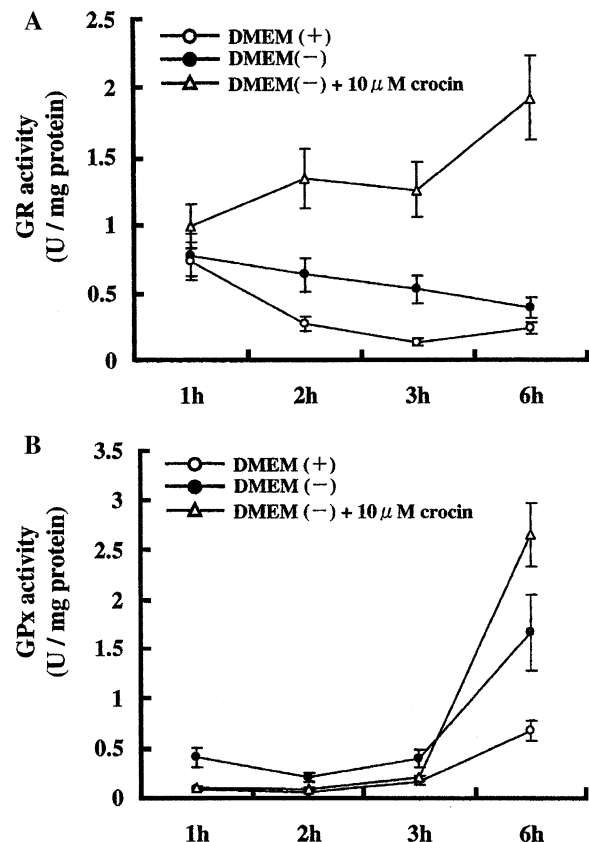
We then investigated the mechanism by which crocin increased the GSH levels. Figure 10a shows the effect of crocin on the time-dependent change in glutathione reductase (GR) activity. The GR activities in serum/glucose-deprived PC-12 cells decreased in a time-dependent fashion, whereas the co-presence of 10  $\mu$ M crocin enhanced GR activity each hour (approximately four-fold elevation at 6 h). Figure 10b shows the time-dependent change of in GPx activity in PC-12 cells cultured in serum/glucose-free DMEM alone or supplemented with 10  $\mu$ M crocin. This result indicates that crocin has no significant effect on the GPx activity in the cells.



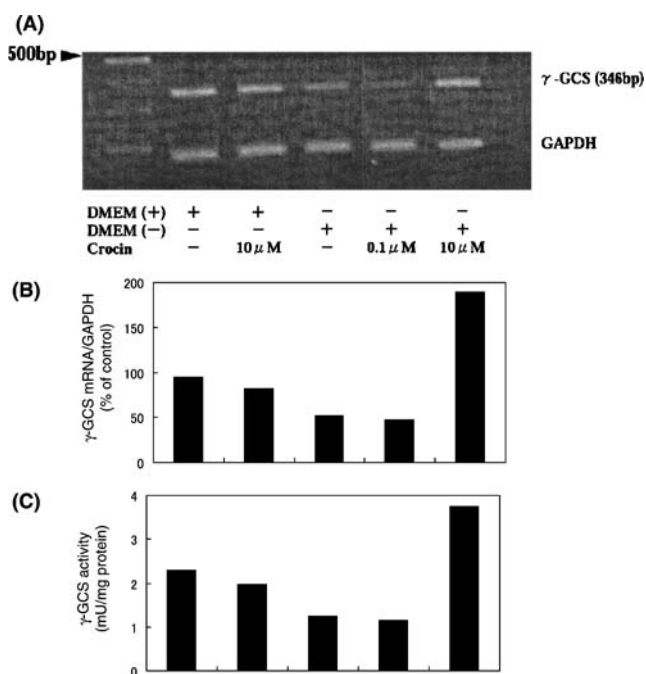
**Fig. 9** Effect of crocin on the intracellular GSH in serum/glucose-deprived PC-12 cells

GSH synthesis is regulated by the rate-limiting enzyme  $\gamma$ -GCS. This enzyme is thought to be regulated by several mechanisms. In mouse endothelial cells, the TNF- $\alpha$ - or IL-1 $\beta$ -induced increase in  $\gamma$ -GCS activity is associated with an increase in mRNA expression [33]. IL-6 also stimulates the expression of  $\gamma$ -GCS mRNA and increases the activity of this enzyme, which leads to increased GSH levels in PC-12 cells [34]. In contrast, Pan and Perez-Polo [35] reported that NGF had an ability to increase the activity of  $\gamma$ -GCS at the transcription level by extending the half-life of  $\gamma$ -GCS mRNA.

