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Lysophosphatidic acid induces interleukin-6 and CXCL15 secretion from MLO-Y4 cells through activation of the LPA_1 receptor and PKCθ signaling pathway

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ABSTRACT

Lysophosphatidic acid (LPA) is a multifunctional phospholipid. Osteocytes are the most abundant cells in bone and can orchestrate bone formation and resorption, in part by producing cytokines that regulate osteoblast and osteoclast differentiation and activity. Interleukin (IL)-6 and IL-8 are two important cytokines that have potent effects on bone fracture healing. Previous studies suggest that platelet-derived LPA may influence fracture healing by inducing osteocyte dendrite outgrowth. However, the biological mechanism through which LPA induces cytokine production in osteocytes is poorly understood. In this study, we report that LPA markedly enhanced IL-6 and CXCL15 (mouse homologue of human IL-8) production in MLO-Y4 cells and that this enhancement was suppressed by the $LPA_{1/3}$-selective antagonist Ki16425, the $G_{i/o}$ protein inhibitor PTX or the protein kinase C (PKC) inhibitor sotrastaurin. We also observed that of all the PKC isoform targets of sotrastaurin, only PKCθ was activated by LPA in MLO-Y4 cells and that this activation was blocked by sotrastaurin, Ki16425 or PTX. Taken together, the results of the present study demonstrate that LPA may be a potent inducer of IL-6 and CXCL15 production in MLO-Y4 cells and that this induction is associated with the activation of LPA_1, $G_{i/o}$ protein and the PKCθ pathway. These findings may help us better understand the mechanism of fracture healing and contribute to the treatment of bone damage.

1. Introduction

Lysophosphatidic acid (LPA) is a simple, ubiquitous bioactive phospholipid that is abundant in serum, where it is produced by activated platelets during blood clot formation [1]. LPA can act as an extracellular signaling molecule to induce various cellular functions, including proliferation, differentiation, survival, migration and cytokine secretion in many different types of cells [2]. To date, at least six specific G protein-coupled receptors (GPCRs), LPA_1-LPA_6, have been shown to be involved in LPA signaling [3]. Because of its diverse and widespread cellular functions, LPA influences many biological processes, such as neurogenesis, vasculogenesis, carcinogenesis and soft tissue wound healing [2]. Bone cells are exposed to high levels of LPA in fracture regions, and LPA has been shown to have multiple effects on osteoblasts and osteoclasts [4], suggesting that this lipid may play an important role in bone healing.

Fracture healing is a complex event that requires the coordinated participation of several bone cell types. In addition to osteoblasts and osteoclasts, osteocytes are also involved in this process [5]. Osteocytes comprise > 90% of cells in bone. This cell population is now recognized as a major orchestrator of bone formation and resorption, and one of the roles of osteocytes is to regulate the cellular functions of osteoblasts and osteoclasts by producing cytokines [6]. Interleukin (IL)-6 and IL-8 are two potent cytokines that influence bone healing and whose functions on osteoblasts and/or osteoclasts have been elucidated [7–9]. In the fracture microenvironment, several cells, such as macrophages and osteoblasts, express IL-6 and IL-8 [9,10]. In recent years, some evidence has suggested that IL-6 and IL-8 can also be produced by osteocytes. In our previous studies, we observed that mechanical loading and some biomolecules, such as serotonin and lipopolysaccharide, enhanced IL-6 secretion from osteocytes [11–13]. In addition, when osteocytes are treated with serum from rheumatoid arthritis patients, their IL-8

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expression is increased [14]. Thus, we hypothesized that some factors in the fracture microenvironment may induce IL-6 and IL-8 secretion from osteocytes and then influence fracture healing.

The ability of LPA to increase IL-6 and IL-8 secretion has been shown for many different types of cells, including maturing dendritic cells, fibroblast-like synoviocytes and oral keratinocytes [15–17]. In bone cells, LPA induces the synthesis of IL-6 and IL-8 in osteoblasts, which are the precursors of osteocytes [18]. Three types of LPA receptors ($LPA_{1,2,4}$) are known to be expressed in osteocytes [19], suggesting that LPA may influence the activity of osteocytes. Furthermore, the results of some studies showed that LPA stimulates osteocyte dendrite outgrowth and promotes its chemotaxis [19,20]. However, little is known regarding whether LPA induces IL-6 and IL-8 production in osteocytes. The MLO-Y4 cell line, which is a mouse osteocyte-like cell line, has been widely used to study the cellular response of osteocytes. In addition, CXCL15 is a mouse homologue of human IL-8 and has been used to evaluate the function of IL-8 in mice [21]. Therefore, in the present study, we investigated the effects of LPA on the production of IL-6 and CXCL15 in MLO-Y4 cells and identified the mechanism of LPA-induced IL-6 and CXCL15 secretion from MLO-Y4 cells.

