科技部補助專題研究計畫成果報告
期末報告

腸泌素訊息減緩β型類澱粉蛋白導致神經毒性之分子機轉研究

計 畫 類 別 ： 個別型計畫
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中 華 民 國 104 年 10 月 30 日

中 文 摘 要 ： 阿茲海默症(Alzheimer’s disease, AD)是一種最常見的神經退化性疾病，臨床上主要具備兩種病理特徵，包括在神經細胞外由Aβ所聚集而成的類澱粉(β amyloid plaques)，及在細胞內由過度磷酸化的tau所組成的神經纖維糾結(neurofibrillary tangles)等。越來越多的證據指出阿茲海默症在本質上是屬於代謝疾病，事實上許多研究皆指出AD病人腦部的神經細胞其胰島素的訊息傳遞均有不正常受到抑制的情形，這些結果顯示了胰島素的訊息抵抗可能是連結代謝症候群與AD兩者間的共同分子機轉，然而胰島素的訊息傳遞與Aβ造成之神經退化之間的確切關聯則不是非常清楚。近來，許多研究已指出，若能透過給予例如DPP-4 (dipeptidyl peptidase-4)的抑制劑來提升GLP-1 (glucagon-like peptide-1)訊息傳遞，將有助於改善胰島素生長因子的訊息抵抗情況，因此被認為是一個具潛力的AD治療方式。根據這些發現，我們假設AD的病人其胰島素訊息傳遞若發生抵抗的現象，便會導致神經退化的情形發生。為了證明此觀點，本計畫將實驗設計分為下列三項來進行討論：(i) 首先我們預計先透過各種不同之細胞實驗模式，釐清胰島素訊息傳遞與Aβ造成神經毒性之間的因果關係。(ii) 其次，我們將透過給予DPP-4抑制劑，來探討提升神經細胞內GLP-1訊息傳遞時是否有益於改善Aβ造成之胰島素訊息傳遞障礙。(iii) 最後，我們將探討神經細胞老化過程與胰島素訊息傳遞之間的關係，並嘗試在老化過程中是否能透過調控胰島素訊息傳遞活性來對抗Aβ所造成之神經毒性。結果發現胰島素及GLP-1訊息傳遞路徑抑制與Aβ所造成之神經退化關係密切，而DPP-4抑制劑確實能有效緩解神經凋亡的情況，我們希望透過本計畫研究結果以瞭解神經細胞內胰島素及GLP-1訊息傳遞的詳細分子機轉，並有助於在未來開發出更有效的AD治療策略與藥物。

中 文 關 鍵 詞 ： 阿茲海默症、β型類澱粉蛋白、胰島素訊息、GLP-1、DPP-4抑制劑

英 文 摘 要 ： Alzheimer’s disease (AD) is the most common neurodegenerative disease. It is characterized by two diagnostic pathological hallmarks like amyloid plaques (APs) which are mainly formed by Aβ peptides accumulation, and neurofibrillary tangles (NFTs) which are composed of hyperphosphorylated tau protein. Growing evidence supports the concept that AD is fundamentally a metabolic disease. In fact, both clinical and experimental data demonstrated insulin signaling was aberrantly distributed in AD neurons. Consequently, neuronal resistance for insulin signaling might represent a molecular link between metabolic syndrome and AD. However, the mechanism how insulin signaling influences the onset and progression of Aβ-induced neurodegeneration remain incompletely understood. As the results, the present study will try to demonstrate a possible mechanism of neuronal insulin resistance in Aβ-induced neurotoxicity. Recently studies have demonstrated some pharmacological agents like dipeptidyl peptidase-4 (DPP-4) inhibitors, which increase the level of glucagon-like peptide-1 (GLP-1) and ameliorate insulin resistance,

have become valuable candidates as disease modifying agents in the treatment of AD. Based on these findings, we postulated that AD neurons degeneration was caused by impairments in brain insulin/IGF-1 signaling and insulin/IGF-1 resistance. To confirm this hypothesis, our results have revealed three findings: (i) Firstly, we investigated the interrelationship between impaired insulin signaling and Aβ neurotoxicity by several in vitro model. (ii) To evaluate the interrelationship between impaired insulin signaling and Aβ neurotoxicity, DPP-4) inhibitors have applied and tested whether upregulated-GLP-1 downstream signaling could alleviate insulin resistance under Aβ treatment. (3) To elucidate whether the aging process is associated with brain insulin and GLP-1 signaling resistance, we have determined the effects and the possible mechanisms involving modulation of insulin/GLP-1 signaling and Aβ-related neurodegeneration. We expect our results can be a contribution to the of the understanding mechanisms involved in neuronal insulin signaling, and shed light onto possible therapeutic approaches to provide novel AD therapeutic strategies and drug targets in future.

英文關鍵詞：Alzheimer's disease, Amyloid β, Insulin signaling, Glucagon-like peptide-1 (GLP-1), DPP-4 (dipeptidyl peptidase-4) inhibitor

科技部補助專題研究計畫成果報告

（□期中進度報告/☑期末報告）

腸泌素訊息減緩β型類澱粉蛋白導致神經毒性之分子機轉研究

計畫類別：☑個別型計畫　□整合型計畫
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計畫主持人：黃建寧教授
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計畫參與人員：李欣樺、王威傑、張琇涵、蕭惠文、蔡善格

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中　華　民　國 104 年 10 月 31 日

中文摘要

阿茲海默症(Alzheimer's disease, AD)是一種最常見的神經退化性疾病，臨床上主要具備兩種病理特徵，包括在神經細胞外由 Aβ 所聚集而成的類澱粉(β amyloid plaques)，及在細胞內由過度磷酸化的 tau 所組成的神經纖維糾結(neurofibrillary tangles)等。越來越多的證據指出阿茲海默症在本質上是屬於代謝疾病，事實上許多研究皆指出 AD 病人腦部的神經細胞其胰島素的訊息傳遞均有不正常受到抑制的情形，這些結果顯示了胰島素的訊息抵抗可能是連結代謝症候群與 AD 兩者間的共同分子機轉，然而胰島素的訊息傳遞與 Aβ 造成之神經退化之間的確切關聯則不是非常清楚。近來，許多研究已指出，若能透過給予例如 DPP-4 (dipeptidyl peptidase-4)的抑制劑來提升 GLP-1 (glucagon-like peptide-1)訊息傳遞，將有助於改善胰島素生長因子的訊息抵抗情況，因此被認為是一個具潛力的 AD 治療方式。根據這些發現，我們假設 AD 的病人其胰島素訊息傳遞若發生抵抗的現象，便會導致神經退化的情形發生。為了證明此觀點，本計畫將實驗設計分為下列三項來進行討論：(i) 首先我們預計先透過各種不同之細胞實驗模式，釐清胰島素訊息傳遞與 Aβ 造成神經毒性之間的因果關係。(ii) 其次，我們將透過給予 DPP-4 抑制劑，來探討提升神經細胞內 GLP-1 訊息傳遞時是否有益於改善 Aβ 造成之胰島素訊息傳遞障礙。(iii) 最後，我們將探討神經細胞老化過程與胰島素訊息傳遞之間的關係，並嘗試在老化過程中是否能透過調控胰島素訊息傳遞活性來對抗 Aβ 所造成之神經毒性。結果發現胰島素及 GLP-1 訊息傳遞路徑抑制與 Aβ 所造成之神經退化關係密切，而 DPP-4 抑制劑確實能有效緩解神經凋亡的情況，我們希望透過本計畫研究結果以瞭解神經細胞內胰島素及 GLP-1 訊息傳遞的詳細分子機轉，並有助於在未來開發出更有效的 AD 治療策略與藥物。

關鍵詞：阿茲海默症、β型類澱粉蛋白、胰島素訊息、GLP-1、DPP-4 抑制劑

Abstract

Alzheimer's disease (AD) is the most common neurodegenerative disease. It is characterized by two diagnostic pathological hallmarks like amyloid plaques (APs) which are mainly formed by Aβ peptides accumulation, and neurofibrillary tangles (NFTs) which are composed of hyperphosphorylated tau protein. Growing evidence supports the concept that AD is fundamentally a metabolic disease. In fact, both clinical and experimental data demonstrated insulin signaling was aberrantly distributed in AD neurons. Consequently, neuronal resistance for insulin signaling might represent a molecular link between metabolic syndrome and AD. However, the mechanism how insulin signaling influences the onset and progression of Aβ-induced neurodegeneration remain incompletely understood. As the results, the present study will try to demonstrate a possible mechanism of neuronal insulin resistance in Aβ-induced neurotoxicity. Recently studies have demonstrated some pharmacological agents like dipeptidyl peptidase-4 (DPP-4) inhibitors, which increase the level of glucagon-like peptide-1 (GLP-1) and ameliorate insulin resistance, have become valuable candidates as disease modifying agents in the treatment of AD. Based on these findings, we postulated that AD neurons degeneration was caused by impairments in brain insulin/IGF-1 signaling and insulin/IGF-1 resistance. To confirm this hypothesis, our results have revealed three findings: (i) Firstly, we investigated the interrelationship between impaired insulin signaling and Aβ neurotoxicity by several *in vitro* model. (ii) To evaluate the interrelationship between impaired insulin signaling and Aβ neurotoxicity, DPP-4) inhibitors have applied and tested whether upregulated-GLP-1 downstream signaling could alleviate insulin resistance under Aβ treatment. (3) To elucidate whether the aging process is associated with brain insulin and GLP-1 signaling resistance, we have determined the effects and the possible mechanisms involving modulation of insulin/GLP-1 signaling and Aβ-related neurodegeneration. We expect our results can be a contribution to the of the understanding mechanisms involved in neuronal insulin signaling, and shed light onto possible therapeutic approaches to provide novel AD therapeutic strategies and drug targets in future.

