Korean J Biol Sci 2: 229-232, 1998

Lysophosphatidylcholine Enhances Chondrogenesis by the Modulation of Protein Kinase C Isoform Expression

Sun Ryung Lee[1], Young-Sup Lee[2], Jang-Soo Chun[1], Jong Kyung Sonn[3]*, and Shin-Sung Kang[1]

Departments of [1,3]Biology and [2]Biochemistry, [1,2]College of Natural Sciences and [3]Teachers' College, Kyungpook National University, Taegu 702-701, Korea

Key Words:
Chondrogenesis
Lysophosphatidylcholine
Protein kinase C isoforms

Lysophosphatidylcholine (LPC) has been reported to be responsible for the sustained activation of protein kinase C (PKC). As chondrogenesis is known to be regulated by PKC, this study was performed to investigate the effects of LPC on chondrogenesis of chick limb bud mesenchymes *in vitro*. LPC treatment of mesenchymes during micromass culture significantly enhanced chondrogenic differentiation. The most effective time of LPC on the stimulation of chondrogenesis was the first day of micromass culture. Analysis of LPC effects on the expression of PKC isoforms revealed that LPC treatment increased expression of PKCα, among the multiple PKC isoforms, in the membrane fraction on day one of culture. The stimulatory effect of LPC on chondrogenesis was abolished if PKCα was down regulated by the prolonged treatment of cells with phorbol ester. The results suggest that LPC promotes chondrogenesis through the activation of PKCα at the early stage of chondrogenic differentiation.

Protein kinase C (PKC) is a family of related ser/thr protein kinases with 11 known isoforms. Individual PKC isoforms are variably dependent on Ca^{2+} and various membrane lipid mediators, and play distinct roles in the control of physiological functions (Goodnight et al., 1994; Newton, 1997). Among the known PKC isoforms, conventional PKC isoforms such as α, βI, βII, and γ and novel PKC isoforms including δ, ε, θ, and η are known to be activated by diacylglycerol (DG) (Nishizuka, 1995). DG, a hydrolysis product of phospholipids, is metabolized quickly and activates PKC transiently (Kaibuchi et al., 1983). Therefore, it has been suggested that other mechanism for the sustained activation of PKC is needed for long-term response of cells such as proliferation and differentiation (Asaoka et al., 1991; Nishizuka, 1995). For example, activation of T-lymphocyte requires sustained activation of PKC either by the repeated treatment of DG (Berry et al., 1990; Asaoka et al., 1991) or by the treatment of lysophosphatidylcholine (LPC) (Asaoka et al., 1992).

PKC has been known to play a role in cartilage differentiation. Prolonged treatment of mesenchymes with phorbol 12-myristate 13-acetate (PMA), which down regulates PKC, inhibits the differentiation of chick limb bud mesenchymes *in vitro* (Kulyk, 1991) and causes dedifferentiation of rabbit costal and articular chondrocytes (Takigawa et al., 1983; Bouakka et al., 1988). PKC activity in micromass cultured mesenchymes is increased as chondrogenesis proceeds and depressed by the chronic treatment of PMA (Sonn and Solursh, 1993). Among the multiple PKC isoforms expressed in chick embryo limb mesenchymes, expression of PKCα and ε isoforms is increased during chondrogenesis, while PKCζ and λ/ι remain at constant levels (Choi et al., 1995). All of the above results were obtained by the negative modulation of PKC during chondrogenesis and an effort to activate PKC for long period has not been made. This study was, therefore, performed to investigate the effects of LPC on the expression and activation of PKC isoforms and the chondrogenic differentiation of mesenchymes *in vitro*. The present report shows that LPC promotes chondrogenesis through the activation of PKCα.

* To whom correspondence should be addressed.
Tel: 82-53-950-5915, Fax: 82-53-950-6809

Materials and Methods

Materials

L-α-myristoyllysophosphatidylcholine (Sigma) was dissolved in chloroform and kept as a stock. The stock solution was dried under N_2 gas just before treatment and resuspended in phosphate-buffered saline (PBS) by vigorous vortexing for 1 min and sonication for 3 min on ice. Antibodies against PKC isoforms were purchased from Transduction Laboratories, and an enhanced chemiluminescence (ECL) kit was purchased from Amersham.

