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Prostaglandin E_2–EP3 Signaling Induces Inflammatory Swelling by Mast Cell Activation

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PGE_2 has long been known as a potentiator of acute inflammation, but its mechanisms of action still remain to be defined. In this study, we employed inflammatory swelling induced in mice by arachidonate and PGE_2 as models and dissected the role and mechanisms of action of each EP receptor at the molecular level. Arachidonate- or PGE_2-induced vascular permeability was significantly reduced in EP3-deficient mice. Intriguingly, the PGE_2-induced response was suppressed by histamine H_1 antagonist treatment, histidine decarboxylase deficiency, and mast cell deficiency. The impaired PGE_2-induced response in mast cell–deficient mice was rescued upon reconstitution with wild-type mast cells but not with EP3-deficient mast cells. Although the number of mast cells, protease activity, and histamine contents in ear tissues in EP3-deficient mice were comparable to those in wild-type mice, the histamine contents in ear tissues were attenuated upon PGE_2 treatment in wild-type but not in EP3-deficient mice. Consistently, PGE_2–EP3 signaling elicited histamine release in mouse peritoneal and bone marrow–derived mast cells, and it exerted degranulation and IL-6 production in a manner sensitive to pertussis toxin and a PI3K inhibitor and dependent on extracellular Ca^{2+} ions. These results demonstrate that PGE_2 triggers mast cell activation via an EP3–$G_{i/o}$–Ca^{2+} influx/PI3K pathway, and this mechanism underlies PGE_2-induced vascular permeability and consequent edema formation. *The Journal of Immunology*, **2014, 192: 1130–1137.**

Four cardinal features, namely rubor (red flare), calor (heat), tumor (swelling), and dolor (pain), characterize acute inflammation. The flare and heat reactions are caused by an increase in local blood flow as a result of vasodilatation, and the swelling is elicited by an increase in vascular permeability and resultant leukocyte recruitment. These processes are triggered by tissue injury and invasion of exogenous materials and organisms (1). Such inflammatory insults are primarily detected by TLRs in immune cells followed by activation of the local cytokine network such as TNF-α and IL-1β. Because these cytokines affect vascular permeability and leukocyte recruitment, such a TLR/cytokine axis in innate immunity is one factor that governs the inflammation process (2, 3). Alternatively, by using various experimental models of acute inflammation, chemical mediators such as bradykinin, histamine, thrombin, and growth factors have been found and characterized (1). Aspirin-like drugs have been used as the first choice of drugs for acute inflammation because of their high potency to suppress the above inflammatory symptoms (4). Because these drugs exert their actions by inhibiting cyclooxygenases (COXs) and thereby inhibit the biosynthesis of PGs, endogenously synthesized PGs are thought to be involved in inflammation reactions (1, 5). Indeed, PGs have been shown to elicit vasodilatation and an increase in local blood flow, leading to red flare and local heat. It is thought that vascular permeability factors such as histamine and bradykinin are thereafter released into the inflammation site, leading to edema formation (1, 6). However, the link between the initial vasodilatation and the subsequent permeability change remains unknown.

PGE_2 is the most abundantly synthesized PG, especially at an inflammation site, and has long been regarded as an important potentiator of acute inflammation (1, 4–7). The diverse actions of PGE_2 are mediated through four receptor subtypes, EP1, EP2, EP3, and EP4 (8, 9). EP1 is coupled to intracellular Ca^{2+} mobilization via G_q, EP2 and EP4 are coupled to stimulation of adenylyl cyclase via G_s, and EP3 is mainly coupled to inhibition of adenylyl cyclase via G_i. Among the four PGE receptors, the EP3 receptor was proposed to participate in arachidonic acid (AA)–induced edema formation (10). These results suggest that PGE_2 may function as a link to an increase in vascular permeability and edema formation (11). However, it remains unsolved as to how PGE_2 increases permeability and edema formation in acute inflammation.

Mast cells (MCs) are immune cells widely distributed in various peripheral tissues and are activated in an Ag-dependent manner (12, 13). Once activated by Ag-induced cross-linking of IgE receptors, MCs release bioactive substances in their granules such as histamine

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Abbreviations used in this article: AA, arachidonic acid; 2-APB, 2-aminoethoxydiphenyl borate; BMMC, bone marrow–derived mast cell; COX, cyclooxygenase; MC, mast cell; MPO, myeloperoxidase; PLC, phospholipase C; PTX, pertussis toxin; WT, wild-type.

and proteases, leading to acute allergic inflammation typically seen in urticaria. To date, many researchers have investigated the roles of PGE receptor signaling in the Ag-dependent activation of MCs and obtained various results because MCs show tissue-specific phenotypes. For example, PGE_2 enhances Ag-induced degranulation in mouse bone marrow–derived MCs (BMMCs) (14) and human peripheral blood–derived MCs (15), whereas it attenuates Ag-induced degranulation in MCs isolated from rat peritoneal cells (16) and human lung (17). A recent study found that MCs can be activated in an Ag-independent fashion; that is, substance P released from blood vessels or peripheral terminals of sensory neurons stimulates MC degranulation (18). Indeed, it was recently reported that MCs play a role in Ag-independent inflammation such as physical or chemical irritation (19, 20). In these models, haptens have been shown to activate TLR signaling in skin cells (21). Because TLR signal activation induces COX gene expression and PG production in many types of cells (22), PGE_2-induced MC activation is a likely mechanism underlying irritant contact dermatitis. Nevertheless, it remains unknown as to whether PGE receptor signaling is involved in such Ag-independent MC activation.

In this study, we conducted a series of experiments to clarify how PGE receptor signaling increases vascular permeability and edema formation. We show that PGE_2 by itself triggers degranulation of dermal MCs via the EP3–$G_{i/o}$–Ca^{2+} influx signaling pathway, and this functions as the underlying mechanism of PGE_2-induced vascular hyperpermeability and subsequent edema formation.

