Role of Protein Kinase C Isoforms in the Regulation of Interleukin-13-induced 15-Lipoxygenase Gene Expression in Human Monocytes

Received for publication, January 14, 2004
Published, JBC Papers in Press, February 2, 2004, DOI 10.1074/jbc.M400413200

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We reported previously that interleukin-13 (IL-13) induces tyrosine phosphorylation/activation of Jak2 and Tyk2 kinases and Stats 1, 3, 5, and 6 in primary human monocytes. We recently revealed that p38 MAPK-mediated serine phosphorylation of both Stat1 and Stat3 is required for the induction of 15-lipoxygenase (15-LO) expression by IL-13. In this study, we present data indicating that another serine/threonine kinase, PKCδ, is also required for IL-13-induced 15-LO expression. PKCδ, a member of the novel protein kinase C (PKC) subclass, was rapidly phosphorylated and activated upon exposure to IL-13. Treatment of cells with rottlerin, a PKCδ inhibitor, blocked IL-13-induced 15-LO mRNA and protein expression, whereas Go6976, an inhibitor of the conventional PKC subclass, had no inhibitory effects. Down-regulation of cellular PKCδ protein levels by PKCδ-specific antisense oligodeoxyribonucleotides also inhibited 15-LO expression markedly. IL-13-induced 15-LO expression resulted in significant inhibition of synthesis of the potent chemotactic factor leukotriene B4, and that process was reversed by rottlerin, presumably through the blockage of PKCδ-dependent 15-LO expression. Furthermore, our data demonstrate that IL-13-mediated activation of PKCδ and p38 MAPK are independent pathways, because inhibition of one kinase activity had no effect on the other, suggesting that the two pathways act in parallel to regulate the downstream targets necessary for 15-LO expression. Inhibition of PKCδ activation by rottlerin also markedly attenuated IL-13-induced Stat3 DNA binding activity. Our findings indicate that PKCδ plays an important role in regulating IL-13-induced 15-LO expression in human monocytes and subsequently modulates the inflammatory responses mediated by 15-LO products.

Th2 lymphocytes secrete IL-4 and IL-13, which have the unique ability to induce the expression of the lipid-oxidizing enzyme 15-LO in primary human monocytes (1–3). To date, no monocyte cell lines have been shown to respond in a similar fashion. 15-LO dioxygenates polyenoic fatty acids to their corresponding hydroperoxide derivatives. These molecules are potent mediators of inflammatory responses and are found in atherosclerotic lesions. It is believed that 15-LO products play an important role in the pathogenesis of atherosclerosis and other inflammatory diseases (4–7). In addition to its proinflammatory actions, 15-LO has also been shown to suppress the inflammatory responses by inhibiting the production of the potent chemotactic factor LTB4. This process has been observed in several cell types, including monocytes (8–10).

Previously, we demonstrated that Jak2 and Tyk2 are upstream tyrosine kinases involved in regulating IL-13-induced expression of 15-LO (3). We have further defined the functional IL-13 receptor complex, the association of Jaks with the receptor constituents, and the tyrosine phosphorylation and activation of Stats in response to IL-13 (11). Tyrosine phosphorylation of Stat molecules facilitates the formation of dimerized Stat complexes, which are then translocated to the nucleus, bind DNA, and regulate target gene expression (12). In addition to tyrosine phosphorylation, the serine phosphorylation of Stat molecules is also necessary for the maximal activation of transcription (13, 14). Several lines of evidence suggest the role of different Ser/Thr kinases in serine phosphorylation of Stat molecules and their transactivation (15–26). Our recent studies demonstrated that p38 MAPK regulates serine 727 phosphorylation of Stat1 and Stat3 and that p38 MAPK activity is required for IL-13-induced 15-LO expression (27).

