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Fermented *Antrodia cinnamomea* Extract Protects Rat PC12 Cells from Serum Deprivation-Induced Apoptosis: The Role of the MAPK Family†

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Antrodia cinnamomea (formerly *A. camphorata*) has recently and commercially been used in the formulation of nutraceuticals and functional foods in Taiwan. Because of its diverse properties, the neuroprotective effect was investigated using a fermented *A. cinnamomea* extract in this study. Serum deprivation-induced apoptosis in neuronal-like pheochromocytoma (PC12) cells was used as a cell stress model, and it was found that *A. cinnamomea* was effective in preventing serum-deprived apoptosis according to results of an MTT assay and Hoechst staining. Serum deprivation resulted in decreased phosphorylation of extracellular signal-regulated kinase (ERK) and increased phosphorylations of c-Jun NH_2-terminal kinase (JNK) and p38, of the family of mitogen-activated protein kinases (MAPKs); however, *A. cinnamomea* reversed these phenomena, supporting the antagonistic effects between ERK and JNK-p38 in regulating cell survival. The previously identified active component of *A. cinnamomea*, adenosine (ADO), also exerted the same effects as *A. cinnamomea* in preventing apoptosis and regulating phosphorylations of MAPKs. Although an inhibitor of the ERK upstream activator blocked *A. cinnamomea*-induced ERK phosphorylations, it failed to block the protection of *A. cinnamomea* and ADO. A protein kinase A (PKA) inhibitor blocked the protection by both *A. cinnamomea* and ADO. Both JNK and p38 inhibitors were effective in preventing the phosphorylations of JNK and p38 and serum deprivation-induced apoptosis. Collectively, *A. cinnamomea* prevented serum deprivation-induced PC12 cell apoptosis through a PKA-dependent pathway and by suppression of JNK and p38 activities.

KEYWORDS: ***Antrodia cinnamomea*; fermentation; PC12 cells; serum deprivation; apoptosis; MAPK**

INTRODUCTION

A. cinnamomea (*1*) is a medicinal fungus of the family Polyporaceae that grows slowly in the inner cavity of the camphor tree, *Cinnamomum kanehirai*. It was recently renamed from *A. camphorata* by Chang and Chou (*2*). It is an indigenous and rare species in Taiwan. *A. cinnamomea* has not only long been utilized to treat a wide variety of diseases, but also has recently drawn the attention of the pharmaceutical industry. Traditionally, it has been used to treat intoxication caused by food, alcohol, and drugs as well as to treat diarrhea, abdominal pain, hypertension, itchy skin, and tumorigenic diseases (*3*). Owing to its many uses, its biological functions have been intensively studied and found to involve anticancer (*4–7*), antihepatotoxic (*8, 9*), antihypertensive (*10*), anti-inflammatory (*11, 12*), antioxidative (*13, 14*), and neuroprotective (*15*) effects. Furthermore, chemical compounds, such as steroids (*16*), polysaccharides (*17–19*), benzenoids (*11, 20*), diterpenes (*15*), triterpenoids (*21*), and maleic/succinic acid derivatives (*22*), may contribute to its biological functions.

Because *A. cinnamomea* is commercially available and is popularly used in the formulation of nutraceuticals and functional foods in Taiwan, it is worthwhile fully characterizing its activities. On the other hand, because the growth rate of natural *A. cinnamomea* in the wild is extremely slow and obtaining fruiting bodies through cultivation is difficult, using a submerged culture method to obtain useful cellular materials or to produce effective substances from cultured mycelia might be an alternative to overcoming the disadvantages of the slow growth of the fruiting bodies (*23*). Thus, fermented mycelia of *A. cinnamomea* were used in this study. In addition, by using different fractionations, our groups have also identified the antiangiogenic (*18, 24*), anti-inflammatory (*25*), antihepatitic (*26*), vasorelaxative (*27*), and neuroprotective (*28*) effects from these fermented mycelia of *A. cinnamomea*. The fraction we used contains no polysaccharides, but does contain nucleosides (*27, 29*), and we

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† The generic name of *Antrodia camphorata* was recently amended to *A. cinnamomea* by Chang and Chou (2004).

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found that adenosine (ADO) is the active component which prevents serum deprivation-induced apoptosis in neuronal-like PC12 cells (*28, 29*).

Neuronal cell death induced by apoptosis is a normal aspect of development in which the death program is triggered by failure of a given neuron to receive limited supplies of target-derived neurotrophic factors. The PC12 cell line is a commonly used model for studies of neuronal differentiation and cell death. Apoptosis may occur when triggered by deprivation of either serum (*30*) or trophic factor/nerve growth factor (NGF) (*31*). The family of mitogen-activated protein kinases (MAPKs), which is largely characterized as proline-directed serine/threonine kinases and which consists of extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38, plays a central role in the signaling pathways of cell proliferation, differentiation, survival, and apoptosis (*32*). Previously, the dynamic balance between growth factor-activated ERK and stress-activated JNK-p38 pathways was shown to be important in determining whether PC12 cells survive or undergo apoptosis (*33*); it was further suggested that the opposing effects of ERK and JNK-p38 MAPKs might generally function in the control of apoptosis in both neuronal and non-neuronal cells.

Currently, because the protective mechanism of *A. cinnamomea* and its active component, ADO (*29*), through the regulation of MAPKs is not clear, in this study, the alterations and functional roles of MAPK family members in response to serum deprivation and *A. cinnamomea* in naive PC12 cells were investigated. In addition, the effective component and the therapeutic potential of *A. cinnamomea* as a neuroprotectant are also discussed.

MATERIALS AND METHODS

Reagents and Cell Culture. All reagents were purchased from Sigma Chemical (St. Louis, MO) except as specified. H-89 was purchased from Biomol (Plymouth Meeting, PA). SB 239063 was purchased from Tocris (Bristol, U.K.). All antibodies were purchased from Cell Signaling (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and horse serum were purchased from HyClone (Logan, UT). Rat PC12 cells purchased from American Type Culture Collection (ATCC; Manassas, VA) were maintained in DMEM supplemented with 10% horse serum and 5% FBS and incubated in a CO_2 incubator (5%) at 37 °C.

