行政院國家科學委員會專題研究計畫期末報告

粒線體功能異常對人體神經細胞的影響(2/2)

The effect of mitochondrial dysfunction on human neurons

計畫編號：NSC 90-2314-B-002-183-

執行期限：90 年 8 月 1 日至 91 年 7 月 31 日

主持人：李旺祚　　台大醫學院小兒科

E-mail: wtlee@tpts5.seed.net.tw

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。最近的研究顯示，抑制興奮性氨基酸的釋放和施予 NMDA 受體的拮抗物，都會減輕粒線體毒物引起的腦病變。在本計畫中，我們以粒線體呼吸鏈複合體 II 的抑制劑(3-Nitropropionic acid, 3-NP，15mg/kg)為實驗藥物，希望探討 NT2-N 細胞的死亡是否與細胞內的鈣離子不平衡有關。當我們加入 1 mM 3-NP 以後，NT2-N 細胞的$[Ca^{2+}]_i$會由原來的 48 ± 2nM 上升到 2 小時後的 140 ± 12nM。如果我們移除細胞外的 Ca^{2+}，阻止 Ca^{2+}的內流，則$[Ca^{2+}]_i$ 不再上升。如果我們同時加入 MK-801 則$[Ca^{2+}]_i$ 的上升幅度明顯減慢，但是如果我們同時再加入 Nifedipine，$[Ca^{2+}]_i$上升的速度並沒有進一步減少。由此證明 NMDA 受體所形成的通道是造成$[Ca^{2+}]_i$上升的主要管道。當我們在無 Ca^{2+}的溶液內加入 1 mM 3-NP 時，最初幾分內$[Ca^{2+}]_i$上升的情形與含 Ca^{2+}溶液中的情形相同。但當時間更久，$[Ca^{2+}]_i$上升的速度便減慢，這證明加入 3-NP 後剛開始幾分鐘 $[Ca^{2+}]_i$上升主要來自於細胞內 Ca^{2+}貯積(從內質網或粒線體)的釋放，但細胞外 Ca^{2+}的內流對維持$[Ca^{2+}]_i$ 的持續上升十分重要。當我們加入 xestospongin C 和 dantrolene 抑制內質網 Ca^{2+}的釋放，則最初幾分鐘內$[Ca^{2+}]_i$ 的上升也被抑制。由此觀之，細胞內的鈣離子不平衡與 NT2-N 細胞的死亡息息相關。

關鍵詞：NMDA 受體, 鈣離子，內質網

Abstract

3-Nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase, induced ATP depletion and both necrosis and apoptosis in human NT2-N neurons. Necrosis occurred predominantly during the first 2 days in a dose-dependent manner, whereas apoptosis was observed after 24 hr or later at a low constant rate in 0.1mM as well as 5mM 3-NP. We assayed the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) during the first 48 hours in 1mM 3-NP, a period during which 10% of the neurons died by necrosis and 3% by apoptosis. During the first 2 hours in 3-NP, all NT2-N neurons showed $[Ca^{2+}]_i$ rise from 48 ± 2 to 140 ± 12 nM (mean ± SEM). After 24 and 48 hours in 3-NP, however, $[Ca^{2+}]_i$ remained above 100nM in only 17% and 25% of the NT2-N neurons, respectively, suggesting that most neurons were able to correct this early rise in $[Ca^{2+}]$, despite severe ATP depletion, and to survive. Activation of NMDA-GluR contributed substantially to 3-NP-induced ATP depletion, and subsequent chronic elevation of $[Ca^{2+}]_i$ in the NT2-N neurons. We also demonstrated that blocking endoplasmic reticulum (ER) Ca^{2+} release enhanced the capacity of these human neurons to maintain $[Ca^{2+}]_i$ homeostasis and resist necrosis while subjected to chronic energy deprivation.

Key Words: 3-nitropropionic acid, mitochondrial permeability transition, caspase, antioxidant, apoptosis, NMDA-GluR.

緣由與目的

3-Nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase (SDH), can produce selective striatal lesions and neurobehavioral changes in rats and non-human primates mimicking those in Huntington's disease (Hamilton and Gould, 1987; Beal et al, 1993; Lee et al, 2000). It was also found to produce apoptotic and necrotic neuronal death in both in vitro and in vivo studies (Pang and Geddes, 1997). Although the pathogenesis of 3-NP neurotoxicity remains controversial, activation of ionotropic glutamate receptors (GluR) had been suggested (Beal et al, 1993; Brouillet et al, 1999).

