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Neuroprotective effects of kobophenol A against the withdrawal of tropic support, nitrosative stress, and mitochondrial damage in SH-SY5Y neuroblastoma cells

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Abstract—This study examined the neuroprotective effects of kobophenol A (kob A), oligomeric stillbene, and a resveratrol tetramer. Neuronal death induced by the withdrawal of tropic support was ameliorated by kob A. The protective effect of kob A against nitrosative/oxidative or mitochondrial damages resulted in the inhibition of the ROS, intracellular calcium ion level, and mitochondrial transmembrane potential changes on SH-SY5Y cells.
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Neuronal death is an important feature of both acute and chronic neurodegenerative diseases.[1,2] Among various neurological disorders, Parkinson's disease is a major cause of morbidity and disability in the adult population. Parkinson's disease is characterized by a selective apoptotic loss of dopaminergic neurons in the tier ventral of the pars compacta of substantia nigra.[3] Although the cause of nigral apoptotic death is not completely understood, the withdrawal of tropic support,[4,5] mitochondrial dysfunction,[6,7] perturbed calcium homeostasis,[2] and nitrosative and/or oxidative stress,[8,9] have been repeatedly observed in clinical settings, as well as in experimental models. Therefore, to prevent pathological damage and/or reverse abnormalities associated with Parkinson's disease and other neurodegenerative disorders, the development of a neuroprotectant is an important issue.

The root of *Caragana sinica* (Buc'hoz) Rehd. (*Leguminosae*) has been used in East Asia as a folk medicine purportedly effective against neuralgia, rheumatism, vascular hypertension, bruises, and contusions.[10–15] Kobophenol A (kob A, $C_{56}H_{44}O_{13}$), a tetrastillbene, is one of the major active compounds of *C. sinica*.[13] Previous chemical studies of kob A reported an antimicrobial activity on *Staphylococcus aureus*[12] and a proliferative activity on cultured osteoblasts in vitro.[14] However, evidence of its pharmacological basis for therapeutic effects has yet to be demonstrated and the effects of kob A on neuronal death caused by nitrosative/oxidative stress remain elusive.

The present study investigated the possibility of kob A as a neuroprotective alternative through whether kob A prevents or reduces the neuronal cell death by the tropic support withdrawal, nitrosative stress, and mitochondrial damaging agents in SH-SY5Y cells that can be differentiated into N-type dopaminergic neurons and are widely used as an in vitro model to study effects and mode of action of drugs on neuronal diseases.[16,17]

To examine the effect of kob A on cell viability, the SH-SY5Y cells were treated with various concentrations of kob A for 24 h in a normal culture medium. The level of cell viability was determined using the MTT assay and was expressed as a percentage of the untreated control (Fig. 1). Kob A augmented cell proliferation as compared with the untreated control. However, concentrations greater than 50 μg/mL were cytotoxic to cells (data not shown). To examine the protective effect of kob A against tropic support withdrawal, the cells were cultured in a serum-starved medium for 48 h in the absence or presence of kob A (Fig. 1). As compared to serum-starved culture condition (cell viability, 64 ± 1.9%), kob A treatment resulted in an increase in cell viability by 105.7 ± 3.7%, 91.4 ± 4.3%, and

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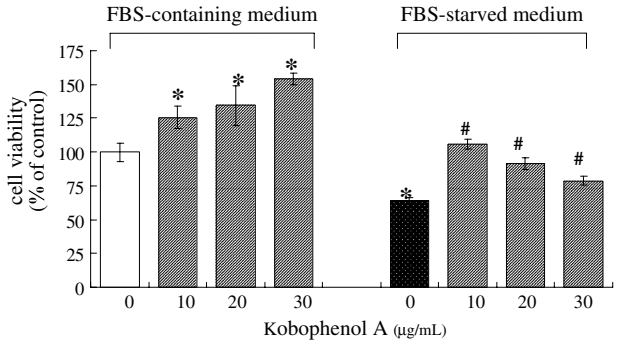

