Indomethacin Causes Prostaglandin D₂-like and Eotaxin-like Selective Responses in Eosinophils and Basophils*

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We investigated the actions of a panel of nonsteroidal anti-inflammatory drugs on eosinophils, basophils, neutrophils, and monocytes. Indomethacin alone was a potent and selective inducer of eosinophil and basophil shape change. In eosinophils, indomethacin induced chemotaxis, CD11b up-regulation, respiratory burst, and L-selectin shedding but did not cause up-regulation of CD63 expression. Pretreatment of eosinophils with indomethacin also enhanced subsequent eosinophil shape change induced by eotaxin, although treatment with higher concentrations of indomethacin resulted in a decrease in the expression of the major eosinophil chemokine receptor, CCR3. Indomethacin activities and cell selectivity closely resembled those of prostaglandin D₂ (PGD₂). Eosinophil shape change in response to eotaxin was inhibited by pertussis toxin, but indomethacin- and PGD₂-induced shape change responses were not. Treatment of eosinophils with specific inhibitors of phospholipase C (U-73122), phosphatidylinositol 3-kinase (LY-294002), and p38 mitogen-activated protein kinase (SB-202190) revealed roles for these pathways in indomethacin signaling. Indomethacin and its analogues may therefore provide a structural basis from which selective PGD₂ receptor small molecule antagonists may be designed and which may have utility in the treatment of allergic inflammatory disease.

Eosinophils and basophils are important effector cells in allergic diseases such as asthma and eczema (1). Many groups have examined the mechanisms initiating and regulating the responsiveness and activation of these cells in the context of allergic disease (1–3). Several agonists mediate eosinophil chemotaxis in vitro and recruitment in vivo. Foremost among these are the chemokines, in particular those acting via CCR3 such as eotaxin/CCL11 (4–9), eotaxin-2/CCL24 (10–12), eotaxin-3/CCL26 (13), and MCP-4/CCL13 (14–16). Additionally, eosinophils from some donors exhibit a significant expression of CCR1 and respond effectively to its ligand MIP-1α/CCL3 (14). Similar results have now been seen by other groups (17), and we have shown that responses of eosinophils to chemokines can be blocked by chemokine receptor antagonists with theoretical benefits for the treatment of allergic inflammation (18). However, activated complement fragments such as C5a and C3a, formylated peptides (formyl-methionyl-leucyl-phenylalanine), and a wide variety of lipid mediators can also induce similar responses in eosinophils (19–22). Recently, interest has arisen in the potential for PGD₂, a mediator known to have actions on eosinophils (23, 24) and which is generated in the asthmatic lung (25), to stimulate eosinophil, basophil, and Th2-type T cell functions in allergic disease through its action on two cell surface receptors, DP and CRTH2 (26–29).

Basophil responses to chemotaxant ligands are more complex. These responses are dependent both upon patterns of receptor expression that overlap with other cell types including eosinophils and monocytes and also the varying affinities of ligands such as MCP-1 and MCP-4 for more than one chemokine receptor (15, 30–34).

Nonsteroidal anti-inflammatory drugs (NSAIDs)1 can have complex anti-inflammatory actions. A recent study revealed that, in addition to their effects on cyclooxygenase function, they also caused varying levels of shedding of the adhesion molecule L-selectin from the surface of neutrophils following a reduction in intracellular ATP (35). Such actions might modulate neutrophil recruitment; however, the ability of NSAIDs to induce similar modulation of eosinophil and basophil function has not been explored. We therefore examined the actions of a large panel of NSAIDs on eosinophil and basophil function. Surprisingly, one NSAID, indomethacin, showed a marked direct action upon leukocytes, inducing selective responses in basophils and eosinophils consistent with a very recent paper that has identified indomethacin as a CRTH2 agonist (36). We have therefore investigated the actions and signaling pathways of indomethacin and related these actions to other stimuli involved in selective recruitment and activation of these allergic inflammatory leukocytes.