2. Materials and methods

2.1. Reagents and antibodies

LPA was purchased from Sigma (MO, USA); Ki16425 and sotrastaurin were both obtained from Cayman (AA, USA); and pertussis toxin (PTX) was purchased from Tocris Bioscience (Bristol, UK). Antibodies against phospho-PKCα (Ser-657), phospho-PKCδ (Tyr-311), and phospho-PKCθ (Thr-538) were purchased from Cell Signaling Technology (MA, USA). Antibodies against phospho-PKC βI (Thr-642), phospho-PKCε (Ser-729), phospho-PKCη (Thr-655) and GAPDH were purchased from Abcam (Cambridge, UK).

2.2. Cell culture

MLO-Y4 cells were generously provided by Prof. Lynda F. Bonewald from the Department of Oral Biology, University of Missouri-Kansas City, Kansas City, MO, USA. The cells were cultured in collagen-coated flasks (rat tail collagen type I; 0.15 mg/ml in 0.02 N acetic acid) in α-MEM (Gibco, NY, USA) supplemented with 5% fetal bovine serum and 5% calf serum (HyClone, USA) in a humidified atmosphere with 5% CO_2 and 95% air at 37 °C. The medium was replaced every two days, and the cells were serum-starved by incubation in α-MEM containing 0.1% fatty acid-free BSA (MP Biomedicals, CA, USA) for 16 h. LPA was dissolved in PBS before use.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, CA, USA). Subsequently, 1 μg RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara, Japan) following the manufacturer's instructions. PCR amplification was performed using SYBR® Premix RX Taq™ II (Takara, Japan) in an ABI PRISMR 7300 Fast Real-Time PCR System. The following primer sets (mouse) were used: IL-6, forward 5′-GAC AAA GCC AGA GTC CTT CAG AG-3′ and reverse 5′-TCC TTA GCC ACT CCT TCT GTG AC-3′; CXCL15, forward 5′-GCT CCT GCT GGC TGT CCT TA-3′ and reverse 5′-CTG CTA TCA CTT CCT TTC TGT TGC-3′; and GAPDH, forward 5′-GAC ATC AAG AAG GTG GTG AAGC-3′ and reverse 5′-GAA GGT GGA AGA GTG GGA GTT-3′. The fold change was calculated as follows: $2^{-\Delta\Delta\ Ct}$, in which, $\Delta\Delta\ Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$, $\Delta Ct = Ct_{targetgene} - Ct_{GAPDH}$.

2.4. ELISA

The IL-6 and CXCL15 concentrations were individually determined using commercially available ELISA kits for murine IL-6 (RayBiotech Inc., GA, USA) and CXCL15 (Senxiong Biotech, Shanghai, China). After incubation, the conditioned medium was collected and centrifuged at 1000 ×*g* for 10 min to pellet any cellular debris. Then, the supernatants were transferred to new tubes and stored at −80 °C. ELISA was performed using the supernatants following the manufacturer's instructions. After the enzymatic reaction was terminated, the optical absorbance of each well was measured using an ELISA reader (HTS7000 +, PE, USA) at 450 nm. Each sample was measured in duplicate.

2.5. Western blotting

After all treatments, MLO-Y4 cells were rinsed three times with cold PBS, and total protein was obtained using a total protein extraction kit (KeyGen Biotech, China). The protein concentration in each sample was quantified using a BCA protein assay kit (Pierce, IL, USA). All of the protein samples were diluted with SDS sample buffer and then boiled for 8 min. Equal amounts of protein from each sample were resolved by SDS-PAGE and transferred to PVDF membranes. Next, the membranes were blocked with 5% BSA in TBST for 1 h at room temperature. The membranes were subsequently incubated at 4 °C overnight with specific primary antibodies, after which they were washed with TBST and incubated with specific secondary antibodies for 1 h at room temperature. Subsequently, the protein bands were visualized using an ECL kit (Amersham Pharmacia Biotech, UK).

2.6. Statistical analysis

All of the experiments were repeated thrice. Data were expressed as the mean ± standard deviation (SD), and the differences between groups were analyzed by one-way ANOVA using SPSS (IBM, NY, USA), with $P < 0.05$ considered significant.

