1. Introduction

Ceramides are critical bioactive lipids derived from sphingosine that induce neuronal cell death by causing mitochondrial dysfunction (Arboleda et al., 2009). There is increasing evidence indicating that ceramides may be involved in several neurodegenerative disorders including Alzheimer’s disease (Cutler et al., 2004; Jana et al., 2009), Parkinson’s disease (Bras et al., 2008; France-Lanord et al., 1997) and cerebral ischemia (Novgorodov and Gudz, 2009). It has been shown that C2-ceramide, a short chain analog of ceramide, mediates the biological activity of ceramide to cause programmed cell death in neuronal cells (Novgorodov and Gudz, 2009). asiatic acid has been shown to exhibit neuroprotective properties both in cultured cells and in vivo (Krishnamurthy et al., 2009; Lee et al., 2000; Mook-Jung et al., 1999; Xiong et al., 2009). However, the underlying signal transduction pathways by which asiatic acid prevents neuronal cell death are largely unknown.

In the present study we investigated the potential cellular and molecular mechanisms by which asiatic acid might counteract mitochondrial dysfunction and C2-ceramide-mediated apoptosis in primary neurons (Fig. 1).

2. Materials and methods

2.1. Materials

Asiatic acid, C2-ceramide, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), poly-o-lysine, 5,5′,6,6′-tetra-chloro-1,1′,3′,3′-tetra-ethylbenzimidazol-carbo-cyanine iodide (JC-1), 7′-dichlorofluorescein diacetate (DCFH-DA), type I collagenase, U0126 and lactate dehydrogenase (LDH) were purchased from Sigma-Aldrich (St. Louis, MO, USA); DMEM medium, fetal bovine serum, B27, and Phenol Red free Neuralbasal medium were obtained from Gibco BRL (Burlington, Ontario, Canada). The Griess assay reagent kit for NO was a product from Beyontime (Jiangsu, China). Antibodies were used as follows: caspase 3, p-ERK1/2, ERK1/2 were products of Cell Signaling Technology (Inc., Danvers, MA, USA); anti-HtrA2/Omi was a kind gift from Prof. Kohji Fukushima of Tohoku University, Japan and all secondary antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Primers were purchased from Sigma-Aldrich.

2.2. Primary neuronal cell preparation and cultures

Primary cultured cortical neurons were obtained from embryonic day (E18) rat fetuses as previously described (Bastianetto et al., 1999; Brewer et al., 2000; Mook-Jung et al., 1999; Xiong et al., 2009). Reagents and chemicals were obtained from the specified vendors. All experiments were performed in accordance with the guidelines and regulations as established by the local institutional and governmental authorities.
oxidation-sensitive fluorescence microscopy (DMI 3000B, Leica, Wetzlar, Germany). which can be hydrolyzed into terase in live cells. Cell cultures were incubated with 1 μM. Calcein-AM is a cell permeable and non-alalyzed either by phase contract microscopy or by dying with calcein-

2.6. Measurement of intracellular ROS generation

which can be hydrolyzed into DCFH, which is oxidized to fluorescent DCF in the presence of an oxidant. Cells were then observed by fluorescence microscopy and photos were captured using a constant exposure time within each experiment. For quantitative analysis, intracellular fluorescence was detected by microplate reader with excitation wavelength at 488 nm and emission wavelength at 520 nm (DTX880, Beckman Coulter, CA, USA). Cells treated with 500 μmol/l H2O2 were used as a positive control. All these experiments were repeated for three times independently.

2.7. ΔΨm assessment by JC-1 staining

2.8. Flow cytometric analysis

To determine cell apoptosis, the fluorescent probes Annexin V/PI were used (Lim et al., 2008). After 24 h treatment with 25 μmol/l C2-ceramide in the presence of Asiatic acid, cells were rinsed with warm PBS before undergoing digestion by 0.02% type I collagenase. Cell suspensions were stained using the Annexin V/PI staining kit following manufacturer's instructions. Flow cytometry was performed on FACSscan (BD Biosciences, San Jose, CA, USA). This experiment was repeated for three times independently.