Figure 1. Effect of kobophenol A on cell viability in the presence or absence of FBS. The SH-SY5Y cells were treated with various concentrations of kobophenol A in the presence of FBS for 24 h or in the absence of FBS for 48 h. The cell viability was then determined using the MTT assay and was expressed as a percentage of the untreated control. Data are represented as means ± SEM from triplicate samples ($n = 5$). $^{*}P < 0.01$ compared with the medium control, $^{\#}P < 0.01$ compared with the FBS-starved control (t-test).

78.7 ± 3.3%, at the concentrations of 10, 20, and 30 μg/mL, respectively. It is possible that cell proliferative effect of kob A might be connected to the protective effect of kob A. Because kob A showed its optimal effect on both the proliferation and the protection of SH-SY5Y cells at the concentration of 30 μg/mL, in most subsequent experiments cells were treated with 30 μg kob A /mL.

To further examine the protective effect of kob A on neuronal death caused by nitrosative/oxidative stress[8,9,17,18] and mitochondrial damage,[6,7,19] MTT assay and Sub G_1 DNA content analysis with propium iodide(PI) staining were performed (Figs. 2 and 3). Sodium nitroprusside (SNP) and paraquat (PQ) were used as the nitrosative/oxidative stress stimulus and mitochondria-damaging agent, respectively. Cellular morphological changes such as round-up and floating were severely induced by SNP and PQ at 12 h and these changes were pronounced with time, whereas kob A co-treatment with SNP or PQ reduced the change of cell morphology (data not shown). The exposure of cells to SNP at 1, 3, and 5 mM for 24 h caused a significant decrease in cell viability to 89.4 ± 1.3%, 38.1 ± 3.2%, and 34.6 ± 9.5% as compared to untreated control, respectively. When the cells were exposed to SNP with kob A for 24 h, cell death was remarkably inhibited and kob A treatment resulted in an increase in cell viability by 108.6 ± 12.4%, 109.8 ± 6.4%, and 88.1 ± 9.5% at the concentrations of 1, 3, and 5 mM SNP, respectively (Fig. 2). While treatment of cells with PQ also caused a significant decrease in cell viability at the concentrations of 2 and 5 mM PQ, exposure to 2 mM PQ with various concentrations of kob A for 24 h resulted in an increase in cell viability (66.3 ± 8.5%, 85.6 ± 2.0%, and 102.5 ± 2.5% of control). However, treatment with kob A has little protection against induction of apoptosis by 5 mM PQ (data not shown). This little protective effect of kob A on 5 mM PQ may be due to rapid or diverse toxic effect of high concentration of PQ or its toxicity surpassing the protective capability of kob A. In addition to changes in survival rate, the protective effect of kob A was further confirmed by flow cytometric analysis. A sub-G1, indicating the amount of apoptotic nuclei, was clearly more pronounced in SNP- or PQ-treated SH-SY5Y cells than in medium-treated cells (Fig. 3). Treatment with SNP or PQ greatly increased the apoptotic cell population to 83.1% and 55.3%, respectively. However, kob A significantly reduced neuronal cell death induced by SNP or PQ in comparison with its vehicle. The family of caspases is the key effector in the execution of apoptotic cell death. Therefore, this study analyzed the activation of caspases in SNP- or PQ-treated cells. The activity of caspase-3 and -9 in these cells was higher than in the cells cultured with medium only. However, the increased activity of caspase-3 and -9 was decreased by kob A treatment in a dose-dependent manner (Fig. 4). Overall, these results demonstrate that kob A exerts an effective protection from nitrosative/oxidative stress.