Figure 11A shows the effect of crocin on the mRNA expression and the activity of  $\gamma$ -GCS in PC-12 cells cultured in serum/glucose-rich or -free DMEM for 6 h. The addition of crocin (10  $\mu$ M) doubled  $\gamma$ -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 (Fig. 11a, b). As shown in Fig. 11c, the crocin-induced increase in  $\gamma$ -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



**Fig. 10** Effect of crocin on activity of GR and GPx in serum/glucose-deprived PC-12 cells



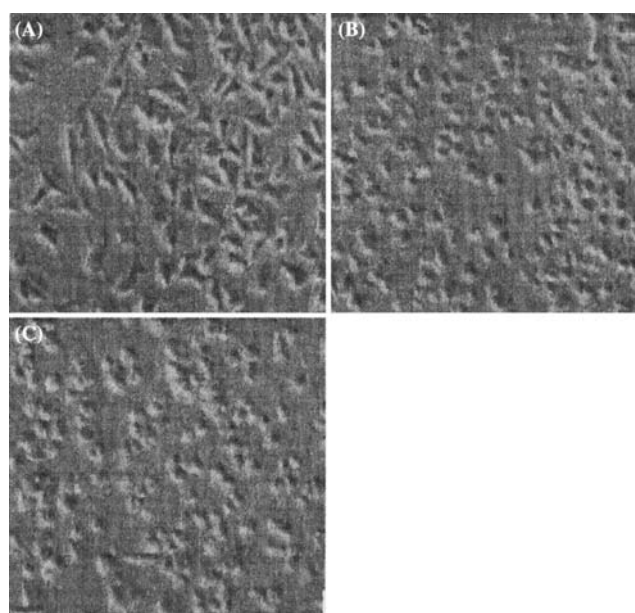
**Fig. 11** Effect of crocin on mRNA expression and activity of  $\gamma$ -GCS in serum/glucose-deprived PC-12 cells

activities of both GR and  $\gamma$ -GCS. To test whether the increase in intracellular GSH levels plays a key role in the crocin's survival-promoting effects on serum/glucose-deprived PC-12 cells, we treated the cells for 6 h with 200  $\mu$ M BSO, a commonly used inhibitor of GSH synthesis [36], and found that BSO inhibited  $\gamma$ -GCS activity in PC-12 cells at that concentration and induced intracellular GSH depletion [34].

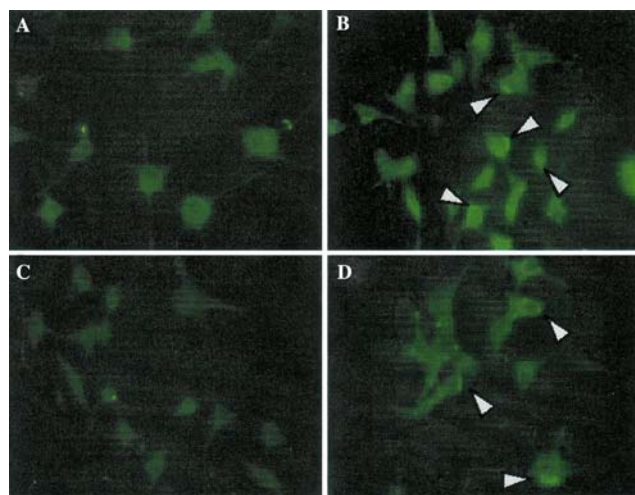
Figure 12 demonstrates that BSO can reverse the anti-apoptotic effect of crocin on the cells. In cells treated with BSO plus crocin, intracellular GSH levels decreased to around those found in the untreated cells, suggesting that crocin can prevent oxidative stress-mediated depletion of GSH in cells by promoting GSH biosynthesis. The GSH-dependent pathway involves activation of N-SMase and appears – at the very least – to be responsible for the earlier ceramide response to induction of apoptosis, as evidenced by the action of crocin presented here.

