Effect of Fetal Bovine Serum Concentration on Lysophosphatidylcholine-mediated Proliferation and Apoptosis of Human Aortic Smooth Muscle Cells

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Abstract: Lysophosphatidylcholine (lysoPtdCho) is produced by the phospholipase A₂-mediated hydrolysis of phosphatidylcholine and can stimulate proliferation and apoptosis of vascular smooth muscle cells. We examined the influence of fetal bovine serum (FBS) concentration in the culture medium on lysoPtdCho-mediated apoptosis and proliferation of human aortic smooth muscle cells (HASMCs) as well as on the activation of extracellular signal-regulated kinases (ERK)1/2. In the presence of 1% FBS, HASMC viability increased after lysoPtdCho treatment at 1 and 10 μM but decreased at 25 and 50 μM. However, lysoPtdCho increased HASMC viability in a dose-dependent manner in the presence of 10% FBS. The activity of caspase 3/7 in HASMCs was increased by 25 μM lysoPtdCho in the presence of 1% FBS, but not 10% FBS. Furthermore, lysoPtdCho at 1 and 10 μM triggered ERK1/2 phosphorylation in the presence of 1% FBS, but not at 10% FBS. Thus, lysoPtdCho-mediated HASMC apoptosis, proliferation, and ERK1/2 activation are dependent on the concentration of FBS.

Key words: vascular smooth muscle cell, lysophosphatidylcholine, proliferation, apoptosis, ERK1/2

1 Introduction

Lysophosphatidylcholine (lysoPtdCho) is produced by phospholipase A₂-mediated hydrolysis of phosphatidylcholine (PtdCho) in cell membranes. Oxidative modification of low-density lipoprotein can also induce the conversion of PtdCho into lysoPtdCho¹,². LysoPtdCho is recognized as a diagnostic biomarker or a disease-related factor. Circulating lysoPtdCho levels are increased in patients with hypalbuminemia³ and heterozygous familial hypercholesterolemia⁴, but decreased in patients with cardiovascular disease⁵,⁶, diabetes mellitus⁷, sepsis⁸, and Alzheimer’s disease⁹. Vascular smooth muscle cells (VSMCs) are the major cell type in the medial layer of arteries, and lysoPtd-Cho reportedly induced inflammatory activation¹⁰,¹¹ as well as apoptosis¹²,¹³, proliferation¹⁴,¹⁵–¹⁶, and calcification in VSMCs¹⁷,¹⁸.

The apoptosis and proliferation of VSMCs are closely associated with each other. The balance between these cellular functions is maintained in healthy vascular walls, but is disrupted in vascular lesions, such as those of atherosclerosis. VSMCs exhibit increased proliferation during early atherogenesis but enhanced apoptosis in advanced atherosclerotic lesions¹⁹,²⁰. Interestingly, increased levels of phospholipase A₂ and lysoPtdCho are present in atherosclerotic plaques²¹,²². LysoPtdCho-mediated release of inflammatory cytokines and growth factors in VSMCs accelerates the progression of atherosclerosis. Therefore, understanding the effect of lysoPtdCho on VSMCs is essential for developing new therapeutic strategies.
erates the progression of atherosclerosis\textsuperscript{31}.

Extracellular signal-regulated kinases 1/2 (ERK1/2) play key roles in regulating the behavior of VSMCs. Notably, ERK1/2 activation increases the proliferation of VSMCs and inhibits their apoptosis\textsuperscript{23, 24}. For example, enhanced ERK1/2 activation protects VSMC from oxidative stress-induced apoptosis\textsuperscript{25}. In the present study, we investigated the lysoPtdCho-mediated apoptosis and proliferation of human aortic smooth muscle cells (HASMCs) and the lysoPtdCho-induced ERK1/2 activation therein in the presence of 1% or 10% fetal bovine serum (FBS) to examine whether these processes are influenced by the concentration of FBS in the culture medium.

