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Yangxueqingnao particles inhibit rat vascular smooth muscle cell proliferation induced by lysophosphatidic acid*

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Abstract: Objective: To observe the effect of Yangxueqingnao particles on rat vascular smooth muscle cell (VSMC) proliferation induced by lysophosphatidic acid (LPA). Methods: The amount of ^{3}H-TdR (^{3}H-thymidine) admixed in cultured rat VSMC was measured and mitogen-activated protein kinase (MAPK) activity and lipid peroxidation end product malondialdehyde (MDA) content of the VSMC were assayed. Results: 1×10^{-9}, 1×10^{-8}, 1×10^{-7} mol/L LPA in a concentration dependent manner, induced the amount of ^{3}H-TdR admixed, MAP kinase activity, and MDA content of the cultured rat VSMC to increase. However, 5%, 10%, and 15% Yangxueqingnao serum preincubation resulted in a decrease of 23.0%, 42.0%, and 52.0% (P<0.01) respectively in the amount of ^{3}H-TdR admixed, a decline in VSMC MAP kinase activity of 13.9% (P<0.05), 29.6% (P<0.01), and 48.9% (P<0.01) respectively, and also, a decrease in MDA content of VSMC of 19.4%, 24.7%, and 43.2% (P<0.01) respectively, in the 1×10^{-7} mol/L LPA-treated VSMC. Conclusions: LPA activates the proliferation and lipid peroxidation of VSMC in a concentration dependent manner. The LPA-induced VSMC proliferation is related to the activity of MAP kinases, enzymes involved in an intracellular signalling pathway. The results of the present study showed that Yangxueqingnao particles can effectively inhibit LPA-induced VSMC proliferation, MAP kinase activation, and reduce lipid peroxidative lesion.

Key words: Yangxueqingnao particles, Lysophosphatidic acid, Vascular smooth muscle cell, Proliferation
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INTRODUCTION

Lysophosphatidic acid (LPA) is a kind of micromolecular lipidic substance involved in intercellular signalling. Several studies in the past few years indicated that LPA could induce the proliferation, migration, and phenotypic modulation of vascular smooth muscle cell (VSMC), and could also cause the intercellular space of vascular endothelial cell to extend, then facilitate development of arterial sclerosis, and so, is an important pathogenetic fact in the cerebral vascular accident (Hayashi *et al*., 2001).

Yangxueqingnao particles are mainly composed of Danggui, Chuanqiong, Baishao, and Xixing. In traditional Chinese medicine, they have the functions of promoting blood flow, removing blood stasis, nourishing blood to calm liver, and dredging the meridian passage. Several studies showed that Yangxueqingnao particles could also lower the elevated LPA level in brain tissue and plasma blood of mice under transient ischemia attack (TIA); and calm down neutral and acidic phospholipids disturbances (Niu *et al*., 2002). The authors are not aware of research on the inhibiting effect of Yangxueqingnao particles on the proliferation and phenotypic modulation of VSMC induced by lysophosphatidic acid (LPA). The present study is aimed at observing the effect of Yangxueqingnao particles on LPA-induced

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rat VSMC proliferation.

MATERIALS AND METHODS

Animals

Clean 200~250 g male Sprague-Dawley (SD) rats aged four weeks were obtained from the Experimental Animal Center, School of Medicine, Zhejiang University.

Main reagents

Dulbecco's Modified Eagle's Medium (DMEM): calf serum, fetal calf serum, and trypsin (GIBCOBRL Co. USA); alkaline phosphatase tagged α-SM-actin single antigen (Sigma, USA, 1:100); tritium-labelled thymin deoxyriboside (^{3}H-TdR, Shanghai Nucleus Technology Exploitation Co. of CAS, China); Yangxueqingnao particles (composed of Danggui, Chuanqiong, Baishao, and Xixing, produced by Tianjin Tianshili Groups, China).

Yangxueqingnao serum preparations

The SD rats were housed into a saline group of four (control serum group) and a Yangxueqingnao serum group of four in the experiment. Each rat was intragastrically administered treatment twice a day [1.2 g/(kg·d)], three days continuously (Li and Wu, 1999). An hour after the last administration, the rats were anesthetized with acetic aldehyde, and their blood was collected from the abdominal aorta by aspiration (the rats were fasted and given water only for 12 h before blood collection). The blood collected was placed undisturbed at room temperature for 4 h, then centrifuged and mixed with serum under the same conditions was finally packed separately and stored at −20 °C.

