Adenosine A<sub>1</sub> and Prostaglandin E Receptor 3 Receptors Mediate Global Airway Contraction after Local Epithelial Injury

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Epithelial injury and airway hyperresponsiveness are prominent features of asthma. We have previously demonstrated that laser ablation of single epithelial cells immediately induces global airway constriction through Ca<sup>2+</sup>-dependent smooth muscle shortening. The response is mediated by soluble mediators released from wounded single epithelial cells; however, the soluble mediators and signaling mechanisms have not been identified. In this study, we investigated the nature of the epithelial-derived soluble mediators and the associated signaling pathways that lead to the L-type voltage-dependent Ca<sup>2+</sup> channel (VGCC)-mediated Ca<sup>2+</sup> influx. We found that inhibition of adenosine A<sub>1</sub> receptors (or removal of adenosine with adenosine deaminase), cyclooxygenase (COX)-2 or prostaglandin E receptor 3 (EP<sub>3</sub>) receptors, epidermal growth factor receptor (EGFR), or platelet-derived growth factor receptor (PDGFR) all significantly blocked Ca<sup>2+</sup> oscillations in smooth muscle cells and airway constriction induced by local epithelial injury. Using selective agonists to activate the receptors in the presence and absence of selective receptor antagonists, we found that adenosine activated the signaling pathway A<sub>1</sub>R→EGFR/PDGFR→COX-2→EP<sub>3</sub>→VGCCs→calcium-induced calcium release, leading to intracellular Ca<sup>2+</sup> oscillations in airway smooth muscle cells and airway constriction.

Keywords: ATP; epidermal growth factor receptor; platelet-derived growth factor receptor; cyclooxygenase-2; L-type voltage-dependent Ca<sup>2+</sup> channels

We have recently demonstrated that laser ablation of a single epithelial cell reproducibly induces rapid and global airway constriction (1). The dynamics of the response suggested that local epithelial injury released a soluble mediator(s) that was transported to underlying smooth muscle cells by diffusion. The soluble mediator(s) evoked multiple Ca<sup>2+</sup> oscillations in smooth muscle cells by stimulating L-type voltage-dependent Ca<sup>2+</sup> channels (VGCCs), thus increasing intracellular Ca<sup>2+</sup> levels via the calcium-induced calcium release (CICR) mechanism. In this study, we investigated the specific nature of the soluble mediator(s) and signaling pathway(s) underlying the VGCC-mediated Ca<sup>2+</sup> influx.

In response to mechanical stimulation, ATP is released from airway epithelial cells and stimulates Ca<sup>2+</sup> waves in the epithelium (2–4); however, the effects of local epithelial injury-induced ATP on airway caliber have not been studied. The level of intracellular ATP is high (millimolar range) for metabolism, but is extremely low in the extracellular space, where it can function as a signaling molecule (5). Extracellular ATP activates two subtypes of purinergic receptors, P2X and P2Y, and both of them are expressed on airway epithelial and smooth muscle cells (6). The P2X receptors are ligand-gated ion channels that mediate Ca<sup>2+</sup> and Na<sup>+</sup> influx, and P2Y receptors are G protein–coupled receptors that regulate phospholipase C pathway, leading to inositol trisphosphate (IP<sub>3</sub>) production and intracellular Ca<sup>2+</sup> release (6). Because ATP has been shown to stimulate small airway constriction in mouse lung tissue slices by activating P2Y receptors (7), it was a likely candidate to be the soluble mediator(s) involved in local epithelial injury–induced smooth muscle contraction. However, in our previous studies, we found that inhibition of P2 purinoreceptor receptors did not block Ca<sup>2+</sup> signaling in smooth muscle cells and airway constriction induced by local epithelial injury. Furthermore, inhibition of P2 purinoreceptor receptors or neutralizing ATP with apyrase did not block extracellular ATP–induced airway contraction. Thus, we previously ruled out the involvement of P2 purinoreceptor receptors on smooth muscle cells in local epithelial injury–induced airway constriction, but we could not completely rule out ATP as a soluble mediator (1).

