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傳統中草藥三七純化物三亞麻油酸在預防血管張力激素 II 誘發的高血壓及心肌肥厚以及氧化壓力的研究

藥用植物三七 Sanchi(川七或田七)，在傳統中醫上，用以治療心血管疾患已有數百年歷史。三亞麻油酸(trilinolein)是從三七根部所萃取出之一種甘油酯。在結構上是以三個亞麻油酸 linoleic acid(不飽和脂肪酸)與甘油(glycerol)氫氣鍵脂化而成。近年來的實驗，證實三亞麻油酸具多項保護心血管系統及改善血液循環的功能：於活體外(in vitro)，三亞麻油酸可促進紅血球變異性，抑制血小板聚集及減少心肌細胞因缺氧產生之傷害；而在活體(in vivo)的實驗中，三亞麻油酸可減少大白鼠心肌梗塞的區域並抑制心室心律不整的產生。以 chemilumine scence 方法測量，發現三亞麻油酸具有比維生素 E (α-tocopherol)更強的抗氧化作用。而目前認為三亞麻油酸的心臟保護作用與其抗氧化能力有關。血管張力素Ⅱ(Angiotensin Ⅱ：AngⅡ)是由血管張力素Ⅰ經由血管張力素轉化(angiotensin converting enzyme)作用而成，是一種張力血管收縮劑，在高血壓及許多心血管疾病病理機轉中，血管張力素Ⅱ扮演重要的角色。Wollert 等人在 1999 年證實 AngⅡ是心肌肥厚的重要因子。它可誘發心肌細胞 β-myosin heavy chain (β-MyHC)的基因表現。而這種胎兒期(fetal-type)的基因表現是心肌肥厚的重要指標(Yue et al., 2000)。在 AngⅡ導致心肌細胞肥厚的訊息傳遞上，mitogen-activated protein kinases (MAPKs)包括 extracellular signal-regulated kinase (ERK)，c-Jun N-terminal kinase (JNK)和 p38 扮演重要的角色。而轉錄因子(transcriptional factors) activator protein-1 (AP-1)和 nuclear factor-kappa B(NF-κB)也已被證實和心肌細胞肥厚產生有關。過去實驗發現，AngⅡ可增加心肌細胞反應性氧自由基族群(reactive oxygen species: ROS)的產生，而給予抗氧化劑可抑制 AngⅡ導致的心肌肥厚。先前的實驗也發現 AngⅡ活化心肌細胞 ERK 路徑與增加 β-MyHC 基因啟動(promoter)活性的作用與 ROS 有關。本研究結果:三亞麻油酸可抑制心管張力素Ⅱ(AngⅡ)導致的心肌細胞 β-MyHC 基因的表現，進而抑制心肌肥厚。而此種抑制作用是與三亞麻油酸抗氧化功能有關，三亞麻油酸能減少 ROS 的產生，降低 MAPKs 活性，及抑制轉錄因子 AP-1, NFκB 導致的肥厚基因表現。

關鍵詞：三亞麻油酸 心管張力素Ⅱ 反應性氧自由基族群

The myocardial protective effects of trilinolein, isolated from the Chinese herb Sanchi (Panax notoginseng), may be related to its antioxidant effects. In the present study, we investigated the effects of trilinolein on angiotensin II-induced cardiomyocyte hypertrophy. Cultured neonatal rat cardiomyocytes were stimulated with angiotensin II, [3H]leucine incorporation and the h-myosin heavy chain promoter activity were examined. We also examined the effects of trilinolein on angiotensin II-induced intracellular reactive oxygen species generation. Trilinolein significantly inhibited angiotensin II-increased protein synthesis, h-myosin heavy chain promoter activity, and intracellular reactive oxygen species generation. Antioxidant N-acetylcysteine also decreased angiotensin II-increased protein synthesis and h-myosin heavy chain promoter activity. Furthermore, trilinolein and N-acetylcysteine decreased angiotensin II- or hydrogen peroxide (H2O2)-activated mitogen-activated protein kinases (MAPKs) phosphorylation, and activator

protein-1 (AP-1)- [or nuclear factor-nB (NF-nB)]-reporter activities. These data indicate that trilinolein inhibits angiotensin II-induced cardiomyocyte hypertrophy and h-myosin heavy chain promoter activity via attenuation of reactive oxygen species generation.

Key Words: Angiotensin II; Trilinolein; Reactive oxygen species

報告內容：

前言、

Trilinolein, isolated from the traditional Chinese herb Sanchi (Panax notoginseng) (Hong et al., 1993), has been used in treating circulatory disorders among the Chinese for hundreds of years. The myocardial protective effect of trilinolein is proposed to be related to an antioxidant effect through potentiation of superoxide dismutase (Chan et al., 1997). However, the cellular and molecular mechanisms of the protective effect of trilinolein in the heart have not been elucidated.