2 Materials and Methods

2.1 LysoPtdCho-mediated proliferation of HASMCs

HASMCs (cat. no. KS-4009; Kurabo Biomedical, Osaka, Japan) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; high glucose; FUJIFILM Wako Pure Chemical Co., Osaka, Japan) containing smooth muscle growth supplement (SMGS; Cascade Biologics, Tokyo, Japan), 10% FBS (Biological Industries, Kibbutz Beit-Haemen, Israel), and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B, all purchased from Gibco, Invitrogen Co., Grand Island, NY, USA). HASMCs at passages 6–7 were cultured in a humidified atmosphere containing 5% CO\textsubscript{2} in air at 37°C. PtdCho and lysoPtdCho (purity ≥ 99%) (Fig. 1A) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in ethanol to prepare stock solutions at a concentration of 40 mM. To examine the effect of lysoPtdCho on the proliferation of HASMCs, cells were cultured in DMEM containing SMGS for 24 h in a 6-well plate at 5 × 10\textsuperscript{5} cells/well. After 24 h, the medium was replaced with DMEM without SMGS, and the cells were further incubated for 24 h. Then, the cells were treated with PtdCho or lysoPtdCho (1, 10, 25, or 50 µM) in DMEM containing 1% or 10% FBS for 24 h. Control groups were treated with phosphate-buffered saline (PBS). Cell viability was determined using Cell Counting Kit-8 (CCK-8; DOJINDO Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions.

2.2 LysoPtdCho-mediated apoptosis of HASMCs

To examine lysoPtdCho-mediated apoptosis, HASMCs were cultured in DMEM without SMGS for 24 h in a 96-well plate at 1 × 10\textsuperscript{5} cells/well. The cells were then treated with lysoPtdCho (0, 10, or 25 µM) in DMEM containing 1% or 10% FBS for 3 h. The activity of caspase 3/7 was determined using the Caspase-Glo\textsuperscript{®} 3/7 Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

2.3 Immunoblotting

HASMCs (1 × 10\textsuperscript{6} cells) were plated in a 60-mm dish and cultured in DMEM supplemented with SMGS, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. When the cells reached >80% conflu-
ience, they were cultured for 24 h in DMEM without SMGS, and subjected to various treatments as described in the text. In the case of 1% FBS, the cells were pre-cultured in DMEM containing 1% FBS for 30 min prior to the lysoPtdCho treatments. The stimulated cells were lysed in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 1% Nonidet P-40, 2 mM ethylenediaminetetraacetic acid, 50 mM NaF, and 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, supplemented with Complete<sup>TM</sup> protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) on ice for 15 min. The cell lysates were centrifuged to remove insoluble materials and immunoblotted with phospho-ERK 1/2 monoclonal antibodies (D13.14.4E; Cell Signaling Technology; Danvers, MA, USA) or actin polyclonal antibodies (1-19; Santa Cruz Biotechnology; Dallas, TX, USA). Reactive proteins were visualized by chemiluminescence.

2.4 Suppression of HASMC proliferation by ERK1/2 inhibition

HASMCs were maintained in DMEM without SMGS for 24 h in a 6-well plate. After 24 h, an ERK1/2 inhibitor (PD184352; 10 μM; Sigma-Aldrich) was added to HASMCs stimulated with lysoPtdCho (1 or 10 μM) in DMEM containing 1% or 10% FBS for 24 h. Cell viability was determined using a CCK-8 kit.

2.5 Statistical analysis

Results are expressed as means and standard deviations. Statistically significant differences between groups were evaluated using a one-way analysis of variance followed by Tukey’s post-hoc test.

3 Results

The viability of HASMCs treated with 1 and 10 μM lysoPtdCho in the presence of 1% FBS was increased, but that of HASMCs treated with 25 and 50 μM lysoPtdCho in the presence of 1% FBS was decreased, compared with that of the PBS-treated control group. In contrast, HASMC viability increased in the range of 10–50 μM lysoPtdCho in the presence of 10% FBS in a dose-dependent manner. There was no change in HASMC viability after PtdCho treatment at concentrations of 1–50 μM (Fig. 1B). These results clearly showed that lysoPtdCho, but not PtdCho, had an impact on the viability of HASMCs.

