Prevention of PC-12 Cell Death by Crocin via Sphingomyelinase-Ceramide Signaling by Increase of Glutathione Biosynthesis

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Abstract

Crocetin glycoside, crocin is a pharmacologically active component of *Crocus sativus* L. (saffron) that has been used in traditional Chinese medicine. Previously we demonstrated that crocin inhibited apoptosis in PC-I2 cells by affecting the function of tumor necrosis factor-α. In this study, we found that depriving cultured PC-12 cells of serum/glucose causes a rapid increase in cellular ceramide levels, followed by an increase in the phosphorylation of c-jun kinase (JNK). The accumulation of ceramide was found to depend on the activation of magnesium-dependent neutral sphingomyelinase (N-SMase), but not on de novo synthesis. The serum/glucose-deprived PC-12 cells also decreased the cellular levels of glutathione (GSH), which is the potent inhibitor of N-SMase. Treating the PC-12 cells with crocin prevented N-SMase activation, ceramide production, and JNK phosphorylation. We also found that the chemical can enhance the activities of GSH reductase and glutamylcysteinyl synthase (γ-GCS), contributing to a stable GSH supply that blocks the activation of N-SMase. Thus our data suggest that crocin combats the serum/glucose deprivation-induced ceramide formation in PC-12 cells by increasing GSH levels and prevents the activation of JNK pathway, which is reported to have a role of the signaling cascade downstream ceramide for neuronal cell death.

INTRODUCTION

It has been evident that glutathione (GSH) is a major non-protein antioxidant that protects neurons from oxidative stress, and the depletion is an early event that precedes the onset of apoptosis induced by various agents (Beaver and Waring, 1995). A possibility of cell death mechanism may be occurred by the activation of magnesium-dependent neutral sphingomyelinase (N-SMase) caused by a drop in GSH levels, followed by a rapid increase in cellular ceramide levels (Liu and Hannun, l997; Liu et al., 1998; Yoshimura et al., 1999). Recently it becomes clear that ceramide stimulates the stress-activated protein kinase/c-jun kinase (SAPK/INK) signaling system that precedes induction of apoptosis (Verheij et al. 1996). Willaime-Morawek et al. (2003) have further investigated the role of JNK pathway in ceramide-induced cell death and demonstrated that p38 kinase and JNK/c-Jun act in parallel to induce neuronal apoptosis by ceramide. Several types of SMases have been identified; these include lysosomal and secreted acidic SMases (A-SMase) and cytosolic, magnesium-independent N-SMase (Levade and Jaffrezou, 1999; Perry, 1999). However, the membrane-bound magnesium-dependent N-SMase may be the SMase most closely linked to the initial production of ceramide induced by depletion of GSH (Liu and Hannun, 1997; Liu et al., 1998; Yoshimura et al., 1999; Lavrentiadou et al., 2001). GSH is synthesized in theγ-glutamyl cycle, in which γ-glutamylcysteinyl synthase (γ-GCS) is the rate-limiting enzyme (Griffith et al., 1979; Pan and Pérez-Polo, 1993). Glutathione peroxidase (GPx) removes H202 and organic peroxides by converting GSH to the oxidized form (GSSG), whereas glutathione reductase (GR) regenerates GSH in the presence of NADPH. Nerve growth factor (NGF)

prevents the PC-12 cell death from oxidative stress by increasing theγ-GCS activity (Pan and Pérez-Polo, 1993). Interleukin-6 (IL-6) is also suggested to prevent PC-12 cell death by increasing the expression ofγ-GCS mRNA, which is followed by an increase in cellular GSH levels (Nakajima et al. 2002). Therefore, chemicals having roles of these neurotrophic factors may be clinically useful.

Crocus sativus L. (saffron) is used for anodyne, traquid and emmenagogue in traditional Chinese medicine. One of the active components, crocin, is a carotenoid pigment, and has the structure of crocetin di-gentiobiose ester. We previously published that crocin exhibits a variety of pharmacological effects in mice including inhibition of skin tumor growth (Konoshima et al., 1998), improvement of learning behavior previously impaired by ethanol (Sugiura et al., 1995), and prevention of long-term potentiation caused by ethanol in rats (Sugiura et al., 1994). Here we have been investigating the effect of crocin on the N-SMase-ceramide signaling pathway. Furthermore, we show that crocin prevents the death of PC-12 cells induced by serum/glucose deprivation. The results suggest GSH-dependent inhibition of N-SMase-ceramide signaling via the enhancement of both GR and γ-GCS activities

MATERIALS AND METHODS

The rat pheochromocytoma cell line (PC-12) was obtained from the RIKEN cell bank, Ibaragi, Japan. Stock cultures of undifferentiated PC-12 cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 10% horse serum, 50 units/ml penicillin, and 100μg/ml streptomycin at 37ºC in a humidified 5% CO2 atmosphere. Prior to experiments, the growing cells were seeded in collagen-coated six-well plates and differentiated to neuronal cells in the above medium plus 50ng/ml NGF at 37ºC for 7 days.

