Lysophosphatidylcholine Enhances Cytokine-Induced Interferon Gamma Expression in Human T Lymphocytes

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Abstract—Accumulation of substantial numbers of activated T lymphocytes, as well as monocyte/macrophages, in focal areas of arterial intima appears to be a hallmark of atherogenesis. Our previous report demonstrated that lysophosphatidylcholine (lyso-PC), a polar phospholipid component that is increased in atherogenic lipoproteins and atherosclerotic lesions, can upregulate the expression of heparin-binding epidermal growth factor–like growth factor and the interleukin (IL)-2 receptor in cultured human peripheral T lymphocytes. In this study, we show that lyso-PC can also enhance interferon gamma (IFN-γ) secretion and gene expression in human T lymphocytes. Lyso-PC–induced upregulation of IFN-γ depended on the presence of IL-2, IL-12, or phytohemagglutinin in culture media and was similarly observed in both CD4+ and CD8+ subsets. Actinomycin D chase by Northern blotting showed that lyso-PC significantly prolonged IFN-γ mRNA half-lives in human T cells. Transient transfection of IFN-γ promoter-reporter gene construct in the human T-cell line Jurkat cells demonstrated that lyso-PC stimulated the transcription of IFN-γ promoter-driven luciferase gene. Analyses of serial deletion mutations of IFN-γ promoter revealed that the lyso-PC–responsive element is located between base pairs −102 and −78 of the transcription initiation site of the IFN-γ gene. Enhanced expression of IFN-γ in T lymphocytes by lyso-PC may play a crucial role in atherogenesis. (Circ Res. 1998;83:508-515.)

Key Words: lysophosphatidylcholine ■ interferon gamma ■ T lymphocyte ■ atherosclerosis

Atherosclerotic lesions, at various stages, are characterized by localized infiltration of T lymphocytes, as well as monocyte/macrophages, in arterial intima.1,2 Most of the T lymphocytes in these lesions appear to be memory cells and in a state of chronic activation, including expression of major histocompatibility complex (MHC) class II antigens and very late antigen-1.3 Interferon gamma (IFN-γ) is a T cell–derived multifunctional cytokine that can induce aberrant MHC class II expression,4,5 inhibit smooth muscle cell (SMC) proliferation and collagen synthesis,5,6 and downregulate scavenger receptor expression,7 which may modulate atherosclerotic progression and affect the stability of advanced plaques.8 Furthermore, IFN-γ and other cytokines, eg, interleukin (IL)-2 and IL-12, have been shown to be expressed in atherosclerotic plaques in vivo.9–11 Thus, potential roles of T lymphocytes have been suggested in atherogenesis; however, molecular mechanisms responsible for activation of T lymphocytes in this disease process have not been fully clarified.

On the other hand, several lines of evidence have suggested that oxidatively modified LDL plays a key role in atherogenesis.12–16 Lysophosphatidylcholine (lyso-PC) is a major phospholipid component increased in both oxidized LDL and β-migrating VLDL.17–19 Concentrations of lyso-PC are elevated in atherosclerotic lesions in animals fed an atherosclerotic diet,20,21 and probucol, an antioxidant that prevents oxidative modification of lipoproteins, can reduce the formation of lyso-PC in atherosclerotic lesions.22 Previous studies have shown that lyso-PC can activate vascular endothelial cells as well as monocyte/macrophages in vitro to induce certain genes relevant to atherogenesis,22–27 in addition to its action as a chemoattractant for T lymphocytes and monocytes.28,29 Our previous report has also shown that lyso-PC selectively upregulates the expression of heparin-binding epidermal growth factor–like growth factor (HB-EGF) and the IL-2 receptor in T lymphocytes and suggested that this polar phospholipid may be an important stimulus for T cells in atherogenesis.30 In the present study, we provide evidence that lyso-PC can transcriptionally and posttranscriptionally enhance other stimuli (IL-2, IL-12, or phytohemagglutinin [PHA]–induced IFN-γ expression in cultured human T lymphocytes.