Fermentation of *A. cinnamomea*. An *A. cinnamomea* isolate, strain B85 from Taitung, Taiwan, was a generous gift from the fungus specialist, Dr. T. T. Chang (Division of Forest Protection, Taiwan Forest Research Institute, Taipei, Taiwan). *A. cinnamomea* was maintained on potato dextrose agar (Sigma) and transferred to fresh medium at 3 week intervals. For liquid culture, 19-day-old seeding mycelium of *A. cinnamomea* on the surface of medium was cut into pieces (approximately 0.7 × 0.7 cm) before being transferred to 30 mL of potato dextrose broth (Sigma) in 125 mL flasks. Flasks were maintained in a stationary condition at 28 °C with shaking at 90 rpm in the dark for 10 days. Thereafter, 300 mL of the shaking-flask culture was inoculated into a 5 L fermentation tank containing 3 L of culture medium (24 g/L PDB, 2 g/L agar, and 20 g/L glucose; pH 5.6) and then cultured at 28 °C for 10 days with an aeration rate of 1 vvm [aeration volume/medium (L)/min] by shaking at 50 rpm to obtain a mucilaginous medium containing mycelia. At the end of the incubation, the mycelia were rapidly washed with 1 L of NaCl (250 mM) using an aspirator−suction system to remove the contaminated culture medium. Samples were then lyophilized and stored at 4 °C. The dry weight of the mycelia was recorded after lyophilization. Several photographs of the fermentation of the mycelial culture of *A. cinnamomea* are available as Supporting Information.

Preparation of the Mycelial Extracts of *A. cinnamomea*. Lyophilized mycelia were extracted with 80 °C water twice in a 1:100 (w/w) ratio for 6 h. Supernatants were collected after centrifugation, whereupon 4 volumes of 95% ethanol were added and then precipitated at 4 °C overnight. The dilute ethanolic supernatants were lyophilized following centrifugation at 8000*g* for 20 min.

MTT Assay. PC12 cells were deprived of serum by three washes of PBS and resuspended in DMEM. This serum-deprived method can be applied to the Western blot analysis. The suspended cells were plated onto 96-well plates (1×10^4 cells/well) and treated with the indicated inhibitor(s) or *A. cinnamomea*. After treatment for 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the medium (0.5 mg/mL) and incubated at 37 °C for another 3 h. After the medium had been discarded, 100 μL of DMSO was then applied to the well to dissolve the formazan crystals, and the absorbances at 570 and 630 nm in each well were measured on a microenzyme-linked immunosorbent assay (ELISA) reader.

Hoechst Staining. PC12 cells growing on 6-well plates (5×10^5 cells/well) were serum-deprived by three washes of 2 mL of PBS and treated with the indicated reagent in DMEM. Twenty-four hours later, cells were fixed with 4% paraformaldehyde and stained with 5 μg/mL Hoechst 33258. The percentage of apoptotic cells was determined by taking fluorescent photomicrographs and counting the number of cells exhibiting nuclear condensation versus the total (1000−1500) cells in each experimental condition. Because some of the dead cells were washed away during this experiment, the apoptotic ratio could have been underestimated in comparison with that of the MTT assay.

Western Blot Analysis. After the indicated reagent(s) treatments, cells growing on 10 cm dishes [(4−5) × 10^6 cells] were lysed in 300 μL of ice-cold lysis buffer (20 mM HEPES, 1 μM DTT, 20 μM EGTA, 10% glycerol, 50 μM β-glycerophosphate, 10 μM NaF, 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, 2 μM aprotinin, 100 μM leupeptin, 2 μM pepstatin, and 0.5 μM OKA). After sonication, cell debris was removed by centrifugation (at 14000 rpm for 10 min), and equal amounts of the supernatant (30−35 μg/well) were loaded and separated by 10% polyacrylamide gel electrophoresis (PAGE) and then electroblotted onto Immobilon PVDF membranes (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk and then sequentially incubated with the first (1:2000) and second (1:5000) antibodies for 1 h each at room temperature. Blots were processed for visualization using an enhanced chemiluminescence system (Pierce, Rockford, IL) and exposed to Kodak XAR-5 film (Rochester, NY) to obtain the fluorographic images. The images were scanned and quantified using an image analyzer (GeneTools, Synoptics, Cambridge, U.K.).

Components of the *A. cinnamomea* B85 Ethanolic Extract. The separated products were analyzed using a photo diode array detector (Agilent G1315B, Foster City, CA) at 260 nm. Separations were obtained with a reversed-phase column (Cosmosil $5C_{18}$-AR-II, 250 × 4.6 mm, Kyoto, Japan) eluted at a flow rate of 1.0 mL/min with a linear solvent gradient elution system composed of eluents A and B (A, 0.0085% H_3PO_4 in H_2O; B, 100% acetonitrile) according to the following profile: 0−15 min, 100−90% A, 0−10% B. Adenosine, cytidine, and thymidine were used as the external standards to identify the active components in these extracts.

Statistical Analysis. Results were analyzed by one- or two-way analysis of variance (ANOVA) according to whichever was appropriate. Differences between means were assessed by the Student–Newman–Keuls method and were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Apoptosis plays an important role during neuronal development, toxicity, and stress, and may underlie various neurodegenerative disorders. To screen potentially therapeutic compounds from Chinese herbal medicines to treat these degenerations, different stress models that induce apoptotic cell death were established. In this study, a cell stress model using serum deprivation was applied, and the possible neuroprotective effect and mechanism of *A. cinnamomea* were examined.

Antiapoptotic Effects of the Fermented *A. cinnamomea* Extract. Using the MTT assay, the ethanolic extract of *A. cinnamomea* was found to protect PC12 cells from serum deprivation-induced toxicity in a dose-dependent manner