After removal of the culture media, the differentiated PC-12 cells were rinsed twice with glucose-free DMEM. To induce cell death, the cells were immersed in serum/glucose-free DMEM without NGF. The experimental cells were put in medium containing crocin at various concentrations. They were incubated at 37°C for set periods ranging from 1 to 24 h.

The amounts of C16-ceramide in PC-12 cells were measured using the liquid chromatography/ion spray ionization mass spectrometry (LC/MS) procedure previously reported (Soeda et al., 2001a).

Western blot analysis of phosphorylated JNK Both treated and untreated cells were centrifuged, then washed with ice-cold PBS. Extraction followed for 10mm in 100 p.1 of a lysis buffer, 10mM HEPES buffer (pH 7.4) 150mM KClI3 mM magnesium acetate/0.3 mM EDTA/l00 μ.M phenylmethylsulfonyl fluoride/10% glyceroll0.5% Nonidet P-40. After centrifugation at 12,000 x g for 20 mm at 4º C, the resulting supernatants were tested for the presence of phosphorylated JNK. Protein concentration was measured with a BCA Protein Assay kit (Sigma). Equal amounts of the proteins (20 p.g each) were separated by 8% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membrane (BIORAD). The transferred protein was reacted with goat polyclonal antibodies against phosphorylated JNK 1 (Thr 183/Tyr 185) obtained from Santa Cruz Biotechnology Inc. The resulting immunocomplex was further reacted with peroxidase-conjugated secondary antibodies and made visible with 4-chloro-l-naphthol as the peroxidase substrate.

Intracellular GSH levels were measured by using a Glutathione Assay kit (Cayman Chemical Co., Ann Arbor, Ml). The cells were centrifuged, then washed with ice-cold PBS, and homogenized in 1 ml of MES buffer (pH 6.0)/i mM EDTA. After centrifugation at 10,000 x g for 15 mm at 4ºC, the supernatants were collected. Following removal of the proteins from the supernatant solutions, GSH levels were measured by the rate of colorimetric change of 5,5’-dithiobis-2-nitrobenzoic acid (Eliman' s reagent) at 405 nm according to the manufacturer's instruction.

Cells were centrifuged, then washed with ice-cold PBS and homogenized in 500 μ1 of a lysis solution containing 5mM EDTA/0.0l% digitoninl0.25% sodium cholate.

After centrifugation at 10,000 x g for 30mm at 4ºC, the enzymes in the supernatants were assayed. The activity of GPx was measured as described previously by Ochi (1990), with a slight modification. Briefly, the assay mixture contained the following components in a total volume of 1 ml: 100mM Tris-HCI buffer (pH 8.0)/0.5 mM EDTA/0.2 mM NADPH/2 mM GSH/l unit/ml GR and 10µ1of the supernatant. The assay mixture was pre-incubated at 37ºC for 5 mm. Immediately after the pre-incubation, the enzyme reaction was initiated at 37ºC by adding 10 µ1 of 7 mM t-butyl hydroxyperoxide. As soon as the reaction was started, the decrease in absorbance at 340 nm was monitored for 2-3 mm on a Shimadzu U-I 600 spectrophotometer. One unit was defined as the enzyme activity oxidizing 1 µmol of NADPH per minute. For the assay of GR (Kum-Tatt et al., 1975), the test medium contained 50mM PB (pH 7.6)/10 µM EDTA/1 µ.M NADPH/20 µM GSSG/0.001 % bovine serum albumin in a total volume of 980 p.1. After pre-incubation at 37ºC for 5 mm, the enzyme reaction was initiated at 37ºC by adding 20 µ1 of the supernatant. The decrease in absorbance at 340 nm was monitored, and the rate of NADPH oxidation was determined.

All cellular RNA was extracted from cells by using an RNeasy mini kit (Qiagen). The levels of µ-GCS mRNA in the cells were determined by RT-PCR. The mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to be coamplified with y-GCS mRNA. The RT-PCR was performed by using the SuperScriptTM One-Step RT-PCR with Platinum Taq (Invitrogen), according to the manufacturer's instructions. The primers used were as follows: y-GCS,
5'-CCTTCTGGCACAGCACGTTG-3' (forward) and
5'-TAAGACGGCATCTCGCTCCT-3' (reverse) (Mousatassim et al., 2000); GAPDH,
5'-GACCTCAACTACATGGTCTACA-3' (forward) and
5'-ACTCCACGACATACTCAGCAC-3' (reverse). PCR started at 50ºC for 30 mm and at 94ºC for 2 mm followed by 27 cycles of denaturation for 1 mm at 94ºC, annealing for 1 mm at 58ºC, and extension for 1 mm at 72ºC. The products were made visible by ethidium bromide staining under IJV radiation and photographed following 1.5% agarose gel electrophoresis. The mRNA bands in the photographs were scanned and quantified by using an NIH Imager.