Materials and Methods

Mice and materials

Specific pathogen-free 8-wk-old C57BL/6 mice and WBB6F_1-*W/W^v* (*W/W^v*) (23) and littermate control WBB6$F_1$$^{+/+}$ mice were obtained from Japan SLC (Hamamatsu, Japan). *Kit$^{W\text{-}sh/W\text{-}sh}$* mice (24) with a genetic background of C57BL/6 were obtained from The Jackson Laboratory (Bar Harbor, ME). *Ptger1$^{-/-}$*, *Ptger2$^{-/-}$*, *Ptger3$^{-/-}$*, and *Hdc$^{-/-}$* mice with a C57BL/6 background were previously generated as described (25–27), and C57BL/6 mice were used as wild-type (WT) controls. We used surviving *Ptger4$^{-/-}$* offspring and their WT littermates with a mixed background of 129Ola and C57BL/6 from heterozygote crosses (28–31). All mice were housed in specific pathogen-free animal facilities and mice at 8–12 wk of age (20 ± 2 g in weight) were used for each experiment. All animal experiments were performed according to the Guidelines for Animal Experiments of Kyoto University, Kumamoto University, and the Tokyo Metropolitan Institute of Medical Science. The following materials were purchased from the sources indicated: AA and La^{3+} from Nacalai Tesque (Kyoto, Japan); recombinant mouse IL-3 from R&D Systems (Minneapolis, MN); PGE_2 and butaprost (an EP2 agonist) from Cayman Chemical (Ann Arbor, MI); *p*-nitrophenyl-β-D-2-acetoamide-2-deoxy-glucopyranoside, LY294002, indomethacin, diphenhydramine, Hoe140, an anti-DNP mouse monoclonal IgE (SPE-7), and DNP-conjugated human serum albumin from Sigma-Aldrich (St. Louis, MO); fura 2-AM from Dojindo (Kumamoto, Japan); pertussis toxin (PTX) from Seikagaku (Tokyo, Japan); U73122, A23187, thapsigargin, and 2-aminoethoxydiphenyl borate (2-APB) from Calbiochem (Merck, Darmstadt, Germany); an anti–phospho-Akt (Ser^{473}) rabbit mAb (193H12) and an anti-Akt rabbit mAb (11E7) from Cell Signaling Technology (Danvers, MA); an anti-actin mouse mAb (C4) from Santa Cruz Biotechnology (Santa Cruz, CA); and SKF-96365 from Tocris Bioscience (Bristol, U.K.). ONO-DI-004 (an EP1 agonist), ONO-AE-248 (an EP3 agonist), and ONO-AE1-437 (an EP4 agonist) were gifts from ONO Pharmaceuticals (Osaka, Japan). The basic profiles of selectivity of each EP-specific agonist were reported previously (32). ONO-AE-208 (an EP4 antagonist) was also provided from ONO Pharmaceuticals. All other chemicals were commercial products of reagent grade.

AA- or PGE_2-induced inflammation

Mice were anesthetized with ether and injected i.v. with 1 mg Evans blue dye in 200 μl saline. Immediately after the dye injection, AA (0.6 mg in 20 μl acetone) was applied to the inner and outer surface of the right ear, whereas the left ear received the vehicle delivered in the same manner. Alternatively, the ears of the mice were intradermally injected with PGE_2 or each EP receptor agonist in 25 μl saline, and 0.57% ethanol in saline was used as the vehicle. Thirty minutes after AA application or the intradermal injection of PGE_2, mice were sacrificed and their ears were collected. The ear biopsies were incubated at 37°C in 1 ml of 3 N KOH. On the following day the mixtures were extracted with 1.24 M phosphoric acid and acetone. Absorbance of the resulting supernatants was measured at 620 nm, and the quantities of leaked dye were calculated by comparison with tissue samples injected with certain amounts of dye. We measured ear thickness with a micrometer for each mouse before and at the indicated times after AA application or PGE_2 injection and expressed the difference as edema formation.

Measurement of myeloperoxidase activity and histamine

The activity of a neutrophil marker enzyme, myeloperoxidase (MPO), was measured according to the procedure reported by Bradley et al. (33) with minor modifications. Briefly, 6 h after the administration of AA to mice, their ears were removed and homogenized with a Polytron homogenizer in 50 mM potassium phosphate buffer (pH 6.0). Centrifuged tissue pellets were re-extracted in potassium phosphate buffer with 0.5% hexadecanoyltrimethyl ammonium bromide, freeze-thawed three times, and centrifuged to collect the supernatants that were then used for measurement of MPO activity. Sixty microliters of supernatant was added to 60 μl assay buffer containing 0.33 mg/ml *o*-dianisidine and 0.001% hydrogen peroxide, and the change of absorbance at 460 nm was measured. One unit of MPO activity was defined as that degrading one micromole of peroxide per minute at 25°C. Ear tissue lysates were centrifuged at 10,000 × *g* for 30 min at 4°C, and the resultant supernatants were subjected to the histamine assay. Specifically, histamine was separated on the cation exchange column WCX-1 (Shimadzu, Kyoto, Japan) by HPLC and detected by the *o*-phthalaldehyde method (34). For the measurement of histamine release from peritoneal MCs, 5 ml PBS was injected into the peritoneal cavity and, after a slight massage, the PBS was collected. Such peritoneal lavage fluid usually contains Kit^+/FcεRIα$^+$ MCs (2–3% of total cells). Peritoneal cells were stimulated with PGE_2 or compound 48/80 for 30 min. After centrifugation, histamine in the supernatants and cellular fractions was measured by the histamine assay.

Counting of tissue mast cells

Ear sections (7 μm) were fixed with formalin, followed by sequential staining with Alcian blue and safranin, and then by staining with acidic toluidine blue (pH 0.5). The number of metachromatic cells was counted in five sections per mouse, and the results from five to six mice were averaged. For examination of degranulation status, tissue MCs were counted as either resting MC or MC undergoing degranulation, depending on whether its toluidine blue–positive granule contents were clustered within a cell or spread out throughout the cell, respectively.

Preparation of BMMCs and reconstitution into MC-deficient mice

Preparation of BMMCs was performed as described previously (35). In brief, bone marrow cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 10 ng/ml mouse IL-3, 50 μM 2-ME, and 0.1 mM nonessential amino acids at 37°C in a fully humidified 5% CO_2 atmosphere. After 4–5 wk of culture, >95% of the viable cells were confirmed to be immature MCs, as assessed by staining of cytospins with acidic toluidine blue (pH 2.5), which results in MC-specific metachromatic staining. For MC reconstitution, WT or *Ptger3$^{-/-}$* BMMCs (1× 10^6 cells/ear) were injected into the ear cutaneous tissues of the *Kit$^{W\text{-}sh/W\text{-}sh}$* mice. After 5 wk, the MC-reconstituted *Kit$^{W\text{-}sh/W\text{-}sh}$* mice were subjected to the PGE_2-induced inflammation assay. Reconstitution and maturation of transferred MCs were confirmed by monitoring the gene expression of *Ptger3* and MC-specific genes (*Fcer1a, Hdc, Mcp4*, and *Cpa3*) using RT-PCR. Flow cytometric analysis was performed as previously described with a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) equipped with CellQuest software (36). The dead cell population was gated out by propidium iodide staining.