Keywords: Alzheimer's disease, Amyloid β, Insulin signaling, Glucagon-like peptide-1 (GLP-1), DPP-4 (dipeptidyl peptidase-4) inhibitor

前言(Introduction)

阿茲海默症(Alzheimer's disease，AD)是一種漸進式神經退化性疾病，又稱為老年失智症，也是目前造成失智症最主要的疾病。流行病學研究指出在 2010 年，全球將近 3560 萬被診斷出老年失智症，且隨著每 20 年，預計將增長近一倍人口罹患老年失智症，導致在 2030 年有 6570 萬罹患老年失智症，而 2050 年將達 1 億 1,540 萬例，那是目前已知病人數的三倍。其中 AD 是最常見老年失智症的一種，其症狀會造成記憶、認知、和語言功能的衰退，及方向感的迷失和出現習慣性的動作等，患者將逐漸不能適應社會，嚴重的情況往往無法理解會話內容，無法獨立照顧自己生活起居，最終癱瘓在床，而需要長期照護，往往伴隨著其他疾病而死亡。一般而言，老化(Aging)是造成 AD 的最主要危險因子，其中在超過六十五歲的總人口中約有 5 至 10％罹患阿茲海默症，而 85 歲以上的人口更是有一半左右是阿茲海默症的患者。依據發病年齡，可分為早發性 AD (Early-onset Alzheimer's Disease)及晚發性 AD (Late-onset Alzheimer's Disease)兩種。其發病年齡在 65 歲前，約 40 至 65 歲間被診斷出來，稱為早發性 AD，其中約一半是屬於體染色體為顯性遺傳疾病而造成家族遺傳型 AD (Familial Alzheimer's disease)，被定義為早發性家族遺傳型 AD (Early-onset Familial Alzheimer's Disease)。而非家族遺傳型 AD 屬於偶發型 AD（sporadic Alzheimer's disease），超過 90%是在 65 歲以後罹病，此稱為晚發性 AD。這些患者最終終究會失去自理能力，因此需要大量的日夜照顧，而這些照顧者大多為家庭成員，往往承受高度情緒和身體的壓力，在此情況之下病患親友的日常生活往往也跟著受到很大的影響。所以，阿茲海默除了是台灣的醫療問題外，也會造成許多嚴峻的社會問題。目前臨床上阿茲海默症的治療情況，大多是以膽鹼酯分解酵素抑制劑(cholinesterase inhibitors)為主。 Cholinesterase 是神經細胞傳導物 acetylcholine 分解反應中的關鍵酵素，這些膽鹼酯分解酵素抑制劑可藉著阻斷 cholinesterase 的作用來抑制 acetylcholine 的分解，進而提高病人腦中 acetylcholine 的含量。但這些藥物雖可暫時延緩記憶的喪失，其最終並不能治癒阿茲海默症，充其量只能減輕症狀而已，因此阿茲海默症目前仍是無法治癒的疾病。

很多研究顯示血管危險因子和阿茲海默症發生率的關聯。包括高血壓、第二型糖尿病、肥胖、抽煙、心臟血管疾病、腦中風等都可能會增加阿茲海默症的風險。最近的研究顯示，胰島素對腦部也很重要，已知胰島素異常和許多神經退化性疾病有關，包括阿茲海默症、Parkinson's disease 和 Huntington's disease 等。特別是第二型糖尿病，在很多研究都發現會造成記憶或認知的衰退，根據統計第二型糖尿病在增加阿茲海默症發生風險方面，相對風險值約增加 1.5 倍，且即使是邊緣性糖尿病，也會顯著升高阿茲海默症發生的風險，而第二型糖尿病與 Apolipoprotein E (ApoE) ε4 基因及高血壓對於升高阿茲海默症的風險又進一步有著交互作用。在發現 ApoE 和阿茲海默症之間的關係之前，許多研究其實已經指出 ApoE 與血液中膽固醇的攜帶與運送方面扮演很重要的角色。ApoE 基因位於人類第 19 對染色體上，這種蛋白質的對偶基因有三種天然的變異型，分別被命

名為 ε2、ε3 和 ε4，是一個與人類心血管疾病有高度關係的基因。基本上，每個人天生都有兩個對偶基因，所以在 ApoE 中一共會有 6 種可能的排列組合，包括 ε2/ε2、ε2/ε3、ε2/ε4、ε3/ε3、ε3/ε4 和 ε4/ε4 等。其中已知 ApoE 的各種基因分型組合與心血管疾病、腦中風以及阿茲海默症的發生率有一定程度的關係，一般認為 ε2 和 ε3 有保護作用，而 ε4 則會增加阿茲海默症的罹患率，因此 ε3/ε4、ε4/ε4 這兩種基因型最具罹患這類疾病的風險，也就是阿茲海默症的高危險群。

1960 年代發現當給病人口服葡萄糖時，血漿中胰島素上升的程度要比靜脈注射葡萄糖更高，此種現象稱為腸促胰素效應(incretin effect)。此種現象大部份是因為兩種腸促胰素 GIP (glucose-dependent insulinotropic polypeptide)及 GLP-1 (glucagon like peptide-1)的 insulinotropic 作用所造成。其中 GLP-1 是由遠端小腸的 L 細胞分泌，GIP 則是由近端小腸的 K 細胞分泌，這些荷爾蒙進入門脈至胰臟作用於 β 細胞使之分泌胰島素，但很快的被淋巴球、巨噬細胞、內皮細胞及各種組織之 dipeptidyl peptidase-4 (DPP-4)所分解，因此 GLP-1 及 GIP 的半衰期非常短(約 2 分鐘)。此外由於目前 GIP 不能成為治療的目標，GLP-1 自然就成為改善胰島素訊息傳遞的利器。GLP-1 的生理作用包括：降低基礎及飯後升糖素之分泌，延後胃排空及作用於下視丘增加飽食感，並可促使細胞新生及減少凋亡等。由於天然的 GLP-1 半衰期非常短，不適合長期使用，因此有二種替代方法，一種是使用較長效且具 DPP-4 抗性的 GLP-1 類似物(mimetic)，也就是所謂 GLP-1 agonist；另一種是使用 DPP-4 酵素的抑制劑來增加內生性活性 incretin 的濃度，也就是所謂 incertin 促進劑(incretin-enhancer)。目前經 FDA 通過的 DPP-4 抑制劑只有 sitagliptin (Januvia®)，而其他的 denagliptin，saxagliptin 及 vildaglitin 等都還在臨床實驗階段。相對的在 AD 方面，越來越多證據已指出失智症的大腦損傷模式與糖尿病狀況有關，特別是與胰島素途徑受阻礙的情況有高度關聯性，而 Incertin 促進劑則能顯著改善此種胰島素途徑受阻礙的情況，然而 DPP-4 抑制劑類藥物雖因此被認為可能具有對抗阿茲海默症的效果，但其詳細的分子機轉，特別是在神經細胞中的訊息傳遞情形目前並不十分明瞭。

根據統計，僅有不到 5%的 AD 患者其發病原因是由於某些基因或蛋白質突變，如 APP、presenilin-1 及 presenilin-2 等變異而導致，另外超過 95%的 AD 患者則是由其他尚未明瞭的病因所造成(sporadic AD)，其原因可能是由於腦內代謝異常或訊息傳遞不正常所引發。研究發現，肥胖與心血管疾病均會顯著提高 AD 的罹患率，而肥胖與心血管疾病事實上皆與現今所謂的代謝徵候群(metabolic syndrome)或糖尿病高度相關。我們都知道糖尿病可分為兩型，第一型糖尿病是胰臟無法正常生產 insulin 所致，而第二型糖尿病的病人的體內雖然會生產 insulin，卻因為 insulin signaling 有問題，身體內各種組織對 insulin 無法起正常反應所致。在 sporadic AD 病人中隨著逐漸老化，腦中接受 insulin 訊號的效率開始越來越差，最終會誘發 Aβ 不正常的累積並加重其毒性反應。其實流行病學早有研究顯示，糖尿病人罹患 AD 的風險比一般人口高得多，另也有許多研究顯示，胰島素顯然有助減少腦部遭到 Aβ 的傷害。此外，許多預防糖尿病的方法都被證實具有

預防 AD 的作用，同時糖尿病用藥也確實有減緩 AD 的效用，因此近年來，有些學者已提出新的說法，認為 AD 應屬於第三型糖尿病，其病因乃是腦中的 insulin receptor downstream signaling 受到阻礙而使然。已知腦內的 insulin signaling pathway 會調節大腦認知功能，包括學習和記憶等等。在人類和動物實驗都已證實 insulin 有增進記憶的作用。例如，給予被動逃避訓練經驗後(passive avoidance training experience)，將 insulin 注入大鼠的第三腦室內，會有較佳的記憶存留(memory retention) 。此外，insulin signaling 和突觸可塑性(synaptic plasticity)及短期記憶形成有關，但確實的分子機制仍不清楚。在腦部中，突觸也是發生 insulin signaling pathway 的重要場所，因此，評估 insulin signaling 的效率可能可以作為神經元促進 synaptic plasticity 與 LTP，進而增進短期記憶有正面的關係。當 insulin 與其 receptor 連結後，會和 insulin receptor substrate (IRS)組成複合體，並促使 phosphatidylinositol 3-kinase (PI3K)磷酸化，進而活化 serine/threonine kinase Akt 以及其下游包括 GSK3β 及 Foxo3a 等蛋白相關的細胞生理反應。近年來的研究指出，insulin 可能在 AD 的病程中扮演著較為重要的角色，特別是與細胞老化過程之間的關係十分密切。眾所皆知，罹患 AD 最大的危險因子便是老化本身，因此若能釐清 insulin signaling 在神經細胞中的生理機制，並深入探討抑制 DPP-4 活性如何改善 Aβ 所產生的神經毒性機轉，將有助益於更深入瞭解 AD 的病程與老化之間的關聯，並有機會藉此在未來開發出新型態的 AD 治療策略。

研究目的(Specific Aims)

胰島素阻抗是引發代謝疾患的重要危險因子。高血糖會增加周邊系統的胰島素使用率，並減少腦部胰島素的運送。胰島素與類胰島素生長因子調控生理系統中神經存活與長壽等關鍵程序，中樞神經系統的學習與記憶功能也受其掌控。由神經發展的觀點來看，胰島素訊息傳遞(Insulin signaling pathway)可能控制著神經退化的關鍵因子。阿茲海默症是神經退化疾病中最常見的疾患，這是一個由膽鹼神經元逐漸失能導致病人開始出現嚴重的行為、運動與認知功能障礙的疾病，最終將導致身體功能逐漸衰弱的神經疾患。逐漸增加的證據顯示，阿茲海默症與代謝疾患如：第二型糖尿病之間在病原學方面可能有相似的機制，如：胰島素阻抗。而由研究第二型糖尿病而發展出的藥物可能可以緩和阿茲海默症的病程發展，並且維持其認知功能。DPP-4 抑制劑是新一代發展出的第二型糖尿病用藥，它的主要功能是增強腸道內生性荷爾蒙 GLP-1 的功能，由此促進胰島素的分泌。但是，抑制 DPP-4 酵素的活性對中樞神經系統的影響機制則尚未有進一步的探討。

