

Role of ERK MAP kinases in responses of cultured human airway smooth muscle cells to IL-1 β

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Laporte, Johanne D., Paul E. Moore, Joseph H. Abraham, Geoffrey N. Maksym, Ben Fabry, Reynold A. Panettieri, Jr., and Stephanie A. Shore. Role of ERK MAP kinases in responses of cultured human airway smooth muscle cells to IL-1 β . *Am. J. Physiol.* 277 (*Lung Cell. Mol. Physiol.* 21): L943–L951, 1999.—We have previously reported that interleukin (IL)-1 β causes β -adrenergic hyporesponsiveness in cultured human airway smooth muscle cells by increasing cyclooxygenase-2 (COX-2) expression and prostanoid formation. The purpose of this study was to determine whether extracellular signal-regulated kinases (ERKs) are involved in these events. Levels of phosphorylated ERK (p42 and p44) increased 8.3- and 13-fold, respectively, 15 min after treatment with IL-1 β (20 ng/ml) alone. Pretreating cells with the mitogen-activated protein kinase inhibitor PD-98059 or U-126 (2 h before IL-1 β treatment) decreased ERK phosphorylation. IL-1 β (20 ng/ml for 22 h) alone caused a marked induction of COX-2 and increased basal PGE₂ release 28-fold ($P < 0.001$). PD-98059 (100 μ M) and U-126 (10 μ M) each decreased COX-2 expression when administered before IL-1 β treatment. In control cells, PD-98059 and U-126 had no effect on basal or arachidonic acid (AA; 10 μ M)-stimulated PGE₂ release, but both inhibitors caused a significant decrease in bradykinin (BK; 1 μ M)-stimulated PGE₂ release, consistent with a role for ERK in the activation of phospholipase A₂ by BK. In IL-1 β -treated cells, prior administration of PD-98059 caused 81, 92 and 40% decreases in basal and BK- and AA-stimulated PGE₂ release, respectively ($P < 0.01$), whereas administration of PD-98059 20 h after IL-1 β resulted in only 38 and 43% decreases in basal and BK-stimulated PGE₂ release, respectively ($P < 0.02$) and had no effect on AA-stimulated PGE₂ release. IL-1 β attenuated isoproterenol-induced decreases in human airway smooth muscle stiffness as measured by magnetic twisting cytometry, and PD-98059 or U-126 abolished this effect in a concentration-dependent manner. These results are consistent with the hypothesis that ERKs are involved early in the signal transduction pathway through which IL-1 β induces PGE₂ synthesis and β -adrenergic hyporesponsiveness and that ERKs act by inducing COX-2 and activating phospholipase A₂.

extracellular signal-regulated kinase; mitogen-activated protein; interleukin-1 β ; prostaglandin E₂; β -adrenergic responses; PD-98059; U-126; magnetic twisting cytometry; cyclooxygenase

β -ADRENERGIC HYPORESPONSIVENESS is a characteristic feature of asthma. Decreased bronchodilator responses

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to β -agonists have been observed in asthmatic airways both in vivo and in vitro as well as in animal models of asthma (2, 3). There is reason to believe that cytokines may contribute to the β -adrenergic hyporesponsiveness of asthma. Cytokines such as interleukin (IL)-1 β and tumor necrosis factor- α are increased in bronchoalveolar lavage fluid from symptomatic asthmatic patients (29, 41). In addition, both IL-1 β and tumor necrosis factor- α have been shown to decrease β -adrenergic responsiveness of a variety of cells and tissues including those in the airways (11, 16, 23, 25, 37, 46).

Cyclooxygenase (COX) activity is the rate-limiting step for the conversion of arachidonic acid (AA) to prostaglandins (PGs) and thromboxane (Tx). COX exists in two isoforms. COX-1 is expressed constitutively in most cells (39), whereas COX-2 is induced by mitogens (19), bacterial lipopolysaccharide (27), and cytokines (4, 32, 39). Laporte et al. (25) have recently reported that the mechanistic basis for IL-1 β -induced decreases in the responsiveness of cultured human airway smooth muscle (HASM) cells to β -agonists involves COX-2-induced prostanoid formation. In particular, Laporte et al. showed that IL-1 β leads to COX-2 expression in HASM cells and that this results in a marked (>10-fold) increase in PGE₂ synthesis. We also demonstrated that exogenous administration of PGE₂ decreases the responses of HASM cells to the β -agonist isoproterenol (Iso), whereas COX-2 inhibitors prevent IL-1 β -induced β -adrenergic hyporesponsiveness. Responses to Iso were assessed by measuring changes in cytoskeletal stiffness by magnetic twisting cytometry (17, 25, 37, 42, 43). Using this technique, Hubmayr et al. (17) have previously shown that HASM cells decrease their stiffness in response to any of a panel of bronchodilator agonists known to cause relaxation of airway smooth muscle, whereas stiffness increases in response to contractile agonists known to increase cytosolic calcium concentration in these cells. Similar results are obtained with vascular smooth muscle cells (26). Although we do not know the precise mechanism by which prostanoids generated in response to IL-1 β lead to β -adrenergic hyporesponsiveness in HASM cells, our results are consistent with the hypothesis that marked increases in PGE₂ lead to phosphorylation and heterologous desensitization of the β -adrenergic receptor (25, 37).

The signal transduction pathway leading to COX-2 expression in IL-1 β -stimulated airway smooth muscle cells has not been described. However, IL-1 β activates a family of protein kinases known as the mitogen-activated protein (MAP) kinases (14, 15, 18, 35, 38). In

other cell types, MAP kinases appear to be involved in COX-2 induction by cytokines, lipopolysaccharide, or growth factors (13, 30, 47). In mammalian cells, at least three subgroups of MAP kinases have been described, including extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38. Two isoforms of ERK, p44 (ERK1) and p42 (ERK2), are expressed in most cell types and are equally active. ERK1 and ERK2 require dual phosphorylation for activation (33). The immediate upstream protein kinase that phosphorylates ERK1 and ERK2 is MAP kinase kinase (MEK) (36). The MAP kinase cascade is one of the major signaling pathways leading from activation of growth factor, hormone, or cytokine receptors to induction of genes via their ability to phosphorylate important transcription factors (8, 21, 22). MAP kinases also phosphorylate other regulatory proteins. For example, ERK activation induces AA metabolism and the formation of prostaglandins by its phosphorylation of cytosolic phospholipase A₂ (PLA₂) (9).