研究目的、

The aims of this study, therefore, were to investigate the effect of trilinolein on the angiotensin II-induced cardiomyocyte hypertrophy and to identify signaling protein kinase cascades that may be responsible for the putative effect of trilinolein. In the present study, we clearly demonstrate that trilinolein inhibits angiotensin II-induced hypertrophy and h-myosin heavy chain gene expression via attenuation of reactive oxygen species generation in cardiomyocytes.

文獻探討、

Trilinolein, isolated from the traditional Chinese herb Sanchi (Panax notoginseng) (Hong et al., 1993), has been used in treating circulatory disorders among the Chinese for hundreds of years. It is a triacylglycerol with linoleic acid as the only fatty acid residue in all esterified positions of glycerol. Trilinolein has various beneficial effects, including reducing thrombogenicity, erythrocyte deformability and arrhythmias, and displaying antioxidant effects in various experimental models (Chan et al., 2002). The antioxidant effect of trilinolein had a concentration–response curve similar to a-tocopherol (Chan et al., 1996b). The myocardial protective effect of trilinolein is proposed to be related to an antioxidant effect through potentiation of superoxide dismutase (Chan et al., 1997). However, the cellular and molecular mechanisms of the protective effect of trilinolein in the heart have not been elucidated. Angiotensin II is an important humoral factor responsible for cardiomyocyte hypertrophy (Wollert and Drexler, 1999), and is associated with the induction of immediate-early genes, such as c-fos (van Wamel et al., 2001), which is followed by activation of fetal-type genes such as h-myosin heavy chain gene (Yue et al., 2000). The recapitulation of fetal isoforms of cardiac genes (i.e., h-myosin heavy chain) has historically been used as an indicator of cardiac hypertrophy (Yue et al., 2000). Mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 have been shown to be implicated in the hypertrophic response of cardiomyocytes to angiotensin II (Shih et

al., 2001; Thomas et al., 2002). Recent evidence indicates that reactive oxygen species function as intracellular messengers to modulate signaling pathways (Cheng et al., 1999; Hirotani et al., 2002). Transcriptional factors such as activator protein-1 (AP-1) (Cheng et al., 2003) and nuclear factor-nB (NF-nB) are reported to be redox-sensitive (Higuchi et al., 2002) and involved in cardiomyocyte hypertrophy (De Keulenaer et al., 2002; Omura et al., 2002; Purcell et al., 2001). Moreover, several studies reported an increase in reactive oxygen species generation after angiotensin II treatment in cardiomyocytes, and inhibition of reactive oxygen species generation by antioxidants leading to the abolishment of cardiomyocyte enlargement (Hirotani et al., 2002; Nakamura et al., 1998). Our previous study demonstrated that angiotensin II activates the ERK pathway and increasing the hmyosin heavy chain promoter activity in part via the generation of reactive oxygen species in cardiomyocytes (Shih et al., 2001). However, no studies exist that address the interference of trilinolein with angiotensin II-induced signaling pathways in cardiomyocytes.

研究方法、

Materials

The chimeric construct h-myosin heavy chain-chloramphenicol acetyltransferase (h-myosin heavy chain-CAT) contained a 1.3 kb HindIII–PstI fragment of the 5V-flanking sequence of h-myosin heavy chain gene linked to the prokaryotic chloramphenicol acetyltransferase (CAT) reporter gene (Wang et al., 1992). PBLCAT2 (containing CAT reporter gene with its promoter) and PBLCAT3 (containing CAT gene only) were constructed as previously described (Cheng et al., 1999). The plasmid AP-1-Luc or NF-nB-Luc containing the firefly luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1 or NF-kB binding element was from Stratagene (La Jolla, CA, USA). 2`,7`-Dichlorofluorescin diacetate was obtained from Serva (Heidelberg, Germany). Hydrogen peroxide was purchased from Acros Organics (Pittsburgh, PA, USA). Angiotensin II, trilinolein, N-acetylcysteine, and all other chemicals of reagent were obtained from Sigma (St. Louis, MO, USA).

