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LYSOPHOSPHATIDYLCHOLINE INDUCES INTEGRIN α6, INTEGRIN β4, RAC-2 AND RAB-11 IN ENDOTHELIAL CELLS

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Lysophosphatidylcholine (lysoPC), a major phospholipid component of oxidized LDL, changes the expression of multiple genes in endothelial cells leading to endothelial dysfunction, and plays an important role in the development of atherosclerosis. We performed a gene expression analysis to identify novel lysoPC-inducible genes in endothelial cells; using macroarray analysis as a screening tool and real time PCR to verify the result of the macroarray. Using this approach, we demonstrated that the mRNA expression of Rac-2, Rab-11, integrin α6 and integrin β4 was upregulated by lysoPC in endothelial cells. The analysis of the mRNA expression profile revealed that lysoPC induced those mRNA expressions in time and concentration dependent manners. Flow cytometric analysis showed that lysoPC selectively increased the surface expression of integrin α6 and integrin β4 on endothelial cells. Thus, we provided an evidence that lysoPC upregulated four genes belonging to the small G protein and integrin family at the mRNA level, and at least integrin α6 and integrin β4 at the protein level in endothelial cells, which might implicate the substantial involvement of lysoPC in the phenotypic changes of endothelial cells.

Key words: atherogenesis, lysophosphatidylcholine, endothelial cells, integrins, small G proteins.

INTRODUCTION

Atherosclerosis is an inflammatory disease in the intima of large- and medium-sized arteries, where both the proliferation and apoptosis of endothelial cells play important roles in the pathogenesis of the disease (1-3). Apoptosis of endothelial cells participate in the migration and proliferation of smooth muscle cells, accompanied by an increased intimal mass (4). Apoptotic cells are procoagulant, suggesting the necessary involvement of apoptotic endothelial cells in the thrombotic events that are frequently observed in atherosclerosis (5). Moreover, the apoptosis of endothelial cells is accompanied by endothelial cell proliferation (6). Since these regenerated endothelial cells do not function properly, showing the reduced production of NO and the increased uptake of LDL (7), they are more inclined to participate in atherogenesis. Furthermore, the proliferation and apoptosis of endothelial cells plays a critical role in neovascularization, and intra-plaque neovascularization is involved in the development and progression of atherosclerosis (8-12). The extent of neovascularization is closely related to the inflammatory reaction and infiltration of macrophages within the plaque (10). These reports indicate the importance of apoptosis and proliferation of endothelial cells, and angiogenesis in the development and progression of atherosclerosis. However, the gene-regulations related to apoptosis and proliferation of endothelial cells, and angiogenesis in atherosclerosis are not fully understood.

Lysophosphatidylcholine (lysoPC) is a bioactive phospholipid, enriched in atherogenic oxidized LDL (13). During LDL oxidation, it is produced from the phosphatidylcholine of LDL by phospholipase A_2-like activity (14), and is increased several fold in atherosclerotic vessels compared to their normal counterparts (13). Its accumulation is also enhanced in the ischemic tissue and in the inflammatory condition (15,16). Several *in vitro* studies have shown that lysoPC modulates multiple gene expression in endothelial cells, highlighting its role in the changes of functions of endothelial cells (17-23). LysoPC has also been reported to affect the proliferation,

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migration and apoptosis of endothelial cells (24-26). The role of lysoPC on the expression of endothelial cell growth, apoptosis and angiogenesis-related genes has not been studied in depth.

To address the role of lysoPC in endothelial cell growth, apoptosis and angiogenesis, we analyzed the expression of 540 genes related to cell growth, apoptosis and angiogenesis in endothelial cells, and screened for lysoPC-regulated genes using a cDNA macroarray technique. We show herein that four genes belonging to the small G protein and integrin family were regulated by lysoPC in endothelial cells. This finding suggests an important role of lysoPC in the changes of functions of endothelial cells through the regulation of this gene expression.