The purpose of this study was to determine whether ERK activation is required for IL-1 β -induced prostanoïd formation and consequent β -adrenergic hyporesponsiveness in HASM cells. To confirm that IL-1 β stimulates ERK in airway smooth muscle and to verify the efficacy of the inhibitors used, we measured ERK phosphorylation in whole cell lysates. We next examined the effect of two highly specific inhibitors of MEK phosphorylation and activation, PD-98059 (10) and U-126 (12), on IL-1 β -induced release of PGE₂ and IL-1 β -induced COX-2 expression. Finally, we examined the effect of PD-98059 and U-126 on IL-1 β -induced changes in HASM cell responses to the β -agonist Iso.

METHODS

Cell culture. Human tracheae were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania (Philadelphia) Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was dissected under sterile conditions, and the trachealis muscle was isolated (31). Approximately 1 g of wet trachealis muscle was obtained from each donor. This tissue was minced; centrifuged; resuspended in 10 ml of buffer containing 0.2 mM CaCl₂, 640 U of collagenase, 10 mg of soybean trypsin inhibitor, and 10 U/ml of elastase; and incubated with enzymes for 90 min in a shaking water bath at 37°C. The cell suspension so generated was filtered through 127- μ m Nytex mesh, and the filtrate was washed with an equal volume of cold Ham's F-12 medium supplemented with 10% fetal bovine serum. For culture, the cells were plated in plastic flasks at 10⁴ cells/cm² in Ham's F-12 medium supplemented with 10% fetal bovine serum, 10² U/ml of penicillin, 0.1 mg/ml of streptomycin, 2.5 mg/ml of amphotericin B, 12 mM NaOH, 1.7 μ M CaCl₂, 2 mM L-glutamine, and 25 mM HEPES. The medium was replaced every 3–4 days. The cells were passaged with 0.25% trypsin and 1 mM EDTA every 10–14 days. Confluent cells were serum deprived and supplemented with 5.7 μ g/ml of insulin and 5 μ g/ml of transferrin 24 h before use. Cells in passages 4–7 from 13 different donors were used in the studies described below.

Western blotting for measurements of phosphorylated ERK and COX-2 expression. We measured MEK1/MEK2 activity of whole cell lysates by determining the level of phosphorylated

ERK by Western blotting with an antibody to phosphorylated p42/p44. COX-2 expression was also measured by Western blot. In both cases, confluent HASM cells were serum deprived and treated with PD-98059 (30–100 μ M for 15 min or 2 h) or U-126 (10 μ M for 15 min or 2 h) and/or IL-1 β (20 ng/ml). IL-1 β treatment was for 15 min in the case of p42/p44 activation and for 20 h in the case of COX-2 expression. The medium was removed, and the cells were washed with PBS and then lysed in 400 μ l of extraction buffer [10 mM Tris·HCl buffer with 50 mM NaCl, 50 mM NaF, 10 mM D-serine, 1 mM EDTA, 1 mM EGTA, 1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 5 μ g/ml of leupeptin, 1 μ g/ml of pepstatin, and 10⁻² U/ml of aprotinin]. Cells were scraped off the flasks, passed through a 25 $\frac{5}{8}$ -gauge needle, and solubilized by sonication.

For p42/p44 Western blots, supernatants of cell lysates were mixed with equal volumes of loading buffer [0.062 M Tris·HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.01% (wt/vol) bromophenol blue] and then were boiled for 5 min. Solubilized proteins (60 μ g/lane for p42/p44 and phospho-p42/p44) were separated by SDS-polyacrylamide gel electrophoresis on a 12% Tris-glycine gel (Novex, San Diego, CA) under nonreducing conditions and transferred electrophoretically to a nitrocellulose membrane in transfer buffer (Pierce, Rockford, IL). For p42/p44 and phospho-p42/p44 Western blots, the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 3 h at room temperature. The blots were probed with rabbit anti-phospho-p42/p44 ERK or anti-p42/p44 ERK antibody (New England Biolabs, Beverly, MA). The phospho-specific antibody recognizes ERK only when phosphorylated at Thr²⁰² and Tyr²⁰⁴. The blots were washed and subsequently incubated (1 h) in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk with horseradish peroxidase-conjugated goat anti-rabbit IgG for 2 h. The proteins were visualized by light emission on film with enhanced chemiluminescent substrate (Pierce). The band visualized at ~42 and 44 kDa was quantified with a laser densitometer. Band density values are expressed in arbitrary optical density units. Western blotting for COX-2 was performed as previously described (25).

PGE₂ release. For these experiments, four flasks of HASM cells from the same passage of the same donor cells were grown to confluence and serum deprived. Ten hours later, two were treated with PD-98059 (30–100 μ M) or U-126 (10 μ M) and the others served as controls. Two hours later, IL-1 β (20 ng/ml) was added to all flasks. Approximately 22 h later, the cell medium was removed, and the cells were washed with PBS. HASM cells were harvested by a brief exposure to 0.25% trypsin and 1 mM EDTA and resuspended in serum-free medium with or without MEK inhibitors and/or IL-1 β . The cells were then plated at 10⁵ cells/well in 24-well plates. The cells were incubated for 4 h, after which time the medium was replaced with 0.5 ml of fresh medium. The cells were either left untreated or AA (10⁻⁵ M) or bradykinin (BK; 10⁻⁶ M) was added. After a 15-min incubation at 37°C, the supernatants were harvested and stored at -20°C until subsequent assay with a PGE₂ enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The antibody to PGE₂ had <1% cross-reactivity to 6-keto-PGF_{1 α} and <0.01% to TxB₂ and other PGs according to the manufacturer's specifications.

