

# RanGAP-Mediated Nuclear Protein Import in Vascular Smooth Muscle Cells Is Augmented by Lysophosphatidylcholine

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## ABSTRACT

The intracellular mechanism responsible for the mitogenic effects of lysophosphatidylcholine (LPC) is unclear. Import of proteins from the cytoplasm into the cell nucleus is integral to the regulation of gene expression and cell growth. We hypothesized that LPC exerts its intracellular effects through alterations in nuclear protein import. Rabbit aortic smooth muscle cells incubated with LPC induced a significant increase in cell proliferation in both quiescent cells ( $63.2 \pm 6.48\%$  of control) and cells grown in 1% fetal bovine serum (FBS) ( $28.3 \pm 7.35\%$  of control). Vascular smooth muscle cells were preincubated with LPC then microinjected with a marker protein for nuclear import. A significant stimulation of nuclear protein transport was observed. Using a conventional nuclear protein import assay in permeabilized cells, a significant stimulation of import ( $72.3 \pm 5.2\%$  of control) was again observed when the cyto-

solic nuclear import cocktail was treated with LPC. This effect was not observed with other lysophosphatidyl species. LPC also activated the extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) pathway, and this was blocked by 2'-amino-3'-methoxyflavone (PD98059), which inhibits the activation of ERK 1/2. The stimulation of nuclear import was also blocked by PD98059. LPC-induced MAPK activation augmented GTP hydrolysis by RanGAP, a RanGTP-ase activating protein and a critical regulatory component of nuclear protein import, and this stimulation was again blocked by PD98059. We conclude that LPC alters gene expression and cell proliferation through striking effects on nuclear protein import via a MAP kinase-induced activation of RanGAP. This may play an important role in cancer and atherosclerosis and other disorders involving accelerated cell growth/proliferation.

Accelerated cell growth and proliferation is an important component of such diseases as atherosclerosis and cancer. It has been suggested that lysophosphatidylcholine (LPC) stimulates cell growth in atherosclerosis and cancer (Chai et al., 1996; Okita et al., 1997; Yamakawa et al., 1998; Fang et al., 2000). LPC is the major form of lysophospholipid in mamma-

lian tissues (Liu et al., 1997) and an amphiphilic metabolite of phosphatidylcholine. It can be generated via phosphatidylcholine hydrolysis by phospholipase A<sub>2</sub> (Prokazova et al., 1998; Yamakawa et al., 1998). During ischemia, LPC rapidly accumulates in the myocardium (Snyder et al., 1981; Sedlis et al., 1983; Liu et al., 1997) through both activation of PLA<sub>2</sub> (Aoyama et al., 2000) and depressed catabolism of LPC (Sedlis et al., 1983; Liu et al., 1997). LPC has been thought to function as a second messenger and signaling molecule. In addition, it has been speculated that the cell proliferation induced by other molecules, such as oxidized low-density lipoprotein, is due to its LPC content (Chai et al., 1996; Yamakawa et al., 1998). However, despite this identification of LPC as a mitogen, the precise intracellular mechanism for these effects is unclear.

The nuclear envelope is a double-membrane barrier be-

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**ABBREVIATIONS:** LPC, lysophosphatidylcholine; NLS, nuclear localization sequence; NPC, nuclear pore complex; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; PD98059, 2'-amino-3'-methoxyflavone; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; LPLC, lysoplasménylcholine; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; DTT, dithiothreitol; GMP-PNP, guanosine-5'-( $\beta$ - $\gamma$ -imido)-triphosphate.

tween the nucleus and the cytoplasm within the cell (Gorlich and Mattaj, 1996; Laskey, 1998; Perez-Terzic et al., 1999). The regulation of gene expression and signal transduction depends heavily on the import of proteins from the cytoplasm into the nucleus. Conversely, mRNA, transfer RNA, ribosomal RNA, and small nuclear RNA must be exported from the nucleus to the cytoplasm to initiate protein expression (Gorlich and Mattaj, 1996; Jans and Hubner, 1996). Nuclear transport is mediated through specific aqueous channels present in the nuclear envelope: the nuclear pore complexes (NPC) (Adam et al., 1991; Pante and Aebi, 1995, 1996; Sweet and Gerace, 1995; Gorlich and Mattaj, 1996; Jans and Hubner, 1996; Laskey, 1998; Perez-Terzic et al., 1999). The large size of most proteins and RNAs does not allow for rapid passive diffusion (Sweet and Gerace, 1995), so they must be transported actively through the NPC (Gorlich and Mattaj, 1996).

The energy-dependent import of proteins into the nucleus (Adam et al., 1991; Moore and Blobel, 1993; Pante and Aebi, 1995; Sweet and Gerace, 1995; Gorlich and Mattaj, 1996; Jans and Hubner, 1996; Pante and Aebi, 1996) is mediated by nuclear localization sequences (NLSs) (Pante and Aebi, 1995; Sweet and Gerace, 1995; Jans and Hubner, 1996), which target nuclear proteins to the NPC (Pante and Aebi, 1995). There are two identifiable steps of NLS-dependent nuclear protein import (Pante and Aebi, 1995; Sweet and Gerace, 1995; Jans and Hubner, 1996; Laskey, 1998). The first involves the binding of the proteins at the NPC (Pante and Aebi, 1995; Sweet and Gerace, 1995; Jans and Hubner, 1996). This step is both energy- and temperature-independent. The second stage of import is energy-dependent (Pante and Aebi, 1995; Sweet and Gerace, 1995; Gorlich and Mattaj, 1996; Laskey, 1998) and can occur only at physiological temperatures (Laskey, 1998). It involves translocation of the protein across the pore in a GTP-dependent fashion through a specific GTP hydrolyzing protein, RanGAP. In view of the critical role that nuclear protein import plays in directing gene expression (Jans and Hubner, 1996), LPC may stimulate vascular proliferation through stimulation of nuclear protein import through an undefined mechanism. This has not been previously addressed. Indeed, the effects of any lipid moiety on nuclear protein import have not been identified. In the present study, therefore, it was hypothesized that the proliferative effects of LPC may occur via an action on nuclear protein import.