2.9. Western blot analysis

2.4. Cell viability assay

2.5. Cell morphology observation

2.6. Measurement of intracellular ROS generation

The production of intracellular ROS was measured using the oxidation-sensitive fluorescent dye, DCFH-DA (Oyama et al., 1994). An increase in the intensity of green fluorescence was used to quantify the generation of intracellular ROS. After pretreatments with Asiatic acid, cells were exposed to 25 μmol/l C2-ceramide for 4 h. Then, cells were rinsed briefly with warm PBS, before further incubation with DCFH-DA (final concentration 5 μmol/l) for 5 min at 37 °C. The cell permeable DCFH-DA was hydrolyzed to DCF, which is oxidized to fluorescent DCF in the presence of an oxidant. Cells were then observed by fluorescence microscopy and photos were captured using a constant exposure time within each experiment. For quantitative analysis, intracellular fluorescence was detected by microplate reader with excitation wavelength at 488 nm and emission wavelength at 520 nm (DTX880, Beckman Coulter, CA, USA). Cells treated with 500 μmol/l H2O2 were used as a positive control. All these experiments were repeated for three times independently.

The fluorescent probe JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 ± 10 nm) to red (610 ± 10 nm) (Zuliani et al., 2003). After pretreatment with Asiatic acid, cells were exposed to 25 μmol/l C2-ceramide for 4 h. Cells were then briefly rinsed with warm PBS before being incubated with 1 μg/ml JC-1 for 15 min at 37 °C. Cell staining was observed using a fluorescence microscopy (DMI 3000B, Leica, Wetzlar, Germany) with a constant exposure time for each experiment. At least three random fields of views were captured from each sample, data were calculated as average number of red stained cells in each view, as counted by Image-Pro Plus 5.0 software (Cybernetics Inc.). The results were expressed as percentage of red stained cells to total cells. All these experiments were repeated for at least three times independently.

2.7. ΔΨm assessment by JC-1 staining

et al., 1993) with minor modifications (Wang et al., 2007). Briefly, fetal rats were sacrificed by decapitation and brain cortex was dissected in Hank’s buffer (137 mmol/l NaCl, 5.4 mmol/l KCl, 0.4 mmol/l KH2PO4, 0.34 mmol/l Na2HPO4·7H2O, 10 mmol/l glucose and 10 mmol/l Hepes) containing 0.125% trypsin. After incubation at 37 °C for 8 min, cortical tissue was dissociated by passing through a series of fire-polished constricted Pasteur pipettes. About 5 × 10^6 cells/ml were seeded onto poly-d-lysine (10 μg/ml) coated 96 well or 6 well plates. Cells were routinely cultured in Neurobasal medium supplemented with 2% B27, 10 U/ml penicillin, 10 U/ml streptomycin and 0.5 mmol/l glutamine at 37 °C in humidified 5% CO2 atm and observed by inverted phase-contrast microscopy. Cell cultures were maintained for 7 d before further treatments. Approximately more than 85% cells were determined as neurons by flowcytometric analysis (NeuN as neuron marker, data not shown).

2.4. Cell viability assay

Cultured cells were exposed to C2-ceramide for 24 h at different concentrations and cell viability was determined by the MTT assay (Mosmann, 1983). After treatment, cells were incubated with 0.5 mg/ml MTT at 37 °C for 4 h. Medium was then discarded and the reaction product formazan was dissolved in DMSO. Absorbance at 570 nm was determined using a microplate reader (DTX880, Beckman Coulter, CA, USA). The cell viability of vehicle-treated controls was set at 100%. The lactate dehydrogenase (LDH) activity in the culture medium was measured according to manufacturer’s instructions. LDH activity was calculated as folds of vehicle-treated control for each group, all these experiments were repeated for three times independently.

2.5. Cell morphology observation

After treatment, cell morphology was observed and qualitative analyzed either by phase contrast microscopy or by dying with calcein-AM. Calcein-AM is a cell permeable and non-fluorescent compound which can be hydrolyzed into fluorescent calcein by intracellular esterase in live cells. Cell cultures were incubated with 1 μmol/l calcein-AM for 1 h at 37 °C before being observed using a fluorescence microscopy (DMI 3000B, Leica, Wetzlar, Germany).