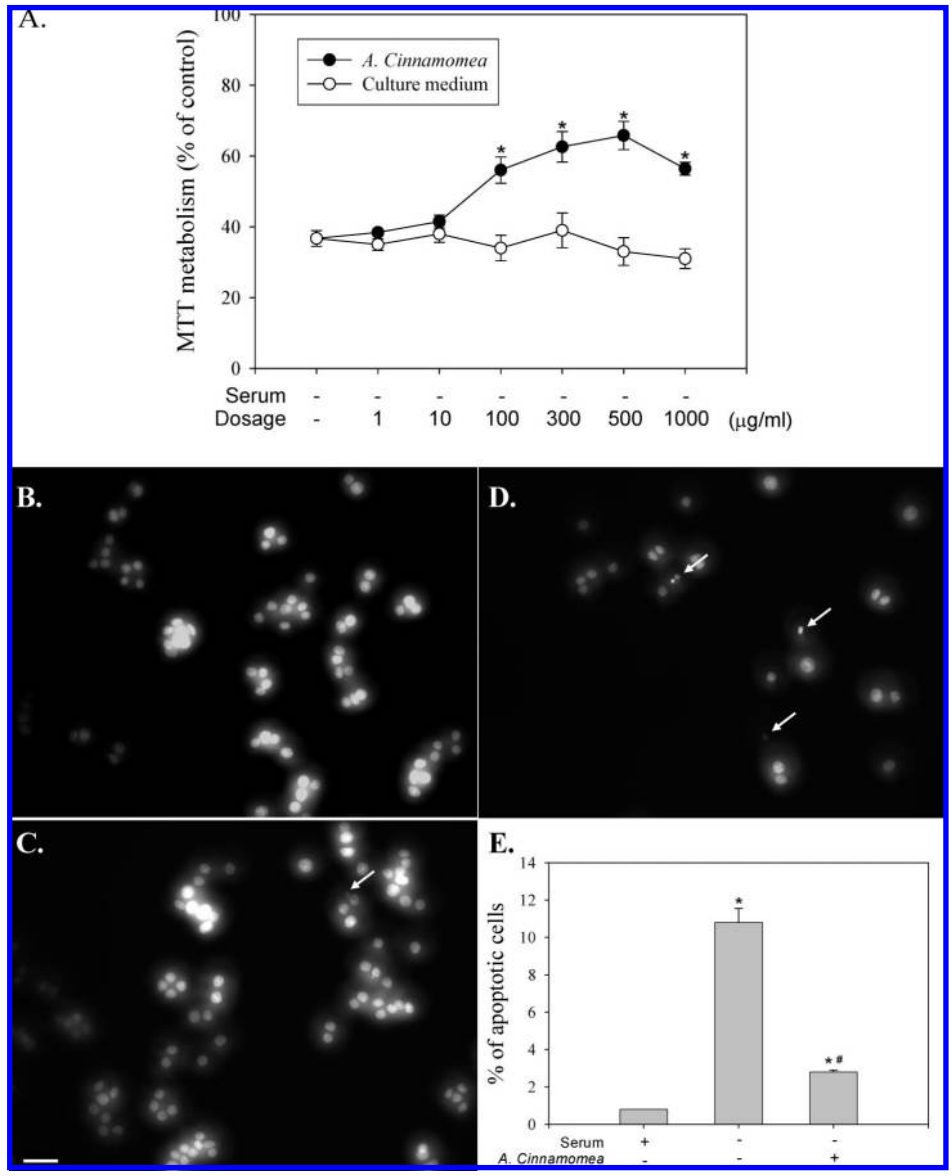

Figure 1. Effects of *Antrodia cinnamomea* in preventing serum deprivation-induced PC12 cell apoptosis. (**A**) Serum-deprived PC12 cells were treated with or without differential dosages of ethanolic extracts of *A. cinnamomea* or its culture medium for 24 h. Cell viability was expressed as a percentage of the results from the MTT assay with respect to the serum-containing control group (100%). (**B**) Cells cultured in serum-containing medium were serum-deprived and treated (**C**) with or (**D**) without *A. cinnamomea* (300 μg/mL). After treatment for 24 h, cells were fixed with paraformaldehyde and stained with Hoechst 33258. White arrowheads indicate apoptotic cells. The bar represents 100 μm. (**E**) Proportions of Hoechst 33258 stained apoptotic cells versus total cells in each group were calculated. Data points represent the mean ± SEM of at least three independent experiments ($n = 3-6$). ∗, $p < 0.05$ compared to the serum-deprived group. #, $p < 0.05$ compared to the corresponding serum-deprived group.

(**Figure 1A**). The mycelial extracts of *A. cinnamomea* were effective in preventing serum deprivation-induced apoptosis, whereas the culture medium was ineffective (**Figure 1A**), demonstrating that the effective component or components are derived from the mycelia instead of being an artifact of the culture conditions. A submaximum dosage (300 μg/mL) was adopted and used for the following experiments. After Hoechst staining, the nuclear morphology was intact in almost all serum-containing cells (**Figure 1B**) and was degraded in a portion of serum-deprived cells (**Figure 1C**). *A. cinnamomea* reversed serum deprivation-induced nuclear degradation (**Figure 1D**), demonstrating that *A. cinnamomea* protects PC12 cells from serum deprivation-induced apoptosis (**Figure 1E**). Other studies using DNA fragmentation (*34*) or annexin-V (*35*) in this similar model also yielded the same conclusions.

Effects of Serum Deprivation on the Phosphorylations of MAPKs. Serine/threonine kinases contain diverse proteins that are responsible for various biological functions, such as apoptosis (*36*). Our previous paper showed that serine/threonine kinases might mediate the neuroprotection of *A. cinnamomea* (*28*), and MAPKs are serine/threonine kinases and have been reported to be responsible for the viability of PC12 cells (*33*). Thus, the significances and alterations of MAPKs in response to serum deprivation and/or *A. cinnamomea* were investigated.

During serum deprivation for 60, 120, and 180 min, phosphorylated ERK1/2 (p-ERK1/2) decreased and phosphorylated JNK1/2 (p-JNK1/2) and p38 (p-p38) increased compared with that of the serum-containing group (**Figure 2**). These results were consistent with the findings of Xia et al. (*33*), who proposed opposite effects of ERK and JNK-p38 MAPKs on apoptosis. Therefore, the effects of *A. cinnamomea* on MAPKs during serum deprivation were subsequently studied.

