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Full Length Research Paper

Regulatory role of RhoA/Rho kinase in the effect of fluvastatin on tumor necrosis factor-α induced expression of tissue factor

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To investigate the regulatory role of RhoA/Rho kinase in the effects of fluvastatin (Flu) on the tumor necrosis factor-α (TNF-α) induced expression of tissue factor (TF) in human umbilical vein endothelial cells (HUVECs) which may provide basis for the prevention of arterial thrombosis, HUVECs in logarithmic phase were divided into TNF-α group, Flu group and TNF-α+Flu group. enzyme-linked immunosorbent assay (ELISA), real time polymerase chain reaction (RT-PCR) and western blot assay were employed to detect the content of TF, messenger ribonucleic acid (mRNA) expression of TF and protein expressions of TF and activated RhoA, respectively. Then, angiotensin II (Ang II) and Y27632 were used to treat these cells which were then divided into control group, TNF-α+Ang II group, AngII group, TNF-α group and TNF-α+Y27632 group. Western blot assay was performed to detect the protein expression of TF. After TNF-α treatment, the content and mRNA and protein expressions of TF were markedly increased when compared with the control group (P<0.05). However, the content and mRNA and protein expressions of TF in Flu group were significantly lower than those in TNF-α group (P<0.05). Our results showed TNF-α could induce TF expression in HUVECs and activate RhoA, which, however, were inhibited by Flu. Ang II treatment could increase the TNF-α induced TF expression in HUVECs which however was inhibited Y27632. Flu could effectively inhibit the TNF-α induced protein and mRNA expressions of TF in HUVECs in which RhoA/Rho signaling pathway may play an important role.

Key words: Tissue factor, endothelial cells, fluvastatin, RhoA/Rho kinase, angiotensin II, Y27632.

INTRODUCTION

Studies have demonstrated that tissue factor (TF) is closely associated with the arterial thrombosi (Steffel et al., 2006; Wolfovitz, 2005). Multiple factors have been expression in endothelial cell (Mitchell et al., 2009; Steffel found to be involved in the regulation of TF synthesis and et al., 2005). Nowadays, increasing studies have been conducted to investigate the TF expression and its procoagulation activity in the treatment of arterial thrombosis (Mitroulis et al., 2011). Statins have proven to have anti-inflammatory, anti-oxidative and anti-thrombotic activities which are independent of its lipid-lowing activity (Liu et al., 2009; Blum and Shamburek., 2009; Haslinger-Löffler, 2008). Mevalonic acid (MVA) is an intermediate metabolite in the synthesis of cholesterol. Statins can competitively inhibit the rate-limiting enzyme of cholesterol synthesis when it also indirectly inhibits the synthesis of intermediate products such as isoprenoid in the MVA pathway. However, these intermediate products are important mediators involved in the cellular signal transduction and complex intercellular communication.

Additionally, these products can also act as lipid adhesion molecules during the post-transcriptional modification of small G proteins such as Rho. It has been revealed that Rho/Rho kinase is associated with the generation of TF (Nagata et al., 2002) and can regulate the expression of TF. The Rho/Rho kinase signaling

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pathway includes Rho protein, Rho kinase, myosin phosphatase, etc. Whether RhoA signaling pathway involves in the effect of Statins on TF expression is still unclear. The present study aimed to investigate the suppressive effects of fluvastatin (Flu) on the activity and protein and messenger ribonucleic acid (mRNA) expressions of TF as well as Rho activation in human umbilical vein endothelial cells (HUVECs) following tumor necrosis factor-α (TNF-α) treatment in which Angiotensin II (Rho kinase agonist) and Y27632 (Rho kinase antagonist) were used. This study was designed to study the role of RhoA/Rho signaling pathway in the anti-thrombosis of Flu.

MATERIALS AND METHODS

Cells and reagents

HUVECs were purchased from the Xiangya School of Medicine, Central South University, and human tissue factor ELISA kit and color development kit (Lot Number: OL 2980650) (AssayPro, USA) were from Shanghai Boatman Biotech. Goat anti-human TNF-α and TF monoclonal antibodies, HRPO conjugated goat anti-rat IgG, HRPO conjugated goat anti-chicken IgG, HRPO conjugated rabbit anti-goat IgG, Ang II, Y27632 (Shenzhen Jingmei, Biotech), cell lysis buffer, Taq DNA polymerase (50 u/ul), diethyl pyrocarbonate (DEPC), RevertAid^{TM} First St /cDNA Synthesis Kit (MBI, USA), WB Marker, reverse transcriptase kit (Fermentas, USA) and fluvastatin sodium powder (Novartis, Switzerland) were used in the present study.