Isolation and incubation of rat VSMC

Tissue-sticking method was applied. Thoracic aorta of the rat was segregated under aseptic condition, placed into DMEM containing 15% fetal calf serum after it was split into pieces, and incubated undisturbed in 5% CO_2 at 37 °C in the incubator. Optical microscope, electron microscope, and α-SM-actin were used to identify and select the well-grown 4th~6th generations of VSMC to be used in the experiment.

Grouping

(1) Control group: only DMEM was added; (2) Normal rat serum group: 15% normal rat serum was added; (3) YXQN (Yangxueqingnao) serum group: 15% Yangxueqingnao serum was added; (4) LPA groups [LPAs were administered in accordance with Ren *et al*.(2003)]: LPAs at concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol/L were added to different samples respectively; (5) LPA+normal rat serum group: preincubated with 15% normal rat serum for 10 min, then LPA at concentration of 1×10^{-7} mol/L was added; (6) LPA+YXQN serum group: preincubated with 5%, 10%, and 15% Yangxueqingnao serum for 10 min respectively, then LPA at concentration of 1×10^{-7} mol/L was added.

Determination of ^{3}H-TdR admixed

Cell densities of the samples were adjusted with DMEM solution containing 10% Fluorescence correlation spectroscopy (FCS) to 1×10^8 cell/L, then the samples transferred to 96-well cell culture plate with 200 μl each well. After incubation of the samples for 48 h, the culture solutions were replaced with DMEM without serum, and after the samples were incubated again for 24 h, the culture solutions were replaced with experimental solution [experiment group (4 wells for each drug concentration)]; then 10% FCS and DMEM were added, and the incubations were resumed for 6~8 h before cell collections. ^{3}H-TdR was admixed to reach a final concentration of 1.85×10^7 Bq/L (Becquerels par/L). Cells were collected onto a vitric filter, and the pulse numbers per minute (connts/min) of each sample were measured by scintillometer.

MAP kinase activity assays

After incubation with different treatments for 24 h, the cells of all the groups above were collected and washed with cold extract. The mixture was centrifuged, 0.5 ml supernatant was collected for mixing with 5 g specific MAP kinase antibody (rabbit anti rat) and incubated at 4 °C for 4 h. The immunocomplex collected was then incubated on agar medium containing anti-rat antibody for 30 min. The precipitated protein obtained was added into 0.5 ml solvent and mixed equably, 150 μl of it was removed and mixed with 20 μl kinase buffer solution, then incubated at 25 °C for 25 min. One hundred μl of the final solution was removed for blotting on cellulose phosphate filter

paper, and the amount of myelin basic protein P admixed above the scintillation fluid was measured and expressed in nmol/g Pr (protein).

Measurement of MDA content

After incubation with different treatments for 24 h, the cells were collected and washed with cold PBS, then 0.2 ml 8.1% sodium dodecyl sulfate (SDS), 1.5 ml 20% acetic acid buffer solution (pH 3.5), 1.5 ml 1% total bile acid (TBA) thiobarbituric solution, and 1 ml distilled water were added in succession. The solution was mixed completely and heated in 90 °C water bath for 40~60 min, then withdrawn from the water-bath and allowed to cool down. Afterwards the mixture was centrifuged at 3500 r/min for 15 min and its optical density (Å) at 534 nm wavelength was measured by spectrophotometer. The MDA content was expressed in nmol/g Pr.

Data analysis

The results were expressed in mean±standard deviation; *t* test was applied for group comparison.

RESULTS

Effect of Yangxueqingnao serum on the amount of ^{3}H-TdR admixed in the rat VSMC treated with LPA

LPA at concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol/L all induced ^{3}H-TdR admixed in VSMC to increase to significantly greater amounts compared to control group ($P<0.01$), and the effects of which were in a concentration dependent manner. Fifteen percent normal rat serum and 15% Yangxueqingnao serum had no effect on the amount of VSMC ^{3}H-TdR admixed. Fifteen percent normal rat serum preincubation had no effect on the increased amount of VSMC ^{3}H-TdR admixed induced by 1×10^{-7} mol/L LPA. However, 5%, 10%, and 15% Yangxueqingnao serum preincubation resulted in a decrease of 23.0%, 42.0%, and 52.0% ($P<0.01$) respectively, in the amount of ^{3}H-TdR admixed in VSMC that treated with 1×10^{-7} mol/L LPA. The inhibition ratios showed dose-effect relationship (Table 1 and Fig.1).