Effects of *A. cinnamomea* and ADO on the Phosphorylations of MAPKs during Serum Deprivation. During serum

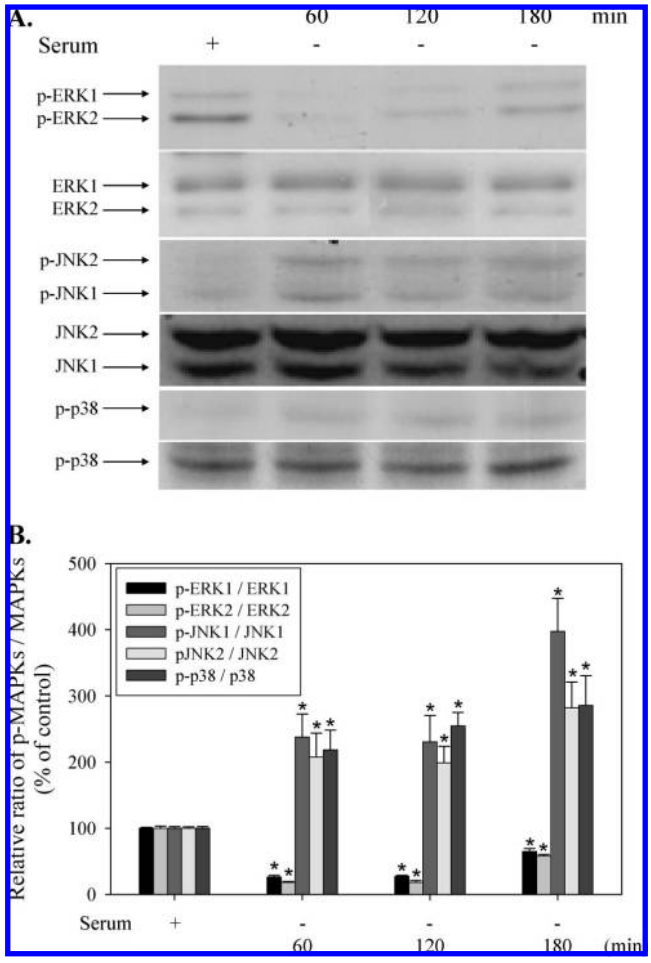

Figure 2. Effects of serum deprivation on the phosphorylations of ERK1/2, JNK1/2, and p38 MAPKs in PC12 cells. (**A**) After serum deprivation for different time intervals, cells were harvested and analyzed by Western blotting. (**B**) Data points that represent the normalized intensity of phosphorylated MAPKs (p-MAPKs) versus nonphosphorylated MAPKs with respect to the serum-contained control group are presented as the mean ± SEM of at least three independent experiments ($n = 3-6$). *, $p < 0.05$ compared to the serum-containing control group.

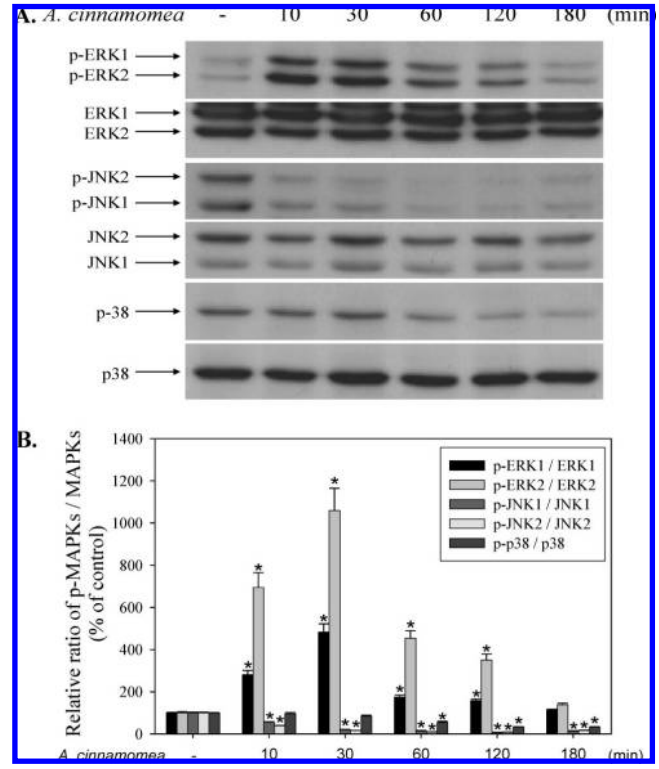

Figure 3. Effects of *Antrodia cinnamomea* on the phosphorylations of ERK1/2, JNK1/2, and p38 MAPKs during serum deprivation. (**A**) Cells that were deprived of serum for 180, 170, 150, 120, 60, or 0 min were treated with *A. cinnamomea* for another 0, 10, 30, 60, 120, or 180 min, respectively. Cell lysates were analyzed by Western blotting. Cells that were deprived of serum for 180 min and treated without *A. cinnamomea* were used as a control group in this experiment. (**B**) Data points that represent the normalized intensity of phosphorylated MAPKs (p-MAPKs) versus nonphosphorylated MAPKs with respect to the serum-deprived control group are presented as the mean ± SEM of at least three independent experiments ($n = 3-6$). *, $p < 0.05$ compared to the serum-deprived control group.

deprivation for 180 min, the drastic time point for alterations of phosphorylated MAPKs, *A. cinnamomea* was administered at different intervals. Results indicated that *A. cinnamomea* reversed serum deprivation-induced dephosphorylation of ERK1/2 and phosphorylation of JNK1/2 and p38 compared with that of the serum-deprived group (**Figure 3**), seemingly further strengthening the opposing effects of ERK1/2 and JNK1/2-p38 MAPKs in regulating apoptosis.

On the other hand, because our previous paper showed that ADO is the active component of *A. cinnamomea* in preventing serum deprivation-induced PC12 cell apoptosis and its protective mechanism has not been determined (*29*), the effects of ADO on the phosphorylations of MAPKs were studied. As expected, ADO not only prevented serum deprivation-induced apoptosis (**Figure 4A**) but also reciprocally regulated phosphorylations between ERK1/2 and JNK1/2-p38 MAPKs (**Figure 4B**,**C**), consistent with the effects of *A. cinnamomea*.

Effects of Kinase Inhibitors on the Protective Effects of ADO and ADO-Induced Phosphorylations of ERK1/2. Furthermore, as our previous paper showed that a PKA inhibitor can block the protective effects of *A. cinnamomea* (*28, 29*) and PKA also mediates ERK1/2 phosphorylations (*37, 38*), we tested if the protective effects of ADO involve the PKA and/or ERK1/2 pathway. H-89, a PKA inhibitor, not only attenuated ERK1/2 phosphorylation, although partially but significantly, but also blocked the protection induced by ADO and *A. cinnamomea* during serum deprivation (**Figure 5A**,**B**), supporting the previous paper's results that ERK1/2 can be activated by PKA and indicating involvement of the PKA and/or ERK1/2 pathway. We thus dissected their relevance and found that although PD 98059, an inhibitor of MEK which activates ERK1/2, was effective in preventing ADO- and *A. cinnamomea*-induced ERK1/2 phosphorylations (**Figure 5A**,**B**), PD 98059 failed to block the protection afforded by ADO and *A. cinnamomea* (**Figure 5C**), demonstrating the irrelevance of the ERK pathway in mediating the protective mechanisms of ADO and *A. cinnamomea*.