## Materials and Methods

**Materials.** DMEM, FBS, and amphotericin B (Fungizone) were purchased from Invitrogen (Burlington, ON, Canada). BODIPY FL-conjugated to bovine serum albumin (BSA) was purchased from Invitrogen. ERK-2 and the MEK-1 inhibitor PD98059 was purchased from Calbiochem (San Diego, CA). Lysophosphatidylcholine (LPC) and PD98059 inhibitor were purchased from Sigma Aldrich Canada Ltd (Oakville, ON, Canada); lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), and lysophosphatidylserine (LPS) were purchased from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). The LPC was analyzed by electrospray ionization mass spectrometry and found to be primarily composed of 16:0 and 18:0 species with minor peaks of 16:1 and 18:1 LPC present. No contaminating lipid classes were detected. Lysoplasmenylcholine (LPLC) was synthesized as described previously (Williams and Ford, 1997). It was purified by reversed-phase high performance liquid

chromatography and was composed entirely of the 16:0 LPLC species as ascertained by electrospray ionization mass spectrometry. All other chemicals were purchased from Sigma-Aldrich Canada Ltd. The following antibodies were used: p44/p42 MAP kinase, phospho-p44/p42 MAPK (Cell Signaling, Danvers, MA) and loading control anti-GAPDH (Abcam, Cambridge, MA). The secondary antibody used was anti-mouse IgG (Chemicon, Temecula, CA).

**Cell Culture.** Aortic vascular smooth muscle cells were obtained from New Zealand white rabbits via an explant technique (Saward and Zahradka, 1997), and were maintained as described previously (Czubryt et al., 2000). For quiescent cell studies, serum-free media was used during cell incubation.

**Cell Proliferation Assay.** Cells were seeded at  $5 \times 10^3$  cells/well in 96-well plates and incubated with 1 to 10  $\mu$ M LPC for 24 h at 37°C in DMEM  $\pm$  1% FBS (Gibco). After 24 h, the number of living cells was determined by a colorimetric enzyme assay (CellTiter 96 Cell Proliferation Assay; Promega, Madison, WI) based on a cytoplasmic enzyme activity present in viable cells. The absorbance of a formazan product in tissue culture media was measured at 500 nm using a microplate reader. This method has been correlated previously in our lab with direct cell counts using a hemocytometer (Hirono et al., 2003).

**Treatment of Import Cocktail.** Import cocktail consisted of import buffer (20 mM HEPES, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, pH 7.5) plus 50% rat liver cytosol, 1 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase, 2 mM DTT, and 1  $\mu$ g/ml each of leupeptin, pepstatin A, and aprotinin. Rat liver cytosol was isolated as described previously (Czubryt et al., 2000). For some experiments, import cocktail was treated with 1 to 50  $\mu$ M LPC  $\pm$  20  $\mu$ M PD98059 for 15 to 60 min at 37°C. The effects of treating import cocktail with 10  $\mu$ M LPE, LPI, LPS, or LPLC for 30 min at 37°C were also tested. For all of the above experiments, import cocktail was treated before the import assay being carried out. Otherwise, the import cocktail was not treated; instead, permeabilized cells were treated directly with either 1 or 10  $\mu$ M LPC for 30 min at 37°C before the addition of import cocktail to initiate nuclear protein import.

**Nuclear Import Assay.** Nuclear protein import was assayed as described previously (Adam et al., 1991), with minor modifications. In brief, rabbit aortic vascular smooth muscle cells grown on coverslips were permeabilized with 40  $\mu$ g/ml digitonin (Sigma) for 5 min. Cells were rinsed with import buffer, and cover slips were inverted over 50  $\mu$ l of import cocktail (import buffer plus 50% rat liver cytosol, 1 mM ATP, 5 mM creatine phosphate, 20 units/ml creatine phosphokinase, and 1  $\mu$ g/ml each of leupeptin, pepstatin A, and aprotinin) plus 5  $\mu$ l of import substrate on parafilm in a humidified box and incubated at 37°C. The import substrate used was BSA coupled to an ALEXA fluorophore (ALEXA-conjugated BSA), to which was attached the simian virus 40 large T antigen NLS (CGGGP-KKKRKVED) (Adam et al., 1991; Czubryt et al., 2000). Cells were then permeabilized in 3.7% paraformaldehyde. Assay results were measured on an MRC 600 UV-VIS confocal imaging system (Bio-Rad Laboratories, Hercules, CA) equipped with a Nikon Diaphot 300 microscope (Nikon, Tokyo, Japan). Transport of the import protein into the nucleus from the cytoplasm was quantified on an SGI workstation (SGI, Mountain View, CA) using GE Healthcare (Little Chalfont, Buckinghamshire, UK) ImageSpace 3.2.1 software.

**Micoinjection.** Coverslips containing smooth muscle cells were placed in a culture dish with 1  $\mu$ M LPC diluted in feeding solution (5% FBS + 1% amphotericin B) and incubated for 30 min at 37°C. Treated coverslips were then rinsed with 1 $\times$  phosphate-buffered saline and placed in a Leyden dish, and 1 ml of prewarmed perfusate buffer (6 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM dextrose, and 6 mM HEPES, pH 7.4) was added. Temperature was maintained at 37°C in a micropertusion chamber. Approximately 10  $\mu$ l of fluorescent substrate (ALEXA-BSA-NLS) in nuclear import buffer was added to a micropipette using a 1-ml syringe, ensuring that no bubbles were present in the tip. Cells to be injected exhibit a small

degree of autofluorescence that allowed visualization of the cell before injection. Using an MS314 micromanipulator (Fine Science Tools), the pipette was inserted into the cell cytoplasm in close proximity to the nucleus. The microinjector used in our study was a PV830 Pneumatic PicoPump (World Precision Instruments), and settings were as follows: injection hold pressure, 40 psi; eject pressure, 60 psi; range, 100 ms. Cells were injected four times, and the pipette was slowly removed. Images of the microinjected cell were acquired as described for nuclear import assays. Final images were analyzed and processed as described above.

