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Sargaquinoic acid supports the survival of neuronal PC12D cells in a nerve growth factor-independent manner

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Abstract

Sargaquinoic acid (designated previously as MC14) was isolated from a marine brown alga *Sargassum macrocarpum*, and has been found to possess a novel nerve growth factor (NGF)-dependent neurite outgrowth promoting activity in PC12D cells. In this study, we explored the neuroprotective effects of MC14 in terms of its survival supporting, antioxidant and neurite-regenerating activities under NGF deficient or deprived conditions. Intriguingly, MC14 did not only promote the NGF-induced survival support on neuronal PC12D cells, but also significantly abated neuronal PC12D cell death even in the absence of NGF. The pharmacological inhibition of phosphatidylinositol-3 kinase (PI3K) by wortmannin significantly suppressed the survival supporting activity of MC14, whereas the NGF receptor (tyrosine kinase A or TrkA) inhibitor K252a showed no detectable effect on MC14 activity. These results demonstrate that MC14 supports survival of neuronal PC12D cells in an NGF-independent manner, and that PI3K may be required for the neuroprotective activity of MC14. In addition, we have shown that MC14 markedly enhanced neurite-regeneration and protected PC12D cells from hydrogen peroxide (H_2O_2)-induced oxidative stress. These pharmacological features suggest that MC14 may be a potentially important neuroprotective agent.

Keywords: Neuronal PC12D cells; Nerve growth factor; Survival supporting activity; Neurite regeneration; Phosphatidylinositol-3 kinase; Sargaquinoic acid

1. Introduction

Nerve growth factor (NGF) plays pivotal roles in supporting neuronal survival, inducing differentiation and repairing injured neurons (Barde, 1994; Connor and Dragunow, 1998; Hefti and Weiner, 1986). Several lines of evidence have suggested NGF's potential to treat neurodegenerative disorders such as Alzheimer's disease. For instance, a number of animal tests have demonstrated that NGF treatment could ameliorate age-related memory impairment in rats, reduce lesion-induced cholinergic neuronal degeneration in the non-human primate brain after fimbrial transection, and prevent neuronal loss in the hippocampus in a cerebral ischemia model of Mongolian gerbil (Burgos et al., 1995; Fischer et al., 1987; Shigeno et al., 1991). The administration of NGF early in the disease process could potentially prevent much of the cholinergic neuronal atrophy observed in the basal forebrain of Alzheimer's disease patients in clinical trials (Saffran, 1992; Seiger et al., 1993). These results underlie the rationale for the use of NGF to treat neurodegenerative diseases. However, the delivery of exogenous NGF has become the greatest obstacle for its therapeutic application since NGF is a large polypeptide molecule that does not penetrate the blood–brain barrier, and is easily metabolized by peptidases when administered peripherally. As an alternative approach, the use of an NGF-potentiating substance with a low molecular weight, or a small chemical that can mimic the effect of NGF has been proposed to be a promising strategy (Brinton and Yamazaki, 1998).

We have been searching for such a neuroactive substance from marine algae (Kamei and Sagara, 2002) and have previously reported the isolation and purification of a novel neuroactive substance (designated as MC14) from the brown alga, *Sargassum macrocarpum* (Tsang et al., 2001a,b). MC14 exhibited a marked NGF-dependent neurite outgrowth promoting activity toward PC12D cells, which can rapidly differentiate into sympathetic neuron-like cells and extend neurites after NGF treatment (Katoh-Semba et al., 1987). By nuclear magnetic resonance and mass spec-

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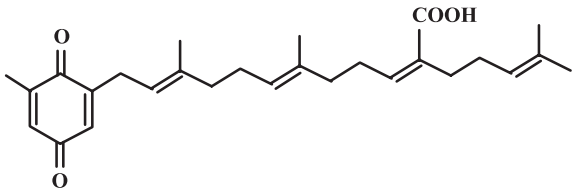

Fig. 1. Chemical structure of MC14.

trometric analyses, the chemical structure of MC14 was elucidated to be sargaquinoic acid with a molecular weight of 424 (Fig. 1) (Tsang et al., 2001a,b). More recently, we have reported that both tyrosine kinase A (TrkA)-mitogen activated protein kinases (MAPK) pathway and an adenylate cyclase-protein kinase A (PKA) pathway are required for the neurite outgrowth promoting activity of MC14 (Kamei and Tsang, 2003). Since MC14 is a small lipid-soluble molecule with potent NGF-potentiating activity, we sought to examine its neuroprotective activity in an attempt to provide a more comprehensive evaluation of MC14 with regard to its therapeutic potential for treating neurodegenerative diseases.