The activity of γ-GCS was measured as described by Ray et al. (1999).
All values are expressed as the mean ± S.D., and the significant levels between groups were assessed by Student's t-test.

RESULTS AND DISCUSSION

Figure 1A gives the chemical structure of crocin. Crocin inhibits the PC-12 cell death induced by serum/glucose deprivation. Figure 1B-E show the effect of crocin on the morphological change and PC-12 cell death induced by serum/glucose deprivation (without NGF). A serum/glucose-rich DMEM (without NGF) kept the normal cell morphology at 24h (Figure 1B). On the other hand cells deprived of serum/glucose appeared round and showed the characteristic morphology of necrotic and/or apoptotic cells (Figure 1C). We confirmed that approximately 60% of the cells had died by using trypan blue staining. The addition of crocin prevented morphological changes resulting in 85% of the cells survived (Figure 1D) by addition of 10 µ.M crocin. Serum (Oppenheim, 1991; Batistatou and Green, 1991; Rukenstein et al., 1991) or NGF (Mesner et al., 1992; Pittman et al., 1993) deprivation induces apoptosis in PC-12 cells. We also observed the fragmented DNA in the cell nuclei by electrophoretic analysis (data not shown). A recent study by Colombaioni et al. (2002) found that serum deprivation increases intra-cellular ceramide levels in undifferentiated HN9.1e cells and induces their apoptosis. These findings suggest that ceramide levels in PC-12 cells may increase in the absence of serum/glucose.

Figure 2 shows the effects of crocin and FB1 on ceramide levels. FB1 inhibits de novo ceramide synthesis in cells at a concentration range of 10-30 µM (Wang et al., 1991; Merrill et al., 1993). When PC-12 cells were kept in serum/glucose-free DMEM for 3 h a 3.5-fold increase of ceramide levels was observed. The suppressive effect of crocin on

ceramide levels was dose-dependent resulting in ceramide levels varied inversely as the crocin concentration. FB1 had no significant effect on the ceramide levels, suggesting that the accumulation of ceramide following a 3 h exposure to serum/glucose-free conditions is not caused by an enhancement of de novo synthesis. During stress-induced cell death in U937 cells and BAE cells, the SM pathway and SAPK/JNK signaling systems may be coordinated (Verheij et al., 1996).

Figure 3A and B show the effect of crocin on JNK phosphorylation. Serum/glucose-free conditions increases INK phosphorylation to approximately 3.7 times that in the control cells. The presence of 10 p.M crocin prevented the increase in phosphorylated JNK. These results suggest that crocin inhibits ceramide accumulation in PC-12 cells, and thereby contributes to the inhibition of ceramide-induced activation of INK that leads to the cell death. The ceramide that was formed early (within 3 h) may be the product of SM hydrolysis by magnesium-dependent N-SMase as it is observed during hypoxic PC-12 cell death (Yoshimura et al., 1998, 1999).

In order to confirm the origin of the accumulated ceramide, we analyzed the activity of magnesium-dependent N-Smase in PC-12 cell homogenate. As shown in Figure 4, addition of 1 or 10 μ.M crocin had no inhibitory effect on N-SMase activity in the reaction medium. However, the addition of GSH at concentrations of 1 and 10 μM inhibited the enzyme activity in a dose-dependent manner. Earlier reports indicate that OSH is a physiological inhibitor of magnesium-dependent N-SMase present in plasma membranes (Liu and Hannun, l997; Liu et al.. 1998; Yoshimura et al., 1999). In cells, the N-SMase, but not A-SMase, is inactive in the presence of physiological concentrations (1-20 mM) of GSH (Liu and Hannun, 1997). Therefore, the results suggest that the SMase activity in the reaction medium is caused by magnesium-dependent N-SMase present in plasma membranes and that the observed N-SMase inhibition by crocin is not caused by its direct action to the enzyme. We hypothesize that crocin might prevent the activation of N-SMase in serum/glucose-deprived PC-12 cells by a OSH-dependent mechanism of inhibition.

For the above hypothesis, we first investigated 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 by half compared with levels in the control cells as indicated in Figure5. However, the addition of crocin to the medium increased the intracellular GSH levels in a dose-dependent manner and maintained higher than normal levels for 3 h. The most significant effect of crocin was seen at a concentration of 10 mM.

