

行政院國家科學委員會專題研究計畫 成果報告

Trilinolein 於平滑肌細胞對血管收縮素所誘發內皮素基因
表現的作用_

計畫類別：個別型計畫

計畫編號：NSC94-2320-B-039-030-

執行期間：94年08月01日至95年07月31日

執行單位：中國醫藥大學中醫學系

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中 華 民 國 95 年 10 月 30 日

一、前言

trilinolein 是三七，一種傳統中藥中所富含之天然抗氧化成份。而三七這種中藥在治療循環系統疾病已經使用了數百年了。此外，trilinolein 在過去一系列實驗證實具有類似維他命 E (α -tocopherol)的抗氧化作用。於動物實驗上，證實可顯著的對抗血栓形成(thrombogenicity)以及減少心律不整之發作。因此，我們有興趣評估 trilinolein 在最近的在血管平滑肌細胞的增生上的作用。並進一步探討其在心血管系統的細胞作用與分子生物機轉。

血管平滑肌細胞是血管系統的細胞，具有調節血管舒張或收縮及其他重要功能。近來實驗發現；血管收縮素(angiotensin II; Ang II)可增加血管平滑肌細胞增生及內皮素-1(endothelin-1; ET-1)的基因表現。有這種作用與細胞內活性氧族群(reactive oxygen species; ROS)有關。所以本實驗即以血管收縮素誘發血管平滑肌細胞增生及內皮素基因表現的模式來探究 trilinolein 對於培養中血管平滑肌細胞內皮素基因表現的影響及其分子生物機轉。

二、實驗材料與方法

Materials.

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents are from Life Technologies, Inc. A rat ET-1 cDNA probe (accession No. M64711) is obtained as previously described (14). A full length of the ET-1 promoter region (4.4 kb) is fused to the chloramphenicol acetyltransferase (CAT) reporter gene (12). PBLCAT2 (containing CAT reporter gene with its promoter) and PBLCAT3 (containing the CAT gene only) are constructed as previously described (15). 2',7'-dichlorofluorescein diacetate (DCF-DA) is obtained from Molecular Probes (Eugene, OR, U.S.A.). H_2O_2 is purchased from Acros Organics (Pittsburgh, PA, U.S.A.). Resveratrol, *N*-acetyl-cysteine (NAC), and all other reagent-grade chemicals, are purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The plasmid

AP-1-Luc containing the firefly luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1 binding element are obtained from Stratagene (La Jolla, CA, U.S.A.).

Culture of Rat Aortic Smooth Muscle Cells.

The investigation conduct is conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of Taipei Medical University. Thoracic aortae from male Sprague-Dawley rats are excised rapidly and immersed in DMEM containing penicillin (100 U/ml) and streptomycin (100 µg/ml). Connective tissue and adherent fat are cleaned away from the specimens. Isolated arteries are cut open, and the endothelium was removed by gently rubbing off the intimal surface with a pair of sharp scissors. Denuded aortae are cut into ~3-mm pieces and placed with the intimal face down into three 35-mm culture dishes (Iwaki, Osaka, Japan). DMEM containing 10% fetal calf serum and penicillin/streptomycin is gently added to the dishes to cover the tissues without disturbing the orientation of the explants. Vascular smooth muscle cells are allowed to proliferate from the tissue (7-10 days), and the tissues are removed using sterilized fine forceps and washed with culture medium. After reaching confluence in three 35-mm dishes, cells are harvested by brief trypsinization and subsequently cultured in T-75 flasks (Iwaki) (passage 1). Cells are routinely propagated in culture dishes to 75–95% confluence, and used between passages 3–12. The purity of smooth muscle cells is evaluated by staining the cells with monoclonal antibodies to α -smooth muscle actin. Vascular smooth muscle cells are grown in DMEM without phenol red containing antibiotics and 10% fetal calf serum until 24 h prior to experimentation, at which time cells are in a defined serum-free medium containing insulin (0.5 µM) and transferrin (5 mg/ml) for all experiments. Cells are

then preincubated with resveratrol for 1 h and then with or without Ang II (100 nM) for different incubation times as indicated, followed by harvesting. Cellular viability under all treatment conditions is determined by cell count, morphology, and trypan blue exclusion.