PKC is a heterogenous family of serine/threonine kinases mediating important intracellular signaling pathways (28–30). PKC isoforms are classified into three major categories, depending on their activation mechanisms as well as Ca2+ and lipid requirements. The conventional PKCs (cPKCs; α, β, and γ) require both Ca2+ and diacylglycerol. The novel PKCs (nPKCs; ε, δ, η, and θ) are Ca2+ independent but require diacylglycerol, whereas the atypical PKC isoforms (aPKCs; ι, λ, and ϶) are independent of both Ca2+ and diacylglycerol. Usually, PKCs are activated in response to the engagement of G protein-coupled receptors, tyrosine kinase receptors, and non-tyrosine kinase receptors. The activation of PKCδ as evidenced by tyrosine and serine/threonine phosphorylation is induced in response to several stimuli, such as H2O2 (31), IL-6 (19, 24), or the proinflammatory mediator thrombin (32). Upon activation, PKCδ phosphorylates a variety of proteins and has been shown to activate various transcription factors (24, 25, 32–34).

In the present study, we investigated the role of PKC isoforms, particularly PKCδ, in IL-13-induced 15-LO expression in primary human monocytes. We provide evidence that activation of PKCδ by IL-13 is required for the induction of 15-LO.
The presence of 10% CO2. For some experiments, monocytes were purified in this manner as indicated previously (3). Adherent cells were collected from the flask after attachment to 15-LO (68 kDa) as determined by migration relative to recombinant standards and molecular mass markers. The bands were then stripped and reprobed with p47phox, a constitutively produced monocyte protein, to assess protein loading (lower panels).

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant human IL-13 was purchased from BIO SOURCE. Antibodies against 15-LO were kindly provided by Dr. Joseph Cornicelli, Parke-Davis. Antibodies to phospho-p38 MAPK, p38 MAPK, phosphoserine 463-PKCδ, and phosphothreonine 505-PKCδ were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to PKCα, p47phox, and β-tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The general PKC inhibitor calphostin C, the PKCδ-selective inhibitor rottlerin, and the εPKC inhibitor Go 6976 were purchased from Biomek (Butler Pike, PA). The inhibitors were dissolved in Me2SO and stored at -20°C as stock solutions.

**Isolation of Human Monocytes**—Human peripheral blood monocytes were isolated from whole blood by the separation of mononuclear cells and real time quantitative RT-PCR was performed according to established protocols (27).

**RNA Extraction and Real Time RT-PCR**—Monocytes were plated in six-well culture plates at 5 x 10⁶/well in 2 ml of medium. Two hours after plating, cells were treated with 500 pm recombinant IL-13 for 4 h. In some experiments, monocytes were pretreated with inhibitors for 30 min followed by IL-13 treatment for 4 h. Total cellular RNA was extracted using the RNeasy mini-kit from Qiagen (Valencia, CA), and real time quantitative RT-PCR was performed according to established protocols (27).

**Immunoprecipitation and Immunoblotting**—Monocytes were plated at 2 x 10⁶/ml in six-well plates, pretreated with the inhibitors (30 min) or antisense ODN whenever required and then treated with IL-13 (500 pm) for different time intervals as indicated. After protein concentration was determined using the Bio-Rad protein assay reagent (Hercules, CA), lysates (50 µg/well) were loaded on an 8% SDS-PAGE gel. The proteins were transferred to a polyvinylidene difluoride membrane and blocked with 5% nonfat milk in phosphate-buffered saline with 0.1% Tween 20 and blotted with appropriate antibodies. The hybridization signal was detected using enhanced chemiluminescence (Pierce). For immunoprecipitation experiments, the lysates were incubated with anti-PKCδ antibodies for 2 h at 4°C with constant rotation. Immune complexes were collected using pre-washed protein A-Sepharose beads (Sigma) at 4°C overnight. Immunoprecipitates were washed three times with lysis buffer. Immune complexes were released by boiling the beads in SDS sample buffer and then subjected to Western blot analysis as described above. In several experiments, immunoblots were stripped and reprobed to assess equal loading.

**Fig. 1. PKCδ isoforms regulate the IL-13 induction of 15-LO expression.** Monocytes were pretreated with calphostin C (A) or Go 6976 (B) for 30 min at various doses, followed by stimulation with 500 pm IL-13 for 24 h. The cells were lysed, and 50 µg of the extract was loaded per lane on 8% SDS-PAGE gels and immunoblotted with antibody to 15-LO. Arrows indicate the position of 15-LO (68 kDa) as determined by migration relative to recombinant standards and molecular mass markers. The bands were then stripped and reprobed with p47phox, a constitutively produced monocyte protein, to assess protein loading (lower panels).