**Western Blot Analysis.** Vascular smooth muscle cells were rendered quiescent for 48 h followed by treatment with 10  $\mu$ M LPC, 10  $\mu$ M LPC + 20  $\mu$ M PD98059, 20  $\mu$ M PD98059, or no treatment for controls. Vascular smooth muscle cells were incubated for 15 min at 37°C. Cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, and 1 mM EGTA, pH 7.5, with 1 mM PMSF, and 1 mM benzamidine), and aliquots of cell lysates were used for determination of protein. Protein samples were prepared using 30  $\mu$ g of protein in 2 $\times$  sample buffer (0.15 M Tris, pH 6.8, 1.2% SDS, 30% glycerol, 15%  $\beta$ -mercaptoethanol, and 1.8% bromophenol blue) and separated on SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes and placed in blocking buffer (10% skim milk powder and 0.05% Tween 20 in TBS) overnight at 4°C, followed by incubation with the 1° antibody in Tris-buffered saline, 1% skim milk, and 0.05% Tween 20. The membrane was then incubated for 1 h in a solution containing 1% skim milk powder, 0.05% Tween 20 and the 2° antibody (i.e., a horseradish peroxidase-conjugated goat anti-mouse Ig). The membrane was then visualized with the ECL detection system (Pierce, Rockford, IL), imaged using a Fluor-S Max MultiImager and quantified using Quantity One imaging software (Bio-Rad Laboratories).

**RanGAP Assay.** RanGAP activity was quantified using a Phos-Free Phosphate Assay Kit (Cytoskeleton Inc., Denver, CO), which measures RanGTP hydrolysis. Approximately 1  $\mu$ g of human recombinant Ran from Sigma-Aldrich Canada Ltd. was loaded with GTP (Sigma-Aldrich) in the presence of loading buffer (10 mM EDTA, 2 mM ATP, 4 mM DTT, and 50 mM HEPES, pH 7.4) for a total of 50  $\mu$ l. Loading mixture was incubated at room temperature for 30 min, then diluted 10-fold into buffer (5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mg/ml BSA, 0.005% Tween 20, and 50 mM HEPES, pH 7.4). Unbound GTP was removed on a NAP-5 column supplied by GE Healthcare and eluted with GAP buffer. Samples containing GAP buffer and 15% rat liver cytosol were pretreated for 10 min at 37°C with 10  $\mu$ M LPC, or 10  $\mu$ M LPC + 20  $\mu$ M PD98059, 20  $\mu$ M PD98059, or no treatment for controls. Reaction mixtures were then incubated with RanGTP for 10 min at 37°C. The assay measures free phosphate as an increase in absorbance at 650 nm.

**Statistical Analysis.** Variation between means was determined by two-tailed Student's *t* test, with *P* < 0.05 considered statistically significant. If more than two groups were tested, then one-way analysis of variance test was used, followed by a Duncan's multiple-range post hoc test, with *P* < 0.05 considered statistically significant.

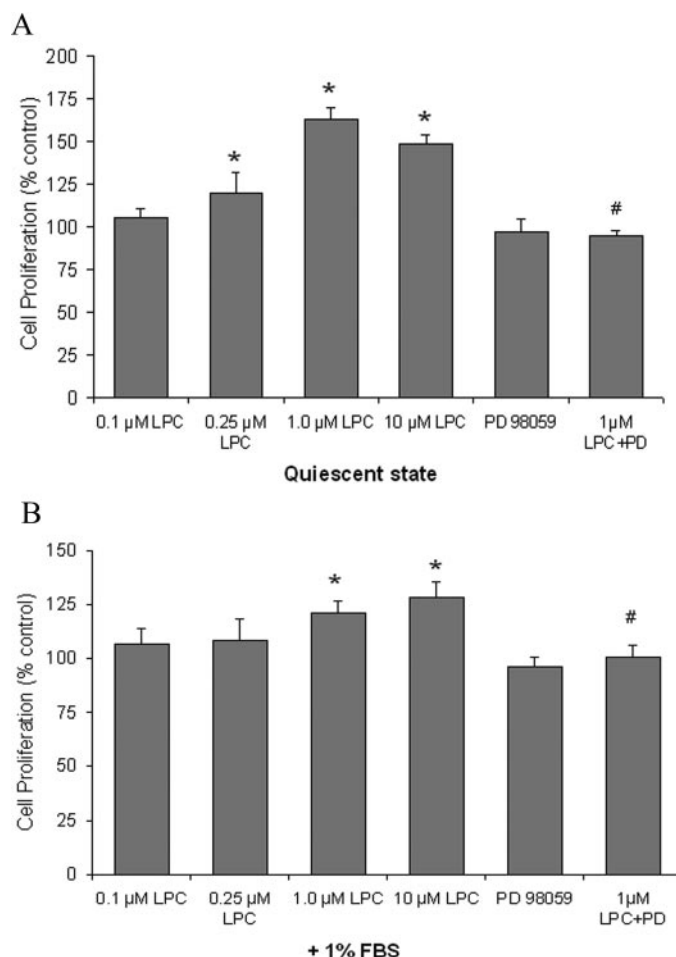
## Results

Both 1 and 10  $\mu$ M LPC induced a strong stimulation of cell proliferation (Fig. 1). This was observed if the cells were starting from a quiescent state or already progressing within the cell cycle in the presence of FBS. The stimulation of cell proliferation was not as great when the cells were exposed to LPC in the presence of FBS because the basal starting point for proliferative activity was already higher than in the quiescent state. These results are consistent with other reports that LPC is a potent mitogen (Chai et al., 1996; Okita et al., 1997; Yamakawa et al., 1998; Fang et al., 2000). The LPC

induced effects on cell proliferation were reduced to control levels with the addition of the inhibitor PD98059 (Fig. 1).