GSH synthesis is regulated by the rate limiting enzyme γ-GCS. This enzyme is thought to be regulated by several mechanisms. In mouse endothelial cells, TNF-α or treated with BSO plus crocin, intracellular GSH levels decreased to near those in the untreated cells (data not shown). These findings suggest 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 least to be responsible for the earlier ceramide response to induction of apoptosis, as evidenced by the presented crocin's action. IL-I 13-induced increase in y-GCS activity is associated with an increase in the mRNA expression. IL-6 also stimulates the expression of y-GCS mRNA and increases the enzyme activity, which leads to increased OSH levels in PC-12 cells (Nakajima et al., 2002). In contrast, Pan and Pérez-Polo (1993) reported that NGF had no ability to increase the activity of y-GCS at the transcription level; rather ft extended the half-life of γ-GCS mRNA.

Figure 6A shows the effect of crocin on the mRNA expression and activity of γ-GCS in PC- 12 cells exposed to serum/glucose-rich or -free DMEM for 6 h. Crocin (10 μM) doubled the γ-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 (Figure 6A and B). As shown in Figure 6C, the crocin-induced increase in y-GCS mRNA expression is reflected in an increase in the enzyme activity of the cells. These results suggest that crocin can increase GSH levels by increasing the activities of both GR and γ-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 with 200 mM BSO for 6h. BSO is a widely used inhibitor of GSH synthesis (Griffith et al., 1979). It inhibits (he y-GCS activity in PC-12 cells at the concentration used and causes the intracellular GSH depletion (Nakajimae al., 2002).