3. Results

3.1. LPA enhances IL-6 and CXCL15 synthesis in MLO-Y4 cells

The results of a previous study suggested that the physiological levels of LPA in blood serum are approximately 1–5 μM, and local LPA concentrations may reach much higher levels after wounding [22]. Therefore, the dose-dependent response of LPA-induced IL-6 and CXCL15 mRNA expression in MLO-Y4 cells was assessed using 1, 5 and 10 μM LPA. The results presented in Fig. 1A and C show that all assayed concentrations of LPA significantly induced IL-6 and CXCL15 mRNA expression. Moreover, no significant difference in IL-6 and CXCL15 mRNA expression was observed between the 5 and 10 μM groups. Next, MLO-Y4 cells were incubated with 5 μM LPA for various durations (0.5, 1, 2, 3, 6 and 24 h). As shown in Fig. 1B, compared to the control cells, cells incubated for 1 h with 5 μM LPA exhibited a rapid increase in IL-6 mRNA expression. This increase was maximal after 1–3 h of exposure to LPA and then gradually decreased from peak levels. The results presented in Fig. 1D show that, after incubation with LPA for 1 h, the expression of CXCL15 mRNA in MLO-Y4 cells began to increase. The maximal CXCL15 mRNA expression was observed at 6 h and then gradually decreased. The ability of LPA to influence IL-6 and CXCL15 protein secretion by MLO-Y4 cells was assessed by ELISA. The results showed that 5 μM LPA increased IL-6 and CXCL15 production in a time-dependent manner (Fig. 1E and F). As early as 2 h after incubation, the secretion of IL-6 was enhanced, and the maximal responses were observed at 24 h (Fig. 1E). Moreover, enhancement of CXCL15 release began 3 h after incubation, peaking by 24 h (Fig. 1F). Thus, LPA can enhance the mRNA and protein expression of IL-6 and CXCL15 in MLO-Y4 cells.

