Inhibitory effects of lithospermic acid on proliferation and migration of rat vascular smooth muscle cells

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Aim: To understand the effects of lithospermic acid (LA), a potent antioxidant from the water-soluble extract of *Salvia miltiorrhiza*, on the migration and proliferation of rat thoracic aorta vascular smooth muscle cells (VSMCs).

Methods: VSMC migration, proliferation, DNA synthesis and cell cycle progression were investigated by transwell migration analysis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, bromodeoxyuridine (BrdU) incorporation assay, and flow cytometric detection, respectively. Intracellular reactive oxygen species (ROS) generation was detected using 2′,7′-dichlorofluorescin diacetate (DCFH-DA). The expression of cyclin D1 protein and matrix metalloproteinase-9 (MMP-9) protein, as well as the phosphorylation state of ERK1/2, were determined using Western blots. The activity of MMP-9 and the expression of MMP-9 mRNA were assessed by gelatin zymography analysis and RT-PCR, respectively.

Results: LA (25–100 μmol/L) inhibited both lipopolysaccharide (LPS)- and fetal bovine serum (FBS)-induced ROS generation and ERK1/2 phosphorylation. By down-regulating the expression of cyclin D1 and arresting cell cycle progression at the G1 phase, LA inhibited both VSMC proliferation and DNA synthesis as induced by 5% FBS. Furthermore, LA attenuated LPS-induced VSMC migration by inhibiting MMP-9 expression and its enzymatic activity.

Conclusion: LA is able to inhibit FBS-induced VSMC proliferation and LPS-induced VSMC migration, which suggests that LA may have therapeutic effects in the prevention of atherosclerosis, restenosis and neointimal hyperplasia.

Keywords: lithospermic acid; ERK1/2; matrix metalloproteinase-9; vascular smooth muscle cells; proliferation; migration
VSMCs were lysed in a lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 150 mmol/L NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L NaF, 1 mmol/L NaVO₄, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L dithiothreitol (DTT), 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1% Triton. Equal amounts of protein from each sample were subjected to SDS-PAGE and blotted on PVDF membrane, which was incubated for 2 h at room temperature with blocking buffer (5% non-fat milk, 0.1% Tween 20, in TBS, pH 7.6) and then probed with primary antibodies overnight at 4 °C. After incubation with the appropriate secondary antibodies, the immunoreactive band was detected by an ECL Western blotting detection system (GE Healthcare).

Cell proliferation assay

The cell proliferation assay was performed using the MTT method as described previously[21]. Briefly, growth-arrested VSMCs were incubated with or without LA for 2 h prior to stimulation with 5% FBS for 48 h, and then the cells were incubated with 0.5 mg/mL MTT for 4 h at 37 °C. Finally the culture medium was removed, and the formazan salt crystals were dissolved with 200 μL dimethylsulfoxide (DMSO) and shaken for 10 min. The absorbance was read at a wavelength of 570 nm using Spectramax M2 microplate Reader (Molecular Devices). To validate the MTT assay, trypan blue exclusion was performed in parallel.

BrdU incorporation assay

DNA synthesis in VSMCs was examined using the BrdU incorporation assay according to the method described previously[22]. Quiescent VSMCs were pretreated with or without LA for 2 h prior to stimulation with 5% FBS for 24 h. Subsequently, 10 μmol/L BrdU was added to the cells and incubated for another 24 h. To immunostain for BrdU, the cells were washed with PBS, fixed in 4% polyformaldehyde and then permeabilized with 0.1% Triton X-100. The cells were then stained with antibody for BrdU followed by incubation with the Alexa Flour 568 goat-anti-mouse IgG (Invitrogen) second antibody. The cell nuclei were stained with Hoechst 33342 and evaluated by fluorescence microscopy with the appropriate fluorescent filters. Results are presented as mitotic index, and defined as the percentage of BrdU-positive nuclei per number of cells.