MATERIALS AND METHODS

Materials

L-α-lysoPC (from egg phosphatidylcholine) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). M199 medium, antibiotics, L-glutamine and fetal calf serum (FCS) were from Invitrogen Corporation (Grand Island, NY).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cords using collagenase (Collaborative Research Inc. Waltham, MA). HUVECs were cultured in M199 medium supplemented with endothelial cell growth supplement (Sigma, Saint Louis, Missouri), heparin (from porcine intestinal mucosa; Sigma), antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin), L-glutamine and 20 % FCS. Confluent cells of 2 to 5 passages were used for this study. For the stimulation of HUVECs, M199 Medium containing 5% FCS, L-glutamine and antibiotics were used in all experiments. This study was approved by the Ethical Committee of Shimane University School of Medicine, and the written informed consent was obtained from all donors of umbilical cord.

Macroarray analysis

Macroarray analysis was performed using a Gene Navigator System (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. After treatment, mRNA was isolated from HUVECs, and 1 μg of mRNA was used to prepare the cDNA, and second strand cDNA was synthesized. This double stranded cDNA was PCR amplified (25 cycles) and biotin-labelled to prepare the cDNA probe. The cDNA probe was hybridized with a gene navigator human cancer cDNA array filter, onto which the cDNA of 540 genes were plotted. After overnight hybridization and detection of the hybridized probe, the array filters were exposed to a CCD camera for half an hour, and the signal intensities were analyzed. The criteria for differentially expressed genes required that the average signal intensity of one gene was at least 2-fold that of the background signal, and signal intensity changes more than +/-2-fold of the control signal intensity were considered to be lysoPC-regulated gene.

Real time PCR

After treating the HUVEC, total RNA was isolated using RNA Stat (TEL-TEST, INC. Friendswood, Tx), according to the manufacturer's protocol. Reverse transcription was performed with 2 μg of total RNA using oligo(dT) and ReverTra Ace (TOYOBO). Real time PCR was carried out with SYBR Green PCR master mix (Applied Biosystem, Warrington, UK) using an ABI Prism 7700 Sequence Detection System (Applied Biosystem). The list of primers for real time PCR is in Table 1.

Northern blot analysis

Northern blot analysis was performed as previously described (27). To prepare cDNA probes for integrin α6, integrin β4, Rac-2, Rab-11, Cdk 4, caspase-4, clusterin and Cox-1, RT-PCR was carried out using total RNA isolated from HUVECs treated with medium alone or 80 μM of lysoPC for 8 hours. The list of primers used for RT-PCR is shown in Table 2. The size of the PCR products were 637, 720, 319, 580, 555, 368, 446 and 725 bp, respectively. For the loading control a 0.9-kb Hind III/Acc I fragment of GAPDH cDNA, purchased from ATCC, was used to prepare the probe. The quality of RNA was evaluated by ethidium bromide staining.

Flow cytometry

HUVECs were harvested with trypsin/EDTA, washed once with PBS containing 1% FCS, and kept at 4℃ for subsequent manipulation. Approximately $1X10^5$ cells were incubated with integrin α3, integrin β1 or integrin β4 mouse monoclonal antibody (Chemocon International, Inc. Temecula, CA), or

