國立台灣大學藥理學研究所博士

乙型雨傘節蛇毒毒素引發小腦顆粒神經細胞毒性之訊息傳遞途徑研究

Studies on Signal Transduction Pathway of b-Bungarotoxin-induced Neurotoxicity in the Cultured Cerebellar Granule Neurons

曾文培

Abstract

The aim of this study is to elucidate the mechanism of neurotoxic effect of β-bungarotoxin on the cultured cerebellar granule neurons. β-BuTX exerted a potent neurotoxic effect upon mature granule neurons. Quantitative analysis of neurotoxicity revealed the time- and concentration-dependency. It is noted that β-BuTX appeared to initially destroy the neurite and then caused the neuronal death mediated by both apoptosis and necrosis processes. Nomarski optics showed that these neurons displayed morphological features of necrotic cells, including cell swelling, destruction of membrane integrity and eventual dissolution of the cell. Staining with the fluorescent dye Hoechst 33258 showed that β-BuTX-treated neuron body had a higher densed staining with smaller apoptotic bodies. Using microspectrofluorimetry and fura-2 to measure [Ca2+]i, we found that β-BuTX markedly increased [Ca2+]i. Furthermore, BAPTA-AM, EGTA, MK 801 and diltiazem not only inhibit the elevated [Ca2+]i but also attenuate the neurotoxicity of β-BuTX.

Moreover, these Ca2+ inhibitors prevent the β-BuTX-induced reactive nitrogenous species production and NO synthase inhibitor (NG-methyl-L-arginine) exhibits the neuroprotection. Therefore, we conclude for the first report that β-BuTX-induced cerebellar granule neuron death is mediated by, at least in part, excessive generation of nitric oxide triggered by [Ca2+]i overloading. The activation of NMDA receptor and L-type calcium channel apparently involved in increasing [Ca2+]i induced by this neurotoxin. We suggest that this potent neurotoxin is a useful tool for us to study the neurotoxic process and thus we will be able to find the neuroprotective agent by using this model system.

Neuronal death signaling through activating NMDA receptor and L-type calcium channel induced by β-bungarotoxin

The aim of this study is to elucidate the mechanism of neurotoxic effect of β-bungarotoxin (β-BuTX, a snake presynaptic neurotoxin isolated from the venom of Bungarus multicintus) on the cultured cerebellar granule neurons. β-BuTX exerted a potent neurotoxic effect upon mature granule neurons in the time-dependency. The mature neurons with an abundant of neurite outgrew were obtained after 7-8 days culture in vitro

(DIV). By means of microspectrofluorimetry and fura-2, we measured intracellular Ca2+ concentration ([Ca2+]i) and founded that [Ca2+]i was increased markedly. BAPTA-AM, EGTA, MK801 and diltiazem prevented not only [Ca2+]i elevation, but also the neurotoxic effect by β-BuTX. The signaling pathway of the elevated [Ca2+]i in β-BuTX-induced neurotoxicity was studied. The results obtained indicated that β-BuTX increased reactive oxygen species production, followed by mitochondrial membrane potential reduction and ATP depletion. All of these events in signaling pathway were blocked by MK801, diltiazem, EGTA and BAPTA-AM. These findings suggested that the neurotoxic effect of β-BuTX was mediated by, at least in part, cascade events including the direct or indirect activation of NMDA receptor and L-type calcium channel followed by Ca2+ influx, oxidative stress, mitochondrial dysfunction and ATP depletion. We, therefore, suggest that this polypeptide neurotoxin resulting from its high potency and irreversible properties is a useful tool to elucidate the mechanism of neurodegenerative diseases.

Suramin prevents neurotoxicity induced by β-bungarotoxin in cultured cerebellar granule neurons