1 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; PTX, pertussis toxin; PMN, polymorphonuclear leukocytes (comprising eosinophils and neutrophils); PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PE, phycoerythrin; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PLC, phospholipase C; PI 3-kinase, phosphatidylinositol 3-kinase; FSC, forward scatter; SSC, side scatter; COX, cyclooxygenase.

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EXPERIMENTAL PROCEDURES

Reagents—All laboratory reagents were from Sigma (Poole, UK) unless otherwise specified. The NSAIDs indomethacin, diclofenac, flufenamic acid, etodolac, piroxicam, and flurbiprofen were also from Sigma. The COX-2-selective NSAID NS-398 was from Cayman (Ann Arbor, MI), and the COX-1 selective NSAID SC-560 was a generous gift from Dr. R. A. Marks (Searle, Skokie, IL). They were dissolved at high concentrations as recommended in water, PBS, MeSO4, ethanol, or ethanol/MeSO4 and then diluted as needed. After relevant vehicle controls were without effect in any assay tested beyond responses seen in buffer-treated cells alone, Dulbecco’s modified PBS (with or without Ca2+ and Mg2+) was from Invitrogen (Paisley, UK). Chemokines were from Peprotech EC (London, UK). Cell Fix and FACSCaliber Flow were from Becton Dickinson Immucytometry Systems (San Jose, CA). Antibodies to CD63 (FITC conjugate) were from Autogen Bio (Wakefield, MA). Antibodies to CD16, CD11b, and CCR3 were from Dako (Ely, UK). Anti-L-selectin (PE) was from eBioscience (San Diego, CA). The anti-human CCR3 monoclonal antibody 7B11 (isotype IgG2a) was a generous gift from Dr. Shixin Qin (Millennium Pharmaceuticals Inc., Cambridge, MA). Relevant isotype-matched control antibodies were used throughout. The p38 mitogen-activated protein kinase (MAPK) inhibitor SB-202190, the MAPK extracellular signal-regulated kinase inhibitor MEK inhibitor U-0126, and the PI 3-kinase inhibitor LY-294002 were purchased from Calbiochem. The phosphatidylinositide-specific phospholipase C (PLC) inhibitor U-73122 (37) and the control small molecule U-73343 were supplied by Biomol (Plymouth Meeting, PA). Boyden chambers and 5-µm pore size polycarbonate filters were from Neuro Probe Inc. (Gaithersburg, MD).

Cell Preparation—Peripheral blood leukocyte preparations of granulocytes (containing eosinophils and neutrophils) and mononuclear cells (including basophils, monocytes, and lymphocytes) were prepared by plasma/Percoll gradients or Histopaque gradients as described (14, 30, 38). Cells prepared by either technique gave similar results in the high sensitivity assays of leukocyte shape change (data not shown). In some experiments, eosinophils were further purified from granulocyte populations by negative magnetic selection using an antibody mixture from StemCell Technologies (Vancouver, Canada), according to the manufacturer’s instructions. Resulting populations of eosinophils were typically >97%, with the majority of contaminating cells being lymphocytes as judged by flow cytometry forward scatter (FSC)/side scatter (SSC) plots.

Leukocyte Shape Change Assays—Eosinophils, monocyte, basophil, and neutrophil shape change was assayed as described in previous work (14, 18, 30). Stimulation of these leukocytes by chemoattractant and chemokinetic agonists results in changes in cell shape that are measured as changes in their ability to scatter light when illuminated in a flow cytometer (Fig. 1) (14, 30). In all experiments, data are displayed as percentage increase in FSC compared with samples treated with buffer alone (Fig. 1A, data not shown). Surprisingly, of the NSAIDs tested, only indomethacin induced a shape change response. This response was observed in eosinophils and basophils but not neutrophils or monocytes (Fig. 2A). Indomethacin induced eosinophil shape change with a maximal response at 100 nM and showed lower potency but the same efficacy as eotaxin/CCL11. The NSAIDs ibuprofen, flurbiprofen, NS-398, SC-560, piroxicam, and etodolac (each 50 nM to 10 µM) and acetylsalicylic acid (5 µM to 1 mM) all failed to induce any detectable shape change response in eosinophils or neutrophils (n = 4–6, data not shown). Similarly, diclofenac and flufenamic acid (each 50 nM to 10 µM) were without effect (n = 3, data not shown).