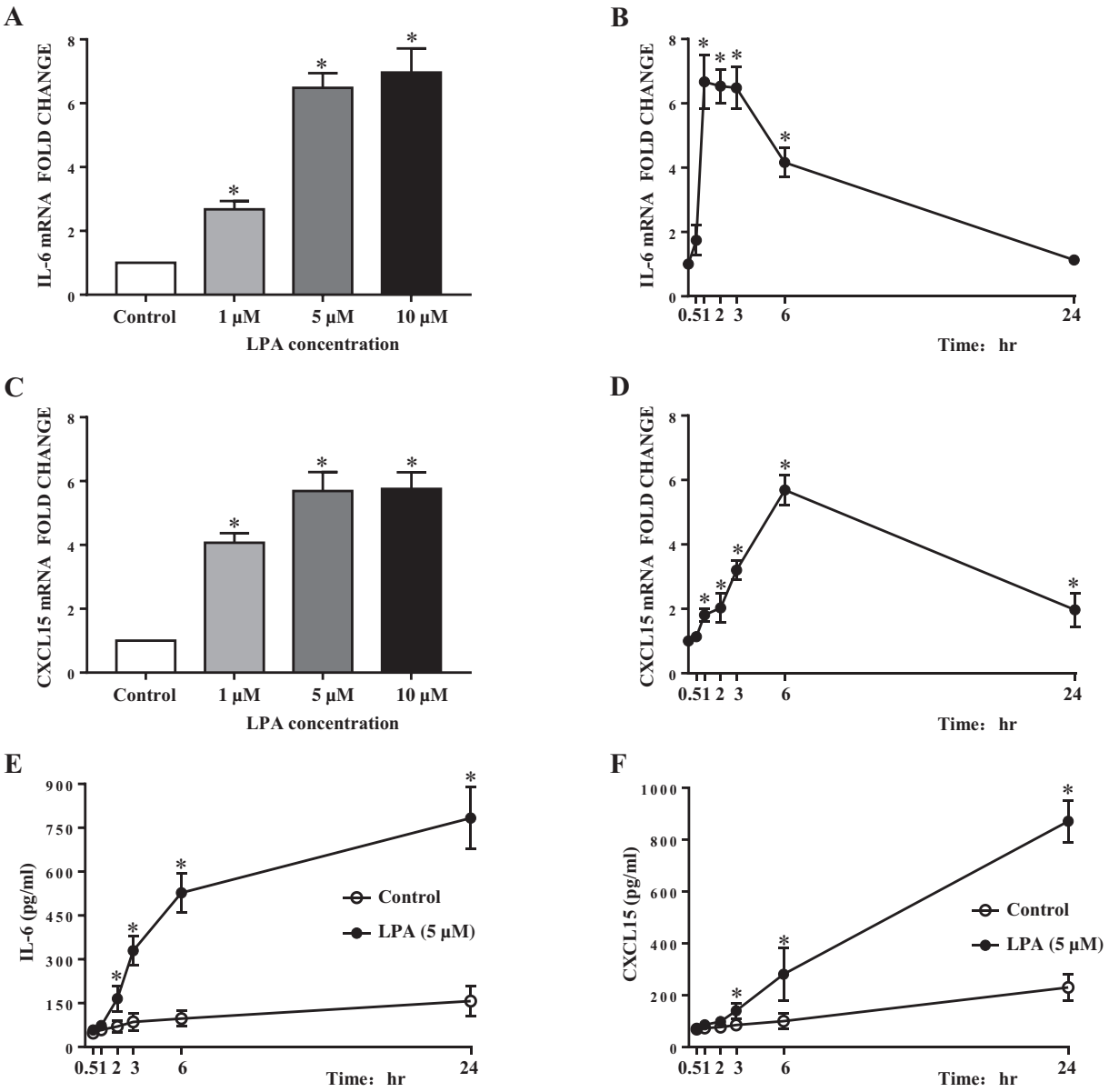

Fig. 1. Effects of LPA on IL-6 and CXCL15 synthesis in MLO-Y4 cells. A, B, C and D: Effects of LPA on IL-6 and CXCL15 mRNA expression. To investigate the effects of different LPA concentrations, starved cells were separately incubated with 1, 5 or 10 μM LPA for 3 h (A) or 6 h (C). To investigate the effects of different incubation times, starved cells were separately incubated with 5 μM LPA for the indicated times (B and D). mRNA expression was quantified by qRT-PCR. The data represent the mean ± SD (n = 3). *, *P* values ≤ 0.05, compared with the controls. E and F: Effects of LPA on IL-6 and CXCL15 secretion. Starved cells were incubated with or without 5 μM LPA for 0.5, 1, 2, 3, 6 and 24 h. Protein secretion was quantified by ELISA. The data represent the mean ± SD (n = 3). *, *P* values ≤ 0.05, compared with the controls.

3.2. LPA_1 receptor and $G_{i/o}$ protein mediate LPA-induced IL-6 and CXCL15 secretion

To determine whether the LPA_1 receptor is involved in LPA-induced secretion of IL-6 and CXCL15, we pretreated MLO-Y4 cells with the $LPA_{1/3}$-selective antagonist Ki16425 for 1 h and then cultured these cells with LPA. The IL-6 and CXCL15 concentrations in the supernatants were detected by ELISA. As shown in Fig. 2A and B, 5 μM Ki16425 significantly suppressed the secretion of these two cytokines by MLO-Y4 cells in response to LPA. In contrast to the strong expression of LPA_1, LPA_3 expression was barely detectable in MLO-Y4 cells [19]. Therefore, LPA_1 is responsible for LPA-induced IL-6 and CXCL15 production in MLO-Y4 cells. Next, we attempted to identify the G protein involved in LPA signaling. PTX is a potent inhibitor of the $G_{i/o}$ protein. Thus, before being stimulated with LPA, MLO-Y4 cells were pretreated with 100 ng/ml PTX for 1 h. Interestingly, when the $G_{i/o}$ protein was blocked, the enhanced effect of LPA on IL-6 and CXCL15 protein secretion was significantly abrogated, indicating that the $G_{i/o}$ protein is required for this activity of LPA in MLO-Y4 cells (Fig. 2C and D).

3.3. PKC mediates LPA-induced IL-6 and CXCL15 secretion

To further investigate the mechanism associated with LPA-stimulated IL-6 and CXCL15 upregulation, we evaluated the effect of the PKC inhibitor sotrastaurin on IL-6 and CXCL15 production. As shown in Fig. 3A, a significant reduction in LPA-induced IL-6 release was observed when cells were cultured with 10 μM sotrastaurin for 1 h before LPA treatment. In addition, the results presented in Fig. 3B show that 10 μM sotrastaurin also efficiently decreased LPA-induced CXCL15 secretion. These data indicate that PKC activation is responsible for the

