行政院國家科學委員會專題研究計畫　期中進度報告

粒線體與神經細胞死亡的研究(1/2)

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執行單位：國立臺灣大學醫學院小兒科

計畫主持人：李旺祚

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中文摘要

粒線體功能異常會引起許多不同年齡層的神經病變。過去的研究顯示，3-nitropropionic acid 所導致的神經細胞死亡與 caspases 的活化有關。動物的研究顯示，安非他命和甲基安非他命也會使紋狀體神經細胞退化死亡。然而這些神經細胞死亡的機轉目前並不很清楚。產生過多的自由基是一個可能的機轉，另外也有研究顯示安非他命和甲基安非他命可能影響粒線體的功能。因此，在本實驗中我們利用鼠腦初級皮質神經細胞培養來探討導致安非他命和甲基安非他命神經毒性的原因。並比較與 3-nitropropionic acid 所導致神經細胞死亡的差異。我們發現安非他命和甲基安非他命都可導致神經細胞死亡，且僅引起輕微 caspase-3 的活化。證明安非他命和甲基安非他命可以導致細胞壞死與細胞凋零。另外安非他命和甲基安非他命也使細胞自由基的產量增加，尤其甲基安非他命所導致的增加更大，證明自由基的產量增加是導致神經細胞死亡的主因之一。由於 caspase-3 的活化發生於粒線體膜電位去極化後，因此主要發生於細胞色素 c 釋放之後。這一點與 3-nitropropionic acid 有所差異。我們將繼續探討安非他命和甲基安非他命的神經毒性，和釐清 caspase-3 的活化是否的確會影響粒線體功能，而更進一步了解 3-nitropropionic acid 與安非他命和甲基安非他命神經毒性差異的地方。

關鍵詞：3-nitropropionic acid，神經毒性，安非他命，甲基安非他命，細胞凋零。

Abstract

3-Nitropropionic acid (3-NP) is an irreversible inhibitor of succinate dehydrogenase. Previous studies had shown that 3-NP can lead to neuronal death following the activation of caspases. In the present study, we first investigate the neurotoxicity of methamphetamine and amphetamine in primary rat cortical neuronal cultures, and will compare the pathogenetic

mechanisms of neuronal death in methamphetamine and amphetamine with those in 3-NP. We found that there was a dose- and time-dependent increase of neuronal death following the application of methamphetamine and amphetamine. Only mild activation of caspase-3 was found following the treatment, indicating that both methamphetamine and amphetamine can result in apoptosis and necrosis. The caspase-3 activation developed following mitochondrial depolarization, which was different from that in 3-NP. Significant elevation of reactive oxygen species was found post the application of the drugs, especially in methamphetamine. It suggests that increase of reactive oxygen species is one of the major pathogenic mechanisms of neuronal death for both amphetamine and methamphetamine. In the following year, works will be focused on the differences of neurotoxicity in both 3-NP and methamphetamine (and amphetamine). Whether caspase-3 activation can really affect mitochondrial function as that in 3-NP neurotoxicity will also be investigated.

Key Words: 3-nitropropionic acid, amphetamine, methamphetamine, caspase, apoptosis.

緣由與目的

In our previous studies, 3-nitropropionic acid (3-NP) was shown to cause neuronal death due to impairment of energy metabolism. Caspase-3 activation developed before mitochondrial depolarization. Therefore, to investigate whether caspase-3 activation can occur before mitochondrial activation in other neurotoxins is very important.

Methamphetamine and amphetamine neurotoxicity have been described to be decreased neurotransmitter levels and neurite degeneration rather than actual cell loss. Recently, however, Sonsalla and colleagues reported that mice treated with repeated doses of methamphetamine exhibited a significant loss of dopamine neurons in the substantia nigra (Sonsalla et al, 1996). Methamphetamine and amphetamine, which can deplete dopamine content of the neurons, had also been shown to aggravate the 3-NP toxicity (Reynold et al, 1998; Eradiri and Starr, 1999). Evidences of metabolic stress following methamphetamine include increased extracellular lactate production and a decrease of striatal ATP contents (Chan et al., 1994). High-dose methamphetamine administration also showed decreased cerebral glucose metabolism weeks or months following drug application (Huang et al., 1999). Previous studies showed that after 7 d of 3-NP treatment, a single low dose of methamphetamine markedly increased the frequency of striatal lesion formation. That effect was mediated via dopamine receptors because it could be blocked by the administration of dopamine receptor antagonists (Reynold et al, 1998). Because dopamine plays an important role in the formation of 3-NP lesions, methamphetamine coadministration may exacerbate the extent of neuronal death induced by 3-NP.