Indomethacin Causes Eosinophil Chemotaxis—We have previously shown that eosinophil shape change can be induced by both chemotactic agonists such as eotaxin/CCL11 and by chemokinetic agonists such as interleukin-8 (14). We therefore investigated the ability of eotaxin/CCL11 and indomethacin to induce eosinophil chemotaxis in micro-Boyden chambers. Each agonist was tested in the presence of 1 µM dextran sulfate sodium (DS) and the bottom chamber of the chemotaxis plate (Fig. 2B). The addition of indomethacin (1 µM) to the top of the chemotaxis chamber alone resulted in some variable migration of eosinophils into the lower chamber although to a lesser degree than seen when indomethacin was added to the bottom chamber alone (Fig. 2C).

Indomethacin Up-regulates Eosinophil CD11b and Down-regulates L-selectin Expression—Our data showed that indomethacin acted as an eosinophil chematoattractant agonist in a
similar manner to the chemokine eotaxin/CCL11. We therefore investigated whether indomethacin could induce modulation of adhesion molecule expression. Fig. 3A shows that treatment of eosinophils in mixed cell suspensions with indomethacin resulted in a reduction in cell surface L-selectin expression, whereas neutrophil L-selectin expression was not significantly affected. Indomethacin also induced an up-regulation of eosinophil CD11b expression (Fig. 3B). In comparison, neutrophil CD11b expression was not affected by indomethacin at any concentration tested (Fig. 3B).

Indomethacin Causes Respiratory Burst but Not Up-regulation of CD63 Expression—Chemokine chemoattractants are typically good stimulators of leukocyte shape change and chemotaxis, variable inducers of respiratory burst, and relatively poor stimulators of degranulation. Classical chemoattractants such as C5a are more potent inducers of these latter responses. In keeping with these data, eosinophil respiratory burst (eotaxin = PGD₂ > indomethacin (Fig. 3C)), although they were less efficacious than C5a and were unable to induce any changes in eosinophil CD63 expres-
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Indomethacin-induced Eosinophil Shape Change Is Inhibited by Cytochalasin B but Not by Pertussis Toxin—Eosinophil shape change as induced by chemotactic factors is dependent upon G protein-coupled receptors mediating regulation of the cell cytoskeleton. The majority of studies have emphasized a role for pertussis toxin (PTX)-sensitive G proteins in the regulation of leukocyte chemokine responses. Pretreatment of eosinophils with PTX (in accordance with protocols previously used to investigate CCR3 signaling (41)) suggested differences in the signaling pathways activated by eotaxin and indomethacin. Eosinophil shape change in response to eotaxin/CCL11 was abolished by PTX, whereas in parallel samples, eosinophils retained their ability to respond to both indomethacin and PGD2 (Fig. 4A).

Previously, we showed chemokine-induced eosinophil shape change to be inhibited by pretreatment of cells with cytochalasin B (14). Here again, we found that eosinophil shape change induced by eotaxin was inhibited by cytochalasin B but not by buffer containing the vehicle control, Me2SO (Fig. 4B). Similarly, indomethacin-induced shape change also showed dependence upon cell microskeletal elements, since it too was inhibited by pretreatment with cytochalasin B.

Experiments using PTX (Fig. 4A) suggested that different signaling pathways were involved in responses of eosinophils to indomethacin and eotaxin/CCL11. In keeping with these data, eotaxin/CCL11-induced shape change was more transient than that induced by indomethacin and was undetectable after prolonged stimulation. In contrast, indomethacin-induced shape change was still detectable after prolonged stimulation, although the concentration-response curve was right-shifted (Fig. 4C).

