行政院國家科學委員會專題研究計畫　期中進度報告

過度糖化最終產物對胰島素訊息傳遞的影響(2/3)

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-038-039-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：臺北醫學大學醫事技術學系

計畫主持人：李宏謨

報告類型：精簡報告

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中　華　民　國 93 年 5 月 26 日

行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：□個別型計畫　　□整合型計畫

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中　華　民　國　９３　年　５　月　３１　日

一、中文摘要

本研究以分化之3T3-L1脂肪細胞，研究AGEs對胰島素所引發的訊息傳遞路徑和葡萄糖運送的影響。過去二年我們以BSA-AGEs(300μg/ml)處理分化過之3T3-L1脂肪細胞24小時後，可發現胰島素所刺激的[^{3}H]2-去氧葡萄糖之運送受到抑制。以AGEs處理3T3-L1脂肪細胞二小時後IRS-1出現doublet，同時大幅誘導IRS-2的蛋白表現。由於AGEs亦可增加PPAR-γ的表現，而PPAR-γ的活化亦可以增加IRS-2的蛋白表現，我們推測IRS-2的upregulation可能導因於AGEs誘導PPAR-γ的表現所致。除此之外，ε-carboxyl-methyllysine (CML)亦可抑制胰島素所刺激的 [^{3}H]2-去氧葡萄糖之運送約40‰。以CML處理3T3-L1細胞二十四小時後，可以發現Akt/PKB的磷酸化和Akt/PKB的酵素活性均有明顯的減低。因為對胰島素敏感的葡萄糖輸送者(insulin-sensitive glucose transporter)，GLUT-4，即在Akt/PKB的下游；很可能 AGEs是透過抑制Akt/PKB的活性而影響glucose transporter 的表現及轉位，並進一步影響 [^{3}H]2-去氧葡萄糖之運送。為了進一步了解Akt/PKB在AGEs之抑制作用中所扮演的角色，我們製備了過度表現Akt/PKB及kinase dead的Akt/PKB突變基因，送入細胞後Akt/PKB的蛋白表現和磷酸化情形都能依照預期的增減。但過度表現Akt/PKB的3T3-L1細胞並無法逆轉AGEs的作用。我們目前的結論是AGEs可以透過調節IRS-1及IRS-2的訊息傳遞來影響 [^{3}H]2-去氧葡萄糖之運送，但和PI 3-kinase的訊息傳遞無關。最近的研究報告顯示protein phosphatase-2C (PP2-C)可以造成PI 3-kinase的p85之去磷酸化並活化[^{3}H]2-去氧葡萄糖之運送(J Biol Chem, 279: 22715-22726)，因此或許AGEs並非調控PI 3-kinase的下游，我們正針對AGEs是否調控PP-2C進一步的研究。除了上述計畫，過去一年我們還執行了一個成果相當promising的計畫，其中有數項重要的新發現，僅將此計畫部分成果摘要於後：

Dipyridamole是一個核甘運送的抑制劑，也是一個非選擇性的phosphodiesterase的抑制劑，因此能夠藉由抑制phosphodiesterase的機制來增加細胞內cAMP以及cGMP的濃度。第四型的phosphodiesterase已經在許多實驗中被證實具有抗發炎的功能，本研究所要探討的主題就是，在RAW 264.7巨噬細胞中Dipyridamole是否可以抑制Lipopolysaccharide (LPS) 誘導的iNOS以及COX-2的表現。以LPS處理RAW 264.7巨噬細胞會造成iNOS以及COX-2以劑量依存性及時間依存性表現。若以Dipyridamole前處理細胞則可以阻斷LPS所誘導的iNOS及COX-2表現。藉由抑制IκB phosphorylation、degradation、p65 NF-κB translocation以及reporter gene的轉錄作用的方式來證明Dipyridamole會抑制NF-κB路徑的活化。另外，Dipyridamole也可以抑制LPS在RAW 264.7細胞中所造成的p38 MAPK以及IKK-β的活化。若進一步以p38 MAPK的抑制劑SB203580前處理細胞，則能抑制LPS誘導的iNOS表現以及IKK-β活化，所以LPS是先活化了p38 MAPK，再活化NF-κB的訊息傳遞路徑。另外，Dipyridamole能夠刺激mitogen-activated protein kinase phosphatase 1 (MKP-1) 的磷酸化及活化而使得p38 MAPK去磷酸化及去活化而失去功能。總而言之，本研究證明在RAW 264.7巨噬細胞中，Dipyridamole會先藉由活化MKP-1的方式使得p38 MAPK去磷酸化而失去功能。然而p38 MAPK去活化後，接著就會抑制IKK-β的活化以及後續由NF-κB所調控的訊息傳遞路徑，因而抑制LPS所誘導的iNOS及COX-2表現。本研究的結果支持 dipyridamole具抗發炎作用的假說。