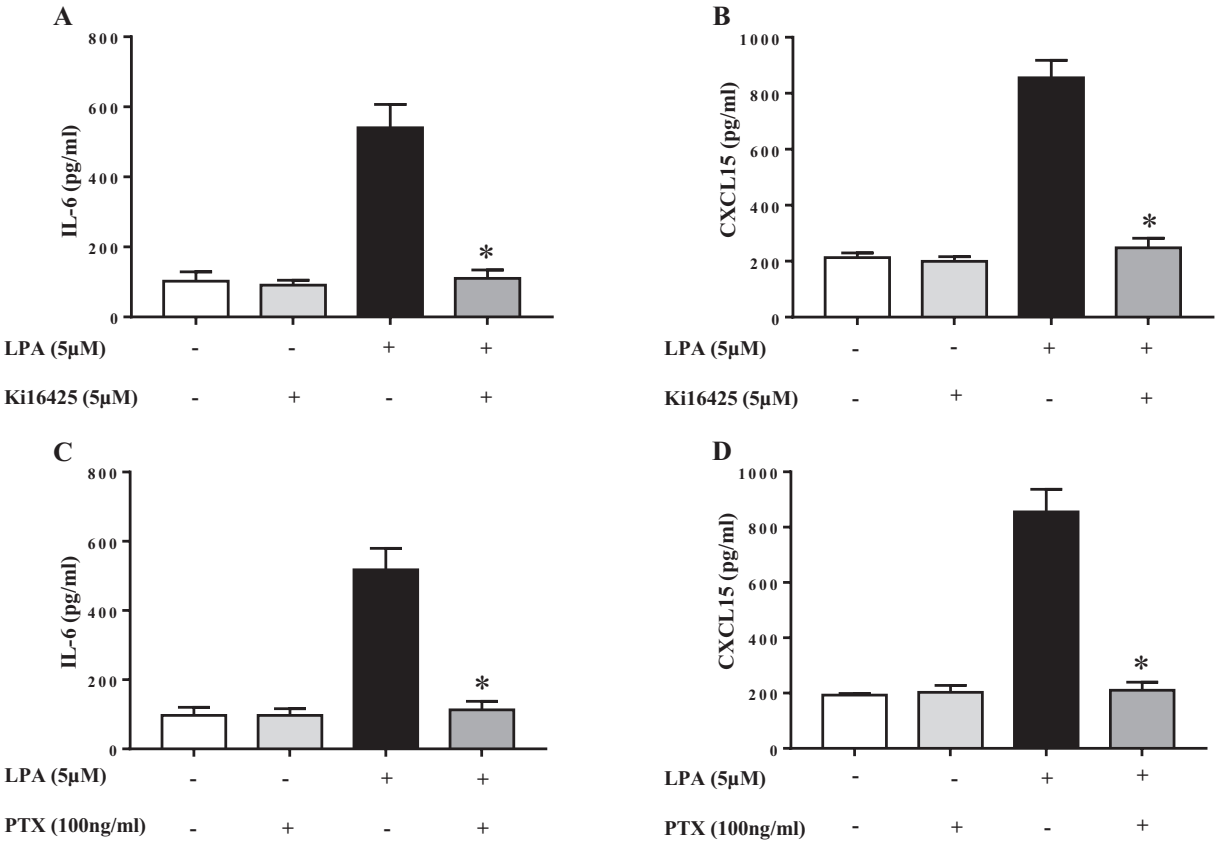

Fig. 2. LPA_1 receptor and $G_{i/o}$ protein mediate LPA-induced IL-6 and CXCL15 secretion in MLO-Y4 cells. A and B: Effects of the $LPA_{1/3}$ antagonist Ki16425 on LPA-induced IL-6 and CXCL15 secretion. Ki16425 was used to treat starved MLO-Y4 cells for 1 h followed by LPA stimulation for 6 h (A) or 24 h (B). The supernatants were harvested, and protein concentrations were analyzed by ELISA. The data represent the mean ± SD (n = 3). *, *P* values ≤ 0.05, compared with LPA treatment alone. C and D: Effect of the $G_{i/o}$ protein inhibitor PTX on LPA-induced IL-6 and CXCL15 secretion. Starved MLO-Y4 cells were pretreated with 100 ng/ml PTX for 1 h before LPA stimulation for 6 h (C) or 24 h (D). The supernatants were harvested, and the protein concentrations were analyzed by ELISA. The data represent the mean ± SD (n = 3). *, *P* values ≤ 0.05, compared with LPA treatment alone.

LPA-induced secretion of these two cytokines from MLO-Y4 cells.

3.4. PKCθ is required for LPA-induced IL-6 and CXCL15 secretion, and LPA_1 and $G_{i/o}$ protein mediate LPA-induced PKCθ activation in MLO-Y4 cells

Sotrastaurin is a selective pan-PKC inhibitor that can inhibit the activation of various PKC isoforms, including PKCα, PKCβI, PKCδ, PKCε, PKCη and PKCθ. To identify the specific PKC isoforms associated with LPA-induced IL-6 and CXCL15 secretion, we evaluated the activation of these PKC isoforms in MLO-Y4 cells in response to LPA treatment. As shown in Fig. 4A, PKCθ was rapidly and markedly activated by LPA, with phosphorylation peaking at 1–2 min. However, no notable phosphorylation was detected for the other PKC isoforms in response to LPA (Fig. 4A). In addition, as shown in Fig. 4B, the LPA-induced activation of PKCθ was abolished by sotrastaurin. These results suggest that PKCθ may be required for LPA-induced IL-6 and CXCL15 secretion. To further assess whether LPA_1 and $G_{i/o}$ protein are involved