Exogenous ATP is rapidly converted to its metabolic products, such as adenosine monophosphate (AMP), ADP, and adenosine by ecto-apyrase enzymes expressed on the cell surface (8). Thus, ATP released from wounded single epithelial cells could potentially stimulate adenosine receptors after degradation to AMP (9) or adenosine (6, 10). Adenosine receptors are G protein–coupled receptors and have four subtypes: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors. Although the four adenosine receptors are all expressed on airway smooth muscle cells (6), adenosine is traditionally considered to indirectly induce bronchoconstriction either through activating A<sub>2B</sub> or A<sub>3</sub> receptors on mast cells or through neural nerves via A<sub>1</sub> receptors (11–14). However, recent studies demonstrated that adenosine could directly activate A<sub>1</sub> and A<sub>2B</sub> receptors to regulate adenylyl cyclase on human tracheal smooth muscle cells (15), and stimulate A<sub>1</sub> receptors on human bronchial smooth muscle cells to increase Ca<sup>2+</sup> signaling (16). Furthermore, adenosine A<sub>1</sub> receptors are colocalized with epithelial growth factor receptors (EGFRs) and induce the transactivation of EGFR and their downstream pathways, such as phosphoinositide 3-kinase and Src kinase in neural cells (17).

CLINICAL RELEVANCE

Our study provides direct evidence that local epithelial injury could contribute to airway hyperresponsiveness in subjects with asthma, and provides potentially new pharmacological targets for asthma treatment.
Prostanoids, which are derived from arachidonic acid, include prostaglandins (PGE$_2$, PGD$_2$, and PGF$_2$), prostacyclins (PGI$_2$), and thromboxane (18). Cyclooxygenase (COX-1 and COX-2) is an enzyme that converts arachidonic acid into the prostanoids. High levels of prostanoids in bronchoalveolar lavage fluid and the increased expression of COX-2 in lung tissue have been detected in subjects with asthma (18). PGE$_2$ is produced by airway epithelial cells (19–21) and by smooth muscle cells (22–25), and has been demonstrated to regulate airway caliber in both epithelial and smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the ATP-induced airway contraction (1). One explanation for these results would be activation of adenosine receptors on smooth muscle cells in local epithelial injury–induced airway contraction; however, we did not completely rule out the involvement of P2 purinoceptor receptors on smooth muscle cells in local epithelial injury–induced airway contraction (1, 11–16, 39–41), we next investigated the role of adenosine receptor agonists (adenosine (38), and found that ADA significantly blocked the local mediator, we incubated lung tissue slices with 5 units/ml adenosine (CGS15943, a non-selective adenosine receptor antagonist (36, 37). Laser ablation of single epithelial cells induced an increase in Ca$^{2+}$ oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca$^{2+}$ wave in epithelial cells (Figure 1C, Figure E1B, and Movie E2). To confirm that adenosine is the soluble mediator, we incubated lung tissue slices with 5 units/ml adenosine deaminase (ADA), an enzyme that catalyzes the deamination of adenosine (38), and found that ADA significantly blocked the local epithelial injury–induced airway contraction (Figure 1C). Because all the four subtypes of the adenosine receptors (A1, A$_2A$, A$_2B$, and A$_3$) have been demonstrated to mediate smooth muscle contraction (5, 11–16, 39–41), we next investigated the roles of these four adenosine receptors. We found that selective inhibition of the A$_3$ receptor with 5–50 μM PSB36 (42, 43) or 4 μM SLV320 (44) significantly blocked Ca$^{2+}$ oscillations in smooth muscle cells and airway contraction induced by local epithelial injury–induced airway contraction in the MATERIALS AND METHODS section of the online supplement.

**Statistical Analysis**

The ratio of lumen area was defined as the minimum cross-sectional area of airways after treatment divided by initial cross-sectional area. Statistical tests of significance of the ratio of lumen area were performed with one-way ANOVA using commercial software (SPSS v. 16; SPSS, Chicago, IL), and a $P$ value less than 0.05 was considered statistically significant.

