98 年研究計畫國科會成果報告書

摘要

我們的先前研究已知鉤藤能減少 kainic acid（KA）誘發 Spragrue-Dawley（SD）大鼠的癲癇發作，鉤藤的這個作用與氧化自由基的抑制或清除有關。又我們發現 KA 誘發癲癇發作大鼠的大腦皮質之 macrophage migration inhibitory factor（MIF)和 Cyclophilin A 都減少，以及 KA 注射 6 週後在海馬區可以觀察到 mossy fiber sprouting。選擇性海馬區神經細胞的損傷和 mossy fiber sprouting 可以作為癲癇形成和自發性癲癇發作產生的原因。因此，本研究目的是探討蛋白質體在慢性癲癇模型的應用。本研究將 SD 大鼠分成正常組（腹腔注射 PBS）、控制組（腹腔注射 KA 12mg/kg）和鉤藤（腹腔注射 KA 後隔日給予口服鉤藤 1.0 g/kg，每週 5 日連續 6 週）等 3 組，大鼠於腹腔注射 PBS 或 KA 後 6 週犧牲取腦。本實驗採集鼠腦海馬區域組織，分別以 17 公分二維電泳進行蛋白圖譜分析，實驗結果顯示，應用影像軟體綜合比較 3 組的海馬體蛋白圖譜，有 4 個蛋白點是只會出現在控制組，未出現在正常組和鉤藤組 更進一步放寬比對條件，發現出現在控制組蛋白表現＞2 fold 正常組和鉤藤組的蛋白點有 5 個，另外為評估鉤藤療效，我們比較正常組及鉤藤組蛋白圖譜，比對結果顯示有共有 271 個蛋白同時出現在這兩組實驗，但其中鉤藤組＞2 fold 正常組的蛋白點只有 3 個。目前我們已經將比對結果確認並應用 in gel digestion 將以上 12 個蛋白點萃取出送驗確認。

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關鍵詞：鉤藤、慢性癲癇模型、Mossy fiber sprouting、Kanic acid、蛋白圖譜

一、前言

癲癇是因慢性的腦損傷導致一群神經細胞同期性、過度放電、反覆發作的慢性疾病。台灣至少有10 萬以上的癲癇患者，至目前為止用抗癲癇藥物或手術治療大約只有75%的癲癇患者可以得到良好控制，其餘的25%則成為困難控制的頑固性癲癇患者(關尚勇，2001)，因此尋求更理想的癲癇治療方法是身為醫師的職責。癲癇是發作性疾病的一種，根據傳統中醫理論屬於肝風內動，因此中醫治療癲癇用具有平肝熄風的中藥或方劑，如天麻、鉤藤、石決明，鎮肝熄風湯、天麻鉤藤飲等（孫孝洪，1992）。Kainic acid（KA）是由*Digenea simplex* 海藻中分離出來類似麩氨酸鹽（glutamate）的物質，具有強的神經興奮毒性作用。大鼠或貓腹腔或腦內注射KA所引起的腦損傷的主要病變部位在海馬區域（hippocampus region），行為出現wet dog shakes，facial myoclonia 和paw tremor 等類似人類複雜性部分性癲癇發作（complex partial seizure），又稱為精神運動性癲癇（psychomotor seizure）(Ben-Ari, 1985；Nitecka et al., 1984；Schwob et al., 1980；（Tanaka et al., 1990；Tremblay et al., 1984；Wuerthele et al., 1978），因此KA能誘發類似人類精神運動性癲癇發作的動物模式，也能提供hippocampus 區域正常和病理功能的更好理解（Ben-Ari,1985）。我們先前的研究結果已知Sprague-Dawley（SD）大鼠腹腔注射KA（12 mg/kg）約20 分鐘後，會發展出wet dog shakes，paw tremor 和facial myoclonia等行為，wet dog shakes 於注射後60-80分鐘時達到頂峰，大約維持至注射後3小時（Hsieh et al., 1999a；Hsieh et al., 1999b；Hsieh et al., 2001a；Hsieh et al., 2001b）。又鉤藤於大鼠能減少KA誘發的癲癇發作，鉤藤的這個作用與氧化自由基的抑制或生成有關（Hieh et al., 1999a）。鉤藤和天麻對於抗癲癇，兩者有協同作用（Hsieh et al., 1999b）。鉤藤於大鼠能抑制hippocampus區域KA誘發的apoptosis、microglia activation，以及inducible nitric oxide synthase（iNOS）和neuronal nitric oxide synthase（nNOS）陽性染色細胞（unpublished）。有研究發現鉤藤對於N-methyl-D-asparate（NMDA）誘發的神經細胞損傷有保護作用（Lee et al., 2003a），以及經由抑制NMDA誘發的apoptosis 來保護NMDA 所引起的細胞毒性（Lee et al., 2003b）。KA治療後會導致癲癇發作閾值（threshold）的全體的減少，造成晚期的自發性癲癇活動（spontaneous seizure activity）（Sperk, 1993）。KA誘發癲癇發作持續狀態後，會出現連續性、再發性的癲癇發作，這種遲發性自發性癲癇發作十分類似人類mesial temporal lobe epilepsy，主要的型態變化在海馬區(Morimoto et al., 2004)。KA誘發癲癇發作的腦損傷在齒狀核會出現sprouting of mossy fiber，這種現象在人類頑固性癲癇小孩死後的組織也有相似mossy fibre pathway 的組織再生（reorganization）（Sperk, 1993）。選擇性神經細胞的損傷和mossy fiber sprouting可以作為癲癇形成和自發性癲癇發作產生的原因（Pitkänen et al., 1999）。雖然有研究指出在電氣刺激杏仁核（amygdala）引發癲癇發作大鼠的海馬區域(hippocampus)之mossy fiber sprouting與神經細胞損傷(neuronal damage)的嚴重度有相互關係，但mossy fiber sprouting並不需要伴隨自發性癲癇