Degranulation and IL-6 production

BMMCs were collected and washed in Tyrode's buffer (10 mM HEPES-NaOH [pH 7.3] containing 130 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μM indomethacin, and 0.1% BSA). The cells were incubated for 30 min in Tyrode's buffer with or without PGE_2. Degranulation of MCs was evaluated by measuring the activity of a granule enzyme, β-hexosaminidase, using *p*-nitrophenyl-β-D-2-acetoamide-2-deoxyglucopyranoside as a substrate. For the measurement of IL-6 production, BMMCs were collected and washed in fresh culture medium.

The cells were then incubated for 3 h in culture medium with PGE_2 (1 μM). The IL-6 in the medium was measured by ELISA (BD Biosciences).

Cytosolic Ca^{2+} and PI3K assay

Cells were loaded with 2 μM fura 2-AM in Tyrode's buffer for 45 min at room temperature and then washed in Tyrode's buffer. For Ca^{2+}-free conditions, the buffer was replaced with Ca^{2+}-free Tyrode's buffer. Fluorescent intensities were measured at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm with a fluorescence spectrometer (CAF-100; Jasco, Tokyo, Japan) as described previously (36). BMMCs were stimulated with PGE_2 or Ag for 10 min in Tyrode's buffer without BSA. For cell lysis, the cell suspension was added directly to an equal volume of 2× Laemmli sample buffer. The proteins were separated on 10% polyacrylamide gels and transferred onto polyvinyl difluoride membranes. Membranes were probed with an anti–phospho-Akt or anti–total Akt Ab. To normalize protein loading, the membranes were stripped and probed for actin.

Statistical analysis

Data are represented as means ± SEM of at least three independent experiments. Two sets of data were compared by Student *t* test. Multiple treatment groups were compared by ANOVA. When significant differences were found, additional comparisons were made with the Dunnett multiple comparison test for comparison with control groups or with the Tukey–Kramer multiple comparison test for comparison of all pairs of columns. A p value < 0.05 was considered statistically significant.

Results

Role of PGE_2–EP3 signaling in AA-induced and PGE_2-induced hyperpermeability

It was previously reported that lipid mediators synthesized by COX pathways are involved in AA-induced inflammation, and that PGE_2 was abundantly synthesized within ear tissues upon AA application (37). Indeed, also in our experimental settings, aspirin significantly suppressed AA-induced hyperpermeability (Fig. 1A). We first identified PGE receptors involved in AA-induced hyperpermeability and edema formation. Mice deficient in each of the four PGE receptors were subjected to the AA-induced and PGE_2-induced inflammation, and vascular permeability was monitored (Fig. 1B). In WT mice, vascular permeability was drastically increased 30 min after topical application of AA. In EP1-, EP2-, and EP4-deficient mice, permeability increased similarly to that in WT mice (Fig. 1B). Moreover, the pretreatment with an EP4 antagonist (10 mg/kg/d orally for 24 h) failed to affect AA-induced responses (Fig. 1C), although this condition was previously shown to block EP4 signaling systemically (29, 31). In contrast, EP3-deficient mice showed significantly attenuated responses (Fig. 1B). When we investigated edema formation, topical application of AA induced an increase in ear thickness in WT mice but failed to do so in EP3-deficient mice (Fig. 1D). These results suggest that PGE_2–EP3 is involved in the initiation of acute inflammation. We then examined neutrophil infiltration by measuring MPO activity. Indeed, in WT mice, MPO activity was augmented 6 h after the application of AA. However, EP3-deficient mice showed only a slight induction of MPO activity (Fig. 1E). Application of AA has been shown to induce the production of a broad range of eicosanoids such as PGE_2 (37). We therefore examined the direct effect of exogenous PGE_2 on vascular permeability. Mice were injected i.v. with Evans blue dye, and immediately thereafter PGE_2 was injected intradermally into the ear. Thirty minutes later, amounts of dye leaked into the ear were measured. PGE_2 exerted transient dye leakage in WT mice, which peaked at 30 min (Fig. 1F). Furthermore, PGE_2 increased vascular permeability in a dose-dependent manner (Fig. 1G). These results suggest that PGE_2 has the potential to induce hyperpermeability. Interestingly, an EP3-specific agonist also induced vascular permeability with a dose-response similar to PGE_2 (Fig. 1H). Because both PGE_2 and an EP3 agonist at a dose of 1.4 nmol were enough to stimulate dye leakage, we compared the effects of the agonists specific for each of the four EPs at this dose. Only an EP3 agonist mimicked the effect of PGE_2 (Fig. 1I), suggesting that the induction of vascular permeability by PGE_2 is mediated by the EP3 receptor. Indeed, the effect of PGE_2 was abolished in *Ptger3*$^{-/-}$ mice (Fig. 1J). These results indicate that the PGE_2–EP3 signal induces hyperpermeability.