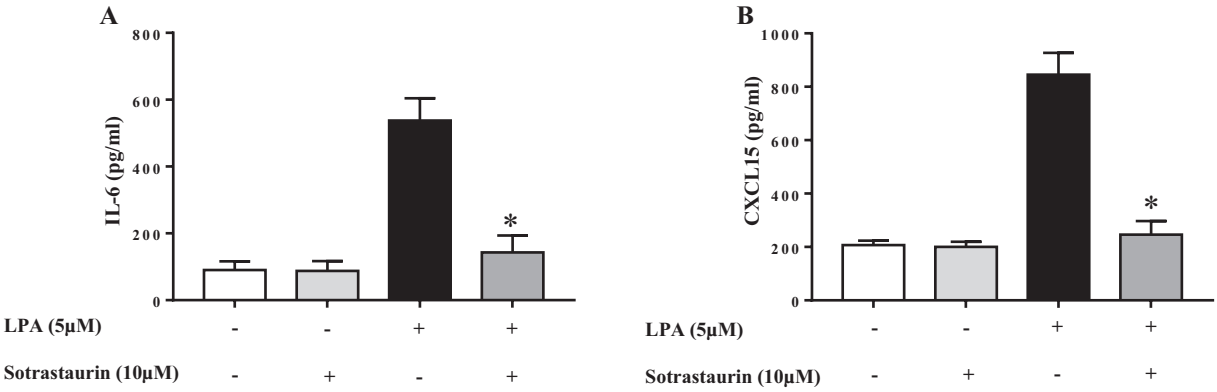

Fig. 3. PKC mediates LPA-induced IL-6 and CXCL15 secretion in MLO-Y4 cells. A and B: Effects of the PKC inhibitor sotrastaurin on LPA-induced IL-6 and CXCL15 secretion. Starved MLO-Y4 cells were pretreated with 10 μM sotrastaurin for 1 h before LPA stimulation for 6 h (A) or 24 h (B). The supernatants were harvested, and the protein concentrations were analyzed by ELISA. The data represent the mean ± SD (n = 3). *, *P* values ≤ 0.05, compared with LPA treatment alone.