Materials and Methods

Cells
Human peripheral T lymphocytes were isolated by 2 different methods: direct positive panning and negative selection. Human mononuclear cells were isolated from healthy donors by Ficoll density gradient centrifugation. T lymphocytes were separated by direct positive panning on anti-CD3 monoclonal antibody–coated flasks (MicroCELLector, Applied Immune Sciences) according to the manufacturer’s instructions. In brief, mononuclear cells were incubated at room temperature in PBS containing 0.5% (vol/vol) human γ-globulin to block Fc receptors for 15 minutes and then

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introduced into anti-CD3 monoclonal antibody–coated flasks. After a 1-hour incubation at room temperature, nonadherent cells were removed, and adherent cells were incubated with RPMI 1640 supplemented with 20% (vol/vol) FBS (Irvine Scientific), penicillin (100 U/mL), streptomycin (100 μg/mL), and IL-2 (50 U/mL) for 24 hours. Cells detached from flasks were collected and maintained in the same culture media. CD4+ and CD8+ subpopulations of T lymphocytes were obtained from peripheral blood mononuclear leukocytes by similar positive-panning methods using anti-CD4 and anti-CD8 monoclonal antibody–coated flasks, respectively. After T lymphocytes were cultured for 7 days, experiments were carried out in RPMI 1640 containing 5% FBS supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 U/mL IL-2.

For negative selection, human T lymphocytes were obtained from an elution volume of column beads coated with anti-immunoglobulin antibody and immunoglobulin (human T-cell enrichment columns, R & D Systems), which can bind B lymphocytes and monocytes. T cells were then suspended in RPMI 1640 containing 5% FBS supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and immediately used for assays.

Reagents
Lyophilophatidylcholine (palmitoyl, C16:0) was purchased from Avanti Polar Lipids. Phorbol 12-myristate 13-acetate (PMA), antigenomycin D, PHA-P, and A23187 were obtained from Sigma Chemical Co. Recombinant mouse IL-12 was obtained from Genzyme, and recombinant human IL-2 was kindly provided by Takeda Chemical Industry. FITC-conjugated anti-CD3 (UCHT1) and anti-CD4 (MT310) antibodies, FITC-conjugated nonimmune mouse IgG1, and phycoerythrin-conjugated anti-CD8 (DK25) were purchased from DAKO.

Enzyme-Linked Immunosorbent Assay
An enzyme-linked immunosorbent assay (ELISA) was performed to measure the amount of IFN-γ in culture media with the use of an ELISA test kit (Life-Technologies, Inc).

Northern Blot Analysis
Total RNA, isolated from T lymphocytes by the acid-guanidinium phenol-chloroform method, was electrophoresed through 1% agarose gels containing formaldehyde and transferred onto nitrocellulose membranes (Schleicher & Schuell, Inc). Northern blots were developed using phosphor images (Fuji). Northern blots were rehybridized with human IL-2 receptor, HB-EGF, IL-2, and IL-4 cDNA. All the blots were rehybridized with human IL-2 receptor cDNA were obtained from Japanese Cancer Research Resources Bank, and human IL-4 cDNA was from American Type Culture Collection.

Reverse Transcription–Polymerase Chain Reaction
Total RNA (1 μg) was reverse-transcribed with antisense primer specific for human IFN-γ (5'-TGACGGGTCACCCACGTGTCCTCATCA3') with the use of AMV reverse transcriptase (Life Science Inc). Reverse-transcribed materials were amplified with Taq DNA polymerase (Takara) by adding a sense primer specific for human IFN-γ (5'-ATGAAATATACGTTATCTTGGCTT-3'). For polymerase chain reaction (PCR), 25 cycles were used at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds. The amplified fragments were separated in a 1% agarose gel and detected by ethidium bromide staining. β-Actin amplification was performed in the same way to allow relative quantification of PCR products (sense primer, 5'-TGACGGGTCACCCACGTGTCCTCATCA3'; antisense primer, 5'-CTGAAGCATTGGGTGTGGA-CGATGGAGGG-3').