Resuscitation and culture of HUVECs and detection of cell viability

The HUVECs at -80℃ were thawed in 37℃ and centrifuged. Then, these cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) complete medium containing 10% newborn fetal calf serum followed by incubation in an atmosphere with 5% CO_2 at 37℃. Cell growth was observed daily, and medium was changed every two days. After digestion with 0.25% trypsin, cells were passaged. Trypan blue staining was performed to detect the cell viability: cell number /ml = total cell number in 4 grids/4×10000×dilution fold. Before experiments, HUVEC was washed twice with D-Hank's solution, digested with trypsin, and 5 ml DMEM was added to suspend cells. Cell suspension was diluted to 5 × 10^6 /ml and seeded in 6-well culture plates (2 ml / well). The medium was drained till the incubation last for 24 h after cell fusion. Cells were washed twice with D-Hank's solution again and 2 ml serum-free medium was added, then different drugs were added for the following experiments.

TNF-α induced mRNA expression of TF and RhoA activity in the presence or absence of Flu

Grouping

HUVECs were grouped as follows: a, control group: cells were incubated in 20 µl of DMEM without bovine serum; b: TNF-α group: Cells were incubated in DMEM containing 20 µl of TNF-α solution (20 ng/mL); c: Flu group: cells were incubated in DMEM containing 20 µl of Flu solution (1 µmol/ml) for 6 h and then with 20 µl of DMEM without bovine serum; d: Flu+TNF-α group: cells were incubated in DMEM containing 20 µl of Flu solution (1 µmol/ml) for 6 h and then in DMEM containing 20 µl of TNF-α solution (20 ng/ml). Incubation was performed for 6 h and cell lysate was collected.

Detection of TF by ELISA

ELISA was performed at room temperature (20 to 30℃). In brief, 50 µl of standard or samples were added to each well followed by incubation for 2 h, 50 µl of biotinylated TF antibody added followed by incubation for 1 h, 50 µl of streptavidin-HRP added followed by incubation for 30 min, 50 µl of substrate added followed by incubation for 12 min and finally 50 µl of stop solution added followed by incubation for 10 min. Absorbance was detected at 450 nm with a microplate reader.

Detection of mRNA expression of TF by RT-PCR

Primers were designed and synthesized in Shanghai Sangong Biotech. Total RNA was extracted and reverse transcribed into complementary deoxyribonucleic acid (cDNA) which was then used for amplification by PCR. The products were subjected to agarose gel electrophoresis and the bands were photographed. The images were analyzed with BIO imaging system and the relative optical density (IA) was obtained which was then normalized by that of GAPDH. Experiment was repeated three times.

Detection of protein expression of TF and RhoA activity by western blot assay

The proteins were extracted from cells following cell lysis and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene fluoride (PVDF) membrane which was blocked with serum, treated with primary antibody, washed with Tris-Buffered Saline Tween-20 (TBST), and treated with secondary antibody. Chemiluminescence method was employed for color development and the expressions were determined by detecting the optical density of bands.

Effects of Ang II and Y-27632 on the expression and activity of TF

Grouping

Then, Angiotensin II (Ang II) and Y27632 were used to treat these cells and then these cell were grouped. a: control: control group: cells were incubated in 20 µl of DMEM without bovine serum; b: TNF-α+Ang II group: cells were incubated in DMEM containing 20 µl of Ang II (10^{-6}mol/L) for 6 h and then in medium with 20 µl of TNF-α solution (20 ng/mL); c: Ang II group: cells were incubated in DMEM containing 20 µl of Ang II (10^{-6}mol/L) for 6 h and then in DMEM without bovine serum; d: TNF-α group: cells were incubated in DMEM containing 20 µl of Y-27632 solution (10^{-5} mol/L) for 6 h and then in DMEM containing 20 µl of TNF-α solution (20 ng/ml). e: TNF-α+Y-27632 group: cells were incubated in DMEM containing 20 µl of Y-27632 solution (10^{-5} mol/L) for 6 h and then in DMEM without bovine serum. Incubation was performed for 6 h and cell lysate was collected.