Magnetic twisting cytometry. We examined the effect of PD-98059 and U-126 on IL-1 β -induced changes in cell stiffness responses to Iso or dibutyryl cAMP. Cells were treated with MEK1/MEK2 inhibitors and/or IL-1 β , harvested, and resuspended in serum-free medium with or without IL-1 β and PD-98059 as described in PGE₂ release. Cells were then

plated at 20,000 cells/well on collagen I (500 ng/cm²)-coated bacteriological plastic dishes (6.4-mm, 96-well Removawells, Immunlon II). Two to six hours later, measurements of cell stiffness were made with magnetic twisting cytometry. Cumulative concentration-response curves to Iso or dibutyryl cAMP were performed as follows. First, three to five measurements of cell stiffness were made under baseline conditions. After these measurements, 2 μ l of a solution containing Iso or dibutyryl cAMP were added to the well that contained 200 μ l of medium. After a 1-min incubation with the agent, two to four measurements of cell stiffness were again obtained. This procedure was repeated with increasing concentrations of the agent. The concentration ranges used were 10⁻⁸ to 10⁻⁵ M Iso and 10⁻⁴ to 3 \times 10⁻³ M dibutyryl cAMP. Only one agonist was studied per well. Details of the methodology for magnetic twisting cytometry are found below.

Details of the magnetic twisting cytometry technique have been previously described (42, 43). Briefly, the principle is as follows. Ferromagnetic beads are first coated with a prescribed ligand [Peptide 2000, Arg-Gly-Asp (RGD); Telios Pharmaceuticals, San Diego, CA], then bound to the surface of the cells through the corresponding receptor system (integrins). Individual wells containing adherent cells bound to RGD-coated ferromagnetic beads in serum-free medium are placed in the magnetic twisting chamber and held at 37°C with a circulating water bath that is built into the system. The attached beads are magnetized with a brief 1,000-gauss pulse so that their magnetic moments are aligned in one direction parallel to the surface on which the cells are plated. The magnetic field vector generated by the beads in the horizontal direction is measured by an in-line magnetometer. Subsequently, a much smaller magnetic field is applied in the vertical direction, generating an applied torque (or twisting stress). This twisting stress (80 dyn/cm²) causes the beads to rotate as a compass needle would, but bead rotation is opposed by reaction forces developed within the cytoskeleton to which the beads are bound through the integrin molecules. Magnetic twisting cytometry uses the applied twisting stress and resulting measured angular rotation of the magnetic bead and expresses the ratio as cell stiffness. Bead rotation increases with the strength of the applied twisting field and is inversely proportional to the resistance of the cell to shape distortion.

Reagents. Tissue culture reagents and drugs used in this study were obtained from Sigma (St. Louis, MO), with the exception of amphotericin B and trypsin-ETDA solution, which were purchased from GIBCO BRL (Life Technologies, Grand Island, NY); IL-1 β , which was obtained from Genzyme (Cambridge, MA); PD-98059, which was obtained from Calbiochem-Novabiochem (La Jolla, CA); and U-126, which was a gift from DuPont Pharmaceuticals (Wilmington, DE). PD-98059 and U-126 were dissolved in DMSO at concentrations such that the concentration of DMSO in the cell wells never exceeded 0.1%. Dibutyryl cAMP was dissolved at 10⁻¹ M in distilled water, frozen in aliquots, and diluted appropriately in medium on the day of use. Iso (10⁻¹ M in distilled water) was made fresh each day. Because Iso is rapidly oxidized, dilutions of Iso in medium were made immediately before addition to the cells.

Statistics. The effect of PD-98059 and U-126 on IL-1 β -induced changes in cell stiffness responses to Iso were examined by repeated-measures ANOVA with treatment (control, IL-1 β , PD-98059, U-126, IL-1 β plus PD-98059, and IL-1 β plus U-126) and experimental day as main effects. Follow-up *t*-tests were used to determine where the treatment effect lay. Changes in basal and BK- and AA-stimulated PGE₂ release induced by IL-1 β were examined by ANOVA, with treatment

and experimental day as main effects. A *P* value < 0.05 was considered significant.

RESULTS

MEK1/MEK2 activity of whole HASM cell lysates was estimated by determining the level of phosphorylated ERK1 and ERK2 (Fig. 1) with Western blotting. ERK1 and ERK2 phosphorylation began to increase within 5 min of addition of IL-1 β to HASM cells, peaked after 15 min, and decreased thereafter. To determine the inhibiting potential of PD-98059 and U-126, two synthetic, cell-permeable, noncompetitive inhibitors of MEK1/MEK2 phosphorylation and activation, we measured the effect of PD-98059 and U-126 on IL-1 β -induced phosphorylation of ERK1 and ERK2. Compared with control lysates, IL-1 β (20 ng/ml for 15 min) increased the level of phosphorylation of ERK1 and ERK2 13- and 8.4-fold, respectively, as assessed by densitometry (*n* = 3 donors). Pretreatment with 100 μ M PD-98059 or 10 μ M U-126 for 2 h before addition of IL-1 β decreased IL-1 β -increased ERK1 and ERK2 phosphorylation (Fig. 2) but did not alter ERK expression (data not shown). ERK1 phosphorylation was inhibited by 88%, whereas ERK2 phosphorylation was inhibited by 77% in cells treated with PD-98059 (100 μ M) compared with that in IL-1 β -treated cells. PD-98059 (30 μ M) decreased the level of phosphorylated ERK1 by 71% and the level of phosphorylated ERK2 by 62%. Pretreatment with U-126 (10 μ M) completely abolished phosphorylation of ERK1 and ERK2 in IL-1 β -treated cells. Fifteen minutes of pretreatment with the MEK1/MEK2 inhibitors also reduced IL-1 β -increased ERK1 and ERK2 phosphorylation (data not shown). ERK1/ERK2 phosphorylation induced by IL-1 β was reduced by 50% in cells treated with PD-98059 (100 μ M) and by 90% in cells treated with U-126 (10 μ M).

IL-1 β (20 ng/ml for 22 h) resulted in marked COX-2 expression (Fig. 3) as previously described (25). Treatment with PD-98059 (100 μ M) for 24 h reduced COX-2 expression (Fig. 3). Similar results were obtained with U-126. In cells from four different donors, PD-98059 and U-126 caused a 66 \pm 13 and 71 \pm 8% reduction, respectively, in the density of the COX-2 band (*P* < 0.02). In contrast, neither PD-98059 nor U-126 had any effect on ERK2 protein expression.