We demonstrated in this paper that a snake presynaptic neurotoxin, β-bungarotoxin (β-BuTX), isolated from the venom of Bungarus multicintus, is a potent neurotoxicant with EC50 of 3 ng/ml (equivalent to 144 pM) on the cultured matured cerebellar granule neurons (CGNs). The neuronal death of CGNs induced by β-BuTX is apparently due to both apoptosis and necrosis processes as revealed by neurite fragmentation, morphological changes and staining the apoptotic bodies with the fluorescent dye Hoechst 33258. Using microspectrofluorimetry and fura-2 to measure intracellular calcium [Ca2+]i, we found that β-BuTX markedly increased intracellular calcium [Ca2+]i correlated with its neurotoxicity in a concentration- and time-dependent manner. The elevated [Ca2+]i is proposed to contribute the neurotoxic effect of β-BuTX, since suramin inhibited both the increased [Ca2+]i and the neurotoxicity. The mitochondria may be the executive site of the elevated intracellular calcium. By means of the fluorescent probes, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and 3,3’-dihexyloxacarbocyanine iodide (DiOC6), respectively, we showed that β-BuTX increased free radical production accompanied by the decreased mitochondrial membrane potential and eventually depleted the cellar ATP content. Suramin also effectively suppressed these detrimental effects of β-BuTX. Based on these findings together with the inhibitory effects of MK801 and EGTA on β-BuTX-neurotoxicity, it is concluded that β-BuTX acts, at least in part, as an activator of both P2X and NMDA receptors on CGNs. Activation of these receptor-cation channels increases an influx of extracellular Ca2+. Ca2+ overload damages mitochondria, leading to mitochondrial membrane depolarization, an increase in ROS production, a depletion of ATP content and eventually neuronal death. Our findings suggest that suramin with its beneficial protective effects on β-BuTX-induced neurotoxicity may provide an effective protective approach against chemical neurotoxic insults.

Activation of NMDA receptor partly involved in β-bungarotoxin-induced neurotoxicity in cultured primary neurons

In this study, we demonstrated that a snake presynaptic toxin, β-bungantoxin (β-BuTX), was capable of binding to NMDA receptors of the cultured primary neurons (cerebellar granule neurons, CGNs). We labeled β-BuTX with fluorescent FITC (FITC-β-BuTX) and showed that the binding of FITC-β-BuTX was inhibited by unlabeled β-BuTX and MK-801 (a NMDA receptor antagonist). Meanwhile, the binding of [3H]-MK801 was also reduced by unlabeled MK-801 and β-BuTX. In addition, β-BuTX produced a very potent neurotoxic effect on the mature CGNs with the EC50 of 3 ng/ml (equivalent to 144 pM), but was less effective in the immature CGNs. We explored the signaling pathway of neuronal death and found that it was apparently due to the excessive production of reactive oxygen species (ROS) induced by β-BuTX. MK-801 and antioxidants (vitamin C, N-acetylcysteine, melatonin, epigallocatechin gallate, superoxide dismutase and catalase) attenuated not only ROS production but also β-BuTX-neurotoxicity. The downstream signaling of ROS was identified as the activation of caspase-3. Caspase inhibitor (z-DEVD-fmk) and antioxidants depressed both caspase-3 activation and neurotoxicity. Based on these findings and our previous reports, we conclude that the binding and activation of NMDA receptors by β-BuTX was crucial step to produce the potent neurotoxic effect. The binding of NMDA receptors resulted in excessive Ca2+ influx, followed by ROS production and activation of caspase-3. This snake toxin is considered not only to be a useful tool for exploring the death-signaling pathway of neurotoxicity, but also provides a model for searching neuroprotective agents.

Long-term lithium treatment prevents neurotoxic effects of β-bungarotoxin in the primary cultured neurons

Lithium is the most commonly used drug for the treatment of manic-depressive illness. The precise mechanisms underlying its clinical efficacy remain unknown. In this paper, we found that long-term exposure to lithium chloride protected cultured cerebellar granule neurons (CGNs) against β-bungarotoxin (β-BuTX)-induced neurotoxicity. This neuroprotection exhibited at the therapeutically relevant concentration of 1.2 mM lithium. Pretreatments for 3-5 days (long-term) were required for protection to occur; but a 3 hr treatment (short-term) was ineffective. In contrast, a longer treatment for 6-7 days or a higher concentration of 3 mM lithium led not only to lost of neuroprotective effect but also to neurotoxic effect. These findings suggest that lithium protection is limited to its narrow window of concentration and apparently relevant to its narrow therapeutic index in clinical application. Measurement of intracellular calcium [Ca2+]i revealed that neurotoxic concentrations of β-BuTX markedly increased [Ca2+]i, which could be attenuated by long-term, but not short-term, lithium treatment. Thus, the protection induced by lithium in CGNs was attributed to its inhibition of calcium overload. In addition, the Ca2+ signaling pathway, including reactive oxygen species production and mitochondrial membrane potential reduction, along with the neurotoxic effect of β-BuTX was blocked by long-term,

but not short-term, lithium treatment. All of these results indicate that a crucial step for lithium protection is modulation of [Ca2+]I homeostasis and lithium neurotoxicity possibly, at least in part, is due to calcium overload. In conclusion, our results suggest that lithium, in addition to its use in treatment of bipolar depressive illness, may have an expanded use in intervention for neurotoxicity.