Abstract

Dipyridamole is a nucleoside transport inhibitor and a non-selective phosphodiesterase inhibitor that increases intracellular level of cAMP and cGMP through phosphodiesterase inhibition. Type 4 phosphodiesterase has been demonstrated to have anti-inflammatory effects in many experimental systems. This study investigates whether dipyridamole inhibits lipopolysaccharide (LPS)-induced inducible nitric oxide (iNOS) and cyclooxygenase (COX-2) expression in RAW 264.7 macrophages. Treatment of RAW 264.7 macrophages with LPS caused dose- and time-dependent increases in iNOS and COX-2 expression. Treatment of cells with dipyridamole blocked the LPS-induced iNOS and COX-2 expression. Dipyridamole inhibited NF-κB activation as demonstrated by inhibition of IκB phosphorylation, IκB degradation, p65 NF-κB transloction and the transcription of reporter gene. Dipyridamole also inhibited LPS-stimulated p38 MAPK and IKK-β activities in RAW 264.7 cells. A p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580, inhibited LPS-stimulated iNOS expression and IKK-β activation suggesting LPS may activate NF-κB signaling pathway via upstream p38 MAPK activation. Furthermore, dipyridamole stimulated a transient activation of mitogen-activated protein kinase phosphatase 1 (MKP-1), a potent inhibitor of p38 MAPK. Taken together, these data suggest that dipyridamole exerts anti-inflammatory effect via activation of MKP-1, which dephosphorylates and inactivates p38 MAPK. Inactivation of p38 MAPK in turn inhibits IKK-β activation and subsequent NF-κB signaling pathway that mediates LPS-induced iNOS and COX-2 expression in RAW 264.7 cells. These results support the notion that dipyridamole may have anti-inflammatory effects.

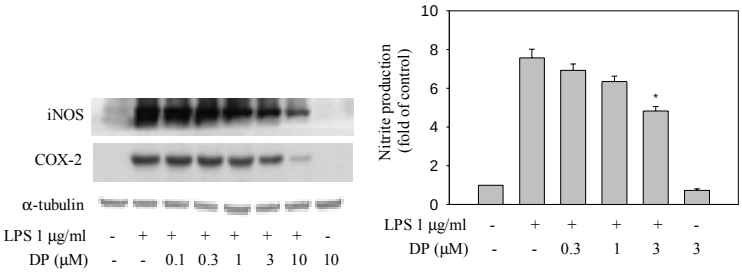
Keywords: Dipyridamole, NOS, LPS, NF-kappa B, RAW 264.7 macrophages.