Table 1. Primers used in real time PCR

Gene Name	Primers
integrin α6	forward 5' GGAAACATGGACCTTGATCGA 3'
	reverse 5' CAGGCCGGGATCTGAAAATA 3'
integrin β4	forward 5' GGCAACATCCATCTGAAACCTT 3'
	reverse 5' CACGAAGTCTCCGTTGAAGCT 3'
integrin β1	forward 5' AGGAGGATTACTTCGGACTT 3'
	reverse 5' TAAATGGGGTGGTGCAGTTC 3'
Rac-1	forward 5' CTTATGGGATACAGCTGGACAAGA 3'
	reverse 5' CCACTTTGCACGGACATTTTC 3'
Rac-2	forward 5' AGCTACACCACCCAACGCCTTT 3'
	reverse 5' CTTCTGTCCACCATCACTTG 3'
Rab-11	forward 5' CCAAGCACAATGTGGTTCCT 3'
	reverse 5' GGGAGAAGAGAAATGCCTTAGATG 3'
tPA	forward 5' AGCGAGCCAAGGTGTTTCA 3'
	reverse 5' GTGGCCCTGGTATCTATTTCACA 3'
Cox-1	forward 5' GGAGACAATCTGGAGCCTCAGT 3'
	reverse 5' TCCATCCAGCACCTGGTACTT 3'
clusterin	forward 5' GGTGGTCGTGAAGCTCTTTGAC 3'
	reverse 5' CGCCACGGTCTCCATAAATTT 3'
PCNA	forward 5' CTAAAATGCGCCGGCAAT 3'
	reverse 5' GCTTCAAATACTAGCGCCAAGGT 3'
Cdk 4	forward 5' TCTGATGCGCCAGTTTCTAAGA 3'
	reverse 5' CACCACTTGTCACCAGAATGTTC 3'

integrin α6 rat monoclonal antibody (Chemicon International) at 4℃ for 30 min at a concentration of 1 μg for $5X10^5$ cells. Cells were washed once with PBS containing 1% FCS, and incubated further with FITC-conjugated goat anti-mouse or goat anti-rat secondary antibody for 30 min at 4℃, and after a wash subjected to flow cytometric analysis for the expressed molecules.

RESULTS

Macroarray analysis for the screening of lysoPC-regulated genes in endothelial cells.

A macroarray filter containing human cDNA of 540 genes related to cell growth, apoptosis and angiogenesis was used for the screening of lysoPC-regulated genes in endothelial cells. The results of macroarray analysis showed that lysoPC regulated the mRNA expression of multiple genes in endothelial cells (Table 3). These included many genes already known to be regulated by lysoPC in endothelial cells. For instance VEGF, eNOS and tPA mRNA expression was upregulated by lysoPC in our experimental model (Table 3), which have been previously shown to be induced by lysoPC in endothelial cells (18,21,28). The results also revealed that the expression of a number of genes that have not been reported to be lysoPC-regulated gene were changed by lysoPC. LysoPC upregulated the mRNA expression of the members of the small G protein

Table 2. Primers used to prepare cDNA probe for Northern blot analysis

Gene Name	Primers
integrin α6	forward 5' AGAGGCAGAAACAGAAAGTG 3'
	reverse 5' AGATATGGGGTGTCAAAGGT 3'
integrin β4	forward 5' CACAGAGGAGACCCAGATTGACAC 3'
	reverse 5' GTGACAGCAAAGATGGGGATGATG 3'
Rac-2	forward 5' TGTGGTGGTGGGAGATGG 3'
	reverse 5' CAGGATGATGGGTGTGCT 3'
Rab-11	forward 5' TTATTGGAGATTCTGGTGTTG 3'
	reverse 5' TTTGGCTTGTTTTCAGTGGTT 3'
Cox-1	forward 5' TGTTCGGTGTCCAGTTCCAAT 3'
	reverse 5' CAAATGTGCTCGGCTTCCAGT 3'
Cdk 4	forward 5' GTGGCTGGAGGGGTTGGTATTGA 3'
	reverse 5' AACTGTGCTGATGGGAAGGC 3'
clusterin	forward 5' GAGACCAGGGAATCAGAGACA 3'
	reverse 5' TACGGAGAGAAGGGCATCAAG 3'
caspase-4	forward 5' AGCAACGTATGGCAGGACAA 3'
	reverse 5' AGAGCAGAAAGCAATGAAGT 3'

Table 3. Results of macroarray analysis of lysoPC-regulated genes

Gene Name	Fold induction	Gene Bank Acc No
Rac-1	2.1	M29870
Rac-2	2.7	M29871
Rab-11	2.9	X56740
integrin α3	1.2	M59911
integrin α6	2.1	X53586
integrin β1	1.3	X07979
integrin β4	2.5	X51841
Cox-1	2.6	M59979
Cox-2	2.2	M90100
VEGF	2.7	M32977
tPA	4.3	M15518
caspase-4	0.42	Z48810
clusterin	0.73	M64722
PCNA	0.42	M15796
Cdk 4	0.25	U37022