**RESULTS**

**Adenosine and A$_1$ Receptor Mediate the Local Epithelial Injury–Induced Airway Contraction**

In our previous studies, we ruled out the involvement of P2 purinoceptor receptors on smooth muscle cells in local epithelial injury–induced airway contraction; however, we did not completely rule out ATP as a soluble mediator, because inhibition of P2 purinoceptor receptors did not block the ATP-induced airway contraction (1). One explanation for these results would be activation of adenosine receptors to stimulate airway contraction from ATP metabolites, such as AMP and adenosine. To test this possibility, we inhibited the adenosine receptors with 2 μM CGS15943, a non-selective adenosine receptor antagonist (36, 37). Laser ablation of single epithelial cells induced an increase in Ca$^{2+}$ oscillations (see Figure E1A in the online supplement), and airway contraction to 70% of the original cross-sectional area (Figures 1A and IC and Movie E1). However, inhibition of adenosine receptors with CGS15943 completely blocked Ca$^{2+}$ oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca$^{2+}$ wave in epithelial cells (Figure 1C, Figure E1B, and Movie E2). To confirm that adenosine is the soluble mediator, we incubated lung tissue slices with 5 units/ml adenosine deaminase (ADA), an enzyme that catalyzes the deamination of adenosine (38), and found that ADA significantly blocked the local epithelial injury–induced airway contraction (Figure 1C).

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**MATERIALS AND METHODS**

**Materials**

Fluo-4/AM, Pluronic F-127, Hanks’ balanced salt solution (HBSS), Dulbecco’s modified Eagle medium, and Antibiotic-Antimycotic were purchased from Invitrogen (Carlsbad, CA). AH6809, AG18, AG1478, AG1296, and 11-deoxy-16,16-dimethyl PGE$_2$ (11-PGE$_2$) were purchased from Cayman Chemical (Ann Arbor, MI). Sulfoxomorpholine, ATP, adenosine 5’-[y-thio]triphosphate tetralithium salt (ATP-γ-S), indomethacin, L-798106, and N6-cyclopentyladenosine (CPA) were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine, 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS15943), 1-butyl-8-(hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1H-purine-2,6-dione (PSB36), 8-[4-[4-(4-chlorophenyl)piperazide-1-sulfonyl]phenyl]-1-propylxanthine (PSB603), SLV320, ZM241385, and MRS1334 were purchased from Tocris Bioscience (Ellisville, MO), Supplemented HBSS (sHBSS) was made from HBSS with Ca$^{2+}$ and Mg$^{2+}$ that included the apical membrane of the epithelial cell. The region of interest was scanned horizontally by the femtosecond laser at 100 μs/μm. By using the “bleach control” program in the LSM 510, we were able to immediately (less than 1 second) switch between the imaging mode and the ablation mode. The femtosecond laser beam was produced from a Coherent Chameleon system (Coherent, Santa Clara, CA) with 800-nm wavelength, 140-fs pulse duration, and 80-MHz repetition rate. The average power at the sample plane was ~600 mW, the pulse energy was ~7.5 nJ per pulse, and the peak power was ~37.5 kW.

Because the selectivity of an inhibitor is dependent on species (e.g., human versus rat) and cell type, for each chemical compound that we used in this study, we have provided detailed information for the concentration used based on the previous studies in rat (see Table E1 in the online supplement). Because molecular methods, such as small interfering RNA or lentivirus-based short hairpin RNA, to confirm the molecular mechanism in our current studies, introduce significant technical challenges in the lung tissue slice model, we applied multiple selective inhibitors to confirm our results.

**Preparation of Lung Tissue Slices**

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine, and were consistent with guidelines published by the National Institutes of Health. The preparation of rat lung tissue slices has been previously described in detail (1), and the procedure is also available in the MATERIALS AND METHODS section of the online supplement.

**Measurement of Intracellular Ca$^{2+}$ Signaling**

To monitor free intracellular Ca$^{2+}$ in both epithelial and smooth muscle cells, lung tissue slices were incubated in sHBSS with 20 μM Fluo-4/AM, 100 μM sulfobromophthalein, and 0.2% Pluronic F-127 for 1 hour at room temperature (35). Subsequently, the slices were kept in sHBSS with 100 μM sulfobromophthalein for another hour at room temperature. The slices were then transferred to a glass-bottom dish (MatTek, Ashland, MA) and held in place with a slice anchor (Warner Instruments, Hamden, CT). Confocal imaging was performed on a Zeiss 510 Meta multiphoton laser scanning microscope (LSM 510; Zeiss, Jena, Germany). Fluo-4 was excited with a 488-nm laser, and the fluorescence images (512 x 512 pixels) were collected.