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Figures

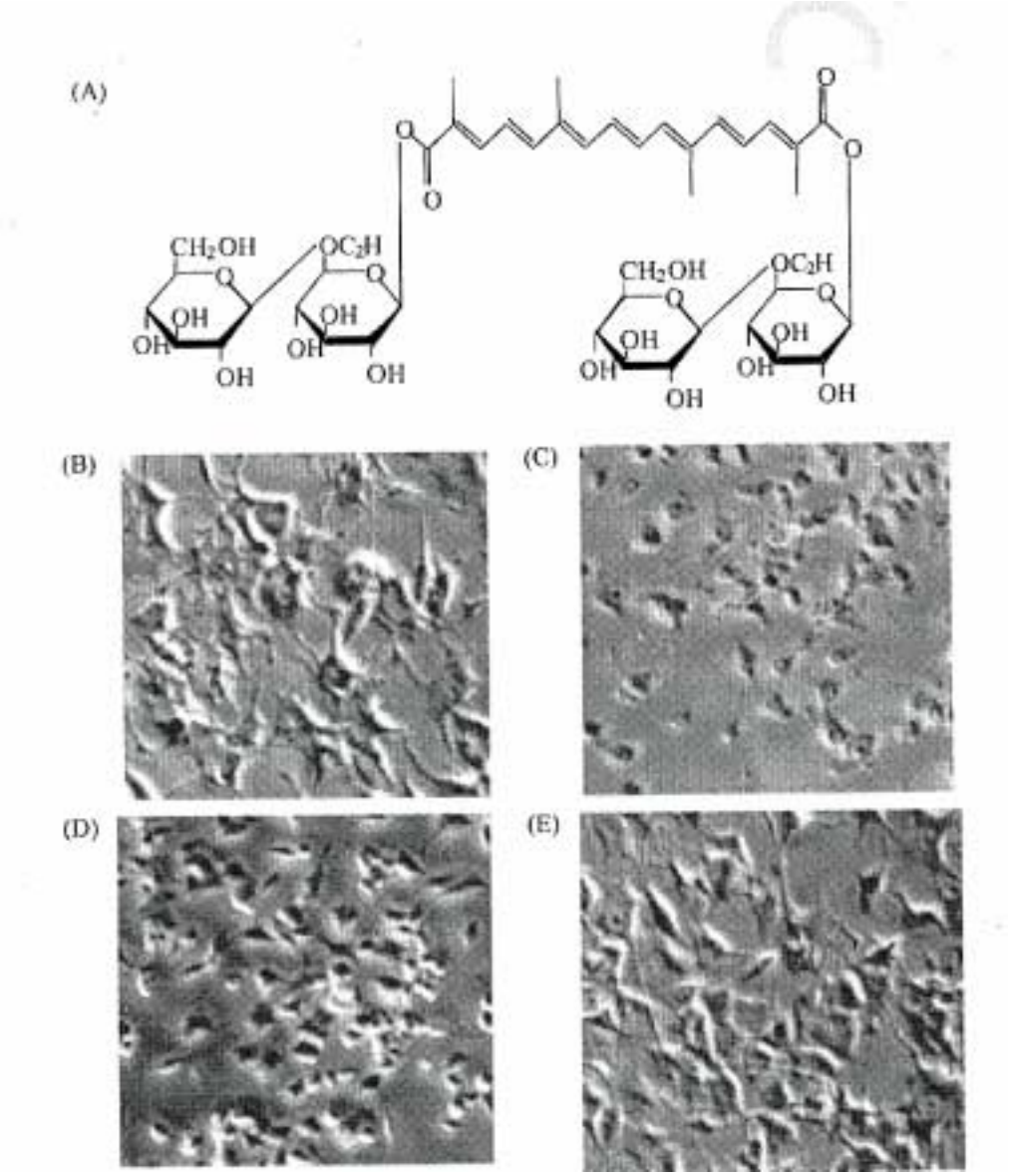

Fig. 1. Structure of crocin (A) and morphological changes of PC-12 cells (B-E) treated by crocin. B: control cells in serum/glucose containing medium C: cells cultured in medium without serum/glucose D: cells cultured in medium supplemented with 0.1 μM crocin without serum/glucose E: cells cultured in medium supplemented with 10 μM crocin without serum/glucose.

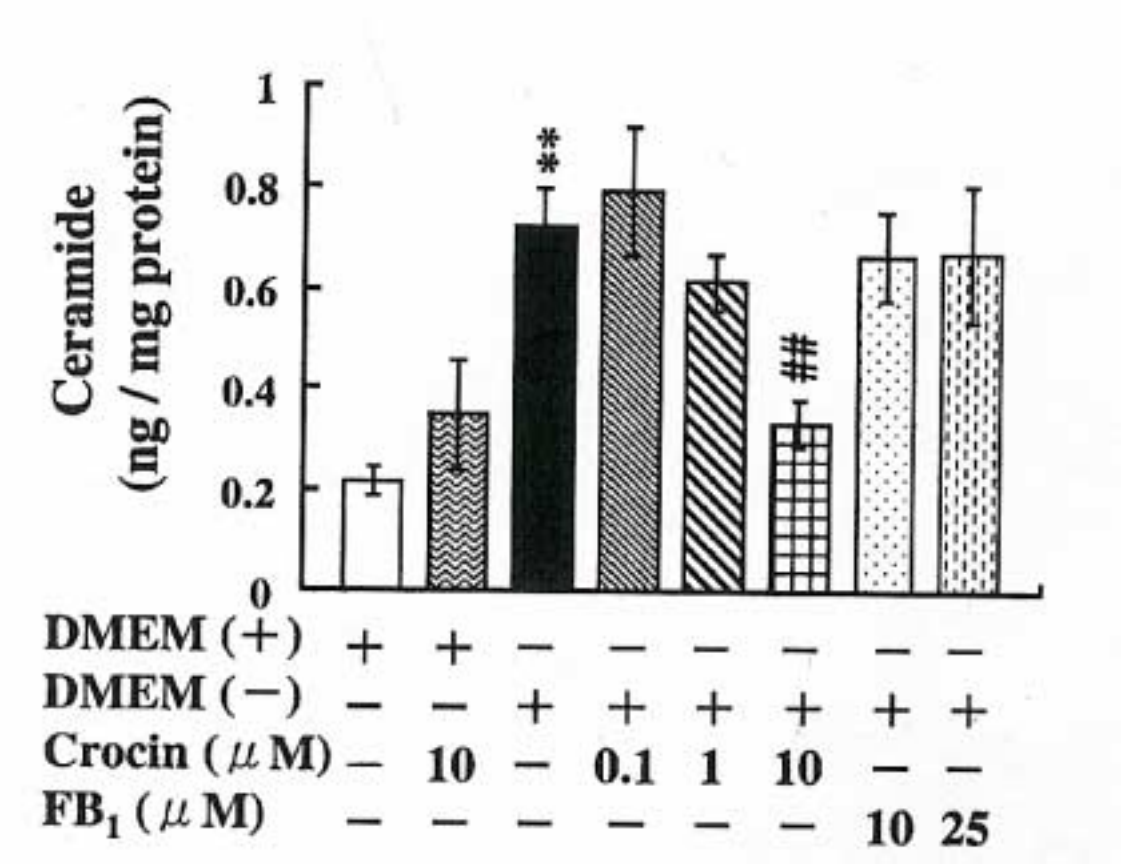

Fig. 2. Effect of crocin and FB1 on the ceramide accumulation in serum/glucose-deprived PC-12 cells.