To investigate the intracellular mechanism responsible for the cell proliferative effects of LPC, we examined the effects of LPC on nuclear protein import. We first examined the *in situ* effects of LPC using a cell microinjection technique. Cells were preincubated with DMEM alone or DMEM + LPC (1  $\mu$ M) and then microinjected with ALEXA<sub>488</sub>-BSA-NLS to visualize nuclear fluorescence. Nuclear fluorescence was measured for 30 min after microinjection of the import substrate. Microinjection of cells treated with LPC resulted in a marked increase in the nuclear accumulation of fluorescent substrate versus control cells (Fig. 2, A and B). High nuclear fluorescence is indicative of increased rates of nuclear transport (Faustino et al., 2002). LPC treatment of cells in multiple experiments induced a statistically significant augmentation of nuclear protein import at all time points examined (Fig. 2C).

The intracellular mechanism whereby LPC stimulated nuclear protein import was unclear. To examine the mechanism of action, a conventional cell permeabilization assay was used (Faustino et al., 2002). The advantage of this technique



**Fig. 1.** Cell proliferation is significantly stimulated by LPC. Vascular smooth muscle cells were rendered quiescent with serum-free media (A) or cultured in the presence of 1% FBS (B). Cells were exposed to various concentrations of LPC for 24 h. LPC stimulated cell growth under both conditions and was significantly inhibited by 20  $\mu$ M PD98059. \*, *P* < 0.05 versus control cells that were not exposed to LPC; #, *P* < 0.05 versus LPC-treated group (*n* = 6–8 independent experiments).



is that it allowed us to examine in greater detail the biochemical characteristics of the action of LPC and to isolate its effects to specific cytosolic components of the nuclear protein import pathway. Consistent with the microinjection results, LPC stimulated nuclear protein import (Fig. 3). Increasing the duration of treatment of import cocktail at 37°C with 10  $\mu$ M LPC resulted in a steady rise in nuclear import, with a peak of  $\sim 70\%$  increase at 30 min (Fig. 3, B and D). This degree of stimulation at 30 min was similar to that observed using the microinjection technique (Figs. 2C and 3D). Longer incubation times resulted in an attenuation of import (Fig. 3, C and D). The effects of different concentrations of LPC on nuclear protein import were studied with a constant pretreatment time of 30 min (Fig. 3E). As little as 1  $\mu$ M LPC significantly stimulated import. Import peaked at 10  $\mu$ M LPC. At concentrations above the critical micelle concentration of LPC ( $>10 \mu$ M), nonspecific detergent side effects were observed. Thus, all subsequent experiments were performed at concentrations of LPC that were  $\leq 10 \mu$ M.

It is possible that other lysophospholipid species may alter import as well. While pretreatment of import cocktail with 10  $\mu$ M LPC caused a striking increase in protein import, pretreatment with any of 10  $\mu$ M LPE, LPI, or LPS produced no

effect (Fig. 4). Pretreatment with 10  $\mu$ M LPLC, however, significantly increased import by  $\sim 30\%$ .

The mechanism by which LPC was altering nuclear protein import was investigated. LPC did not stimulate import when incubated with isolated nuclei, suggesting that the nuclear pore complex itself did not directly mediate its effects (data not shown). This stimulation of import occurred only after pretreatment of the cytosolic fraction and demonstrates that the effects of LPC are dependent upon an action of a specific cytosolic component. It is known that LPC can activate the ERK MAPK pathway (Jing et al., 2000) and that ERK1/2 activation can stimulate nuclear protein import (Faustino et al., 2006). The possibility that LPC may stimulate nuclear protein import through ERK1/2 activation was tested using PD98059, an inhibitor of ERK1/2 activation by the MEK1/2. PD98059 was included with 10  $\mu$ M LPC and the rate of nuclear import was measured. LPC alone caused a significant increase in nuclear fluorescence, but the addition of PD98059 significantly reversed this augmentation of nuclear protein import (Fig. 5).

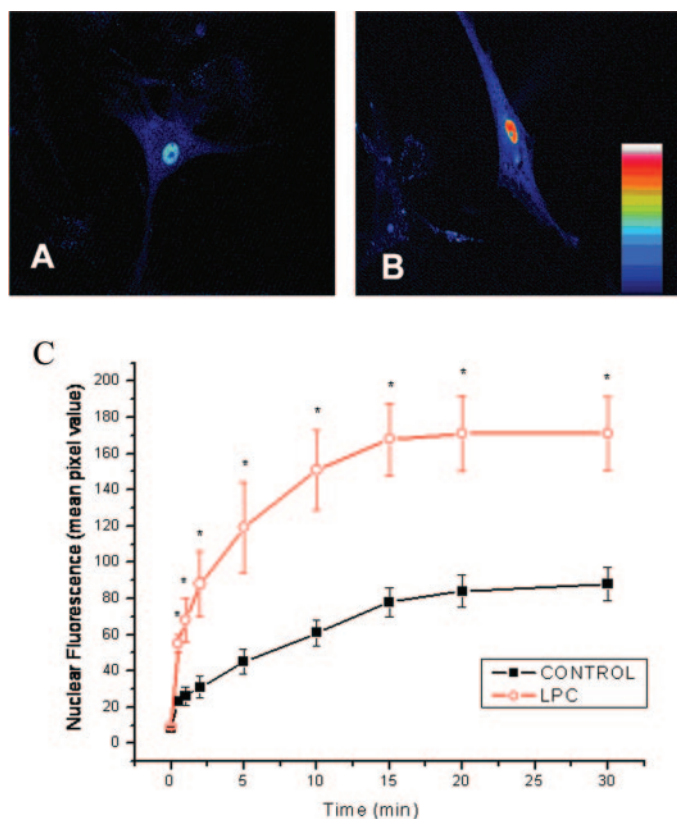
The observation that LPC-stimulated import was sensitive to PD98059 suggests that LPC induced an activation of ERK 1/2. As shown in Fig. 6, LPC induced an increase in phospho44/42 and this was inhibited by PD98059. PD98059 had no effect on its own. This demonstrates a direct activation of ERK 1/2.