2.6. Measurement of intracellular ROS generation

The production of intracellular ROS was measured using the oxidation-sensitive fluorescent dye, DCFH-DA (Oyama et al., 1994). An increase in the intensity of green fluorescence was used to quantify the generation of intracellular ROS. After pretreatments with Asiatic acid, cells were exposed to 25 μmol/l C2-ceramide for 4 h. Then, cells were rinsed briefly with warm PBS, before further incubation with DCFH-DA (final concentration 5 μmol/l) for 5 min at 37 °C. The cell permeable DCFH-DA was hydrolyzed to DCF, which is oxidized to fluorescent DCF in the presence of an oxidant. Cells were then observed by fluorescence microscopy and photos were captured using a constant exposure time within each experiment. For quantitative analysis, intracellular fluorescence was detected by microplate reader with excitation wavelength at 488 nm and emission wavelength at 520 nm (DTX880, Beckman Coulter, CA, USA). Cells treated with 500 μmol/l H2O2 were used as a positive control. All these experiments were repeated for three times independently.
the SuperSignal West Pico Substrate (Pierce, Rockford, IL, USA) and exposed to film. Digital images were quantified using densitometric measurement by Quantity-One software. β-actin was detected as housekeeping protein.

2.10. NO assay

The NO concentration in culture medium after treatment was detected using the Griess assay (Huygen, 1970) reagents kit following manufacturer’s instruction. At least three independent experiments were included.

2.11. 3-NT detection by immunocytochemistry

Cells were fixed for 10 min by 3.7% formaldehyde at room temperature. After 2 rinses with PBS, cells were blocked in PBS containing 3% bovine serum albumin and 0.1% Triton X-100 then incubated with anti-3-NT antibody (1:500 in block buffer) overnight at 4 °C. The cells were further incubated with rhodamine conjugated secondary antibody at room temperature for 1 h. Immunostained cells were observed using a fluorescence microscopic camera device. All images were captured using a constant exposure time within each experiment. Cells treated with 500 μmol/l H2O2 for 12 h were used as a positive control. Relative fluorescence intensity was determined by Image-Pro plus 5.0 software (Cybernetics Inc.). At least three random fields of views were captured from each sample, data were calculated as average. Simultaneously, fixed cells were incubated with 1 μg/ml DAPI for 1 min in room temperature. The nuclei were observed at excitation wavelength 350 nm. Nuclei exhibiting clear brightly condensed chromatin or fragmented nuclei were regarded as apoptotic cells (Dusek et al., 2006).

2.12. Statistics

All data were expressed as mean ± S.D from at least three independent experiments. Differences between groups were examined...
for statistical significance using one-way ANOVA analysis with post hoc of Dunnett test. In all cases, $P<0.05$ was considered significant.

3. Results

3.1. Asiatic acid prevents C$_2$-ceramide-induced cytotoxicity

Treatment of primary rat neuronal cells with 25 $\mu$mol/l C$_2$-ceramide resulted in a significant decrease in cell viability as determined by the LDH leakage and MTT assay. Pre-incubation of neurons with Asiatic acid counteracted the C$_2$-ceramide-induced cell death in a dose-dependent manner with the maximal protection at 1.0 $\mu$mol/l (Fig. 2A, B).

Furthermore, qualitative observation by both fluorescence microscopy (Fig. 2C) and phase contrast microscopy (Fig. 2D) that C$_2$-ceramide treatment caused a decrease in cell number and loss of neuritis which could be partly ameliorated by 1.0 $\mu$mol/l Asiatic acid. These data indicated that Asiatic acid is neuroprotective against C$_2$-ceramide-induced cell death.

3.2. Asiatic acid counteracts C$_2$-ceramide-induced programmed cell death

C$_2$-ceramide caused neuronal cells apoptosis. To test whether Asiatic acid exerts its neuroprotection by virtue of anti-apoptosis effects, apoptosis rate was determined by flow cytometry. C$_2$-ceramide significantly increased the percentage of apoptotic cells, whilst the percentage of necrotic cells (PI high stained, Annexin V low stained cells) increased from 5% to 14% ($P<0.05$). Pretreatment of neuronal cells with 1.0 $\mu$mol/l Asiatic acid significantly decreased the rate of apoptotic cells but not necrotic cells (Fig. 3A).