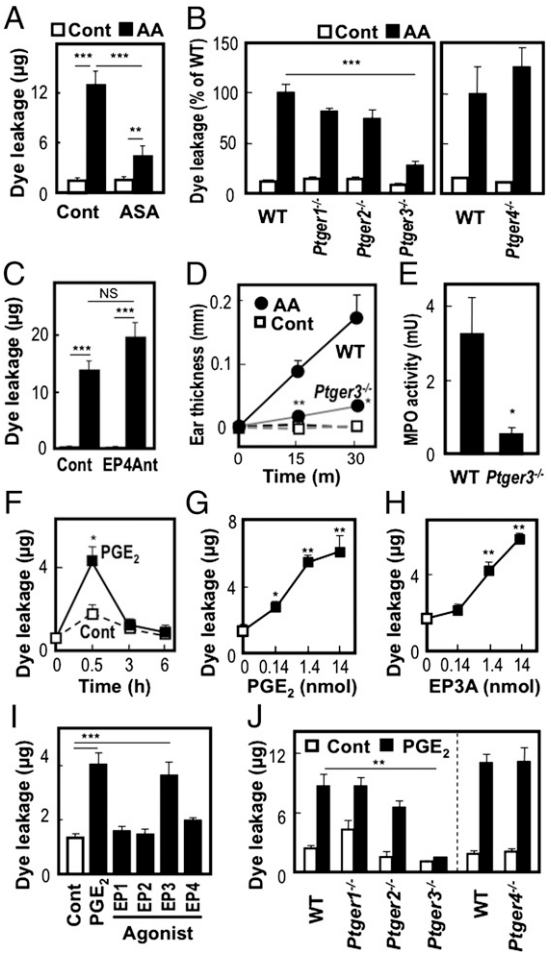

FIGURE 1. PGE_2–EP3 signaling plays a role in AA- or PGE_2-induced hyperpermeability. (**A**) AA-induced (0.6 mg) hyperpermeability was attenuated by aspirin (ASA, 100 mg/kg i.p., 30 min before stimulation). (**B**) The AA-induced response was examined in WT and mice deficient in each EP. Vascular permeability was measured by Evans blue dye extravasation 30 min after AA stimulation. Leaked dye was measured based on extract absorbance at 620 nm. The responses in mutant mice were expressed as percentage of the amount of leaked dye in WT mice upon AA treatment. (**C**) The EP4 antagonist ONO-AE-208 failed to alter the AA-induced response. An EP4-specific antagonist (10 mg/kg/d) was administered in the drinking water for 24 h, and then AA was applied into the ears (0.6 mg). (**D**) AA-induced edema formation was monitored in *Ptger3*$^{-/-}$ mice compared with WT mice. (**E**) Accumulation of neutrophils was determined in WT and *Ptger3*$^{-/-}$ mice by measuring MPO activity in ear extracts. (**F**) Topical injection of PGE_2 (1.4 nmol) transiently increased vascular permeability in WT mice. (**G** and **H**) Dose responses of PGE_2 (G) and an EP3 agonist (H) on vascular permeability. WT mice were intradermally injected with vehicle or various doses of PGE_2 or EP3 agonist, and 30 min later the dye leakage was measured. (**I** and **J**) The effect of PGE_2 is mediated by the EP3 receptor. WT mice were intradermally injected with vehicle, PGE_2, or an agonist specific for each EP (1.4 nmol each), and only an EP3-agonist mimicked the effect of PGE_2 (I). The PGE_2-induced response was hardly detected in *Ptger3*$^{-/-}$ mice (J). Values are represented as means ± SEM (n = 4–10). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

PGE_2-induced vascular hyperpermeability depends on EP3 receptors on MCs

To uncover the mechanisms involved in PGE_2-elicited hyperpermeability, we tested the effect of various chemicals known to affect inflammation. Although PGE_2 is known to sensitize bradykinin-induced responses, a bradykinin B_2 receptor antagonist failed to alter the PGE_2-induced response (Supplemental Fig. 1A), indicating that there is little contribution of bradykinin B_2 signaling in PGE_2-elicited hyperpermeability. Alternatively, we found that a histamine H_1 blocker, diphenhydramine, inhibited the action of PGE_2 in WT mice (Supplemental Fig. 1B), suggesting that histamine mediates PGE_2-induced hyperpermeability.

MC-derived inflammatory mediators such as histamine and proteases have been shown to increase vascular permeability. Indeed, MCs were reported to be essential for Ag-independent inflammation, such as AA-induced inflammation and irritant contact dermatitis (19, 37). We therefore examined the effects of genetic depletion of MCs (*W/W^v*) on AA-induced hyperpermeability. As expected, AA-induced responses were significantly attenuated in *W/W^v* mice, although the proinflammatory actions of AA still remained (Fig. 2A). Indeed, when we examined the MC granule statuses in AA-added ear tissues, AA application remarkably induced the MC degranulation (Fig. 2B). These results suggest that AA-induced responses are at least partly due to MC degranulation. Interestingly, aspirin significantly attenuated AA-induced MC degranulation (Fig. 2B), suggesting that some COX products may elicit MC degranulation. Indeed, when we investigated the PGE_2-induced response in *W/W^v* mice, PGE_2 completely failed to stimulate vascular permeability (Fig. 2C). These results indicate that PGE_2–EP3-mediated vascular hyperpermeability depends on MCs. Indeed, we found that the EP3 receptor is abundantly expressed in BMMCs, as reported previously (38). To confirm that the action of PGE_2 is mediated by EP3 expressed in MCs, we employed *Kit$^{W\text{-}sh/W\text{-}sh}$* mice, another strain of MC-deficient mice, and reconstituted them with WT BMMCs or BMMCs deficient in the EP3 receptor (*Ptger3$^{-/-}$*). After 5 wk, reconstitution and maturation of the transferred MCs in ear tissues were confirmed by monitoring gene expression of MC-specific genes (Supplemental Fig. 2), and these mice were subjected to the PGE_2-induced inflammation assay (Fig. 2D, 2E). As observed in *W/W^v* mice, PGE_2-induced permeability changes were abolished in *Kit$^{W\text{-}sh/W\text{-}sh}$* mutants but were significantly recovered by reconstitution with WT BMMCs, but such a recovery was not seen in mice reconstituted with *Ptger3$^{-/-}$* BMMCs (Fig. 2D). Moreover, similar results were obtained when we measured ear thickness as an index of edema formation (Fig. 2E). If PGE_2 increases vascular permeability by acting on the EP3 receptor on MCs, the inability of PGE_2 to induce hyperpermeability in *Ptger3$^{-/-}$* mice might be due to impaired migration, differentiation, and/or maturation of *Ptger3$^{-/-}$* MCs. When we compared the number and maturation indexes of MCs between WT and *Ptger3$^{-/-}$* mice, we failed to detect any difference in the number and histological appearance of MCs, as well as in protease activity and histamine content (Fig. 3). We thus concluded that PGE_2–EP3 signaling directly triggers MC activation.

PGE_2 induces histamine release from MCs by acting on the EP3 receptor

Histamine is one of the bioactive mediators released from MCs and is responsible for the extravasation reaction (25). To investigate whether PGE_2-induced vascular hyperpermeability is mediated by