### Antioxidant effect of crocin in preventing neuronal cell death

The effects of crocin on PC-12 cells deprived of serum/glucose in comparison with those of  $\alpha$ -tocopherol have been reported [37]. Depriving the PC-12 cells of serum/glucose caused changes in the morphology and peroxidation of their membrane lipids and decreased



**Fig. 12** Combination effect of BSO and crocin on morphological changes and PC-12 cell death induced by serum/glucose deprivation. **a** 10  $\mu$ M crocin, **b** 200  $\mu$ M BSO, **c** 200  $\mu$ M BSO plus 10  $\mu$ M crocin

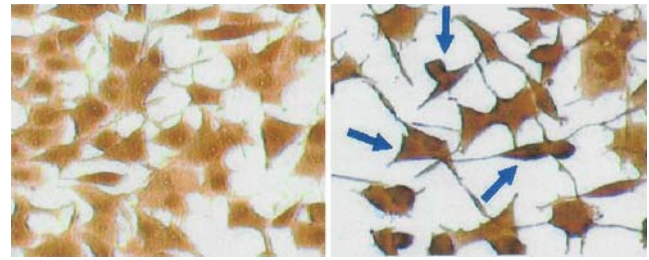


**Fig. 13** Annexin V staining of PC-12 cells exposed for 3 h in serum/glucose-deprived DMEM. **a** Control cells in DMEM (+), **b** cells in DMEM (-) alone, **c** Cells in DMEM (-) plus 10  $\mu$ M crocin, **d** cells in DMEM (-) plus 10  $\mu$ M  $\alpha$ -tocopherol. Arrows indicate ring-shape stains

intracellular superoxide dismutase (SOD) activity. Figure 13 shows Annexin V staining of the PC-12 cells cultured for 3 h in serum/glucose-free culture medium. Although phosphatidylserine (PS) residues are normally present in the inner membrane, the oxidative stress transferred these into the outer membrane leaflet. PS externalization is known as an early sign of apoptotic induction. Annexin binds to the negatively

charged PS, and the conjugated FITC shows a ring-like stain along the cellular boundary. The cells deprived of serum/glucose show strong ring-like stains (Fig. 13b) compared to the control cells (Fig. 13a). Figure 13c and d show the effects of 10  $\mu\text{M}$  crocin and  $\alpha$ -tocopherol on the serum/glucose-deprived cells. Crocin kept the cell's morphology more intact than  $\alpha$ -tocopherol. In PC-12 cells deprived of serum/glucose for 6 h, the level of peroxidized membrane lipids 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  $\alpha$ -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  $\mu\text{M}$ ). Crocin did not inhibit caspase-8 activity in the cell lysates and its inhibitory effect may be caused indirectly by the antioxidant activity.

There are eight isomers of vitamin E in nature, such as  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherols and  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocotrienols; all function as an antioxidant and a stabilizer of cell membranes [38]. Vitamin E suppresses the generation of free radicals by terminating the propagation of radical chain reactions [39] and it can block PC-12 cell death under the presence of anti-SOD antibodies [40]. Biochemically, the hydroxyl group in the vitamin E molecule may donate a proton to saturate and detoxify the unpaired electron [41]. In Alzheimer's disease models, vitamin E prevents the accumulation of oxidative metabolites, which induce amyloid  $\beta$  protein toxicity [42]. Based on the protective activity of vitamin E against amyloid  $\beta$  protein toxicity, it has been used in clinical trials for the treatment of Alzheimer's disease [43]. In a multicenter, double-blind, placebo-controlled study on 341 patients with moderately severe Alzheimer's disease, a daily dose of approximately 1,350 mg  $\alpha$ -tocopherol led to a slight – but nevertheless significant – delay in reaching institutionalization or death. These results suggest that oxidative events play an important role in initiating early neurodegenerative processes in Alzheimer's disease. Recently, Mishima et al. [44] have demonstrated that  $\alpha$ -tocotrienol is more potent as an antioxidant than  $\alpha$ -tocopherol in the prevention of cerebral infarction in mice. The key difference in the structures is the three double bonds in  $\alpha$ -tocotrienol's lipophilic tail. If antioxidant activity varies directly as the number of double bonds, then crocin, which has seven double bonds in a molecule, should be an effective antioxidant. Oral



**Fig. 14** PC-12 cells treated with 10  $\mu\text{M}$  crocin (left, 0 min) and immunostained with anti-crocin MAb (right, 30 min). Arrows indicate cells stained strongly by anti-crocin MAb

administration of saffron's extract at concentrations varying from 0.1 to 5 g/kg was nontoxic in mice [45]. Crocin prevented cerebral infarction in mice, as did  $\alpha$ -tocotrienol and  $\gamma$ -tocopherol.