二、緣由與目的

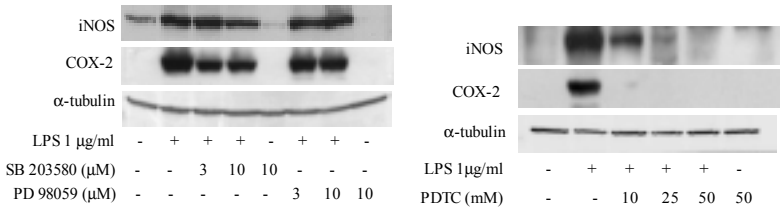
由於原計畫已接近完成，去年度開始我們開始探討糖尿病併發症另一個層面的問題。去年我們發現一種新型口服降血糖藥 rosiglitazone，除降血糖外還可以有抑制 iNOS 基因表現的抗發炎作用，並發表於今年四月 Kidney International (65) 1664-1675。這項研究成果鼓舞我們繼續探討常用來治療腎絲球體腎炎，並能成功的控制血尿和蛋白尿的藥物-dipyridamole 是否也具抗發炎作用。我們發現 dipyridamole 可以抑制 LPS 在 Raw 264.7 巨噬細胞誘導的 iNOS 和 COX-2 表現；我們接著研究其分子機制，發現 dipyridamole 可以活化一種 phosphatase, MKP-1，並造成 p38MAPK 的去磷酸化而抑制其活性。由於 p38MAPK 在 IKK 的上游，此一抑制作用可以間接影響 IkB 的磷酸化、抑制 IkB 的降解及後續 NFkB 的活化及 NFkB 所調控的 reporter 基因表現。我們證實了 dipyridamole 可以和 glucocorticosteroid 一樣經由 MKP-1 達到免疫抑制的效果，我們認為此一發現將有助於解釋 dipyridamole 的臨床藥效，具臨床和學術的價值，因此將試圖投稿 J. Immunol。

三、結果與討論

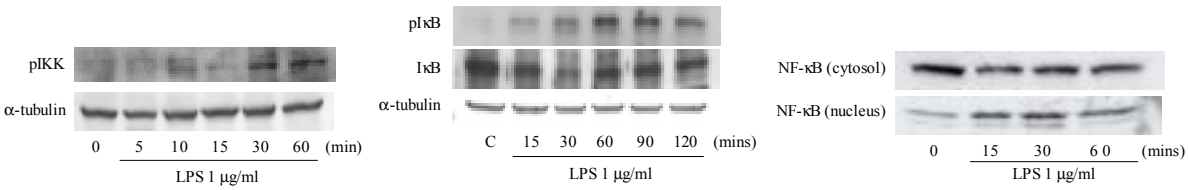
3.1. Dipyridamole inhibits LPS-induced iNOS and COX-2 expression and nitrite accumulation in RAW 264.7 cells.



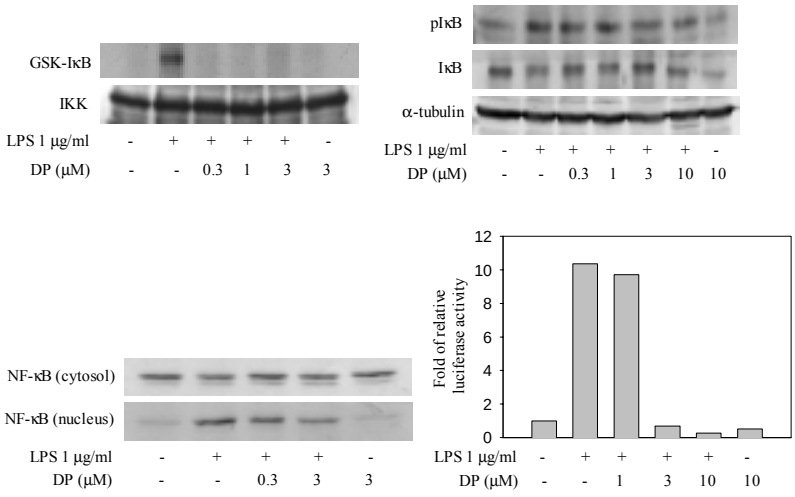
3.2. LPS-induced iNOS and COX-2 expression is mediated through p38MAPK and NFkB pathways.



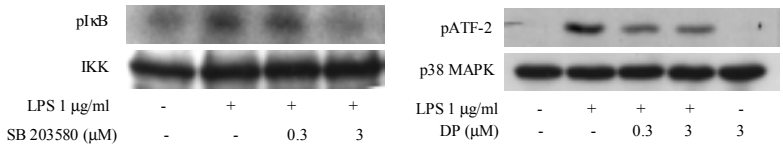
3.3. Effect of LPS on IKK phosphorylation, IkB phosphorylation, IkB degradation and NF-κB translocation in RAW 264.7 cells.