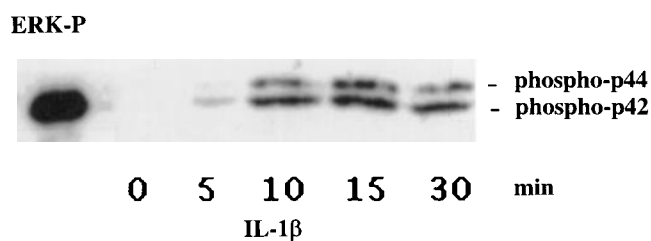


Fig. 1. Representative time course of mitogen-activated protein (MAP) kinase kinase-1 (MEK1; phospho-p44)/MEK2 (phospho-p42) phosphorylation by interleukin (IL)-1 β (20 ng/ml). Levels of phosphorylated extracellular signal-regulated kinase-1 (ERK1) and ERK2 MAP kinases were determined by Western blotting. This result is representative of experiments on cells from 3 different donors. ERK-P, phospho-ERK2 standard.

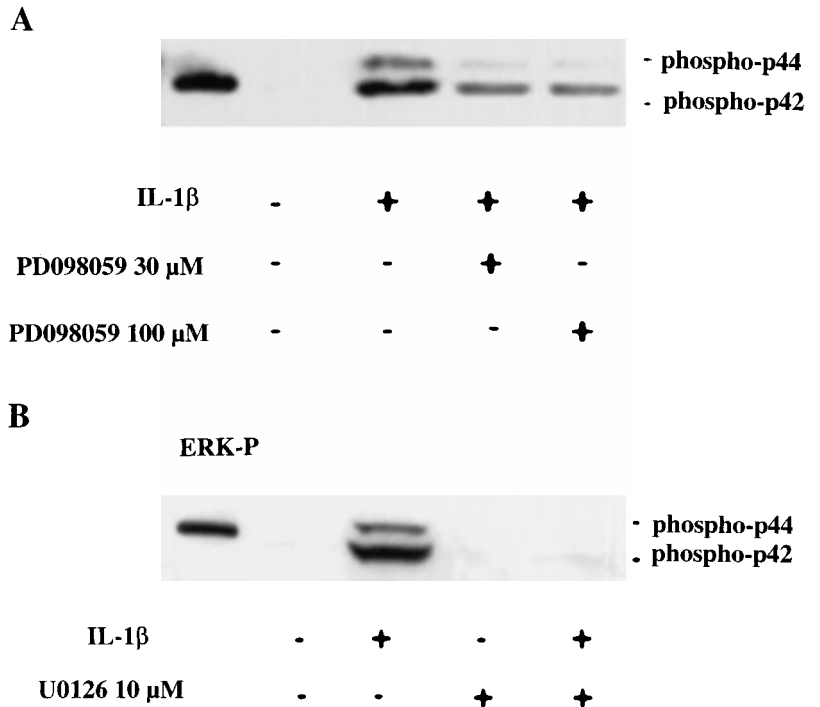


Fig. 2. Western blot showing effect of PD-98059 (for 2 h; A) and U-126 (for 2 h; B) before addition of IL-1 β on MEK1/MEK2 activity in IL-1 β (20 ng/ml for 15 min)-treated human airway smooth muscle (HASM) cells. Similar results were obtained on cells from 3 other donors. +, Presence; -, absence.

We also examined the effect of PD-98059 and U-126 on PGE₂ release induced by IL-1 β (20 ng/ml for 22 h). Compared with control cells, IL-1 β caused a significant, 28-fold increase in basal PGE₂ release ($P < 0.001$) as previously described (25). Most of this increased PGE₂ release results from COX-2 activity (25). PD-98059 (30 or 100 μ M) administered 2 h before IL-1 β reduced basal PGE₂ release by 65 ($P < 0.001$) and 81% ($P < 0.01$), respectively. The inhibitory effects of PD-98059 were not due to cytotoxicity because the release of lactate dehydrogenase into the culture medium was not altered by 24 h of incubation with 100 μ M PD-98059 (data not shown). U-126 (10 μ M) administered 2 h before IL-1 β reduced basal PGE₂ release by 92% ($P < 0.05$).

To further evaluate the role of MEK inhibition in IL-1 β -induced prostanoid release, we measured the effect of prior administration of MEK inhibitors (100 μ M PD-98059 or 10 μ M U-126 for 24 h) on BK (10⁻⁶ M)- and AA (10⁻⁵ M)-stimulated PGE₂ release in control and IL-1 β (20 ng/ml for 22 h)-stimulated cells (Figs. 4 and 5). AA-stimulated PGE₂ release requires both the

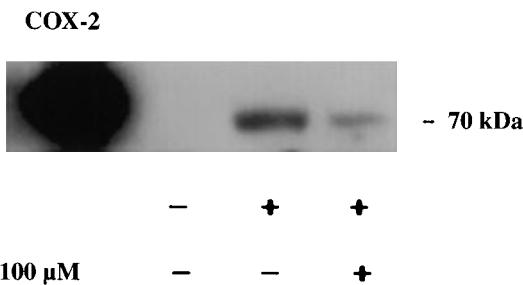


Fig. 3. Western blot showing cyclooxygenase-2 (COX-2) expression. HASM cells were pretreated for 24 h with PD-98059 (100 μ M) before stimulation with IL-1 β (20 ng/ml) for 22 h. This result is representative of experiments on cells from 4 different donors. No. on right, molecular mass.