Therefore, in the first part of the project, we will investigate the neurotoxicity of amphetamine and methamphetamine, and clarify their relationship with 3-NP.

研究方法、結果與討論

Neuron culture

Embryos of 18-day pregnant Wistar rats were used. The neural tissue was dissociated by trituration, resuspended in 10% fetal bovine serum in MEM defined medium and plated onto poly-D-lysine coated culture dishes or plates (Lee et al., 2002). The cultures were placed in a humidified 5% CO_2 atmosphere incubator for 7 days. The medium will be changed very one week. All the experiments will be done on 7-10 days in vitro (DIV).

Assay for cell survival

The neuronal death was evaluated with Hoechst 33258 staining. The apoptotic nuclei of the experimental groups were counted as the percentage of the controls.

Measurements of mitochondrial membrane potentials

The dye Mitotracker red (Molecular Probes) was used as a measure of mitochondrial transmembrane potential according to methods described previously. In brief, cells were incubated for 30 min in the presence of 100 nM of the dye and then were washed in Locke's solution. Cellular fluorescence was imaged using a confocal laser scanning microscope with excitation at 514 nm and emission at 535 nm, and the average pixel intensity/cell was determined using Imagespace software (Molecular Dynamics).

4) The measurement of caspase activation

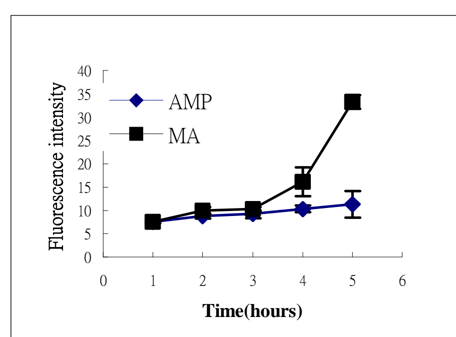
Caspase-3-like protease activity was assessed in individual cells by a method described previously that employed biotinylated *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), a pseudosubstrate and inhibitor of caspase-3. At designated time points following exposure of cultures to 3-nitropropionic acid, cells were exposed for 10 min to Locke's solution containing 0.01% digitonin. Cells were then incubated for 20 min in the presence of 10 µg/ml biotinylated DEVD-CHO (Calbiochem), washed three times with PBS (2 ml/wash), and fixed for 30 min in a cold solution of 4% paraformaldehyde in PBS. Cells were then incubated for 30 min in PBS containing 5 µg/ml Oregon Green-streptavidin (Molecular Probes) and were washed twice with PBS. Images of cellular fluorescence, corresponding to conjugates of activated caspase-3 with DEVD-biotin, were acquired using a confocal laser scanning microscope, and levels of fluorescence (average pixel intensity/cell) will be

quantified.

Western blotting

The changes of Bcl-2 and Bax following treatment were performed.

In indicated time points after treatment with methamphetamine or amphetamine, relative levels of specific proteins in neurons were determined by western blot analysis. In brief, 50 μg of solubilized proteins were separated by electrophoresis in a 10-12% sodium dodecyl sulfate/polyacrylamide gel and then transferred to a nitrocellulose sheet. After blocking with 5% milk and a 3-h incubation in the presence of primary antibody, the nitrocellulose sheet