The PC12 cell line has been extensively employed as a model system to study the mechanisms of apoptosis as well as the neuroprotective activity of neurotrophic factors on neuronal cells. Neuronally differentiated PC12 cells require NGF for survival in serum-free medium, while deprivation of NGF induces apoptotic cell death (Mesner et al., 1992, 1995). In this study, we used PC12D cells (a subline of PC12 cells) to investigate the survival supporting activity of MC14 in NGF-deprived and NGF-deficient serum-free medium. In addition, the signaling pathways involved in the survival supporting activity of MC14 were investigated by using several pharmacological inhibitors. These results will provide insight into the signal transduction pathways regulating neuronal survival and the action of MC14 on neuronal cells. On the other hand, it has been reported that NGF protected cells against hydrogen peroxide (H_2O_2)-induced apoptosis (Jackson et al., 1990). The protective effect of MC14 against H_2O_2-induced oxidative stress was investigated. Furthermore, the NGF-dependent neurite regenerating activity of MC14 to PC12D cells was studied in order to obtain a comprehensive evaluation of the therapeutic potential of MC14 as a neuroprotective agent.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM; high glucose) and horse serum were purchased from Gibco RBL. Nerve growth factor (2.5S NGF), penicillin, streptomycin, ampicillin, fetal brovin serum, H_2O_2 (30%), chelerythrine chloride and wortmannin were purchased from Sigma. PC12D cells were kindly provided by Dr. M. Sano of Aichi Colony Development Disorder Research Center, Japan. The inhibitors K252a and 2′-amino-3′-methoxyflavone (PD98059) were purchased from Calbiochem. SC-3010 (TTYADFIASGRTGRRNAIHD), a peptide inhibitor of protein kinase A, was purchased from Santa Cruz Biotechnology. MC14 was isolated and purified from *S. macrocarpum* as described (Tsang et al., 2001a,b).

2.2. Cell culture and preparation of neuronal PC12D cells

PC12D cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal brovin serum, 100 U/ml penicillin, 200 μg/ml streptomycin and 25 μg/ml ampicillin (complete medium) in a water-saturated atmosphere of 5% CO_2 at 37 °C. PC12D cells were differentiated into neuronal PC12D cells by incubating in complete medium supplemented with 50 ng/ml NGF for 48 h. Neuronal PC12D cells were harvested and washed in serum-free DMEM by three cycles of centrifugation–resuspension.

2.3. Assay of the survival supporting activity of MC14

Neuronal PC12D cells (5×10^4 cells/well) were seeded on poly-L-lysine coated 96-well microplate. NGF and/or MC14 were added to the cultures in serum-free DMEM medium. After 24 h incubation, the percentage of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a microplate reader (BIO-RAD, model 450) as described by Hansen et al., 1989. Cell viability of each culture was expressed as a percentage relative to positive control (100 ng/ml NGF, a predetermined optimal concentration for supporting cell survival in serum-free medium).

2.4. Pharmacological inhibitor treatment of neuronal PC12D cells

After the neuronal PC12D cells were seeded on 96-well plate as described above, an aliquot of each inhibitor was added to the cell cultures, and were further incubated for 1 h before the addition of NGF or/and MC14. The concentrations of each inhibitor used in the tests could effectively block their corresponding kinase activities as previously described (Bohm et al., 1995; Hiruma et al., 1999; Parran et al., 2003; Schonhoff et al., 2001; Shibata et al., 2003). After 24 h treatment, cell viability was determined by MTT assay. The following stock concentrations of inhibitors dissolved in the indicated solvents were used in the experiments: PD98059 (20 mM, dimethyl sulfoxide (DMSO)); K252a (200 μM, methanol); chelerythrine chloride (0.5 mg/ml, DMEM); wortmannin (20 μM, DMSO). Each inhibitor was further diluted with serum-free DMEM to a suitable working concentration. An aliquot of each inhibitor was then added to the test wells in less than 3% v/v of the medium, at which no solvent effect was observed on the neuronal PC12D cells.

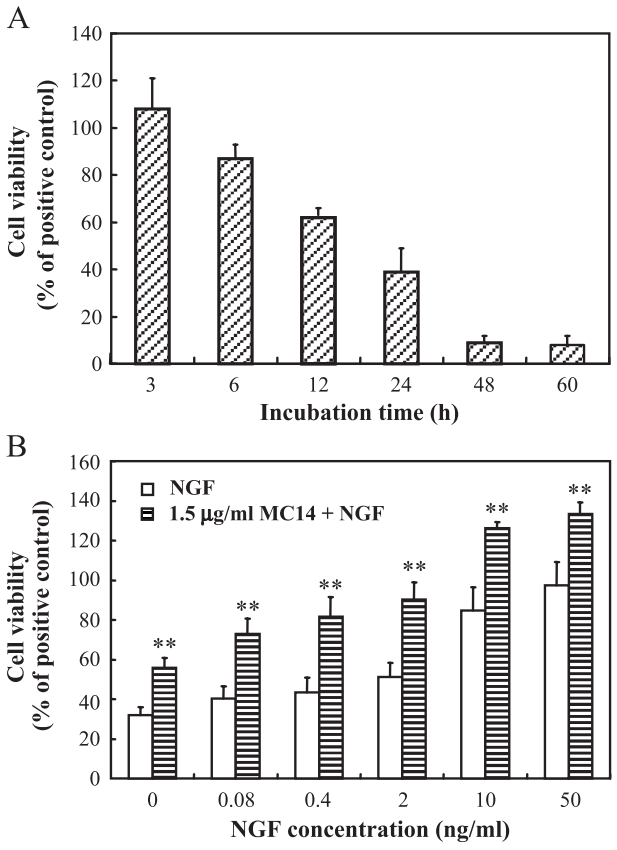

Fig. 2. Survival supporting activity of MC14 in the absence or presence of NGF in serum-free medium. (A) Viability of neuronal PC12D cells incubated in NGF-deprived serum-free medium for a period of 60 h. (B) Neuronal PC12D cells were incubated with the indicated concentrations of NGF in the absence or presence of 1.5 μg/ml MC14 in serum free medium for 24 h. Cell viability was determined by MTT assay and is expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Data are given as mean ($n=4$) ± S.D. Significant difference from MC14-untreated control: $^{**}P<0.01$ (Student's *t*-test).