Flow cytometry analysis of cell cycle

Quiescent VSMCs were pretreated with or without LA for 2 h, followed by 5% FBS treatment for 24 h. Cells were then trypsinized, collected, and washed twice with cold PBS. Cells pellets were fixed in 70% ethanol and stored at 4 °C. Next, the fixed cells were treated with RNase A (10 μg/mL). DNA was stained with propidium iodide (50 μg/mL) for 30 min at 37 °C, and 1×10⁴ cells were analyzed by flow cytometry.
rates of G0/G1, S and G2/M phases were determined using the computer program ModiFit LT.

**Cell migration assay**

VSMCs migration was examined using transwell cell culture chamber with gelatin-coated polycarbonate membrane as described previously[21]. Growth-arrested cells were harvested, washed, and resuspended in the media (DMEM+0.1% FBS). Approximately 1×10⁵ cells were placed in the upper chamber of a 24-well transwell that was precoated with 0.1% gelatin. LA was added to both the upper and the lower compartments and was present throughout the duration of the experiment. Migration was induced by the addition of LPS (100 ng/mL) to the lower chamber. After incubation at 37 °C for 24 h, the non-migratory cells were removed from the upper surface of the membrane by scraping them with cotton swabs. The membrane was fixed with 90% ethanol and stained with 0.1% crystal. Migrated cells were counted at 200× magnification in five randomly chosen microscope fields per filter.

**Gelatin zymography**

Gelatinase activity in the conditioned medium collected from cell cultures was measured with zymography, as described previously[23]. Conditioned medium were electrophoresed in a polyacrylamide gel containing 0.1% (w/v) gelatin. After being washed at room temperature for 1.5 h with 2.5% Triton X-100, the gel was subsequently incubated at 37 °C for 24 h in a buffer containing 10 mmol/L CaCl₂, 200 mmol/L NaCl, and 50 mmol/L Tris-HCl (pH 7.5), and then stained with 0.2% coomassie brilliant blue. The gelatinolytic regions were observed as white bands against a blue background and band densities were quantified using Smart View Analysis program.

**Statistical analysis**

Results are expressed as mean±SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) or Student’s t-test. A P-value of < 0.05 was considered significant.

**Results**

**LA inhibits ROS generation in FBS- or LPS-stimulated VSMCs**

In this study we investigated the effects of LA on FBS- and LPS-induced ROS generation. Compared with the control group, FBS (5%) or LPS (100 ng/mL) significantly increased ROS generation in VSMCs (Figure 2). However, the increased intracellular ROS generation as induced by FBS or LPS was clearly inhibited by LA in a concentration-dependent manner.

**LA inhibits LPS- and FBS-induced ERK1/2 phosphorylation in VSMCs**

It has been reported that ERK1/2 activation plays a critical role in the migration and proliferation of VSMCs[24]. Therefore, we examined the effect of LA on ERK1/2 activation. Our results indicated that exposure of VSMCs to either FBS or LPS for 15 min markedly induced the phosphorylation of ERK1/2. Conversely, LA could completely abrogate ERK1/2 activation in FBS- or LPS-stimulated VSMCs (Figure 3A and 3B).

**LA inhibits FBS-induced VSMCs proliferation and DNA synthesis**

We next studied the effect of LA on the proliferation of VSMCs using the MTT assay. When growth-arrested cells were treated with LA (25, 50, 100 μmol/L) in the presence of 0.1% FBS, no significant difference was observed in cell viability (Figure 4A, left panel), suggesting that LA did not show significant cytotoxicity up to 100 μmol/L. The absence of cytotoxicity was further confirmed with a trypan blue exclusion assay (data not shown). Moreover, we found that FBS induced a 2.21-fold increase in the proliferation of VSMCs. Treatment of cells with LA 2 h before stimulation with FBS reduced cell proliferation in a concentration-dependent manner (Figure 4A, right panel). We further examined the effect of LA on DNA synthesis, as induced by 5% FBS in VSMCs using the BrdU incorporation assay. BrdU incorporation was markedly increased in VSMCs exposed to 5% FBS for 48 h (P<0.01), indicating an increase in DNA synthesis. However, this effect was abolished in VSMCs pretreated with LA (Figure 4B).