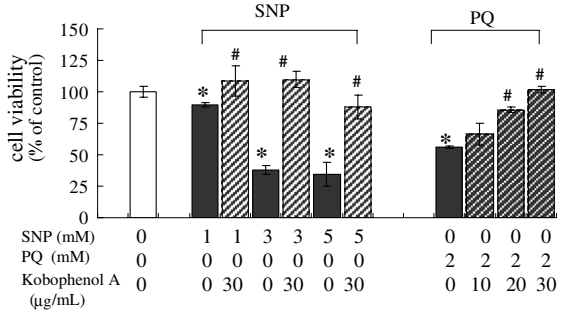

Figure 2. Effect of Kobophenol A on SNP- or PQ-induced cytotoxicity in SH-SY5Y cells. The cells were treated with SNP or PQ in the presence or absence of kobophenol A. Twenty-four hours later, the cell viability was determined using the MTT assay and was expressed as a percentage of the medium control. Data are represented as means ± SEM from triplicate samples ($n = 5$). $^{*}P < 0.01$ compared to medium control, $^{\#}P < 0.01$ compared with SNP- or PQ-treated control (t-test).

In order to elucidate the protective mechanism of kob A, we performed the following experiment. Because apparent morphological change such as cell round-up was not seen until 12 h treatment, the incubation time was adjusted to 8 h from 24 h to measure the production of reactive oxygen species (ROS), intracellular calcium level,[20] and mitochondrial transmembrane potential (MMP).[6,7] ROS is a cell-damaging and/or apoptosis-inducing mediator.[8,9] Following SNP and PQ treatment, the production of ROS was determined by CM-H_2-DCFDA (5 μM) and was expressed as a percentage of the medium control. The ROS level was 109.3 ± 0.7% and 107.3 ± 0.3% at 3 mM SNP and 2 mM PQ, respectively. However, SNP and PQ with kob A treatment reduced ROS production by 103.4 ± 0.1% ($P < 0.01$) and 103.6 ± 0.3% ($P < 0.05$), respectively. Our results indicate that kob A has the capacity to scavenge ROS or to inhibit the excessive production of ROS. This modulatory effect of kob A on the formation of ROS might play a role in protecting the cells from SNP or PQ-induced cytotoxicity and be related to its antioxidant property.

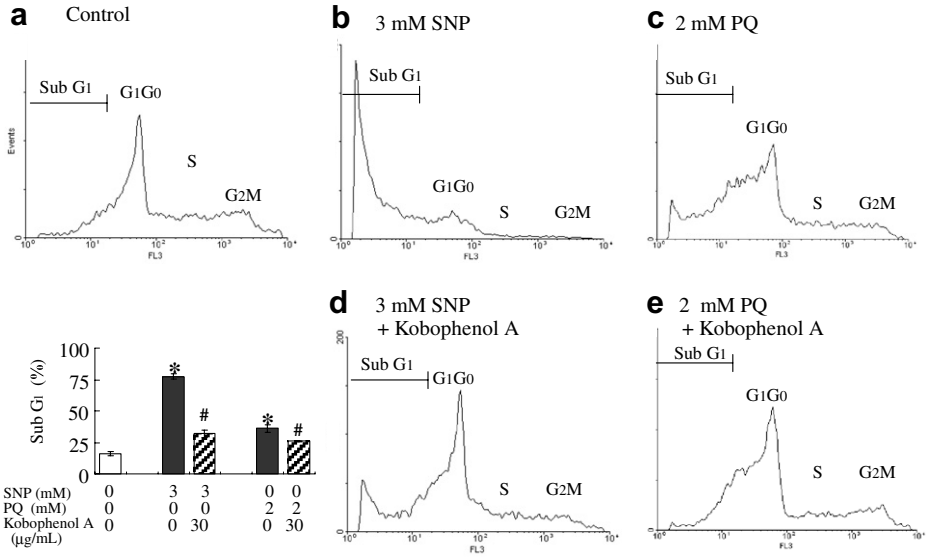

Figure 3. Effect of kobophenol A on the rate of Sub G_1 DNA content induced by SNP or PQ. After treatment of cells with SNP or PQ in the presence or absence of kobophenol A, Sub G_1 DNA content was measured by staining with PI using flow cytometry. Data are represented as means ± SEM from triplicate samples. $^{*}P < 0.01$ compared with the medium control, $^{\#}P < 0.01$ compared with SNP- or PQ-treated control (*t*-test).