Detection of protein expression of TF by Western blot assay

The proteins were extracted from cells following cell lysis and then subjected to SDS-PAGE. The proteins were transferred onto PVDF membrane which was blocked with serum, treated with primary antibody, washed with TBST, and treated with secondary antibody. Chemiluminescence method was employed for color development and the expressions were determined by detecting the optical density of bands.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 statistic software package. Normality test was done first. Quantitative data with normal distribution were expressed as means ± standard deviation ($\bar{x} \pm s$). Comparisons of means between groups were performed with t test. Comparisons of data with normal distribution among multiple groups were done with analysis of variance. A value of P<0.05 was considered statistically significant.

RESULTS

Effects of Flu on TNF-α induced expression and activity of TF

HUVECs were incubated with Flu at 0, 0.01, 0.1, 1, 10 or 100 μmol/L for 6 h and then with TNF-α. ELISA was done to detect the TF content. When compared with control group, the TF content was reduced by Flu in a dose dependent manner (dose-effect relationship). Flu at 0.1~100 μmol/L could significantly inhibit the TNF-α induced TF expression and activity in HUVECs (P<0.05). When the concentration of Flu was higher than 1 μmol/L, increase of Flu concentration did not further decrease the TF expression (P>0.05) (Table 1).

Effects of Flu on the TNF-α induced release, mRNA and protein expressions of TF and RhoA activation in HUVECs

There was no difference in the total protein concentration between HUVECs of different groups (P>0.05). In addition, no marked differences in the TF content, mRNA and protein expressions of TF and RhoA activity between control group and Flu group (P > 0.05. The levels of these parameters in TNF-α group were markedly higher than those in control groups (P<0.05). In TNF-α+Flu group, the TF content and mRNA and protein expressions of TF were significantly higher than those in control group (P<0.05) but lower than those in TNF-α group (P<0.05) (Table 2, Figures 1, 2, and 3).

Effects of Ang II and Y-27632 on the activity and expression of TF

Western blot assay was performed to detect the protein expression of TF following Ang II and/or Y27632 treatment. Results showed when compared with control, the protein expression of TF in TNF-α+Ang II group, TNF-α group, Ang II group and TNF-α+Y27632 group was markedly increased (P<0.05). When compared with TNF-α group, the protein expression of TF was significantly raised in TNF-α+Ang II group (P<0.05), and that dramatically decreased in TNF-α+Y27632 group (P<0.05). The TF level in TNF-α group was higher than that in Ang II group although significant difference was not found (P>0.05) (Figure 4).

DISCUSSION

TF is a transmembrane glycoprotein. When the blood vessel wall is injured or the endothelial cells are stimulated, the endothelial cells can synthesize and express a large amount of TF which then activate coagulation cascade resulting in blood clotting. Numerous animal and clinical studies have confirmed that TF plays an important role in the transformation of atherosclerotic plaque into thrombus (Holy and Tanner, 2010; Breitenstein et al., 2010). Statins can exert multiple regulatory activities independent of its lipid-lowing activity. Studies have found that Statins can inhibit the expression of TF on the monocytes *in vitro* and *in vivo* (Zawadzki et al., 2007). Our study also confirmed that in HUVECs, Flu could suppress the TNF-α induced secretion of TF accompanied by decrease of mRNA and protein expressions of TF in these cells.

It has been revealed that Rho/Rho kinase is associated with the generation of TF (Nagata et al., 2002) and can regulate the expression of TF. The Rho/Rho kinase signaling pathway includes Rho protein, Rho kinase, myosin phosphatase, etc. Rho protein is a small GTP-binding protein molecule (small G protein) and a member of Ras superfamily. Evidence reveals Rho could exert multiple biological effects in the signaling pathway through acting on the cytoskeleton or other target proteins. The prenylation of the carboxyl terminal of Rho protein leads to the activation of Rho protein which then binds to GTP and translocates to the cell membrane exerting its biological effects. With the in depth understanding of factors involving in the formation of atherosclerosis, increasing attention has been paid to the Rho/Rho kinase pathway. Evidence shows Rho/Rho kinase involves in the regulation of cell adhesion and migration, contraction of smooth muscle cells and cytokinesis, and these processes have been confirmed to be involved in the occurrence and development of atherosclerosis (Hiroki et al., 2004). Animal and clinical studies reveal Rho/Rho kinase is closely related to the atherosclerosis, hypertension, myocardial ischemia and other cardiovascular diseases (Dai and Wu, 2011; Yao et al., 2010).