Differential calcium signaling of β-bungarotoxin and 8-(N, N-diethylamino) octyl-3, 4, 5-trimethoxybenzoate (TMB−8) linking the neuronal death in Primary Neurons

Ca2+ is an important secondary messenger in regulating various cellular functions. However, excessive intracellular Ca2+ ([Ca2+]i) is detrimental to cellular survival. Whether [Ca2+]i derived from different Ca2+ compartments can lead to differential cell death is still not known. In this paper, we compared the elevation of [Ca2+]i profile in relevance to neuronal death induced by β-bungarotoxin (β-BuTX; a presynaptic neurotoxin, increasing [Ca2+]i via activating NMDA receptor and L-type Ca2+-channel) and TMB-8 (an prototypic intracellular Ca2+ antagonist), respectively in cultured rat cerebellar granule neurons (CGNs). We found that β-BuTX (5 nM) increased [Ca2+]i level was comparable to that induced by TMB-8 (100 μM) with a potency ratio of 20,000. The relationship between [Ca2+]i elevation and neuronal death induced by β-BuTX and TMB-8, respectively were apparently paralleled in a linear regression line; but β-BuTX exhibited about two folds more efficient than TMB-8 at equivalent [Ca2+]i elevation state. Prior exposure of TMB-8 (100 μM) exhibited neuroprotective effect against β-BuTX (5 nM); in accordance with shifting [Ca2+]i profile toward that of TMB-8. The morphological changes also supported the neuroprotective effect of TMB-8 against β-BuTX. Pretreatment with various selective inhibitors for preventing neurotoxic effects of β-BuTX and TMB-8, we found that thapsigargin (an inhibitor of endoplasmic reticulum Ca2+-ATPase) prevented the neurotoxic effect of TMB-8 but had no effect on β-BuTX; whereas diltiazem (an inhibitor of L-type Ca2+ channel) only inhibited β-BuTX, but not TMB-8-induced neurotoxicity. These findings support our working hypothesis that differential Ca2+ signaling derived from different Ca2+-compartmentation leads to differential neurotoxicity.

The results presented here show that (1) overstimulation of NMDA receptor and/or activation of L-type calcium channel deregulated homeostasis of [Ca+2]i and induced neuronal death; (2) massive Ca+2 influx induced ROS production; (3) ROS disturbed mitochondrial function and induced collapse of $\Delta\Psi$,which made ATP depletion. β-BuTX-induced Ca+2 influx is sufficient to make mitochondrial dysfunction; and Ca+2 chelators decrease the incidence of neuronal death. The complex relationship between elevation of [Ca+2]i and mitochondrial function is a basis to formulate a model of β-BuTX-induced neuronal death essence; this model proposes that neuronal survival relies on a balance between mitochondrial function and [Ca+2]i homeostasis. Massive Ca+2 influx induces an imbalance in mitochondrial homeostasis, leading to mitochondrial dysfunction, which triggers neuronal death (Fig. 0-1). We turn now to explore the experimental foundation of the model.

中文摘要

神經細胞在體內生長需靠 neurotrophic factors 及神經的傳入刺激才能良好生存，在體外培養的神經細胞則需使用含有血清的高鉀(25mM K+)培養液，成熟神經細胞乃由小腦取出離體後培育 7-8 天(DIV)而得，已具有密致的神經突生長(neurite outgrew)。小腦顆粒神經細胞(cerebellar granule neurons, CGN)為高度均一的神經培養模式，一直被用來研究神經細胞生長發育及凋亡的機轉。鈣離子為調控各種細胞功能之重要二級傳遞物質，細胞內鈣離子對細胞各種生理、及生化作用
扮演著重要的角色。然而許多研究指出，細胞內負荷過量的鈣離子是造成病理變化或壓力引起的細胞傷害的主要原因。