The generation and utilization of GTP is a critical regulatory point within the sequence of steps necessary for protein to be imported into the nucleus (Gorlich et al., 1996). Its involvement in the stimulatory effects of LPC was tested. Incubation of GMP-PNP (a nonhydrolyzable analog of GTP) with 1  $\mu$ M LPC in the permeabilized cell assay system abolished the LPC-mediated increases in nuclear protein import (data not shown). Exogenously added cytosol that had been pretreated with 1  $\mu$ M LPC plus 1 mM GMP-PNP for 30 min at 37°C was not able to augment nuclear import ( $88.6 \pm 1.9\%$  of control;  $n = 3$  separate experiments examining 92 cells). RanGAP is an important protein that activates hydrolysis of Ran-bound GTP. It was, therefore, a logical site to investigate as a potential mechanism for the effects of LPC. As shown in Fig. 7, incubation of cytosol with 10  $\mu$ M LPC induced a significant increase in RanGAP activity above control levels, as reflected by the generation of inorganic phosphate through its GTPase activity. This stimulation was inhibited by PD98059 and this drug had no effect on its own.

## Discussion

LPC significantly stimulated cell proliferation and nuclear protein import. The stimulatory effects of LPC on both proliferation and nuclear import were relatively large and similar in magnitude ( $>70\%$ ). The stimulation of import by LPC was observed irrespective of the methodology used to measure import. The effects of LPC were observed in situ in cells using the microinjection technique. This demonstrates its physiological relevance in these cells.

A source for concern in our study was that higher concentrations of LPC tend to form micelles, causing membrane breakdown via a "detergent-like" action (Katz and Messineo, 1981). A 16-carbon LPC chain (as was used in our study) would not be expected to induce critical micelle formation until LPC concentrations exceeded 10  $\mu$ M. The small inhib-