（spontaneous seizure）的發生（Nissinen et al., 2001），以及在杏仁核和海馬區域電刺激點燃（kindling）大鼠的mossy fiber sprouting 不因損傷產生而靠著神經細胞的活化（neuronal activation）（Adams et al., 1997）。有的研究發現在Ihara 癲癇大鼠的全身性強直性一陣攣性癲癇發作（generalized tonic and clonic convulsion）和海馬齒狀回（dentate gyrus）分子層（molecular layer ）的mossy fiber sprouting，兩者之間有正相關的關係（postive correlation），因此推論mossy fiber sprouting 是一種由於突觸間組織再生（synaptic reorganization）的續發性型態轉變，也就是由於癲癇放電之構造上的神經可塑性（structural neuroplasticity）（Amano et al., 1999）。在顳葉性癲癇有海馬硬化（temporal lobe epilepsy with hippocampal sclerosis）的患者發現海馬區有嚴重的神經細胞喪失，astrogliosis 和mossy fiber sprouting，這些型態上的轉變是一種新的功能性突觸的形成（the formation of new functional synapses），如此推測海馬區域神經細胞的喪失隨後進行性mossy fiber sprouting 可以是提供癲癇形成（epileptogenesis）的重要因素。又mossy fiber sprouting 伴隨著突觸密度增加，說明mossy fiber terminal 不僅是構造的，而且可能也是功能的，這些功能性glutamatergic mossy fiber terminals與顆粒細胞的樹狀突（dendrites of granule cell）間發生突觸可以作為癲癇發作的開始（Proper et al., 2000）。在癲癇大鼠，Aberrant mossy fiber sprouting 增加了顆粒細胞和增加癲癇發作敏感性之間的興奮性連接（excitatory connection），以及aberrant mossyfiber sprouting 的發展和癲癇發作的進展，兩者之間有相互關係，因此mossy fiber sprouting 可以形成一個興奮性環繞（excitatory circuit）而提供顳葉癲癇的慢性狀態（Rao et al., 2006）。在小鼠以pilocarpine 誘發癲癇持續狀態（status epilepticus）4-8 weeks 後，可以看到豐富的mossy fiber sprouting 進入內分子層（inner molecular layer），而所有小鼠發生自發性癲癇發作。Pilocarpine誘發癲癇持續狀態所引起的行為和組織變化和顳葉性癲癇的發生有密切的一致性。慢性癲癇動物模型（chronic epilepsy animal model）可作為癲癇形成的進行之研究（Shibley and Smith, 2002）。在人類頑固性顳葉性癲癇患者的顳葉皮質的brain-derived neurotrophin factor （BDNF）的蛋白質濃度明顯的比控制組增加2 倍，BDNF 的增加和neuropeptide Y（NPY）呈現有意義的相互關係，推測BDNF 參與人類癲癇的形成包括NPY 的誘導（induction）在內（Takahashi et al., 1999）。有研究在pilocarpine治療大鼠的杏仁核注入BDNF，結果在海馬齒狀回的顆粒層的mossy fiber axon 可以看到NPY 和BDNF 表現增加，以及mossy fiber sprouting 和對pilocarpine發作敏感，因此支持BDNF在海馬可以促進、強化地驅動，癲癇發作活動的假說（Scharfman et al., 2002）。在大鼠的腹腔KA後12 到24 小時，可以在不同區域的腦組織增加BDNF（Katoh-Semba et al., 1999）。又在成人小鼠的dorsal hipoocampus注入KA 增大的顆粒細胞（granule cells出現BDNF 和TrK B，這個增加從KA 注射後的2到16週，維持到達12個月（Inoue et al., 1998）。癲癇發作大鼠的腦組織呈現BDNF 和NPY 兩者之間的動態（dynamic）和時間