Studies show statins have anti-inflammatory, anti-oxidative and anti-thrombotic activities which are independent of its lipid lowing activity. These activities of statins may be associated with Rho/Rho kinase signaling

Table 1. Effects of Flu of different concentrations on TNF-α induced TF expression.

Flu concentration (μmol/L)	0.01	0.1	1	10	100	0
TF content (pg/ml)	8.34±0.77 [ab]	6.69±0.59[ab]	4.99±0.24[a]	4.68±0.26[a]	4.21±0.46[a]	9.64±0.58[b]

a P<0.05 vs control; b P<0.05 vs 1 μmol/L Flu.

Table 2. TF content, mRNA and protein expressions of TF and RhoA activity in different groups.

Group	Total protein concentration	TF content (pg/ml)	TF mRNA expression	TF protein expression	RhoA activity
Control group	13.2	2.30±0.20	0.02±0.01	0.06±0.02	0.04±0.02
TNF-α group	12.4	9.64±0.58△	0.88±0.26△	0.94±0.32△	0.36±0.04△
Flu group (1 μmol/L)	12.7	2.52±0.19	0.03±0.02	0.07±0.01	0.08±0.03
TNF-α+Flu group	12.8	4.99±0.24△▲	0.48±0.15△▲	0.67±0.23△▲	0.21±0.02▲

△P<0.05 vs control; ▲P<0.05 vs TNF-α.

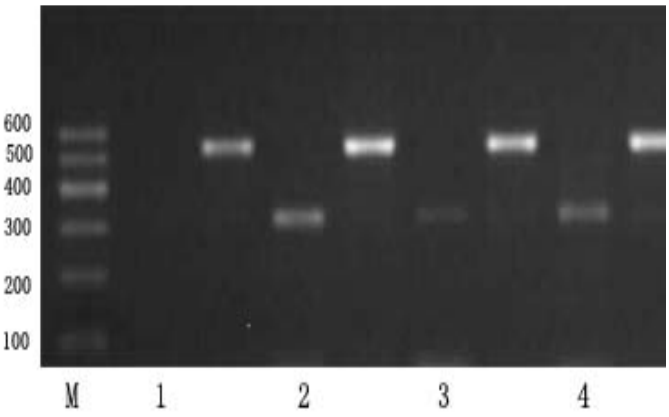

Figure 1. Effect of Flu on the TNF-α induced mRNA expression of TF. M: mark; 1: control; 2: TNF-α group; 3: Flu group; 4. TNF- +Flu group. The lengths of internal reference and TF were 400 bp and 311 bp, respectively. Bands between M, 1, 2, 3 and 4 were internal reference.

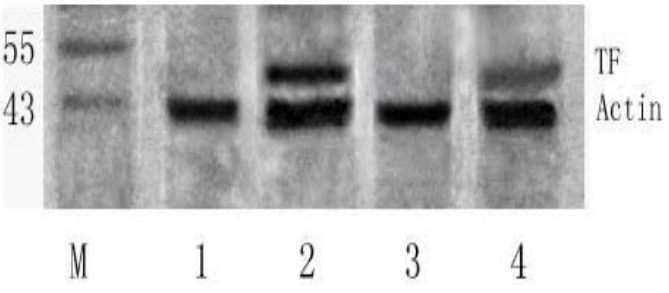

Figure 2. Effect of Flu on the TNF-α induced protein expression of TF. M: mark; 1: control; 2: TNF-α group; 3: Flu group; 4. TNF-α+Flu group. The molecular weight of β-actin and TF were 43 bp and 47 bp, respectively.