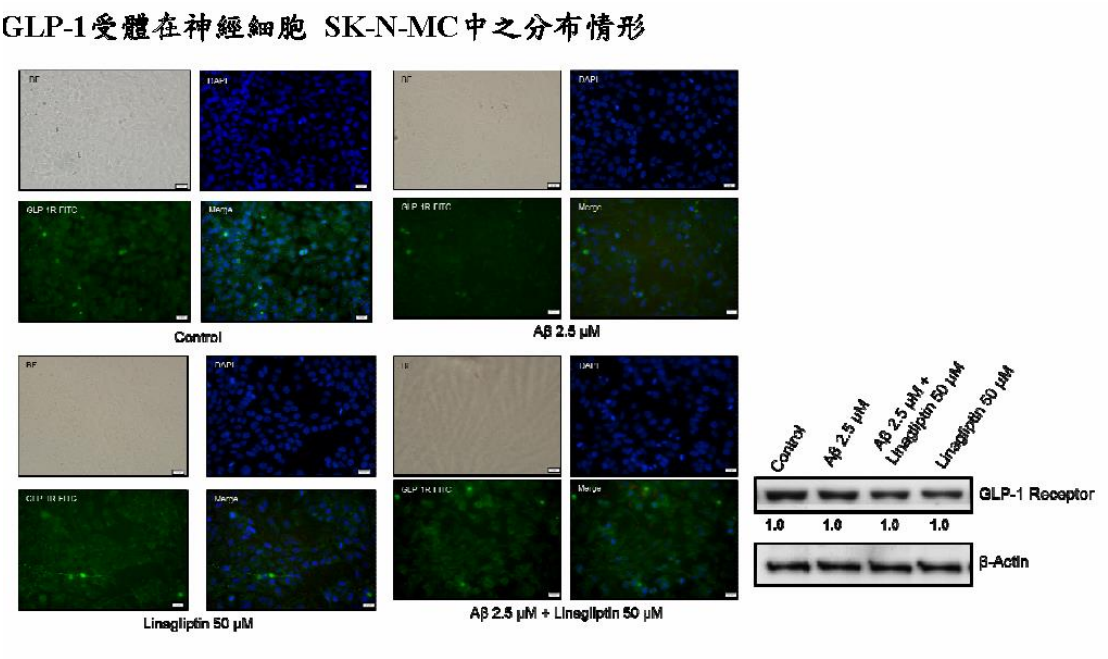
研究方法及架構(Methods and Designs)

在本研究中，我們測試了 DPP-4 抑制劑：Linagliptin 在神經元細胞中的功能，並進一步了解其對神經退化性疾病是否有正向影響。我們使用細胞存活率測定 DPP-4 抑制劑：Linagliptin 是否減少乙型類澱粉蛋白(amyloid β, Aβ)在神經元細胞

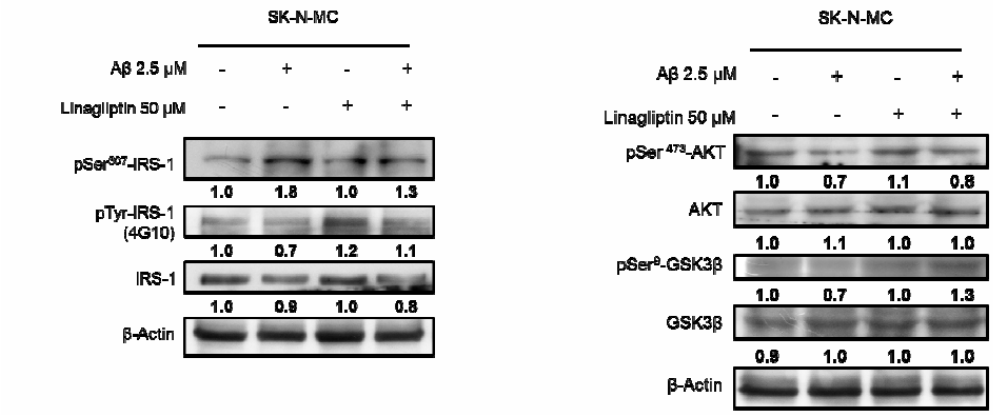
SK-N-MC 中造成的傷害，然後使用免疫螢光染色 (Immunofluorescence staining assay)偵測 SK-N-MC 中 GLP-1 receptor 的表現情形。以 Western Blot 偵測 DPP-4 抑制劑：Linagliptin 在神經元細胞中對胰島素訊息傳遞路徑相關蛋白的影響，以免疫螢光染色測試 DPP-4 抑制劑：Linagliptin 對自噬小體 (Autophagosome)與粒線體 (Mitochondria)的影響，最後以 Western Blot 偵測自噬作用(Autophagy)與 SOD-1 等蛋白表現的影響。

結果(Results)

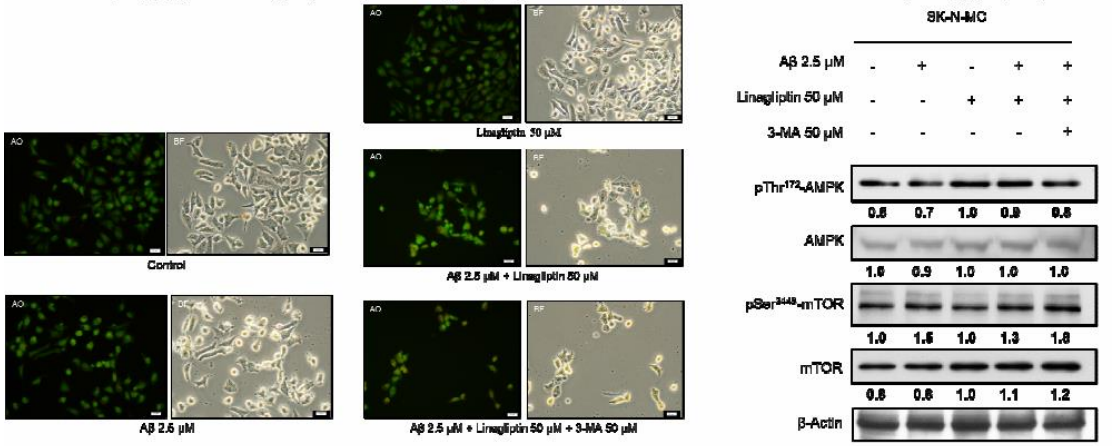
GLP-1受體在神經細胞 SK-N-MC中之分布情形



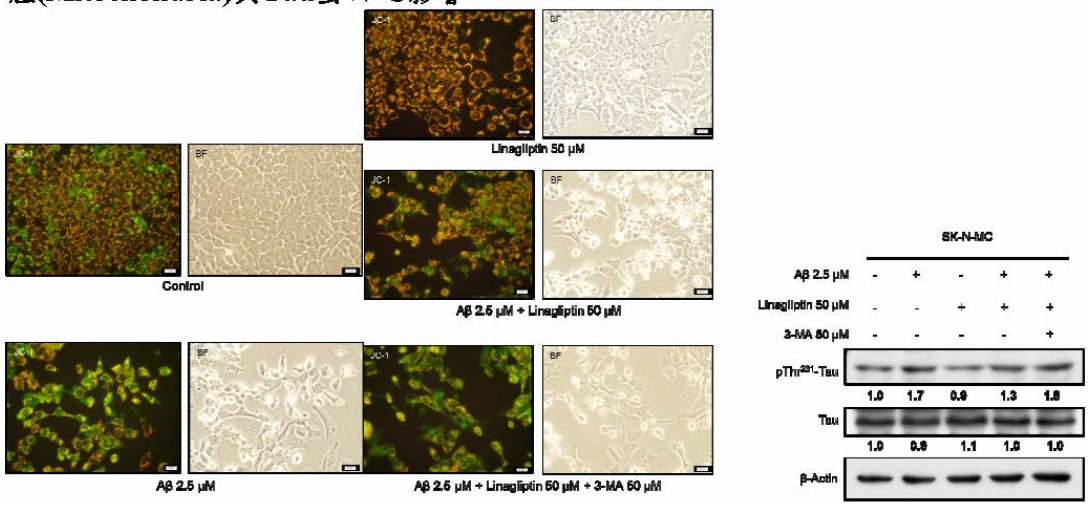
DPP-4抑制劑 Linagliptin可以促進神經細胞 SK-N-MC之胰島素訊息傳遞路徑功能