**LA efficiently abrogates cyclin D1 protein expression in VSMCs and arrests FBS-stimulated cells in the G1-phase**

It is known that cyclins, CDKs, and their inhibitors regulate cell cycle progression. Changes in the cyclin D1/CDK com-

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*Figure 2.* Effects of LA on (A) FBS (5%) and (B) LPS (100 ng/mL) induced ROS generation in VSMCs. Serum-starved VSMCs were pretreated with LA for 2 h before stimulation with FBS (5%) or LPS (100 ng/mL) for 30 min at 37 °C. The cells were then stained with DCFH-DA (10 μmol/L) and analyzed by a fluorescence plate reader. Relative fluorescence intensities were calculated using untreated control cells as a standard. Results are presented as mean±SEM. n=6. bP<0.05, cP<0.01 vs control. dP<0.05, eP<0.01 vs FBS or LPS.
plex, for example, can either stop or promote cell proliferation\cite{25}. We investigated the potential effect of LA on cyclin D1 protein expression. Our results showed that FBS significantly increased the expression of cyclin D1, while LA completely abolished this serum action (Figure 5).

Proliferation cells pass through several cell cycle checkpoints, mainly the G1 to S and G2 to M transitions. The former checkpoint is considered to be the most important step in DNA replication. Accordingly, flow cytometric assessment was performed to determine the effect of LA on cell cycle progression. As shown in Figure 6, the percentage of G0/G1 or S phase cells in the 5% FBS-stimulated group were 59.07\%±3.25\% and 16.69\%±2.81\%, respectively. LA at concentrations of 50 to 100 μmol/L effectively increased the proportion of G0/G1 phase cells.

Figure 3. Effects of LA on (A) FBS- and (B) LPS-induced ERK1/2 activation in VSMCs. Cells were treated with LA for 2 h prior to FBS (5\%) or LPS (100 ng/mL) stimulation for 15 min. Whole cell lysates were prepared and subsequently used for detection of p-ERK1/2 and ERK1/2 by Western blotting. The bar graphs show the ratio of densities of phosphoproteins to total proteins for the blots. n=3. cP<0.01 vs control, dP<0.05, eP<0.01 vs FBS or LPS. Values are presented as mean±SEM.

Figure 4. Effects of LA on FBS-induced proliferation and DNA synthesis in VSMCs. (A) Proliferation activities were measured by MTT assay in the absence (left) or presence (right) of 5\% FBS. Relative proliferation activities were expressed using untreated control cells as a standard. n=6. (B) DNA synthesis was measured by BrdU incorporation assay. The left part of the diagram shows BrdU incorporation of quiescent and FBS-stimulated VSMCs. On the right side, a concentration-dependent decrease of BrdU incorporation in LA-treated VSMCs is shown. n=6. Values are presented as mean±SEM. cP<0.01 vs control. dP<0.05, eP<0.01 vs FBS.

Figure 5. Inhibitory effect of LA on FBS-stimulated cyclin D1 protein expression. Growth-arrested VSMCs were pretreated with LA (50 or 100 μmol/L) for 2 h and then stimulated with 5\% FBS for 24 h. Expression of cyclin D1 protein was examined by Western blotting. β-actin was used as a loading control. Values are presented as mean±SEM. n=3. bP<0.05 vs control. dP<0.05, eP<0.01 vs FBS.
tion of cells in the G₀/G₁ phase and simultaneously decreased the proportion of cells in the S phase (Figure 6).