LysoPC-regulated gene expression in endothelial cells. HUVECs were treated with medium alone (control) or 80 μM of lysoPC for 8 h and mRNA was isolated and used for macroarray analysis as described in the Materials and Methods. The criteria for selecting a gene was that the average signal intensity of that gene was at least 2-fold that of the background signal; signal intensity changes more than +/-2-fold of the control signal were considered to be lysoPC-regulated genes.

family Rac-1, Rac-2 and Rab-11, and the members of the integrin family, integrin α6 and integrin β4, in endothelial cells (Table 3). LysoPC also increased the expression of members of Cox family, Cox-1 and Cox-2 (Table 3), and Cox-2 was previously reported to be induced by lysoPC in endothelial cells (29). Furthermore, lysoPC reduced the mRNA expression of some of the genes to less than half of the control expression in endothelial cells, such as caspase-4, cyclin dependent kinase (Cdk) 4 and proliferating cell nuclear antigen (PCNA) (Table 3).

Analysis of the mRNA expression of lysoPC-regulated genes in endothelial cells with real time PCR.

To further investigate the mRNA expression and to confirm the results of the macroarray, we performed real time PCR to analyze the mRNA expression of lysoPC-regulated genes screened out by macroarray analysis. Consistent with the results of macroarray, the results of real time PCR revealed that lysoPC increased the mRNA expression of Rac-2, Rab-11, integrin α6 and integrin β4 3.9, 3.5, 4.4 and 7.7-fold, respectively, compared to the medium treated condition in endothelial cells (Figure 1A). We also checked the mRNA expression of tPA, which was previously reported to be induced by lysoPC in endothelial cells (18). The results showed that the mRNA expression of tPA was increased 8.4 fold by lysoPC (Figure 1A), confirming the results of macroarray analysis (Table 3) and a previous report (18). On the other hand, lysoPC increased the mRNA expression of Rac-1, but that increment was 1.8 fold (Figure 1A), although it was shown to be 2.1 fold in macroarray analysis (Table 3). Moreover, there was a 2.6-fold increase of Cox-1 mRNA expression by lysoPC shown in macroarray analysis (Table 3), but the results of real time PCR revealed that it was only 1.3 fold of the control (Figure 1B). Again, the results of real time PCR indicated that the mRNA expression of PCNA and Cdk 4 was not affected by lysoPC in endothelial cells (Figure 1B), which were shown to be downregulated in the results of macroarray analysis (Table 3). Furthermore, to detect false negative results, the mRNA expression of some genes shown to be nonregulated by lysoPC in macroarray analysis, such as integrin β1 and clusterin were analyzed by real time PCR. Similar to the results of macroarray, the mRNA expression of integrin β1 and clusterin was not regulated by lysoPC in endothelial cells (Figure 1B).

mRNA expression profile of lysoPC-regulated genes in endothelial cells.

After detecting the lysoPC-regulated genes by macroarray analysis and real time PCR, we decided to analyze the mRNA expression profile of newly identified lysoPC-regulated genes in endothelial cells by Northern blot analysis. The results showed that lysoPC increased the expression of integrin α6,