DNA Synthesis

To measure synthesis of new DNA, cells (1×10^5 /well) are plated in six-well (35-mm) dishes 24 h before experiments as previously described (11). Cells are incubated with [3 H]thymidine (5μ Ci/ml). Following the treatment as indicated, cells are harvested by incubation at 4 °C with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH, and radioactivity is determined by scintillation counting. Data are presented as the mean \pm SEM of 9-12 determinations for six different cell preparations and normalized to the untreated sample \times 100 (*i.e.* percentage of control).

Assay of Intracellular ROS

The level of ROS are measured using a previously described method (12). Prior to the chemical or Ang II treatment, smooth muscle cells were incubated in culture medium containing a fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Eugene, OR, U. S. A.) at a concentration of 30 μ M for one hour to establish a stable intracellular level for the probe. The same concentration of DCF-DA is maintained during either chemical or Ang II treatment. Subsequently, the cells are washed with PBS, removed from Petri dishes by brief trypsinization, and then assessed for their 2',7'-dichlorofluorescein (DCF) fluorescence intensity. The DCF fluorescence intensity of the cells is an index of intracellular levels of ROS; and it can be determined by fluorescence spectrophotometry with excitation and emission wavelengths at 475 and 525 nm, respectively. For counting cell numbers, cells are harvested and counted in an automatic cell counter (S.ST.II/ZM, Coulter Electronics Ltd., Miami, FL, U.S.A.). The cell number in each sample is counted and utilized to

normalize the DCF fluorescence intensity.

RNA Isolation and Northern Blot Analysis

Total RNA is isolated from cells by the guanidine isothiocyanate/phenol chloroform method as previously described (12). The RNA (10 μ g/lane) is separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher & Schuell, Inc, Germany) by a vacuum blotting system (VacuGene XL, Pharmacia, Sweden). Following hybridization with the ³²P-labeled ET-1 cDNA probes, the membrane is washed with 0.1x SSC containing 1% SDS at 42°C for 30 min and then exposed to x-ray film at -70°C. Blots of specific mRNA bands are detected by autoradiography and analyzed with a densitometer (Computing Densitometer 300S, Molecular Dynamics). Blots are stripped and reprobbed for 18S cDNA probe (obtained from American Type Culture Collection) to control for loading. The level of expression of ET-1 mRNA is quantitated and was normalized to the 18S signal.

Transfection and Chloramphenicol Acetyltransferase Assays

For the transient transfections, cells are transfected with different expression vectors by the calcium phosphate method (15). DNA concentration for all samples is adjusted to equal amount with empty vector pSR α in each experiment. To correct for variability in transfection efficiency, 5 μ g of pSV- β '-galactosidase plasmid DNA is cotransfected in all the experiments. The CAT and β '-galactosidase assays are performed as previously described (15). The relative CAT activity is corrected by normalizing the respective CAT value to that of β -galactosidase activity. Cotransfected β -galactosidase activity varied by <10% within a given experiment and is not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) are included in each assay.

Western Blot Analysis

Rabbit polyclonal anti-phospho-specific ERK antibodies are purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-ERK antibodies are purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Western blot analysis is performed as previously described (12).

Luciferase assay

Smooth muscle cells plated on six-well (35-mm) dishes are transfected with the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc) (Stratagene, La Jolla, CA, U.S.A.). Following incubation for 24 h in serum-free DMEM, smooth muscle cells are cultured under various different conditions as indicated for a period of 48 h. Smooth muscle cells are assayed for luciferase activity with a luciferase reporter assay kit (Stratagene). The specific firefly luciferase activity, as is the case for AP-1 transcriptional activity, is normalized for transfection efficiency to its respective β -galactosidase activity and expressed as activity relative to the control.

三、實驗結果

1. Effects of trilinolein on the angiotensin II-induced cell proliferation in smooth muscle cells.

以往的研究報告顯示，血管平滑肌增生是許多心血管病變的重大指標，因此本實驗以 thymidine incorporation 法來測量大鼠平滑肌細胞的增生。如圖 1 顯示單獨給予不同劑量的 trilinolein (1, 10, 100 μ M) 對血管平滑肌細胞增生並無顯著影響。此外，低劑量 trilinolein (1 μ M) 對血管緊張素 (angiotensin II) 所誘發的血管平滑肌細胞增生並無顯著影響，但中高劑量 trilinolein (10, 100 μ M) 則明顯抑制 angiotensin II 所誘發的血管平滑肌細胞增生。

2. Trilinolein inhibits angiotensin II-induced endothelin-1 expression in smooth

muscle cells.