（temporal）的連結轉變，牽涉到癲癇的形成，推測BDNF 和NPY 兩種物質之間在神經網路興奮的調節有功能上的連結（Vezzani et al., 1999）。由於BDNF 在海馬內注射，急性期能導致過渡興奮（hyperexcitability）和癲癇發作活動而促進癲癇形成，以及誘發自發性癲癇放電和引起glutamate 釋放等，但慢性的連續注射exogenous BDNF 則會減少TrKB 的表現和TrK 磷酸化作用，導致喪失對BDNF 的反應而抑制癲癇形成，所以認為BDNF 對癲癇形成的效應不在於mossy fiber sprouting 而在於對excitability（Xu et al., 2004）。延長癲癇發作引發長期間NPY 在mossy fiber 的表現，NPY 從再發的mossy fiber 末端被自發性的釋放（Nadler et al., 2007）。NPY 扮演一個有意義的角色於過渡興奮的情況（condition of hyperexcitability）調節海馬的功能（Vezzani et al., 2002）。在KA 治療大鼠於癲癇發作的急性期引發細胞外海馬NPY 表現增加，NPY 濃度的增加有可能是減輕癲癇發作的一個內源性機制（Husum et al., 1998）。當基因體序列開啟生物系統研究新的門後，後基因時代（post-genomic era）必須尋求如何轉譯這個DNA 序列的訊息進入活細胞（living cells）、組織、和有機體（organization）的理解，其中最主要的目標是去描繪蛋白質的功能、生物醫學的路徑和網路（Hust and Grant 2001）。蛋白質體（proteomics）與基因體（genomics）相類似，它是蛋白質層次（protein level）大範圍基因表現（gene expression）的研究，特別在蛋白質的功能和構造，它的優點勝過基因表現影像，能夠快速分析蛋白質的標記（protein markers）（Wilkins et al., 1996;Wilkins 2002; Butterfield et al. 2006）。蛋白質體能從單一樣本中包括蛋白質的分開、鑑定和量化（quantification）做分析，它主要由兩個階段所組成：1)蛋白質的分離（separation of proteins），通常用two-dimensional gel electrophoresis（2-DE）來分離蛋白質；2)蛋白質的分析和鑑定（protein analysis and identification），主要是利用質譜分光計檢法（mass spectrometry）來分析和鑑定蛋白質（Lubee et al., 2003; Butterfield et al. 2006）。蛋白質的決定比RNA 更貼近它的功能，類似基因體圖譜（mapping of genome）一般，將發展出人類和動物腦的蛋白質圖譜（Quadroni and James 1999; Celis et al., 1998）。有研究指出蛋白質體也能用於腦缺血ㄧ再灌流損傷時所產生氧化緊迫（oxidative stress）之相關抗氧化蛋白及它的同分異構物（isoforms）如superoxide dismutase 1（SOD1）和SOD2（Lubee et al., 2003）之識別。此外，蛋白質體也能用來識鑑別藥物活性的生物標記（biomarkers）作為監測治療和毒性的反應（Wilkins 2002）。有研究指出2-DE 配合Matrix-assisted laser desorption ionization time of flight-time of flight mass spectrometry (MALDI-TOF-TOF MS)和Nanoscale capillary LC-MS/MS 能夠做完整的蛋白質分析，提供蛋白質構造的訊息，鑑定和轉譯後修飾的分析（analysis of posttranslational modifications）（Liu and Schey, 2005; Lo et al., 2007 a,b），其應用於蛋白質分析的範圍甚為包括血漿（plasma）、血液（blood）、血球（blood cells）、腦脊髓液（cerebrospinal fluid）、組織（tissue）包括腦組織、細胞（cells）、尿液（urine）和唾液（saliva）等（Aldred et al., 2004）。