Effects of JNK and p38 Inhibitors on Serum Deprivation-Induced Apoptosis and Their Related Enzyme Activities. In addition, if serum deprivation-induced JNK1/2 and p38 activations indeed mediate PC12 cell apoptosis, then their inhibitors should have some degree of protective effects. Indeed, the JNK and p38 inhibitors, dicumarol and SB 239063, not only attenuated the respective serum deprivation-induced JNK1/2 and p38 phosphorylations (**Figure 6A**,**B**) but also blocked serum deprivation-induced apoptosis (**Figure 6C**), although partially but significantly. This finding partly supports the proapoptotic natures of JNK and p38 in this system.

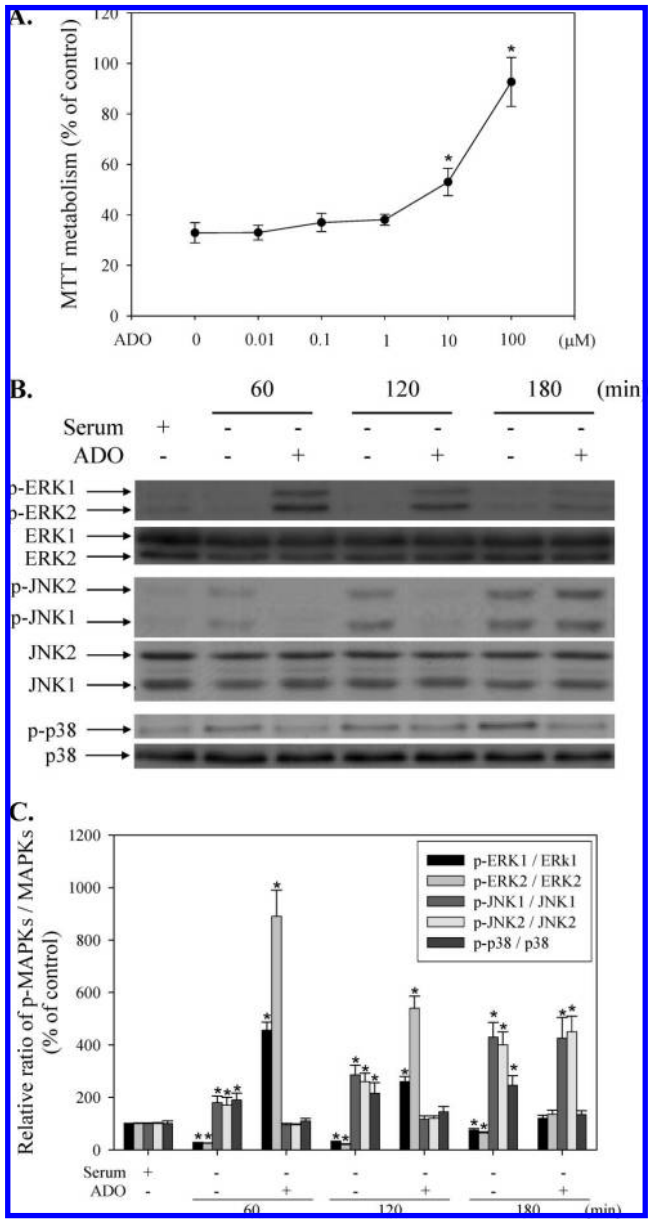

Figure 4. Effects of adenosine (ADO) on PC12 cell viability and the phosphorylations of ERK1/2, JNK1/2, and p38 MAPKs during serum deprivation. (**A**) Serum-deprived PC12 cells were treated with or without ADO at different dosages for 24 h. Cell viability was expressed as a percentage of the results from the MTT assay with respect to the serum-containing control group (100%). Data points represent the mean ± SEM of at least three independent experiments ($n = 3-6$). *, $p < 0.05$ compared to the serum-deprived group. (**B**) Cells that were deprived of serum were treated with or without *A. cinnamomea* for 60, 120, or 180 min. Cell lysates were analyzed by Western blotting. Serum-containing cells were used as a control group in this experiment. (**C**) Data points that represent the normalized intensity of phosphorylated MAPKs (p-MAPKs) versus nonphosphorylated MAPKs with respect to the serum-deprived control group are presented as the mean ± SEM of at least three independent experiments ($n = 3-6$). *, $p < 0.05$ compared to the serum-containing control group.

Roles of MAPKs in Regulating Serum Deprivation-Induced Apoptosis. The finding that the MEK inhibitor was unable to block the protective mechanisms of *A. cinnamomea* and ADO (**Figure 5C**) contradicts the assertion by Xia et al. (*33*) and counters the idea that MEK/ERK signaling plays a critical role in cell survival (*39*). However, this may be controversial. A MEK inhibitor has been shown to protect against damage resulting from focal cerebral ischemia (*40*), and H_2O_2-induced apoptosis is mediated by ERK1/2 phosphorylations in mouse fibroblast cells (*41*). Furthermore, it was also found that NGF-mediated PC12 cell survival does not require ERK1/2 activation (*42*), supporting our finding (**Figure 5**). Thus, although the significance of ERK phosphorylation induced by *A. cinnamomea* (or ADO) is currently unknown (**Figure 7**), the sustained increase in ERK activity, which has been suggested to promote cell growth and/or differentiation instead of cell survival (*42*), may partly explain this question. However, its resolution requires further investigations.

Although ample evidence suggests a correlation between activation of the p38 pathway and apoptosis (*43*), others have shown it to be antiapoptotic (*44*). Recent evidence has indicated a number of links between stress kinases (p38) and cell-cycle checkpoint pathways (*45*). Because serum deprivation results in significant growth arrest in PC12 cells (*46*) and apoptosis (*47*), it is possible that serum deprivation-induced p38 activation may contribute to regulating the cell cycle.