2.5. Assay of H_2O_2-induced cell death and protective effect of MC14

PC12D cells were seeded on poly-L-lysine coated 96-well culture plate at a density of 5×10^4 cells/well in complete medium for 24 h. Then, NGF and MC14 were added to the cell cultures. After incubation for 2 h, H_2O_2 (freshly prepared from 30% stock solution prior to each experiment) was added to the cultures at a final concentration of 400 μM for producing oxidative stress (Lee et al., 2000). Cell viability was determined by MTT assay after the H_2O_2 treatment for 6 h.

2.6. Assay of neurite regeneration-promoting activity of MC14

Neurites of the neuronal PC12D cells were sheared mechanically as described (Twiss and Shooter, 1995). The neurite-sheared cells were washed three times with serum-free medium, seeded on a poly-L-lysine coated 96-well plate at a cell density of 5×10^3 cells/well in complete medium containing 0–50 ng/ml NGF with or without 3 μg/ml MC14. The proportion of cells with regenerated neurites was determined after 48 h. Cells with neurites twice the length of their cell body were counted as neurite-regenerated cells.

2.7. Statistical analysis

Each datum point represents the mean ± S.D. ($n=4$). Significant difference from the control was determined by Student's *t*-test. $P<0.05$ was considered to be a significant difference.

3. Results

3.1. Survival supporting activity of MC14 under serum-deprived conditions

We first determined the behavior of neuronally differentiated PC12D cells (referred as 'neuronal PC12D' hereafter) in NGF-deprived serum free medium by measuring their viability using the MTT assay. As a positive control, cells were treated with 100 ng/ml NGF, which was predetermined

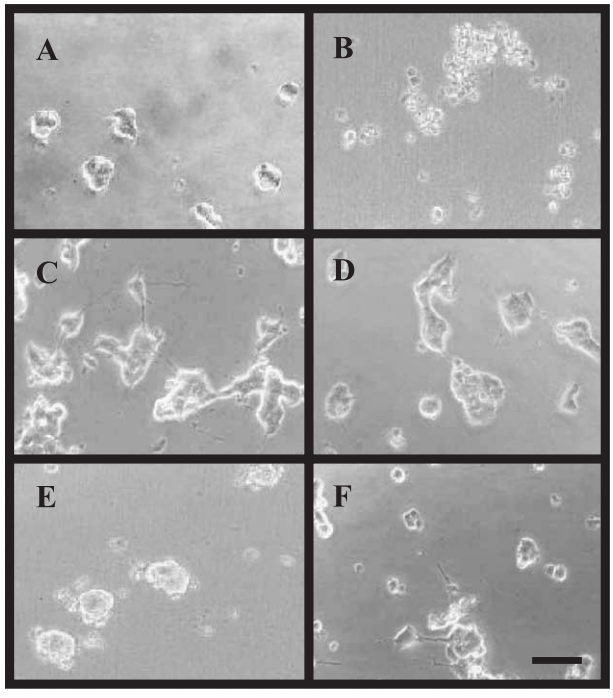

Fig. 3. Morphologies of neuronal PC12D cells after various treatments for 24 h in serum free medium. (A) Neuronal PC12D cells prior to NGF-deprived treatment. The neurites of neuronal PC12D cells were removed during the harvesting and washing processes. (B) Neuronal PC12D cells cultured in the serum free medium only. (C) Neuronal PC12D cells cultured in 100 ng/ml NGF. (D) Neuronal PC12D cells treated with 1.5 μg/ml MC14 only. (E) Neuronal PC12D cells treated with 0.4 ng/ml NGF only. (F) Neuronal PC12D cells treated with 0.4 ng/ml NGF and 1.5 μg/ml MC14. Scale bar: 20 μm.

to be capable of supporting the survival of almost 100% of cells (see below). As shown in Fig. 2A, the number of viable cells decreased by more than 50% after an incubation time of 24 h in NGF-deprived serum free medium. This result confirmed that the kinetics of cell death of neuronal PC12D cells resembled that of sympathetic neuron after NGF withdrawal under serum free conditions (Mesner et al., 1995).

The survival promoting effect of MC14 was examined at a predetermined optimal effective concentration of MC14 (1.5 μg/ml) in the presence of 0–50 ng/ml NGF in serum free medium. As shown in Fig. 2B, the number of viable cells was significantly enhanced compared to those treated with NGF alone. In addition, the percent of surviving cells reached 140% at 50 ng/ml NGF. It is notable that the treatment of MC14 prevented neuronal cell death even at an extremely low concentration of NGF, as more than 40% of cells were rescued by MC14 at 0.08 ng/ml of NGF. Interestingly, the survival supporting activity of MC14 did not seem to require NGF since MC14 alone also significantly enhanced cell viability ($P<0.01$). In addition, the activity of MC14 appeared to be primarily additive with increasing NGF concentration since the increase in cell viability due to MC14 remained relatively constant from 0.08 to 50 ng/ml NGF. This result demonstrates that MC14 does not require NGF for its survival supporting activity, and that two parallel signaling pathways might be involved in effecting MC14 and NGF on neuronal PC12D cells.