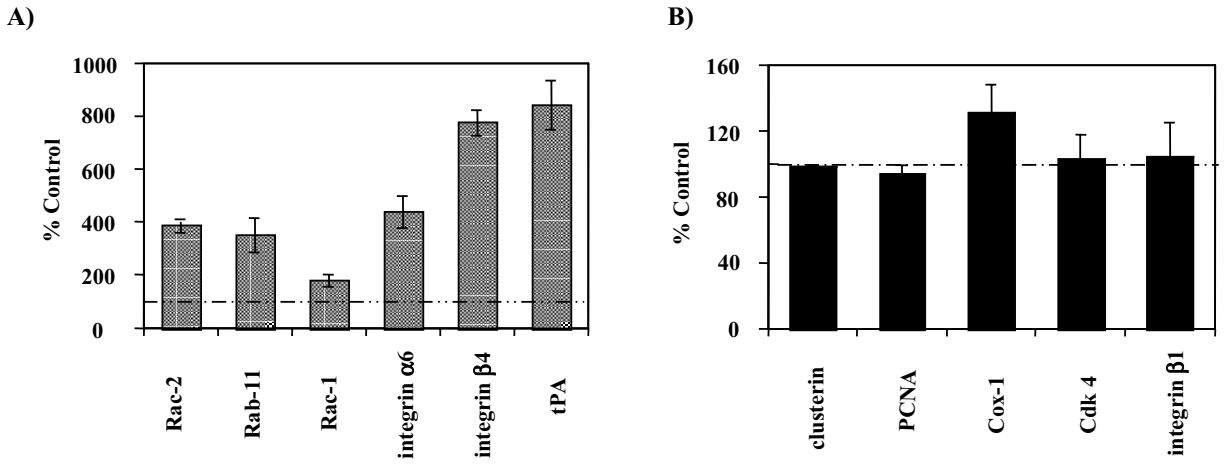

Fig. 1. Results of real time PCR. HUVECs were treated with medium alone or 80 μM of lysoPC for 8 h and the total RNA was isolated, reverse transcribed and real time PCR was performed, as described in the Materials and Methods, to verify the gene expression of lysoPC-regulated genes, determined by macroarray analysis. Results are expressed as the % of the control, considering the amount of mRNA of medium-treated condition to be 100%. (A) shows the real time PCR results of lysoPC-induced genes, and (B) shows the results of gene expression unaffected by lysoPC.

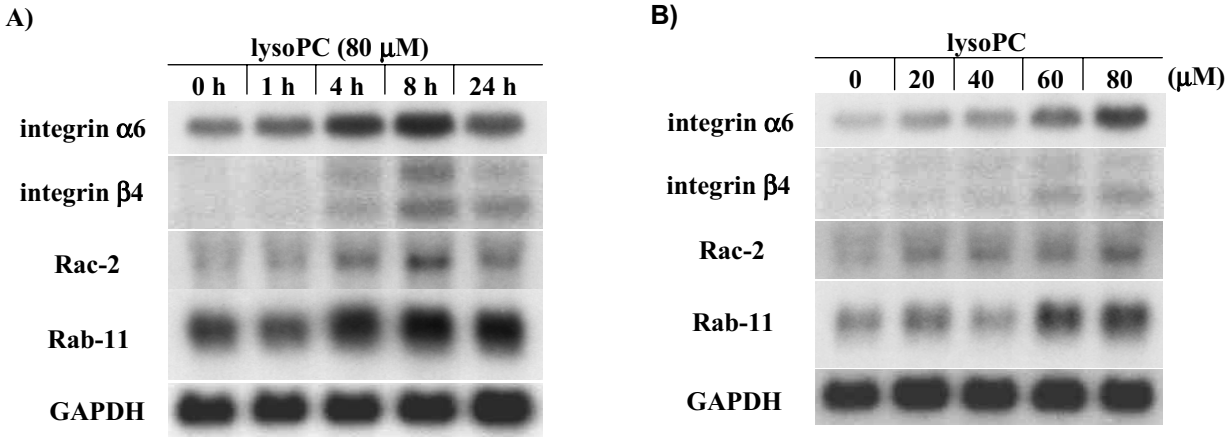

Fig. 2. The mRNA expression profile of lysoPC-regulated genes in endothelial cells. HUVECs were treated with lysoPC for the indicated time (A) or indicated dose (B), total RNA was isolated and Northern blot analysis was performed to analyze the level of mRNA expression, as described in the Materials and Methods. (A) shows the time course, and (B) shows the dose response analysis of lysoPC-regulated genes. Signal intensities of mRNA were normalized with the corresponding GAPDH mRNA.

integrin β4, Rac-2 and Rab-11 mRNA in endothelial cells in a dose and time dependent manner (Figure 2A and B). Integrin α6 mRNA was expressed at the basal condition, increased at 1 h, reached a peak at 8 h and decreased at 24 h (Figure 2A). The basal expression of integrin β4 mRNA was very small, its expression was induced at 4h, reached a peak at 8h and again decreased at 24 h (Figure 2A). Rac-2 and Rab-11 mRNA were expressed at the basal condition, and Rab-11 showed the strongest basal expression among the four molecules. LysoPC increased those expressions at 4h, reached a peak at 8h and then decreased at 24 h (Figure 2A).