Mitochondrial membrane potential ($\Delta\Psi$m) was determined by JC-1 staining. As depicted in Fig. 3B, control cells exhibited an intact $\Delta\Psi$m while C$_2$-ceramide-treated cells showed an apparent $\Delta\Psi$m loss, shown by switching fluorescence from red to green. This fluorescence shift could be observed both in cell bodies (arrow) and neurites...
ΔΨm loss could be partly counteracted by Asiatic acid pretreatment (Fig. 3C).

Mitochondria are sensitive to changes in cellular redox state, and ROS may cause mitochondrial dysfunction. DCFH-DA staining revealed that C2-ceramide treatment induced ROS generation which could be suppressed by 1.0 μmol/l Asiatic acid pretreatment (Fig. 3D). C2-ceramide caused an upregulation of Bax protein while Bcl-2 protein remained unchanged. 1.0 and 0.1 μmol/l Asiatic acid pretreatment prevented the increase in Bax but showed little impact on Bcl-2 protein levels after 4 h incubation of C2-ceramide (Fig. 4A and B). Thus C2-ceramide caused a significant decrease in the Bcl-2/Bax ratio, which was partially offset by Asiatic acid in a concentration dependent manner.

Multiple mitochondrial proteins are released into the cytoplasm in neuronal cells following C2-ceramide incubation (Stoica et al., 2005). Western blotting demonstrated that cytoplasmic HtrA2/Omi protein was elevated in response to C2-ceramide treatment for 16 h (Fig. 4A and C). Pretreatment with Asiatic acid reduced this cytosolic release of HtrA2/Omi and subsequent caspase 3 activation, determined after 24 h of C2-ceramide incubation, in a concentration dependent manner, with the maximal effect at 1.0 μmol/l (Fig. 4A). Taken together, these data indicate that Asiatic acid pretreatment diminished C2-ceramide-induced programmed cell death.

3.3. Asiatic acid counteracts C2-ceramide-induced 3-NT formation but not NO production

Besides mitochondrial dysfunction, ROS and subsequent nitrotyrosine (3-NT) contribute to cell apoptosis as well. Treatment of rat neuronal cells with C2-ceramide significantly increased 3-NT formation. However, pretreatment of neuronal cells with 1.0 μmol/l Asiatic acid slightly reduced this 3-NT level (Fig. 5A and B). Simultaneous DAPI staining indicated condensed and fragmented nuclei in C2-ceramide treated cells, which was partly ameliorated by Asiatic acid pretreatment.

3-NT formation may be enhanced due to both NO and ROS generation in C2-ceramide treated cells. Under the conditions investigated, iNOS was dramatically up-regulated in response to C2-ceramide treatment for 24 h. However, iNOS protein levels were not regulated by Asiatic acid (Fig. 5C). Coincident with the induction of iNOS, C2-ceramide induced the production of NO as determined by the Griess reaction (Fig. 5D). NO formation was not changed by treating the neuronal cells with Asiatic acid either. These results suggest that Asiatic acid may suppress C2-ceramide-induced 3-NT formation mainly by inhibiting ROS rather than NO generation.

3.4. Involvement of ERK1/2 signaling pathway in Asiatic acid-mediated neuroprotection

MAPK signaling pathways were previously proved to be involved in C2-ceramide-induced cell death (Stoica et al., 2005; Willaime et al., 2001). Therefore, it is interesting to test whether these signalings also participate in Asiatic acid’s action. After 4 h treatment with C2-ceramide, ERK1/2 protein levels were found to be down-regulated. This C2-ceramide-mediated ERK1/2 down-regulation was partly reversed by Asiatic acid. However p38 and JNK were not altered by treatment with C2-ceramide and Asiatic acid (Fig. 6A).