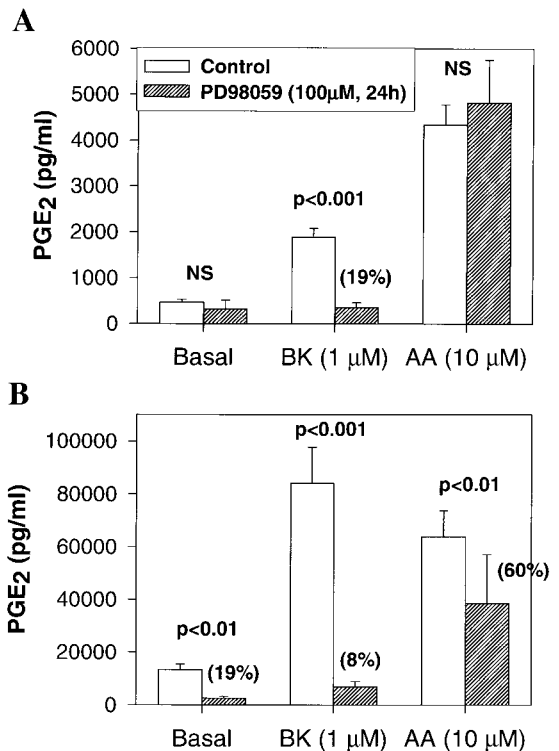


Fig. 4. Release of PGE₂ by control cells (A) and HASM cells treated with IL-1 β (20 ng/ml for 22 h; B). Some cells were pretreated with PD-98059. Basal and bradykinin (BK)- and arachidonic acid (AA)-stimulated-PGE₂ release over a 15-min period was assessed. Results are means \pm SE from 8 HASM cell wells on 7 experimental days; ns. in parentheses, PGE₂ concentration in PD-98059-treated cells as percentage of control cells. Cells were obtained from 3 different donors. NS, not significant. P values compare cells with and without PD-98059 treatment.

COX and PGE₂ synthase enzymes but not PLA₂, whereas in the case of BK-stimulated PGE₂ release, PLA₂ must also be activated. Both BK and AA caused significant increases in PGE₂ release in control and IL-1 β -pretreated cells compared with basal release (Figs. 4 and 5). In control cells, PD-98059 (100 μ M for 24 h) and U-126 (10 μ M for 24 h) caused a marked and significant reduction in BK-stimulated PGE₂ release, consistent with a report (10) that ERK is involved in BK activation of PLA₂. In contrast, PD-98059 and U-126 had no significant effect on AA-stimulated PGE₂ release (Figs. 4A and 5A). In IL-1 β -treated cells (Figs. 4B and 5B), PD-98059 and U-126 significantly reduced both BK- and AA-stimulated PGE₂ release, but the effect on BK-stimulated release was greater than on AA-stimulated release.

To examine the extent to which effects of ERK on COX-2 expression versus PLA₂ activation contributed to IL-1 β -induced PGE₂ release, we also examined the effect of a very short preincubation with PD-98059 (100 μ M) on BK- or AA-stimulated PGE₂ release in IL-1 β -treated cells. In this case, PD-98059 (100 μ M) was added to the cells for only 15 min before the addition of BK or AA and not throughout the 22-h period of IL-1 β pretreatment, thus being unable to influence COX-2 expression. Short-term (15-min) PD-98059 treatment still caused a significant reduction in basal and BK-stimulated PGE₂ release in IL-1 β -treated cells ($P < 0.02$), although the magnitude of the effect was not as great as with the 24-h pretreatment (Fig. 6). In contrast, although long-term (24-h) PD-98059 treatment caused an ~50% reduction in AA-stimulated PGE₂ release in IL-1 β -treated cells (Fig. 4), short-term (15-min) PD-98059

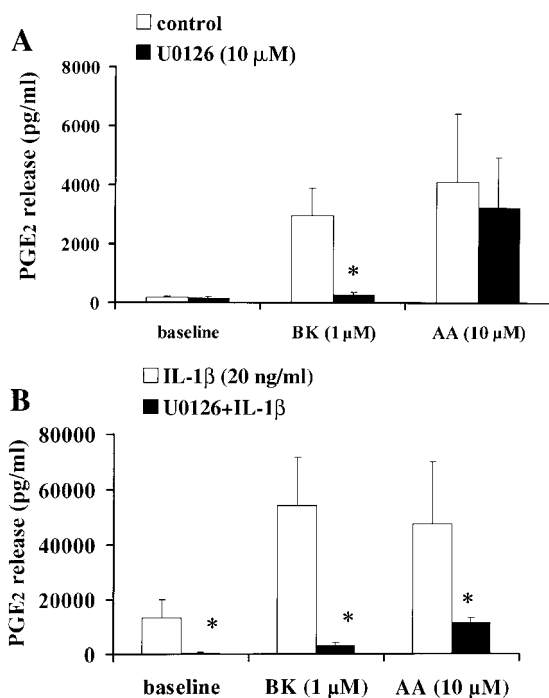


Fig. 5. Release of PGE₂ by control cells (A) and HASM cells treated with IL-1 β (20 ng/ml for 22 h; B). Some cells were pretreated with U-126 for 24 h. Basal and BK- and AA-stimulated PGE₂ release over a 15-min period was assessed. Results are means \pm SE from 8 HASM cell wells on 5 experimental days. Cells were obtained from 3 different donors. * $P < 0.01$ vs. cells not treated with U-126.

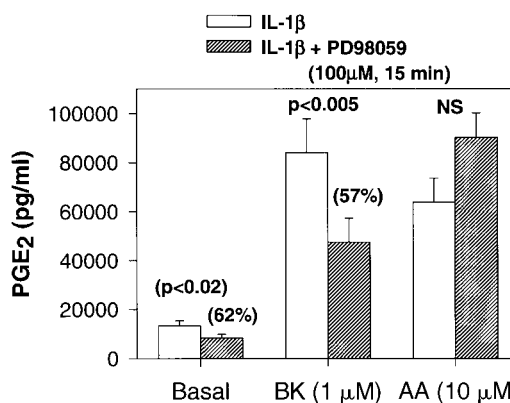


Fig. 6. Release of PGE₂ by HASM cells treated with IL-1 β (20 ng/ml for 22 h). Basal and BK- and AA-stimulated PGE₂ release over 15 min was assessed. Some cells were pretreated with PD-98059 for 15 min before this collection period. Results are means \pm SE from 7–12 HASM cell wells obtained on 6 experimental days; nos. in parentheses, PGE₂ concentrations in PD-98059-treated cells as percentage of control cells. Cells were obtained from 3 different donors. P values compare cells with and without PD-98059 treatment.

treatment had no significant effect on AA-stimulated PGE₂ release in IL-1 β -treated cells (Fig. 6).