Micromass culture

Hamburger-Hamilton (Hamburger and Hamilton, 1951) stage 23/24 chick embryo wing buds were dissected and rinsed with Ca^{2+}-Mg^{2+}- free saline G. Micromass

cultures of wing buds were prepared as described by Ahrens et al. (1977). Briefly, cells were dissociated by incubation with 0.1% trypsin- collagenase for 10 min at 37℃. The reaction was stopped by adding F12 medium containing 10% fetal calf serum (FCS) and antibiotics. The cells were collected by centrifugation and resuspended with culture medium. Cell suspension was filtered through two layers of Nytex 20 filters and cell number was counted with hemocytometer. Cell density was adjusted to 2×10^7 cells/ml and 10 μl of cell suspension was dropped onto 35 mm culture dish. After cell attachment to the dish by incubation for 1 h at 37℃, cells were flooded with culture medium.

Quantitative analysis of chondrogenesis

Cultures were fixed with Kahle's fixative for 5 min and stained with alcian blue at pH 1.0 for overnight. Dye bound to sulfated glycosaminoglycans was extracted by incubation with 4 M guanidine HCl overnight and absorbance was measured at 600 nm.

Western blot analysis

Mesenchymes were micromass cultured for the indicated time periods and washed three times with saline G and PBS, respectively. Total cell lysates were prepared by extracting proteins with an extraction buffer (20 mM Tris, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μM β-mercaptoethanol, and 0.5% Triton X-100). After sonication for six sec, cell homogenates were centrifuged at 100,000×g for 60 min and the supernatant was saved as a total cell lysate. To separate cytosolic and particulate membrane fractions, micromass cultured mesenchymes were scraped in the extraction buffer without Triton X-100. The cells were sonicated twice for six sec and centrifuged at 100,000×g for 60 min. The supernatant was designated as a cytosolic fraction. The pellet was extracted with extraction buffer containing 0.5% Triton X-100, centrifuged at 100,000×g for 60 min, and the supernatant was saved as the particulate membrane fraction. Proteins from total extract, cytosolic, or particulate membarne fractions were separated by 7.5% SDS-polyacrylamide electrophoresis and transferred to nitrocellulose paper (Schleicher & Schuell). The membranes were blocked with Tris buffered saline containing 3% nonfat dry milk and 0.1% Tween 20 for 1 h and incubated with anti-PKC isoform specific antibodies (PKCα, ε, ζ, and λ/ι) for 2 h at room temperature. After washing three times for 10 min, horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG was applied to the membrane and incubated for 2 h at room temperature. PKC isoforms were detected with ECL kit. Protein content was determined using a Bio-Rad DC protein assay kit with bovine serum albumin as a standard.

Results and Discussion

LPC is known to modulate various cellular functions. For instance, activation of human T lymphocytes was enhanced by LPC treatments (Asaoka et al., 1991, 1992). To investigate the effects of LPC on chondrogenic differentiation, LPC was treated for three days to mesenchymes during micromass culture. LPC promoted chondrogenesis *in vitro* in a concentration dependent manner as checked by reading the absorbance of alcian blue bound to extracellular matrix (Fig. 1A). To examine the effective time period of LPC on chondrogenesis, cells were treated with LPC during various periods of culture. As shown in Fig. 1B, the degree of enhancement of chondrogenesis by LPC was similar if LPC was present at the first day of culture. However, LPC showed little effect on chondrogenesis when treated after the first day of culture. These results show that the most effective period of LPC's effects on chondrogenesis is the early stage of differentiation.