The role of JNK activation in mediating apoptosis is also controversial, as it has been suggested to have a proapoptotic role, an antiapoptotic role, or no role in this process (*48*). Knowledge of the proapoptotic JNK pathway originated from a study of NGF withdrawal-induced apoptosis in sympathetic neurons (*49*). Currently, we found that *A. cinnamomea* suppressed JNK activation (**Figure 3**) and that a JNK inhibitor attenuated serum-deprived apoptosis (**Figure 6**), indicating a role for JNK in mediating apoptosis (**Figure 7**). Previously, transfection of a JNK dominant negative mutant rescuing PC12 cells from serum-deprived apoptosis supported this notion (*34*). However, it should be noted that neither JNK nor p38 necessarily regulates similar events, such as apoptosis. For instance, JNK and p38 exert opposing effects on cardiomyocyte hypertrophy (*50*).

The dephosphorylation of p38 (or JNK) occurs through MAPK phosphatases (*32*), and the upstream pathway of ERK consists of Ras/Raf/MEK; therefore, *A. cinnamomea* may directly or indirectly activate MAPK phosphatases and the upstream pathway of ERK that results in p38 (or JNK) suppression and ERK activation. In addition, previous studies have shown that PKA functions as a negative regulator of JNK (*51*) and that cyclic AMP, a PKA activator, inhibits p38 activation in PC12 cells (*52*), indicating a PKA-mediated event. Because we found the PKA-dependent pathway of *A. cinnamomea* regulates apoptosis (*28*), the suppressions of JNK and p38 phosphorylations by *A. cinnamomea* (**Figure 3**) may be mediated by PKA (**Figure 7**). However, confirming these possibilities requires further investigations.

Because JNK and p38 inhibitors only partially rescued PC12 cells from cell death (**Figure 6**) and their protective effects were smaller than that of the extract of *A. cinnamomea*, other signaling pathways might be involved in the ability of *A. cinnamomea* to promote cell survival. Therefore, although the protective mechanism of *A. cinnamomea* is serine/threonine kinase-dependent and ERK-independent, there are still other serine/threonine kinases [such as PKA (*36*) (**Figure 5**), PKB, and PKC] that are also known to regulate apoptosis (*36*) and may be responsible for the protective signaling pathways of *A. cinnamomea*.

Collectively, ERK being a protective signal and JNK-p38 MAPKs being a proapoptotic signal do not always hold true and may depend on the nature of the cell type, the death stimulus, the duration of activation, and probably, most importantly, the activities