Yangxueqingnao serum inhibited mitogen-activated protein kinase activation induced by LPA

LPA at concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol/L all induced MAP kinase activity to incr-

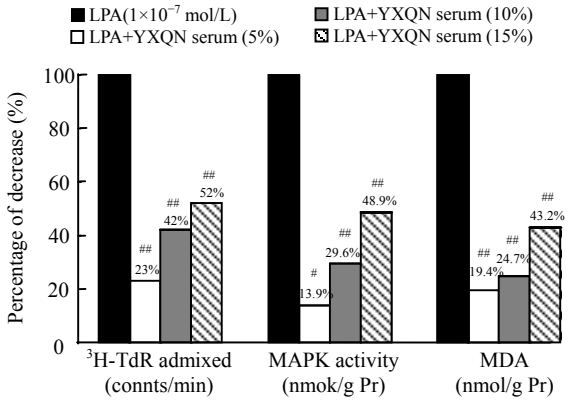

Fig.1 Effect of Yangxueqingnao (YXQN) serum on the amount of ^{3}H-TdR admixed, MAP kinase activity and MDA content. $^{\#}P<0.05$, vs LPA (1×10^{-7} mol/L) group; $^{\#\#}P<0.01$, vs LPA (1×10^{-7} mol/L) group

Table 1 Effect of Yangxueqingnao (YXQN) serum on the amount of ^{3}H-TdR admixed, MAP kinase activities, and MDA content increased in the LPA-treated rat VSMC ($\bar{x}\pm s$, $n=4$)

Group	^{3}H-TdR admixed (connts/min)	MAPK activity (nmol/g Pr)	MDA content (nmol/g Pr)
Control	826±112	53.12±19.32	5.32±1.09
Normal rat serum (15%)	767±131	61.15±20.72	5.73±0.86
LPA (1×10^{-9} mol/L)	1287±154**	263.23±32.12**	8.65±2.21**
LPA (1×10^{-8} mol/L)	1873±189**	342.35±28.43**	10.54±1.98**
LPA$^{▲}$ (1×10^{-7} mol/L)	2935±247**	466.18±43.36**	14.37±2.13**
LPA$^{▲}$+normal rat serum (15%)	2896±272**	489.22±38.79**	15.25±2.54**
YXQN serum (15%)	802±109	59.32±17.48	5.17±1.43
LPA$^{▲}$+YXQN serum (5%)	2259±178$^{**\#\#}$	401.34±26.44$^{**\#}$	10.43±1.95$^{**\#\#}$
LPA$^{▲}$+YXQN serum (10%)	1702±162$^{**\#\#}$	328.26±23.54$^{**\#\#}$	8.16±1.73$^{**\#\#}$
LPA$^{▲}$+YXQN serum (15%)	1408±137$^{**\#\#}$	238.63±26.21$^{**\#\#}$	6.82±1.59$^{**\#\#}$

**Significantly ($P<0.01$) higher compared to control group; $^{\#\#}$Significantly ($P<0.01$) lower compared to LPA (1×10^{-7} mol/L) group; $^{▲}$LPA at concentration of 1×10^{-7} mol/L

ease (P<0.01), and the effects of which were in a concentration dependent manner. Fifteen percent normal rat serum and 15% Yangxueqingnao serum had no effect on the VSMC MAP kinase activity (P>0.05). Fifteen percent normal rat serum preincubation had no effect on the VSMC MAP kinase activity stimulated by 1×10^{-7} mol/L LPA, but 5%, 15%, and 15% Yangxueqingnao serum preincubation led to a decline in MAP kinase activity of 13.9% (P<0.05), 29.6% (P<0.01), and 48.9% (P<0.01) respectively, in the 1×10^{-7} mol/L LPA-stimulated VSMC. The inhibition ratios showed dose-effect relationship (Table 1 and Fig.1).