Western blot analysis confirmed ERK1/2 dephosphorylation by the specific MEK inhibitor U0126 (Fig. 6B). Furthermore, our MTT cell viability data indicated that pretreatment of rat neuronal cells with U0126 partly counteracted the Asiatic acid-mediated neuroprotection in C2-ceramide stimulated cells. To confirm whether C2-ceramide-mediated mitochondrial dysfunction might be partly related to ERK1/2 dephosphorylation, ΔΨm and ROS levels were determined in the absence and presence of U0126. It was shown that the neuroprotective effects of Asiatic acid regarding membrane potential and ROS production were abolished in the presence of U0126 (Fig. 6C and D). Furthermore, U0126 counteracted the Asiatic acid-mediated down-regulation of Bax in the presence of C2-ceramide (Fig. 6B). These data indicated that Asiatic acid protects against C2-ceramide-induced neuronal cell death by, at least partly, preventing ERK1/2 dephosphorylation.

Fig. 4. Asiatic acid prevents C2-ceramide-induced mitochondria-dependent apoptosis-related proteins upregulation. 7-d-old primary cultured rat cortical neuronal cells were pretreated with indicated concentrations of Asiatic acid for 24 h. (A) Apoptosis-related proteins were detected by western blot. Bcl-2/Bax, cytoplasmic HtrA2/Omi protein levels and caspase 3 protein were determined 4, 16 and 24 h after C2-ceramide incubation, respectively. (B) Bcl-2, Bax, Bcl-2/Bax ratio and (C) HtrA2/Omi were semi-quantified by densitometric measurements. Bar charts present mean ± S.D. *P < 0.05, **P < 0.01, n=3.

(arrow head). ΔΨm loss could be partly counteracted by Asiatic acid pretreatment (Fig. 3C).

Mitochondria are sensitive to changes in cellular redox state, and ROS may cause mitochondrial dysfunction. DCFH-DA staining revealed that C2-ceramide treatment induced ROS generation which could be suppressed by 1.0 μmol/l Asiatic acid pretreatment (Fig. 3D). C2-ceramide caused an upregulation of Bax protein while Bcl-2 protein remained unchanged. 1.0 and 0.1 μmol/l Asiatic acid pretreatment prevented the increase in Bax but showed little impact on Bcl-2 protein levels after 4 h incubation of C2-ceramide (Fig. 4A and B). Thus C2-ceramide caused a significant decrease in the Bcl-2/Bax ratio, which was partially offset by Asiatic acid in a concentration dependent manner.

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4. Discussion

Previous studies suggest that Asiatic acid may exhibit neuroprotective properties (Krishnamurthy et al., 2009; Lee et al., 2000; Mook-Jung et al., 1999; Xiong et al., 2009), but the underlying mechanisms have yet not been systematically elucidated. On the basis of the current data, we suggest that Asiatic acid may protect neurons from C2-ceramide-induced cell death by antagonizing mitochondria-dependent apoptosis.

Mitochondrial dysfunction is characterized by ΔΨm loss and ROS generation (Gross et al., 1999). Loss of ΔΨm is considered to be an early event in the apoptotic process (Green and Reed, 1998), which is believed to be an irreversible initiation of mitochondria-dependent apoptosis. Under the conditions investigated, we found that Asiatic acid prevented the ΔΨm loss caused by C2-ceramide, indicating that the protective effects of Asiatic acid may be mitochondria dependent. A previous study demonstrated that Asiatic acid attenuated ΔΨm loss and subsequent cytochrome c release in a mouse model of ischemia (Krishnamurthy et al., 2009). Together with our data, it can be characterized that preventing mitochondrial dysfunction could be an important mechanism underlying neuroprotection of Asiatic acid.

The Bcl-2 family of proteins is essential in controlling ΔΨm (Adams and Cory, 1998). Pro-apoptotic Bax and anti-apoptotic Bcl-2 are considered to be critical regulators of programmed cell death in neurons (Chao and Korsmeyer, 1998). To explore whether or not Asiatic acid prevents C2-ceramide-induced ΔΨm loss by regulating the Bcl-2 family proteins, the Bcl-2/Bax ratio was determined. We found that modulation of Bax rather than Bcl-2 may be associated with changes in ΔΨm in response to C2-ceramide treatment. These results are in agreement with a previous report suggesting that Bax contributes to C2-ceramide induced cell injury in cortical neurons (Willaime-Morawek et al., 2005). Therefore, we propose that Asiatic acid-mediated neuroprotection may be related to the attenuation of C2-ceramide-induced up-regulation of Bax which in turn may prevent ΔΨm loss.