Culture of cardiac myocytes

Primary cultures of neonatal rat ventricular myocytes were prepared as previously described (Cheng et al., 1999). Briefly, ventricles from 1- to 2-day-old neonatal Sprague–Dawley rats were cut into chunks of approximately 1 mm^3 using scissors and subjected to trypsin (0.125%, Gibco) digestion in phosphate-buffered saline (PBS). Trypsin digested cells were collected by centrifugation at 1200 rpm for 5 min. The cell pellet was re-suspended in a medium containing 80% F10 nutrient mixture, 20% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) and then plated into a Petri dish. The non-attached myocytes in the medium were collected and then plated on culture dishes (10- or 3-cm diameter) with cell density at $1x10^7$ cells/10-cm dish or $2x10^6$ cells/3-cm dish. After 2 days in culture, cells were transferred to medium containing 90% Dulbecco's modified Eagle's medium (DMEM) nutrient mixture, 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Myocytes cultures obtained were >80% pure as revealed by their contractile characteristics under light microscopy. Serum-containing medium from these cultured myocytes was replaced with serum-free medium

and exposed to agents as indicated. This study protocol complies with European Community guidelines for the use of experimental animals and was approved by our institutional ethics committee.

Protein synthesis measurement ([^{3}H]leucine incorporation)

To measure synthesis of new protein, cardiomyocytes cultured in six-well plates were incubated with 1.0 μCi/ml [^{3}H]leucine in serum-free medium. After addition of agent indicated, cells were harvested by incubation at 4 ◦ C with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH, and radioactivity was determined by scintillation counting.

Transfections

Cardiac cells were transiently transfected with different expression vectors by the calcium phosphate method as previously described (Cheng et al., 1999). DNA concentration for all samples was adjusted to equal amount in each experiment. Briefly, cardiomyocytes ($1x10^7$ cells/10-cm diameter dish or $2x10^6$ cells/3-cm diameter dish) were maintained in culture for 48 h prior to transfection. The indicated expression vectors were mixed with calcium phosphate, and immediately added to the cardiomyocyte cell culture. After incubation for 5 h, cells were then washed three times with PBS and incubated with 10% serumDMEM. After 24 h, cells were washed with serum-free medium and incubated in serum-free medium for an additional 24 h. Cells were then treated with different agents. To correct for transfection efficiency, 5 μg of pSV–galactosidase plasmid, which contains a beta-galactosidase gene driven by the simian virus 40 promoter and enhancer, was cotransfected into cells.

Chloramphenicol acetyltransferase (CAT) assays and b-galactosidase assays

The CAT and h-galactosidase assays were performed. The relative CAT activity was determined by normalizing the CAT value to its respective h-galactosidase activity. Cotransfected beta-galactosidase activity was observed to vary by less than 10% within a given experiment and was not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) were included in every assay.

Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species was measured by previously described method (Shih et al., 2001). Prior to the chemical or angiotensin II treatment, cardiomyocytes were incubated in culture medium containing dichlorofluorescein diacetate of 30 μM for 1 h to establish a stable intracellular level of the probe. The same concentration of dichlorofluorescein diacetate was maintained during the chemical or angiotensin II treatment. Subsequently, the cells were washed with PBS, removed from Petri dishes by brief trypsin digestion, and then measured for the dichlorofluorescein fluorescence intensity. Dichlorofluorescein diacetate penetrating the cells is initially converted by cellular esterase, and is in turn oxidized to dichlorofluorescein in the presence of reactive oxygen species. The dichlorofluorescein fluorescence intensity of the cells is an index of intracellular levels of reactive oxygen species, and it can be determined by fluorescence spectrophotometry with excitation and emission wavelengths at 475 and 525 nm, respectively. The cell number in each sample was counted and utilized to normalize the fluorescence intensity of dichlorofluorescein.

Western blot analysis

Rabbit polyclonal anti-phospho-specific p38 MAPK, anti-phospho-specific extracellular signal-regulated kinases (ERK), and anti-phospho-specific c-Jun N-terminal kinase (JNK) antibodies were purchased from New England Biolabs (Beverly, MA, USA). Anti-ERK, anti-p38 MAPK, and anti-JNK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western blot analysis was performed as previously described (Cheng et al., 2001).

Luciferase assay

Cardiomyocytes plated on 3-cm-diameter culture dishes were transfected with the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc) or NF-nB binding sites (NF-nB-Luc) (Strategene, La Jolla, CA, USA). After incubation for 24 h in serum-free DMEM, myocytes were cultured under different treatments as indicated for 48 h. Myocytes were assayed for luciferase activity with a luciferase reporter assay kit (Strategene). The firefly luciferase activities as AP-1 or NF-nB transcriptional activities were normalized for transfection efficiency to its respective h-galactosidase activity and expressed as relative activity to control

Statistical Analysis

Results are expressed as mean $\pm$ SEM of at least three experiments unless designated otherwise. Statistical analysis was performed using analysis of variance (ANOVA) and Student's t test as appropriate. A value of $p<0.05$ was considered to be statistically significant.