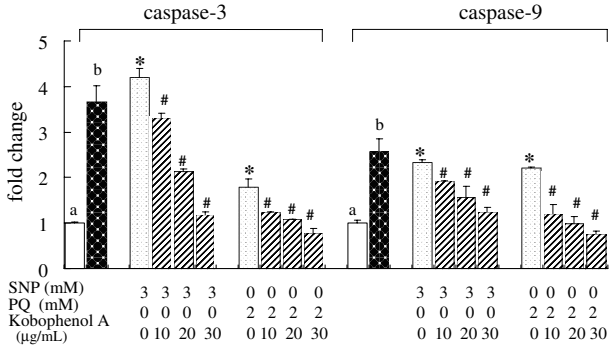

Figure 4. Effect of kobophenol A on the activity of caspase-3 and caspase-9. SH-SY5Y cells were treated with SNP or PQ in the presence or absence of kobophenol A. Eighteen hours later, cells were harvested in lysis buffer and enzymatic activities of caspase-3 and -9 were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrates, Ac-DEVD-pNA and Ac-LEHD-*p*NA, respectively. The release of chromophore pNA was monitored spectrophotometrically (405 nm). Data are represented as means ± SEM from triplicate samples. a and b indicate untreated control and positive control (etoposide, 10 μM), respectively. $^{*}P < 0.01$ compared with the medium control, $^{\#}P < 0.01$ compared with SNP- or PQ-treated control (*t*-test).

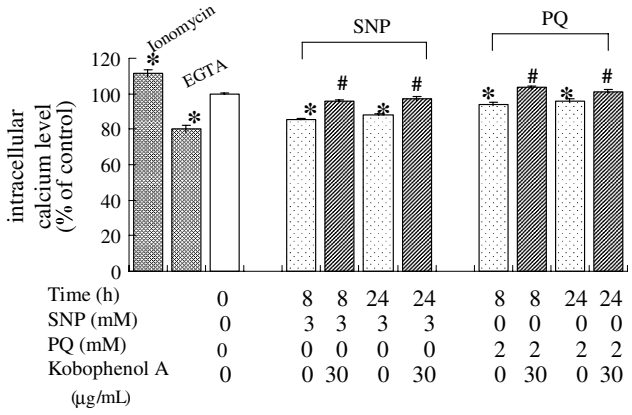

Figure 5. Effect of kobophenol A on intracellular calcium level induced by SNP or PQ. Using Fluo-3 (5 μM), the intracellular calcium level was determined by flow cytometry. Ionomycin (10 μM) was used as a positive control and EGTA (5 mM) was used as a negative control. The SH-SY5Y cells were exposed to SNP or PQ in the presence or absence of kobophenol A. Eight or twenty hours later, intracellular calcium level was measured and expressed as a percentage of the medium control. Data are represented as means ± SEM from triplicate samples. $^{*}P < 0.01$ as compared with the medium control, $^{\#}P < 0.01$ compared with SNP- or PQ-treated control (*t*-test).