以往的實驗結果顯示,內皮素在 angiotensin II 所誘發的血管平滑肌細胞增生過程中,扮演重要的調節角色。本實驗分別以 Northern blotting assay 以及 ELISA 法測定 endothelin-1 的 mRNA 表現量以及蛋白質含量。如圖 2 顯示單獨給予不同劑量的 trilinolein (1, 10, 100 μ M) 對 endothelin-1 的 mRNA 表現量以及蛋白質含量並無顯著影響。此外,低劑量 trilinolein (1 μ M) 對血管緊張素 (angiotensin II) 所誘發的 endothelin-1 的 mRNA 表現量以及蛋白質含量並無顯著影響,但中高劑量 trilinolein (10, 100 μ M)則明顯抑制 angiotensin II 所誘發的 endothelin-1 的 mRNA 表現量以及蛋白質含量。

3. Effect of trilinolein on angiotensin II-induced superoxide formation in smooth muscle cells.

以往的文獻報告顯示,氧游離基 (superoxide) 在 angiotensin II 所誘發的內皮素產生過程中,扮演重要的調節角色。如圖 3 顯示單獨給予不同劑量的 trilinolein (1, 10, 100 μ M) 對 NADPH oxidase 活性以及 superoxide 量並無顯著影響。此外,低劑量 trilinolein (1 μ M) 對血管緊張素 (angiotensin II) 所誘發的 NADPH oxidase 活性以及 superoxide 量並無顯著影響,但中高劑量 trilinolein (10, 100 μ M)則明顯抑制 angiotensin II 所誘發的 NADPH oxidase 活性以及 superoxide 量。

4. Inhibitory effect of trilinolein on angiotensin II-increased ERK phosphorylation in smooth muscle cells.

許多文獻報告顯示 ERK 磷酸化在 angiotensin II 誘發的內皮素產生過程中,重要訊息路徑。本實驗以 Western blotting assay 測定 ERK 的磷酸化。如圖 4 顯示單獨給予 trilinolein (10 μ M) 對 ERK 的磷酸化並無顯著影響。此外, trilinolein 則明顯抑制 angiotensin II 所誘發的 ERK 的磷酸化。還有抗氧化劑 NAC 也會明顯抑制 angiotensin II 所誘發的 ERK 的磷酸化。外加 H₂O₂ 則會明顯誘發的 ERK 的磷酸化。

5. Effects of trilinolein, or *N*-acetyl-cysteine on angiotensin II – or H₂O₂-increased AP-1-mediated reporter activity.

以往的文獻報告顯示 AP-1 在血管平滑肌增生過程中,扮演重要角色。本實驗以 chloramphenicol acetyltransferase (CAT) report gene assay 法測量 AP-1-mediated reporter gene 的活性。如圖 5 顯示 endothelin-1 以及 H₂O₂ 都會活化 AP-1-mediated

reporter gene, 而 trilinolein (10 μ M) 以及抗氧化劑 NAC 會明顯抑制 AP-1-mediated reporter gene 的活化。

四、結論

trilinolein 有明顯的對抗血管平滑肌細胞增生的作用。而這種作用與 trilinolein 的抗氧化能力有密切的關係。本實驗進一步釐清 trilinolein 對抗血管平滑肌增生的作用機轉。Trilinolein 會抑制 angiotensin II 所誘導產生的氧游離基、內皮素釋出、ERK 磷酸化以及 AP-1-mediated reporter gene 的活化。這些可以作為預防以及治療心血管疾病之新藥開發的重要參考。