Plasmid Constructions and Transient Transfection
DNA fragments containing −539 to +1 bp, −102 to +1 bp, −77 to +1 bp, and −37 to +1 bp (base pairs are numbered relative to the transcription start site) of the 5'-flanking region of the IFN-γ gene were generated by PCR and were confirmed by DNA sequencing. The plasmids, pIFN (−539) Luc, pIFN (−102) Luc, pIFN (−77) Luc, and pIFN (−37) Luc, were constructed by subcloning these fragments into the ScaI/HindIII sites of pGL2-basic vector (Promega). To construct pIFN (−311) Luc and pIFN (−175) Luc, a −539- to +1-bp fragment was digested by HindIII and SryI, respectively, followed by subcloning into the SalI/HindIII site of the pGL2-basic vector. Transient transfections of Jurkat cells were carried out with the use of DMRIE-C reagent (Life-Technologies, Inc). For each treatment, 2×10⁶ cells were incubated with 1 μg of tested plasmid DNA (pIFN Luc) and 50 ng of pRL-TK vector (Promega), which contains the herpes simplex virus thymidine kinase promoter upstream of the Renilla luciferase coding region as an internal control, in the presence of DMRIE-C reagent (3 μL) in 1 mL of serum-free RPMI 1640 for 3 hours. Complete medium (4 mL of RPMI 1640 containing 10% FBS) was then added, and cells were incubated for additional 18 hours. To decrease variations in transfection efficiency, cells were transfected in single batches, which were then separated into different drug-treatment groups.

Luciferase Assay
After being treated with test stimuli for 6 hours, transfected cells were processed (dual-luciferase reporter assay system, Promega), and firefly and Renilla luciferase activity was measured with a luminometer (Lumat LB9507). To control the transfection efficiency, each value of experimental firefly luciferase activity was normalized with control Renilla luciferase activity.

Statistical Analysis
Statistical significance of the differences among means of groups was determined using the paired Student t test.

Results
Lyso-PC Increases Amounts of IFN-γ Secreted into Cell Culture Media IL-2–Dependently
Human T lymphocytes isolated by direct positive panning on anti-CD3 antibody–coated flasks were cultured in IL-2–containing media for a week before the experiments, as described in Materials and Methods. The purity of cultured human T lymphocytes was >96%, as determined by flow cytometry using anti-CD3 antibody (data not shown). To examine the effect of lyso-PC on secretion of IFN-γ, T cells were treated with lyso-PC in IL-2–containing media, and cell-conditioned media was analyzed by ELISA. As shown in Figure 1, treatment with lyso-PC increased the amount of secreted IFN-γ in a dose- and time-dependent fashion. Lyso-PC, as low as 37.5 μmol/L, caused a significant increase in the amount of secreted IFN-γ, and a maximal effect was observed at 50 μmol/L after 6 hours of treatment (Figure 1A). In response to 50 μmol/L lyso-PC, IFN-γ that had accumulated in conditioned media remained increased for at least 24 hours (Figure 1B).

To examine whether exogenous IL-2 is necessary for lyso-PC–induced production of IFN-γ, T cells were treated with lyso-PC in the absence of IL-2. After being cultured in IL-2–containing media for 7 days, T cells were stimulated...
Lyso-PC Selectively Increases the Amount of mRNA for IFN-\(\gamma\)

To determine whether upregulated expression of IFN-\(\gamma\) depends on increased amounts of mRNA, Northern blot analysis was performed. As shown in Figure 2, treatment with 37.5 \(\mu\)mol/L of lyso-PC in IL-2–containing medium for 6 hours resulted in significant increases in mRNA levels for IFN-\(\gamma\) (4.4-fold increase). These effects of lyso-PC on the expression of IFN-\(\gamma\) mRNA were dose dependent up to 50 \(\mu\)mol/L. Time-course experiments revealed that increased levels of mRNA were detected as early as 3 hours, peaked at 6 hours, and declined after 10 hours (Figure 3). As demonstrated in our previous study,\(^29\) lyso-PC also increased mRNA levels for HB-EGF and the IL-2 receptor (Figure 3). Lyso-PC–induced gene expression in T cells appeared to be specific to certain genes and distinct from other stimuli, since lyso-PC treatment did not affect mRNA levels for IL-2 or IL-4, whereas treatment with PMA (50 \(\mu\)mol/L) in combination with A23187 (300 ng/mL) elevated the levels of mRNA for IL-2 and IL-4 (Figure 3).