Recently, impairment of calcium homeostasis with elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) had been suggested to be important in 3-NP neurotoxicity in rats (Deshpande et al, 1997; Fukuda et al, 1998; Greene et al, 1998; Brouillet et al, 1999). In primary neuronal cultures, both neurons and astrocytes showed a gradual 3-NP-induced elevation of ($[Ca^{2+}]_i$, which can be blocked by inhibiting reverse operation of Na^+-Ca^+ exchanger in astrocytes and removal of extracellular Ca^{2+} in neurons (Deshpande et al, 1997; Fukuda et al, 1998). However, studies also showed that 3-NP did not change the basal $[Ca^{2+}]_i$ of neurons after in vitro 3-NP exposure (Greene et al, 1998). Increasing evidences also support that elevated $[Ca^{2+}]_i$ and alteration of mitochondrial function secondary to defective energy metabolism would lead to changes of redox state, and overproduction of free radicals (Beal et al, 1995; Schulz et al, 1996; Keller et al, 1998), which may lead to the cell death.

In the past, in vitro studies about 3-NP toxicity were performed in rat primary neuronal cultures of different cell types, not human neurons. The mixed neuronal/glial cultures with different culture conditions

in primary neuronal cultures may get results not applicable to human neurons, which may have different gene expressions. Human NT2-N neurons are derived from the Ntera2/cl.D1 teratocarcinoma cell line (Pleasure et al, 1992). They can develop separate axonal and dendritic compartments, and several CNS proteins mimicking normal neuronal differentiation in the brain. NT2-N neurons can also constitutively express functional NMDA and non-NMDA glutamate receptors, and thus are susceptible to NMDA- and non-NMDA-mediated excitotoxicity. Because they are clonal available in large quantity, and can survive for a long period without glial population, NT2-N neurons are especially useful for investigation of mechanisms of neuronal toxicity arising from glutamate receptor activation.

Therefore, to clarify the effect of impaired energy production on the survival of human neurons, in the present study we developed a model of 3-NP-induced neurotoxicity in post-mitotic human NT2-N neurons.

研究方法、結果與討論

Cell culture

The processes for preparation and maintenance of NT2-N neurons were as described previously (Pleasure et al., 1992). In brief, after treating the cells with 10 u M retinoid acid (Sigma, St. Louis, MO, USA) for 5 weeks, they were plated on 6-well, 12-well and 24-well plates or small chambers coating with polylysine and Matrigel (Becton Dickinson, Bedford, MA, USA).

The neurons were fed weekly for 3 weeks with Dulbecco's modified Eagle medium-high glucose/5% fetal bovine serum (10% for those used in the calcium measurements), 100IU/ml penicillin, 100 ug/ml streptomycin, and mitotic inhibitors (10 uM uridine, 10 uM 5-fluoro-2'-deoxyuridine, and 1 uM cytosine arabinoside; all from Sigma). The NT2-N neurons used in the present study were between 4 and 5 weeks after termination of retinoid acid treatment. All the experiments were done 2-4 days after the last feeding.

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ measurements were performed on all isolated NT2-N neurons in randomly chosen fields at room temperature (23-25^{0}C) as previously described (Itoh et al, 1998). Briefly, the NT2-N neurons cultured on coverslips were loaded with 5 μM fura-2/AM with 0.02% (w/v) pluronic F-127 in the standard recording solution (NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.8, Na_2HPO_4 0.8, HEPES 10, and D-glucose 25 (in mM), adjusted to pH 7.4 with NaOH) for 45 min. The coverslip was attached to a perfusion chamber (RC-21B; Warner Instrument, CT, US.A) on the stage of an upright epifluorescence microscope (Optiphot, Nikon, Japan), and emission fluorescence images at 510 nm were alternatively taken at 340 and 380 nm excitation wavelengths. $[Ca^{2+}]_i$ values were calculated from the emission ratio (R) at 340 and 380 nm excitation wavelengths, using the formula described by Grynkiewicz (1985): $[Ca^{2+}]_i = bK_d$ (R-Rmin)/(Rmax-R). K_d is the effective dissociation constant of fura-2, and b is the ratio of fluorescence intensity at 380 nm for Ca^{2+}-free and Ca^{2+}-saturated dye. R_{min} and R_{max} are the emission ratios obtained under Ca^{2+}-free and Ca^{2+}-saturated conditions, respectively. These parameters were obtained by in vitro calibration.