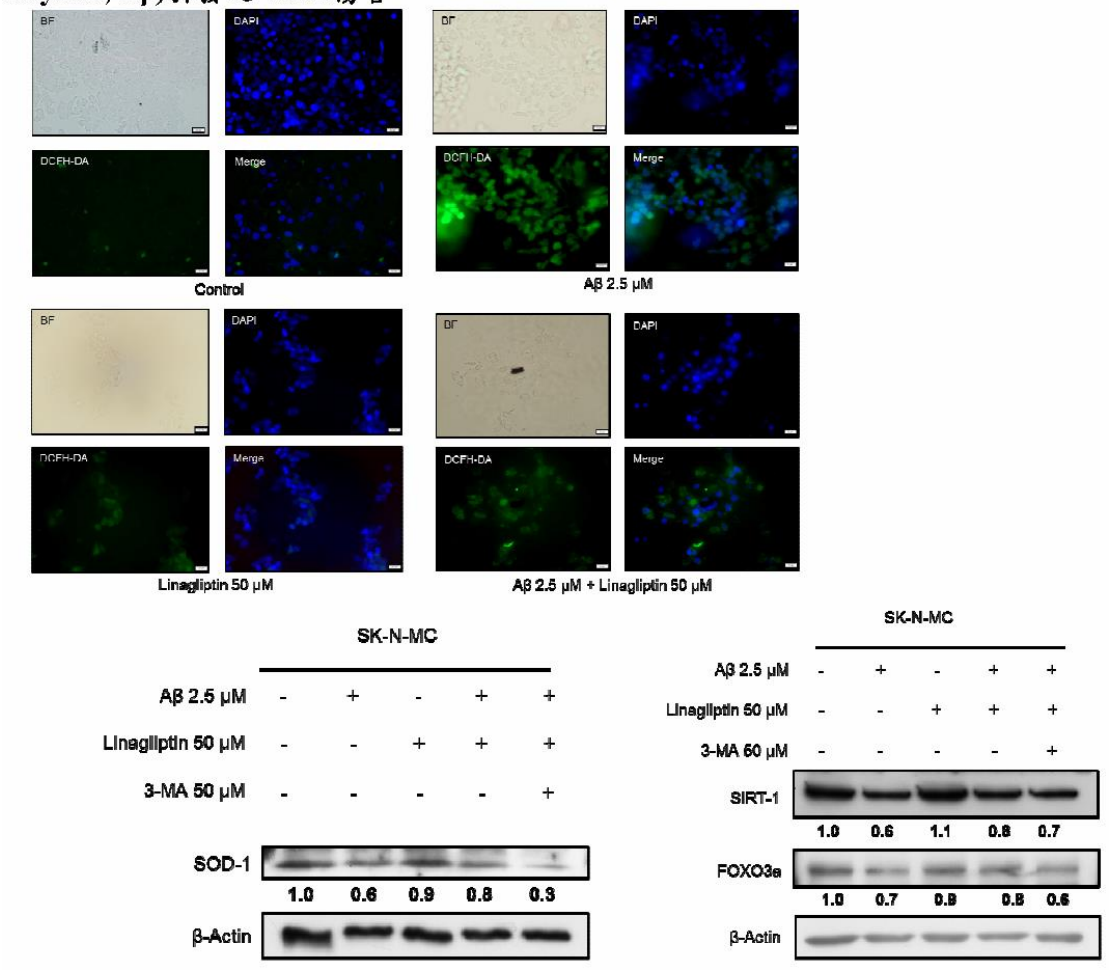
DPP-4 抑制劑 Linagliptin 可以提高神經細胞 SK-N-MC 之 Autophagy 能力



DPP-4 抑制劑 Linagliptin對於阿茲海默症 (Alzheimer's disease, AD) marker：粒線體(Mitochondria)與Tau蛋白之影響



DPP-4抑制劑 Linagliptin可以減少神經細胞 SK-N-MC受到乙型類澱粉胜肽 (β-amyloid, Aβ)引發之 ROS傷害



討論(Discussion)

根據本研究中對於 DPP-4 抑制劑 Linagliptin 在神經細胞 SK-N-MC 中所發現的功能，我們提出了簡單的模式圖做為參考。整體來說，DPP-4 抑制劑 Linagliptin 可以透過改善胰島素訊息傳遞的功能活化 Akt 訊息傳遞，使細胞得已存活。另外，DPP-4 抑制劑 Linagliptin 也可以透過提高細胞自噬能力、穩定細胞結構等方式維持神經細胞功能正常，透過活化 SIRT-1 與減少 ROS 表現減緩 Aβ 對神經細胞的傷害，由於 Aβ 是造成阿茲海默症的主要影響因子，而 DPP-4 抑制劑 Linagliptin 可以減少 Aβ 對細胞的傷害，但由於 Aβ 的病理機制牽扯複雜，要進一步了解其中的相互關聯還需要進一步的實驗做釐清。

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附錄：本計畫研究成果代表著作之抽印本

Kornelius E, Lin CL, Chang HH, Li HH, Huang WN, Yang YS, Lu YL, Peng CH, Huang CN*. DPP-4 inhibitor linagliptin attenuates Aβ-induced cytotoxicity through activation of AMPK in neuronal cells. *CNS Neurosci. Ther.* ***21****:549-57, 2015.* (SCI) (IF=3.931, Ranking=43/254; **16.9%** of PHARMACOLOGY & PHARMACY)

CNS Neuroscience & Therapeutics

ORIGINAL ARTICLE

CNS Neuroscience & Therapeutics

DPP-4 Inhibitor Linagliptin Attenuates Aβ-induced Cytotoxicity through Activation of AMPK in Neuronal Cells

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Keywords
Alzheimer's disease; AMP-activated protein kinase; Amyloid-β; Linagliptin; Sirtuin 1.

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SUMMARY

Aim: It is now clear that insulin signaling has important roles in regulation of neuronal functions in the brain. Dysregulation of brain insulin signaling has been linked to neurodegenerative disease, particularly Alzheimer's disease (AD). In this regard, there is evidence that improvement of neuronal insulin signaling has neuroprotective activity against amyloid β (Aβ)-induced neurotoxicity for patients with AD. Linagliptin is an inhibitor of dipeptidylpeptidase-4 (DPP-4), which improves impaired insulin secretion and insulin downstream signaling in the in peripheral tissues. However, whether the protective effects of linagliptin involved in Aβ-mediated neurotoxicity have not yet been investigated. **Methods:** In the present study, we evaluated the mechanisms by which linagliptin protects against Aβ-induced impaired insulin signaling and cytotoxicity in cultured SK-N-MC human neuronal cells. **Results:** Our results showed that Aβ impairs insulin signaling and causes cell death. However, linagliptin significantly protected against Aβ-induced cytotoxicity, and prevented the activation of glycogen synthase kinase 3β (GSK3β) and tau hyperphosphorylation by restoring insulin downstream signaling. Furthermore, linagliptin alleviated Aβ-induced mitochondrial dysfunction and intracellular ROS generation, which may be due to the activation of 5′ AMP-activated protein kinase (AMPK)-Sirt1 signaling. This upregulation of Sirt1 expression was also observed in diabetic patients with AD coadministration of linagliptin. **Conclusions:** Taken together, our findings suggest linagliptin can restore the impaired insulin signaling caused by Aβ in neuronal cells, suggesting DPP-4 inhibitors may have therapeutic potential for reducing Aβ-induced impairment of insulin signaling and neurotoxicity in AD pathogenesis.

Introduction

Impaired insulin signaling is a physiological condition that cells lost the ability to respond to insulin. This failure to respond is called insulin resistance and plays a central in the development of the metabolic disorders such as diabetes, obesity, hypertension, and dyslipidemia. Particularly, defective brain insulin signaling has been implicated in the development of Alzheimer's disease (AD), the most common cause of dementia [1]. In fact, abnormal insulin sensitivity is shown to be associated with AD-related pathological features. For example, type 2 diabetes (T2D) is identified as a major risk factor for AD, suggesting defective insulin signaling may account for pathogenesis of neurodegeneration [2]. Moreover, patients with AD show significantly reduced expression of insulin receptors and insulin receptor substrate (IRS) in the brain that contributes to the severity of cognitive impairment [3]. All these findings suggest that neuronal insulin signaling is altered in the AD brain resembling T2D [4]. Actually, AD pathogenesis is initially triggered by the presence of extracellular amyloid-β (Aβ) proteins, which are found to cause oxidative stress and neurotoxicity in the brain [5]. It is well known that insulin and its receptors are widely expressed in neurons and glial cells throughout the brain [6], and evidence is also presented that insulin can be produced locally within the brain [7]. In addition, Aβ has been reported to impair synaptic insulin sensitivity in cultured neurons, which may impair synaptic functions associated with pathogenesis of AD [8]. This indicates insulin signaling may serve as an important regulatory role in neurons. However, the molecular basis that links between insulin signaling and Aβ-induced neurotoxicity remains unclear.

At a molecular level, the serine phosphorylation of insulin receptor substrate (IRS) can block the insulin signaling. This results in the inhibition of IRS tyrosine phosphorylation, suppressing the downstream phosphatidylinositol 3-kinase (PI3-kinase)

signaling and subsequent inactivation of the kinase Akt [9]. It is known that activated Akt inactivates glycogen synthase kinase 3β (GSK3β) by phosphorylating its Ser^9 residue, which is one of the important enzymes induce tau hyperphosphorylation and neurotoxicity [10]. For this reason, impairment of insulin signaling may result in a high activity of GSK3β, which leads to an enhanced tau hyperphosphorylation, a crucial step in AD pathogenesis. Hyperphosphorylated tau and Aβ cooperatively impair mitochondrial membrane potential and further increase in accumulation of intracellular reactive oxygen species (ROS), which ultimately result in neurodegeneration [11]. Therefore, it is not surprising that pharmaceuticals found to be effective treatment of impaired insulin signaling have also shown benefits in the prevention or reduction of AD [12].

The enzyme dipeptidyl peptidase-4 (DPP-4) is a ubiquitous membrane-bound prolyl peptidase that was responsible for the degradation of incretin hormones [13]. Incretins are a group of gut-derived hormones that potentiate insulin secretion and related cellular signaling [14]. However, incretins are rapidly metabolized and inactivated by DPP-4. As a result, DPP-4 inhibitors such as linagliptin have a relevant effect of increasing the half-life in retaining the physiological effects of endogenous incretins [15]. Interestingly, it has been recently shown that incretins may be good candidates for treating AD [16]. For example, glucagon-like peptide-1 (GLP-1), the major incretin in humans, has been shown to elicit neuroprotective properties against AD pathological processes [17]. Similar to insulin, GLP-1 is produced in the brain mediating many neuronal functions, including neuroprotection, improvement of learning and memory ability, and potentiation of insulin signaling [18]. Therefore, GLP-1 signaling have demonstrated the potential to serve as therapeutic or preventive strategies against diabetes-related AD [19]. As DPP-4 inhibitor effectively increases GLP-1 levels, it may also exert protective effects against AD-related Aβ-induced neurotoxicity. Linagliptin is a recently approved DPP-4 inhibitor and widely considered as the first-line treatment for T2D patients. It has been demonstrated greater inhibitory effects than other DPP-4 inhibitors such as alogliptin, saxagliptin, sitagliptin, or vildagliptin [20]. Moreover, linagliptin also significantly improves insulin secretory dysfunction and sensitivity in animal studies [21]. This indicates linagliptin may have beneficial effects on impaired insulin signaling in neuronal cells. However, whether linagliptin is involved in Aβ-induced neurotoxicity is still largely unknown. In this study, we postulated that neuronal insulin resistance may be one of the underlying neurotoxic mechanisms by Aβ, whereas linagliptin can protect neuronal cells by restoring impaired insulin signaling, and thereby contribute to the alleviation of Aβ-induced neurotoxicity.