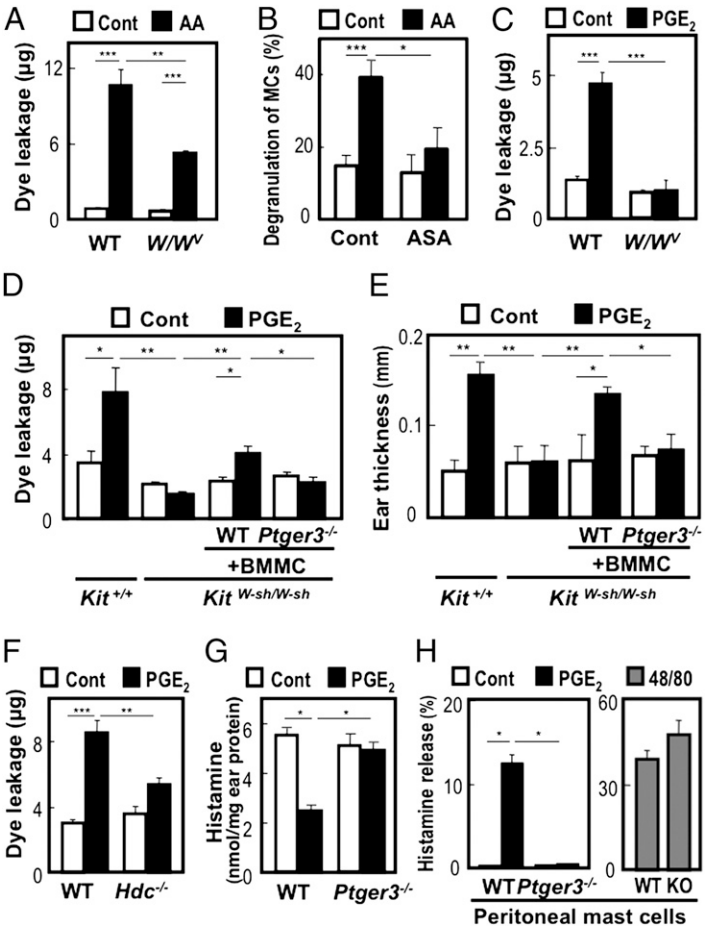

FIGURE 2. PGE_2-induced hyperpermeability depends on EP3 receptors on the MCs, as well as histamine. (**A**) AA (0.6 mg) was topically applied to the ears of WT and *W/W^v* mice, and 30 min later vascular permeability was measured. (**B**) Effects of aspirin (ASA, 100 mg/kg i.p. 30 min before AA application) on AA-induced mast cell degranulation. MC degranulation 30 min after AA stimulation was assessed by examining MCs on the ear sections stained with toluidine blue. (**C**) PGE_2 was intradermally injected into the ear of WT and *W/W^v* mice, and vascular permeability was measured 30 min later. (**D** and **E**) PGE_2-induced dye extravasation (D) and edema formation (E) were abolished in *Kit$^{W\text{-}sh/W\text{-}sh}$* mice, and were recovered in mice reconstituted with WT but not *Ptger3$^{-/-}$* BMMCs. (**F**) PGE_2 was intradermally injected into the ears of WT and HDC-deficient (*Hdc$^{-/-}$*) mice, and vascular permeability was measured 30 min later. (**G**) PGE_2 was intradermally injected into the ears of WT and *Ptger3$^{-/-}$* mice, and 30 min later histamine content in tissue was measured. (**H**) PGE_2 induces histamine release from peritoneal resident MCs of WT but not *Ptger3$^{-/-}$* mice. Resident cells were recovered from the peritoneal cavity of WT and *Ptger3$^{-/-}$* (KO) mice and then stimulated by PGE_2 (*left*) or compound 48/80 (*right*), and histamine levels in the supernatants and cell pellets were measured. Values are represented as means ± SEM (n = 3–10). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

MC-derived histamine, we examined the effects of PGE_2 on mice deficient in histidine decarboxylase ($Hdc^{-/-}$), an enzyme responsible for histamine biosynthesis in vivo. In $Hdc^{-/-}$ mice, PGE_2-induced vascular hyperpermeability was almost abolished (Fig. 2F). Once histamine is released into the extracellular space, it is immediately converted into an inactive form by the action of diamine oxidase (39) and is rapidly cleared from tissue. Indeed, the histamine content in pinna tissue was significantly decreased to 50% upon PGE_2 injection in WT mice (Fig. 2G), suggesting that PGE_2 initiates histamine release from MCs. In contrast, histamine content in $Ptger3^{-/-}$ mice was constant even after PGE_2 application. These results suggest that PGE_2-induced hyperpermeability depends on the EP3 receptor, MCs, and histamine. These results also suggest that PGE_2 by itself triggers MC degranulation by acting on the EP3 receptor. To confirm this, we prepared peritoneal cells from WT mice, which include MCs (~2–3%), and examined whether PGE_2 induces histamine release from peritoneal MCs. As expected, PGE_2 alone induced histamine release from peritoneal MCs (Fig. 2H). No apparent histamine release was detected in $Ptger3^{-/-}$ cells. There were no apparent differences in histamine contents (Fig. 3D), percentage of MCs in total peritoneal cells (WT, 2.43 ± 0.38% versus $Ptger3^{-/-}$, 2.44 ± 0.52%), and compound 48/80–induced histamine release between WT and $Ptger3^{-/-}$ peritoneal cells (Fig. 2H). These results demonstrate that PGE_2–EP3 signaling triggers histamine release from MCs.

PGE_2 triggers not only degranulation but also IL-6 production in BMMCs

To investigate the mechanism of PGE_2-induced MC activation, we used mouse BMMCs in which EP3 is abundantly expressed (38). We first compared the basic characteristics of WT and $Ptger3^{-/-}$ BMMCs but failed to detect any difference in toluidine blue staining, expression level of FcεRI or c-Kit, histamine content, or degranulation upon Ag or secretagogue stimulation (Supplemental Fig. 3 and data not shown), suggesting that $Ptger3^{-/-}$ BMMCs differentiate normally. We next examined whether PGE_2 alone triggers degranulation of WT BMMCs. PGE_2 rapidly induced degranulation in WT BMMCs, which was saturated in 3 min (Fig. 4A). PGE_2 induced degranulation in a concentration-dependent fashion. In contrast, in $Ptger3^{-/-}$ BMMCs, such a PGE_2-induced response was hardly detected although crosslinking of FcεRI induced degranulation in a manner similar to WT cells (Fig. 4B). Because MC activation includes the release of a wide variety of cytokines (12, 13), we next examined the effect of PGE_2 on IL-6 release from BMMCs. In WT cells, PGE_2 induced IL-6 production (Fig. 4C). In contrast, PGE_2 failed to induce IL-6 release in $Ptger3^{-/-}$ BMMCs, although crosslinking of FcεRI stimulated IL-6 release to levels similar to WT cells (Fig. 4D). These results indicate that PGE_2 triggers not only degranulation but also cytokine release via the EP3 receptor in BMMCs. Indeed, an EP3 agonist mimicked the effects of PGE_2 on degranulation and IL-6 release (Fig. 4E, 4F). Because the EP3 receptor was shown to be coupled to PTX-sensitive G proteins, mainly G_i (40), we investigated whether PTX affects PGE_2-induced responses in WT BMMCs. PTX inhibited PGE_2-induced degranulation and IL-6 production (Fig. 4G, 4H) but failed to suppress Ag/IgE-induced responses. These results indicate that PGE_2–EP3 signaling induces MC activation in a PTX-sensitive manner.