The morphological changes of neuronal PC12D cells after various treatments are shown in Fig. 3. At 100 ng/ml NGF, almost no shrunken cells were observed and some cells even showed extended neurites. In contrast, a substantial number of cells shrunk in serum free medium, while MC14 exhibited survival supporting activity in neuronal PC12D cells in the absence or presence of a deficient level of NGF in serum free medium.

To dissect the intracellular signaling pathways responsible for the observed survival supporting effect of MC14, several specific inhibitors were used to specifically shut

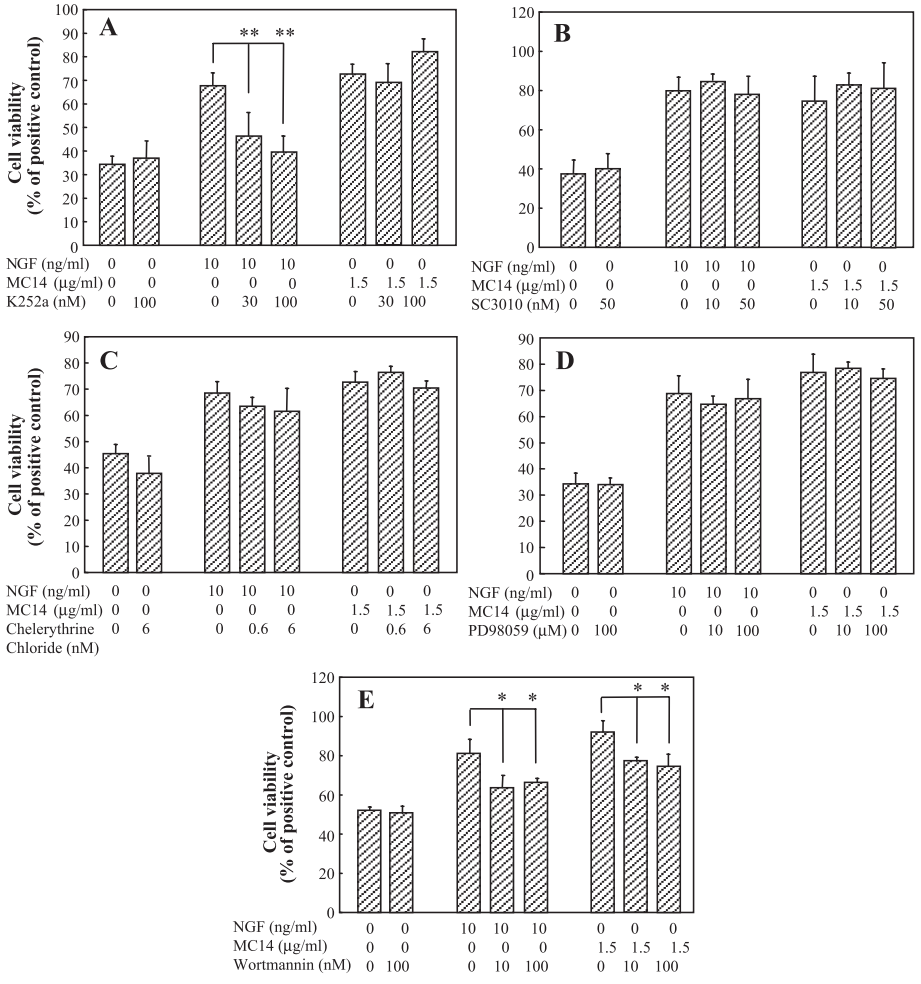

Fig. 4. Effect of various signaling protein inhibitors on MC14 or NGF treated neuronal PC12D cells. Neuronal PC12D cells were pretreated with or without (A) K252a, (B) SC3010, (C) chelerythrine chloride, (D) PD98059 and (E) wortmannin for 1 h before the addition of 1.5 μg/ml MC14 or 10 ng/ml NGF in serum free medium. After 24 h incubation, cell viability was determined by MTT assay, and is expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Data are given as mean ($n=4$) ± S.D. Significant difference from inhibitor-untreated control: *$P<0.05$ **$P<0.01$ (Student's t-test).

down some of the representative intracellular signaling pathways. First, we examined whether MC14 acts on the NGF-receptor or other downstream intracellular kinases. The action of NGF is initiated by binding to its specific high affinity receptor, TrkA. Upon NGF binding, TrkA autophosphorylates and initiates the intracellular signaling cascade. We used K252a, an inhibitor of phosphorylative events associated with TrkA signal transduction pathway activation, to pretreat the cells, followed by the evaluation of the survival supporting effect of MC14. As a control, we tested the effect of K252a on NGF-treated cells. Treatment with 30 nM K252a significantly suppressed the NGF-induced survival supporting effect, while 100 nM K252a completely blocked NGF action. In contrast, the inhibitor failed to block the survival supporting activity of MC14 (Fig. 4A). Treatment with K252a alone had no effect on cell survival under these conditions. This result indicates that TrkA is not involved in the signaling pathway induced by MC14 for its survival supporting activity. However, these concentrations of K252a have been reported to inhibit other enzymes in addition to TrkA, such as PKA, PKC, PKG, calmodulin-dependent protein kinase II and myosin light chain kinase (Tanaka, 2001). To verify that the inhibition of other possible K252a-sensitive kinases are not involved in survival supporting activity of MC14, we further examined the effects of specific inhibitors of PKA, PKC and MAPK by using SC-3010, chelerythrine chloride and PD98059, respectively. The concentrations used for each inhibitor were sufficient to specifically abolish the activity of their respective protein kinases as described previously (Bohm et al., 1995; Schonhoff et al., 2001; Hiruma et al., 1999). As shown in Fig. 4B–D, none of them caused a significant reduction of viable cells induced by MC14. Taken together, these results demonstrate that the neuronal survival supporting activity of MC14 may not require the NGF receptor TrkA, PKA, PKC and MAPK mediated signaling pathways. The results further support the contention that the mechanism of survival supporting activity of MC14 on neuronal PC12D cells is apparently via an NGF-independent intracellular signaling pathway.