**Laser Ablation**

The procedure for femtosecond (fs) laser ablation has been previously described in detail (1). Briefly, the laser ablation was performed on the LSM 510 with an Achroplan 40×/0.8 NA water-immersion objective. A single epithelial cell was ruptured by focusing the Mode-locked Ti: Sapphire femtosecond laser beam over a triangular region of interest (~6 μm$^2$) that included the apical membrane of the epithelial cell. The region of interest was scanned horizontally by the femtosecond laser at 100 μs/μm. By using the “bleach control” program in the LSM 510, we were able to immediately (less than 1 second) switch between the imaging mode and the ablation mode. The femtosecond laser beam was produced from a Coherent Chameleon system (Coherent, Santa Clara, CA) with 800-nm wavelength, 140-fs pulse duration, and 80-MHz repetition rate. The average power at the sample plane was ~600 mW, the pulse energy was ~7.5 nJ per pulse, and the peak power was ~37.5 kW.
To test the role of adenosine receptors in mediating ATP-induced airway contraction, we investigated the effects of ATP, its nonhydrolyzable analog, ATP-γ-S, and adenosine on airway caliber in the presence and absence of adenosine receptor antagonist CGS15943. We found that 10 μM adenosine, ATP, or ATP-γ-S induced airway contraction (Figure 1D). Inhibition of adenosine receptors with CGS15943 significantly blocked airway contraction induced by adenosine and ATP, but not by ATP-γ-S (Figure 1D).

**EP3 Receptors Participate in the Local Epithelial Injury–Induced Airway Constriction**

PGE2 has been shown to be released by mechanical scratch of the guinea pig tracheal mucosa (19), indicating that epithelial cell damage could possibly increase PGE2 levels in lung tissue. Thus, we investigated the role of PGE2 in local epithelial injury–induced airway contraction by inhibiting COX-2, an enzyme that mediates PGE2 production. We found that inhibition of COX-2 with 20 μM indomethacin, a nonselective COX inhibitor (50–52), or with 10 μM NS-398, a selective COX-2 inhibitor (24, 53, 54), completely blocked Ca2+ oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca2+ wave in epithelial cells (Figures 2A, Figure E1D, and Movie E4).

Because there are four EP receptors presented on airway smooth muscle cells (55), we next attempted to determine whether a selective EP receptor(s) mediates the local epithelial injury–induced airway contraction. We found that 10–25 μM L-798106, a selective EP3 antagonist (56–59), and 100 μM AH6809, a nonselective rat EP1, EP2, and EP3 receptor inhibitor (59–61), completely blocked Ca2+ oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca2+ wave in epithelial cells (Figure 2B, Figure E1E, and Movie E5).

To investigate the order in which A1 and EP3 receptors were activated, we assessed the effects of CPA, a selective A1R agonist (45, 62), and 11-PGE2, a stable synthetic analog of PGE2 that selectively activates EP3 receptor (61, 63), on airway caliber in the presence and absence of EP3 receptor antagonist L-798106. We found that both 10 μM CPA and 11-PGE2 induced airway contraction (Figure 2C). Inhibition of EP3 receptors with L-798106 significantly blocked airway contraction induced by CPA or 11-PGE2, but not by 25 mM KCl, a VGCC agonist (Figure 2C). However, inhibition of A1R with 50 μM PSB36 did not block airway contraction with 11-PGE2 (Figure 2C). Thus, the role of A1R is upstream of EP3 and VGCC in the activation of smooth muscle contraction after airway epithelial injury.