Our previous observations that PMA treatment inhibits chondrogenesis and its most effective period is the first day of culture (Sonn and Solursh, 1993; Choi et al., 1995) suggest that down regulation of PKC for the first day of culture may negatively regulate chondrogenic differentiation. The consistency of the effective time of PMA and LPC action and their opposite effect on chondrogenesis suggest that PKC may mediate the effects of LPC on chondrogenesis. We have previously shown that mesenchymes derived from chick limb buds express multiple PKC isoforms such as α, γ, ε, ζ, and λ/ι isoforms (Choi et al., 1995). However, anti-PKCγ antibody (Transduction Laboratories) that has been used in the previous study turned out to cross-react with PKCα, according to the manufacturer. When PKCγ-specific antibodies from Santa Cruz Biotech. or Gibco BRL were used, no immunoreactive band was detected in mesenchymes cultured up to five days (data not shown). Thus, it appears that PKCγ is not expressed to significant level in mesenchymes during chondrogenesis. Among the expressed PKC isoforms, down regulation of PKCα and ε was sufficient to inhibit chondrogenesis (Choi et al., 1995). We, therefore, examined whether LPC affects chondrogenesis through the modulation of PKC isoform expression such as α, ε, ξ, and λ/ι. As shown in Fig. 2, the expression of PKCα and ε was increased during chondrogenesis while PKCζ and λ/ι remained constant throughout the culture period. Addition of LPC to the cultures increased the expression of PKCα on day one of culture. The expression of other isoforms examined in this experiment was not affected by the LPC treatment.

Because the activation of PKC requires stable association of cytosolic PKC to the membrane (Nishizuka, 1995; Newton, 1997), translocation of cytosolic PKC to the particulate membrane was examined during LPC-induced stimulation of chondrogenesis. PKCα was expressed at low levels in both cytosolic and membrane

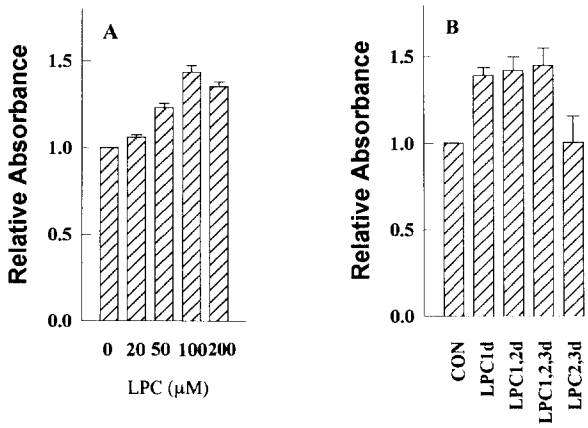

Fig. 1. LPC promotes chondrogenesis *in vitro*. A, Various concentrations of LPC were added to the micromass cultures of chick limb bud mesenchymes. Following culture for three days, the cells were stained with alcian blue. Bound alcian blue was extracted with 4 M guanidine HCl and absorbance was measured at 600 nm. B, LPC (100 μM) was added to the cultures for one day (1d), one and two days (1,2d), two and three days (2,3d), or whole culture periods (1,2,3d).

fractions and the expression levels were increased in both fractions as chondrogenesis proceeds. The expression pattern of PKCε was similar to that of PKCα except that more PKCε was expressed in day one of culture and expression of PKCε in membrane fraction was lower than that of PKCα in day two and three of culture. PKCζ and λ/ι were expressed in day one of culture in both fractions and the levels of expression were kept constant throughout the culture periods (Fig. 3). Treatment of LPC caused increased expression of PKCα only in the membrane fraction in day one of culture. However, LPC did not affect the distribution of other PKC isoforms such as ε, λ/ι, and ζ. The stimulatory effect of LPC on chondrogenesis was abolished when mesenchymes were treated with PMA for prolonged period (Fig. 4). Under this condition, PKCα was down regulated (Choi et al., 1995). The results strongly suggest that promotion of chondrogenesis by LPC is closely related to the increased expression and

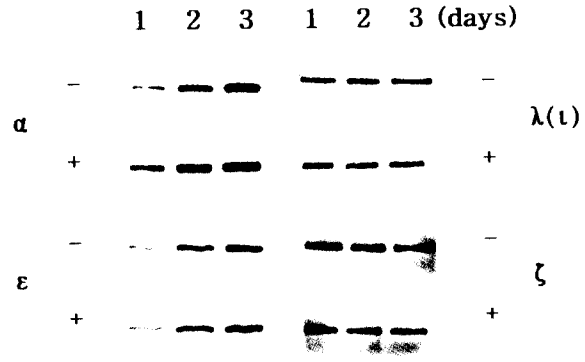

Fig. 2. LPC promotes the expression of PKCα during chondrogenesis. Cells were cultured in the absence (-) or presence (+) of LPC for the indicated periods and expression of PKC isoforms was determined by Western blotting.