乙型雨傘節蛇毒毒素(β-BuTX) 為一種由雨傘節 Bungarus multicintus 之毒素分離出之突觸前神經蛇毒，此毒素抑制運動神經末端(motor nerve terminals)釋放乙醯膽鹼(acetylcholine)，但其毒性分子作用機轉尚未完全明瞭。由於運動神經末端太細微，難以傳統方法尋求 β-BuTX 之分子作用機轉。β-BuTX 對中樞神經系統或小腦顆粒神經細胞作用也尚未研究清楚。為尋求可能作用目標，我們發現 β-BuTX 對成熟小腦顆粒神經細胞為一強效神經毒物，其百分之五十致效劑量(EC50)為 3 ng/ml (相當於 144 pM) ，但對未成熟小腦顆粒神經細胞則較無毒性。本研究中，我們探討乙型雨傘節蛇毒毒素(β-BuTX)引發小腦顆粒神經細胞毒性之作用機轉，希望能藉由此作用機制的闡明，瞭解神經退化的訊息傳遞途徑。

鈣離子活化一氧化氮生成對 β-BuTX 引發小腦顆粒神經細胞毒性扮演重要角色

β-BuTX 對成熟小腦顆粒神經細胞有強效神經毒性，定量分析此神經毒性，呈現時間依存性及劑量依存性。值得注意的是 β-BuTX 一開始先破壞神經突(neurite)生長、神經突斷裂(neurite fragmentation)、型態改變，繼而經由凋亡(apoptosis)及壞死(necrosis)途徑引起神經細胞死亡。Nomarski 光學顯微法(Nomarski optics)顯示神經細胞表現壞死之型態特徵，包括細胞腫大(swelling)、破壞細胞膜完整性、最後造成細胞分解(dissolution)。以螢光染料 Hoechst 33258 染色，顯示 β-BuTX 處理過之神經細胞體(neuron body)呈現較亮而密集之凋亡細胞體(apototic bodies)。使用顯微光譜螢光計量法(microspectrofluorimetry)與 fura-2 測量細胞內鈣離子濃度，我們發現 β-BuTX 顯著增加細胞內鈣離子濃度，而此鈣離子濃度增加與其時間依存性及劑量依存性之神經毒性有關聯性。再者，BAPTA-AM (細胞內鈣離子敖合劑)、EGTA (細胞外鈣離子敖合劑)、MK801 (NMDA 受體拮抗劑)及 diltiazem (L 型鈣離子通道阻斷劑)不只抑制細胞內鈣離子濃度之增加，也減弱 β-BuTX 之毒性。進一步發現，鈣離子抑制劑避免 β-BuTX 引發之反應性含氮物質(reactive nitrogenous species)生成，前處理一氧化氮合成酵素(NO synthase)抑制劑(NG-methyl-L-arginine)會呈現神經保護作用。因此，我們所得的結論是 β-BuTX 引發小腦顆粒神經細胞之神經毒性的訊息傳遞途徑可能部分經由細胞內鈣離子濃度過載，造成過多一氧化氮的釋放，最後致使細胞凋亡。至於此毒素導致鈣離子過載活化之機理乃是我們繼續探究的問題，我們推測活化 NMDA 受

體(NMDA receptors)及 L 型鈣離子通道(L-type calcium channel)與增加細胞內鈣離子濃度可能有關。

β-BuTX 活化 NMDA 受體及 L 型鈣離子通道引發神經細胞毒性

本篇探討 β-BuTX 引發神經毒性之增加細胞內鈣離子濃度的訊息途徑。結果顯示，β-BuTX 增加之反應性含氧物質(reactive oxygen species)生成，再造成粒腺體膜電位降低及 ATP 排空。BAPTA-AM、 EGTA、MK801 及 diltiazem 可抑制以上所有反應，顯示 β-BuTX 引發小腦顆粒神經細胞之神經毒性的訊息傳遞途徑可能部分經由直接或間接活化 NMDA 受體(NMDA receptors)及 L 型鈣離子通道(L-type calcium channel)而造成鈣離子內流、氧化性壓力、粒腺體功能失常、ATP 排空等一連串反應。因此我們提出，此多生 神經毒素由於強效性及不可逆性質，對研究神經退化疾病機轉為一有用工具。

