The Inhibitory Effect of Trilinolein on Norepinephrine-Induced β-Myosin Heavy Chain Promoter Activity, Reactive Oxygen Species Generation, and Extracellular Signal-Regulated Kinase Phosphorylation in Neonatal Rat Cardiomyocytes

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Key Words
Norepinephrine • Trilinolein • Reactive oxygen species • Extracellular signal-regulated kinase • β-Myosin heavy chain gene

Abstract
The myocardial protective effects of trilinolein, isolated from the traditional Chinese herb Sanchi (Panax notoginseng), are thought to be related to its antioxidant activity. However, the intracellular mechanism underlying the protective effect of trilinolein in the heart remains unclear. In the present study, we investigated the effect of trilinolein on norepinephrine (NE)-induced protein synthesis in cardiomyocytes. Cultured neonatal rat cardiomyocytes were stimulated with NE, then protein content, [3H]-leucine incorporation, and β-myosin heavy chain (β-MyHC) promoter activity were examined. The effect of trilinolein on NE-induced intracellular reactive oxygen species (ROS) generation was measured with a redox-sensitive fluorescent dye (2',7'-dichlorofluorescin diacetate) and extracellular signal-regulated kinase (ERK) phosphorylation by Western blotting. Trilinolein inhibited NE-increased protein synthesis, β-MyHC promoter activity, and intracellular ROS. Both trilinolein and the antioxidant, N-acetyl-cysteine, decreased NE- and H2O2-induced protein synthesis, β-MyHC promoter activity, and ERK phosphorylation. These data indicate that trilinolein inhibits NE-induced protein synthesis via attenuation of ROS generation in cardiomyocytes.

Introduction
Trilinolein, isolated from the traditional Chinese herb Sanchi (Panax notoginseng) [14], has been used to treat circulatory disorders in Chinese patients for hundreds of years. Trilinolein is a triacylglycerol with linoleic acid as the only fatty acid residue in all of the esterified positions...
of glycerol. The chemical structure is given in figure 1. Trilinolein has various beneficial effects, including the ability to reduce thrombogenicity, erythrocyte deformability, and arrhythmias, and it has been shown to possess antioxidant activity in various experimental models [7]. The antioxidant effects of trilinolein produce a concentration-response curve similar to that of α-tocopherol [5]. The myocardial protective effect of trilinolein is thought to be related to antioxidant activity through potentiation of superoxide dismutase (SOD) [6]. However, the cellular and molecular mechanisms of the protective effect of trilinolein in the heart are unknown.

Cardiac hypertrophy is an adaptive response of the heart to cardiovascular diseases [11]. Although the hypertrophic response appears to be initially a compensatory mechanism that can increase cardiac output, sustained hypertrophy may cause dilated cardiomyopathy, heart failure and even sudden death. Cardiac sympathetic stimulation is known to occur in the earlier stage of congestive heart failure [22]. However, sympathetic stimulation, which is similar to what is produced by the presence of norepinephrine (NE), has been previously reported to induce cardiac hypertrophy [13, 17, 26]. Despite the diverse stimuli that lead to cardiac hypertrophy, a prototypical final molecular response of cardiomyocytes to hypertrophic signals has recently been reported to involve an increase in protein synthesis and reexpression of the β-myosin heavy chain (β-MyHC) embryonic cardiac gene [23]. Numerous studies have proposed that the extracellular signal-regulated kinase (ERK) cascade is involved in the hypertrophic response [2, 18, 25]. Initial findings indicated that G-protein-coupled receptor hypertrophic agonists stimulate ERK activation [10]. Subsequent studies focusing on the role of the Ras/Raf/ERK pathway have provided considerable support for this hypothesis [1, 24, 30]. Recent evidence indicates that reactive oxygen species (ROS) may function as intracellular messengers to modulate signaling pathways [9, 13]. Studies have demonstrated that NE causes hypertrophy in part via the generation of ROS in cardiomyocytes [13, 26, 29]. However, whether trilinolein scavenges the NE-induced intracellular ROS and thereby affects the ERK pathway as well as the subsequent cardiac hypertrophic responses remains unclear. In this study, we clearly demonstrate that trilinolein can inhibit NE-induced ERK phosphorylation and β-MyHC gene expression in neonatal cardiomyocytes. These effects could be associated with its ability to attenuate the generation of intracellular ROS.

Materials and Methods

Materials

The chimeric construct, β-MyHCCAT, contains a 1.3-kb Hind III-Pst I fragment of the 5′-flanking sequence of the β-MyHC gene linked to the prokaryotic chloramphenicol acetyltransferase (CAT) reporter gene [28]. PBLCAT2 (containing the CAT reporter gene with its promoter) and PBLCAT3 (containing the CAT gene only) were constructed as previously described [9]. 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, Ore., USA). H2O2 was purchased from Acros Organics (Pittsburgh, Pa., USA). NE, trilinolein, N-acetyl-cysteine (NAC), and all other chemicals were reagent grade and were obtained from Sigma (St. Louis, Mo., USA).

Culture of Cardiomyocytes

Primary cultures of neonatal rat ventricular myocytes were prepared as previously described [9]. Briefly, ventricles from 1- to 2-day-old neonatal Sprague-Dawley rats were cut into pieces of approximately 1 mm³ using scissors and subjected to trypsin (0.125%, Gibco) digestion in phosphate-buffered saline (PBS). Trypsin-digested cells were collected by centrifugation at 1,200 rpm for 5 min. The cell pellet was resuspended in medium containing 80% F10 nutrient mixture, 20% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) and plated into a Petri dish. Nonattached myocytes in the medium were collected and plated on 10-cm- or 3-cm-diameter culture dishes at cell densities of 1 × 10⁶ or 2 × 10⁵ cells/dish, respectively. After 2 days in culture, cells were transferred to medium containing a 90% DMEM nutrient mixture, 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Myocyte cultures thus 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. All procedures used in the present study comply with the ethical standards recommended by the Helsinki Declaration and conform to the guidelines approved by our institutional committee on experimental animals.

Protein Content

Total protein content of myocytes was measured by the bicinchoninic acid method according to the manufacturer’s instructions (Pierce Chemical, Rockford, Ill., USA). Briefly, cells after treatment were washed twice with PBS, lysed with 200 μl of 0.15 M NaOH, and the protein concentration was determined.

Fig. 1. The chemical structure of trilinolein.