Eosinophil Responses to Indomethacin Involve Specific Kinases—Chemotaxattractant agonists activate multiple intracellular signaling pathways in eosinophils, including PLC, PT 3-kinase, and p38 MAPK. We therefore investigated whether indomethacin- and eotaxin-induced eosinophil responses were modulated by antagonists of these pathways. Fig. 5 shows that eosinophil shape change responses induced by eotaxin/CCL11 were markedly attenuated by inhibition of PLC using U-73122. Similarly, responses of eosinophils to indomethacin and PGD2 were inhibited by U-73122, but responses to eotaxin/CCL11, indomethacin, and PGD2 were not inhibited by the control compound, U-73343.

Eosinophil shape change induced by submaximal concentrations of eotaxin/CCL11 was inhibited by both the PI 3-kinase inhibitor LY-294002 and the p38 MAPK inhibitor SB-202190 (Fig. 6, A and B). However, responses seen at concentrations of eotaxin/CCL11 inducing maximal shape change were not in-

Fig. 3. Actions of indomethacin on eosinophils and neutrophils. PMNL were stimulated with indomethacin for 30 min. Samples were stained to analyze L-selectin (A) and CD11b (B) expression and double-stained with anti-CD16 to identify neutrophils and eosinophils. Data were quantified as percentage change from basal (buffer-treated) expression levels and are the mean of four experiments ± S.E. In separate experiments, induction of respiratory burst (C) was investigated by treating purified eosinophils with agonists for 20 min, and CD63 up-regulation (D) was investigated by stimulating eosinophils in mixed cell suspensions for 30 min. Changes in anti-CD39 binding or dihydrorhodamine 123 fluorescence are shown as the mean of four experiments (CD63 up-regulation) and three experiments (respiratory burst) ± S.E.

Fig. 4. Modulation of eosinophil shape change by pertussis toxin and cytochalasin B and time dependence of signaling. Eosinophils in mixed PMNL populations were pretreated with PTX (1 μg/ml for 60 min (A, i–iii) or cytochalasin B (5 μg/ml for 5 min; B, i–ii) and stimulated with indicated ligands for 4 min, and shape change was measured as described. C depicts the eosinophil shape change induced in mixed cell populations by stimulation with eotaxin/CCL11 or indomethacin for the times indicated. Data shown are the mean of six experiments ± S.E. (A and B) and four experiments (C, indomethacin) or three experiments (C, eotaxin) ± S.E., and significant inhibition of shape change in the presence of PTX or cytochalasin B is indicated by p < 0.05 (*).
hibited by either LY-294002 or SB-202190. The up-regulation of eosinophil CD11b expression induced by eotaxin/CCL11 was also inhibited by both LY-294002 and SB-202190 (Fig. 6C) and also by MEK inhibitor U-0126 (Fig. 6C), although interestingly U-0126 had no effect on eotaxin/CCL11-induced eosinophil shape change (Fig. 6A).

Indomethacin-induced responses in eosinophils were similarly modulated by inhibitors of PI 3-kinase, p38 MAPK, and MEK but with two exceptions (Fig. 6). First, inhibition of p38 MAPK reduced both the potency and efficacy of indomethacin in assays of eosinophil shape change, whereas the p38 MAPK inhibitor suppressed eotaxin-induced eosinophil shape change potency alone. Second, the MEK inhibitor U-0126 caused an insignificant inhibition of eosinophil shape change induced by submaximal indomethacin concentrations and had no effect on indomethacin-induced up-regulation of eosinophil CD11b expression.