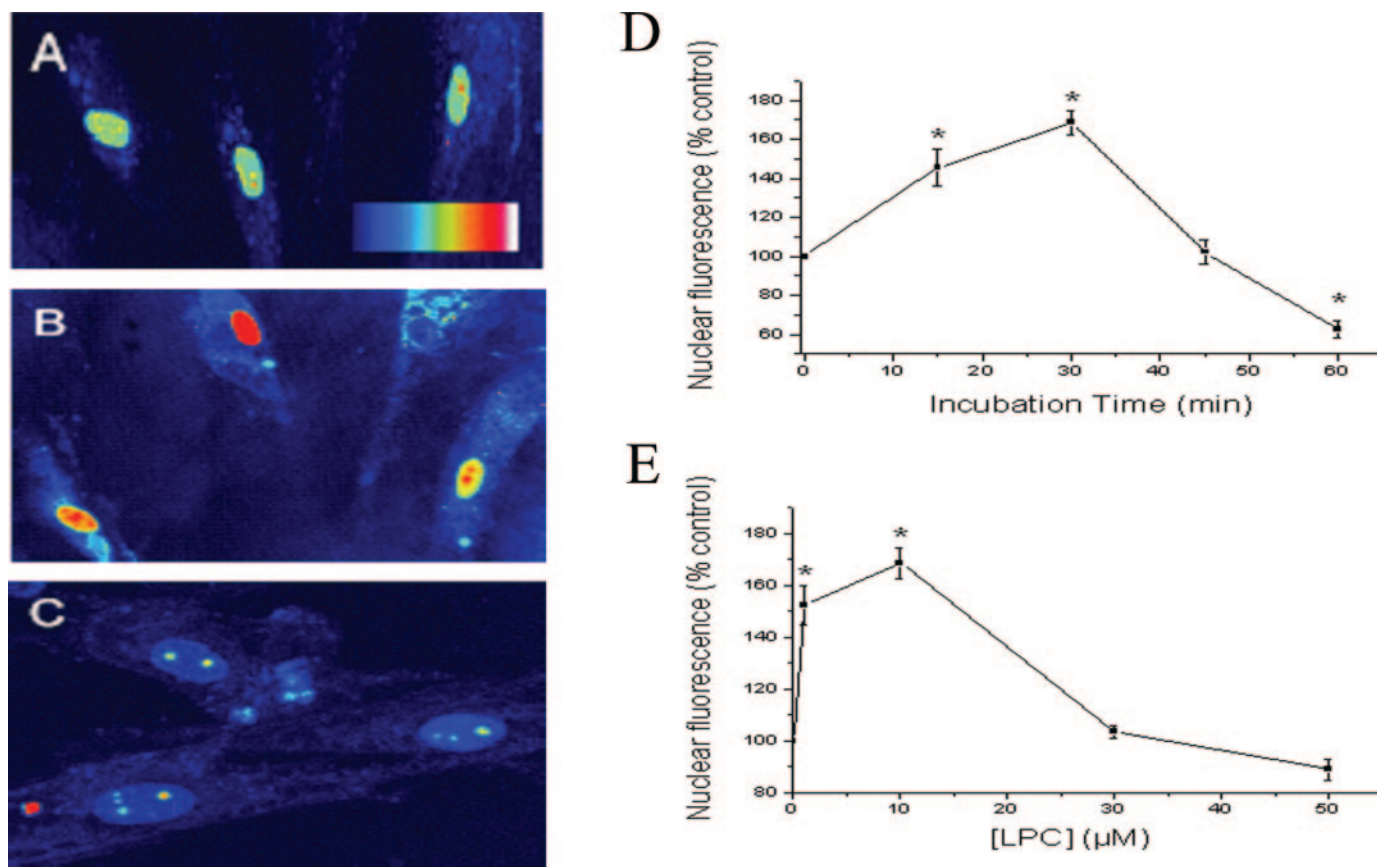


**Fig. 2.** Nuclear protein import in vascular smooth muscle cells is significantly increased after treatment with 1  $\mu$ M LPC. A, cells incubated in DMEM alone were microinjected with ALEXA<sub>488</sub>-BSA-NLS substrate and used as controls. B, cells incubated in DMEM plus a final concentration of 1  $\mu$ M LPC were microinjected and exhibit augmented nuclear fluorescence. C, comparison of nuclei from control and LPC-treated cells over 30 min after injection show a sustained increase in the rate of nuclear protein import in vascular smooth muscle cells. Nuclear fluorescence was reported as mean pixel value of the nucleus  $\pm$  S.E.M. Values at preinjection ( $t < 0$  min) are due to autofluorescence. (\*,  $P < 0.05$  versus control,  $n = 3$ ).

itory effects demonstrated by  $>10\ \mu\text{M}$  LPC and during longer incubation times were probably the result of nonspecific detergent effects. It is highly unlikely, therefore, that the stimulatory effects at 1 to  $10\ \mu\text{M}$  LPC were associated with a nonspecific detergent action. Furthermore, the lack of an effect of other lysophospholipids at  $10\ \mu\text{M}$  makes it unlikely that the effects we observed were due to a nonspecific detergent action. Alternatively, the specificity of the stimulatory effects of the different lysolipids may be thought to be due to some slight differences in their hydrophobicity. For example, the LPI and LPS are composed predominantly of 18:0 molecular species, whereas LPE is composed of  $\sim 25\%$  16:0 and  $75\%$  18:0. In contrast, the LPC used is  $\sim 75\%$  16:0 and  $25\%$  18:0. Thus, the LPC is slightly less hydrophobic. However, it should be noted that the LPLC that was employed in the study contained 100% 16:0, which would make it similar or even less hydrophobic compared with the mixed LPC used. One would expect that if hydrophobicity were important for efficacy of these lysophospholipids, then 16:0 LPLC would be more efficacious than the mixed molecular species LPC used. We speculate that the difference in monoacyl LPC and LPLC on nuclear protein import was probably due to the disparate molecular impact of the ester-linked and vinyl ether-linked aliphatic groups on the putative phosphocholine binding

pocket. These data are consistent with recent findings that the phosphocholine head group was necessary for the stimulation of DNA synthesis in smooth muscle cells (Chai et al., 2000).

Several lines of evidence suggest that the mechanism of action of LPC on nuclear protein import involves an intracellular pathway. The data acquired from the two different techniques for measuring nuclear protein import represent the first line of evidence in support of this hypothesis. In the microinjection experiments, LPC was applied to the outside of the cells, and then the cells were injected with the protein import substrate. With this method, it is difficult to determine whether the effects of LPC are due to an interaction with the extracellular surface of the cell or to an intracellular mechanism of action. Either option is possible. LPC is known to passively cross the plasma membrane into the cell (Van Der Luit et al., 2003). Alternatively, LPC can interact with receptors on the cell surface to initiate a cascade of cell signaling to induce its effects (Chai et al., 1996). However, the cell permeabilization assay selectively examines the effects of LPC on the cell cytosol. The plasma membrane is effectively perforated by digitonin treatment of the cell, and LPC is preincubated only with the cytosolic fraction. The stimulation of LPC in this assay system, therefore, clearly



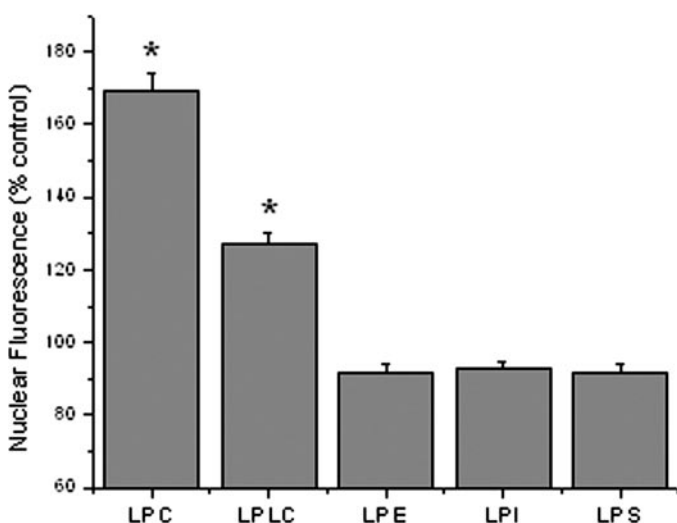
**Fig. 3.** Nuclear protein import as a function of duration of incubation with  $10\ \mu\text{M}$  LPC. A, control cells were incubated with nuclear import cocktail untreated with LPC. Inset, color scale indicates areas of low fluorescence (blue) and high fluorescence (red/white). High nuclear fluorescence is representative of increased rates of import. Permeabilized cells were also treated with  $10\ \mu\text{M}$  LPC for 30 min (B) or 60 min (C) at  $37^\circ\text{C}$  before the import assay. Nuclei in B demonstrate increased import at 30 min, whereas those in C, show decreased import at 60 min. Nuclear protein import was assessed as a function of incubation time in a series of experiments. D, exogenous cytosol was treated for various timepoints with  $10\ \mu\text{M}$  LPC before adding to the permeabilized cell assay system. Nuclear fluorescence quantitated by confocal microscopy was normalized relative to controls. Error bars represent S.E.M. for  $n = 4-8$  separate experiments (57–146 cells per experiment). \*,  $P < 0.05$  versus control. E, nuclear protein import as a function of treatment of import cocktail with different concentrations of LPC for 30 min at  $37^\circ\text{C}$ . Error bars represent S.E.M. for  $n = 3-8$  separate experiments (53–146 cells per experiment). \*,  $P < 0.05$  versus control.