Laporte et al. (25) have previously reported that prostanoids are implicated in IL-1 β -induced decreases in HASM cell responses to β -agonists. Because our data indicated that the ERK MAP kinases were involved in IL-1 β -induced COX-2 expression and prostanoid release, we sought to determine whether ERK is also involved in IL-1 β -induced β -adrenergic hyporesponsiveness. To do so, we examined the effect of PD-98059 (100 μ M for 24 h) on IL-1 β -induced changes in HASM cell stiffness responses to Iso. The results are shown in Fig. 7A. Neither PD-98059, IL-1 β , nor their combination had any effect on baseline cell stiffness (129.3 ± 16.0 dyn/cm² in control cells, 123.0 ± 11.4 dyn/cm² in IL-1 β -treated cells, 116.0 ± 13.0 dyn/cm² in PD-98059-treated cells, and 115.2 ± 7.3 dyn/cm² in PD-98059 plus IL-1 β -treated cells). In control cells, Iso caused a dose-related decrease in cell stiffness (Fig. 7A). Repeated-measures ANOVA indicated a significant effect of drug treatment on Iso-induced changes in cell stiffness ($P < 0.01$). Follow-up analysis indicated that the treatment effect lay in the response to IL-1 β (20 ng/ml), which reduced the capacity of Iso to decrease cell stiffness as previously described (25, 37). Compared with control treatment, PD-98059 (100 μ M) alone had no effect on the cell stiffness responses to Iso. However, PD-98059 (100 μ M) abolished the effects of IL-1 β on cell stiffness responses to any concentration of Iso. A lower concentration of PD-98059 (30 μ M) did not abolish the IL-1 β response but did significantly reduce the effect of IL-1 β at 10^{-7} and 10^{-6} M Iso (Fig. 7B). A still lower concentration of PD-98059 (10 μ M) was without effect (data not shown). Similar results were obtained with U-126 (Fig. 8). Neither U-126, IL-1 β , nor their combination had any effect on baseline cell stiffness (114.4 ± 10.9 dyn/cm² in control cells, 119.0 ± 10.1 dyn/cm² in IL-1 β -treated cells, 122.5 ± 9.57 dyn/cm² in PD-98059-treated cells, and 119.4 ± 10.5 dyn/cm² in PD-98059 plus IL-1 β -treated cells). Compared with control treatment, U-126 (10 μ M) alone had no effect on the cell

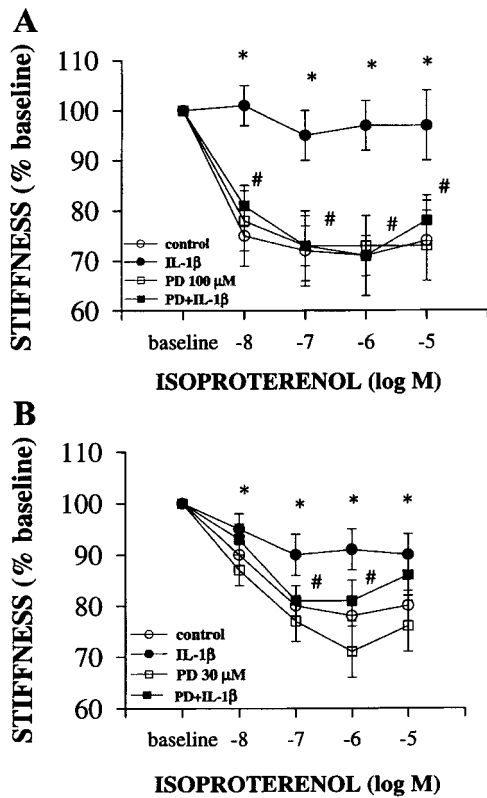


Fig. 7. Effect of 100 (A) and 30 (B) μ M PD-98059 (PD) for 24 h on IL-1 β (20 ng/ml for 22 h)-induced changes in cell stiffness response to isoproterenol (Iso). Results are expressed as percent of baseline stiffness values before addition of Iso and are means \pm SE from 7 control wells, 11 IL-1 β -treated wells, 9 PD-treated wells, and 11 PD+IL-1 β -treated wells on 7 experimental days. Cells were obtained from 3 different donors. * $P < 0.05$ compared with control wells. # $P < 0.01$ compared with IL-1 β -treated wells.

stiffness response to Iso. However, U-126 abolished the effect of IL-1 β on the cell stiffness responses to any concentration of Iso (Fig. 8).

Shore et al. (37) have previously reported that IL-1 β decreases HASM cell stiffness responses to Iso but has

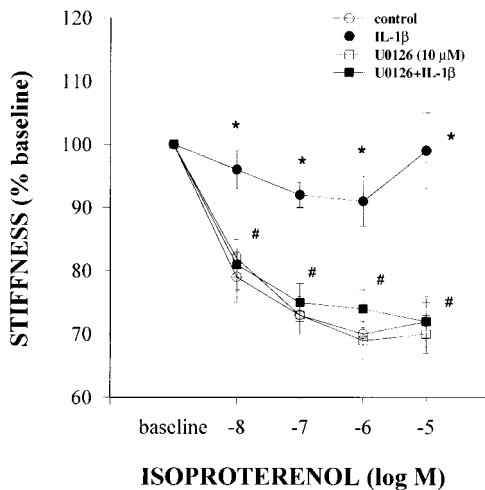


Fig. 8. Effect of U-126 for 24 h on IL-1 β (20 ng/ml for 22 h)-induced changes in cell stiffness response to Iso. Results are means \pm SE from 8 control wells, 9 IL-1 β -treated wells, 7 U-126-treated wells, and 8 U-126+IL-1 β -treated wells on 4 experimental days. Cells were obtained from 4 different donors. * $P < 0.001$ compared with control wells. # $P < 0.05$ compared with IL-1 β -treated wells.