**Fig. 2. Activation of PKCδ in IL-13-treated monocytes.** Monocytes were treated with IL-13 for various times as indicated. Cells were lysed, and 100 µg of total protein was immunoprecipitated (IP) with an anti-PKCδ antibody and subjected to Western blot analysis with anti-phospho-PKCδ antibodies. Arrows indicate phospho-PKCδ (upper and middle panels) and total PKCδ (lower panel) (A). PKCδ kinase activity was assessed using cell lysates from cells either untreated or treated with IL-13 for 15 min (B). 100 µg of total protein was immunoprecipitated with anti-PKCδ antibody. PKCδ activity was measured using the immune complex protein kinase assay with histone H1 as the substrate as described under “Experimental Procedures.” The upper panel is the phosphorimage, the lower panel shows the Western blot result using anti-PKCδ antibody.
1 mM phenylmethylsulfonyl fluoride, and 1/1000 protease inhibitor mixture (Sigma). Cell lysates were immunoprecipitated with an antibody against PKC and collected onto protein A-Sepharose beads. The immune complexes were washed three times with the phosphorylation lysis buffer, twice with kinase assay buffer, and then resuspended in 30 μl of kinase assay buffer. Histone H1 (10 μg) was added as a substrate for PKC and incubated at 30 °C for 10 min before adding 20 μM ATP, 10 μCi of [γ-32P]ATP, and 20 μg of phosphatidylserine. The incubation was continued at 30 °C for an additional 30 min. The reactions were terminated by adding 5/1000 sample buffer. After boiling for 5 min, the reaction mixtures were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The phosphorylated form of histone H1 was detected using a PhosphorImager. The membranes were also subjected to Western blot analysis to assess the amount of PKC in the immune complexes.

LTB4 Production by Monocytes—Monocytes (1 × 10⁶ cells/ml) were plated in duplicate in 12-well culture plates and either pretreated with 5 μM rottlerin for 30 min or left without rottlerin before IL-13 treatment for 72 h. Cells were then challenged with the Ca2⁺-ionophore A23187, at a final concentration of 5 μM for 15 min. At the end of the incubation, cell supernatants were collected and assayed for LTB₄ using the LTB₄ Biotrak enzyme immunoassay (EIA) system (Amersham Biosciences) following the manufacturer’s protocol.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay (EMSA)—To assess the role of PKC in IL-13-induced Stat3 DNA binding activity, EMSA was performed using nuclear extracts and a specific Stat3 probe (Santa Cruz Biotechnology). Monocytes were pretreated with 5 μM rottlerin for 30 min followed by the addition of IL-13 for another 30 min. Nuclear proteins were extracted, and the EMSA was performed following our previously published protocol (11, 27).

RESULTS
Role of PKC Isoforms in Regulating IL-13-induced 15-LO Expression—Our previous studies indicated that, in addition to protein tyrosine kinases, serine/threonine kinases are also required in the IL-13 induction of 15-LO expression (27). PKCs are a major class of serine/threonine kinases and are involved in regulating the expression of a variety of different genes. We, therefore, assessed the possible involvement of PKC in IL-13-mediated 15-LO expression. Monocytes were treated with calphostin C, a general inhibitor for PKC (40), before exposure to IL-13. Calphostin C substantially inhibited IL-13-induced 15-LO expression at the doses shown to be specific for inhibiting PKCα and PKCβ activity (41) (Fig. 1B). These data suggest
that PKC isoform(s) other than cPKCs are required for IL-13-induced 15-LO expression.