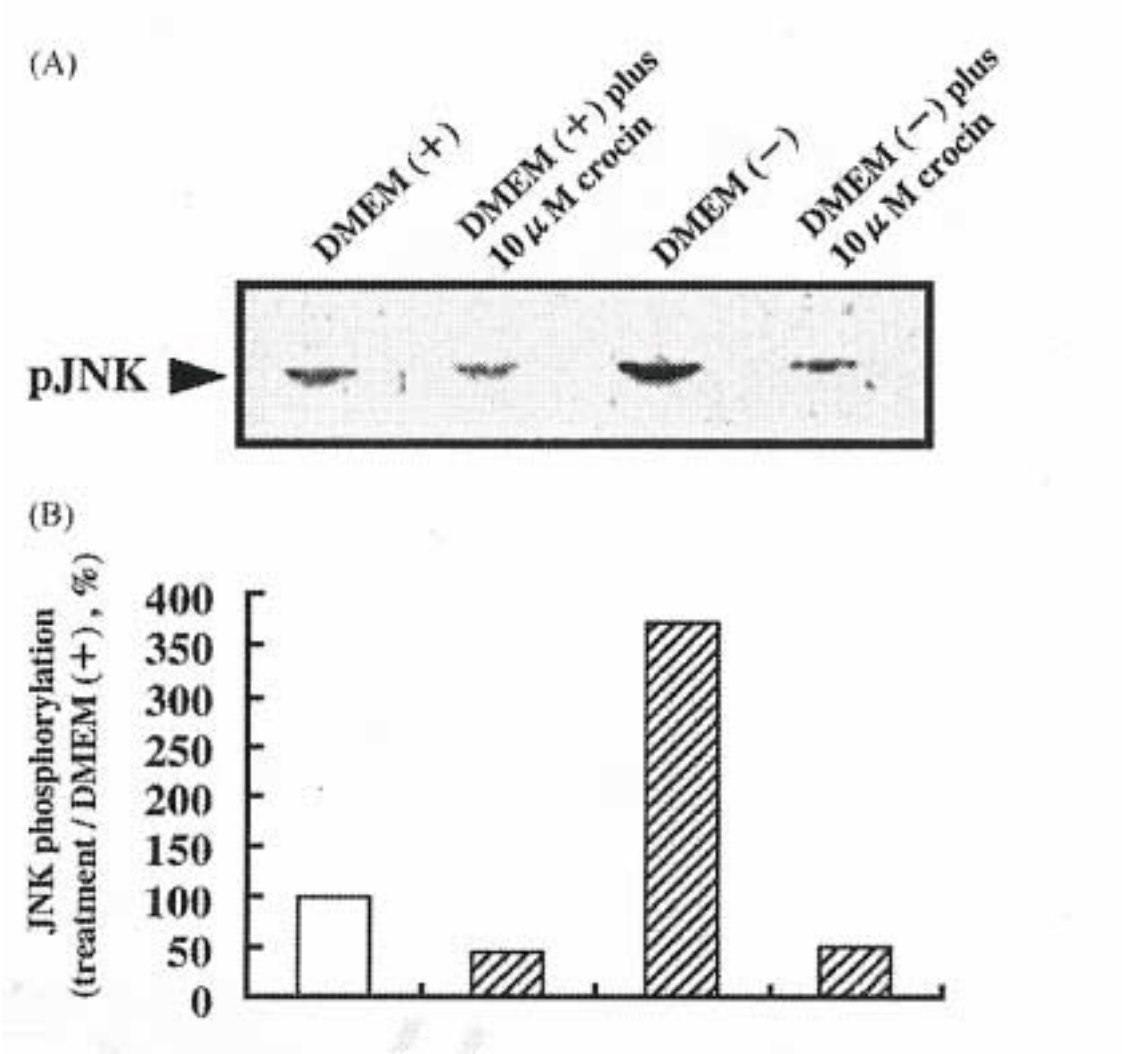

Fig. 3. Effect of crocin on the phosphorylation of JNK in serum/glucose-depending PC-12 cells. A: western blotting. B: JNK phosphorylation in serum/glucose and crocin containing medium.