我們先前的研究，在大鼠利用腹腔注射KA 誘發癲癇發作後，其腦組織的 proteomic analysis of biomarker 的分析， 結果2-DE 和MASS assay 都顯示癲癇發作大鼠的大腦皮質 macrophage migration inhibitory factor（MIF）和Cyclophilin A 都減少；Western blot 顯示癲癇發作大鼠大腦皮質的MIF 和Cyclophilin A 的蛋白質表現，以及RT-PCR 也顯示MIF 和Cyclophilin A 的基因表現減少（Hsieh et al., 2007）。另外，我們第一年的研究發現於SD 大鼠腹腔注射KA（12 mg/kg），6週後在海馬區顆粒層可以清楚的看到mossy fiber sprouting，而每週服用5日，每日口服鉤藤1.0 g/kg，連續6週可以明顯的減低mossy fiber sprouting。因此，本研究的目的在探討蛋白質體在慢性癲癇模型的應用。

二、材料與方法

（一）動物的裝備

雄性 SD 大鼠，重量介於 200-300 克之間，每隻大鼠均於 PBS 或 KA 注射前 15 分鐘及注射後 3 小時施行後腦波和肌電圖，以及動物行為觀察。將 9 隻 SD 大鼠隨機分成三組，每組 3 隻如下

1. 正常組：SD 大鼠腹腔注射 PBS 容液 1.0 ml/kg，6 週後將大鼠犧牲取腦。
2. 控制組：SD 大鼠腹腔注射 KA (12 mg/kg) ，6 週後將大鼠犧牲取腦。
3. 鉤藤組：同控制組，但 KA 注射後開始隔日口服鉤藤 1.0 g/kg/日，每週 5 日，連續 6 週後將大鼠犧牲取腦。

（二）實驗流程

A. 蛋白質抽出（Protein extractions）

For two-dimensional gel electrophoresis（2-DE）, to remove blood by PBS, all tissues were homogenized with 0.5 ml lysis buffer containing protease inhibitor cocktail (Sigma, USA) by homogenizer (MagNA Lyser, Roach). The protein lysates were centrifuged at 8000 rpm for 20min at 4℃ and supernatants were collected to do precipitation of acetone for protein purification. The protein pellet was denatured with sample buffer (8M urea, 4% CHAPS, 65mM DTE, 0.5% ampholytes). The denatured proteins were incubated at 4℃ 2~3 hour before centrifuge 13,000 rpm for 15 mins and measured the protein concentrations, respectively (Lo et al. 2007a,b).

B. 二維膠電泳（Two-dimensional gel electrophoresis, 2-DE）

We took 200μg protein extraction per sample for 2D-gel separation. The extracted sample was diluted with rehydration buffer consisted of a 8M urea, 4% CHAPS, 65mM DTE, 0.5% ampholytes, and a trace of bromophenol blue to 350 microliter finally. Then, applying the rehydration solution (containing sample) into 11 cm

immobilized pH gradient pH 4-7 IPG strip (ReadyStrip IPG strip, Bio-Rad) overnight. Electrophoresis in the first dimension was focused for a total of 60 kVhr (PROTEAN IEF cell, Bio-Rad) at 20℃ and then stored at -20℃ until SDS-PAGE electrophoresis. Before SDS-PAGE electrophoresis, IPG strips were equilibrated with 3 ml of a equilibrium solution containing 50mM Tris-HCl (pH 8.8), 6M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue, and DTE (1 % w/v) for 20 min, and followed with second equilibration for 20 min in the same equilibrium solution containing iodoacetamide (2.5% w/v) instead of DTE. Finally, strip was transferred to the top of 12% polyacrylamide gels and held in position with molten 0.5% agarose in running buffer containing 25mM Tris, 0.192 M glycine, 0.1% SDS. Gels were run at 16mA/gel for 30 min followed by 50mA/gel for 4~5 hour (Lo et al., 2007a,b).