結果與討論（含結論與建議）、

Result

Effect of trilinolein on angiotensin II-increased [^{3}H]leucine incorporation and b-myosin heavy chain promoter activity

The effect of angiotensin II on protein synthesis was analyzed by measurement of [^{3}H]leucine incorporation into the cardiomyocytes. Cardiomyocytes cultured in serum-free condition were treated with angiotensin II (1, 10, 100, and 1000 nM) for 48 h. Angiotensin II (10, 100, and 1000 nM) significantly increased protein synthesis in cardiomyocytes (see Fig. 1A). Trilinolein was solubilized in dimethyl sulfoxide (DMSO) at 10 mM stock. No significant difference was observed between control and vehicle-[DMSO (0.1%)]-treated groups on the protein synthesis in cardiomyocytes (data not shown). As shown in Fig. 1B, cardiomyocytes were treated with angiotensin II (100 nM) in the absence or presence of different concentrations of trilinolein (0.01–10 μM). Trilinolein inhibited angiotensin II-increased [^{3}H]leucine incorporation in a concentration-dependent manner in cardiomyocytes (see Fig. 1B). We then examined the effect of trilinolein on angiotensin II-increased beta-myosin heavy chain promoter activity. As shown in Fig. 1C, treatment with angiotensin II (100 nM) for 48 h increased beta-myosin heavy chain promoter activity. Trilinolein (1 and 10 μM) significantly inhibited angiotensin II-increased beta-myosin heavy chain promoter activity (see Fig. 1C). These data indicate that trilinolein inhibits angiotensin II-increased protein synthesis and beta-myosin heavy chain promoter activity in cardiomyocytes.

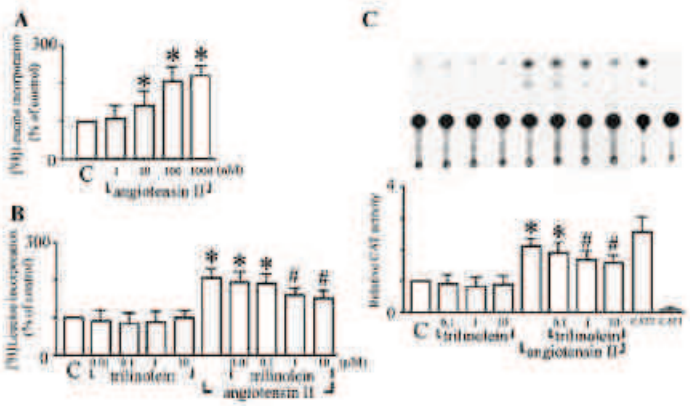

Fig. 1. Effect of trilinolein on angiotensin II-induced protein synthesis and β-myosin heavy chain promoter activity. (A) Different concentration of angiotensin II (1–1000 nM) on protein synthesis. (B) Effect of trilinolein (0.01–10 μM) on angiotensin II-induced protein synthesis. Cardiomyocytes were pretreated with trilinolein (0.01–10 μM) for 30 min and then stimulated with angiotensin II (100 nM). (C) Effect of trilinolein (0.01–10 μM) on angiotensin II-induced β-myosin heavy chain promoter activity. Cardiomyocytes were pretreated with trilinolein (0.01–10 μM) for 30 min and then stimulated with angiotensin II (100 nM). C (control), no drugs; CAT2 and CAT3 are shown as the positive and negative controls. CAT activities are shown as %incorporation after normalizing to that of β-galactosidase activities. Data are represented as fold increase relative to control groups. The results are shown as mean ± S.E.M. *$P < 0.05$ vs. control; *$P < 0.05$ vs. angiotensin II alone. The experiment was repeated three times with reproducible results.

Trilinolein inhibits angiotensin II-increased intracellular reactive oxygen species levels

Our previous study demonstrated that angiotensin II increases the generation of reactive oxygen species in cardiomyocytes (Shih et al., 2001). We next examined whether trilinolein prevents the angiotensin II-increased intracellular reactive oxygen species generation. Cardiomyocytes were treated with trilinolein (0.01, 0.1, 1, and 10 μM) in the absence or presence of angiotensin II (100 nM). The addition of trilinolein (1 and 10 μM) to cultured cardiomyocytes significantly inhibited angiotensin II-induced reactive oxygen species levels as measured after angiotensin II treatment for 1 h (see Fig. 2A). The pretreatment of trilinolein (10 μM) or N-acetylcysteine (10 mM) to cultured cardiomyocytes also significantly inhibited angiotensin II or hydrogen peroxide (H_2O_2)-induced reactive oxygen species levels (see Fig. 2B). These findings clearly demonstrate that trilinolein inhibits angiotensin II-increased intracellular reactive oxygen species levels in cardiomyocytes.