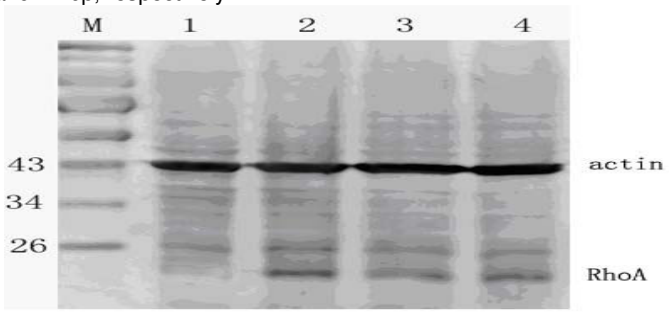

Figure 3. Effect of Flu on the TNF-α induced protein expression of RhoA. M: mark; 1: control; 2: TNF-α group; 3: Flu group; 4. TNF-α+Flu group. The molecular weight of β-actin and RhoA were 26 Kd and 22 Kd, respectively.

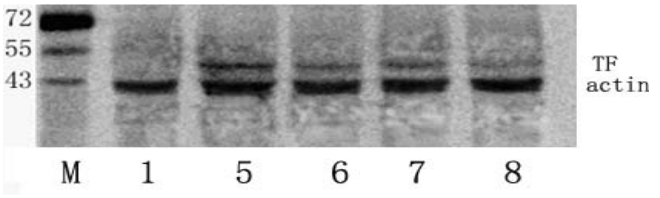

Figure 4. Protein expression of TF following Ang II and/or Y-27632 treatment. M: mark; 5: TNF-α+Ang II group; 6: Ang II group; 7: TNF-α group; 8. TNF-α+ Y-27632 group. The molecular weight of β-actin and TF were 43 Kd and 47 Kd, respectively.

Statins can inhibit the prenylation of Ras and Rho leading to inactivation of Rhp/Rho kinase which then delays the development of atherosclerosis (Chiba et al., 2008; Chen et al., 2008). Eto et al. (2002) investigated the pathway

(Bussolati et al., 2005; Vieira et al., 2005). mechanisms underlying the thrombin induced TF expression. Their results showed thrombin could act on Rho/Rho kinase pathway to induce the expression of TF in HAECs and HASMCs, and this expression was inhibited by the addition of Y27632, a specific inhibitor of Rho kinase, in a dose dependent manner. Our results also showed TNF-α could induce the TF expression in a RhoA dependent manner, and Flu could inhibit the synthesis and expression of TF in the endothelial cells in a dose dependent manner accompanied by suppression of RhoA activation. Based on these findings, we further confirmed that RhoA specific inhibitor (Y27632) suppressed the TF expression and RhoA activator (Ang II) promoted its expression. These findings suggest RhoA/Rho kinase pathway involves in the TNF-α induced TF expression and can be activated by TNF-α. RhoA/Rho kinase may involve in the generation of TF and specific agonist and antagonist of Rho/Rho kinase can promote and inhibit TF expression, respectively. In addition, Flu can suppress the TF expression in a Rho/Rho kinase dependent manner.

Taken together, Flu can inhibit the TNF-α induced TF expression in which Rho protein plays an important role. The activation of protein kinase can regulate the TNF-α induced TF expression in HUVECs and the Flu suppresses the TF expression at least partially in a RhoA/Rho kinase dependent manner. The RhoA/Rho kinase pathway may involve in the regulation of TF expression by Flu at the G protein level. The importance and evaluation of RhoA/Rho kinase pathway in biological activities of Flu provide evidence for the molecular mechanism of the anti-thrombotic effect of statins and provide basis for the prevention and treatment of atherosclerosis and the improvement of prognosis of cardiovascular diseases.

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Abbreviations: Flu, Fluvastatin; **TNF-α,** tumor necrosis factor-α; **TF,** tissue factor; **HUVECs,** human umbilical vein endothelial cells; **ELISA,** enzyme-linked immunosorbent assay; **RT-PCR,** real time polymerase chain reaction; **mRNA,** messenger ribonucleic acid; **MVA,** mevalonic acid; **cDNA,** complementary deoxyribonucleic acid; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PVDF,** polyvinylidene fluoride; **TBST,** TRIS-Buffered Saline Tween-20; **DMEM,** Dulbecco's modified eagle's medium.

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