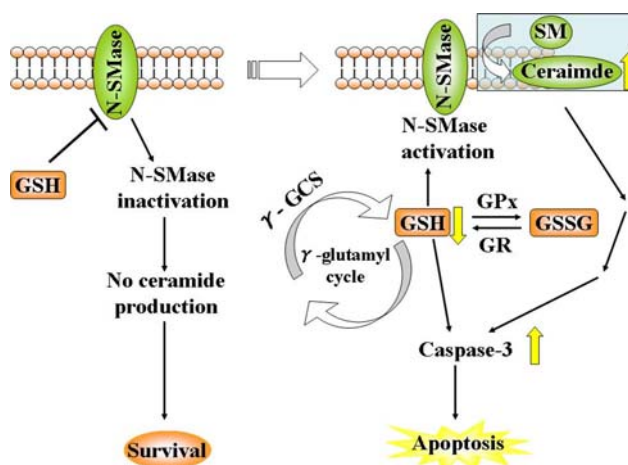
In order to confirm the localization of crocin in PC-12 cells, we immunostained cells using the anti-crocin Mab prepared in our laboratory [2]. Figure 14 shows the time course of crocin (10  $\mu\text{M}$ ) distribution in PC-12 cells over a 30-min time span. Although we concluded that crocin is localized in the cell membrane, as is  $\alpha$ -tocopherol, and that it is incorporated into the cells in a time-dependent, as we reported previously [37], the role of crocin is still obscure.

## Conclusion

It is well known that saffron is very safe because oral administration of saffron's extract at concentrations of up to 5 g/kg is still nontoxic in mice [45]. In the early stage of our investigation on the pharmacological activity of crocin, we reported that crocin is the actual active component involved both in the improvement of learning and memory [46] and with the preventive effect of LTP blocked by ethanol *in vivo* [17]. We also 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 [47]. This action of crocin may underlie the antagonism against ethanol-induced memory impairment. Thus, crocin can be used as a new pharmacological tool for studying the mechanism of ethanol inhibition of NMDA receptor functions. Based on these findings, crocin would be the compound of preference to be used for the central nervous system together with general use for anodyne, tranquil and emmenagogue.

In view of this accumulating data, we re-started our investigation on neuronal cell death. Oxidative stress is considered to play an important role in a variety of neurodegenerative disorders of the central nervous





**Fig. 15** Prevention mechanism of apoptosis by crocin

system, such as Alzheimer's disease, Parkinson's disease, Huntington's disease and ischemia [41]. We first demonstrated that crocin prevented TNF- $\alpha$ -induced apoptotic morphological changes and DNA fragmentation of PC-12 cells that was related to the caspase family [19]. In PC-12 cells, 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. Crocin can prevent the oxidative stress-mediated depletion of GSH in cells by promoting GSH biosynthesis. The GSH-dependent pathway involves activation of N-SMase and appears to be responsible for the earlier ceramide response to the induction of apoptosis. Figure 15 summarizes the occurrence of apoptosis in terms of GSH biosynthesis, ceramide release and the caspase family, as discussed already. When the effects of 10  $\mu$ M crocin and  $\alpha$ -tocopherol on the serum/glucose-deprived cells were compared, crocin was found to be more active in maintaining normal cell morphology than  $\alpha$ -tocopherol. In PC-12 cells deprived of serum/glucose, the level of peroxidized membrane lipids increased in comparison to the control cells and the decrease in SOD activity was also greater. Therefore, we conclude that crocin may have potential for treating neurodegenerative damage induced by oxidative stress. Future publications will report on the effect of crocin on the reduction of the infarcted area caused by occlusion of the middle cerebral artery in mice.

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