The release of mitochondrial intra-membrane proteins following ΔΨm loss is considered to be a critical step in the apoptotic process.

Fig. 5. Asiatic acid partly prevents C2-ceramide-induced 3-NT formation in neuronal cells. 7-d-old primary cultured rat cortical neuronal cells were pretreated with indicated concentrations of Asiatic acid for 24 h followed by further 24 h incubation with 25 μmol/l C2-ceramide. (A) 3-NT formation in neuronal cells (arrows) was determined by immunofluorescence (upper panel), which could be partly abolished with 1.0 μmol/l Asiatic acid. Cell nuclei were stained by DAPI staining. Nuclei showed clearly bright chromatin condensation which was regarded as apoptotic cells indicated by arrows (lower panel). (B) The 3-NT immune-reactivity was quantified by measuring fluorescence intensity. (C) After 24 h exposure with 25 μmol/l C2-ceramide, iNOS protein levels were examined by Western blotting. Semi-quantitative analyses of iNOS levels normalized to β-actin are shown. (D) NO production in supernatants from cultured samples as determined by the Griess assay. Data were expressed as mean ± SD from at least three independent experiments. **P<0.01, ***P<0.001.
HtrA2/Omi is a pro-apoptotic mitochondrial protein. We found C2-ceramide induced ΔΨm loss subsequent with significant HtrA2/Omi release into cytoplasm, both can be partly inhibited by Asiatic acid treatment. These data indicated that Asiatic acid prevented C2-ceramide-induced ΔΨm loss and thus the release of pro-apoptotic proteins. Furthermore, enhanced HtrA2/Omi is associated with oxidative injury to cells (Ding et al., 2009). Thus the Asiatic acid-mediated HtrA2/Omi release inhibition may protect cells from oxidative stress as well.

C2-ceramide has been implicated in the induction of neuronal cell death predominantly by inducing caspase-dependent apoptosis (Movsesyan et al., 2002). In this study, Asiatic acid pretreatment of neuronal cells prevented the C2-ceramide induced apoptotic rate as determined by flow cytometry (Annexin V positive staining), whilst Asiatic acid was not effective in reducing C2-ceramide-induced cell necrosis (PI positive staining alone). Asiatic acid attenuated C2-ceramide-induced caspase-3 up-regulation indicating that the anti-apoptotic effects of Asiatic acid are via antagonizing caspase3-dependent cell death. Lines of investigations demonstrated the time course of apoptosis (Susin et al., 1999), in which mitochondrial dysfunction, including ΔΨm loss and ROS generation are considered as early-phase while caspase activation and cell nucleus condensation as late-phase events. Although the present data cannot provide direct evidences to prove that these events also happened in the indicated sequence, it was shown that these events happened in distinct time points, which are consistent with previous reports.

3-NT is formed due to the reaction of tyrosine with peroxynitrite (ONOO−). Peroxynitrite is generated by the reaction of ROS with NO. 3-NT formation leads to protein dysfunction in the brain (Yamakura et al., 1998) and has been found in a number of neurodegenerative diseases.
disorders, including Alzheimer’s disease (Castegna et al., 2003; Mihm et al., 2001; Reed et al., 2009). Overproduction of NO by iNOS is critical to 3-NT generation (Bagasra et al., 1995; Ryu and Mclarnon, 2006). Our data suggest that C2-ceramide induces iNOS upregulation which results in NO production and possibly 3-NT formation. This increase in 3-NT may in turn contribute to cell death. Asiatic acid slightly inhibited 3-NT formation, yet Asiatic acid did not suppress iNOS expression and subsequent NO release into the cell culture medium. However, Asiatic acid inhibited C2-ceramide-induced ROS generation. Therefore, we hypothesize that an Asiatic acid-mediated reduction in ROS formation, rather than a reduction in NO generation, may be related to decreased 3-NT formation in Asiatic acid-treated neuronal cells.