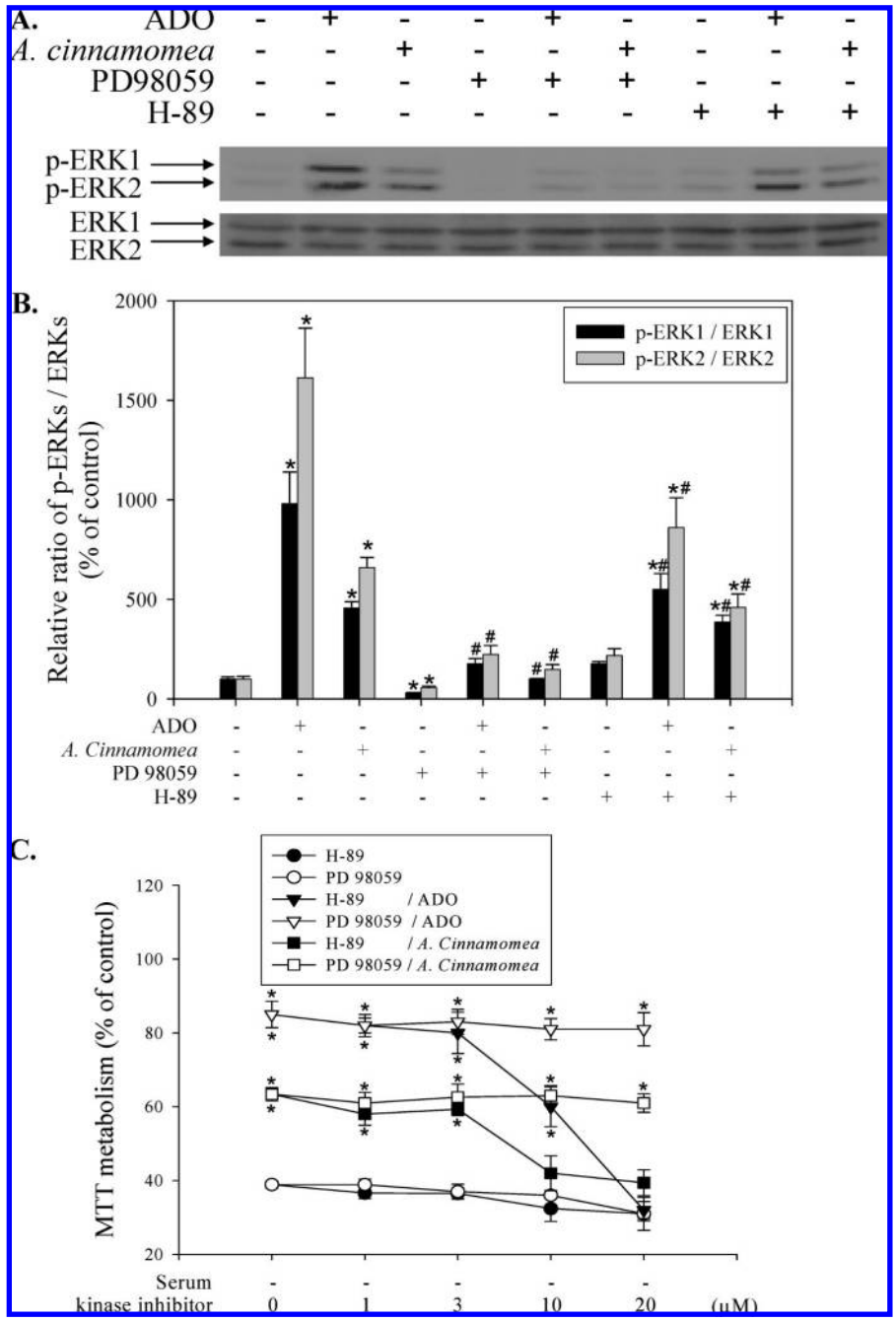

Figure 5. Effects of MEK or PKA inhibitors on the phosphorylation of ERK1/2 and cell protection mediated by adenosine (ADO). (**A**) After serum deprivation, cells were pretreated with or without kinase inhibitors for 30 min, followed by the addition or exclusion of *Antrodia cinnamomea*. After *A. cinnamomea* treatment for 60 min, cells were harvested and analyzed by Western blotting. Cells that were only deprived of serum were used as a control group in this experiment. (**B**) Data points that represent the normalized intensity of phosphorylated ERK1/2 versus nonphosphorylated ERK1/2 with respect to the serum-deprived control group are presented as the mean ± SEM of at least three independent experiments ($n = 3-6$). ∗, $p < 0.05$ compared to the serum-deprived control group. #, $p < 0.05$ compared to the corresponding ADO-containing group. (**C**) Serum-deprived cells were pretreated with or without different dosages of H-89 or PD 98059 for 30 min, and then *A. cinnamomea* was added for 24 h. Cell viability was expressed as a percentage of the results from the MTT assay with respect to the serum-containing control group (100%). Data points represent the mean ± SEM of at least three independent experiments ($n = 3-6$). ∗, $p < 0.05$ compared to the serum-deprived group.

of other signaling pathways (*39, 43, 48*). Thus, the opposing effects of ERK and JNK-p38 MAPKs in apoptosis may be valid in NGF-differentiated (*33*) but not in naive PC12 cells.

Role of ADO as an Active Component of *A. cinnamomea* and Its Molecular Targeting in Regulating Apoptosis. On the other hand, ADO is abundant in diverse foodstuffs including many medicinal mushrooms, such as *Ganoderma lucidum* (*53*) and *Cordyceps* spp. (*54*). Previously, we tested the effects of other fungi, including *Antrodia taxa*, *Antrodia xantha*, *Antrodia malicola*, *Antrodiella liebmanni*, *Fomitopsis pinicola*, and *Agaricus murrill*, which contain no detectable ADO and exert no protection against serum deprivation-induced apoptosis (data not shown). Again, we also identified other nucleosides, such as thymidine and cytidine, in *A. cinnamomea* but found them to have no protective effect as does ADO, further suggesting the importance of ADO.

In addition, four ADO receptors (ARs) (A_1, A_{2A}, A_{2B}, and A_3) have been identified (*55*) and are regarded as therapeutic

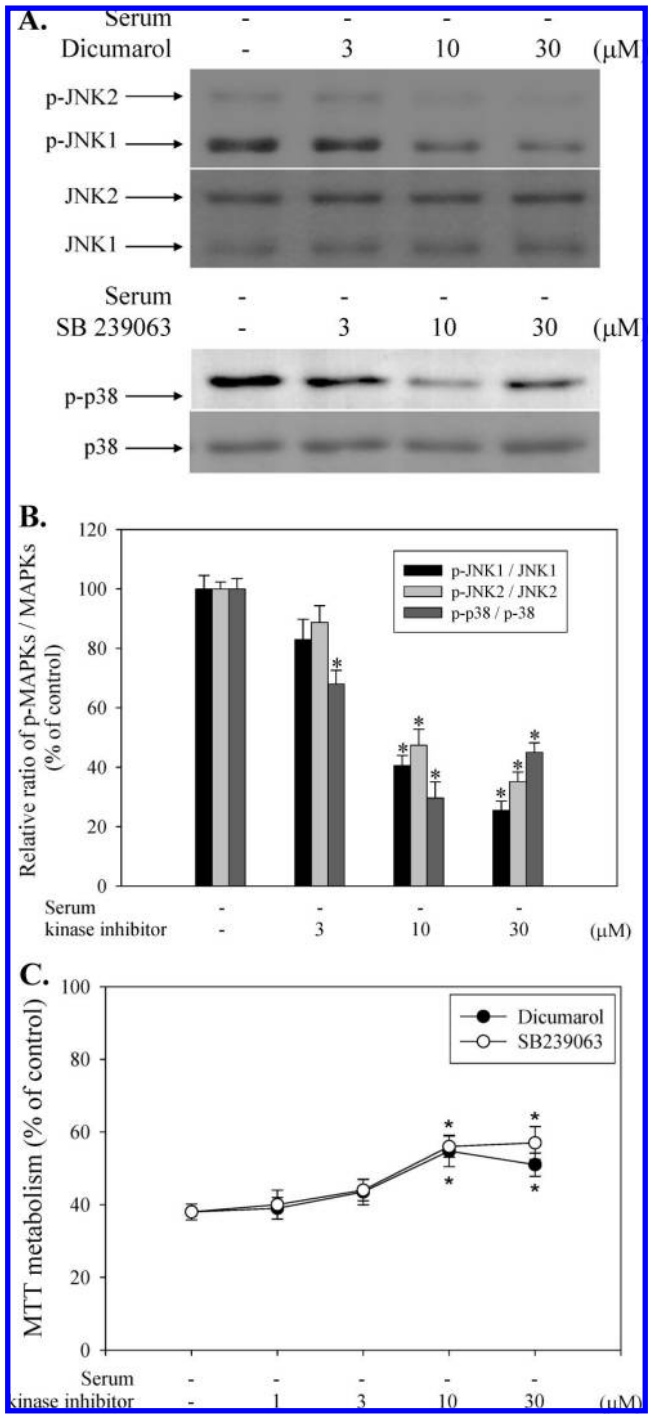

Figure 6. Effects of dicumarol, a JNK inhibitor, and SB 239063, a p38 inhibitor, on the phosphorylations of JNK1/2 and p38 and on serum-deprived apoptosis. (**A**) After serum deprivation and kinase inhibitor (dicumarol or SB 239063) treatment for 3 h, cells were harvested and analyzed by Western blotting. Cells that were only deprived of serum were used as a control group in this experiment. (**B**) Data points that represent the normalized intensity of phosphorylated JNK1/2 (p-JNK1/2) or p38 (p-p38) versus nonphosphorylated JNK1/2 or p38 with respect to the serum-deprived control group are presented as the mean ± SEM of at least three independent experiments ($n = 3-6$). ∗, $p < 0.05$ compared to the serum-deprived control group. (**C**) Serum-deprived cells were pretreated with or without different dosages of dicumarol or SB 239063 for 24 h. Cell viability was expressed as a percentage of the results from the MTT assay with respect to the serum-containing control group (100%). Data points represent the mean ± SEM of at least three independent experiments ($n = 3-6$). ∗, $p < 0.05$ compared to the serum-deprived group.