Lyso-PC was unable to induce IFN-\(\gamma\) gene expression in the absence of IL-2 (Figure 4). These results observed with Northern blotting appear to be in parallel with those observed with ELISA.

Lyso-PC Enhances IFN-\(\gamma\) mRNA in CD4\(^+\) and CD8\(^+\) T Lymphocytes

To further examine the effects of lyso-PC on different subsets of T lymphocytes, CD4\(^+\) and CD8\(^+\) subpopulations of T cells were separately isolated and stimulated by lyso-PC in IL-2–containing media. Purity of cultured human CD4\(^+\) and CD8\(^+\) T lymphocytes were \(\geq 95\%\) and \(\geq 98\\%\), respectively, as determined by flow cytometry (data not shown). As shown in Figure 4, lyso-PC was unable to induce IFN-\(\gamma\) gene expression in the absence of IL-2. In this condition, lyso-PC did not affect IFN-\(\gamma\) secretion in T cells (Figure 1B), indicating that continuous activation by IL-2 is necessary for lyso-PC–induced secretion of IFN-\(\gamma\).
Figure 5. Effects of lyso-PC on CD4+ and CD8+ subsets of T lymphocytes. Cultured CD4+ or CD8+ T lymphocytes were treated with or without lyso-PC (50 μmol/L) in RPMI 1640 with 5% FBS and 50 U/mL of IL-2 for 6 hours. Total cellular RNA was isolated, and Northern blot analysis was performed. Each lane contained 10 μg of total RNA. Comparable data were obtained in 3 independent experiments.

**Lyso-PC–Enhanced IFN-γ Gene Induction Is Independent of PKC Activation**

To determine whether lyso-PC–induced IFN-γ gene induction depends on PMA-regulatable protein kinase C (PKC) activation, T lymphocytes were pretreated with 200 nmol/L PMA for 24 hours to deplete PKC activities, and then the cells were stimulated with lyso-PC or PMA in combination with calcium ionophore in IL-2-containing media. As shown in Figure 6, prolonged preexposure to PMA significantly inhibited IFN-γ gene expression induced by PMA in combination with the calcium ionophore. In contrast, pretreatment with PMA did not block lyso-PC–induced IFN-γ gene expression but rather enhanced the effect of lyso-PC. These results indicate that IFN-γ gene induction elicited by lyso-PC appears to be independent of PMA-regulatable PKC activation.

**Lyso-PC Stabilizes mRNA for IFN-γ**

To clarify whether increased amounts of IFN-γ mRNA by lyso-PC treatment result from stabilization of IFN-γ mRNA or transcriptional activation of IFN-γ gene (or both), we first measured mRNA half-lives by Northern analysis using actinomycin D. After peripheral T cells were incubated with or without lyso-PC in the presence of IL-2 for 6 hours, actinomycin D (5 μg/mL) was added to block new RNA synthesis, and mRNA levels for IFN-γ were chased up to an additional 3 hours. As shown in Figure 7, mRNA half-lives of IFN-γ were significantly prolonged in lyso-PC–treated cells (<45 minutes in sham-treated cells and >3 hours in lyso-PC–treated cells), showing that lyso-PC stabilized mRNA for IFN-γ.