Several recent reports have demonstrated that phosphatidylinositol 3-kinase (PI3K) is a key signaling molecule in regulating the survival of sympathetic neurons and neuronal PC12 cells (Franke et al., 1997; Crowder and Freeman, 1998; Ashcroft et al., 1999). This prompted us to examine the role of PI3K on MC14-induced survival supporting activity. Before the addition of MC14, neuronal PC12D cells were preincubated with wortmannin at concentrations that significantly inhibit PI3K as described previously (Shibata et al., 2003). As shown in Fig. 4E, treatment of cells with wortmannin significantly suppressed the survival supporting effect of MC14 by more than 18% ($P<0.05$). However, the possibility that other signaling pathways may be regulated by MC14 should not be ruled out since the inhibition of PI3K only partially blocked its survival supporting effect.

3.2. Protective effect of MC14 against H_2O_2-induced cytotoxicity

Next, we investigated whether MC14 could mimic the other neuroprotective activities of NGF, such as the protective effect against oxidative stress induced by H_2O_2. The result of H_2O_2-induced cytotoxicity on PC12D cells showed that cell viability was reduced by more than 80% after the treatment of 400 μM H_2O_2 for 6 h, compared with control cells incubated in complete medium only. As shown in Fig. 5, preincubation of PC12D cells with MC14 in the presence of 0.4–50 ng/ml NGF for 2 h substantially attenuated cell death induced by H_2O_2 treatment, compared with those treated with NGF alone. Results showed that 27–47% of the number of viable cells were enhanced by the treatment of MC14, suggesting that MC14 could significantly promote the protective activity of NGF against H_2O_2-insult on PC12D cells. Similar to serum free conditions, pretreatment of cells with MC14 alone could enhance cell viability by 2.7-fold (Fig. 5). This result clearly demonstrates that MC14 significantly protects PC12D cells against oxidative stress even in the absence of NGF.

3.3. Neurite regenerating activity

We previously found that MC14 is a potent neurite outgrowth promoting substance on naive PC12D cells in the presence of NGF (Tsang et al., 2001a,b). This observation prompted us to further investigate whether MC14 also possesses the neurite regenerating effect on neuronal PC12D cells. The neurites of neuronal PC12D cells were sheared and treated with or without MC14 in the presence of 0.02–50 ng/ml NGF for 48 h. Measurement of the proportion of neurite-regenerated cells showed that the treatment with

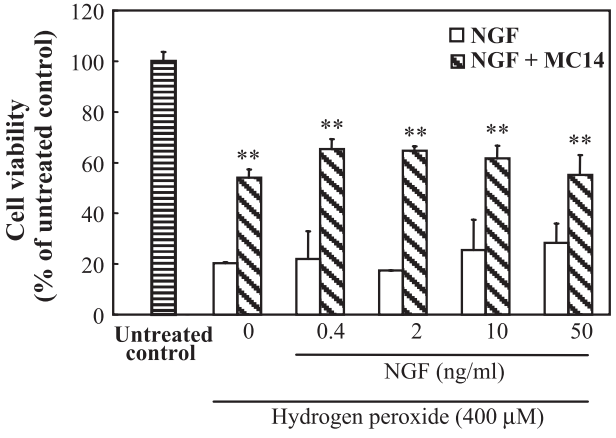

Fig. 5. Protective effect of MC14 against H_2O_2-induced oxidative stress on PC12D cells. PC12D cells were treated with 3 μg/ml MC14 and the indicated concentration of NGF for 2 h before the addition of 400 μM H_2O_2. After 6 h incubation with H_2O_2, cell viability was determined by MTT assay, and is expressed as a percentage relative to the H_2O_2-untreated control (complete medium, 100%). Data are given as mean ($n=4$) ± S.D. Significant difference from the corresponding NGF-only control: $^{**}P<0.01$ (Student's *t*-test).

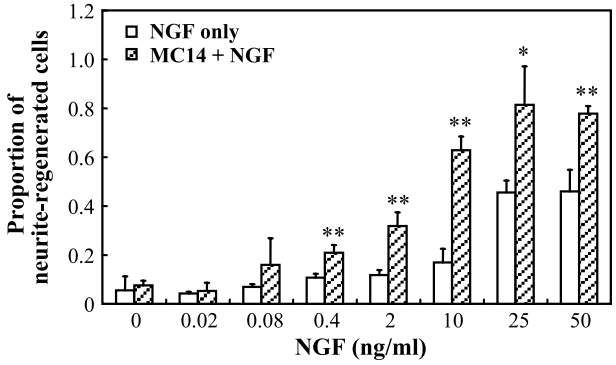

Fig. 6. Neurite-regeneration promoting activity of MC14 on neurite-sheared neuronal PC12D cells. The neurite-sheared cells were treated with or without 3 μg/ml MC14 in the indicated concentrations of NGF for 48 h. Data are given as mean ($n=4$) ± S.D. Significant difference from the NGF-only control: *$P<0.05$ and **$P<0.01$ (Student's t-test).