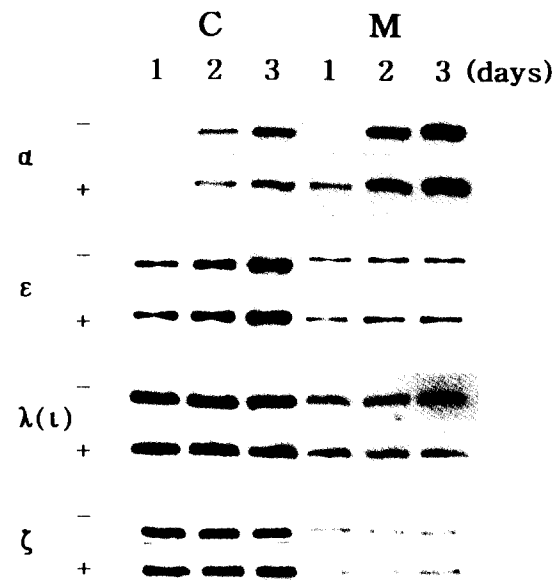

Fig. 3. LPC treatment increases membrane-bound PKCα during chondrogensis. Cells were cultured in the absence (-) or presence (+) of LPC for the indicated periods and cell extracts were separated into cytosolic (C) and membrane (M) fractions. Distribution of PKC isoforms was determined by Western blotting.

translocation of PKCα. Our result is also consistent with the observation that only conventional PKCs, among the 11 isoforms of PKC, use LPC as an activator (Nishizuka, 1995).

Mounting evidence indicates that LPC controls various cellular processes in a PKC-dependent manner. For example, LPC activates phospholipase D in human endothelial cells (Cox and Cohen, 1996), stimulates the expression of MCP-I gene in human umbilical vein endothelial cells (Takahara et al., 1996), promotes

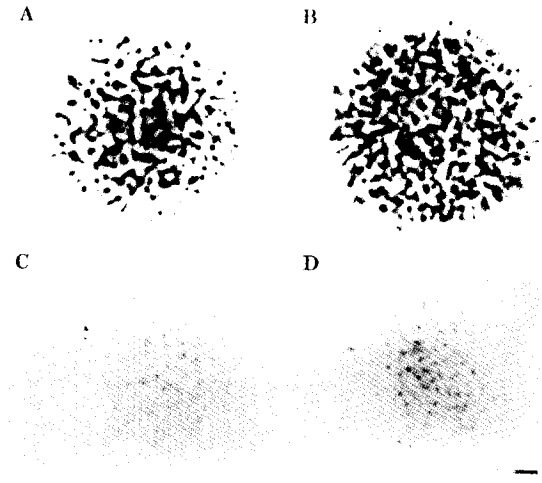

Fig. 4. LPC-induced enhancement of chondrogenesis requires PKC. Cells were cultured in the presence of vehicle alone (A), 100 μM LPC (B), 20 nM PMA (C), or 100 μM LPC and 20 nM PMA (D) for 3 days and stained with alcian blue. Scale bar=1 mm.

P-selectin expression (Murohara et al., 1996), and modulates Na current in cardiac myocytes (Watson and Gold, 1997). However, all the data presented in the above papers were from short-term treatment of LPC or indirect evidences. In this paper, the increase of PKCα expression was observed after treatment of LPC for one day, which is long enough to promote chondrogenesis. Taken together, our current results indicate that LPC promotes chondrogenesis through the activation of PKCα at the early stage of differentiation. In fact, it should be noted that the enhancement of chondrogenesis by LPC could be due to general increase in the cell proliferation rate by the activation of PKC signaling pathway. Further characterization should be performed to distinguish these possibilities.