**Adenosine A1R Activates EP3 Receptors via EGFR/PDGFR Pathway**

It has been demonstrated that adenosine A1 receptor mediates the transactivation of the EGFR in rat cortical neurons (17), whereas activation of receptor tyrosine kinases, such as EGFR and PDGFR, could increase the production of PGE2 in human or guinea pig tracheal smooth muscle cells (23, 24). Thus, we hypothesized that receptor tyrosine kinases are necessary for adenosine A1R–mediated EP3 receptor activation. To assess the role of receptor tyrosine kinases in local epithelial injury–induced airway contraction, we blocked receptor tyrosine kinases with 100 μM AG18, an inhibitor of EGFR and PDGFR (64–66), and 10 μM AG1478 (24, 67, 68) or 10 μM AG1296 (68–70), which are selective inhibitors of EGFR and PDGFR, respectively. We observed that inhibition of EGFR or PDGFR significantly blocked Ca2+ oscillations in smooth muscle cells and airway contraction induced by local epithelial injury, but did not block the Ca2+ wave in epithelial cells (Figures 3A, Figure E1F, and Movie E6).
To demonstrate whether the receptor tyrosine kinase pathway is involved in adenosine A$_1$R–mediated activation of EP$_3$ receptors, we stimulated A$_1$ and EP$_3$ receptors with CPA and 11-PGE$_2$, respectively, in the presence and absence of EGFR inhibitor, AG1478. We found that inhibition of EGFR with 10 μM AG1478 significantly blocked airway contraction induced by 10 μM CPA, but not by 10 μM 11-PGE$_2$ or 25 mM KCl (Figure 3C). Thus, the role of EP$_3$ and VGCC in the activation of smooth muscle contraction is downstream of EGFR.

**DISCUSSION**

Local epithelial injury induces airway hyperresponsiveness (1); however, the underlying mechanism has not been identified. In this study, we investigated the underlying mechanisms of the rapid (<10 s) communication between local epithelial injury and airway constriction by combining a lung tissue slice model with a femtosecond laser ablation technique. We first identified adenosine as the soluble mediator initiating local epithelial injury–induced airway contraction via A$_1$R activation. We then revealed a novel signaling pathway that includes the sequence A$_1$R→EGFR/PDGFR→COX-2→EP$_3$→VGCCs→CICR, leading to increase in Ca$^{2+}$ oscillations in airway smooth muscle cells and initiation of airway constriction. For the first time, we show a sequential link between adenosine A$_1$R, receptor tyrosine kinases, including EGFR and PDGFR, and prostaglandin receptor EP$_3$ in airway smooth muscle cells.

ATP regulates multiple biological responses, such as airway hyperresponsiveness in the lungs (6). As an energy source, ATP is maintained at a very high level in the cytoplasm of airway epithelial cells. Upon epithelial injury, the local concentration of ATP can rapidly increase to 125 μM (38) and initiate a Ca$^{2+}$ wave in airway epithelium by activating P2Y receptors on epithelial cells (4). However, the effects of local epithelial injury–induced ATP release on airway caliber have not been studied. In this study, we found that local epithelial injury–induced ATP activated both airway epithelial and smooth muscle cells. Locally, ATP activates P2 purinergic receptors on neighboring epithelial cells; however, over the time (<5–10 s [1]) it takes to diffuse to the underlying smooth muscle, ATP is rapidly (<1 s [71]) degraded to adenosine, leading to the stimulation of adenosine A$_1$ receptors on smooth muscle cells (Figure 4). The hypothesis that ATP is rapidly degraded into adenosine is supported by the results that ATP-induced airway contraction is blocked by inhibition of adenosine receptors (Figure 1D), but not by inhibition of P2 purinergic receptors (1). In the present study, we confirmed the role of adenosine in the local epithelial injury–induced airway contraction by both inhibiting A$_1$ receptors and neutralization of adenosine with ADA; however, we cannot rule out the involvement of AMP, because AMP, which has been shown to activate A$_1$ receptors (9), might also be decreased by ADA.