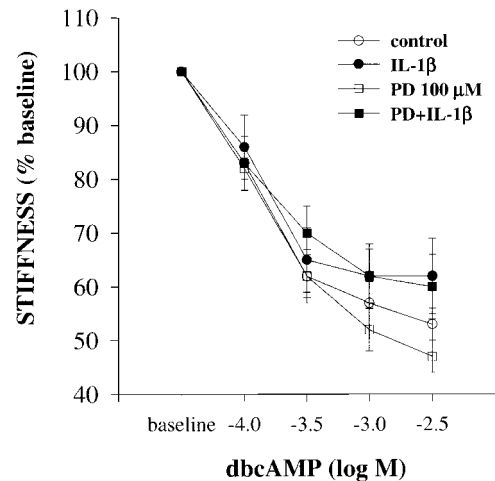


Fig. 9. Effect of PD (for 24 h) on IL-1 β (20 ng/ml for 22 h)-induced changes in cell stiffness response to dibutyryl cAMP (dbcAMP). Results are expressed as percent of baseline stiffness before addition of Iso and are means \pm SE from 8 control wells, 8 IL-1 β -treated wells, 8 PD-treated wells, and 10 PD+IL-1 β -treated wells on 4 experimental days. Cells were obtained from 4 different donors.

no effect on the cell stiffness responses to dibutyryl cAMP, suggesting that the effect of IL-1 β lies upstream from PKA activation. To ensure that the effects of PD-98059 (Fig. 9) were not the result of nonspecific effects of the drug on the ability of HASM cells to decrease cell stiffness, we also examined the effect of PD-98059 and IL-1 β on the cell stiffness responses to dibutyryl cAMP. Dibutyryl cAMP induced a concentration-related decrease in cell stiffness. Neither IL-1 β , PD-98059 (100 μ M), nor their combination had any effect on the cell stiffness responses to dibutyryl cAMP (Fig. 9). Furthermore, there was no significant effect of U-126 on the cell stiffness responses to dibutyryl cAMP in HASM cells (data not shown).

DISCUSSION

Our results indicate that the addition of IL-1 β to airway smooth muscle cells increased phosphorylation of ERK1 and ERK2 (Fig. 1). Pretreatment of HASM cells with the selective MEK1/MEK2 inhibitors PD-98059 and U-126 reduced this increased phosphorylation (Fig. 2). IL-1 β significantly increased COX-2 expression and PGE₂ release, and the MEK1/MEK2 inhibitors significantly inhibited these events (Figs. 3–6). PD-98059 and U-126 also blocked the effects of IL-1 β on the HASM cell stiffness responses to the β -agonist Iso (Figs. 7 and 8) without affecting the responses to dibutyryl cAMP (Fig. 9). Taken together, these results support the hypothesis that ERK1/ERK2 activation is involved in IL-1 β -induced prostanoid release and β -adrenergic hyporesponsiveness in HASM cells.

Our results indicate that IL-1 β causes a marked increase in the level of phosphorylated ERK1/ERK2 in HASM cells (Fig. 1). Other investigators (13, 14) have reported that IL-1 β activates p42 and p44 MAP kinases in other cell types, with a time course similar to that reported here. In HASM and other airway smooth muscle cells, the ERK MAP kinases have also been shown to be activated by seven-transmembrane-domain receptor ligands and by growth factors such as

platelet-derived growth factor, epidermal growth factor, and insulin growth factor I (5, 8, 22, 44). PD-98059 caused a marked inhibition of IL-1 β -induced ERK phosphorylation in these HASM cells (Fig. 2) at concentrations similar to those reported as being effective in other cell types (18, 44). Nevertheless, even 100 μ M PD-98059 did not completely block the IL-1 β -induced phosphorylation of ERK1/ERK2 in these cells. We also used a second MEK1/MEK2 inhibitor, U-126 (10 μ M), which completely inhibited the IL-1 β -induced ERK1 and ERK2 phosphorylation in HASM cells, consistent with the results of Favata et al. (12) who reported that U-126 has an \sim 100-fold higher affinity for MEK enzymes compared with PD-98059.

Several groups (32, 40), including ours (25), have reported that IL-1 β induces COX-2 expression and increases PGE₂ release in cultured HASM cells. In this study, we demonstrated that the signal transduction pathway leading from IL-1 β stimulation to COX-2 expression and increased PGE₂ release includes ERK activation. In IL-1 β -treated cells, we observed a partial inhibition of COX-2 expression (Fig. 3) and a marked reduction in PGE₂ release (Figs. 4 and 5) in cells treated with the MEK1/MEK2 inhibitors PD-98059 and U-126. ERK has also been shown to be involved in COX-2 expression induced by lipopolysaccharide in a rat macrophage cell line and in human monocytes treated with PD-98059 (18, 30). ERK is also involved in COX-2 expression or PGE₂ release induced by the TxA₂ analog U-46619 or by fibroblast growth factor in porcine aortic smooth muscle cells (20). The observation that PD-98059 and U-126 did not completely block the expression of COX-2 induced by IL-1 β (Fig. 3) is consistent with reports by other investigators (18) and suggests that other pathways may also be implicated in IL-1 β -induced COX-2 expression. For example, the p38 and JNK MAP kinases are also activated by IL-1 β , and in other cell types, p38 inhibitors can block IL-1 β -induced COX-2 expression (13, 38). It is possible that the inability of PD-98059 to completely block IL-1 β -induced COX-2 expression is related to the fact that the inhibitor did not completely suppress ERK phosphorylation. However, ERK phosphorylation was virtually abolished by U-126, whereas this inhibitor also did not completely abolish COX-2 expression.

We do not know the precise mechanism by which ERK activation leads to COX-2 expression. Once activated, ERK translocates to the nucleus and phosphorylates the transcription factor complex TCF/Elk1 (45). Elk1 and serum response factor (SRF) form a complex and bind to a serum response element in the promoter region of some genes. *COX-2* gene does not contain a serum response element in its promoter region (47). However, *COX-2* gene does have putative activator protein (AP)-1 sites in its regulatory region. This could be important because it has been shown that ERK-activated ternary complex factor/Elk1 does induce c-Fos transcription (28). c-Fos forms heterodimers with the c-Jun family to form AP-1. Thus ERK activation could lead to COX-2 expression via AP-1 activation. The ERK pathway may also phosphorylate an as yet unidentified transcription factor that also participates in the expression of COX-2.

