行政院國家科學委員會專題研究計畫　成果報告

Pentoxifylline 抑制白蛋白刺激近端腎小管 monocyte chemoattractant protein-1 表現的機轉

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中文摘要

關鍵詞：白蛋白，單核球細胞化學吸引蛋白質-1，pentoxifylline，近端腎小管細胞

目前已知腎臟間質病變與蛋白尿的多寡是慢性腎病惡化的重要指標，而且尿液中的蛋白質已被證實可以引起間質發炎，進而引起腎病的繼續惡化。單核球細胞化學吸引蛋白質-1【Monocyte chemoattractant protein-1 (MCP-1)】是目前已知最強的單核發炎細胞化學吸引蛋白質。動物實驗發現 MCP-1 會在許多慢性腎炎的腎臟大量表現，而且阻斷 MCP-1 的作用可以改善腎臟病的惡化。白蛋白是尿蛋白中的主成分, 在近端腎小管細胞培養的實驗中發現白蛋白可以刺激 MCP-1 分泌，而降低慢性腎病鼠尿蛋白的治療則可以減少腎臟內 MCP-1 的表現。過去吾人已發現 pentoxifylline (PTX)可以改善實驗鼠的急性腎炎及慢性腎衰竭。PTX 能夠改善腎病的原因之一可能與其能降低炎性反應有關。事實上，吾人已發現 PTX 能夠減少腎臟內的單核發炎細胞浸潤，以及減少 MCP-1 的表現；更有趣的是在近端腎小管細胞培養的實驗中，PTX 也可以降低白蛋白所刺激的 MCP-1 基因表現。然而 PTX 是透過何種機制來降低白蛋白刺激 MCP-1 表現則是未知。本研究探討 PTX 抑制白蛋白刺激近端腎小管 MCP-1 表現的機轉。吾人將 NF-κB、AP1、及 SP1 等 binding site 分別進行 mutation，吾人發現 AP1B 及 SP1 會影響 MCP-1 的 basal transcription，而且 NF-κB、AP1B、SP1 與白蛋白刺激近端腎小管 MCP-1 gene 表現有關。吾人正進行 EMSA 以了解 PTX 是否透過抑制 NF-κB、AP1B、或 SP1 等轉錄因子的活化來抑制白蛋白刺激 MCP-1 基因的表現。

英文摘要

Key words：Albumin，Monocyte chemoattractant protein-1，pentoxifylline，renal proximal tubular cells

Interstitial inflammation and proteinuria are hallmarks of progressive chronic renal disease, and proteinuria has been implicated as an effector of progressive renal scarring, particularly through induction of interstitial inflammation. Increasing concentrations of albumin in cultured proximal tubular cells has been shown to upregulate the expression of monocyte chemoattractant protein-1 (MCP-1), one of the most powerful mononuclear cell attractants characterized so far. In our previous studies, pentoxifylline (PTX) has been shown to attenuate experimental mesangial proliferative glomerulonephritis and progressive chronic renal disease. One of the possible mechanisms for the renoprotection may be linked to the effect of PTX against inflammation. Indeed, we have shown that PTX attenuates mononuclear cell infiltration possibly through MCP-1 downregulation in renal cortex as well as in albumin-stimulated proximal tubular cells. However, the detail mechanism by which

PTX inhibits MCP-1 induction in albumin-stimulated proximal tubular cells is not known. In promoter activity assay, we found that the basal activity of MCP-1 promoter was reduced after AP1B, or SP1 binding site mutation. Furthermore, the increased promoter activity of MCP-1 by human serum albumin was suppressed in HK2 cells transfected with NF-κB, AP1B, or SP1 mutated construct. We will further demonstate the target molecule of PTX in suppressing the albumin-induced MCP-1 gene expression. We will also study whether PTX affects the albumin-activated signal pathways involving the activation of AP1 and SP1 binding.