五、參考文獻

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Figure 1

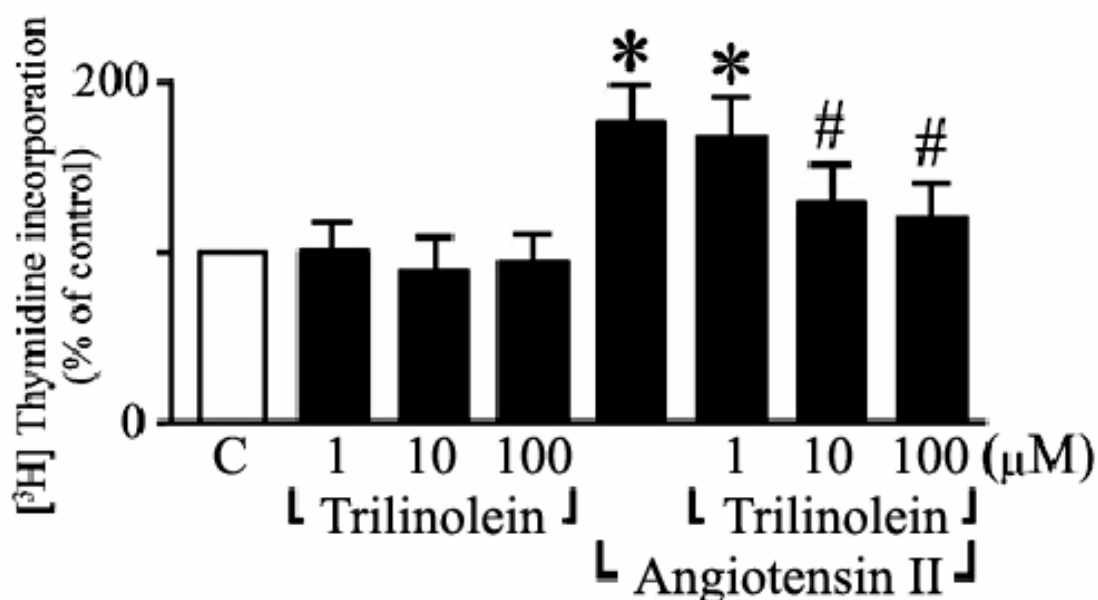


圖 2