Calcium is one of the important second messengers in neurons and is involved in various cell signaling pathways.[20] Perturbation or dysregulation of calcium homeostais is another important cause of neuronal death.[2] When cells were exposed to SNP for 8 h, the intracellular calcium level was 85.6 ± 0.3% as compared with the medium control. However, by the co-treatment with kob A, the intracellular calcium level was preserved up to 96.1 ± 0.4% (Fig. 5). Similar results were also seen when cells were treated with 3 mM SNP in the presence of kob A for 24 h. It has been known that the reduction of intracellular calcium is related to the NO-induced anoikis.[21] Anoikis is an adhesion-related apoptosis and is initiated by the action of antiadhesive substances such as NO. Based on this finding the reduction of intracellular calcium level induced by NO appears to be inhibited by kob A treatment. However, kob A did not affect the NO production by SNP (data not shown). Therefore, the present data suggest the inhibitory effect of kob A on intracellular calcium level decreased by SNP may not be due to the reduction of NO production but to other activity of kob A. The intracellular calcium level induced by 2 mM PQ treatment was 90.1 ± 1.0% and 91.6 ± 1.0% at 8 and 24 h, respectively. However, when

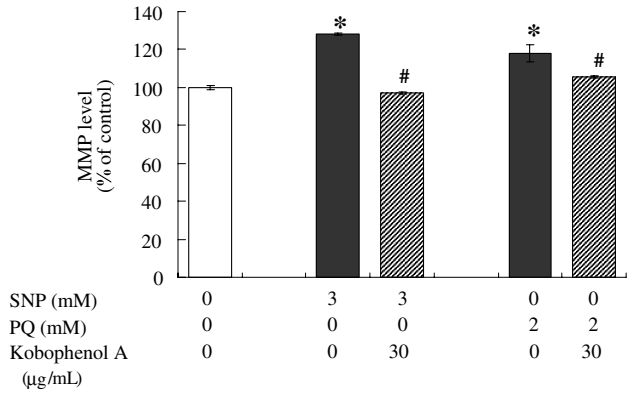

Figure 6. Effect of kobophenol A on the mitochondrial transmembrane potential (MMP) change. Using JC-1 (1 μg/mL), the MMP was determined by flow cytometry. The SH-SY5Y cells were treated with SNP or PQ in the presence or absence of kobophenol A. Eight hours later, MMP level was measured and expressed as a percentage of the medium control. Data are represented as means ± SEM from triplicate samples. $^{*}P < 0.01$ compared with the medium control, $^{\#}P < 0.01$ compared with SNP- or PQ-treated controls (*t*-test).

exposed to PQ with kob A, the intracellular calcium level was 99.1 ± 0.7% and 98.3 ± 0.9% at 8 and 24 h, respectively (Fig. 5). Our data suggest that kob A treatment showed a strong tendency to retain the physiological level of intracellular calcium in SH-SY5Y cells.

The mitochondria are important cell organelles involved in the production and regulation of cellular energy. Excessive production of NO and ROS has an influence on the mitochondrial transmembrane potential (MMP) and induces the depolarization of the MMP.[19,22] When the MMP was measured by JC-1 it resulted in an increase by 128.3 ± 0.4% and 119.3 ± 1.8% with 3 mM SNP and 2 mM PQ treatment at 8 h, respectively (Fig. 6). However, SNP and PQ treatment with kob A resulted in MMP levels of 100 ± 0.3% and 104.1 ± 0.9%, respectively (Fig. 6). Unexpectedly, the MMP level was increased (hyperpolarization) by SNP- or PQ-treatment. To confirm this result, the MMP was measured at 8 h using another MMP-specific fluorescent dye, Mito Tracker Red. Similar results were observed (data not shown). The results of MMP analysis correlated well with the above reported, since mitochondrial hyperpolarization has been related to ROI hyperproduction.[23] Thus, the hyperpolarization of mitochondria by SNP- or PQ-treatment appears to be blocked by kob A.

Taken together, kob A has a protective effect against neuronal death caused by tropic support withdrawal, nitrosative/oxidative stress, and mitochondrial damage. This protective effect might be related to the ability of kob A to maintain the normal state of ROS production, intracellular calcium level, and MMP. Thus kob A appears to be a potential therapeutic agent for treating various neurological disorders including dopaminergic neuronal death. However, a detailed study on the molecular mechanisms responsible for the neuroprotective activity of kob A is required.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.078.

