YC-1 對於人類血管內皮細胞的生長抑制作用

The anti-proliferation effect of YC-1 in human vascular endothelial cells.

中文摘要

本篇論文的主旨在研究一藥物 guanylylcyclase activator—YC-1 對於人類臍帶靜脈內皮細胞(human umbilical vein endothelial cell, HUVEC)在體外(in vitro)生長的影響，並且探討其可能的作用機制。我們使用的實驗材料爲初代培養之人類臍靜脈內皮細胞，並且其繼代培養次數皆維持在 7 代之內。實驗研究發現，YC-1 在 5 μM 的濃度下即對 HUVEC 的增生產生抑制作用，且其作用強度以劑量相關（dose-dependent）的方式增加。以 H3-thymidine incorporation 的實驗觀察發現，加藥 24 小時後，YC-1 明顯的抑制內皮細胞 DNA 的合成。利用流式細胞儀（flow cytometry）分析細胞週期，發現 YC-1 會造成內皮細胞的細胞週期停滯在 G0/G1 phase。利用 western blot 發現，內皮細胞以 YC-1 處理時，可以觀察到和細胞週期停滯（cell cycle arrest）有關的蛋白 p21 及 p27 比對照組有明顯的增加，但是 cyclin A，cyclin D1，cyclin D3，cyclin E，cdk2 及 cdk4 的表現量卻沒有改變。我們利用 kinase assay 觀察 cdk2 以及 cdk4 的活性，發現在 YC-1 處理之下，cdk2 的 kinase 活性有明顯的被抑制現象，但 cdk4 的 kinase 活性反而增加；同時利用 anti-cdk2 及 anti-cdk4 antibody 進行免疫沉澱法（immunoprecipitation）發現加藥組中與 cdk-cyclin complex 結合的 p21 量的確比對照組高，因而抑制了 cdk2 的活性。根據這個結果推測，YC-1 可能經由抑制了對內皮細胞增生最重要的訊號傳遞路徑，MAP kinase pathway，而產生其作用，實驗結果卻發現，YC-1 反而會促進 p44/42 MAP kinase 的磷酸化（phosphorylation）。我們接著懷疑，YC-1 對內皮細胞的作用，是否經由活化 guanylylcyclase，而增加細胞內 cGMP 的量所造成？於是我們以 guanylylcyclase inhibitor—ODQ，methylene blue，以及 protein kinase G inhibitor(PKG) inhibitor—KT5823 來處理內皮細胞，結果發現它們並不能阻斷 YC-1 的作用；同時，我們也利用一具細胞膜通透性的 cGMP 類似物—8-bromo-cGMP，來模擬內皮細胞內 cGMP 大量增加時的情形，結果，在所測試的劑量之下，8-bromo-cGMP 都不會有抑制內皮細胞 DNA 合成以及細胞增生的情況。過去曾有論文指出，YC-1 除了會增加 cGMP 外，亦會增加胞內的 cAMP。當我們以 cAMP 類似物 8-bromo-cAMP 處理內皮細胞，發現 8-bromo-cAMP 對細胞增生有較強的抑制作用；但是當我們加入 adenylylcyclase inhibitor—2’,5’-DDA 以及 protein kinase A inhibitor(PKA) inhibitor—KT5720，它們卻無法阻斷 YC-1 的作用。另外，過去亦發現 YC-1 會活化 eNOS 從而增加 NO 生成，因此我們給予 NOS 抑制劑 L-NAME，實驗結果發現 L-NAME 亦無法阻斷 YC-1 對內皮細胞 DNA 合成的抑制作用及抗細胞增生作用。從以上結果顯示，YC-1 是經由誘導胞內 p21 及 p27

的增加，而抑制 cdk2 的 kinase activity，繼而導致內皮細胞細胞週期的停滯；但此作用並非透過抑制 MAP kinase 活性，或經由活化 cGMP、cAMP 、NO pathways 而來。

英文摘要

The aim of this thesis is to examine the antiproliferation effect of guanylyl cyclase activator, YC-1, in human umbilical vein endothelial cells (HUVEC) and it’s possible underlying mechanism. Our data demonstrates that YC-1 caused a concentration-dependent inhibition in HUVEC proliferation. The results of 3H-Thymidine incorporation showed that YC-1 significantly decreases endothelial cell DNA synthesis. Flow cytometric analysis demonstrated that treatment of HUVEC with YC-1 arrested the cell at the G0/G1 phase of the cell cycle. Western blot analysis showed that treatment of HUVEC with YC-1 for 18h increased the levels of p21 and p27 protein, while the levels of cyclin A, cyclin D1, cyclin D3, cyclin E, cdk2 and cdk4 protein were not changed. Kinase assay showed that YC-1 increased the p21/cdk2 association, which in turn inhibited the cdk2 enzyme activity. Interesting, YC-1 increases the level of phospho-p44/42 MAP kinase. To examine whether guanylyl cyclase activation is involved in the YC-1-mediated antiproliferation in HUVEC, effects of YC-1 were measured in the presence or absence of guanylyl cyclase inhibitors (ODQ and methylene blue) or PKG inhibitor (KT5823). The results showed that YC-1 inhibited HUVEC proliferation to the same extent, regardless whether ODQ, methylene blue, or KT5823 was present or not. Moreover, administration of the cell membrane permeable cGMP analogue, 8-bromo-cGMP, which mimics the cGMP effect, does not cause any retardation in endothelial cell proliferation even at the concentration of 100uM. Since YC-1 has been shown to increase the intracellular cAMP level, we examined the antiproliferation effect of cAMP analogue, 8-bromo-cAMP, in endothelial cells and found that it strongly induced antiproliferation in endothelial cells. However, adenylyl cyclase inhibitor (2’,5’-DDA) and protein kinase A (PKA) inhibitor (KT5720) did not change the antiproliferation effect caused by YC-1. YC-1 also has been shown to activate NOS, whichin turn increases NO concentration in endothelial cell, we tested if the NOS inhibitor, L-NAME, can block the antiproliferation effect of YC-1 in HUVEC. Our results revealed that L-NAME did not reverse the YC-1 ability in HUVEC. In conclusion, the results of the present study suggest that YC-1 interrupts the cell cycle progression and proliferation of human endothelial cells by increasing the protein levels of p21 and p27 via apathway independent of p44/42 MAP kinase, cGMP, cAMP, or NO.