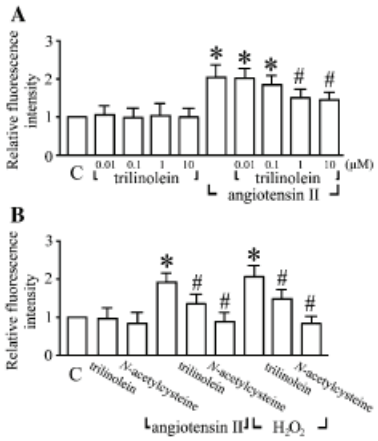

Fig. 2. Effect of trilinolein on angiotensin II-induced reactive oxygen species generation. (A) Effect of trilinolein (0.01–10 μM) on angiotensin II-induced reactive oxygen species generation. Angiotensin II-increased intracellular reactive oxygen species levels were revealed by fluorescent intensities of dichlorofluorescein. (B) Cardiomyocytes from either control (C; column 1) or treated with 10 μM trilinolein or 10 mM *N*-acetylcysteine in the absence or presence of angiotensin II (100 nM) or H_2O_2 (25 μM) for 1 h. Fluorescence intensities of cells are shown as relative intensity of experimental groups compared to untreated control cells. The results show mean ± S.E.M. *$P < 0.05$ vs. control; #$P < 0.05$ vs. angiotensin II (100 nM) treated cells. The experiment was repeated six times with reproducible results.

Trilinolein inhibits angiotensin II-activated MAPKs phosphorylation in cardiomyocytes

We further investigated whether trilinolein inhibits MAPKs pathway in angiotensin II-treated cardiomyocytes. We examined the phosphorylation of ERK, JNK, and p38 in cardiomyocytes exposed to angiotensin II (100 nM) in the presence of trilinolein (10 μM) or NAC (10 mM). As shown in Fig. 3A-C, cardiomyocytes exposure to angiotensin II (100 nM) or H_2O_2 (25 μM) for 30 min activated the phosphorylation of ERK, JNK, and p38. However, cardiomyocytes pretreated with trilinolein or N-acetylcysteine showed significantly decreased angiotensin II- or H_2O_2-induced ERK, JNK, and p38 phosphorylation. These findings imply that trilinolein inhibits angiotensin II-activated MAPKs signaling pathway in cardiomyocytes.

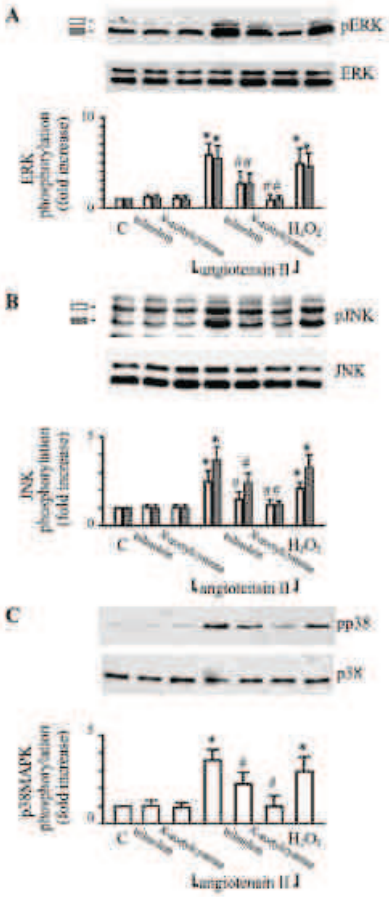

Fig. 3. Effect of trilinolein on angiotensin II-increased MAPKs phosphorylation. (A–C) Effect of trilinolein on angiotensin II-induced phosphorylation of ERK, JNK, and p38MAPK. Cells were preincubated with either the trilinolein (1 μM), or *N*-acetylcysteine (10 mM) for 30 min and stimulated with angiotensin II (100 nM) or H_2O_2 (25 M) for 30 min. Phosphorylation of ERK, JNK, or p38MAPK was detected by Western blotting using anti-phospho-ERK, anti-phospho-JNK, and anti-phospho-p38MAPK antibodies. Both trilinolein and *N*-acetylcysteine inhibited angiotensin II-induced activation of ERK, JNK, or p38MAPK. Phosphorylation of ERK, JNK, or p38MAPK was detected, and densitometric analyses were performed. The results are shown as mean ± S.E.M. ($n=4$ per group). $^{*}P<0.05$ vs. control (Student's *t*-test); $^{\#}P<0.05$ vs. angiotensin II (or H_2O_2) alone (ANOVA).