MC14 in the presence of 0.4–50 ng/ml NGF resulted in significant enhancement of neurite-regeneration compared with the respective MC14-untreated control (Fig. 6). In the presence of 10 ng/ml NGF, the treatment of cells with MC14 led to a four-fold enhancement of neurite regeneration. This result suggests that MC14 does not only promote differentiation of PC12D cells but also neurite regeneration from the neuronal PC12D cells.

4. Discussion

MC14, a low molecular weight quinonic compound isolated from a brown alga *S. macrocarpum*, has been previously shown to be capable of promoting the NGF-induced neurite outgrowth from PC12D cells (Tsang et al., 2001a,b). This neuronal differentiation promoting activity is NGF-dependent as MC14 by itself does not induce neurite outgrowth. In this report, we demonstrate the novel biological activities of MC14, in which NGF is dispensable. Under serum free conditions, the cultures of sympathetic neurons and the differentiated neuronal PC12 cells exhibit the typical aspects of apoptosis, including the requirement of protein synthesis, aggregation of chromatin, DNA fragmentation and conservation of mitochondria (Martin et al., 1998). The present study shows that the cell death of neuronal PC12D, a subline of PC12, is also induced by NGF-withdrawal in serum-free medium. The kinetics of cell viability of neuronal PC12D cells resembles that of sympathetic neurons and neuronal PC12 cells (Mesner et al., 1995). The viability of neuronal PC12D cells substantially decreases within 24 h when cultured in serum-free medium. It has been well documented that NGF can rescue neuronal cells in the serum free medium (Shigeno et al., 1991; Yao and Cooper, 1995; Pean et al., 2000). This prompted us to further investigate the effect of MC14 on promoting NGF-induced survival supporting activity. Intriguingly, our findings show that MC14 enhances the survival of neuronal PC12D cells in the presence of NGF. MC14 also promotes cell proliferation at relatively high concentrations of NGF. To our surprise, MC14 alone also significantly alleviates the death of neuronal PC12D cells under serum free conditions. Our findings provide several lines of evidence suggesting that the neuroprotective action of MC14 is in fact independent of NGF. Firstly, MC14 is effective in supporting neuronal PC12D cells in the absence of NGF. Secondly, the increase in cell viability contributed by MC14 seems to be constant from 0.08 ng/ml NGF to 50 ng/ml NGF, indicating that MC14 and NGF might support cell viability by two parallel signaling pathways. Thirdly, several specific signaling protein inhibitors were used to further support this suggestion. We confirmed that TrkA, a specific NGF receptor, is required for the neuronal supporting activity of NGF to neuronal PC12D cells. This is consistent with its role in other neuronal cells (Culmsee et al., 2002; Hetman and Xia, 2000). However, our finding clearly shows that the inhibition of NGF receptor by K252a fails to block the survival supporting activity of MC14, strongly suggesting that NGF is not required for the survival supporting action of MC14. Finally, NGF appears to have only a minimal protective effect on the viability of PC12D cells in the presence of hydrogen peroxide, and that minimal effect does not substantially change with increasing doses of NGF (Fig. 5). In contrast, MC14 alone shows a substantial protective effect which does not change significantly with increasing concentration of NGF. Therefore, the protective effect of MC14 on cell viability is apparently due solely to its antioxidant activity without substantial interplay with NGF. Taken together, these biochemical and physiological analyses suggest that MC14 supports the survival of neuronal PC12D cells in an NGF-independent manner.

To investigate the mechanism of action of MC14, we focused on some representative signal transduction pathways responsible for cell survival. It has been reported that several intracellular signaling molecules, including PI3K, MAP kinases and PKC, are involved in mediating cell viability (Shirakawa and Mizel, 1989; Yao and Cooper, 1995; Virdee and Tolkovsky, 1995; Crowder and Freeman, 1998; Ashcroft et al., 1999). Shirakawa and Mizel (1989) reported that PKA and PKC trigger the nuclear translocation of the activated nuclear factor-κB (NF-κB), a transcription factor responsible for supporting cell survival. It has also been demonstrated that NGF-mediated survival in rat sympathetic neurons is associated with sustained activation of p42/p44 MAPK, suggesting a role of the MAPK cascade in promoting neuronal survival (Twiss and Shooter, 1995). In the present study, however, we show that the specific inhibitors of PKA, PKC and MAPK fail to abolish the MC14-induced viability enhancement in neuronal PC12D cells. Consistent with these findings, we demonstrate that 100 nM K252a, a concentration at which other kinases including PKA and PKC are also inhibited (Tanaka, 2001), does not affect the MC14-induced survival supporting activity. These results suggest that PKA, PKC, and MAPK

mediated signaling pathways are apparently not required for the survival supporting action of MC14. Interestingly, PKA and MAPK mediated signaling pathways have been implicated previously to be involved in the NGF-dependent neurite outgrowth promoting activity of MC14 to PC12D cells (Kamei and Tsang, 2003). Intriguingly, the inhibition of PI3K leads to a considerable suppression of the survival supporting effect of MC14, suggesting that PI3K may play an important role in the survival supporting activity induced by MC14. Our finding suggests that MC14 may act upstream of PI3K, or directly target to PI3K to activate this protein kinase. Indeed, cumulative evidence has shown that the survival of many neuronal cell types is regulated by PI3K (Franke et al., 1997; Crowder and Freeman, 1998; Ashcroft et al., 1999; Heaton et al., 2000; Kuruvilla et al., 2000). Taken together, our present findings, together with those of others, support the important role of PI3K for mediating cell survival. In addition, MC14 may regulate more than one signaling pathway for controlling diverse neuronal responses.