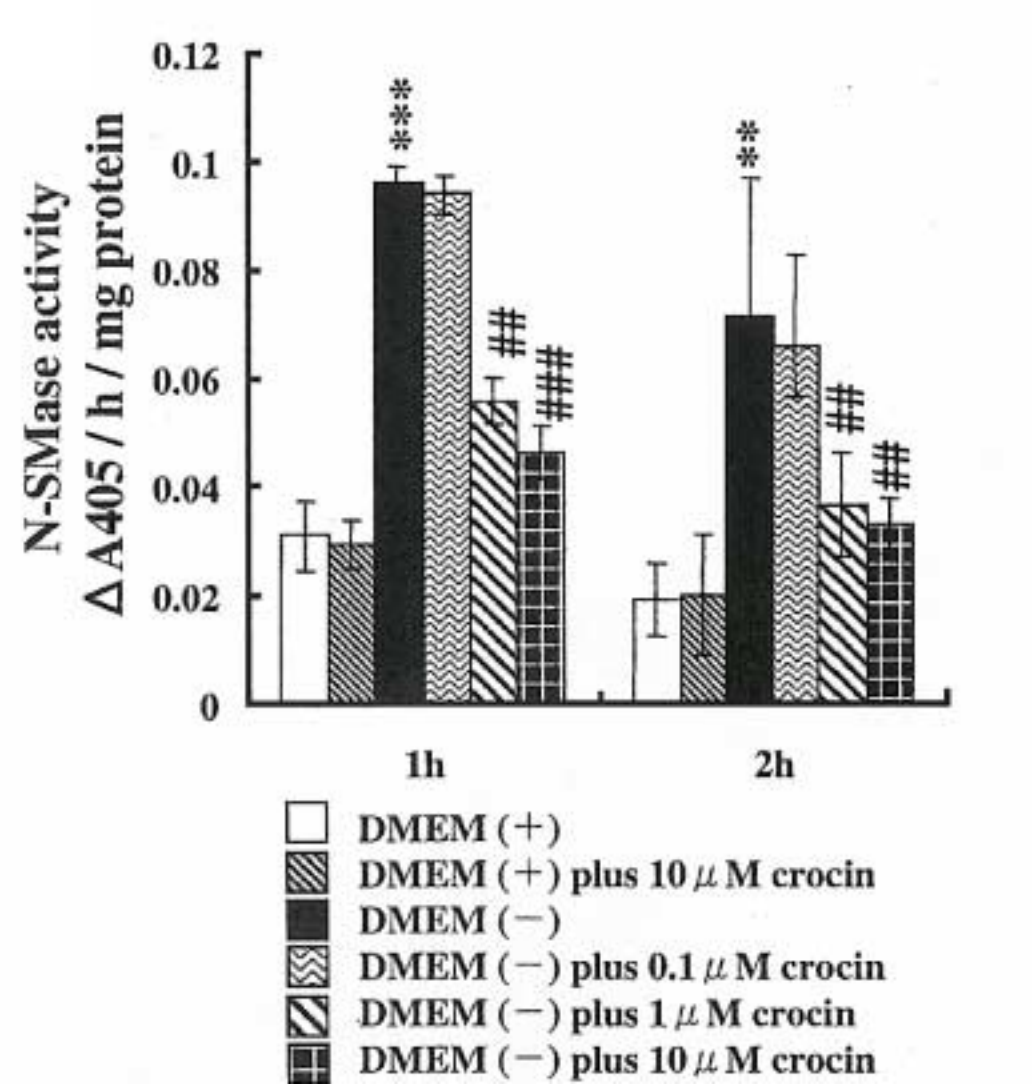

Fig. 4. Effect of crocin on activation of magnesium-dependent N-SMase in serum/glucose-deprived PC-12 cells.