Several studies suggest that the MAPK signaling pathway is involved in regulating C2-ceramide-induced cell death (Stoica et al., 2005; Willaime et al., 2001). U0126, a selective MEK inhibitor which deactivated ERK1/2, counteracted the protection elicited by Asiatic acid in apoptosis, indicating that Asiatic acid may be neuroprotective by virtue of antagonizing C2-ceramide-induced ERK1/2 dephosphorylation. To test the hypothesis that mitochondrial dysfunction may be related to ERK1/2 dephosphorylation, ΔΨm, ROS levels and Bax expression were examined in the absence and presence of U0126 treatment. The neuroprotective effects of Asiatic acid in terms of ΔΨm, ROS formation and Bax regulation could be partly antagonized by U0126. Thus ERK1/2 seems to be an important molecular target of Asiatic acid affecting ΔΨm and ROS generation and consequently C2-ceramide induced cell death. The mechanisms of how Asiatic acid impacts on ERK1/2 phosphorylation are not fully understood. It can be speculated that Asiatic acid might preserve the activities of upstream kinases of ERK1/2 and subsequently preserve the p-ERK1/2 level. Further investigation is needed to address this issue. Out data suggested a role of p-ERK1/2 in Bax level regulation. It was reported that the ERK1/2 signaling may directly inhibit Bax expression in cardiomyocytes (Nebigil et al., 2003). Accordingly, our data also supported a role of ERK1/2 in Bax regulation. In addition, it was reported that ERK1/2 may phosphorylate other Bcl-2 family proteins and further inhibited the interactions with Bax and ultimately induce cell death (Harada et al., 2004). Therefore, it cannot be excluded the indirect involvement of ERK1/2 in Bax regulation.

Notably, the regulation of p-ERK1/2 could be observed as early as 4 h after Asiatic acid treatment, indicating that ERK1/2 regulation may be an early event mediating neuroprotection by Asiatic acid. It also worth to note that U0126 alone did not induce cell death. Taken together with the fact that U0126 reversed the neuroprotective effects of Asiatic acid, our data indicated that ERK1/2 deactivation may be necessary but not sufficient for neuronal cells survival. In addition, there are some controversy regarding with MAPK regulation manners following C2-ceramide exposure (Stoica et al., 2005; Willaime et al., 2001), together with our results, these data figured out a complex regulation manner of MAPKs response to C2-ceramide. These differences may result from a variety of culture conditions and C2-ceramide concentrations. Nevertheless, all of these investigations found C2-ceramide decreased p-ERK1/2 significantly, which may reflect the truth.

Interestingly, in most of our assays, treatment with 10 μmol/l Asiatic acid exhibited no protective effect against C2-ceramide in our neuronal cells (data not shown), whereas 0.1 and 1.0 μmol/l Asiatic acid exhibited clear neuroprotective activity. Pharmacokinetic studies in dogs suggest that Asiatic acid can reach a maximal plasma concentration of 1.6 μmol/l after oral administration (Zheng and Wang, 2009). In human studies, chronic oral administration of total triterpene fraction from C. asiatica yield approximately 3.1 μmol/l plasmic Asiatic acid (Grimaldi et al., 1990). These concentrations are close to that indicated to be effective in our research. Nevertheless, it need to be established if and to what extent Asiatic acid crosses the blood–brain barrier.

In conclusion, we provide the first evidence that Asiatic acid protects against C2-ceramide-induced injury in neuronal cells by countering mitochondria-dependent apoptosis as well as 3-NT formation. Furthermore, this neuroprotective activity of Asiatic acid may be regulated by the ERK1/2 signaling pathway (Fig. 7). Given the association between ceramide and neurodegenerative disorders, we propose that the cell culture model carried out in the present study could be used as a screening tool for natural compounds with potential neuroprotective activity.

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**References**


Willaine-Morawek, S., Arbez, N., Mariani, J., Brugg, B., 2005. IGF-I protects cortical neurones against ceramide-induced apoptosis via activation of the PI-3K/Akt and ERK pathways; is this protection independent of CREB and Bcl-2? Brain Res. Mol. Brain Res. 142, 97–106.