**IL-13 Induces Phosphorylation and Activation of PKCδ in Human Monocytes**—We recently reported that IL-13-induced 15-LO gene expression requires p38 MAPK activity (27). Another study suggests that thrombin-induced intercellular adhesion molecule-1 (ICAM-1) gene expression in endothelial cells involves PKCδ-dependent activation of p38 MAPK (32). To determine whether PKCδ is involved in the regulation of IL-13-induced 15-LO expression, we first examined PKCδ activation following IL-13 treatment utilizing antibodies that specifically recognize phosphoserine at position 643 or phosphothreonine at position 505 of PKCδ. After treatment with IL-13 for different times, the cells were lysed, and the whole cell lysates were immunoprecipitated with anti-PKCδ antibody followed by immunoblot analysis using the anti-phospho-PKCδ antibodies. As shown in Fig. 2A, IL-13 induced both serine (643) and threonine (505) phosphorylation in a time-dependent manner. The phosphorylated forms of PKCδ were detected as early as 5 min, and the peak of phosphorylation was at 10 min after IL-13 treatment, with the signal diminishing afterward. To further determine whether phosphorylation of PKCδ resulted in increased kinase activity, we performed a PKCδ kinase assay in which PKCδ from monocytes was immunoprecipitated using an anti-PKCδ antibody, and the phosphorylation of the substrate histone H1 was assessed. IL-13 treatment for 15 min resulted in a remarkable induction of PKCδ kinase activity as evidenced by increased phosphorylation of its substrate, histone H1 (Fig. 2B). These results suggest that the activation of PKCδ in IL-13-treated cells is one of the early
15-LO gene expression, we treated cells with the PKC inhibitor rottlerin (42) and 15-LO expression (Fig. 3) had no detectable effect on IL-13-induced 15-LO mRNA or protein expression.

To further test the functional role of PKCδ in regulating 15-LO gene expression, we treated cells with the PKCδ-specific antisense or control sense ODN before IL-13 stimulation. Total cellular RNA or proteins were extracted for real time RT-PCR or Western blot analysis, respectively. Our results, presented in Fig. 4, A and B, indicate that PKCδ antisense ODN inhibited both 15-LO mRNA and protein expression by ~50%, whereas PKCδ sense ODN caused no inhibition of 15-LO expression. These data confirm the results presented in Fig. 3, A and B, where pharmacological inhibitors were employed.

**Induction of 15-LO Expression Blocks LTβ4 Production—** Previous studies suggest that LTβ4 production in human monocytes is inhibited by IL-4-induced 15-LO expression (10). We reproduced this observation with IL-13-induced 15-LO expression and tested whether a blockade of IL-13-induced 15-LO expression by the PKCδ inhibitor rottlerin could restore LTβ4 production. For these studies, monocytes were either left untreated or were treated with 5 μM rottlerin for 30 min, followed by IL-13 for 72 h. Cells were then challenged by the Ca++ ionophore A23187, and LTβ4 production in supernatants was determined. As presented in Fig. 5, treatment of monocytes with A23187 induced LTβ4 production by >3-fold, and IL-13 pretreatment significantly inhibited the A23187-induced LTβ4 production (p = 0.0302). Furthermore, when cells were treated with rottlerin at the dose that almost completely inhibited IL-13-induced 15-LO expression, it blocked the IL-13 inhibitory effects on LTβ4 production (p = 0.0215 when compared with IL-13/A23187 group). These data suggest the potential importance and biological function of PKCδ in regulating inflammatory responses via 15-LO expression induced by Th2 cytokines.

**PKCδ Does Not Regulate p38 MAPK Phosphorylation/Activation—** We recently found that, in human monocytes, IL-13 induces p38 MAPK activation, which regulates the Ser-727 phosphorylation of both Stat1 and Stat3 (27). As mentioned previously, a prior study suggests that PKCδ may be one of the upstream kinases regulating p38 MAPK activation (32). To determine whether PKCδ activity is required for p38 MAPK activation in IL-13-treated monocytes, we treated cells with rottlerin for 30 min before IL-13 stimulation. Total cell lysates were extracted, and p38 MAPK phosphorylation/activation was evaluated. As presented in Fig. 6A, pretreatment with rottlerin at a dose (5 μM) that substantially inhibited PKCδ activation (42) and 15-LO expression (Fig. 3) had no detectable effect on IL-13-induced p38 MAPK phosphorylation. These data suggest that p38 MAPK is not the downstream target of PKCδ in the IL-13-induced signaling pathway in human monocytes. To determine whether p38 MAPK was upstream of PKCδ, we treated cells with the p38 MAPK inhibitor SB202190 prior to IL-13 stimulation and evaluated PKCδ phosphorylation. Pretreatment with SB202190 at a dose that significantly inhibited p38 phosphorylation led to various cellular processes.