Effect of Yangxueqingnao serum on MDA content in the VSMC

LPA at concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol/L all induced the MDA content to increase (P<0.01), and the effects of which were in a concentration dependent manner. Fifteen percent normal rat serum and 15% Yangxueqingnao serum had no effect on the MDA (P>0.05) in VSMC. Fifteen percent normal rat serum preincubation had no effect on the increased MDA concentration induced by 1×10^{-7} mol/L LPA. However, 5%, 10%, and 15% Yangxueqingnao serum preincubation caused the MDA concentration of VSMC treated with 1×10^{-7} mol/L LPA to decrease 19.4%, 24.7%, and 43.2% (P<0.01), respectively. The inhibition ratios showed dose-effect relationship (Table 1 and Fig.1).

DISCUSSION

Lysophosphatidic acid (LPA) is so far the structurally simplest and smallest phosphatide that has been found. It has hormone-like and growth-factor-like effects that in many instances its diverse biologic activity can affect cell growth, cell proliferation, cell differentiation, intracellular signal transmission and platelet aggregation in several aspects (Fueller *et al*., 2003). Recent studies revealed that LPA could induce phenotypic modulation of vascular smooth muscle cells (VSMC), stimulate VSMC of the arterial media to migrate into intima and proliferate, and also induce the contraction of vascular smooth muscle, thus lead to vessel lumen narrowing; besides, LPA could also cause the interspaces of vascular endothelial cells to increase to accelerate the occurrence of such process (Hayashi *et al*., 2001). Seewald *et al*.(1997) reported that LPA could promote in vitro growth of rabbit VSMC. Gennero *et al*.(1999) obtained similar result in human VSMC in vitro, and discovered the growth-factor-like effect of LPA that inhibited cell migration. Many studies indicated that LPA as a key factor in the atherosclerosis-triggered thrombosis is one of the core molecules in the pathophysiology of ischemia cardio-cerebral vascular diseases.

Yangxueqingnao particles are developed from the "Decoction of Four Drugs" which is one of the famous classical recipes in traditional Chinese medicine. It can improve cerebromeningeal microcirculation, inhibit platelet aggregation, and release vasospasm. Currently, the recipe is mostly used for treating headache, including migraine headache, neurogenic headache, etc. However, there were studies indicating that Yangxueqingnao particles could also lower the elevated LPA level in brain tissue and plasma blood of mice suffering transient ischemia attack (TIA), as well as calming down neutral and acidic phospholipids disturbances (Niu *et al*., 2002). The present study is aimed at exploring further the mechanism of the action of Yangxueqingnao particles, viz. at observing if Yangxueqingnao particles can inhibit the proliferation of VSMC induced by LPA likewise, and at clarifying fully the clinical application value of Yangxueqingnao particles.

Our study showed that LPA dose-dependently stimulated the amount of ^{3}H-TdR admixed, MAP kinase activity, and lipid peroxidation end product MDA content of VSMC to increase, which was accorded with conclusions reached in other studies (Niu *et al*., 2003; Ren *et al*., 2003). These parameters were not affected when the specimen was incubated with Yangxueqingnao serum alone, but as different concentrations of LPA were added separately, Yangxueqingnao could depress the effects of LPA in promoting cell proliferation, stimulating MAP kinase activity and causing MDA content of VSMC to increase in a concentration dependent manner. MAP kinase which is a key endocellular enzyme has been found lately to be correlated with cell proliferation, is the common pathway or crossing point of the nucleus reactions including cell proliferation, cell differentiation, etc., due to exocellular signal. Its activity could

represent cell proliferation level, i.e., stronger activity denotes higher proliferation level (Gennero *et al*., 1999). The main function of LPA is to stimulate proliferation and smooth muscle contraction. The present study suggest that Yangxueqingnao serum inhibits the LPA-induced proliferative effects partially by depress the VSMC's MAP kinase activation stimulated by LPA. This study finding that Yangxueqingnao serum could markedly reduce the cellular formation of lipid peroxidation products (MDA) induced by LPA, implies that the medicated serum has anti-lipid-peroxidation effect; but it remains unclear if such effect is one of its anti-proliferative effects.

In conclusion, this study indicates that Yangxueqingnao particles having inhibitive effect on smooth muscle cell proliferation induced by LPA, thereby may be antiosclerosis. This contributes will lead to wider application of Yangxueqingnao particles in clinical use, and further development of Chinese traditional medicine of our country.

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