**Indomethacin Pretreatment Can Modulate Eosinophil CCR3 Expression**—We have previously shown that G protein-coupled signaling can result in cross-desensitization and internalization of other chemoattractant receptors in human neutrophils (42). Fig. 7 shows that eotaxin/CCL11 induced internalization of its own receptor, in keeping with previous data (43). Interestingly, pretreatment with either indomethacin or PGD$_2$ also resulted in a significant decrease in eosinophil CCR3 expression (Fig. 7), whereas the chemoattractant PAF had no effect.

**Indomethacin Enhances Responsiveness to Eotaxin**—Since indomethacin can be used to treat inflammatory diseases, we determined whether stimulation with this compound would alter eosinophil responses to eotaxin. We pretreated eosinophils with 100 nM indomethacin (the EC$_{50}$ of CCR3 down-regulation (Fig. 7)) for 30 min and then removed the indomethacin by a single wash step prior to stimulation of the cells with eotaxin/CCL11. The data shown in Fig. 8 show that the efficacy of eotaxin/CCL11-induced shape change was increased by indomethacin pretreatment, whether the data were shown as mean FSC (Fig. 8A) or responses were corrected for the minor increase in eosinophil FSC base line arising from the indomethacin pretreatment (Fig. 8B).

**DISCUSSION**

In preliminary studies, we found that lipid-derived mediators play a role in the regulation of eosinophil chemokine responsiveness, and others have shown that NSAIDs such as indomethacin modulate expression of adhesion molecules that are relevant in neutrophil recruitment (35). We examined the actions of NSAIDs to determine whether treatment of leukocytes with these compounds could modulate chemokine responsiveness. Surprisingly, we found that indomethacin induced a direct rapid shape change response in eosinophils in a manner similar to the chemoattractant agonists eotaxin/CCL11 and PGD$_2$, reaching a maximum shape change response at indomethacin concentrations of 100 nM, but had no effect on neutrophils. In addition, we observed that indomethacin also induced shape change responses in basophils but not monocytes. This effect was confined to indomethacin alone out of a large panel of NSAIDs, including the indomethacin-related NSAID, etodolac (44). From these data, we postulated that indomethacin might act as a chemoattractant, and our data show that it did induce concentration-dependent chemotaxis of purified eosinophils, as did the other eosinophil-stimulating agonists eotaxin/CCL11 (Fig. 2) and PGD$_2$ (data not shown).

In a recent study, Gomez-Gaviro et al. showed that indomethacin induced neutrophil L-selectin shedding (35) but with an IC$_{50}$ in excess of 30 $\mu$g/ml (84 $\mu$g). In order to explore the difference between NSAID actions on neutrophils and eosinophils in more detail, we examined the ability of indomethacin to...

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$^2$ V. E. L. Stubbs, A. Hartnell, T. J. Williams, A. Heinemann, and I. Sabroe, unpublished data.
modulate adhesion molecule expression. At the concentrations we tested (10 μM SB-202190, 10 μM U-0126, or 20 μM LY-294002), we were unable to detect significant indomethacin-induced changes in neutrophil L-selectin expression. In contrast, indomethacin caused small but significant decreases in eosinophil L-selectin levels. Indomethacin was also efficacious in the up-regulation of eosinophil CD11b expression, yet had no significant effect on neutrophil CD11b expression, again mirroring effects of eosinophil-selective chemotactants such as eotaxin/CCL11 and PGD2 (26, 45, 46). One other group has described an ability of indomethacin to induce up-regulation of neutrophil CD11b expression, but again this effect was maximal only at high concentrations (100 μM) (47). Thus, our data potentially separate two actions of indomethacin: one that may be common to other NSAIDs, is dependent upon changes in intracellular ATP levels, and mediates L-selectin shedding (35) and one that is specific to indomethacin and is consistent with chemotactant-like actions.

