行政院國家科學委員會補助專題研究計畫成果報告

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行政院國家科學委員會專題研究計畫期中報告
粒線體與神經細胞死亡的研究

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一、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 與許多神經毒物包括安非他命和甲基安非他命一樣會導致神經細胞死亡。而這些神經細胞的死亡與 caspases 的活化有關。另外產生過多的自由基也是一個可能的機轉。過去的研究顯示，caspase 的活化通常因為粒線體功能的異常所引起。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這些神經毒物神經毒性的原因。並比較這些神經細胞的死亡導致 caspases 活化的差異。我們第一年的研究發現安非他命和甲基安非他命所導致神經細胞死亡可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，並造成細胞壞死與細胞凋零。然而安非他命和甲基安非他命僅引起輕微 caspase-3 的活化。我們進一步發現，施與少量 3-nitropropionic acid 可使經安非他命和甲基安非他命處理的細胞 ATP 大量下降，證明安非他命和甲基安非他命會影響粒線體功能。我們接著比較發現安非他命和甲基安非他命處理的細胞其 caspase-3 的活化發生於粒線體膜電位去極化後，而 3-nitropropionic acid 處理的細胞其 caspase-3 的活化發生於粒線體膜電位去極化前即已進行。證明 3-nitropropionic acid 與安非他命和甲基安非他命在 caspase-3 的活化上是不一樣的。由於 3-nitropropionic acid 本身為粒線體毒物且會影響 Kreb cycle，是否因而誘發其他與粒線體無關的 caspase-3 活化值得進一步研究。相反的，安非他命和甲基安非他命所導致神經細胞死亡主要與自由基的產量增加有關，並進而影響粒線體膜電位去極化。是否其他間接影響粒線體膜電位去極化的神經毒物其 caspase-3 活化都與安非他命和甲基安非他命一樣發生於粒線體膜電位去極化後值得進一步研究。

關鍵詞：安非他命、甲基安非他命、神經毒物、粒線體

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 pathogenic 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. Significant elevation of reactive oxygen species was also found after the application of the drugs, especially in methamphetamine. Addition of low concentration of 3-NP significantly decreased the cellular ATP in amphetamine-treated neurons, indicating the effect of the drug on mitochondrial function. However, compared with that in 3-NP neurotoxicity, only mild activation of caspase-3 was found following the treatment of amphetamine. We further found that the caspase-3 activation in amphetamine and methamphetamine developed following mitochondrial depolarization. On the contrary, the caspase-3 activation in 3-NP developed before mitochondrial depolarization, indicating both a mitochondrial-dependent and independent pathways in 3-NP neurotoxicity. Because 3-NP is a direct mitochondrial toxins while amphetamine and methamphetamine are both indirect mitochondrial toxins, whether that's one of the reasons leading to different caspase-3 activation needs further investigation.

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

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 was involved in the pathogenesis of neuronal death. Therefore, to investigate whether caspase-3 activation can occur in other neurotoxin-induced neurotocity 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.

In the present study, we use amphetamine, methamphetamine and 3-NP to investigate the mechanisms of neuronal death, and activation of caspase-3.

研究方法、結果與討論

Neuron culture and drug treatments

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 5-7 days in vitro (DIV).

Amphetamine (AMP), methamphetamine (MA), and 3-NP were added at indicated time points.

Assay for cell survival and cellular ATP

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

The cellular ATP was measured by commercialized kit with luciferase reaction.

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).

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, p53 and Bax following treatment were performed.

In indicated time points after treatment with methamphetamine or amphetamine or 3-NP, 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, and the application of 3-NP greatly increases the neurotoxicity

Both AMP and MA induced dose- and time-dependent apoptosis and necrosis in neurons. Addition of 0.1mM 3-NP did not induce significant neuronal death or decrease of cellular ATP. However, the combination of 0.1 mM 3-NP and AMP or MA, which greatly decreased the cellular ATP content, increased the neuronal death, indicating MA and AMP can have induce mitochondrial stress

Reactive oxygen species (ROS) production is dose- and time-dependent, and precedes the mitochondrial depolarization in MA and AMP group

The ROS production increased after the application of MA and AMP, and was greater in MA group. There was a dramatic increase of ROS production at 4 hrs in MA group. The increase of ROS preceded the change of mitochondrial membrane potentials, indicating that mitochondrial depolarization in MA and AMP neurotoxicity is related to ROS production.

Caspase-3 was only mildly activated

The activation of caspase-3 following the application of AMP and MA was mild and delayed compared with that in 3-NP application. In MA and AMP groups, the caspase-3 activation occurred following the mitochondrial depolarization. However, in 3-NP group, the caspase-3 activation developed before the mitochondrial depolarization. It indicated that the mitochondrial depolarization in MA and AMP group may be related to ROS overproduction. However, in 3-NP group, the caspase-3 activation may have dual pathways involving both mitochondria-dependent and independent pathways.

Methamphetamine, amphetamine, and 3-NP lead to a decrease of Bcl-2 expression while there is no change in Bax and p53

There was a dose- and time-dependent decrease of Bcl-2 expression in neurons post the exposure of MA, AMP and 3-NP. There was no significant change in Bax and p53 proteins..

DISCUSSION

In the present studies, both methamphetamine and amphetamine lead to a dose- and time-dependent increase of neuronal death The percentage of cell death was higher in those treated with methamphetamine. There was also a significant increase of ROS, especially in methamphetamine group. The mitochondrial depolarization developed following the ROS production in methamphetamine and amphetamine, indicating the mitochondrial depolarization in amphetamine and methamphetamine is related to ROS overproduction. There was also a mild and delayed activation of caspase-3 in methamphetamine and amphetamine group, which developed following the mitochondrial depolarization. On the contrary, the activation of caspase-3 in 3-NP developed before mitochondrial depolarization, indicating a mitochondria-independent pathway. 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.

Because the cellular ATP depletion and neuronal death were greatly enhanced by low-dose 3-NP in methamphetamine and amphetamine application, it indicated that both amphetamine and methamphetamine produced a significant mitochondrial stress, which may be related to ROS production. On the contrary, 3-NP is one of mitochondrial toxins, which act directly on mitochondrial Whether the differences in the mechanisms of mitochondrial stress lead to the differences in caspase-3 activation needs further investigation.

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