References and notes

1. Yuan, J.; Tankner, B. A. *Nature* **2000**, *407*, 802.
2. Mattson, M. P. *Nat. Rev. Mol. Cell Biol.* **2000**, *1*(2), 120.
3. Eve, D. J.; Nisbet, A. P.; Kingsbury, A. E.; Hewson, E. L.; Daniel, S. E.; Lees, A. J.; Marsden, C. D.; Forster, O. J. *Brain Res. Mol. Biol* **1998**, *63*, 62.
4. Harada, J.; Sugimoto, M. *Brain Res.* **1999**, *842*, 311.
5. Yamada, M.; Tanabe, K.; Wada, K. *J. Neurochem.* **2001**, *78*, 940.
6. Yang, W. S.; Tiffany-Castiglioni, E. *J. Toxicol. Environ. Health* **2005**, *68*, 1939.
7. Andersen, J. K. *Neurotoxicol. Res.* **2003**, *5*, 307.
8. Li, L.; Feng, Z.; Porter, A. G. *J. Biol. Chem* **2004**, *299*, 4058.
9. Ghatan, S.; Larner, S.; Kinoshita, Y.; Hetman, M.; Patel, L.; Xia, Z.; Youle, R.; Morrison, R. S. *J. Cell Biol* **2000**, *150*, 335.
10. Jung, P. S.; Shin, M. K. *Hyangyak Daesageon*; Younglim Press: Seoul, 1990, pp.669–670.
11. Sung, S. H.; Kang, S. Y.; Lee, K. Y.; Park, M. J.; Kim, J. H.; Park, J. H.; Kim, Y. C.; Kim, J. W.; Kim, Y. C. *Biol. Pharm. Bull.* **2002**, *25*, 125.
12. Liang, G.; Cheng, K.; Zhou, H.; Hu, C. Q. *Chem. Pharm. Bull.* **2004**, *52*, 1489.
13. Kurihara, H.; Kawabata, J.; Ichikawa, S.; Mishima, M.; Mizutani, J. *Pytochemistry* **1991**, *30*, 649.
14. Luo, H. F.; Zhang, L. P.; Hu, C. Q. *Tetrahedron* **2001**, *57*, 4849.
15. JC-1, CM-H_2-DCFDA and Mito tracker Red were purchased from Molecular Probe (Eugene, Oregon). Colorimetric caspase-3 and -9 substrates were purchased from Calbiochem (San Diego, CA). Unless otherwise indicated, all chemicals were purchased from Sigma–Aldrich Chemical Co. The intracellular calcium level was assessed by Fluo-3 (5 μM), an intracellular calcium specific fluorescent dye, using flow cytometry.
16. Guarneri, P.; Cascio, C.; Piccoli, T.; Guarneri, R. *J. Neuro. Res.* **2000**, *60*, 656.
17. Ding, Q.; Dimayuga, E.; Martin, S.; Bruce-Keller, A. J.; Nukala, V.; Cuervo, A. M.; Keller, J. N. *J. Neurochem.* **2003**, *86*, 489.
18. Turpaev, K. T. *Biochemistry* **2002**, *67*, 339.
19. Kindler, D. D.; Thiffault, C.; Solenski, N. J.; Dennis, J.; Kostecki, V.; Jenkins, R.; Keeney, P. M. *Mol. cell. Neurosci.* **2003**, *23*, 559.
20. Zayzafoon, M. J. *Cell. Biochem.* **2006**, *97*, 56.
21. Monteiro, H. P.; Silva, E. F.; Stern, A. *Nitric oxide* **2004**, *10*, 1.
22. Carreras, M. C.; Franco, M. C.; Peralta, J. G.; Poderoso, J. J. *Mol. Aspects Med.* **2004**, *25*, 125.
23. Pierre, S.; Jolivet, G.; Devinoy, E.; Houdebine, L. M. *Mol. Endocrinol.* **1994**, *8*, 1720.