**PKCδ Is Required for IL-13 Signaling Pathways Leading to 15-LO Expression—** To investigate the possible role of PKCδ in regulating IL-13-induced 15-LO expression, monocytes were pretreated with rottlerin, a PKCδ-selective inhibitor, or Go 6976 for 30 min and then incubated with IL-13 for an additional 4 h. The quantitative real time RT-PCR experiments showed that the induction of 15-LO mRNA by IL-13 was ~60-fold (26) by 4 h, and pretreatment with rottlerin had profound inhibitory effects by reducing the fold-induction to 25 (>60% inhibition compared with that of the IL-13 treated group) (Fig. 3A). The mRNA levels of glyceraldehyde-3-phosphate dehydrogenase were nearly identical in all the samples, indicating specificity of the response. Rottlerin was also effective in inhibiting IL-13-induced 15-LO protein expression as assessed by Western blot analysis (Fig. 3B). In contrast, Go 6976 had no effect on 15-LO mRNA or protein expression.

FIG. 5. Effects of IL-13 and rottlerin on LTβ4 production in human monocytes. Monocytes were treated with 5 μM rottlerin and/or IL-13 for 72 h followed by A23187 for 15 min as indicated. Supernatants were collected at the end of incubation, and LTβ4 production was assayed by using the LTβ4 EIA as described under “Experimental Procedures.” Data are presented as mean ± S.D. of three independent experiments. Mean values were compared using one-tailed Student’s t test (*, p < 0.0302; **, p < 0.0215).

![Figure 5](http://www.jbc.org/)

**PKCδ Regulates IL-13-induced 15-LO Expression**

A Probe: anti-phospho-p38MAPK

\[ P-p38 \]

Strip and reprobe: anti-p38MAPK

\[ p38 \]

IL-13

Rottlerin

- - + + (μM)

**FIG. 6. PKCδ and p38 MAPK independently regulate the IL-13-induced 15-LO expression.** A, monocytes were pretreated with various doses of rottlerin for 30 min before exposure to IL-13 for 15 min. Cell lysates were subjected to Western blot analysis using anti-phospho-p38 MAPK antibody. The membrane was stripped and reprobed with anti-p38 MAPK antibody. Arrows indicate the position of the phospho and total p38 MAPK. B, cells were pretreated with SB202190 for 30 min with different doses followed by IL-13 stimulation for 10 min. Whole cell lysates were immunoprecipitated with an antibody against PKCδ. The immunoprecipitates were then subjected to immunoblot analysis with anti-phosphoserine 643 and anti-phosphothreonine 505 PKCδ antibody (upper and middle panels). The blot was stripped and reprobed to detect total PKCδ (lower panel).
PKCδ Regulates IL-13-induced 15-LO Expression

The early activation of PKCδ suggests that it may be one of the upstream Ser/Thr kinases providing a co-stimulatory signal for IL-13-induced gene expression. There is increasing evidence supporting the critical role of PKCδ-mediated Stat1 and Stat3 Ser-727 phosphorylation in cytokine-induced gene expression (19, 24, 32). Because IL-13 treatment induces p38 MAPK activation and subsequent Stat1 and Stat3 Ser-727 phosphorylation in human monocytes (27), we investigated whether p38 MAPK was related to PKCδ in the IL-13-mediated signal transduction pathways. Our results with pharmacological inhibitors reveal that IL-13-induced p38 MAPK phosphorylation/activation is neither a downstream event nor an upstream kinase of PKCδ. Hence, these two different pathways leading to phosphorylation of downstream targets are independent, parallel pathways, but both are indispensable for IL-13-induced 15-LO expression. The finding that two distinct Ser/Thr kinases regulate Stat3 DNA binding activity is novel and intriguing (27). We propose that, although neither of PKCδ or p38 MAPK regulates the phosphorylation/activation of the other, they may be present in a signaling complex that is required for and controls the important phosphorylation events of their downstream targets like Stat3. Current studies in the laboratory are focused on delineating the details of this unique process.

In summary, our current study indicates that activation of PKCδ by IL-13 plays an important role in IL-13-induced 15-LO expression in human monocytes. Although PKCδ-mediated 15-LO expression is independent of p38 MAPK, the two Ser/Thr kinases may provide co-stimulatory signals to downstream targets regulating 15-LO expression and subsequently modulate the inflammatory responses mediated by 15-LO products.

Acknowledgment—We thank Claudine Horton for critical reading of the manuscript.

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