Materials and Methods

Materials

Chemicals such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), and JC-1 were purchased from Sigma (München, Germany). Amyloid-β (Aβ) 1-42 was acquired from AnaSpec Inc. (San Jose, CA, USA). We purchased antibodies against Akt, p-Akt, GSK3β, p-GSK3β and IRS-1, caspase 3, SOD1, and poly(ADP-ribose) polymerase (PARP) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Sirt1 antibody from GeneTex (Irvine, CA, USA), β-actin antibody from Novus Biologicals (Littleton, CO, USA), and p-IRS-1 antibodies from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies were used at a dilution of 1:1000 in 0.1% Tween-20 and secondary antibodies were used at 1:5000 dilutions. Pure linagliptin was provided by Boehringer Ingelheim Pharmaceuticals (Biberach, Germany). All the chemicals were prepared by dissolving phosphate buffer saline solutions stored at −20°C until needed for use in experiments.

Cell Culture and Viability Assay

Human neuroblastoma SK-N-MC cells were obtained from the American Type Culture Collection (Bethesda, MD, USA). Cells were maintained in minimal Eagle's medium (MEM; Gibco, Carlsbad, CA, USA), supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37°C, 5% CO_2. The Aβ solutions were prepared as described previously [22]. Briefly, $A\beta_{1\text{-}42}$ lyophilizates were dissolved at 10 mM in 10% 60 mM NaOH and 90% 10 mM phosphate buffer (pH 7.4) as a stock reagent, and stored at −78°C until use. For viability assay, cells were seeded in 96-welled plates at a density of 1×10^4 cells/well overnight and then treated as indicated. After 24 h, the tetrazolium salt MTT was added to the medium following the manufacturer's instructions. Only viable cells could metabolize MTT into a purple formazan product, of which the color density (OD) was further quantified by an EZ Read 400 microplate reader (Biochrom, Holliston, MA, USA) at 550 nm. Cell viability was determined by the percentage of OD of the treated cells divided that of the untreated controls.

mRNA Expression Analysis by Reverse Transcription Quantitative PCR

Total mRNA was extracted from the samples after treatment for the indicated conditions by utilizing the kit Qiagen RNeasy Kit (Qiagen, Germantown, MD, USA), and was quantified spectrophotometrically. RNA reverse transcription was performed at 25°C for 10 m for primer binding, 37°C for 120 min for reverse transcriptase, and 85°C for reverse transcriptase denaturation using the TProfessional Thermocycler (Biometra). Real-time quantitative PCR (qPCR) was performed for quantification of mRNA by using an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR amplifications of target mRNA genes were carried out in conjunction with Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Each cDNA sample was tested in triplicate. The following temperature parameters were 95°C/10 min, 40 cycles of 95°C/15 s, 60°C/1 min and dissociation stage was 95°C/15 s, 60°C/15 s and 95°C/15 s. The following primer pairs were used, forward 5′-ACACCTGTGCGGCTCACA-3′ and reverse 5′-TCCCGGCGGGTCTTG-3′ for insulin, forward 5′-TGCTTCCGGA GCTGTGATCT-3′ and reverse 5′- CGGACAGAGCGAGCTGACTT-3′ for IGF-1, forward 5′- ATTGCTTGGCTGGTGAAAGG-3′ and reverse 5′- TGTCTGCGGCCAAGTTCTTC-3′ for proglucagon, and forward 5′-TGGTATCGTGGAAGGACTCATGAC-3′ and reverse 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′ for GAPDH. Values of

relative mRNA expression were obtained by using the software SDS (Sequence Detection Systems 7300 Real Time PCR System; Applied Biosystems), and the values were standardized by comparing with values from relative expression of GAPDH.

Western Blot Analysis

After treatment, cells were harvested and homogenized in a protein extraction lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.5% Nonidet P-40; 0.5 mM phenylmethylsulfonyl fluoride; and 0.5 mM dithiothreitol), and centrifuged at 12,000 g for 30 min at 4°C. The supernatants were used as cell extracts for immunoblotting analysis. SDS-solubilized samples were then loaded onto SDS-polyacrylamide gels. Equal protein amounts of total cell lysates were resolved by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and then probed with a primary antibody followed by a secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Millipore). The relative expression of proteins was quantified densitometrically by using the software QuantityOne (BioRad, Hercules, CA, USA) and was calculated according to the reference bands of β-actin. Each blot represents at least in three independent experiments.

Microscopic Observation and Nucleus Morphology

Changes in cell nucleus morphology, characteristic of apoptosis, were examined in cells grown on coverslips, using a microscope. The cells were fixed in 4% paraformaldehyde after 24 h of treatment with the indicated compounds. For phase-contrast inverted microscopy, images of cells were captured with no specific staining procedure. For nucleus morphology microscopy, cells were fixed in ice-cold methanol, and incubated for 15 min at room temperature with 1 ng/mL of 4′,6-diamidino-2-phenylindole (DAPI) stain, and observed under a fluorescence microscope (DP80/BX53; Olympus, Tokyo, Japan). Apoptosis was quantified by averaging cell counts in twenty random 400× fields. Values were expressed as the percentage of apoptotic cells relative to total number of cells.

Measurement of Reactive Oxygen Species

To evaluate the levels of intracellular ROS, cells were seeded onto glass coverslips and incubated with 10 μM of 2′, 7′-dichlorodihydrofluorescin diacetate (DCFH-DA, a general oxidative stress indicator) for 0.5 h at 37°C under 5% CO_2 after treatment. After incubation, the staining medium was discarded and cells were washed twice with immediately with PBS, after which the intensity of fluorescence was imaged by a fluorescence microscopy (DP72/CKX41; Olympus) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. One representative image of three different experiments is shown.

Analysis of Mitochondrial Membrane Potential

The vital mitochondrial cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria, was used to investigate mitochondrial function. Cells were treated in fresh medium containing 1 μM JC-1 and were incubated at 37°C for 30 min. The staining medium was then discarded and the cells were washed. Cells then imaged using an inverted fluorescence microscope (DP72/CKX41; Olympus) excited at 488 nm. In normal cells, JC-1 continues to exist as aggregates and produces a red fluorescence (~590 nm). During the induction of apoptosis, the mitochondrial potential collapses and JC-1 forms a monomer producing green fluorescence (~525 nm).

Blood Samples from Patients

A total of 14 Chinese patients with non-diabetic AD or diabetic AD were recruited from Chung Shan Medical University Hospital, Taichung, Taiwan. T2D was diagnosed according to the 1985 World Health Organization criteria using diagnostic values of fasting plasma glucose ≥7.0 mmol/L, and/or 2 h plasma glucose ≥11.1 mmol/L with or without 75 g oral glucose tolerance test, depending on the presence or absence of symptoms. AD was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria. From each subject, 20 mL of venous peripheral ethylenediamine tetra-acetic acid (EDTA) blood was obtained, and total RNA was isolated by utilizing the kit Qiagen RNeasy Kit (Qiagen) which was quantified spectrophotometrically in accordance with the manufacturer's instructions. The aforementioned protocol was approved by the Chung Shan Medical University Hospital Institutional Review Board (IRB) protocols (CSMUH No: CS13233). Informed consent was obtained from all participants according to the Declaration of Helsinki and was approved by the IRB.

Statistical Analysis

All data are presented as means ± standard error of the means (SEM). Statistical analysis of data was performed using analysis of variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons with SPSS statistical software (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

Results

Effects of Linagliptin on Viability of SK-N-MC Neuronal Cells

The influence of DPP-4 inhibitor linagliptin treated in neuronal cells is largely unknown. To evaluate the effects of linagliptin on cell viability, SK-N-MC neuronal cells were exposed to 10 to 100 μM of linagliptin for 24 h, and the cytotoxic effects were determined by MTT assay (Figure 1A). The results showed that linagliptin concentrations ranging from 10 to 50 μM did not induce significant cytotoxicity, whereas treatment with 100 μM linagliptin slightly reduced cell viability. In accordance, treated with 50 μM of linagliptin displays no significant time-dependent change within a 48-h period (Figure 1B). This indicates no detectable toxic effect is present at a concentration <50 μM of linagliptin. Thus, in subsequent experiments, we investigated the mode of action of linagliptin at a concentration of 50 μM. It is known

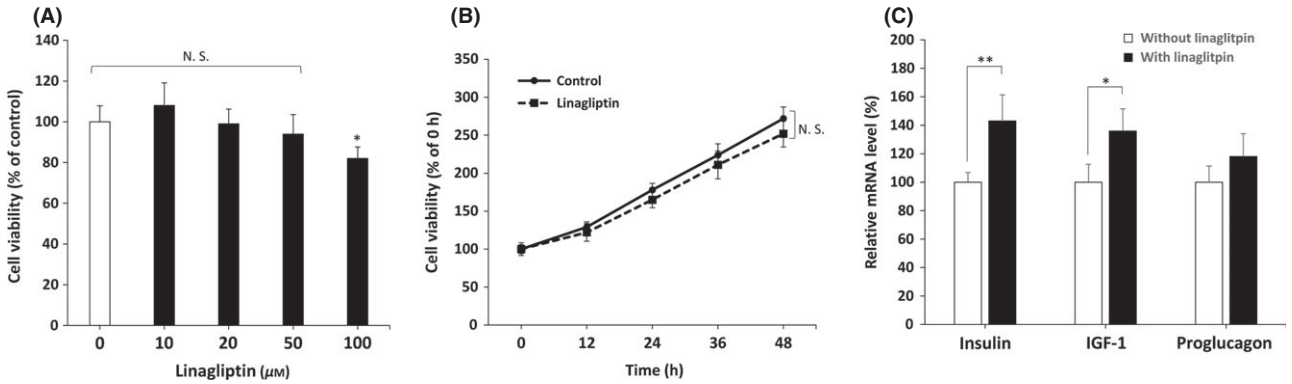

Figure 1 Effects of linagliptin on cell viability and incretin-related mRNA expression in SK-N-MC neuronal cells. (**A**) Dose effects of linagliptin on SK-N-MC cells by MTT assay. Linagliptin shows no significant cytotoxicity <50 μM. (**B**) 50 μM of linagliptin causes no significant alteration of cell viability within a 48-h period. (**C**) SK-N-MC cells are treated with or without 50 μM of linagliptin for 24 h. The mRNA levels of incretin-related target genes including insulin, insulin-like growth factor-1 (IGF-1), and glucagon-like peptide 1 (GLP-1) are measured by using real-time qPCR, and the results are presented as means ± standard error of the means (SEM) of three independent experiments. $*P < 0.05$ and $**P < 0.01$ by using multiple comparisons of Dunnett's post-hoc test. N. S., no significant difference.