報告內容

簡介（Introduction）

Interstitial inflammation and proteinuria are hallmarks of progressive chronic renal disease, and proteinuria has been implicated as an effector of progressive renal scarring, particularly in the tubulointerstitial compartment (Remuzzi and Bertani, 1998). Experimental and human data in recent years have suggested that proteins filtered through the glomerular capillary barrier in excessive amount have an intrinsic renal toxicity linked to their over-reabsorption by proximal tubular cells and activation of tubular-dependent pathways of interstitial inflammation (Remuzzi and Bertani, 1998; Abbate and Remuzzi, 1999; Lin et al., 2002). Thus, in models of overload proteinuria, repeated injections of albumin in the rat increased glomerular barrier permeability and caused massive proteinuria and tubular changes with heavy macrophage and T lymphocyte infiltration into the renal interstitium (Eddy et al., 1989). Increasing concentrations of albumin in cultured rat and human proximal tubular cells upregulated the expression of monocyte chemoattractant protein-1 (MCP-1), one of the most powerful mononuclear cell attractants characterized so far (Lin et al., 2002; Wang et al., 1997b; Wang et al., 1999; Morigi et al., 2002). The promoter of MCP-1 gene contains binding sites for the transcription factors activator protein (AP-1), nuclear factor-κB (NF-κB), and sequence-specific transcription factor (Sp1), which have been shown to be important for its expression induced by cytokines or viral proteins (Roebuck et al., 1999; Lim and Garzino-Demo, 2000). There is evidence that albumin induces MCP-1 expression through activation of NF-κB, predominantly containing p50, p65, and c-Rel (Wang et al., 1999; Morigi et al., 2002). Moreover, exposure to excess proteins in proximal tubular cells induces the formation of H_2O_2 through a PKC-dependent pathway, which is responsible for NF-κB activation and consequent induction of MCP-1 (Morigi et al., 2002). Whether AP-1 and Sp1 also mediate the stimulation signal of albumin to MCP-1 induction is not reported before. Pentoxifylline (PTX) is a phosphodiesterase inhibitor used clinically

to treat patients with peripheral vascular diseases (Ward and Clissold, 1987). We previously found that PTX attenuates mononuclear cell infiltration in glomeruli of rats with acute Thy 1 glomerulonephritis and in interstitium of rats with remnant kidney, possibly by downregulating MCP-1 expression (Lin et al., 2002; Chen et al., 1999). Furthermore, we also found that PTX reduces the MCP-1 induction in proximal tubular cells stimulated with albumin (Lin et al., 2002). PTX is reported to inhibit MCP-1 production in peripheral mononuclear cells as well as T cell activation via c-Rel inhibition (Krakauer, 1999; Wang et al., 1997a). However, the detail mechanism by which PTX inhibits MCP-1 induction in albumin-stimulated proximal tubular cells is not known.

材料及方法 (Subjects and Methods)

(A) Cell culture

HK2, a human proximal tubular cell line, was cultured for this study.

(B) Northern analyses

Northern and Western blot analyses was performed as previously for analyses of MCP-1 gene (Tsai et al., 1995; Lin et al., 2002; Chen et al., 2003).

(C) Plasmids

PhMCP213, which covers positions –213 to +6 of the human MCP-1 promoter that contains the binding sites for transcription factors NF-κB, AP-1, and SP1, is kindly gifted by Prof. Garzino-Demo (Lim and Garzino-Demo, 2000). The DNA fragments obtained from phMCP213 digested with *Nhe*I and *Bgl*II was cloned into the *Nhe*I-*Bgl*II site of pGL3-Basic (Promega) to produce pGL3-hMCP213 plasmid construct. Site-directed mutation was introduced with a QuikChange site-directed mutagenesis kit (Stratagene). Briefly, 5 ng of pGL3-hMCP213 plasmid DNA and 125 ng of each of two complementary oligonucleotides containing the desired mutation (**TABLE**) was incubated with 2.5 U of *Pfu* DNA polymerase and cycled 18 times with the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 10 min. The reaction mix was cooled on ice for 2 min and digested with 10 U of *Dpn*I at 37°C for 1 h, and 1 ml of the reaction mix was transformed into Epicurian Coli XL1-Blue supercompetent cells. Plasmids were isolated from colonies grown overnight at 37°C on ampicillin-resistant agar plates, and sequencing was carried out to identify those that contain the mutated sequences of interest.

TABLE: c*is* elements on the hMCP-1 promoter region to be analyzed in this study

cis-acting element	Position	Sequence	Bases changed (boldface) in site-directed mutagenesis	Construct
AP1	-156	5’-ACTTATCACTCATGGAA-3’	5’-ACTTA**GACAGAC**TGGAA-3’	pΔAP1A
	-129	5’-TCCTGCTTGACTCCGCCC-3’	5’-TCCTGCT**GTCAGAA**GCCC-3’	pΔAP1B
NF-κB	-148	5’-ACTCATGGAAGATCCCTCCT-3’	5’-ACTCAT**ATCGTGGAAA**TCCT-3’	pΔNF-κB