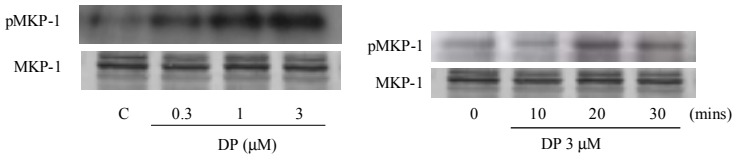
3.4. Dipyridamole inhibits LPS-induced IKK activity, IkB phosphorylation, IkB degradation, NF-κB translocation and NF-κB responsive luciferase reporter gene expression in RAW 264.7 cells.



3.6. Effect of SB203580 on LPS-stimulated on IKK-β activity in RAW 264.7 cells.
Dipyridamole inhibits LPS-activated p38 MAPK in RAW 264.7 cells.



3.7. Dipyridamole increases MKP-1 phosphorylation in RAW 264.7 cells.



討論：

Dipyridamole is a non-selective phosphodiesterase inhibitor, which has been shown to improve proteinurea in membranous glomerulonephritis, mesangial IgA glomerulonephritis, and segmentary and focal hyalinosis glomerulonephritis (Harmankaya et al., 2001). Inhibition of proteinuria in glomerulonephritis has been attributed to platelet response (Camara et al., 1991). However, there is no consistent evidence supporting dipyridamole as an effective antithrombotic agent in cardiovascular and renal diseases. Because many PDE inhibitors suppress the LPS-stimulated cytokine production (Yoshigawa et al., 1999), we have sought to resolve the question of whether dipyridamole exerts an anti-inflammatory effect. LPS is a bacterial endotoxin, which induces the expression of a number of proteins associated with inflammation. LPS-induced iNOS and COX-2 expression are key mediators in inflammatory responses. In the present study, we investigate whether dipyridamole has an effect on LPS-induced iNOS and COX-2 expression. We present evidences showing that dipyridamole inhibits iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells. Because inhibition of p38 MAPK by a pharmacological specific inhibitor, SB203580, is enough to suppress the IKK-β activity, these data suggest that dipyridamole inhibits p38 MAPK activation leading to an inhibition of IKK-β and the NF-κB signaling pathway, and subsequently suppresses LPS-induced iNOS and COX-2 expression in RAW 264.7 macrophages. In addition to these novel findings, we demonstrated that dipyridamole stimulates MKP-1 activation, which lead to p38 MAPK and IKK-β inactivation and NF-κB specific transcription.

Our data agree with many other reports showing phosphodiesterase inhibitors are immunoregulators and can be used as an anti-inflammatory agent (Bielekova et al., 2000; Burnouf et al., 2002). Dipyridamole exerts beneficial effects on glomerulonephritis (Camara etal., 1991) and blocks the lipopolysaccharide (LPS)-induced increase in monocyte-associated tissue factor activity (Brozna et al., 1990). LPS-activation of p38MAPK and NF-kB signal transduction pathways may contribute to inflammatory responses and disease progression. In

this study, we demonstrated that dipyridamole activates MKP-1, which in turn inactivates these proinflammatory signaling pathways. Our results support the notion, that dipyridamole can serve as a anti-inflammatory agent as well.

The results of our inhibitor studies suggest that the LPS-induced iNOS and COX-2 expressions is a consequence of the activation of p38 MAPK, IKK, and NF-κB in RAW 264.7 cells. Activation of the transcription factor, NF-κB, is responsible for the altered transcription of iNOS and COX-2 in macrophages and many other cell types (Huttunen et al., 1999; Mohamed et al., 1999; Li et al., 2000). Consistently, we have found that NF-κB activation plays an important role in LPS-induced iNOS expression. This notion is supported by the data herein: *1)* Pretreatment of cells with PDTC inhibited LPS-mediated iNOS induction; *2)* Treatment of RAW cells with LPS increased p65 NF-κB translocation from the cytosol to the nucleus; *3)* LPS treatment phosphorylated and degraded IκB in the cytosol; and 4) Treatment of RAW cells with LPS enhanced NF-κB-specific transcription as demonstrated by the expression of reporter gene, luciferase activity.