that the main action of linagliptin is to stimulate insulin action by the incretin hormones such as glucagon-like peptide 1 (GLP-1). To determine the effects of linagliptin on insulin signaling-related gene expression in SK-N-MC cells, we performed relative expression qPCR assays to measure levels of mRNA transcripts. As shown in Figure 1C, adding linagliptin to SK-N-MC cells resulted in upregulation of insulin and insulin-like growth factor-1 (IGF-1) but no significant effects in proglucagon (the pro-hormonal precursor mRNA of GLP-1), indicating linagliptin may exert its pharmacological action by stimulating insulin/IGF-related signaling to neuronal cells.

Linagliptin Protects SK-N-MC Cells Against Aβ-induced Neurotoxicity

Recent studies have demonstrated the DPP-4 inhibitory properties indicating neuroprotective effects against AD pathological hallmarks such as Aβ accumulation, tau phosphorylation and neuroinflammation [23]. To assess whether linagliptin exerts these similar beneficial effects, cell viability assay was conducted to determine the neuroprotective effect of linagliptin on Aβ-induced cell death. As shown in Figure 2A, incubation of SK-N-MC cells with 2.5 μM of Aβ for 24 h markedly underwent a ~50% decrease of MTT reduction. However, linagliptin alleviated cell death at concentrations ranging from 10 to 50 μM of SK-N-MC cells in a dose-dependent manner during Aβ treatment. To precisely determine which mode of cell death is induced by Aβ, we examined the expressions of cleaved caspase 3 and poly (ADP-ribose) polymerase (PARP), two typical markers of apoptosis by western blotting. As shown in Figure 2B, Aβ markedly increased cleavage of caspase 3 and PARP, indicating the enhanced apoptosis occurs mainly in Aβ treatment. On the contrary, co-treated with linagliptin was shown to effectively inhibit caspase 3 and PARP activation by Aβ. These results were also confirmed by DAPI staining that treatment with linagliptin significantly reduced nuclei fragmentation as shown in Figure 2C. Taken together, this support the idea that the addition of linagliptin may effectively attenuate Aβ-induced apoptosis in neuronal cells.

Linagliptin Restores Aβ-induced Insulin Signaling Blockade in Neuronal Cells

Because the mechanism of DPP-4 inhibitors is to increase endogenous incretin levels, we proposed that linagliptin might exert neuroprotective effects by enhancing GLP-1 downstream signaling. To determine whether linagliptin-mediated neuroprotection operates via alteration of GLP-1 receptor expression in the SK-N-MC cells, we performed immunocytochemical staining. As shown in Figure 3A, both Aβ and linagliptin treatment did not markedly alter the cellular distribution and expression of GLP-1 receptor in SK-N-MC cells. Similarly, the constant expression of GLP-1 receptor was also confirmed by western blotting (Figure 3B). Several lines of evidence have indicated that linagliptin can enhance insulin action and plays an important role in improving insulin sensitivity in peripheral tissues [24]. To further elucidate the molecular mechanism of linagliptin-mediated neuroprotection, Western blot analysis was conducted to detect the levels of phospho insulin receptor substrate-1 (IRS-1) at residue Ser^{307} and Tyr in SK-N-MC cells. As shown in Figure 3C, Aβ treatment for 24 h caused a significant increase in Ser^{307} IRS-1 phosphorylation, which is recognized as a hallmark of insulin resistance. Accordingly, Aβ also prevented IRS-1 phosphotyrosine (4G10 clone) expression. By contrast, co-treated with linagliptin returned the Tyr phosphorylation of IRS-1 and its downstream target Akt to basal levels, showing that the neuronal insulin signaling can be activated by linagliptin during Aβ treatment. To gain insight into the downstream effects of Akt in the presence of linagliptin, we investigated the glycogen synthase kinase 3β (GSK3β), a direct phosphorylation target of Akt. After 24 h of Aβ treatment, the Ser^{9} phosphorylation of GSK3β was markedly inhibited, which indicates the Aβ-suppressed Akt pathway leads to activation of GSK3β (Figure 3D). However, linagliptin could reduce GSK3β activity by increasing Akt-mediated GSK3β Ser^{9} phosphorylation during Aβ treatment. This linagliptin-mediated neuroprotection was also confirmed by inhibiting Thr^{231} phosphorylation of one GSK3β's downstream substrate tau, which was recognized as one of the crucial pathological hallmarks of AD. To further investigate the role of insulin

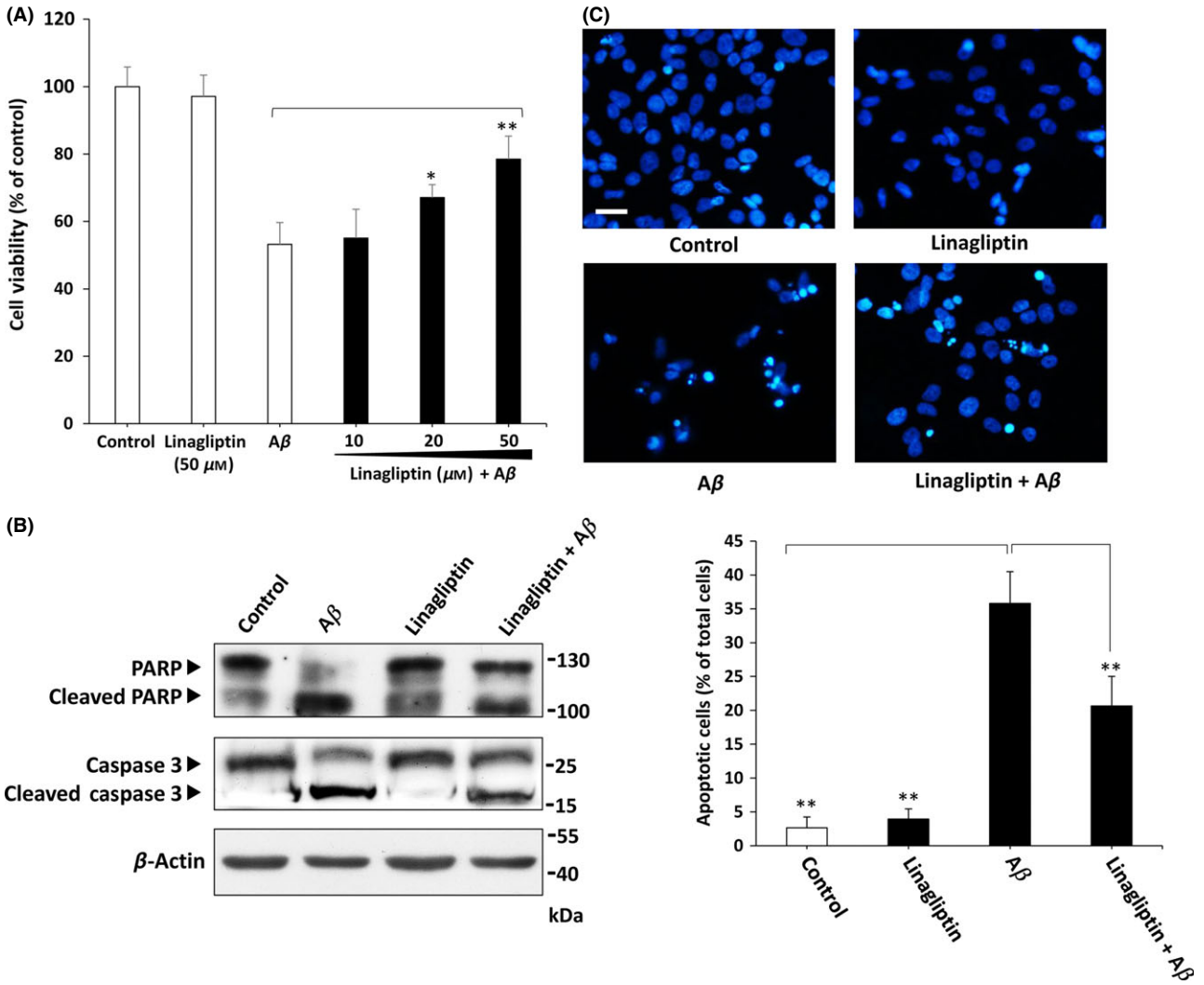

Figure 2 Linagliptin protects against Aβ-induced SK-N-MC cell death. (**A**) MTT assays indicate 2.5 μM of Aβ markedly induces cell death after 24 h of incubation. However, linagliptin significantly prevents Aβ-induced neurotoxic effects in a dose-dependent manner. (**B**) Western blotting results demonstrate that linagliptin (50 μM) treatment suppresses both caspase 3 and PARP activation induced by Aβ (2.5 μM). (**C**) Linagliptin (50 μM) markedly reduces 2.5 μM of Aβ-induced nucleus fragmentation. Apoptosis is determined by fragmented morphology in the nucleus for DAPI fluorescence. The numbers of apoptotic cells are quantified by averaging cell counts in twenty random 400× fields. Other data were performed in three independent experiments, and values are presented as mean ± SEM. Significant differences was determined by using the multiple comparisons of Dunnett's post-hoc test for $*P < 0.05$ and $**P < 0.01$ compared to Aβ only groups. Scale bar represents 50 μm.

signaling in linagliptin-mediated neuroprotection, the PI3-kinase inhibitor LY294002 was used as negative control. As shown is Figure 3D and 3E, LY294002 significantly blocked the linagliptin-restored Akt signaling and cell viability during Aβ treatment. This indicates that Aβ-impaired insulin signaling may trigger neuronal apoptosis; however, linagliptin effectively reduces Aβ-induced cytotoxicity by returning the blocked neuronal insulin signaling.