**Lyso-PC Stimulates Transcription of IFN-γ Promoter–Luciferase Fusion Gene: Identification of a cis-Acting Element**

To gain insight into lyso-PC–induced IFN-γ gene transcription, the 5’-flanking region (between −539 and +1) of the IFN-γ gene was linked to a promoterless luciferase gene, and...
the construct was transiently transfected into Jurkat cells. In sham-treated cells, transfection of the IFN-γ promoter–luciferase fusion gene resulted in a 14-fold increase in the luciferase activity compared with the transfection of promoterless pGL2-basic vector. In lyso-PC–treated cells, a 27-fold increase in the luciferase activity was observed compared with the promotorless vector. Taken together, lyso-PC treatment showed a 1.9-fold increase in transcriptional activity of IFN-γ promoter [Figure 8, lane showing pIFN (−539) Luc].

To identify the cis-regulatory elements responsible for lyso-PC–induced transcription of the IFN-γ gene, a series of 5’ deletion mutants of IFN-γ promoter were linked to the luciferase reporter gene, followed by transient transfection and luciferase assay. When compared with the transcriptional activity in the wild-type full-length promoter [pIFN (−539) Luc], none of the deletion mutants, which lack nucleotides between −539 and −312 bp, −539 and −176 bp, or −539 and −103 bp, significantly reduced lyso-PC–induced transcriptional activity. A dramatic reduction in lyso-PC–induced transcriptional activity was observed when nucleotides between −539 and −78 bp were deleted (Figure 8). These results show that the lyso-PC–responsive element in the IFN-γ promoter appears to be located between −102 and −78 bp upstream from the transcription start site. This element contains a sequence homology with the consensus activator protein-1 (AP-1)/cAMP response element (CREB)–activating transcription factor (ATF) binding element, which has been shown to be responsible for transcription of IFN-γ induced by PMA plus ionomycin. A subsequent deletion to position −35 bp totally abolished both the basal and lyso-PC–induced transcriptional activities. Taken together, this proximal element between −78 and −35 also is responsible for transcription of the IFN-γ gene.

**Figure 8.** Identification of the responsive elements for lyso-PC–enhanced transcriptional activation of IFN-γ. Various lengths of the 5’ flanking region of the IFN-γ gene, linked to the firefly luciferase gene, were transiently transfected into Jurkat cells, along with pRL-TK as an internal control for transfection efficiency. Transfected cells were treated with or without lyso-PC (50 μmol/L) in RPMI 1640 with 5% FBS for 6 hours, and luciferase activity was measured. Transcriptional activities relative to those of sham-treated cells transfected with pIFN (−539) Luc are shown. Data are expressed as mean±SEM from 4 independent experiments.

**Discussion**

The activation of T lymphocytes has been implicated in the initiation and progression of atherosclerosis. T cell–derived cytokines, including IFN-γ, appear to play key roles in this process. We have previously shown that lyso-PC can selectively upregulate the expression of HB-EGF and IL-2 receptors in cultured T lymphocytes, suggesting that this polar phospholipid may be an important factor in the modulation of T-cell functions in atherogenesis in vivo. In the present study, we provide evidence that lyso-PC, in concert with IL-2 or IL-12, enhances IFN-γ production in cultured human T lymphocytes by both transcriptional and posttranscriptional mechanisms. We also have identified the 5’ cis-acting element responsible for lyso-PC–induced transcription of the IFN-γ gene.
Recent studies have implicated crucial roles of inflammatory infiltrates, such as macrophages and T lymphocytes, in the rupture of advanced atherosclerotic plaques, which appears to be the pathogenesis of acute coronary events in humans. T lymphocytes are abundant in the shoulder areas of atheromatous plaques, which correspond to vulnerable sites for the rupture of fibrous cap and thrombus formation. Histopathological studies have revealed that ruptured fibrous caps usually are infiltrated by larger numbers of macrophages and T lymphocytes and contain fewer SMCs and less amounts of collagen and glycosaminoglycan than intact fibrous caps. Ruptured plaques are also characterized by higher levels of MHC class II antigen expression in inflammatory infiltrates and adjacent SMCs. Since IFN-γ is a key mediator that can dramatically inhibit interstitial collagen gene expression and proliferation of SMCs and induce the expression of MHC class II antigen, T cell–derived IFN-γ may play an important role in the rupture of atherosclerotic plaques. Atherosclerotic lesions in IFN-γ receptor/apolipoprotein E compound knockout mice showed a marked increase in collagen content and a reduction in lipid accumulation, both of which are characteristic of stable atherosclerotic plaques. These observations in mice further support the hypothesis that IFN-γ may affect the instability of atheromatous plaques. Given the fact that expression of IFN-γ can be upregulated in T cells by lyso-PC generated during oxidative modification of lipoproteins, lipid-lowering and antioxidant therapy might act to stabilize vulnerable atherosclerotic plaques by reducing the production of IFN-γ.