We previously demonstrated that $[Ca^{2+}]_i$ measurements could be done in NT2-N neurons up to 3 hr, if emission images were taken at a 2.5 min interval to minimize damage to the cells by UV exposure as well as photobleaching of loaded fura-2 (Itoh et al., 1998). Even when the frequency of UV exposure was reduced, however, we still observed significant death of NT2-N neurons at 24 hr after UV exposure. Therefore, in order to evaluate chronic changes in $[Ca^{2+}]_i$ after 24 hr or 48 hr with 3-NP, we used sister cultures treated identically to those for acute experiments.

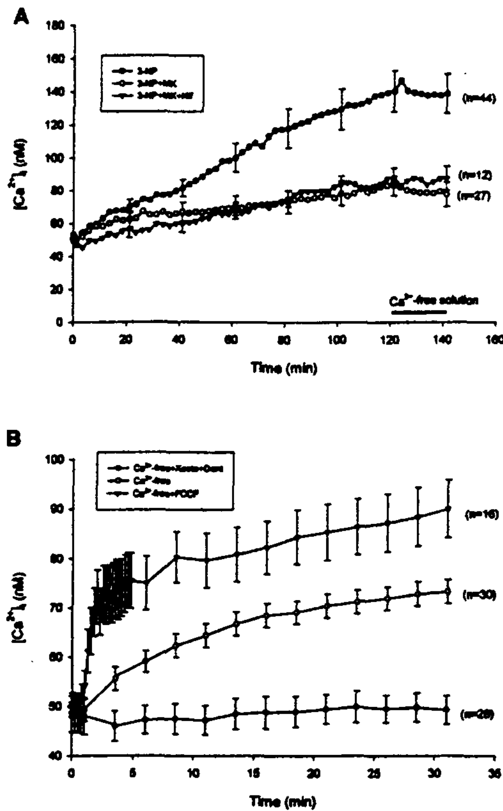
Statistics

In most instances (as indicated in the text and figure legends), the results were expressed as means $\pm$ SEM, with statistical significances calculated by paired t-test or ANOVA. Chi square testing was done for analysis of statistical significance of the data in Table 2.

RESULTS

3-NP elicits a consistent early rise in NT2-N neuronal $[Ca^{2+}]_i$

Loss of calcium homeostasis is considered to be a key mechanism in NMDA-GluR-mediated neuronal excitotoxicity (). During the first 2 hours after addition of 1mM 3-NP in a Ca^{2+}-containing medium, there was a gradual increase in $[Ca^{2+}]_i$ in all NT2-N neurons, from a basal $[Ca^{2+}]_i$ levels of 48 $\pm$ 2 nM to 140 $\pm$ 12 nM (mean $\pm$ SEM). This $[Ca^{2+}]_i$ increase was prevented by removal of Ca^{2+} from the medium. Thus, influx of extracellular Ca^{2+} is required for 3-NP to induce a sustained increase in NT2-N neuronal $[Ca^{2+}]_i$. The rate of the rise in neuronal $[Ca^{2+}]_i$ for the first 2 hr was markedly slower in the presence of MK-801. The L-type VGCC inhibitor, nifedipine, did not further augment the inhibitory effect of MK-801 on this rise in $[Ca^{2+}]_i$. We concluded, therefore, that NMDA-GluR played a predominant role in the early rise in $[Ca^{2+}]_i$ in 3-NP-treated NT2-N neurons.