Chemokine agonists are generally effective stimuli of cell recruitment but exhibit varying efficacy in their ability to cause degranulation and respiratory burst. We showed that the classical eosinophil chemotactant and activator, C5a, induced potent activation of eosinophils as seen by up-regulation of CD63 expression (a marker of degranulation) and induction of respiratory burst. Eotaxin/CCL11, PGD2, and indomethacin were unable to cause up-regulation of CD63, emphasizing their primary actions as mediators of cell recruitment. In keeping with the known actions of eotaxin/CCL11 (45), we showed that indomethacin, eotaxin/CCL11, and PGD2 were all able to induce respiratory burst, although with less efficacy than C5a.

Relatively few chemotactant receptors are expressed selectively by eosinophils and basophils but not by neutrophils.
and monocytes (14, 30, 46). Indomethacin showed similar actions to eotaxin/CCL11. However, PTX abolished shape change responses to eotaxin but had no effect on indomethacin-induced shape change, suggesting actions on a different receptor. Recent studies have identified a role for PGD2 as a selective eosinophil-, basophil-, and Th2-type T cell-stimulating activity. Eosinophils express two receptors for PGD2, DP and CRTH2 (26–29). Of these two receptors, CRTH2 is selectively responsible for eosinophil chemotaxis (26, 27), actin polymerization, and CD11b up-regulation in response to PGD2 (26). We therefore hypothesized that indomethacin was acting via a PGD2 receptor, in keeping with its PGD2-like activities on eosinophils, and the PTX-resistant signaling seen in response to both indomethacin and PGD2. During the preparation of this manuscript, a recent paper reported that indomethacin is indeed a selective agonist for CRTH2 but is inactive at DP (36). This paper characterized the actions of indomethacin in detail using receptor transfectants and also found, as we have, that indomethacin can cause eosinophil chemotaxis, although it did not investigate other actions of indomethacin on eosinophils. In contrast, one other recent publication identified actions of PGD2 on eosinophil CRTH2 as inducing chemokinesis rather than chemotaxis (28). We found that indomethacin-induced signaling resulted in some chemokinetic responses, although these were variable between experiments, and in all experiments the addition of indomethacin to the bottom chamber of the chemotaxis plate resulted in greater leukocyte migration than when indomethacin was added to the top of the plate alone. These data suggest that indomethacin primarily induces eosinophil chemotaxis but also a variable degree of eosinophil chemokinesis. Thus, our data strongly support a role for CRTH2 as a receptor that can induce eosinophil recruitment. As also noted by Hirai et al. (36), these data contribute to an understanding of various actions of indomethacin in vivo, such as its ability to induce, or failure to suppress, eosinophilic inflammation at multiple tissue sites (48, 49).

Interestingly, PGD2 signaling is thought to act via the PTX-
sensitive G protein-coupled receptor CRTH2 and the PTX-resistant receptor DP (27). Likewise, the actions of indomethacin at CRTH2 have been shown to be PTX-sensitive in receptor transfectants and Th2-type T cells (36). However, in primary human eosinophils, we observed that neither PGD2 nor indomethacin responses were inhibited by PTX under conditions where this toxin completely inhibited eotaxin/CCL11-induced eosinophil shape change. These data suggest that CRTH2 in eosinophils may perhaps be coupled, at least in part, to the PTX-resistant G protein Goα13 which is also involved in eosinophil chemotactic responses to other ligands, although interpretation of PTX-dependence in investigation of agonist-mediated responses must be carried out with caution (50). An alternative hypothesis, that the actions of both indomethacin and PGD2 on eosinophils are mediated predominantly via the PTX-resistant DP receptor rather than CRTH2, is unlikely, given the published specificity of indomethacin and the previous data on the selective roles of CRTH2 in mediating eosinophil PGD2 responses (26–28, 36). In keeping with this, signaling via DP has been shown to modulate eosinophil apoptosis only (28), and we found that the DP-selective agonist BW245C was unable to cause eosinophil shape change (n = 7, data not shown).