rules out the involvement of the cell surface in the effects of LPC on nuclear protein import and instead implicates a target in the cytosol as the primary mechanism for its action.

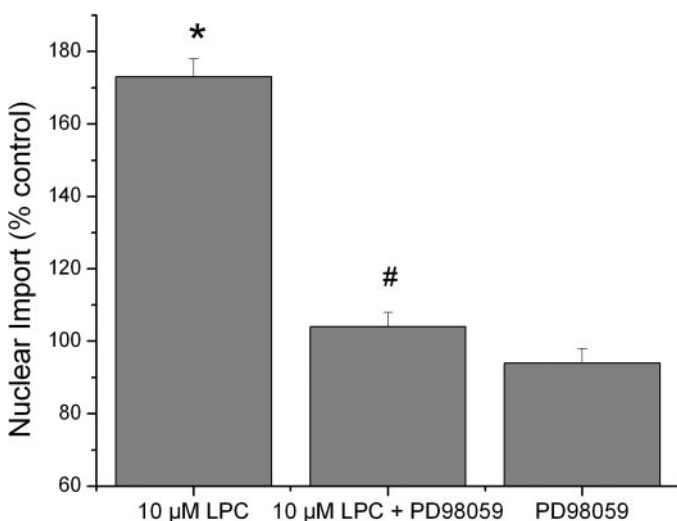
Our experiments have identified the cytosolic target for LPC. We have demonstrated that LPC activates the ERK MAPK pathway. This is consistent with previous observations (Jing et al., 2000). We have found MAPK activation, specifically ERK1/2, can increase nuclear protein import (Faustino et al., 2006). Thus, it was a logical step to test the possibility that the action of LPC might be mediated through this pathway. The stimulation of nuclear protein import by LPC was blocked by PD98059, strongly suggesting that ERK1/2 activity is involved in the process. However, the downstream target for the LPC-induced ERK effects on nu-

clear transport was not as easily anticipated. Because the import substrate we constructed does not contain a phosphorylation substrate site within its NLS sequence (Czubryt et al., 2000), the effects of LPC clearly did not involve phosphorylation of the imported protein itself. Instead, a variety of cytosolic proteins shares a docking sequence for ERK MAP kinase (Jacobs et al., 1999). RanGAP was selected as a candidate protein upon which to focus our attention. Our data demonstrate that LPC stimulated RanGAP activity (Fig. 7). The involvement of ERK in the LPC-induced stimulation of RanGAP activity was demonstrated by its sensitivity to PD98059.

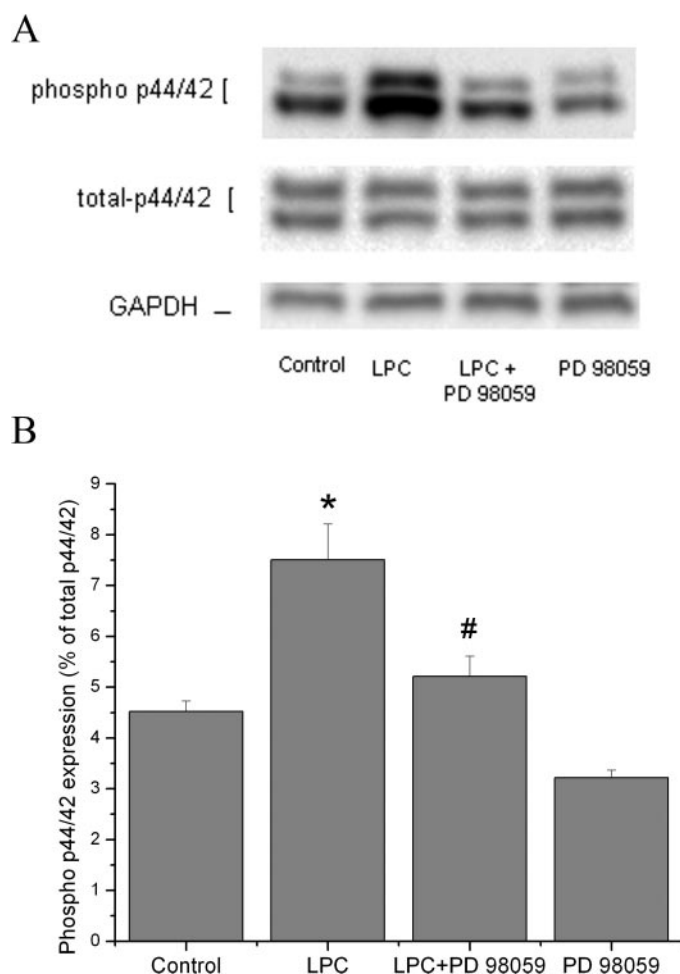
The significance of this cascade of signaling events is clear. RanGAP is an upstream regulator of Ran (Bischoff et al., 1994; Nishimoto, 2000) and, in concert with RanBP1, is essential in maintaining high levels of RanGDP in the cytosol (Bischoff et al., 1994). Because of the exclusively cytoplasmic localization of RanGAP and RanBP1, a gradient is created across the nuclear envelope with low concentrations of RanGDP in the nucleus. In contrast, RanGTP concentrations are high in the nucleus and low in the cytosol (Izaurralde et al., 1997). We have shown that LPC stimulates ERK 1/2 activity, which in turn will stimulate RanGAP activity. This



**Fig. 4.** Nuclear protein import as a function of treatment of import cocktail with LPC or other lysolipids (10  $\mu$ M) for 30 min at 37°C. Nuclear fluorescence (import) determined by confocal microscopy was normalized relative to controls. Error bars represent S.E.M. for 77 to 146 cells in three to eight separate assays. (\*,  $P < 0.05$  versus control).