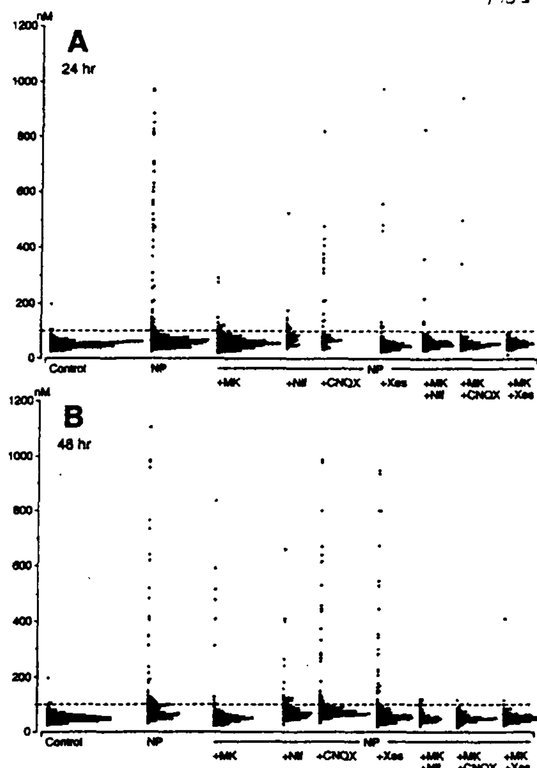
Roles of ER and mitochondria in buffering $[Ca^{2+}]_i$ during the first 30 minutes of treatment of NT2-N neurons with 3-NP

When NT2-N neurons were exposed to 1mM 3-NP in a Ca^{2+}-free medium, there was still an early rise in $[Ca^{2+}]_i$ (Fig 1B). During the first few minutes, the rate of this rise was nearly identical to that in a Ca^{2+}-containing medium. This demonstrates that, during this brief initial time period, the increase in $[Ca^{2+}]_i$ caused by 3-NP was the result of release of Ca^{2+} from internal storage (ER and/or mitochondria). With longer periods of observation, the increase in $[Ca^{2+}]_i$ decelerated, confirming the requirement for extracellular Ca^{2+} to sustain an 3-NP-induced $[Ca^{2+}]_i$ elevation. The rise in $[Ca^{2+}]_i$ in Ca^{2+}-free medium was totally blocked by addition of xestospongin c plus dantrolene. These drugs inhibit IP3-sensitive ER Ca^{2+} release and Ca^{2+} activated ER Ca^{2+} release, respectively (Wei and Perry, 1996; Gafni et al, 1997; Yu et al, 1999; Mattson et al, 2000). We concluded, therefore, that release of Ca^{2+} from ER was largely responsible for the 3-NP-induced initial rise in $[Ca^{2+}]_i$.

Release of Ca^{2+} from mitochondria may substantially increase the rise in $[Ca^{2+}]_i$ caused by activation of neuronal NMDA-GluR (Mody and MacDonald, 1995; Stout et al, 1998). To evaluate the role of mitochondria in buffering $[Ca^{2+}]_i$ during the early phase of exposure of NT2-N neurons to 3-NP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), a proton ionophore which blocks mitochondrial retention of Ca^{2+} (Luo et al, 1997; Khodorov et al, 1999) was added to the Ca^{2+}-free medium. This elicited a rapid, approximately 20nM, increase in $[Ca^{2+}]_i$. The slope of the rise in $[Ca^{2+}]_i$ that followed during the next 30 minutes in these 3-NP-treated neurons was very similar to that in the absence of FCCP. We interpret these results to indicate that, in a Ca^{2+}-free medium, the NT2-N neuronal mitochondria contained a small amount of Ca^{2+}, sufficient, when released, to raise $[Ca^{2+}]_i$ by 20nM, and that, during the remainder of the observation period, mitochondrial Ca^{2+} uptake did not substantially contribute to $[Ca^{2+}]_i$ homeostasis.

Chronic effects of 3-NP on NT2-N neuronal Ca^{2+} homeostasis

We measured $[Ca^{2+}]_i$ following 24 or 48 hours of incubation with 1 mM 3-NP in a Ca^{2+}-containing medium (chronic studies in a Ca^{2+}-free medium were not performed, because chronic incubation in Ca^{2+}-free medium was toxic to these neurons even in the absence of 3-NP (data not shown). There were 2 populations of neurons in these 3-NP-treated cultures: the majority (83% at 1 day, 75% at 2 days) had restored $[Ca^{2+}]_i$ to below 100nM, whereas in the remaining neurons (17% at 24 hours, 25% at 48 hours), $[Ca^{2+}]_i$ remained above 100nM. The distributions of $[Ca^{2+}]_i$ values in these neurons are shown in Fig 2. Inspection of this figure indicates that the increase in neurons with $[Ca^{2+}]_i$ above 100nM between 24 and 48 hours of treatment with 3-NP without other additives (column 2 in the figure) was entirely accounted for by neurons in which $[Ca^{2+}]_i$ was barely above 100nM. The proportion of neurons in which $[Ca^{2+}]_i$ was above 100nM at 24 or 48 hours was markedly reduced by treatment with MK-801, and was also significantly reduced by treatment with xestospongin c, but not by treatment with CNQX or nifedipine. Combination treatments with MK-801 and xestospongin C or MK-801 plus CNQX were more effective in diminishing the incidence of neurons with $[Ca^{2+}]_i$ greater than 100nM than treatment with MK-801 alone.