Trilinolein inhibits angiotensin II-increased AP-1 or NFkB binding activity in cardiomyocytes
We evaluated the effects of trilinolein on AP-1 or NF-kB activations which are involved in angiotensin II-induced reactive oxygen species-mediated signaling pathways (Shih et al., 2001). The effects of trilinolein on angiotensin II-induced AP-1 or NF-kB functional activity were assessed in a reporter gene assay. Angiotensin II had no effect on luciferase activity of the background vector not containing AP-1 (or NF-kB) binding site (see Fig. 4A,C). Angiotensin II significantly increased both AP-1- and NF-kB-luciferase activities in a concentration-dependent

manner (see Fig. 4A,C). These results indicated that angiotensin II activated AP-1 and NF-kB. We next examined the effects of trilinolein and N-acetylcysteine on AP-1 or NF-kB activities. Either trilinolein (10 μM) or N-acetylcysteine (10 mM) significantly attenuated angiotensin II- or H_2O_2-induced AP-1 or NF-kB promoter activation (see Fig. 4B,D). These results suggest that trilinolein inhibits angiotensin II-increased AP-1 or NF-kB activation.

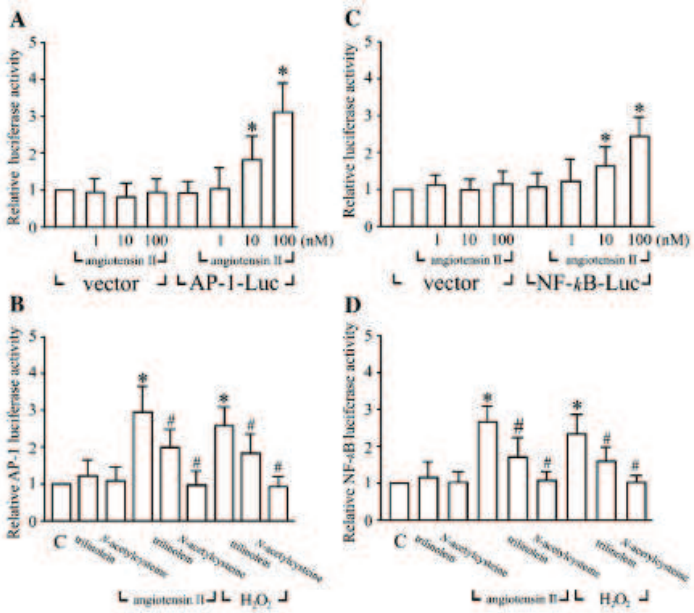

Fig. 4. Trilinolein inhibits the transcriptional activation of AP-1 or NF-κB in cardiomyocytes treated with angiotensin II. (A) Cardiomyocytes, transfected with vector or AP-1-Luc, were treated for 48 h with indicated concentrations of angiotensin II. Luciferase activity was expressed as relative activity to untreated controls (column 1). Values are mean ± S.E.M. of the data for three experiments performed in triplicate. *P < 0.05 vs. untreated. (B) Cardiomyocytes, transfected with AP-1-Luc, were incubated for 48 h with no drug, 10 μM trilinolein, or 10 mM *N*-acetylcysteine in the absence or presence of angiotensin II (100 nM) or H_2O_2 (25 μM). *P < 0.05 vs. untreated. #P < 0.05 vs. angiotensin II (or H_2O_2) alone (ANOVA). (C) Cardiomyocytes, transfected with vector or NF-κB-Luc, were treated for 48 h with indicated concentrations of angiotensin II. Luciferase activity was expressed as relative activity to untreated controls. Values are mean ± S.E.M. of the data for three experiments performed in triplicate. *P < 0.05 vs. untreated. (D) Cardiomyocytes, transfected with NF-κ B-Luc, were incubated for 48 h with either no drug, 10 μM trilinolein, or 10 mM *N*-acetylcysteine in the absence or presence of angiotensin II (100 nM) or H_2O_2 (25 μM). *P < 0.05 vs. untreated. #P < 0.05 vs. angiotensin II (or H_2O_2) alone (ANOVA).

Trilinolein inhibits angiotensin II-increased protein synthesis and beta-myosin heavy chain promoter activity via attenuation of reactive oxygen species generation