SP1	-125	5’-TGACTCCGCCCTCTCT-3’	5’-TGACTCCG**GGG**TCTCT-3’	pΔSP1
Consensus AP1		5’-CGCTTGATGACTCAGCCGGAA-3’		
Mutated AP1		5’-CGCTTGATGACTTGGCCGGAA-3’		
Consensus NF-kB		5’-AGTTGAGGGGACTTTCCCAGGC-3’		
Mutated NF-kB		5’-AGTTGAGGCGACTTTCCCAGGC-3’		
Consensus SP1		5’-ATTCGATCGGGGCGGGGCGAGC-3’		
Mutated SP1		5’-ATTCGATCGGTTCGGGGCGAGC-3’		

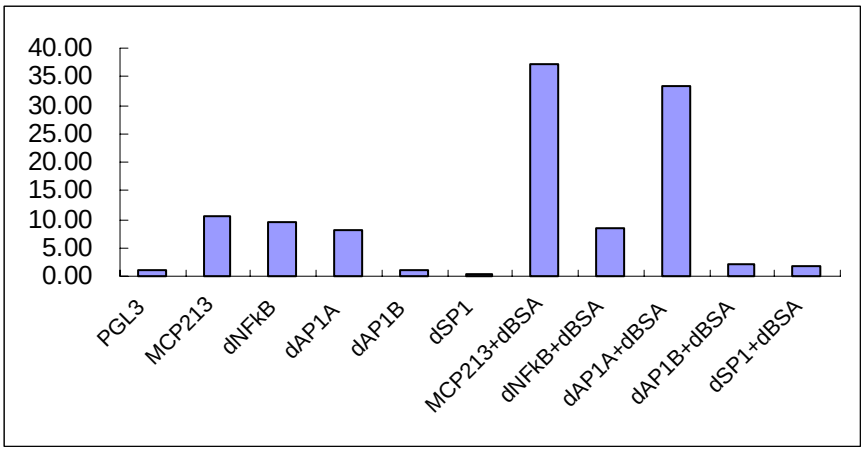
(D) Cell transfection and luciferase activity assay

For analysis of promoter activity of human MCP-1 with various mutations, these reporter constructs as well as pGL3-basic will be transfected into HK2 by lipofectamin 2000 following the manufacturer’s instructions (Invitrogen, Life Technologies). Control vector with Renilla luciferase expression (phRL-TK) was cotransfected to normalize the transfection efficiency. After 24 hours of transfection, the cells were incubated with or without HSA and PTX (Sigma). Luciferase activities will be quantified with the Duo-Glo Luciferase Assay System (Promega).

結果 (Results)

(A) The DNA fragments obtained from phMCP213 digested with *Nhe*I and *Bgl*II was cloned into the *Nhe*I-*Bgl*II site of pGL3-Basic (Promega) to produce pGL3-hMCP213 plasmid construct. Site-directed mutation was introduced with a QuikChange site-directed mutagenesis kit (Stratagene). The mutated constructs, pΔNF-κB, pΔAP1A, pΔAP1B, and pΔSP1, were confirmed by sequencing.

(B) In promoter activity assay, we found that the basal activity of MCP-1 promoter was reduced after AP1B, or SP1 binding site mutation. Furthermore, the increased promoter activity of MCP-1 by HSA was suppressed in HK2 cells transfected with NF-κB, AP1B, or SP1 mutated construct.



討論 (Discussion)

In addition to NF-κB involving in the activation of MCP-1 gene by urinary albumin in human proximal tubular cells, we further demonstrate that mutation at the binding site for AP1, or SP1 also blocks the activation of MCP-1 gene by urinary albumin in human proximal tubular cells. PTX is reported to inhibit MCP-1 production in peripheral mononuclear cells as well as T cell activation via c-Rel inhibition (Krakauer, 1999; Wang et al., 1997a). We will further demonstate the target molecule of PTX in suppressing the albumin-induced MCP-1 gene expression. We will also study whether PTX affects the albumin-activated signal pathways involving the activation of AP1 and SP1 binding.

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計畫成果自評

1. 研究內容與原計畫相符程度及達成預期目標約百分之五十。
2. 研究結果讓吾人進一步了解 pentoxifylline 保護腎臟的機轉。
3. 吾人正進行 EMSA 以了解 PTX 是否透過抑制 NF-κB、AP1B、或 SP1 等轉錄

因子的活化來抑制白蛋白刺激 MCP-1 基因的表現。