Acknowledgements

This work was supported by grants from the Korea Ministry of Education for the Basic Science Research Center (BSRI-4402) and from Korea Science and Engineering Foundation (951-0507-049-2).

References

Ahrens PB, Solursh M, and Reiter RS (1977) Stage-related capacity for limb chondrogenesis in cell culture. *Dev Biol* 60: 69-82.

Asaoka Y, Oka M, Yoshida K, and Nishizuka Y (1991) Metabolic rate of membrane-permeant diacylglycerol and its relation to human resting T-lymphocyte activation. *Proc Natl Acad Sci USA* 88: 8681-8585.

Asaoka Y, Oka M, Yoshida K, Sasaki Y, and Nishizuka Y (1992) Role of lysophosphatidylcholine in T-lymphocyte activation: involvement of phospholipase A2 in signal transduction through protein kinase C. *Proc Natl Acad Sci USA* 89: 6447-6451.

Berry N, Ase K, Kishimoto A, and Nishizuka Y (1990) Activation of resting human T cells requires prolonged stimulation of protein kinase C. *Proc Natl Acad Sci USA* 87: 2294-2298.

Bouakka M, Legendre P, Jouis V, Langris M, Beliard R, Loyau G, and Bocquet J (1988) Calcium ionophore and phorbol myristate acetate synergistically inhibited proteoglycan biosynthesis in articular chondrocytes by prostaglandin independent mechanism. *Biochem Biophys Res Commun* 153: 690-698.

Choi B, Chun J-S, Lee Y-S, Sonn J-K, and Kang S-S (1995) Expression of protein kinase C isozymes that are required for chondrogenesis of chick limb bud mesenchymal cells. *Biochem Biophys Res Commun* 216: 1934-1040.

Cox DA and Cohen ML (1996) Lysophosphatidylcholine stimulates phospholipase D in human coronary endothelial cells: role of PKC. *Am J Physiol* 271: H1706-H1710.

Goodnight J, Mischak H, and Mushinski JF (1994) Selective involvement of protein kinase C isozymes in differentiation and neoplastic transformation. *Adv Cancer Res* 64: 159-209.

Hamburger V and Hamilton HW (1951) A series of normal stages in the development of the chick embryo. *J Morphol* 88: 49-92.

Kaibuchi K, Takai Y, Sawamura M, Hoshijima M, Fujikura T, and Nishizuka Y (1983) Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J Biol Chem* 258: 6701-6704.

Kikkawa U and Nishizuka Y (1986) The role of protein kinase C in transmembrane signalling. *Annu Rev Cell Biol* 2: 149-178.

Kulyk WM (1991) Promotion of embryonic limb cartilage differentiaiton *in vitro* by staurosporine, a protein kinase C inhibitor. *Dev Biol* 146: 38-48.

Murohara T, Scalia R, and Lefer AM (1996) Lysophosphatidylcholine promotes P-selectin expression in platelets and endothelial cells. Possible involvement of protein kinase C activation and its inhibition by nitric oxide donors. *Circ Res* 78: 780-789.

Newton AC (1997) Regulation of protein kinase C. *Curr Opin Cell Biol* 9: 161-167.

Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 9: 484-496.

Sonn JK and Solursh M (1993) Acitivity of protein kinase C during the differentiation of chick limb bud mesenchymal cells. *Differentiation* 53: 155-162.

Takahara N, Kashiwagi A, Maegawa H, and Shigeta Y (1996) Lysophosphatidylcholine stimulates the expression and production of MCP-1 by human vascular endothelial cells. *Metabolism* 45: 559-564.

Takigawa M, Fukuo K, Takano T, and Suzuki F (1983) Restoration by parathyroid hormone and dibutyryl cyclic AMP of expression of the differentiated phenotype of chondrocytes inhibited by a tumor promoter, 12-o-tetradecanoylphorbol-13-acetate. *Cell Differ* 13: 283-291.

Watson CL and Gold MR (1997) Lysophosphatidylcholine modulates cardiac I(Na) via multiple protein kinase pathways. *Circ Res* 81: 387-395.

[Received March 10, 1998; accepted April 21, 1998]