NF-κB can be activated in response to a broad range of stimuli and conditions, including interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (Bowie and O'Neill, 2000). LPS triggers a signaling pathway resulting in the production of inflammatory cytokines, which include TNF α and IL-1. Thus, LPS activation of NF-κB may well be an indirect effect due to the release of cytokines. Indeed, LPS induces TNF α secretion through nuclear factor kappa B in human vascular muscle cells.

The intracellular signaling mechanisms by which LPS induce NF-κB remain to be determined. LPS may activate protein tyrosine kinase, p21 Ras, protein kinase C-βII (PKC βII), or p42/44MAPK. In agreement, we previously found that protein tyrosine kinase, p21 Ras, and p38 MAPK are involved in LPS-induced iNOS expression in A549 cells (Lin et al., 2001). LPS stimulation of human monocytes activates several intracellular signaling pathways that include the p38 mitogen-activated protein kinase (MAPK) pathway (Guha and Mackman, 2001). Lipoteichoic acid activation of NF-κB is mediated through protein tyrosine kinase (Kengatharan al., 1996). Activation of NF-κB in human monocytes involves PKC and PI-3K (D'Addario et al., 1999; Diaz-Guerra et al., 1999). Thus, LPS may activate tyrosine kinase, PI-3K, PKC, and p38 MAPK, which in turn activate IκB kinase (IKK), resulting in NF-kappaB (p50/p65) translocation and the induction of many genes encoding inflammatory mediators including iNOS. However, given the addition of MEK-specific inhibitor, PD 98059, fails to normalize the LPS-induced iNOS expression, and the facts that many other pathways could contribute to the activation of NF-κB, which may play a role in the NF-κB dependent induction of iNOS expression. It is possible that other signaling pathways other than those highlighted in the present study may also contribute to the activation of LPS in RAW264.7 cells.

In conclusion, our results suggest that dipyridamole may have anti-inflammatory effects. Our results clearly demonstrated that dipyridamole inhibits LPS-induced inflammatory

mediator expression in RAW 264.7 macrophages through p38 MAPK, IKK, and NF-κB dependent mechanisms. Whether dipyridamole exerts these effects in clinical treatment warrant further investigation.

四、計畫成果自評

我們以過度糖化最終產物研究糖尿病併發症的細胞及分子機轉。過去三年我們發現 AGEs 可以又發 iNOS 及 COX-2 的表現這些發現分別發表於 Life Science (69): 2503-2515 **(SCI)**。及 European Journal of Pharmacology 438(3): 143-52. (2002) **(SCI)**。PI-3 Kinase、NFkB 的研究結果則也已發表於 Molecular Cellular Endocrionology (194) 9-17 (2002) **(SCI)**。另外有關 PI-PLC, PC-PLC、PKC 亞型的研究，已發表於 *Biochem. Pharmacol.* (66)203-212 (2003) **(SCI)**；除此之外，我們也研發出的血中 AGEs 自動分析法發表於 Journal of Clinical Biochemistry *35, (3): 189-195* (2002) **(SCI)**。今年(2004)我們也在腎臟學門排名第二的 Kidney International (65) 1664-1675 發表了 rosiglitazone，一種 PPAR-γ 的 ligand,可以抑制 AGEs 所誘導的 iNOS 表現。總計過去三年多我們共已發表十四篇文章於 SCI 期刊，除此之外，我們還有三篇文章已經投稿，另外本篇報告的內容將試圖投稿於 J. Immunol. (SCI)。另外，今年四月本計畫主持人並擔任由國科會魏處長和本校許校長共同主辦的第二屆粒線體醫學暨研究研討會之秘書長，共吸引了二百多名國內外的學者參加，大會圓滿成功。

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