Suramin 阻止 β-BuTX 引發小腦顆粒神經細胞毒性

Suramin 為一種無色染料，過去被用來當抗錐蟲藥及癌症治療之臨床試驗用藥，此藥有許多藥理作用，包括為非選擇性之 P2 膘呤受體阻斷劑(non-selective blocker of P2 purinoceptors)，也被認為可能為 NMDA 受體拮抗劑。因此本實驗欲探究是否已知 suramin 對 NMDA 受體之拮抗作用， 可用來對抗 β-BuTX 引發之神經毒性，而具神經保護作用。既然 suramin 抑制鈣離子濃度增加及神經毒性，鈣離子濃度增加可能導致 β-BuTX 之神經毒性反應。粒腺體可能為增加之鈣離子濃度的作用位置。分別藉由螢光染料 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) 及 3,3'-dihexyloxa carbocyanine iodide (DiOC6)，我們測得 β-BuTX 增加自由基生成，伴隨粒腺體膜電位降低及最終 ATP 排空。Suramin 亦有效地抑制這些 β-BuTX 之有害反應。基於這些發現及 MK801 與 EGTA 抑制 β-BuTX 之毒性，我們所得的結論是 β-BuTX 對小腦顆粒神經細胞作用的訊息傳遞途徑可能部分為 P2X receptors 與 NMDA receptors 之活化劑(activator)。活化此受體陽離子通道(receptor-cation channels)增加細胞外鈣離子內流，鈣離子過載損害粒腺體，造成粒腺體膜去極化，增加反應性含氧物質生成，而使 ATP 排空，最後導致神經死亡。我們的發現指出，suramin 對 β-BuTX 之毒性有保護作用，可提供對抗化合物神經毒性侵害之有效保護性研究。

β-BuTX 引發小腦顆粒神經細胞毒性與其活化 NMDA 受體之相關性

我們的研究結果顯示，β-BuTX 能結合至小腦顆粒神經細胞之 NMDA 受體。我們以螢光 FITC 標記 β-BuTX (FITC-β-BuTX)，發現 FITC-β-BuTX 之結合可被未標記之 β-BuTX 及 MK801 (NMDA 受體拮抗劑)抑制。同時，未標記之 MK801 及 β-BuTX 也會抑制[3H]-MK801 之結合。我們探討 β-BuTX 引發小腦顆粒神經細胞毒性之作用

機轉，發現由於 β-BuTX 產生過多之反應性含氧物質而致。MK801 及抗氧化物 (vitamin C, N-acetylcysteine, melatonin, epigallocatechin gallate, superoxide dismutase 與 catalase) 減弱反應性含氧物質之生成及 β-BuTX 引發之神經毒性反應。反應性含氧物質之下游訊息為 caspase-3 之活化，caspase 抑制劑(z-DEVD-fmk) 及抗氧化物抑制 caspase-3 之活化與神經毒性。基於這些發現，我們所得的結論是 β-BuTX 結合並活化 NMDA 受體為強效神經毒性反應之第一步驟，而結合 NMDA 受體會造成反應性含氧物質生成及 caspase-3 之活化，進而導致細胞凋亡。

長期鋰離子前處理減弱 β-BuTX 引發之小腦顆粒神經細胞毒性

鋰鹽為最常用來治療躁鬱症(manic-depressive illness)之藥物，突顯其臨床功效之正確機轉尚未明瞭。本研究，我們發現長期處理氯化鋰可對抗 β-BuTX 對小腦顆粒神經細胞引發之神經毒性。與臨床上相關濃度之 1.2 mM 鋰鹽表現神經保護作用。此神經保護作用需 3-5 天前處理(長期)氯化鋰，3 小時(短期)則無效。相較之下，較長時間前處理 6-7 天或較高濃度 3 mM 鋰鹽不僅失去神經保護作用，也引發神經毒性。這些發現顯示鋰鹽之神經保護作用僅限於狹窄濃度範圍，並明顯與臨床應用之狹窄治療範圍指標相關。測量細胞內鈣離子濃度，指出神經毒性濃度之 β-BuTX 顯著增加細胞內鈣離子濃度，長期前處理鋰鹽可減弱此鈣離子濃度增加，而短期前處理則否。因此，鋰鹽之神經保護作用歸因於其抑制鈣離子濃度過載。此外，長期前處理鋰鹽可減弱許多反，包含減弱反應性含氧物質之產生、粒腺體膜電位降低之鈣離子訊息途徑，及減弱 β-BuTX 引發之神經毒性，而短期前處理則否。以上結果指出，鋰鹽神經保護作用之重要步驟為調控細胞內鈣離子濃度之恆定，鋰鹽之神經毒性作用之訊息途徑可能部分是由於細胞內鈣離子濃度過載。總之，我們的結果指出鋰鹽除了用來治療躁鬱症，也許可另用於干預神經毒性作用。