LA inhibits LPS-induced migration of VSMCs, and attenuates the enzymatic activity and expression of MMP-9

Finally, we examined the effect of LA on the migration of VSMCs. VSMC migration was significantly increased from stimulation with LPS (100 ng/mL) for 24 h (P<0.01; Figure 7A). However, LA inhibited the migration of VSMCs in a concentration-dependent manner (Figure 7A). We then investigated the effect of LA on MMP-2 and MMP-9 activity using gelatin zymography. As shown in Figure 7B, the medium from control cells showed very weak proteolytic activity at 92 kDa (corresponding to MMP-9) and high proteolytic activity at 72

**Figure 6.** Cell cycle distribution of (A) quiescent and (B) FBS-stimulated VSMCs. (C) and (D) show VSMCs treated with 50 and 100 μmol/L LA, respectively, in the presence of FBS stimulation. It demonstrates LA-induced cell cycle arrest in G₀/G₁ phase. G₀/G₁ phase is represented by the first peak, S phase in diagonal and G₂/M by the second peak. Results are expressed as the percentage of total cells in G₀/G₁, S, or G₂/M phase of the cell cycle. Values are presented as mean±SEM. n=3.

**Figure 7.** Effects of LA on LPS-induced VSMC migration as well as MMP-9 enzymatic activity and expression. (A) Cells were treated as indicated and migration assays were performed as described in the Methods section. The diagram shows counted cells on the bottom side of the transwell chamber membrane at different LA concentrations. n=6. Cells were pretreated with LA for 2 h prior to 100 ng/mL LPS stimulation for 24 h. (B) The conditioned media were prepared and used for gelatin zymography. n=6. (C) The total cellular proteins were prepared and used for Western blotting. n=3. (D) The MMP-9 mRNA levels were measured by RT-PCR. GAPDH was used as an internal control. n=6. Values are presented as mean±SEM. 

\[ P<0.05, \ \ \ P<0.01 \] vs control. 

\[ P<0.05, \ \ \ P<0.01 \] vs LPS.
kDa (corresponding to MMP-2). LA suppressed LPS-induced MMP-9 activity in a concentration-dependent manner (Figure 7B). However, the level of MMP-2 secretion was not affected by LPS or LA (Figure 7B). Furthermore, we performed Western blots to confirm the decrease of MMP-9 protein. As shown in Figure 7C, LA demonstrated an inhibitory effect on LPS-induced MMP-9 protein expression in VSMCs. Consistent with the results from zymography and Western blotting, we observed that MMP-9 mRNA expression, as induced by LPS, was inhibited by LA (Figure 7D).

Discussion
LA, a naturally occurring polyphenolic compound, is found in various plants and more abundantly in S. miltiorrhiza and Lithospermum. Our study demonstrated that LA inhibited FBS-induced VSMC proliferation and LPS-induced VSMC migration.

ROS play a key role in the proliferation of VSMCs and produce inflammation in the vessel wall. Therefore, ROS are regarded as a risk factor for vascular diseases. It was reported that FBS induced ROS production, leading to cell proliferation via activation of ERK1/2 in smooth muscle cells.[26–28]. LPS, a bacterial endotoxin that functions as a proinflammatory mediator, is considered to be a strong stimulator of atherosclerosis.[29]. Furthermore, LPS has been shown to induce ROS generation and ERK1/2 phosphorylation in VSMCs.[30]. In our study, LPS and FBS induced ROS generation in VSMCs, which was consistent with previous reports. LA presented inhibitory effects on the generation of ROS in a concentration-dependent manner.

The ROS-ERK1/2 pathway has been implicated in the control of VSMC proliferation and migration.[31] Addition of exogenous ROS can activate mitogen-activated protein (MAP) kinases, including ERK1/2, p38 MAP kinase, JNK, and ERK5, which are important for cell growth, inflammation, apoptosis, and cell differentiation, respectively.[31,32]. These findings suggest that ERK1/2 could represent a link between ROS and VSMC proliferation as well as migration in atherosclerosis, restenosis and neointimal hyperplasia. In our study, LA inhibited FBS- and LPS-induced ERK1/2 activation in a concentration-dependent manner.