To further determine whether trilinolein affects the angiotensin II-induced protein synthesis and h-myosin heavy chain promoter activity via attenuation of reactive oxygen species generation, the effects of trilinolein on protein synthesis and h-myosin heavy chain promoter activity were examined under H_2O_2 stimulation. As demonstrated in Fig. 5A, neither trilinolein nor N-acetylcysteine alone had an effect on the basal protein synthesis. However, cardiomyocytes treated with angiotensin II (100 nM) or H2O2 (25 μM) for 48 h led to the increase in protein synthesis, as compared to unstimulated control cells (see Fig. 5A). In the presence of trilinolein (or N-acetylcysteine), angiotensin II- or H_2O_2- increased protein synthesis was significantly inhibited. Furthermore, neither trilinolein nor N-acetylcysteine alone had an effect on the basal h-myosin heavy chain promoter activity (see Fig. 5B). However, cardiomyocytes treated with angiotensin II (100 nM) or H_2O_2 (25 μM) for 48 h led to the increase in CAT activity, as compared with unstimulated control cells (see Fig. 5B). In the presence of trilinolein (or N-acetylcysteine), angiotensin II- or H_2O_2-increased beta-myosin heavy chain promoter activities were significantly inhibited. These data implicate that trilinolein inhibits the angiotensin II-induced protein synthesis and hmyosin heavy chain promoter activity via attenuation of reactive oxygen species generation in cardiomyocytes.

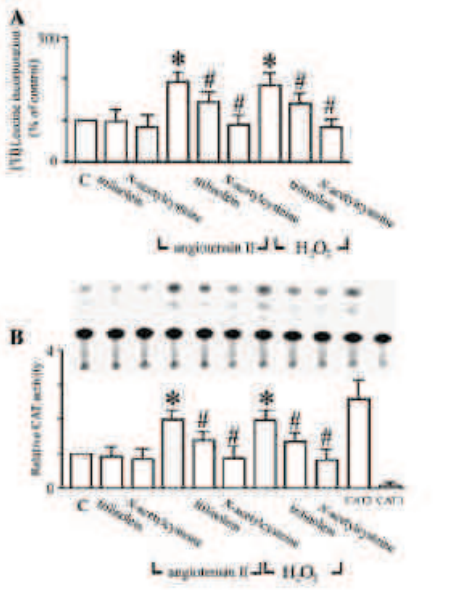

Fig. 5. Trilinolein modulates angiotensin II-increased protein synthesis and β-myosin heavy chain promoter activity. (A) Trilinolein modulates angiotensin II (or H_2O_2)-increased protein synthesis. (B) Trilinolein modulates angiotensin II (or H_2O_2)-increased β-myosin heavy chain promoter activity. Cardiomyocytes were transfected with 15 g of β-myosin heavy chain-*CAT* chimeric gene. Some cells were pretreated with trilinolein or *N*-acetylcysteine for 30 min. Cardiomyocytes were then treated with angiotensin II (10 nM) or H_2O_2 (25 μM) for 48 h. CAT2 and CAT3 are shown as positive and negative controls of a CAT assay system. The results are show as mean ± S.E.M. $^*P < 0.05$ vs. control; $^\#P < 0.05$ vs. angiotensin II alone. The experiment was repeated three times with reproducible results.

Discussion

Cardiac hypertrophy can be induced by pressure or volume overload, injury, or neurohormonal activation. In its early stages, hypertrophy is a compensatory response, but if prolonged, the heart may undergo a transition to heart failure. Therefore, to prevent the process of cardiac hypertrophy induced by extracellular signals is important for any proposed therapy to regulate the myocardial hypertrophic response. The major outcome of this work is that trilinolein inhibits angiotensin II-induced hypertrophy and h-myosin heavy chain gene expression via its ability to attenuate reactive oxygen species generation in cultured neonatal rat cardiomyocytes. These findings that trilinolein inhibits angiotensin II-induced protein synthesis and beta-myosin heavy chain gene expression are supported by the following observations. First, trilinolein inhibited angiotensin II-increased protein synthesis and beta-myosin heavy chain promoter activity in cultured cardiomyocytes. Second, trilinolein decreased angiotensin II-generated intracellular reactive oxygen species levels. Third, either trilinolein or antioxidant N-acetylcysteine inhibited the MAPK phosphorylation, AP-1 (or NFkB) activation, h-myosin heavy chain promoter activity, and protein synthesis induced by angiotensin II or H_2O_2.

Trilinolein has shown various beneficial effects and antioxidant effects in experimental models (Chan et al., 2002). Pre-treatment with the antioxidants trilinolein and various other saturated and unsaturated lipid compounds (0.01 nM–1 μM) quenched free radical-generated luminal chemiluminescence following the addition of phorbol myristic acetate in medium containing leukocytes (Chan et al., 1996a). Trilinolein showed concentration dependent antioxidant activity at concentrations between 0.1 nM and 1 μM, with a maximal free radical reduction of 48.0%, whereas trolox, a water-soluble analogue of vitamin E, showed a maximal mean reduction of 39.2% (Chan et al., 1996a). The antioxidant effect had a concentration–response curve similar to