PGE_2 induces extracellular Ca^{2+} influx and PI3K activation via the EP3/G_i pathway

It has been shown that various stimuli induce MC degranulation through intracellular Ca^{2+} mobilization (41–43). To investigate whether a similar signaling pathway is stimulated by EP3 activation, we examined the effects of PGE_2 on intracellular Ca^{2+} concentration in BMMCs. PGE_2 stimulated intracellular Ca^{2+} mobilization in WT BMMCs in a dose-dependent manner. In contrast, such a PGE_2-induced Ca^{2+} response was not observed in $Ptger3^{-/-}$ BMMCs, although thapsigargin-induced Ca^{2+} responses were observed at levels similar to those in WT cells (Fig. 5A, 5B). Among the agonists specific for each EP, only an EP3 agonist elicited significant Ca^{2+} mobilization in WT BMMCs (Fig. 5C). Pretreatment with PTX abolished PGE_2-induced but not Ag/IgE-induced Ca^{2+} mobilization in WT BMMCs (Fig. 5D), indicating that PGE_2–EP3 signaling induces Ca^{2+} mobilization via the PTX-sensitive G protein, $G_{i/o}$. In MCs, PTX-sensitive G proteins such as G_{i1} and G_{i2} are

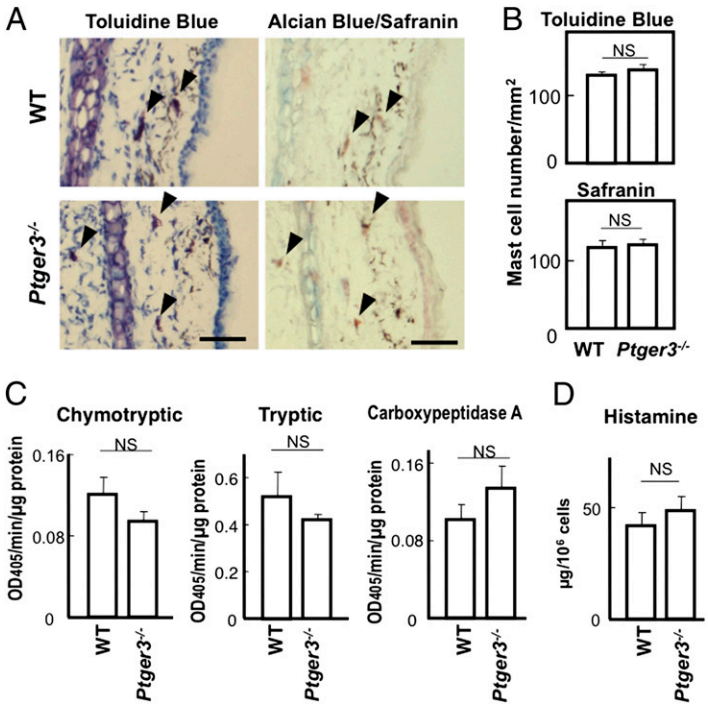

FIGURE 3. MCs in $Ptger3^{-/-}$ mice are normal in morphology (**A**), cell number (**B**), MC protease activity (**C**), and histamine content (**D**). (A and B) MCs in the ear dermis of WT and $Ptger3^{-/-}$ mice were stained with toluidine blue (general MCs) or Alcian blue-safranin (mature MCs), and the number of MCs is shown as counts per square millimeter. Scale bars, 100 μm. (C and D) MC protease activity (C) and histamine content (D) were measured in the peritoneal cells of WT and $Ptger3^{-/-}$ mice. Values are represented as means ± SEM (n = 5).