We replaced a single growth factor with 5% FBS to induce cell proliferation. FBS contains a range of growth factors, including PDGF, FGF, transforming growth factor, serotonin, and thrombin.[33]. Consequently, it was used to mimic the multiple factors present in the environment in vivo. Our results indicated that LA inhibited FBS-induced VSMC proliferation in a concentration-dependent manner. Cell cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of cell-cycle regulatory proteins provide an important mechanism for inhibiting cell growth. Cyclin D1 is a primary target for the mitogenic effects of ROS and it plays an important role for G1/S transition in cell cycle progression.[34]. Our results indicated that LA suppressed FBS-induced cyclin D1 protein expression and arrested cells in the G1 phase. Traversing the G1-S phase boundary is coupled to DNA synthesis, followed by entry into S, and finally mitosis in the M phase. BrdU, a thymidine analog, is widely used as a marker of DNA synthesis, and is able to incorporate into cellular DNA during the S-phase.[35]. In our study, LA (50 μmol/L) exerted strong inhibition on BrdU incorporation into DNA, which indicated that LA significantly inhibited FBS-induced DNA synthesis. However, LA exerted only 30%–40% inhibition on proliferation at the same concentration. The discrepancy may result from the following reason. As the cell cycle comprises a range of highly ordered events that lead to mitosis and the formation of new cells, regulating any of these conserved processes is critical to cell growth and replication. DNA synthesis during S phase is only one step in cell cycle progression. Besides DNA replication, cell proliferation is also affected by mitotic activity, which is regulated by many mitotic kinases and checkpoints.[36]. LA may interfere with the mitotic process directly and result in a loss of coordination between DNA synthesis and subsequent events required for cell division.

It was reported that LPS increased neointimal hyperplasia in balloon-injured rabbit aortas.[37]. When injury occurs, VSMCs migrate from the tunica media to the intima, which leads to neointima formation.[38]. Our results indicated that LA significantly inhibited LPS-induced VSMC migration. It should be noted that the increase in VSMC migration might be a result of the proliferation. However, we can rule out this possibility as LPS (100 ng/mL) did not show any effect on the proliferation of VSMCs (data not shown).

VSMC migration requires the breakdown of the extracellular matrix. One possible mechanism by which VSMCs break down the extracellular matrix is by secreting matrix metalloproteinases (MMPs).[39]. It was reported that MMPs, specifically MMP-2 and MMP-9, were important for the migration of VSMCs.[39]. LPS has been shown to upregulate MMP-9 via ERK1/2 signaling.[40,41]. In our study, LPS increased MMP-9 expression and enzymatic activity while LA attenuated these effects in a concentration-dependent manner. Therefore, LA inhibits LPS-induced VSMC migration by attenuating MMP-9 expression and enzymatic activity.

In addition to ERK1/2 signaling, p38 and JNK also play important roles in regulating cell migration.[42]. Accordingly, we cannot rule out the possibility that these two signaling pathways are involved in the anti-migratory effect of LA.

In summary, our results demonstrated that LA inhibited ROS generation and ERK1/2 activation, and exerted inhibitory effects against the proliferation and migration of VSMCs in vitro. The anti-proliferative effect of LA is attributed to its direct down-regulation of cyclin D1 expression and subsequent cell cycle arrest in G1 phase. The anti-migratory effect of LA on VSMCs is attributable to its ability to inhibit MMP-9 expression as well as enzymatic activity. These results suggested that LA, a water-soluble polyphenolic antioxidant, may have therapeutic potential against atherosclerosis, restenosis and neointimal hyperplasia.
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Author contribution
Yi-ping WANG, Li CHEN, and Wen-yi WANG designed the research; Li CHEN performed the research and analyzed the data; Li CHEN, Wen-yi WANG, and Yi-ping WANG wrote the manuscript.

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