alpha-tocopherol (Chan et al., 1996b). In rat hearts pre-treated with 0.1 μM trilinolein and subjected to 60 min of hypoxia, Cu2 +.Zn2 +-superoxide dismutase activity was increased compared with the baseline and compared with hearts subjected to 60 min of hypoxia without trilinolein (Chan et al., 1997). Pre-treatment with trilinolein was associated with better preservation of left ventricular function during hypoxia and a more rapid return to recovery during the subsequent normoxic perfusion. The myocardial protective effect of trilinolein may be related to an antioxidant effect through potentiation of superoxide dismutase, particularly Cu2 +.Zn2 +-superoxide dismutase during hypoxia (Chan et al., 1997). However, previous studies showed a role for trilinolein at concentrations >0.1 μM (Chan et al., 1997). Because the trilinolein concentration in Sanchi is ~10–20 μM, it is plausible that the effect of trilinolein described at such concentrations would be observed in vivo.

However, we would suggest that trilinolein activities should be evaluated at physiologic concentrations, i.e., ~1 μM. Furthermore, because the effects of trilinolein are observed after prolonged uptake of Sanchi, a long incubation time should be analyzed together with brief incubations. It is well known that angiotensin II causes hypertrophy of cardiomyocytes (Wollert and Drexler, 1999), and multiple intracellular pathways in angiotensin II signaling have been reported. Upon angiotensin II stimulation, reactive oxygen species are generated via a membrane bound NADPH oxidase and act as second messengers within cardiac cells (Sano et al., 2001). In particular, it has been demonstrated that activation of MAPK is redox-sensitive (Sano et al., 2001; Shih et al., 2001; Tanaka et al., 2001) and suppression of reactive oxygen species inhibits angiotensin II-induced hypertrophy (Hirotani et al., 2002; Nakamura et al., 1998). In the present study, trilinolein inhibited the phosphorylation of ERK, JNK, and p38. One possible explanation for the antihypertrophic effect of trilinolein in cardiomyocytes may thus be its ability to act as an antioxidant. However, further experiments will be necessary to identify the detailed mechanisms by which trilinolein exerts its antigrowth effects in cardiomyocytes.

Transcriptional regulation is critical for molecular signaling in cellular response involving interactions between the proteins of the general transcriptional apparatus and proteins that bind gene-specific enhancer elements. Our results also indicate that trilinolein inhibits angiotensin II-induced AP-1 or NF-kB activation. Possibly, both AP-1 and NF-kB might play a key role in cardiomyocyte hypertrophy (Purcell et al., 2001; Takemoto et al., 1999). It has been reported that induction of the brain natriuretic peptide promoter by mechanical strain depends on activation of NF-kB in cardiomyocytes (Liang and Gardner, 1999). Direct inhibition of AP-1 activity also significantly decreased cardiac hypertrophy (Omura et al., 2002). The inhibitory effect of the trilinolein on angiotensin II-induced AP-1 or NF-kB activation suggested that scavenging of angiotensin II-induced reactive oxygen species by trilinolein leads to inhibition of AP-1 or NF-kB.

We have demonstrated that angiotensin II induces the expression of beta-myosin heavy chain gene in part via the generation of reactive oxygen species in cardiomyocytes (Shih et al., 2001). In the present work, we provide evidence suggesting an inhibitory effect of trilinolein on angiotensin II-induced hypertrophy and h-myosin heavy chain gene expression in cardiomyocytes. We also demonstrated the antioxidant effect of trilinolein on angiotensin II-increased reactive

oxygen species generation. In further Western blot analysis, we found that MAPK phosphorylation was increased by angiotensin II and inhibited by trilinolein or the antioxidant N-acetylcysteine, suggesting that it could be a potential site of action for trilinolein in cardiomyocytes. However, any site upstream of MAPK also could be implicated.
The present study delivers important new insights to the molecular mechanisms of action of trilinolein in cardiomyocytes. For the first time we have definitively shown that trilinolein significantly influences important angiotensin II-activated pathways in cardiomyocytes. Moreover, they suggest that trilinolein acts predominantly via the MAPK pathway to reduce angiotensin II-mediated cardiomyocyte hypertrophy. It is plausible that the angiotensin II signaling pathway consists of redox-sensitive steps and trilinolein treatment could modulate the redox state of the cell. In summary, our data show that trilinolein inhibits angiotensin II-induced hypertrophy and h-myosin heavy chain gene expression in part via attenuation of reactive oxygen species generation and the suppression of MAPK pathway in cardiomyocytes. These findings support the therapeutic potential of trilinolein in the prevention of cardiomyocyte hypertrophy that may contribute to the proposed beneficial effects of trilinolein in cardiovascular disease.

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計畫成果自評、

研究內容與原計畫完全相符、順利達成預期目標、研究成果之學術價值甚高、部分結果已投稿至國外 SCI 學術期刊發表。