We found that induction of eosinophil shape change in response to both eotaxin/CCL11 and indomethacin was inhibited by an antagonist of phosphatidylinositol-specific PLC, whose isoforms have major and complex roles in the regulation of leukocyte chemotaxis (51, 52). PLC has also been shown to play a role in heterologous receptor desensitization (53) and may therefore be involved in the indomethacin-induced modulation of CCR3 expression we observed here. Eotaxin/CCL11-induced eosinophil responses have also been shown to involve signaling pathways dependent upon PI 3-kinase and p38 MAPK (21, 54), both of which can couple into pathways regulating actin polymerization and cytoskeletal change responses such as underpinning eosinophil shape change (22, 54) and leukocyte integrin-dependent adhesion (55). The roles of these pathways in the regulation of CRTH2-induced signaling have not been explored to date. We found that both indomethacin-induced and eotaxin/CCL11-induced eosinophil responses were reduced by specific inhibitors of these pathways, thus providing the first evidence for their roles in CRTH2-mediated signaling in primary human cells. The inability of the PI 3-kinase inhibitor to prevent agonist-induced shape change to high concentrations of chemottractant may be consistent with the hypothesis that the PI 3-kinase product phosphatidylinositol 3,4,5-trisphosphate is not essential for chemotaxis but rather exerts a primarily regulatory role in this process (51). Interestingly, our data also show that eosinophil signaling via CRTH2 may be more dependent upon p38 MAPK than that induced by eotaxin/CCL11 acting via CCR3. An illustration of the proposed receptors and signaling pathways for eosinotaxis, PGD2, mast cell, and NSAIDs in eosinophils is shown in Fig. 9.

We have also observed that indomethacin may modulate responses of eosinophils to other ligands. Pretreatment of eosinophils with 100 nM indomethacin enhanced the efficacy of eotaxin/CCL11 and suggests that indomethacin may enhance eosinophil responses to proinflammatory ligands. However, at higher concentrations, indomethacin treatment had marked effects upon CCR3 expression. Such effects could be mediated by release of CCR3 ligands from the eosinophil after indomethacin stimulation but are also in keeping with the processes of heterologous desensitization between seven-transmembrane G protein-coupled chemotactant receptors observed in leukocytes by ourselves and others (42, 53). The consequences of these effects on eosinophil responses to ligands such as eotaxin remain to be fully explored.

Indomethacin has other potential actions in eosinophils, most notably through its anti-inflammatory ability to inhibit COX-1 and COX-2 isoenzymes (Fig. 9). Published IC50 values for indomethacin on these enzymes vary within low to high nanomolar ranges (56–59), and thus inhibition of eosinophil COX isoenzymes within the experiments here may have occurred. However, no other NSAID from an extensive panel of drugs including nonselective NSAIDs and drugs with specific actions on either COX-1 or COX-2, tested across a broad range of concentrations, induced any similar responses in human eosinophils. These data, in combination with the observed similarities in signaling between PGD2 and indomethacin in this study and the identification of indomethacin as a ligand for the eosinophil-expressed PGD2 receptor, CRTH2 (36), strongly suggest that the proinflammatory actions of indomethacin on eosinophils and basophils are not mediated by COX inhibition.

Therefore, we have shown that indomethacin is a potent chemoattractant ligand acting on eosinophils, with actions also upon basophils but not neutrophils or monocytes. We have characterized the actions of indomethacin on eosinophils and found that it exerts similar effects to PGD2, supporting evidence that most chemotactant-like PGD2 signaling is mediated in eosinophils by CRTH2. We have also shown that signaling of this receptor shows different sensitivity to PTX compared with signaling in response to eotaxin and that it is coupled into pathways including PLC, p38 MAPK, and PI 3-kinase in primary human eosinophils. Many studies have examined the actions of indomethacin in inflammatory disease, focusing on its roles as a cycloxygenase inhibitor, but the independent actions of indomethacin as a potent eosinophil- and basophil-stimulating compound may alter our understanding of the actions of this drug. Development of structural analogues of indomethacin may generate both new CRTH2 activators and perhaps antagonists that will be of use in the treatment of human allergic inflammatory disease.

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