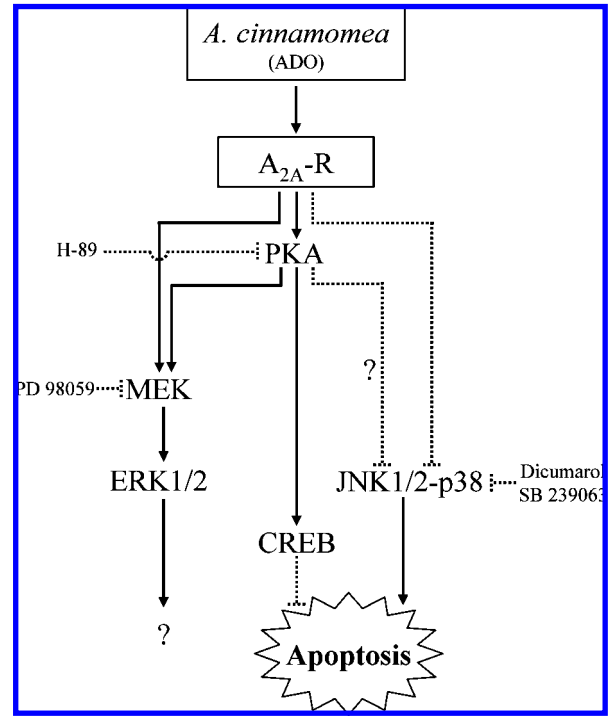

Figure 7. Mechanisms of *A. cinnamomea* in preventing serum deprivation-induced apoptosis. *A. cinnamomea* [or adenosine (ADO), the active component of *A. cinnamomea*] could target the A_{2A} receptor (A_{2A}-R) to prevent serum-deprived apoptosis through the PKA/CREB pathway (*28, 29*). *A. cinnamomea* resulted in increased phosphorylation of ERK1/2 and decreased phosphorylation of JNK1/2-p38 (**Figure 3**). ADO exerted effects similar to those of *A. cinnamomea* on these MAPKs (**Figure 4**). Although PD 98059, an inhibitor of MEK which activates ERK1/2, blocked *A. cinnamomea*-mediated ERK1/2 activation (**Figure 5A**), it could not block protection by *A. cinnamomea*(**Figure 5**), indicating the absence of ERK1/2 in mediating apoptosis in this model. Therefore, the significance of *A. cinnamomea*-increased phosphorylation of ERK1/2 is not known and requires further investigation. Furthermore, H-89, a PKA inhibitor, blocked the protection by *A. cinnamomea* (**Figure 5C**) and partially attenuated *A. cinnamomea*-induced ERK1/2 activation (**Figure 5A**), further confirming the importance of PKA-mediated protection of *A. cinnamomea* and suggesting PKA-dependent and -independent pathways of *A. cinnamomea* in regulating ERK1/2 activity. *A. cinnamomea*-induced dephosphorylations of JNK1/2 and p38 may partly contribute to the antiapoptotic effect (**Figure 3**), because both a JNK inhibitor (dicumarol) and a p38 inhibitor (SB 239063) blocked serum deprivation-induced apoptosis and phosphorylation of JNK1/2-p38 (**Figure 6**). Because PKA-dependent negative regulation of JNK (*52*) and p38 has been demonstrated, it is also possible that *A. cinnamomea*-induced suppression of the phosphorylations of JNK1/2 and p38 is mediated by PKA. The solid and dashed lines, respectively, indicate stimulatory and inhibitory effects.

targets in different milieu (*56*). Each of them has a unique pharmacological profile, tissue distribution, and effector coupling (*56*). By acting through different ARs, ADO plays a number of diverse roles in normal physiological and pathological functions (*57*). It is also recognized as exhibiting neuromodulation and neuroprotection (*58–60*). For instance, ADO is effective in preventing hypoxic/ischemic injury (*61, 62*) and exhibits myocardial protection (*57*). In contrast, ADO has also been found to induce apoptosis in leukemia (*63*), endothelial (*64, 65*), and smooth muscle (*66*) cells. Those results tend to contradict our findings. Currently, the mechanisms of these discrepancies are not clear. The dosing regimens and tissues that are coupled with different targets or machineries may account for these discrep-

ancies. For instance, 1 mM ADO was used in HL-60 cells instead of a lower range (*63*). ADO was revealed to induce apoptosis in hamster kidney (*67*), human arterial smooth muscle (*66*), and human hepatoma (*68*) cells through action on A_1R, $A_{2B}R$, and A_3R, respectively. Furthermore, apoptotic events may involve the regulation of tyrosine phosphatase (*65*), Bcl-2 (*69*), or *S*-adensyl-L-homocysteine hydrolase (*64*). However, on the basis of our current studies (**Figure 5**) and a previous paper (*29*), ADO may act through the $A_{2A}R$/PKA pathway to prevent serum deprivation-induced apoptosis (**Figure 7**).

Furthermore, we previously used ADO deaminase to degrade ADO, thus proving that ADO is an active component in *A. cinnamomea*'s efficacy against serum deprivation-induced apoptosis (*29*); however, because most AR agonists are ADO-based derivatives, we could not rule out the existence of ADO-based derivatives as protective agonists. This viewpoint was supported by the following findings. A novel N^6-substituted adenosine isolated from *Armillaria mellea* has cerebral-protective effects (*70*). Several adenosine derivatives derived from *Atriolum robustum* were shown to inhibit cyclic-AMP formation through A_1R activation (*71*). Therefore, the finding of ADO in *A. cinnamomea* seemingly refines these implications; other ADO-based derivatives serving as neuroprotectants may still exist. Further investigations are required to resolve this question.

Taken together, in this study, we not only demonstrate that *A. cinnamomea* prevents serum-deprived PC12 cell apoptosis, suggesting a novel therapeutic potential in treating neurotoxicity or an opportunity for exploration of neuroprotectants, but also indicate that the protective mechanism may be mediated through a PKA-dependent pathway and by suppression of JNK and p38 activities (**Figure 7**).

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Supporting Information Available: Fermentation of the mycelial culture of *A. cinnamomea*. This information is available free of charge via the Internet at http://pubs.acs.org.

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