C. 蛋白質斑點的偵察和資料分析（Detection of protein spots and data analysis）

Gels were routinely stained with silver nitrate and then scanned by GS-800 imaging Densitometer with PDQuest software version 7.1.1 (Bio-Rad). Protein spots were quantified and compared using the PDQuest software. Consistently and significantly overexpressed spots were got the averages in each protein and selected for analysis with Nanoelectrospray mass spectrometry (nano LC-MS/MS) (Lo et al., 2007a,b)

D. Enzyme digestion and nano LC-MS/MS analysis.

Each interested protein spot was cuted with pipette tip and transfer into a microcentrifuge tube (0.6 ml). The gel pieces were washed twice with 50μL of 50% acetonitrile (ACN)：50% 200mM ammonium bicarbonate for 5 min, shrink with 100% acetonitrile until the gels turned white, then dry the gels for 5 min in a speed vac. At room temperature, The gel pieces were rehydrated in 15μL of 50mM ammonium bicarbonate (37℃, 4 min), and add an equivalent volume (15μL) of trypsin (Promega, Madison, WI) solution (20 ng/μL in 50mM ammonium bicarbonate), incubate at 37℃ for 4 hr or 30℃ for at least 16 hr. After digestion, vortex and spin down the gel pieces, the supernatants were the peptides solution and storage at -20℃ until mass analysis.

Nanoscale capillary LC-MS/MS was used to analyze the meaningful proteins involved in the reaction. All analysis was performed using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTARXL quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA, USA). The nanoscale capillary LC separation was performed on a RP C18 column (15 cm×75 m i.d.) with a flow rate of 200 nL/min and a 60 min linear gradient of 5-50% buffer B. Buffer A contained 0.1% formic acid in 5 % aqueous ACN; buffer B contained 0.1% formic acid in 95% aqueous ACN. Data

acquisitions were performed by Automatic Information Dependent Acquisition (IDA; Applied Biosystem /MDS Sciex). The IDA automatically finds the most intense ions in a TOF MS spectrum, and then performs an optimized MS/MS analysis on the selected ions. The product ion spectra generated by nanoLC-MS/MS were searched against NCBI databases for exact matches using the ProID program (Applied Biosystem/MDS Sciex) and the MASCOT search program. Carbamidomethyl cysteine was set as a fixed modification, while serine, threonine, tyrosine phosphorylation and other modifications were set as variable modifications. All phosphopeptides identified were confirmed by manual interpretation of the spectra (Lo et al., 2007a,b).

（三）資料分析

分析各組鼠腦海馬 mossy fiber sprouting 區蛋白質的表現，根據蛋白質之表現推測 KA 誘發慢性癲癇形成蛋白質的角色及鉤藤效用之機制。

三、結果

本實驗完成正常組、控制組和鉤藤等 3 組，大鼠於腹腔注射 PBS 或 KA 後 6 週犧牲取腦。本實驗採集腦中的，分別以 17 公分二維電泳進行蛋白圖譜分析，實驗結果顯示，應用影像軟體綜合比較 3 組的海馬體蛋白圖譜，有 4 個蛋白點是只會出現在控制組，未出現在正常組和鉤藤組 更進一步放寬比對條件，發現出現在控制組蛋白表現＞2 fold 正常組和鉤藤組的蛋白點有 5 個，另外為評估鉤藤療效，我們比較正常組及鉤藤組蛋白圖譜，比對結果顯示有共有 271 個蛋白同時出現在這兩組實驗，但其中鉤藤組＞2 fold 正常組的蛋白點只有 3 個。目前我們已經將比對結果確認並應用 in gel digestion 將以上 12 個蛋白點萃取出送驗確認。

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