Linagliptin Protects Cells Against Aβ-Induced Intracellular ROS Accumulation and Mitochondria Dysfunction

Previous studies show strong evidence that Aβ-induced ROS accumulation and mitochondrial dysfunction are both potential pathogenic markers in AD [25]. To determine whether linagliptin protects cells from Aβ-induced oxidative stress, we measured intracellular ROS levels by a 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluorometric method. As expected, our results showed that linagliptin suppresses Aβ-induced ROS intracellular accumulation, which in turn protects cells from oxidative stress (Figure 4A). It is also suggested that increased ROS levels may inhibit AMP-activated protein kinase (AMPK) activity, which is likely to promote the development of insulin resistance [26]. To test whether such mechanism is also involved in SK-N-MC cells, the phosphorylation of AMPK was determined by immunoblotting in Figure 4B. Our results showed that Aβ significantly down-regulates the Thr^{172} phosphorylation of AMPK, whereas this effect was counteracted by co-treatment of linagliptin. Further-

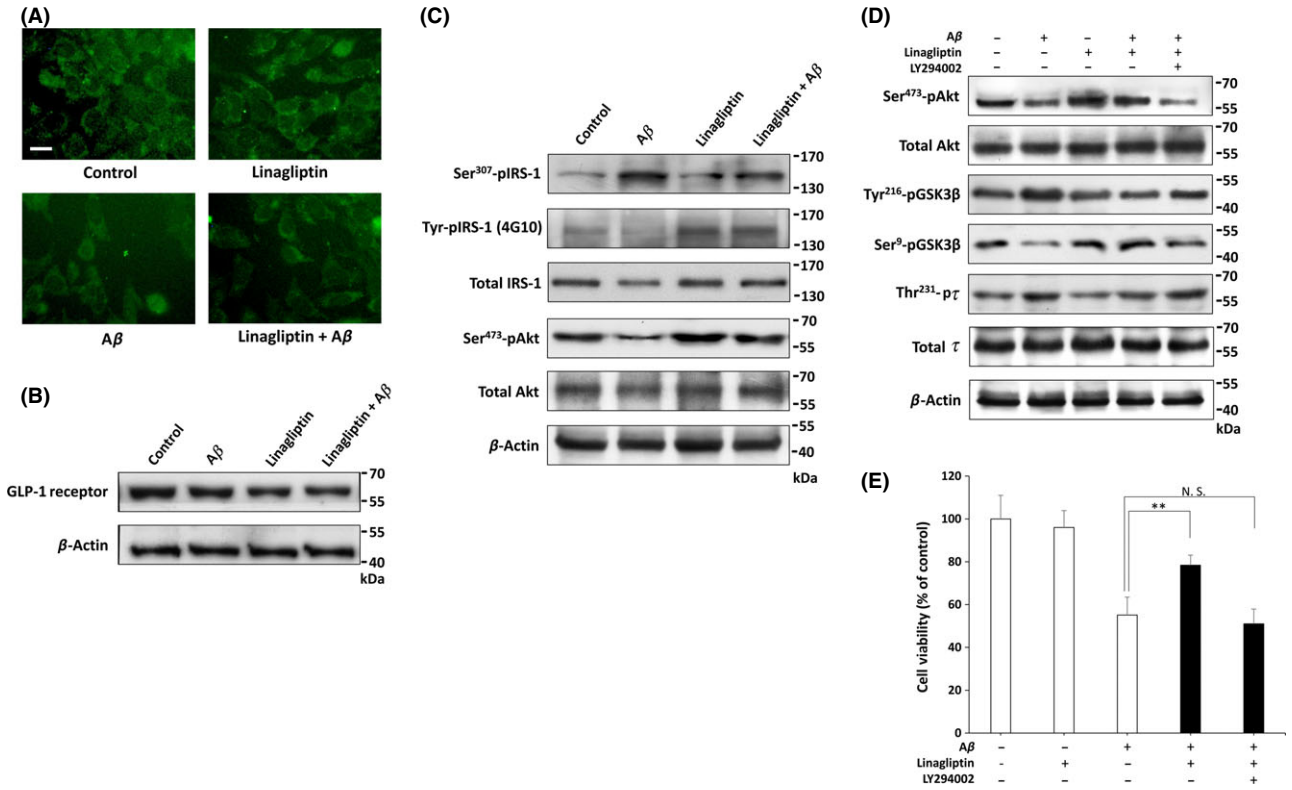

Figure 3 Linagliptin alleviates Aβ-impaired insulin downstream signaling in SK-N-MC neuronal cells. (**A**) Immunofluorescence images show that the cellular distribution of GLP-1 receptor is not altered by treatment with Aβ (2.5 μM), linagliptin (50 μM) or in combination for 24 h. (**B**) Western blotting also reveals that the expression of GLP-1 receptor is not altered by Aβ (2.5 μM) or linagliptin (50 μM) treatment for 24 h in SK-N-MC cells. (**C**) Immunoblotting reveals that phosphorylation of Tyr-IRS-1 and Ser^{473}-Akt are inhibited when cells are exposed to Aβ (2.5 μM) for 24 h, and this inhibition is effectively restored by linagliptin (50 μM). (**D**) Western blotting shows that 50 μM of linagliptin-activated Akt leads to the Ser^{9} phosphorylation of GSK3β, resulting in the inhibition of tau Thr^{231} phosphorylation by Aβ (2.5 μM) for 24 h. (**E**) Cell viability is determined by MTT assay, and the linagliptin-mediated neuroprotective effects are abolished by the co-treatment of LY294002 (20 μM), a specific inhibitor of PI3-kinase. All data were performed in three independent experiments, and values are presented as mean ± SEM. Significant differences was determined by using the multiple comparisons of Dunnett's post-hoc test for $*P < 0.05$ and $**P < 0.01$. Scale bar represents 20 μm.

more, western blot analysis of AMPK downstream target sirtuin 1 (Sirt1) and superoxide dismutase 1 (SOD1) protein expressions also provided evidence that levels of these antioxidative pathways are increased significantly by linagliptin compared to Aβ only groups. As discussed previously, the pathogenic role of Aβ in mediating ROS accumulation was often accompanied by mitochondrial dysfunction. To further examine the details of linagliptin-mediated neuroprotection, we performed JC-1 staining to assess the mitochondrial membrane potential. As shown in Figure 4C, JC-1 aggregates were found in healthy mitochondria by a red fluorescence in nontreated controls. However, exposure of cells to Aβ resulted in significant increases in green fluorescence, indicating a loss of mitochondrial membrane potential. On the contrary, co-treatment with linagliptin reduced the deteriorating effects of Aβ on mitochondrial membrane potential. We further confirmed that insulin signaling inhibition results in an oxidative stress damage by Aβ, as the PI3-kinase inhibitor LY294002 significantly attenuated linagliptin-mediated antioxidative effects, suggesting these benefits may depend on linagliptin-improved insulin sensitivity in neuronal cells.

Peripheral Blood Leukocyte Sirt1 mRNA Expression is Partially Returned by Linagliptin in Diabetic Patients with AD

Sirt1 is an important modulator in humans in the protection against oxidative events. A previous study reported that the Sirt1 mRNA expression level is suppressed in blood samples obtained from patients with AD or T2D [27,28]. However, it remains unclear whether the reduction of Sirt1 mRNA is more susceptible to diabetic patients with AD. To evaluate Sirt1 inhibition in diabetic AD patients, peripheral leukocytes were isolated and Sirt1 mRNA levels were determined with fourteen human subjects with clinically diagnosed AD (six pure AD, four diabetic AD without linagliptin treatments, and four diabetic AD with linagliptin treatments at least for 6 months). A detailed overview of the patient's characteristics is summarized in Table 1. In line with our preliminary expectation, both mini-mental state examination (MMSE) scores and Sirt1 mRNA expressions were lower in patients with diabetic AD as compared to pure patients with AD. However, the MMSE scores and expressions of Sirt1 mRNA were significantly