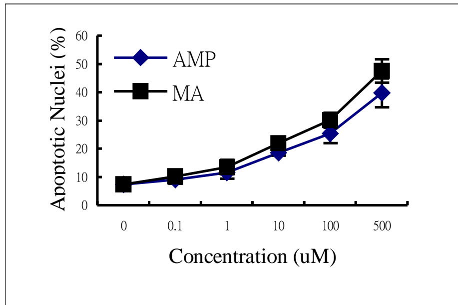


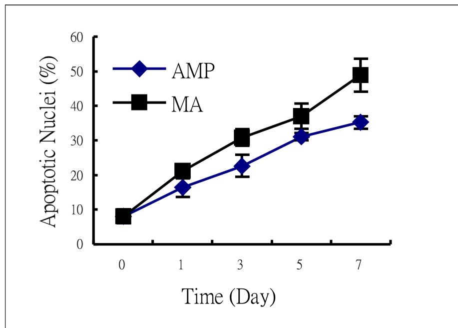
were further processed using horseradish peroxidase conjugated secondary antibody and a chemiluminescence system (Amersham). The primary antibodies include Bcl-2 and Bax (Chemicon).

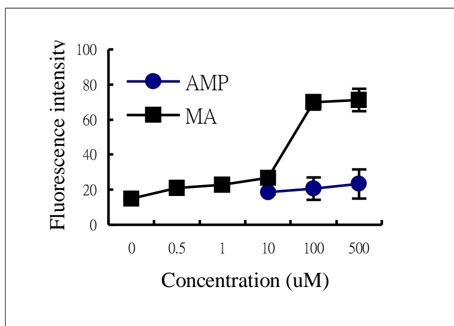
RESULTS

Amphetamine and methamphetamine toxicity are dose- and time-dependent

Both AMP and MA induced dose- and time-dependent apoptosis and necrosis in neurons. The dose of AMP and MA used in the time course figure is 10 uM.





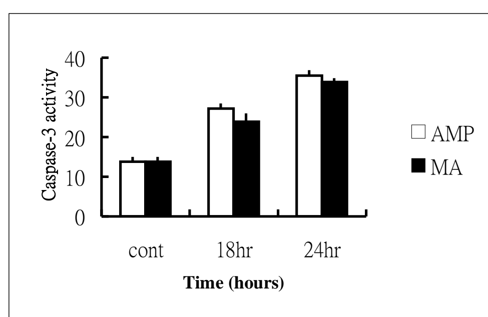


Reactive oxygen species (ROS) production is dose- and time-dependent

The ROS production increased after the application of amphetamine and methamphetamine, and was greater in methamphetamine group. There was a dramatic increase of ROS production at 4 hrs in methamphetamine group.

Caspase-3 was only mildly activated

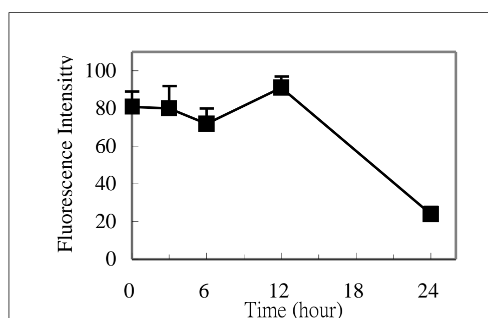
There was only a mild elevation of caspase-3 activity following the application of AMP and MA, indicating that apoptosis is only part of the mechanism of neuronal death. Caspase-3 activation was found after the depolarization of mitochondrial membrane potentials.



Methamphetamine and amphetamine lead to a decrease of Bcl-2 expression

There was a dose- and time-dependent decrease of Bcl-2 expression in neurons post the exposure of methamphetamine and amphetamine (data not shown).

DISCUSSION



In the present studies, both methamphetamine and amphetamine lead to a dose- and time-dependent increase of neuronal death with mild activation of caspase-3. The percentage of cell death was higher in those treated with methamphetamine. There was also a significant increase of ROS, especially in methamphetamine group following the application of the drugs. Taken together, it indicated that both methamphetamine and amphetamine can lead to cortical neuronal death, in both apoptosis and necrosis (Cadet et al., 1997). The pathogenesis of neuronal death may be related to the increase of ROS (De Vivo et al., 1989; Jayanthi et al., 1998).

Because the activation of caspase-3 developed following the mitochondrial depolarization, it may be activated following the release of cytochrome c from the mitochondria. That was different from what occurred following the application of 3-NP.

In the following year, we will continue to clarify the differences of neurotoxicity in

3-NP and methamphetamine (and amphetamine). Whether caspase-3 activation can really affect mitochondrial function will also be investigated.

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