**Fig. 5.** Inhibition of the ERK1/2 pathway reverses the effects of LPC on nuclear protein import. Permeabilized smooth muscle cells show a marked increase in nuclear fluorescence after pretreatment with 10  $\mu$ M LPC for 30 min. This was significantly inhibited in the presence of 20  $\mu$ M PD98059, a specific MEK inhibitor. PD98059 alone gave results similar to control nuclear protein import data. Fluorescence values were normalized to control cells and reported as mean  $\pm$  S.E.M. (\*,  $P < 0.05$  versus control; #,  $P < 0.05$  versus LPC-treated cells).



**Fig. 6.** A, stimulation of p44/42 phosphorylation by LPC was determined by Western blot analysis. B, LPC significantly increased phospho-p44/42 expression compared with control. Addition of the inhibitor PD98059 successfully attenuated the LPC induced activation. Results are normalized to total p44/42 levels and reported as mean  $\pm$  S.E.M.,  $n = 4$ . (\*,  $P < 0.05$  versus control; #,  $P < 0.05$  versus LPC).



will increase the hydrolysis of GTP-bound Ran, leading to a greater amount of RanGDP. The data obtained using the nonhydrolyzable GTP analog GMP-PNP strengthens the association of GTP with the import process. As more RanGDP becomes available, this will cause a net increase in RanGTP, enabling more importins ( $\alpha$  and  $\beta$ ) to be recycled to the cytoplasm per unit time. The number of nuclear import events then increases as more importins become available for nuclear transport. Our report is the first to document such an effect on nuclear import by any lipid, and we have identified a unique interaction via the RanGAP pathway.

The results have important functional implications during atherosclerosis. Proliferation of smooth muscle cells is a critical event in atherosclerosis (Chai et al., 1996). Atherosclerotic molecules like oxidized LDL have been suggested to induce cell proliferation in a plaque through its LPC content (Chai et al., 1996; Yamakawa et al., 1998). As shown in the present study and elsewhere (Chai et al., 1996; Yamakawa et al., 1998), LPC can also directly induce smooth muscle cell proliferation. LPC may achieve this action by stimulating the expression of a variety of genes important in atherogenesis and cell proliferation (Kume and Gimbrone, 1994; Aoyama et al., 2000). Indeed, the relevance of the present study may extend beyond the vasculature. The effects of LPC on nuclear protein import reported here may also partly explain the proliferative effects of LPC in cancer (Okita et al., 1997; Fang et al., 2000) and other disorders like ischemia. In ischemia, LPC is known to accumulate (Snyder et al., 1981; Sedlis et al., 1983; Liu et al., 1997) to  $\sim 10$  to  $20 \mu\text{M}$  under in vivo conditions (Liu et al., 1997).

In summary, LPC may be a far more important signaling molecule in effecting changes in gene expression than previously appreciated. Relatively high concentrations of LPC are thought to be generated in vivo (Liu et al., 1997). In addition, nuclei are unusually enriched in lysolipids (Baker and Chang, 2000). Thus, they are in sufficient concentrations and in an ideal location within the cell to produce a striking

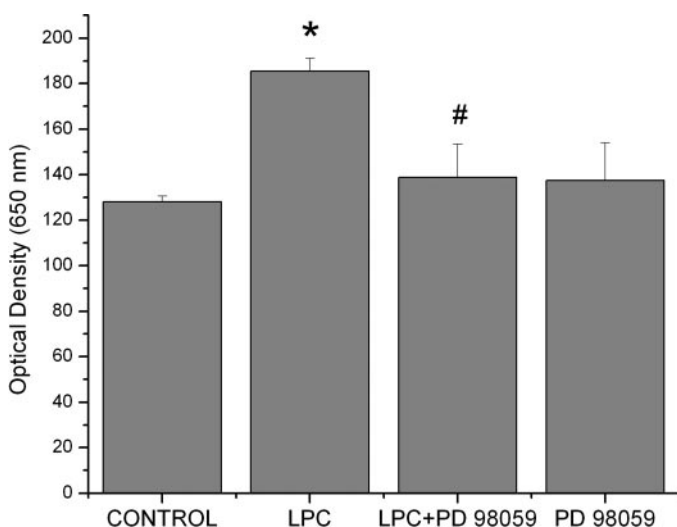
stimulation of nuclear protein import through an effect on the RanGAP signaling pathway. This could have important mechanistic implications for cell proliferation in atherosclerosis and other proliferative disorders, such as cancer.

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**Fig. 7.** RanGAP activity after treatment with  $10 \mu\text{M}$  LPC. Rat liver cytosol was incubated with or without LPC, and RanGAP activity was assayed by measuring RanGTP hydrolysis. Treatment with  $10 \mu\text{M}$  LPC significantly increased RanGAP activity. Incubation with PD98059 significantly attenuated the LPC activated hydrolysis of RanGTP. Results were reported as mean  $\pm$  S.E.M.,  $n = 5$ ,  $P < 0.05$  versus control; #,  $P < 0.05$  versus LPC.

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