The mechanism that leads to the activation of free-radical generation and subsequent cell damage appears to be a common theme in most of the neurodegenerative diseases, such as the involvement of glutamate-mediated excitotoxicity in Huntington's disease, neurotoxicity of β-amyloid in Alzheimer's disease, and the selective vulnerability of dopaminergic neurons in Parkinson's diseases (Gilgun-Sherki et al., 2001). It has been suggested that oxidative stress may be an important component in the death of neurons. In the present study, we show that MC14 can substantially protect PC12D cells against H_2O_2-induced oxidative stress, suggesting that MC14 might attenuate oxidative stress-induced neurodegeneration. Although the molecular mechanism of its antioxidant activity is still unclear, we postulate that the MC14 molecule may directly scavenge the free radical by its unsaturated isoprenoid moiety, or indirectly activate the enzymes such as catalase and phospholipase to induce the free radical detoxifying mechanisms (Jackson et al., 1990; Lee et al., 2000). On the other hand, MC14 exhibits marked neurite regenerating activity to neurite-sheared neuronal PC12D cells. These activities of MC14 suggest that it may be a promising neuroprotective agent.

An interesting finding in this study reveals that the mode of action of MC14 varies depending on the developmental stage of PC12D cells. When the naive PC12D cells are committed to differentiation by NGF treatment, the action of MC14 is to promote neurite outgrowth, via an NGF-dependent PKA and MAPK mediating pathways (Kamei and Tsang, 2003). With respect to the differentiated neuronal PC12D cells, MC14 supports cell survival via the PI3K-mediated signaling pathway in an NGF-independent manner. These findings imply that MC14 has distinct effects on neuronal cells, and apparently acts on different signaling pathways depending on their developmental stages.

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References

Ashcroft, M., Stephens, R.M., Hallberg, B., Downward, J., Kaplan, D.R., 1999. The selective and inducible activation of endogenous PI3-kinase in PC12 cells results in efficient NGF-mediated survival but defective neurite outgrowth. Oncogene 18, 4586–4597.

Barde, Y.A., 1994. Neurotrophins: a family of proteins supporting the survival of neurons. In: Gerald, D. (Ed.), Molecular Neurobiology: Mechanisms Common to Brain, Skin, and Immune System. Wiley-Liss Press, New York, pp. 45–56.

Bohm, M., Moellmann, G., Cheng, E., Alvarez-Franco, M., Wagner, S., Sassone-Corsi, P., Halaban, R., 1995. Identification of $p90^{RSK}$ as the probable CREB-Ser^{133} kinase in human melanocytes. Cell Growth Differ. 6, 291–302.

Brinton, R.D., Yamazaki, R.S., 1998. Advanced and challenges in the prevention and treatment of Alzheimer's disease. Pharm. Res. 15, 386–398.

Burgos, I., Cuello, A.C., Liberini, P., Pioro, E., Masliah, E., 1995. NGF-mediated synaptic sprouting in the cerebral cortex of lesioned primate brain. Brain Res. 692, 154–160.

Connor, B., Dragunow, M., 1998. The role of neuronal growth factors in neurodegenerative disorders of the human brain. Brain Res. Rev. 27, 1–39.

Crowder, R.J., Freeman, R.S., 1998. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J. Neurosci. 18, 2933–2943.

Culmsee, C., Gerling, N., Lehmann, M., Nikolova-Karakashian, M., Prehn, J.H.M., Mattson, M.P., Krieglstein, J., 2002. Nerve growth factor survival signaling in cultured hippocampal neurons is mediated through TrkA and requires the common neurotrophin receptor P75. Neuroscience 115, 1089–1108.

Fischer, W., Wictorin, K., Bjorklund, A., Williams, L.R., Varon, S., Gage, F.H., 1987. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329, 65–68.

Franke, T.F., Kaplan, D.R., Cantley, L.C., 1997. PI3K: downstream AKTion blocks apoptosis. Cell 88, 435–437.

Gilgun-Sherki, Y., Melamed, E., Offen, D., 2001. Oxidative stress induced neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology 40, 959–975.

Hansen, M.B., Nielsen, S.E., Berg, K., 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119, 203–210.

Heaton, M.B., Kim, D.S., Paiva, M., 2000. Neurotrophic factor protection against ethanol toxicity in rat cerebella granule cell cultures requires phosphatidylinositol 3-kinase activation. Neurosci. Lett. 291, 121–125.

Hefti, F., Weiner, W.J., 1986. Nerve growth factor and Alzheimer's disease. Ann. Neurol. 20, 275–281.

Hetman, M., Xia, Z., 2000. Signaling pathways mediating anti-apoptotic action of neurotrophins. Acta Neurobiol. Exp. 60, 531–545.

Hiruma, H., Maruyama, H., Katakura, T., Simada, Z.B., Nishida, S., Hoka, T., Takenaka, T., Kawakami, T., 1999. Axonal transport is inhibited by a protein kinase C inhibitor in cultured isolated mouse dorsal root ganglion cells. Brain Res. 826, 135–138.