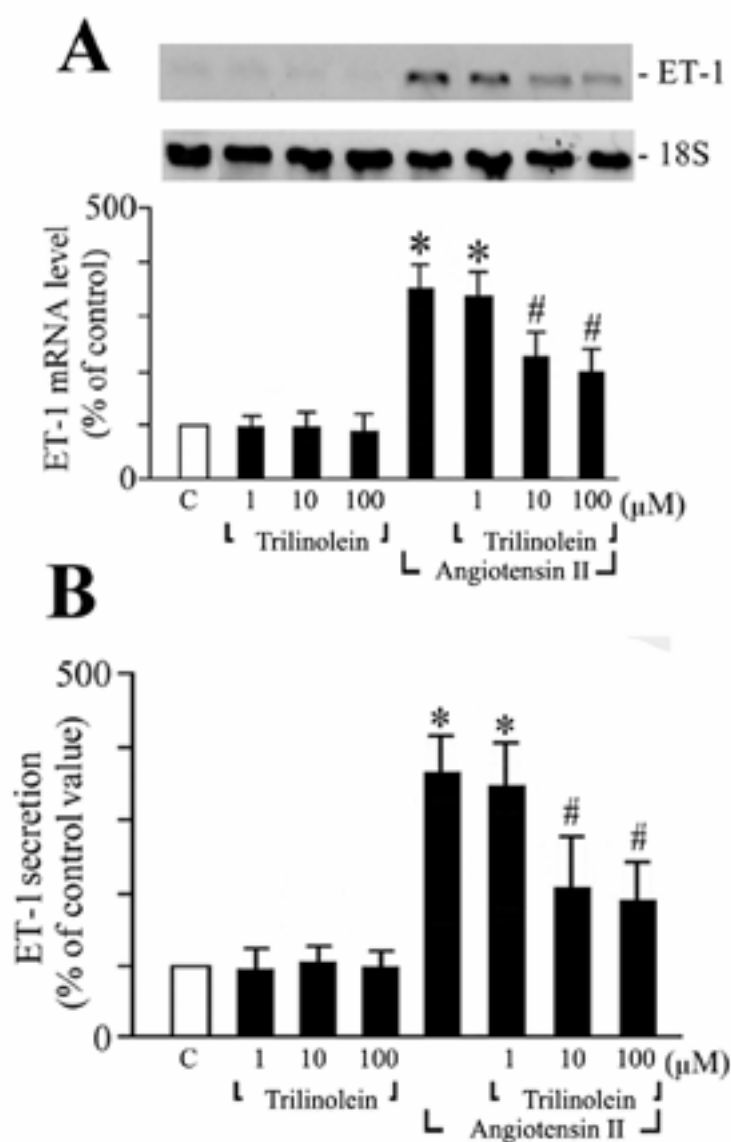


圖 3

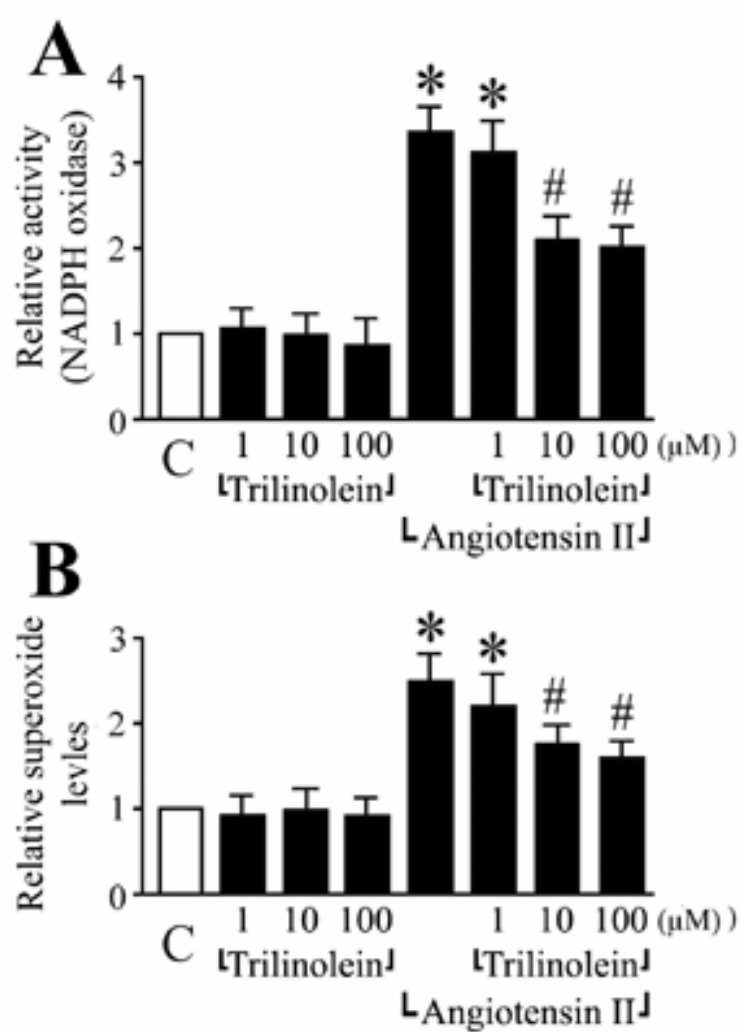


圖 4

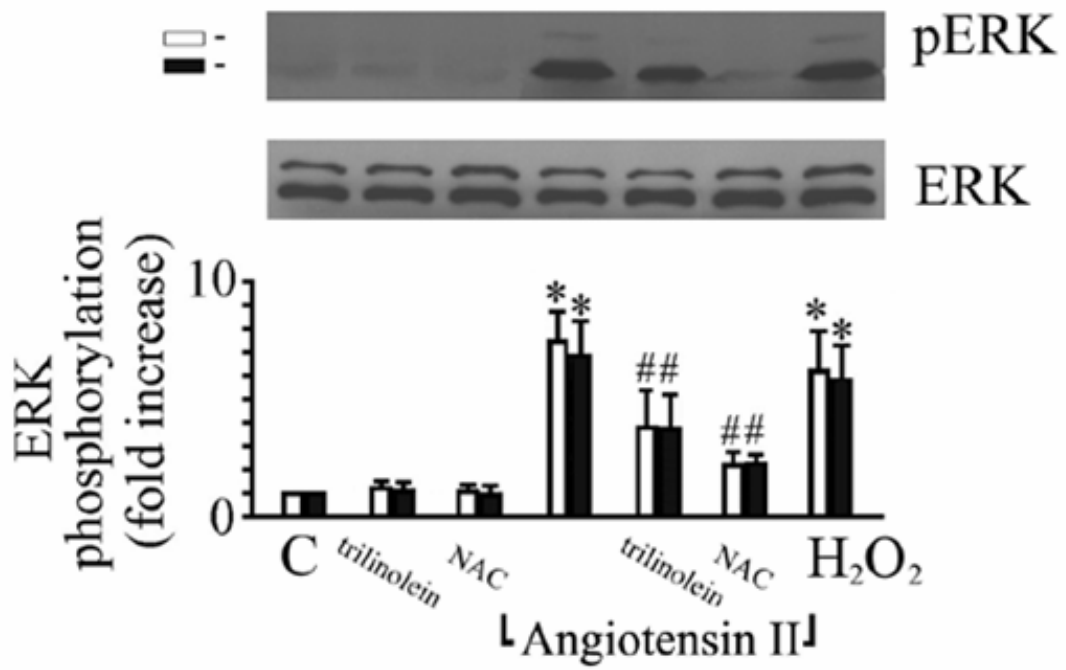


圖 5