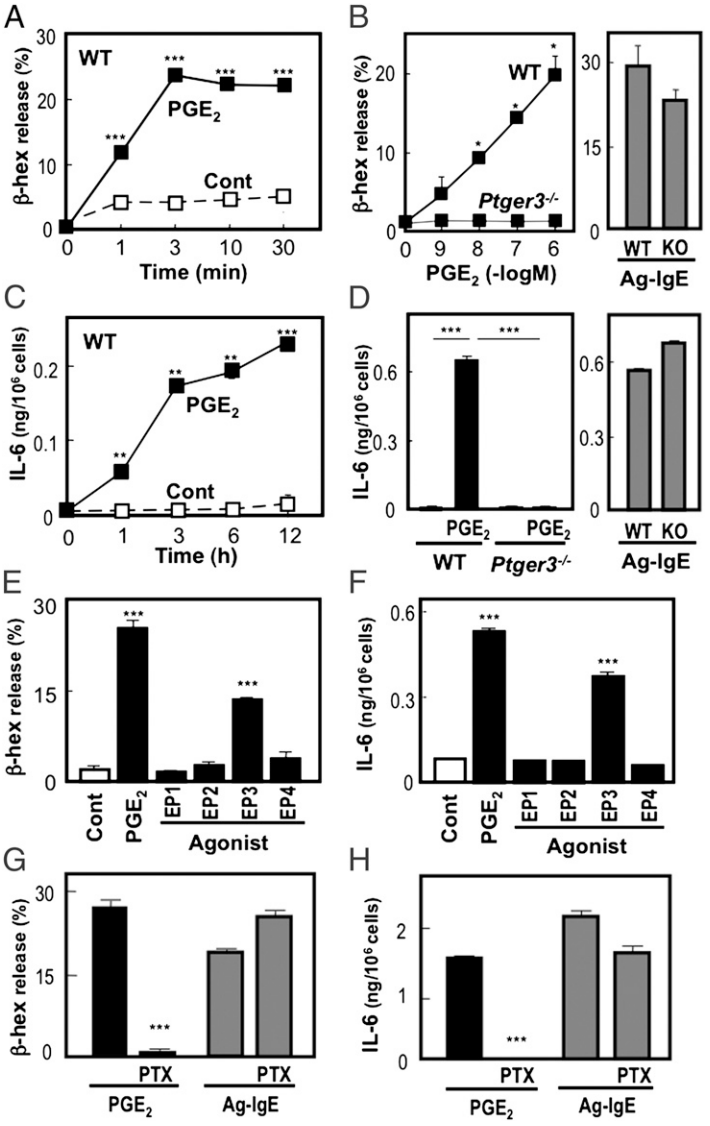

FIGURE 4. PGE_2–EP3 signaling triggers degranulation and IL-6 production in BMMCs in a PTX-sensitive manner. (**A**) WT BMMCs were stimulated with vehicle and PGE_2 (1 μM), and β-hexosaminidase (β-hex) released from the cells was measured at the indicated time points after PGE_2 stimulation. (**B**) *Left*, WT or *Ptger3*$^{-/-}$ BMMCs were stimulated with vehicle or the indicated concentrations of PGE_2, and degranulation was measured 30 min after stimulation. *Right*, No significant difference was detected in the Ag-IgE–induced degranulation between WT and *Ptger3*$^{-/-}$ (KO) BMMCs. (**C**) WT BMMCs were stimulated with vehicle and PGE_2 (1 μM), and IL-6 levels in the medium were measured at the indicated time points. (**D**) *Left*, WT or *Ptger3*$^{-/-}$ BMMCs were stimulated with PGE_2, and IL-6 levels were measured 3 h after stimulation. *Right*, Ag-IgE–induced IL-6 responses were observed in WT and *Ptger3*$^{-/-}$ (KO) cells. (**E** and **F**) WT BMMCs were stimulated with PGE_2 or agonists specific for each EP, and degranulation (E) and IL-6 levels (F) were measured. (**G** and **H**) Pretreatment of cells with PTX (0.3 μg/ml) abolished PGE_2-induced degranulation (G) and IL-6 release (H) but not Ag-IgE–elicited responses (Ag-IgE). WT BMMCs were pretreated with or without PTX for 6 h and subjected to PGE_2 or Ag-IgE stimulation. Values are represented as means ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

thought to induce phospholipase C (PLC) activation and Ca^{2+} release from the endoplasmic reticulum store. Indeed, when we treated WT BMMCs with the PLC inhibitor U73122, the PGE_2-induced Ca^{2+} response was abolished (Fig. 5E). Endoplasmic reticulum Ca^{2+} store depletion–induced Ca^{2+} influx via the Stim/Orai pathway has been shown to be important for MC activation (44). We hence examined the effects of inhibitors of Orai channels, La^{3+}, 2-APB, and SKF-96365, on PGE_2-induced Ca^{2+} responses. These inhibitors significantly attenuated PGE_2-induced Ca^{2+} responses (Fig. 5E). We further investigated whether Ca^{2+} entry from the extracellular space is actually required for PGE_2-induced MC activation. Both PGE_2-induced degranulation and IL-6 production were abolished by the depletion of extracellular Ca^{2+} (Fig. 5F, 5G), indicating that PGE_2–EP3 signaling induces MC activation via Ca^{2+} influx from an extracellular source. Recent studies demonstrated that MC degranulation processes are divided into two events: the Ca^{2+}-independent microtubule-dependent translocation of granules to the plasma membrane, and Ca^{2+}-dependent membrane fusion and exocytosis (45). The former event is mediated by PI3K activation and production of phosphatidylinositol-3,4,5-trisphosphate leading to phosphorylation of the Ser^{473} residue within the C-terminal region of Akt (46, 47). We therefore examined PI3K activation by detecting Akt phosphorylated at Ser^{473} upon PGE_2 stimulation of WT and *Ptger3*$^{-/-}$ BMMCs. As reported previously, FcεRI aggregation induced Akt phosphorylation within 10 min in WT and *Ptger3*$^{-/-}$ cells (Fig. 6A). Interestingly, PGE_2 also rapidly stimulated Akt phosphorylation in WT but not *Ptger3*$^{-/-}$ cells. Pretreatment of the WT cells with PTX abolished PGE_2-induced but not IgE-mediated Akt phosphorylation. These results suggest that PGE_2 induces PI3K activation via an EP3-$G_{i/o}$–dependent pathway. Because the depletion of extracellular Ca^{2+} failed to affect IgE-mediated and PGE_2-induced PI3K activation, PGE_2-induced PI3K activation appears to be Ca^{2+}-independent, as reported previously (47). To examine whether PI3K activation is involved in PGE_2-induced MC activation, we finally examined the effect of a PI3K inhibitor, LY294002. Both degranulation and IL-6 release were inhibited by LY294002 (Fig. 6B, 6C). These results indicate that Ca^{2+} influx along with PI3K activation is required for PGE_2–EP3-induced MC activation.

Discussion

Edema formation is a prominent feature of the inflammatory response and is due to the movement of fluid and plasma proteins into the extracellular space from leaky blood vessels. It is thought that an increased vascular permeability is induced by a range of injurious stimuli and by several chemical mediators generated during an inflammatory response (1). Almost 40 y ago, Williams and Morley (6) proposed the "two mediator hypothesis," which states that the magnitude of inflammatory swelling is dependent on two