Next, we investigated the mRNA expression profile of Cox-1, Cdk 4 and caspase-4 to determine whether lysoPC had any effect on their mRNA expression at any time point. Our results showed that lysoPC did not affect the mRNA expression of Cox-1, Cdk 4 and caspase-4 at any time point we have checked (Figure 3). Furthermore, lysoPC did not show any effect on the mRNA expression of clusterin, consistent with the macroarray analysis and real time PCR (Figure 3).

Flow cytometric analysis of integrin expression on endothelial cells.

To investigate the protein expression of lysoPC-regulated genes, the surface expression of integrins on endothelial cells was investigated by flow cytometry. Flow cytometric analysis showed that lysoPC selectively increased the surface expression

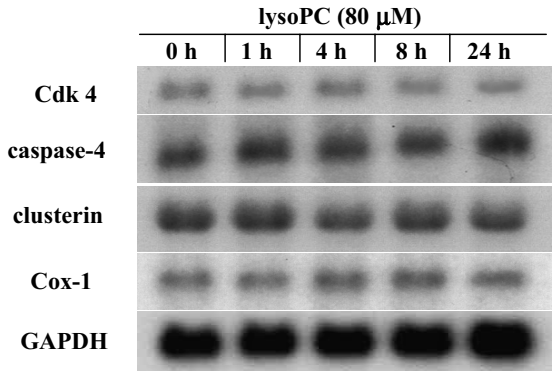

Fig. 3. mRNA expression profile of genes not regulated by lysoPC in endothelial cells. HUVECs were treated with lysoPC (80 μM) for the indicated time and total RNA was isolated; Northern blot analysis was performed to analyze the level of mRNA expression, as described in the Materials and Methods. Signal intensities of mRNA were normalized with the corresponding GAPDH mRNA.

of integrin α6 and integrin β4 in endothelial cells (Figure 4). We also checked the surface expression integrin α3 and integrin β1, which were shown to not be regulated by lysoPC in macroarray analysis (Table 3), and here also lysoPC did not affect their surface expression in endothelial cells (Figure 4).

DISCUSSION

The aim of this study was to investigate the effect of lysoPC on the expression of cell growth-, apoptosis- and angiogenesis-related genes in endothelial cells. The macroarray is a very useful technique