To investigate lysoPtdCho-mediated apoptosis, the activity of caspase 3/7 in HASMCs was measured after the cells were treated with lysoPtdCho in the presence of 1% or 10% FBS. Caspase 3/7 activity was increased in HASMCs treated with 25 μM lysoPtdCho in the presence of 1% FBS, but not 10% FBS, compared with that in the PBS-treated group (Fig. 2). These results are consistent with the effect of lysoPtdCho on cell viability (Fig. 1B).

4 Discussion

VSMC proliferation and migration are required for vascular wall repair, but aberrant proliferation is associated with the development of atherogenesis<sup>15</sup>,<sup>20</sup>. Increased lysoPtdCho levels in atherosclerotic plaques can induce aberrant proliferation of VSMCs and promote atherosclerosis<sup>1</sup>,<sup>21</sup>,<sup>22</sup>. Several studies have reported that the proliferation of VSMCs treated with low concentrations of lysoPtdCho (1–10 μM) was increased, while the viability of VSMCs treated with ≥ 25 μM lysoPtdCho in FBS-free or low-FBS (≤ 1%) media was decreased<sup>15</sup>–<sup>16</sup>. Our findings demonstrated that lysoPtdCho at 1 and 10 μM lysoPtdCho increased HASMC proliferation, but lysoPtdCho at 25 and 50 μM decreased HASMC viability in the presence of 1% FBS (Fig. 1B), which are consistent with those of previous studies. It was also reported that VSMC proliferation was increased in the presence of 10% FBS after treatment with 10 and 20 μM lysoPtdCho<sup>20</sup>. Our study showed that HASMC prolifer-
The activation of caspase-8 and -9 triggers the activation of executioner caspases-3, -6, and -7 in apoptotic cells.

The enhancement in caspase 3/7 activity in HASMCs treated with 25 μM lysoPtdCho in the presence of 1% FBS clearly showed that lysoPtdCho induced HASMC apoptosis under these conditions. However, lysoPtdCho (25 μM) had no effect on caspase 3/7 activity in the presence of 10% FBS (Fig. 2).

LysoPtdCho stimulates ERK1/2 activity in VSMCs, followed by the proliferation of VSMCs. In the present study, ERK1/2 activation was induced in HASMCs treated with 1 and 10 μM lysoPtdCho in the presence of 1% FBS, but not 10% FBS, compared with that in PBS-treated HASMCs (Fig. 3A). Furthermore, ERK1/2 inhibition induced HASMC apoptosis in the presence of 1% FBS, but not 10% FBS (Fig. 3B).

These results may indicate that high levels of lysoPtdCho (≥ 25 μM) more efficiently simulate apoptotic signals than proliferative signals in HASMCs under the low FBS environment.

FBS contains several growth factors associated with VSMC proliferation, such as insulin-like growth factors, epidermal growth factors, and fibroblast growth factors. The levels of these growth factors can be influenced by disease stages. For example, early atherosclerosis is triggered by inflammation in the intimal area of arteries. Inflammatory responses stimulate the secretion of growth factors by immune cells (e.g., monocytes and macrophages) and enhance VSMC proliferation, lipid deposition, and endothelial dysfunction, which result in atherosclerotic plaque formation. However, advanced atherosclerotic lesions show decreased levels of growth factors and increased apoptosis. Therefore, the medium containing different FBS concentrations may be a useful tool for investigating the diverse cellular response in normal or abnormal cells.

5 Conclusion
LysoPtdCho participates in apoptosis and ERK1/2-mediated proliferation of VSMCs. In the present study, we investigated the influence of FBS concentration in the medium on lysoPtdCho-mediated apoptosis and proliferation of HASMCs as well as on the activation of ERK1/2. Our results indicated that lysoPtdCho-mediated apoptosis and...
proliferation of HASMCs and lysoPtdCho-induced ERK1/2 activation in HASMCs are influenced by the concentration of FBS in the medium.

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Conflicts of Interest
The authors declare no conflict of interest.

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