Jackson, G.R., Apffel, L., Werrbach-Perez, K., Perez-Polo, J.R., 1990. Role of nerve growth factor in oxidant-antioxidant balance and neuronal

injury. I. Stimulation of hydrogen peroxide resistance. J. Neurosci. Res. 25, 360–368.

Kamei, Y., Sagara, A., 2002. Neurite outgrowth promoting activity of marine algae from Japan against rat adrenal medulla pheochromocytoma cell line, PC12D. Cytotechnology 40, 99–106.

Kamei, Y., Tsang, C.K., 2003. Sargaquinoic acid promotes neurite outgrowth via protein kinase A and MAP kinases-mediated signaling pathways in PC12D cells. Int. J. Dev. Neurosci. 21, 255–262.

Katoh-Semba, R., Kitajima, S., Yamazaki, Y., Sano, M., 1987. Neurite growth from a new subline of PC12 pheochromocytoma cells: cyclic AMP mimics the action of nerve growth factor. J. Neurosci. Res. 17, 36–44.

Kuruvilla, R., Ye, H., Ginty, D.D., 2000. Spatially and functionally distinct roles of the PI3K effector pathway during NGF signaling in sympathetic neurons. Neuron 27, 499–512.

Lee, S.D., Lee, B.D., Han, J.M., Kim, J.H., Kim, Y., Suh, P.G., Ryu, S.H., 2000. Phospholipase D2 activity suppresses hydrogen peroxide-induced apoptosis in PC12 cells. J. Neurochem. 75, 1053–1058.

Martin, D.P., Schmidt, R.E., Distefano, P.S., Lowry, O.H., Carter, J.G., Johnson Jr., E.M. 1998. Inhibitions of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J. Cell Biol. 106, 829–844.

Mesner, P.W., Winters, T.R., Green, S.H., 1992. Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. J. Cell Biol. 119, 1669–1680.

Mesner, P.W., Epting, C.L., Hegarty, J.L., Green, S.H., 1995. A timetable of events during programmed cells death induced by trophic factor withdrawal from neuronal PC12 cells. J. Neurosci. 15, 7357–7366.

Parran, D.K., Barone, S.J., Mundy, W.R., 2003. Methylmercury decreases NGF-induced TrkA autophosphorylation and neurite outgrowth in PC12 cells. Dev. Brain Res. 141, 71–81.

Pean, J.M., Menei, P., Morel, O., Montero-Menei, C.N., Benoit, J.P., 2000. Intraseptal implantation of NGF-releasing microspheres promote the survival of axotomized cholinergic neurons. Biomaterials 21, 2097–2101.

Saffran, B.N., 1992. Should intracerebroventricular nerve growth factor be used to treat Alzheimer's disease? Perspect. Biol. Med. 35, 471–486.

Schonhoff, C.M., Bulseco, D.A., Brancho, D.M., Parada, L.F., Ross, A.H., 2001. The Ras-ERK pathway is required for the induvtion of neuronal nitric oxide synthase in differentiating PC12 cells. J. Neurochem. 78, 631–639.

Seiger, A., Nordberg, A., Holst, V.H., Backman, L., Ebendal, T., Alafuzoff, I., Amberla, K., Hartvig, P., Herlitz, A., Lilja, A., Lundqvist, H., Langstrom, B., Meyerson, B., Persson, A., Viitanen, M., Winblad, B., Olson, L., 1993. Intracranial infusion of purified nerve growth factor to an Alzheimer patient: the first attempt of a possible future treatment strategy. Behav. Brain Res. 57, 255–261.

Shibata, A., Laurent, C.E., Smithgall, T.E., 2003. The c-Fes protein-tyrosine kinase accelerates NGF-induced differentiation of PC12 cells through a PI3K-dependent mechanism. Cell. Signal. 15, 279–288.

Shigeno, T., Mima, T., Takaura, K., Graham, D.I., Kato, G., Hashimoto, Y., Furukawa, S., 1991. Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. J. Neurosci. 11, 2914–2919.

Shirakawa, F., Mizel, S.B., 1989. In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. Mol. Cell. Biol. 9, 2424–2431.

Tanaka, K., 2001. Alteration of second messengers during acute cerebral ischemia-adenylate cyclase, cyclic AMP-dependent protein kinase, and cyclic AMP response element binding protein. Prog. Neurobiol. 65, 173–207.

Tsang, C.K., Sagara, A., Kamei, Y., 2001a. Neurite outgrowth promoting substance from a marine alga, *Sargassum macrocarpum*. In: Lindner-Olsson, E., Chatzissavidou, N., Lullau, E. (Eds.), Animal Cell Technology: From Target to Market. Kluwer Academic Publishing, Dordrecht, pp. 20–22.

Tsang, C.K., Sagara, A., Kamei, Y., 2001b. Structure–activity relationship of a neurite outgrowth-promoting substance purified from the brown alga, *Sargassum macrocarpum*, and its analogues on PC12D cells. J. Appl. Phycol. 13, 349–357.

Twiss, J.L., Shooter, E.M., 1995. Nerve growth factor promotes neurite regeneration in PC12 cells by translational control. J. Neurochem. 64, 550–557.

Virdee, K., Tolkovsky, A.M., 1995. Activation of p44 and p42 MAP kinases is not essential for the survival of rat sympathetic neurons. Eur. J. Neurosci. 7, 2159–2169.

Yao, R., Cooper, G.M., 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267, 2003–2005.