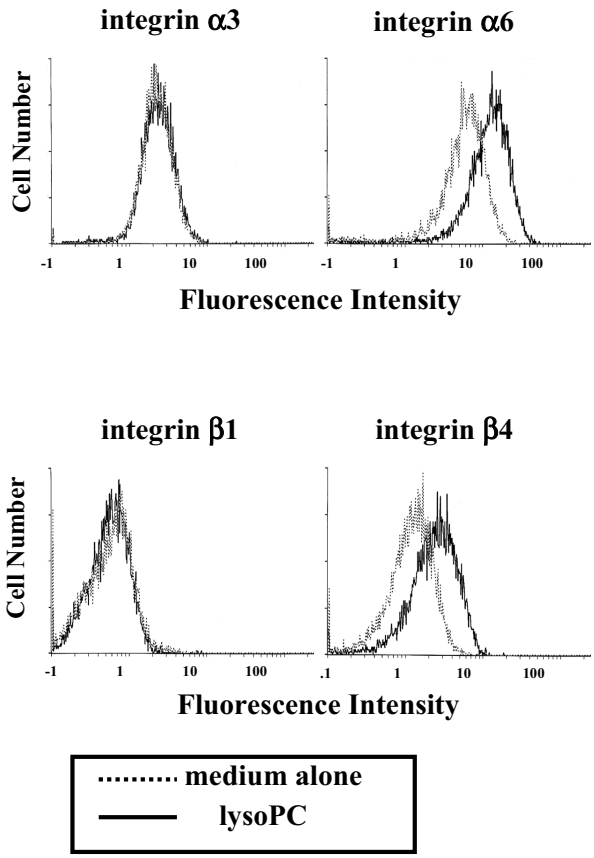

Fig. 4. Role of lysoPC on the surface expression of integrins on endothelial cells. HUVECs were treated with medium alone or with lysoPC (80 μM) for 16 h and flow cytometric analysis was performed to analyze the surface expression of integrins, as described in the Materials and Methods.

that can be used to analyze the expressional change of a large number of genes at one time. However, it was reported previously that macroarray analysis shows false positive and false negative results, and the amplification of an expressed sequence may introduce a distortion of the original expression pattern (30). Therefore, we used the macroarray as a screening tool to detect lysoPC-induced cell growth-, apoptosis- and angiogenesis-related genes in endothelial cells, and the mRNA expression of lysoPC-induced genes, screened out by macroarray, was further analyzed using real time PCR and Northern blot analysis to confirm the inducibility of these genes by lysoPC. Using this model we have shown that the expression of members of the small G protein family, Rac-2 and Rab-11, and those of the integrin family, integrin α6 and integrin β4, are induced by lysoPC at the mRNA level.

Small G protein Rac takes part in the assembly of the active NADPH oxidase complex, leading to the production of reactive oxygen species (ROS) (31,32). The organization of NADPH oxidase complexes of endothelial cells and neutrophils are similar (33), and Rac-1 and Rac-2 take part in the assembly of NADPH oxidase complex in endothelial cells and in neutrophils, respectively (31,32). LysoPC induces the production of ROS involving the NADPH oxidase complex, and increases the growth of endothelial cells (25). However, the involvement of Rac in the assembly of NADPH oxidase complex and ROS production by lysoPC is not clear. In our experiments, the mRNA expression of Rac-2 showed the strongest induction by lysoPC among the small G proteins, and Rac-1 mRNA reproducibly showed some induction (1.8 fold) in endothelial cells, suggesting the possibility of the involvement of Rac in lysoPC-induced NADPH oxidase assembly and production of ROS.

We showed that lysoPC induced Rab-11 expression at the mRNA level. Members of the Rab family play an essential role in the regulation of membrane transport between intracellular compartments (34). Rab-11 regulates protein recycling through recycling endosomes (34). For example, the overexpression of Rab-11 established angiotensin II type$_1$ A (AT_1 A) receptor recycling and promoted the re-distribution of AT_1 A receptor from early to recycling endosomes (35). AT_1 A receptor activation leads to ROS production in the vessel wall involving NADPH oxidase. Moreover, AT_1 A receptor activation induces endothelial cell growth and angiogenesis, and is implicated in the development of atherosclerosis (36), implying the possibility of the involvement of Rab-11 in the pathogenesis of the disease.

We also confirmed that lysoPC increased the mRNA and protein expression of integrin α6 and integrin β4 in endothelial cells. A recent report shows that integrin α6β4 signalling promotes the onset of pathological angiogenesis (37). Again, integrin β4 is involved in endothelial cell growth and apoptosis (38), which are important features in the process of angiogenesis. Considering these reports, our results suggested that lysoPC modulates angiogenesis by inducing the expression of the integrin α6 and integrin β4 gene, which influences

the growth and rupture of atherosclerotic plaques.

In conclusion, we presented the evidence that lysoPC induced the expression of the members of the small G protein family, Rac-2 and Rab-11, and those of the integrin family, integrin α6 and integrin β4, in endothelial cells. These findings suggest its involvement in the mechanisms of NADPH oxidase activation and ROS production, and draw attention to the role of lysoPC in endothelial cell growth and angiogenesis, and help us to better understand the development of atherosclerosis.

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