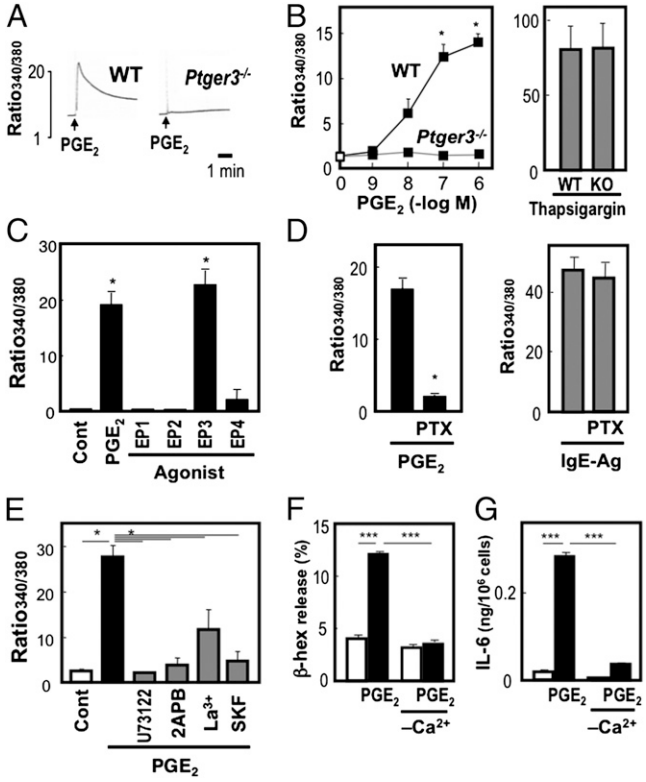

FIGURE 5. PGE_2–EP3 signaling induces MC activation through an influx of extracellular Ca^{2+}. (**A** and **B**) WT or *Ptger3*$^{-/-}$ BMMCs preloaded with fura 2-AM were stimulated with PGE_2, and the fluorescence ratio was monitored. A typical response to PGE_2 (1 μM) in WT or *Ptger3*$^{-/-}$ cells is shown in (A). PGE_2 induced intracellular Ca^{2+} mobilization in a dose-dependent manner in WT but not in *Ptger3*$^{-/-}$ (KO) cells (B, *left*), although both cells showed similar responses upon thapsigargin stimulation (B, *right*). (**C**) Among the EP-specific agonists, only an EP3 agonist mimicked the effect of PGE_2 on intracellular Ca^{2+} concentration. (**D**) PTX (0.3 μg/ml) abolished PGE_2-induced Ca^{2+} mobilization but not the Ag-IgE–induced response. (**E**) PGE_2-induced Ca^{2+} mobilization is dependent on PLC and store-operated Ca^{2+} channels. WT BMMCs were pretreated with U73122 (an inhibitor of PLC), 2-APB, La^{3+}, and SKF-96365 (inhibitors for store-operated Ca^{2+} channels) and then stimulated with PGE_2 (1 μM). (**F** and **G**) PGE_2-induced degranulation [(D) 30 min after stimulation] and IL-6 production [(E) 3 h after stimulation] were abolished by the depletion of extracellular Ca^{2+} ($–Ca^{2+}$). Values are represented as means ± SEM (n = 3–4). $*p < 0.05$, $***p < 0.001$.

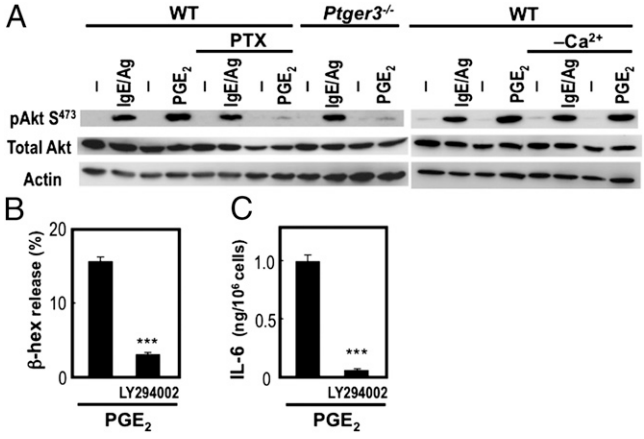

FIGURE 6. PGE_2–EP3 signaling induces MC activation via Akt Ser^{473} phosphorylation. (**A**) PI3K activation was examined by monitoring Akt Ser^{473} phosphorylation in WT and *Ptger3*$^{-/-}$ BMMCs stimulated with PGE_2 or Ag-IgE. Representative immunoblot images of pAkt Ser^{473}, total Akt, and β-actin are shown. Both PGE_2 and Ag-IgE stimulation induced PI3K activation, which is insensitive to the depletion of extracellular Ca^{2+} ($–Ca^{2+}$). Only the PGE_2-induced response was abolished by PTX treatment (0.3 μg/ml) and EP3 deficiency (*Ptger3*$^{-/-}$). —, Vehicle-treated as a control for PGE_2 or Ag-IgE stimulation. (**B** and **C**) PGE_2-induced degranulation (B, 30 min after stimulation) and IL-6 production (C, 3 h after stimulation) were abolished by pretreatment with LY294002 (an inhibitor of PI3K, 50 μM). Values are represented as means ± SEM (n = 3). $***p < 0.001$.

factors: the degree of vasodilatation, which is induced by PGs including PGI_2 and PGE_2, and the extent of endothelial cell permeability, which is induced by a variety of permeabilizing substances, including bradykinin and histamine. Furthermore, according to this hypothesis, when both factors are present they synergize to produce a greater net swelling response. Although this hypothesis has been frequently cited and is currently embraced (1, 4–7), the link between the initial vasodilatation and the subsequent permeability change remains unclear. Recently, it was shown that EP2 and EP4 receptors are involved in edema formation mainly by eliciting vasodilatation and the subsequent increase in local blood flow in UV-induced skin inflammation (48). Therefore, PGE_2 may elicit acute inflammation via two different actions: by stimulating vasodilatation via the EP2 and EP4 receptors on smooth muscle cells, and by stimulating vascular permeability via the EP3 receptor on MCs. Furthermore, in the present study we also show that PGE_2–EP3 signaling triggers IL-6 release from MCs. IL-6 has been shown to stimulate the local production of neutrophil-attracting chemokines such as IL-8 and GLO-α (49) and to induce the endothelial expression of adhesion molecules such as VCAM-1 and E-selectin (50), both of which stimulate leukocyte rolling. Indeed, MPO activity, a hallmark of neutrophil recruitment, was significantly reduced in EP3-deficient mice with AA-induced inflammation (Fig. 1C). These results indicate that EP3-mediated MC activation plays a critical role not only in permeability change but also in neutrophil infiltration. Thus, EP3 signaling in MCs appears to function as an underlying mechanism of PGE_2-induced vasodilatation and subsequent edema formation.

Potential involvement of PGE_2-induced MC activation in various kinds of inflammatory diseases is significant from a clinical perspective. In particular, Ag-independent acute inflammatory reactions such as physical or chemical irritation may be attributed to this PGE_2 action. Indeed, MCs were recently found to play critical roles in Ag-independent irritant contact dermatitis (19, 20), and haptens have been shown to activate TLR signaling in skin cells (21). Because TLR signal activation induces COX gene expression and PG production in many types of cells (22), PGE_2-induced MC activation is a likely mechanism underlying irritant contact dermatitis. Therefore, we propose that EP3-mediated MC activation is involved not only in the previously reported PG-related acute inflammation responses but also in Ag-independent innate immune reactions. Moreover, in addition to degranulation and cytokine release, PGE_2 has been shown to stimulate chemotaxis (or chemokinesis) and adhesion activities of MCs via the EP3 receptor (39, 51, 52). If this is the case, PGE_2–EP3 signaling could function as a sensor of MCs against microenvironmental changes.

In summary, the present study demonstrates that PGE_2 directly triggers MC degranulation and cytokine release via the EP3/G_i pathway, resulting in enhancement of vascular permeability and leukocyte recruitment in an MC-dependent manner (Supplemental Fig. 4). Thus, EP3-mediated MC activation may be potentially involved in Ag-independent innate immune reactions.

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Disclosures

The authors have no financial conflicts of interest.

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