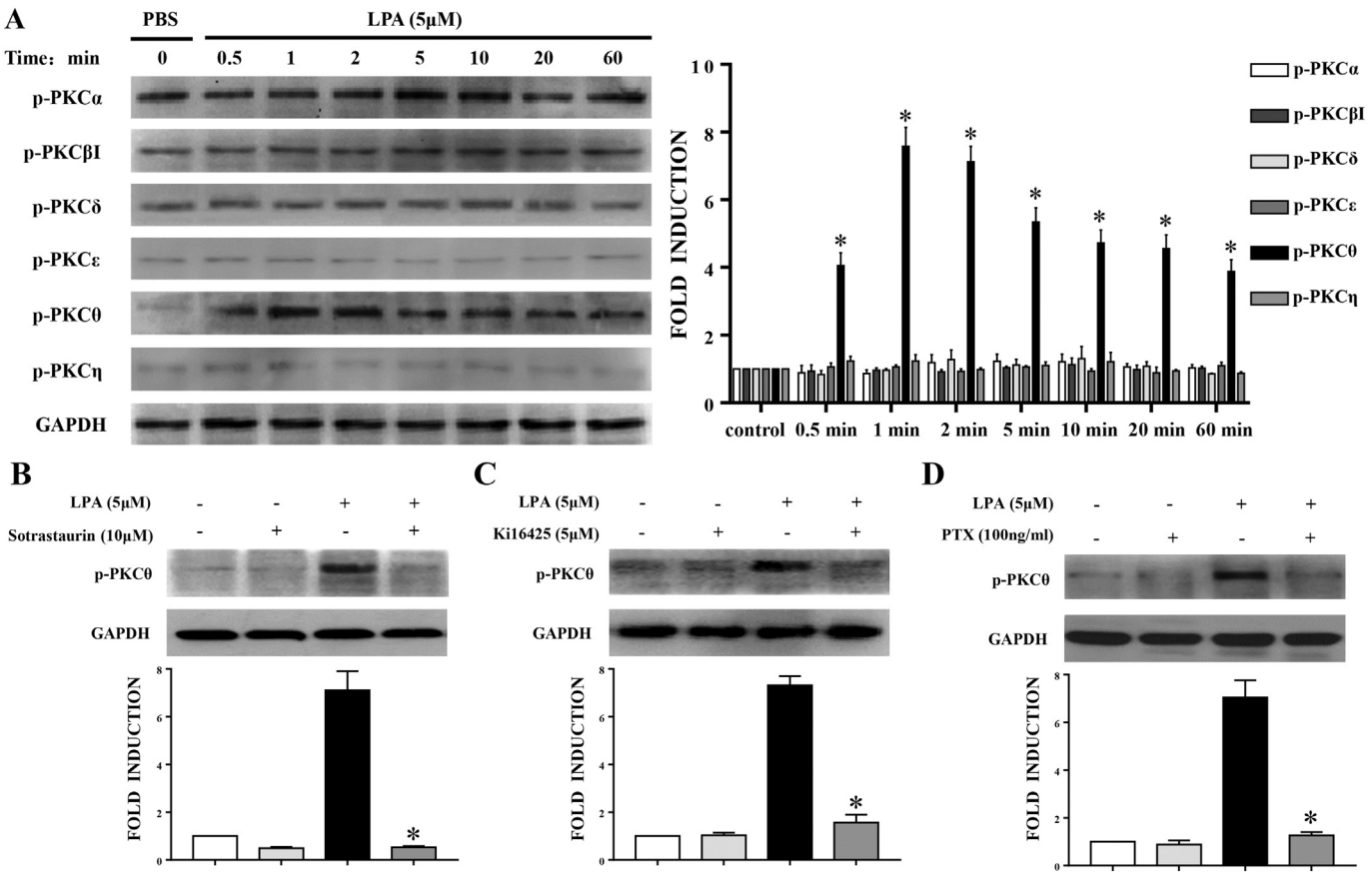

Fig. 4. PKCθ is activated by LPA in MLO-Y4 cells, and the effects of the PKC inhibitor sotrastaurin, the $LPA_{1/3}$ antagonist Ki16425 and the $G_{i/o}$ protein inhibitor PTX on LPA-induced PKCθ activation. A: Dynamic phosphorylation of PKC isoforms in MLO-Y4 cells in response to LPA stimulation. Starved MLO-Y4 cells were stimulated with LPA for various durations as indicated. The phosphorylation of various PKC isoforms was detected by Western blot analysis using specific antibodies against various phosphorylated sites of PKC isoforms. Expression of GAPDH served as an internal control. Left, representative immunoblot; right, quantification of Western blot. The data represent the mean ± SD (n = 3). *, *P* values ≤ 0.05, compared with the PBS control. B, C and D: Effects of the PKC inhibitor sotrastaurin, the $LPA_{1/3}$ antagonist Ki16425 and the $G_{i/o}$ protein inhibitor PTX on LPA-induced PKCθ phosphorylation. Starved cells were separately pretreated with sotrastaurin, Ki16425 and PTX for 1 h before stimulation with LPA for 1 min. The phosphorylation of PKCθ was detected by Western blot analysis. GAPDH served as an internal control. Top, representative immunoblot; bottom, quantification of Western blot. The data represent the mean ± SD (n = 3). *, *P* values ≤ 0.05, compared with LPA treatment alone.

in the LPA signal leading to PKCθ activation, we examined the effects of Ki16425 and PTX on LPA-induced PKCθ activation. As shown in Fig. 4C and D, the pretreatment of cells with Ki16425 and PTX significantly blocked the LPA-induced activation of PKCθ. These results demonstrate that LPA_1 and $G_{i/o}$ protein both mediate LPA-induced PKCθ activation in MLO-Y4 cells.

4. Discussion

In the present study, we demonstrated that LPA induces the production of IL-6 and CXCL15 in MLO-Y4 cells. Furthermore, our data show that the secretion of LPA-induced IL-6 and CXCL15 is mediated by its cognate receptor LPA_1, $G_{i/o}$ protein and PKCθ.

Significantly increased levels of IL-6 and IL-8 occur around broken bones in the early phases of fracture healing [9], and IL-6 gene and protein expression in the damaged bone are notably upregulated following injury [23,24]. In addition, it likely that osteocytes are exposed to LPA arising from the hematoma in regions adjacent to fractures. Our results show that LPA induces IL-6 and CXCL15 synthesis in MLO-Y4 cells. Moreover, osteocytes comprise the overwhelming majority of cells in bone. Therefore, the results of our study suggest that osteocytes may be an important source of IL-6 and IL-8 in the fracture microenvironment, and LPA may be a potent regulator of IL-6 and IL-8 production in this area. IL-6 and IL-8 are proinflammatory cytokines involved in the acute inflammatory response associated with fracture healing [9]. These cytokines are also known to induce the formation of osteoclasts that resorb the necrotic bone fragments and necrotic ends of fractured bone during the early phase of bone healing [25,26]. Furthermore, IL-6 is involved in the recruitment of osteoprogenitor cells and their osteoblastic differentiation, which helps to initiate new bone formation [25,27]. Thus, the results of our study indicate that LPA may influence fracture healing by inducing IL-6 and IL-8 secretion from osteocytes.