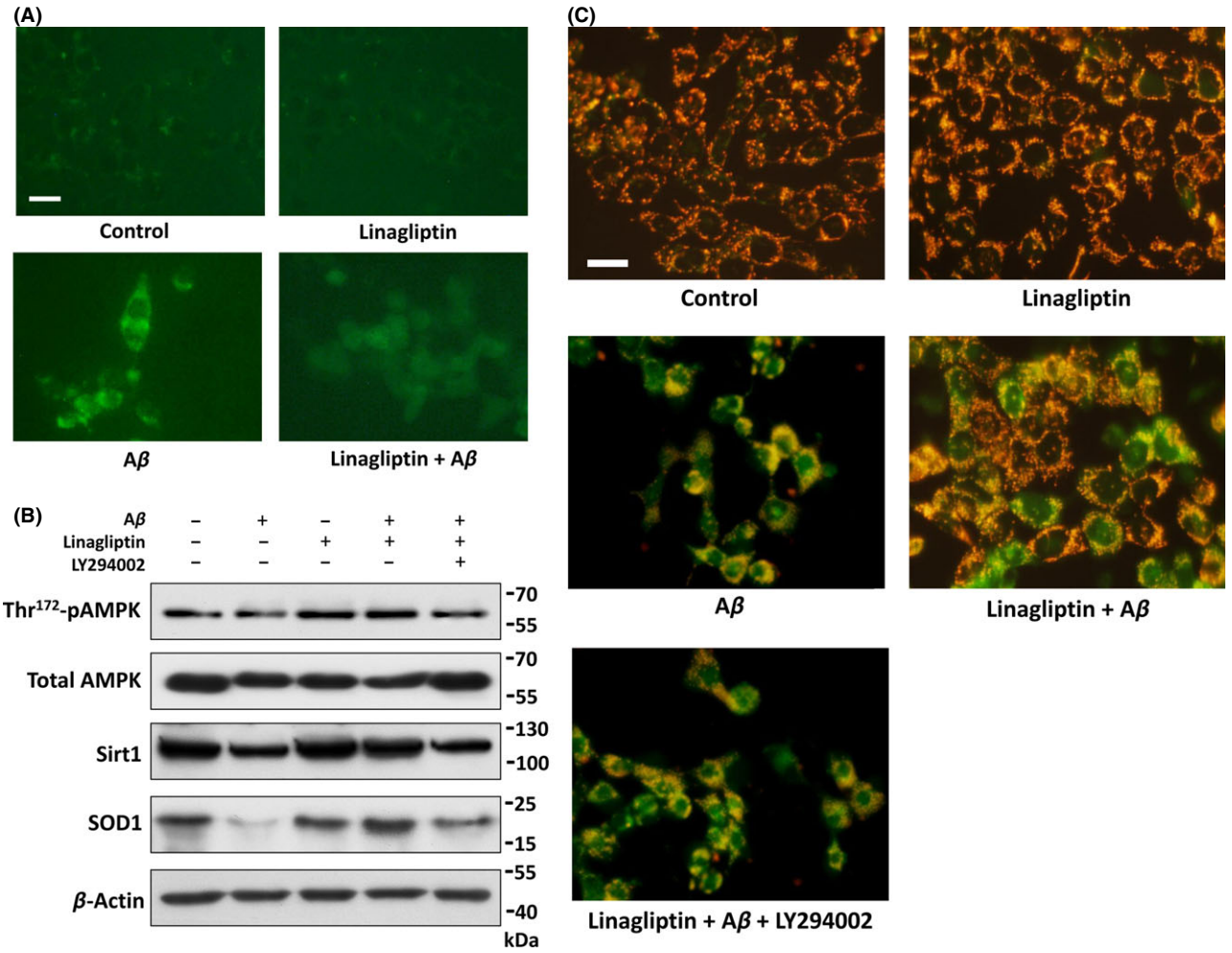

Figure 4 Linagliptin reduces Aβ-induced intracellular ROS accumulation and improves mitochondria dysfunction. (**A**) Effects of linagliptin (50 μM) in reducing 2.5 μM of Aβ-induced intracellular ROS accumulation determined by dichlorofluorescin diacetate (DCFH-DA) staining under microscope. (**B**) Effects of linagliptin (50 μM), Aβ (2.5 μM), and LY294002 (20 μM) on Thr^{172} phosphorylation of AMPK, and the protein levels of AMPK, Sirt1 and SOD1 by immunoblotting. (**C**) JC-1 immunofluorescent staining. Green fluorescence represents Aβ-induced mitochondrial dysfunction by dissipation of mitochondrial membrane potential. Red fluorescence indicates that co-treatment with linagliptin (50 μM) preserved an intact mitochondrial membrane potential compared with the group treated with Aβ (2.5 μM) alone. LY294002 (20 μM), a specific inhibitor of PI3-kinase. Scale bar represents 20 μm.

Table 1 Characteristics of patients with AD, and diabetic AD treated with or without linagliptin

Characteristics	Non-diabetic AD (n = 6)	Diabetic AD no linagliptin (n = 4)	Diabetic AD treated with linagliptin (n = 4)
Age, years	81.3 ± 5.4	83.5 ± 4.5	79.5 ± 3.4
Sex (male/female)	3/3	2/2	2/2
MMSE score	20.6 ± 2.0*	15.5 ± 3.4	20.3 ± 4.1
Sirt1 mRNA (folds)	2.6 ± 0.66*	1.0 ± 0.29	1.88 ± 0.28*

AD, Alzheimer's disease; MMSE, mini-mental status examination. Values shown are means ± SD. *$P < 0.05$ compared to diabetic AD groups.

restored in diabetic patients with AD treated with linagliptin (Table 1). This observation consists the idea that patients with diabetic AD expressed reduced Sirt1 by inhibition of incretin signaling, which may contribute to pathogenesis of neurodegeneration.

Discussion

Interestingly, accumulating evidence indicates a strong link between T2D and AD, highlighting the key role of insulin signaling in the pathogenesis of these diseases. Although the underlying mechanism remains largely unknown, now the evidence for it has become very significant [29]. In fact, Steen *et al*. have firstly proposed a connection between increased insulin resistance in the brain with AD and termed it as "type 3 diabetes" [30], hinting that insulin-based therapies may be useful in the treatment of AD. Traditionally, the majority of insulin in the brain is generated from pancreatic β-cells and transported across the blood–brain barrier (BBB). However, insulin can be locally synthesized and released by neurons [31]. Moreover, GLP-1 and its receptor are also known to ubiquitously express in central nervous system (CNS), particularly in hypothalamus, cortex and hippocampus that typically vulnerable in patients with AD [32]. Therefore, it is not surprising

that the locally produced GLP-1 may be upregulated by treatment with a DPP-4 inhibitor which stimulates insulin downstream effects related to neuronal functions. Regarding the insulin protective effects in CNS, we provided evidence that linagliptin can protect neuronal cells against Aβ-induced neurotoxicity likely by blocking DPP-4 makes GLP-1 levels rise, which increases insulin release and restores insulin signaling impairment. As brain GLP-1 has been suggested to be neuroprotective [33], it is possible that DPP-4 inhibitors such as linagliptin may represent a promising strategy against Aβ-induced neurodegeneration.

Insulin resistance and mitochondrial dysfunction are the two common features both in AD and T2D [34]. As previous mentioned, mitochondrial dysfunction leads to impairment of insulin sensitivity by reduced activity of AMPK, an important cellular fuel sensor and regulator [35]. Aβ was found to cause ROS accumulation and oxidative damage in the brain, which is believed to play a pivotal role in the development of insulin resistance [36]. Interestingly, GLP-1 has been reported to stimulate AMPK activation in preventing the ROS production and *vice versa* [37]. Additionally, AMPK activation has also been suggested to enhance insulin sensitivity by GLP-1 agonist liraglutide [38]. This indicates that AMPK may play a key role in response to Aβ exposure by DPP-4 inhibition. In accordance with these findings, we found that the Thr^{172} phosphorylation of AMPK could be reduced during the incubation of cells with Aβ, and this inhibition was prevented by linagliptin co-treatment. Moreover, we also observed that linagliptin protects mitochondrial function and suppresses intracellular ROS accumulation depends on insulin signaling pathways. These observations were further confirmed by Sirt1, a well-known longevity factor, is in fact upregulated by linagliptin. By linagliptin treatment, AMPK can trigger its downstream target Sirt1, which was reported previously in triggering antioxidant pathways such as SOD [39]. Considering the important roles of the Aβ-induced oxidative stress in AD pathogenesis, our research unveils a new neuroprotective mechanism by which linagliptin suppresses oxidative damage and preserves mitochondria function through restoration of neuronal insulin signaling.

Recently, Kosaraju *et al.* [40,41] observed that inhibition of DPP-4 ameliorates streptozotocin-induced memory loss and neuronal death in rats, indicating the possibility of using of these agents for the treating diabetes-associated AD. Their results revealed a significant improvement in a dose-dependent attenuation of Aβ production, tau hyperphosphorylation and cognitive deficits by upregulation of GLP-1 signaling. These robust therapeutic effects of DPP-4 inhibitors demonstrate a unique mechanism for Aβ-related pathology observed in AD. However, previous study has demonstrated that linagliptin does not pass through the BBB easily [42], whereas GLP-1 could be able to effectively penetrate into the brain [43]. Because linagliptin has been suggested to have direct neuroprotective effects, we postulate that linagliptin treatment may increase levels of brain blood GLP-1 and confers its neuroprotection. This is further supported by the fact that linagliptin-mediated neuroprotection occurs directly at the neuronal level because the brain expression of GLP-1 receptors is exclusively in neurons [44]. However, further evaluation is necessary to confirm the neuroprotective effect of linagliptin in patients with AD. Collectively, in the present study we provided evidence for the view that linagliptin inhibits neurotoxicity induced by Aβ. This protection appears to be associated with the insulin signaling-dependent AMPK activation and the Sirt1-elicited antioxidant pathways such as SOD1. To our knowledge, this is the first report demonstrating the AMPK-Sirt1 molecular mechanism of linagliptin against Aβ-induced insulin signaling impairment and oxidative damage. Our report therefore provides new insights that incretin-based agents such as linagliptin may be a potential useful therapeutic approach to AD.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/28

科技部補助計畫	計畫名稱: 腸泌素訊息減緩β型類澱粉蛋白導致神經毒性之分子機轉研究
	計畫主持人: 黃建寧
	計畫編號: 103-2314-B-040-011- 學門領域: 腎臟科新陳代謝及內分泌

無研發成果推廣資料

103年度專題研究計畫研究成果彙整表

計畫主持人：黃建寧	計畫編號：103-2314-B-040-011-
計畫名稱：腸泌素訊息減緩β型類澱粉蛋白導致神經毒性之分子機轉研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數（含實際已達成數）	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	CNS Neurosci. Ther. 21:549-57, 2015
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	3	2	100%	人次	
		博士生	2	1	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	2	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	1	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
其他成果（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體		無					

效益事項等，請以文字敘述填列。）	

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形：
   論文：■已發表 □未發表之文稿 □撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以100字為限）
   計畫研究成果代表著作: Kornelius E, Lin CL, Chang HH, Li HH, Huang WN, Yang YS, Lu YL, Peng CH, Huang CN*. DPP-4 inhibitor linagliptin attenuates Aβ-induced cytotoxicity through activation of AMPK in neuronal cells. CNS Neurosci. Ther. 21:549-57, 2015. (SCI) (IF=3.931, Ranking=43/254; 16.9% of PHARMACOLOGY & PHARMACY)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）
   本研究結果發現胰島素及GLP-1訊息傳遞路徑抑制與Aβ所造成之神經退化關係密切，而DPP-4抑制劑確實能有效緩解神經凋亡的情況，我們希望透此研究結果能幫助瞭解神經細胞內胰島素及GLP-1訊息傳遞的詳細分子機轉，並有助於在未來利用糖尿病相關藥物(如本計畫中所使用的DPP-4 inhibitors)，開發出更有效的AD治療策略與藥物。