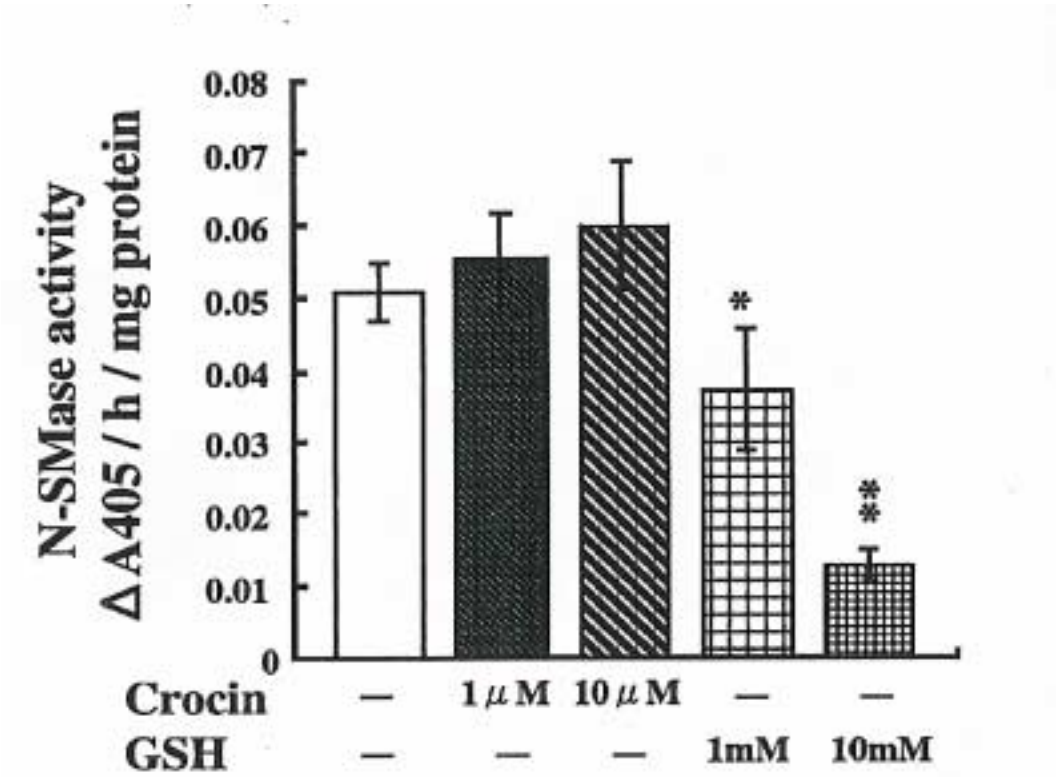

Fig. 5. Effect of crocin and GSH on activation of magnesium-dependent N-Smase.

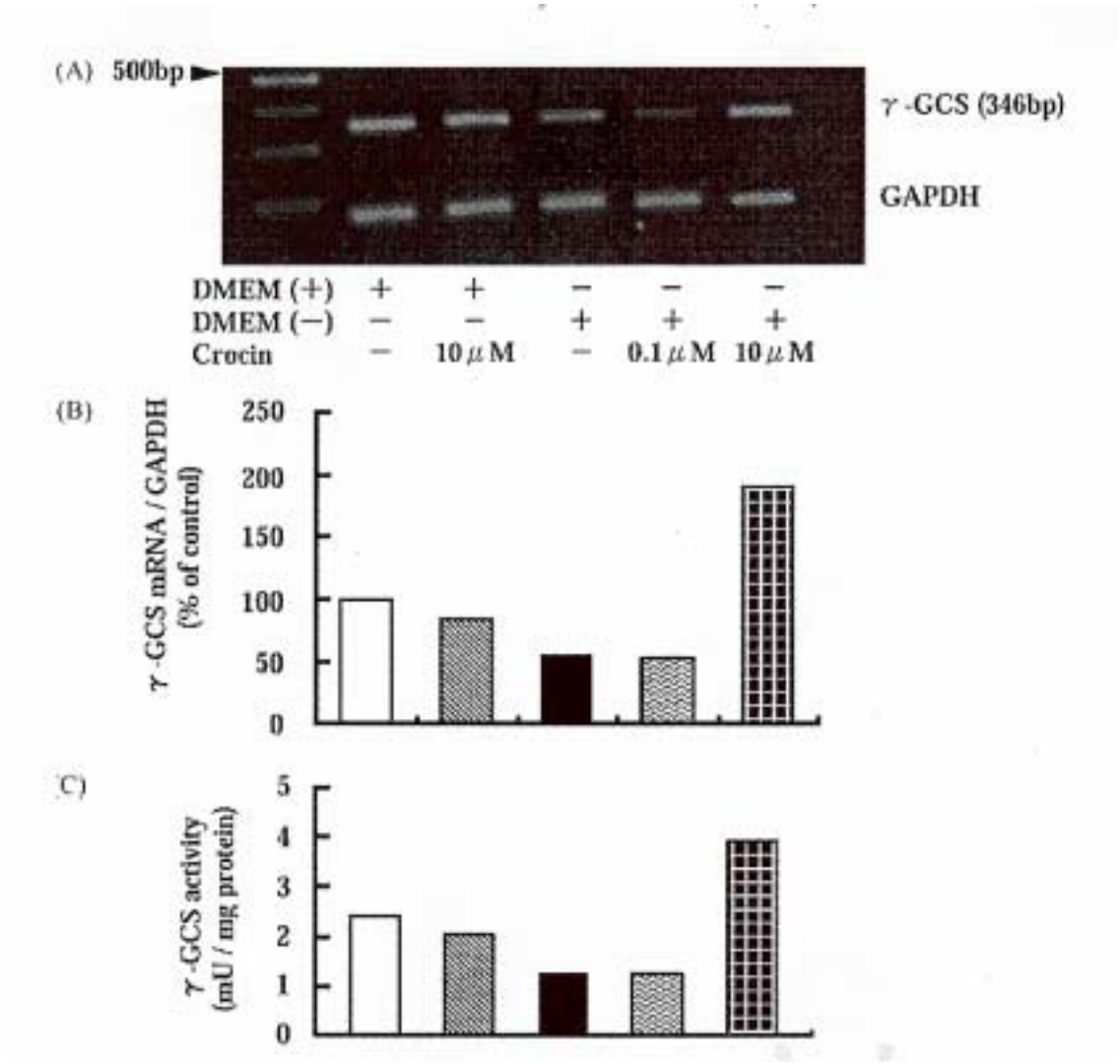

Fig. 6. Effect of crocin on mRNA expression and activity of γ-GCS in serum/glucose-deprived PC-12 cells.