Our previous report has shown that the effect of lyso-PC on HB-EGF and IL-2 receptor expression depends on IL-2 in the culture medium. The present study shows that lyso-PC can enhance IFN-γ expression in T cells costimulated with IL-2, IL-12, or PHA. Although IL-2 has been shown to act synergistically with IL-12 in upregulating IFN-γ expression, lyso-PC further enhanced IFN-γ expression in T cells stimulated with IL-2 in combination with IL-12. Since IL-2 and IL-12 appear to be expressed in atherosclerotic lesions, lyso-PC further enhanced IFN-γ expression in T cells stimulated with IL-2 in combination with IL-12. IL-2 and IL-12 receptor by lyso-PC conceivably may further enhance stimulatory effects of IL-2 on T cells and might result in augmented production of IFN-γ in response to lyso-PC. Interestingly, this pattern of T-cell activation is qualitatively distinct from that elicited by PMA in combina-
tion with the calcium ionophore (Figure 4). Our previous studies have demonstrated that endothelial activation by lyso-PC also is selective and qualitatively different from that induced by bacterial endotoxin, IL-1, or tumor necrosis factor.22,23 Although signal transduction mechanisms involved in lyso-PC–induced gene expression remain to be clarified, PMA-regulatable PKC does not appear to be responsible, and intracellular CAMP can inhibit lyso-PC–induced gene expression in cultured vascular endothelial cells.43 The present results also provide evidence that lyso-PC–induced IFN-γ expression does not result from PKC activation (Figure 6).

Our results demonstrate that the transcriptional activity of the IFN-γ promoter is significantly enhanced by lyso-PC in Jurkat cells. A series of deletion mutants of the IFN-γ promoter has identified a lyso-PC–responsive element situated in a position from −102 to −78 bp relative to the transcription initiation site. Penix et al36 reported that the IFN-γ promoter region located between −108 and −40 bp upstream from the transcription start site was required for the transcriptional activity by PMA in combination with the calcium ionophore. This promoter region contains 2 elements that are conserved across species,44 both of which have some homology with the consensus sequences for AP-1 and CREB-ATF binding sites.36 The lyso-PC–responsive element in the IFN-γ promoter appears to correspond to the distal element of these 2 conserved elements. Previous studies have also identified that these AP-1/CREB-ATF binding elements are crucial for the transcriptional activity induced by PMA in combination with the calcium ionophore.46 In these reports, transcription factors, such as c-Jun and c-Fos, have been shown to bind to the AP-1/CREB-ATF sites of the IFN-γ promoter.46 In contrast to these results, our preliminary data of electrophoretic mobility shift assays indicated that transcription factors, other than c-Jun or c-Fos, might bind to this AP-1/CREB-ATF site (data not shown); however, identification of the transcription factors involved in this process remains to be clarified.

In summary, the present data demonstrate that lyso-PC can enhance cytokine-induced IFN-γ expression in human T lymphocytes. Furthermore, we have shown that lyso-PC can both transcriptionally and posttranscriptionally upregulate IFN-γ gene expression, and we have identified the 5′ cis-acting element crucial for lyso-PC–induced transcription of the IFN-γ gene. Further studies related to transcriptional regulatory mechanisms involved in IFN-γ expression by this lipid stimulus might provide new insight into the pathogenesis of atherosclerosis and plaque rupture.

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References


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