β-BuTX 與 TMB−8 之不同鈣離子訊息對神經細胞毒性影響

是否不同鈣離子來源所造成之細胞內鈣離子濃度變化會致使不同之細胞死亡仍舊未知。本研究中，我們比較 β-BuTX (經由活化
NMDA 受體(NMDA receptors) 及 L 型鈣離子通道(L-type calcium channel)而增加細胞內鈣離子濃度)與 TMB−8 (一種典型的細胞內鈣離子拮抗劑)對小腦顆粒神經細胞增加細胞內鈣離子濃度與引發神經毒性之關聯。我們發現 5 nM 之 β-BuTX 增加細胞內鈣離子濃度與 100 μM 之 TMB−8 增加相當，但 β-BuTX 強度(potency)高出兩萬倍。β-BuTX 及 TMB−8 個別對細胞內鈣離子濃度之增加與引發神經細胞死亡呈平行之線性回歸關係，但對相同細胞內鈣離子濃度之增加狀態下，β-BuTX 比 TMB−8 效力多兩倍。然而前處理 TMB−8 (100 μM)可對抗 β-BuTX (5nM)而有神經保護作用，同時將細胞內鈣離子濃度之量變曲線轉移向 TMB−8 之曲線型式。型態上之改變也支持了 TMB−8 可對抗 β-BuTX 而有神經保護作用。前處理各種選擇性的抑制劑來觀察對

β-BuTX 及 TMB−8 之對抗神經毒性作用，發現 thapsigargin (一種內質網鈣離子幫浦抑制劑)可對抗 TMB−8 神經毒性作用，但對 β-BuTX 無效；而 diltiazem(一種 L 型鈣離子通道抑制劑)只抑制 β-BuTX 神經毒性，對 TMB−8 無效。此發現支持我們的假說，不同鈣離子來源所造成之細胞內鈣離子濃度變化會造成不同之神經毒性。綜合以上有關 β-BuTX 對小腦顆粒神經細胞致毒作用之訊息傳遞的研究，所得的結論為：(1)過度興奮 NMDA 受體與活化 L 型鈣離子通道導致鈣離子恆定失調引發神經毒性；(2)大量鈣離子內流引發反

應性含氧物質及一氧化氮之生成；(3)反應性含氧物質損害粒腺體功能，使粒腺體膜電位降低，ATP 排空，最後導致神經死亡；(4) β-BuTX 活化 caspase-3，引發細胞凋亡；(5) 一氧化氮增加導致神經細胞死亡。由於大量鈣離子內流引發粒腺體恆定失調，導致損害粒腺體功能，進而釋放反應性含氧物質，造成神經細胞死亡。這與許多臨床疾病如缺血性神經傷害　神經退化疾病之病因有關聯。因此，尋求調控細胞內鈣離子濃度恆定之藥物及抗氧化劑，可運用於臨床疾病治療之開發價值。我們認為 β-BuTX 對研究神經毒性過程為一有用工具，此蛇毒不僅可用以探求神經毒性死亡訊息途徑，藉由此作用機制的闡明，瞭解神經退化的原因，並且藉此模式尋求神經保護藥物之開發，期能應用於臨床神經疾病之治療。

Key words：b-Bungarotoxin; Cerebellar Granule Neurons; Intracellular calcium; Reactive oxygen species; Nitric Oxide; Apoptosis; Neurotoxicity; Siganl transduction

關鍵字：乙型雨傘節蛇毒毒素; 小腦顆粒神經細胞; 細胞內鈣離子濃度; 活性氧化物質; 一氧化氮; 細胞凋亡; 神經毒性; 訊息傳遞;