The inhibitory effects of MEK1/MEK2 inhibitors on COX-2 expression and PGE₂ release were not due to cytotoxicity because lactate dehydrogenase release into the culture medium was not altered by 24 h of incubation with 100 μ M PD-98059 and because we observed no effect of PD-98059 on the expression of another protein, ERK2. We cannot exclude the possibility that the effects of PD-98059 or U-126 might be the result of nonspecific effects on enzymes other than MEK1/MEK2. However, other investigators (1, 12) have demonstrated that PD-98059 and U-126 at the concentrations used in this study do not inhibit activation of MKK-4, protein kinase C, cdk2, JNK, MKK3, or p38. In contrast, PD-98059 has been reported to inhibit COX activity in platelets (6). Although it is theoretically possible that such nonspecific effects of PD-98059 might have contributed to its effects on IL-1 β -induced PGE₂ release in this study, we do not think that this is likely. First, another MEK inhibitor, U-126, with a different chemical structure, had effects similar to those of PD-98059 on PGE₂ release. Second, neither PD-98059 nor U-126 treatment had any significant effect on AA-stimulated PGE₂ release in control cells, indicating that they did not alter COX-1 activity or PGE₂ synthase activity in HASM cells (Figs. 4 and 5). PD-98059 and U-126 did reduce AA-stimulated PGE₂ release in IL-1 β -treated cells (Figs. 4 and 5), but most of this effect is likely to have been the result of the effects of the compounds on COX-2 expression (Fig. 3) rather than on activity because when PD-98059 was administered to IL-1 β -treated cells too late to influence COX-2 expression, it had no effect on AA-stimulated PGE₂ release (Fig. 6).

The reduction in COX-2 expression caused by PD-98059 and U-126 also contributed to its ability to decrease basal and BK-stimulated PGE₂ release in IL-1 β -treated cells (Figs. 4B and 5B). However, the effect of long-term PD-98059 and U-126 treatment on basal and BK-stimulated PGE₂ release was greater than the effect of the inhibitors on AA-stimulated PGE₂ release (Figs. 4B and 5B). In addition, PD-98059 also inhibited basal and BK-stimulated PGE₂ release even when the drug was given only 15 min before the supernatants for PGE₂ analysis (Fig. 6). Furthermore, PD-98059 and U-126 also inhibited BK-stimulated PGE₂ synthesis in control cells that do not express COX-2 (Figs. 4A and 5A). Because AA generation of PGE₂ requires only COX and PGE₂ synthase, whereas BK-stimulated PGE₂ release also requires generation of free AA through PLA₂ activity, the most likely explanation for these data is that ERK activation is important for BK-stimulated PLA₂ activation. In support of this hypothesis, several studies have shown that the ERK MAP kinases are required for activation of cytosolic PLA₂ by G protein-coupled receptors such as the BK receptor in other cells systems (24, 34). For example, Pyne et al. (34) showed that PD-98059 decreased the phosphorylation of PLA₂ induced by BK and abolished the stimulatory effect of BK on PGE₂ release in guinea pig airway smooth muscle cells.

Laporte et al. (25) have previously reported that the mechanism by which IL-1 β causes decreased HASM cell responses to β -agonists involves COX-2-generated

prostanoid release. In particular, we showed that exogenous administration of PGE₂ mimics the effects of IL-1 β , whereas the inhibition of COX-2 with either NS-398 or indomethacin blocks the effects of IL-1 β . Our results suggested that marked increases in PGE₂ lead to increased basal cAMP, consequent PKA activation, and subsequent phosphorylation and heterologous desensitization of the β -adrenergic receptor (25, 37). Because the results of this study indicated that ERK activation was required for IL-1 β -induced PGE₂ release, we reasoned that ERK activation should also be involved in IL-1 β -induced β -adrenergic hyporesponsiveness. Our results support that hypothesis. In particular, we demonstrated that the MEK1/MEK2 inhibitors PD-98059 and U-126 caused a concentration-dependent inhibition of the effects of IL-1 β on the cell stiffness changes induced by Iso (Figs. 7 and 8). In particular, PD-98059 (100 μ M) virtually abolished the responses to IL-1 β , whereas 30 μ M had only a partial effect. These results are consistent with the effect of PD-98059 on basal PGE₂ release: 100 μ M caused an 81% inhibition of basal PGE₂ release in IL-1 β -treated cells, whereas 30 μ M had a smaller effect (65% inhibition).

Cytoskeleton stiffness as measured here is an index of the ability of cells to resist distortions of shape in response to shear stress applied through magnetic beads linked to the cytoskeleton via integrin receptors. Actin and myosin form part of the cytoskeleton, and cross-bridge formation appears to increase cytoskeletal stiffness because the application of a variety of contractile agonists to smooth muscle cells results in increased stiffness, whereas bronchodilating agonists reduce stiffness (17, 37). The observation that transfection of NIH/3T3 fibroblasts with a tonically active myosin light chain kinase results in increased myosin phosphorylation and also increases cell stiffness compared with cells transfected with an empty plasmid (7) also supports the idea that actomyosin interactions affect cell stiffness. Changes in cell adhesion to the extracellular matrix can also influence cytoskeleton stiffness, and Hubmayr et al. (17) have previously reported that HASM cells plated on high-density collagen are more spread and develop more pronounced decreases in cell stiffness in response to Iso than cells plated on a low-density collagen matrix. Although it is possible that PD-98059 might have influenced cell adhesion and consequently cell stiffness responses to Iso, we believe that such an explanation is very unlikely. First, changes in cell adhesion influence basal cell stiffness (17, 42), but neither IL-1 β , MEK1/MEK2 inhibitors, nor their combination altered the baseline stiffness in these experiments. Second, such changes would have been expected to alter cell stiffness in response to any dilating agonist, but the responses to dibutyryl cAMP were unaffected by IL-1 β , PD-98059, or their combination (Fig. 9).

In summary, our results indicate that IL-1 β activates ERK and that ERK activation results in increased PGE₂ expression through effects on both PLA₂ activation and COX-2 expression. Our results also indicate that ERK activation is required for IL-1 β -induced β -adrenergic hyporesponsiveness. The observation that

ERK activation is required for IL-1 β -induced PGE₂ formation in conjunction with previous results by Laporte et al. (25) indicating that prostanoid formation is necessary for IL-1 β -induced β -adrenergic hyporesponsiveness suggests that the mechanism by which ERK is involved in IL-1 β -induced β -adrenergic hyporesponsiveness is through its effects on PGE₂ formation. Understanding the role of MAP kinases in the mechanism by which cytokines lead to β -adrenergic-receptor dysfunction may provide new avenues for pharmacological intervention for asthma.

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