The induction of cytokine production in different cell types by LPA is mediated by specific LPA receptors. In breast cancer cells, LPA_1 overexpression was observed to markedly increase LPA-induced IL-6 and IL-8 secretion [28]. The LPA-induced secretion of IL-6 and IL-8 by fibroblast-like synoviocytes may be dependent on LPA_1 and LPA_3 [16]. In colon cancer cells, LPA-induced IL-8 synthesis has been demonstrated to primarily involve LPA_2 [29]. The results of these previous studies have indicated that LPA_1, LPA_2 and LPA_3 may be involved in LPA signal transduction to increase IL-6 and/or IL-8 synthesis. Our results in MLO-Y4 cells do not preclude the possibility that LPA_2 and LPA_4 may also play a role in LPA-dependent IL-6 and CXCL15 production. However, because the $LPA_{1/3}$ antagonist Ki16425 significantly blocked the LPA-induced secretion of IL-6 and CXCL15, and because there is almost no LPA_3 expression in MLO-Y4 cells, our results indicate that the effect of LPA on MLO-Y4 cells is associated with LPA_1. Moreover, LPA_1 is a GPCR that can bind to and activate three types of G proteins,

namely, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$. An earlier study showed that LPA-induced dendrite outgrowth of MLO-Y4 cells may be dependent on $G_{i/o}$ protein-coupled LPA_1 [19]. In our study, we observed that LPA-induced IL-6 and CXCL15 secretion by MLO-Y4 cells was inhibited by PTX, suggesting that $G_{i/o}$ protein mediates LPA-induced IL-6 and CXCL15 secretion in MLO-Y4 cells and that it is likely coupled to LPA_1 in this process. This possible signaling mechanism is consistent with the finding in osteoblasts that LPA_1 and $G_{i/o}$ protein are also involved in this LPA-mediated activity [18].

Previous studies have demonstrated that PKC activation contributes to LPA-induced IL-6 and/or IL-8 secretion in dendritic cells, aortic smooth muscle cells and breast cancer cells [15,30,31]. The present study showed that the PKC inhibitor sotrastaurin inhibited LPA-induced IL-6 and CXCL15 secretion in MLO-Y4 cells, suggesting that PKC also has a role in this process. PKC has multiple isoforms, and the effects of LPA in different cells may be associated with specific PKC isoforms [32,33]. PKCθ has been observed to be an important signaling molecule in LPA signaling [34]. The PKCθ signaling pathway was also found to be involved in IL-6 or IL-8 production in various cell types. In breast cancer cells, PKCθ expression increased the level of IL-6 gene expression [35]. In colonic lamina propria $CD4^+$ T cells, thymocyte-selected $CD4^+$ T cells, skeletal muscle cells, alveolar epithelial cells and human pterygium epithelial cells, the secretion of IL-6 was regulated by PKCθ [36–40]. PKCθ knockdown inhibited upregulation of IL-8 gene expression in memory $CD4^+$ T cells [41], and the inhibition of PKCθ activation reduced both IL-6 and IL-8 secretion in human mast cells [42]. Furthermore, LPA was shown to induce IL-6 and/or IL-8 production in many different kinds of cells [15–17,43]. Thus, LPA-mediated induction of IL-6 and IL-8 production may be related to PKCθ phosphorylation. In our study, of all the targets of sotrastaurin, only PKCθ was activated by LPA in MLO-Y4 cells. Moreover, we observed that the pretreatment of cells with sotrastaurin blocked LPA-induced PKCθ activation. Given that LPA-induced IL-6 and CXCL15 secretion was reduced by sotrastaurin, these results indicate that PKCθ is required for LPA-induced IL-6 and CXCL15 production in MLO-Y4 cells. PKCθ pathways are known to be activated by GPCRs and $G_{i/o}$ protein [34]. Our results showed that Ki16425 and PTX blocked LPA-induced PKCθ activation, suggesting that PKCθ is a downstream molecule of LPA_1 and $G_{i/o}$ protein in LPA signaling in MLO-Y4 cells. Because some PKC isoforms are not targeted by sotrastaurin, such as PKCγ and PKCζ, we did not assess their roles in the present study, but their relationship with LPA-induced IL-6 and CXCL15 production in MLO-Y4 cells will be a focus of future research.

In summary, the results of our study provide evidence that LPA enhances IL-6 and CXCL15 secretion in MLO-Y4 cells. Moreover, this activity of LPA is mediated by LPA_1 and $G_{i/o}$ protein via activation of PKCθ. The results of our study provide new insight into the mechanisms of fracture healing and suggest that LPA may play an important role in facilitating bone fracture repair.

Declaration of Competing Interest

None.

Acknowledgements

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