CONCLUSIONS

In conclusion, the present study showed that 3-NP can induce a concentration- and time-dependent neuronal death in NT2-N neurons secondary to ATP depletion. The percentage of apoptosis and necrosis in NT2-N neurons is related to the concentration of the 3-NP. The 3-NP-induced neuronal death may be related to free radical production, caspase activation, and induction of MPT. However, the activation of NMDA-GluRs plays the most important role in the neuronal death. In the second year of the project, we will continue to measure the change of intracellular calcium, and investigate the relationship between the elevation of intracellular calcium and neuronal death.

參考文獻

1. Beal M.F., Brouillet E., Jenkins B.G., Henshaw R., Rosen B. and Hyman B.T. (1993) Neurochemical and histologic characterization of Striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J Neurosci 13,4181-4192.
2. Borlongan C.V., Koutouzis T.K. and Sanberg P.R. (1997) 3-Nitropropionic acid animal model and Huntington's disease. Neurosci Biobehvio Rev 21,289-93.
3. Brouillet E., Hantraye P., Ferrante R.J., Dolan R., Leroy-Willig A., Kowall N.W. and Beal M.F. (1995) Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. Proc. Natl. Acad. Sci. USA, 92:7105-7109.
4. Brownell A.L., Jenkins B.G., Elmaleh D.R., Deacon T.W., Spealman R.D. and Isacson O. (1998) Combined PET/MRS brain studies show dynamic and long-term physiological changes in a primate model of Parkinson disease. Nature Medicine 4,1308-1312.
5. Fix A.S., Horn J.W., Wightman K.A., Johnson C.A., Long G.G., Storts R.W., Farber N., Wozniak D.F. and Olney J.W. (1994) Neuronal vacuolization and necrosis induced by the noncompetitive N-methyl-D-aspartate (NMDA) antagonist MK(+)801 (dizocilpine maleate): A light and electron microscopic evaluation of the rat retrosplenial cortex. Exp Neurol 123:204-215.
6. Greenamyre J.T., Olsen J.M.M., Penney J.B. and Young A.B. (1985) Autoradiographic characterization of N-methyl-D-aspartate-, quisqualate- and kainate-sensitive glutamate binding sites. J Pharmacol Exp Ther 233,254-263.
7. Guimaraes A.R., Schwartz P., Prakash M.R., Carr C.A., Berger U.V., Jenkins B.G., Coyle J.T. and Gonzalez R.G. (1995) Quantitative in vivo 1H nuclear magnetic resonance spectroscopic imaging of neuronal loss in rat brain. Neuroscience 69,1095-1101.
8. Guyot M.C., Hantraye P., Dolan R., Palfi S., Maziere N. and Brouillet E. (1997) Quantifiable bradykinesia, gait abnormalities and Huntington's disease-like striatal lesions in rats chronically treated with 3-nitropropionic acid. Neuroscience 79,45-56.
9. Jenkins B.G., Brouillet E., Chen Y.C.I., Storey E., Schulz J.B., Kirschner P., Beal M.F. and Rosen B.R. (1996) Non-invasive neurochemical analysis of focal excitotoxic lesions in models of neurodegenerative illness using spectroscopic imaging. J Cereb blood Flow Metab 16,450-461.
10. Leach M.J., Baxter M.G. and Critchley M.A. (1991) Neurochemical and behavioral aspects of lamotrigine. Epilepsia 32(suppl), S4-8.
11. Novelli A., Reilly A.J., Lysko P.G. and Henneberry R.C. (1988) Glutamate becomes neurotoxic via N-methyl-D-aspartate receptor when intracellular enregy levels are reduced. Brain Res 451,205-212.
12. Pang Z., Umberger G.H. and Geddes J.W. (1996) Neuronal loss and cytoskeletal disruption following intrahippocampal administration of the metabolic inhibitor malonate: lack of protectiion by MK-801. J Neurochem 66, 474-484.