ATP has been demonstrated to induce airway smooth muscle contraction through either directly activating P2X or P2Y on mouse airway smooth muscle cells (7) or indirectly stimulating P2Y receptors on epithelial cells, which release prostaglandins in guinea pig trachea (20). We can eliminate the direct effect of ATP on airway smooth muscle cells, because inhibition of P2X or P2Y on smooth muscle cells did not block the local epithelial injury–induced smooth muscle contraction (1). Furthermore, we can rule out the indirect effect of ATP on airway epithelial cells, because inhibition of P2 purinergic receptors on epithelial cells significantly decreased the Ca$^{2+}$ wave in the epithelium, but did not block the local epithelial injury–induced airway contraction (1). Thus, we have identified a novel pathway in which adenosine derived from local epithelial injury–released ATP stimulates A$_1$ receptors on smooth muscle cells to initiate airway contraction in rat lung tissue slices.

The levels of adenosine in bronchoalveolar lavage fluid are increased in asthma, and hyperresponsiveness to adenosine is a hallmark of asthma (72). Adenosine is traditionally thought to induce airway contraction indirectly by activation of adenosine receptors,
Inhibition of COX-2 with indomethacin and NS-398 completely blocked the local epithelial injury–induced airway contraction, demonstrating the involvement of prostanoids in this process. To confirm this, we found that inhibition of EP3 receptors also blocked the airway contraction induced by local epithelial injury. Although PGE2 is traditionally considered an agonist of EP3 receptors, PGI2 has recently been shown to activate EP3 receptors (59), indicating that either PGE2 or PGI2 could be released upon local epithelial injury. Our results are consistent with the studies in which activation of receptor tyrosine kinases leads to COX-2 expression (75–77) and PGE2 generation (24). Our results are also consistent with the studies showing that smooth muscle cell contraction can be stimulated by self-generated prostanoids (23).

VGCC-mediated Ca2+ influx induces a large amount of intracellular Ca2+ release, which regulates numerous cellular functions, including smooth muscle contraction. In our previous study, we demonstrated that inhibition of VGCCs with nifedipine completely blocked local epithelial injury–induced airway contraction. We further showed that inhibition of A1 and EP3 receptors did not block KCl-induced airway contraction (Figures 2C and 3C), whereas inhibition of VGCCs blocked A1 and EP3 receptor agonist-induced airway contraction (data not shown).

These results suggest that A1R-, EGFR-, and EP3 receptor-mediated pathways are upstream and dependent on VGCCs.

We acknowledge the use of relatively high concentrations of some chemical inhibitors in comparison to reported negative log dissociation constants or half maximal inhibitory concentration (IC50) (Table E1), and thus off-target effects for one or more of the compounds are possible. However, in our present study, we report a series of compelling observations (multiple agonists and antagonists for each receptor) that are all consistent with the revealed molecular pathway underlying local epithelial injury–induced airway contraction. Thus, it is essentially impossible that all potential off-target effects would lead to the same set of conclusions.

In conclusion, we have identified a novel sequence of events that provides the underlying mechanism by which local airway epithelial injury can induce global airway smooth muscle contraction. Airway epithelial cell injury releases ATP, which is rapidly degraded to adenosine. Adenosine can diffuse to the underlying smooth muscle cells through gap junctions and activates A1 receptors on the airway smooth muscle cells. Although adenosine has been shown to activate A1 and A2B receptors on human airway smooth muscle cells and regulate adenyl cyclase and Ca2+ signaling (15, 16), the underlying mechanism leading to mobilization of Ca2+ is not clear.

In this study, we show that inhibition of EGFR/PDGFR completely blocks the local epithelial injury–induced airway contraction. We further demonstrate that inhibition of EGFR significantly blocks the A1R agonist–induced airway contraction, but not EP3 agonist–induced contraction (Figure 3C). The results indicate that receptor tyrosine kinases, including EGFR and PDGFR, are necessary for A1R-mediated EP3 activation. Our results are consistent with those of previous studies in which adenosine A1 receptors mediated the transactivation of EGFRs in neural cells (17). Because receptor tyrosine kinases regulate many cellular functions, such as cell migration, differentiation, proliferation, apoptosis, and inflammation (73, 74), it is possible that local epithelial injury–induced receptor tyrosine kinase activation may have an even broader impact on pulmonary pathology. Multiple signaling pathways could be involved in EGFR/PDGFR–induced COX-2 production, such as phosphoinositide 3-kinase/Akt/NF-κB (24), mitogen-activated protein kinase/mitogen-activated protein kinase (23, 75), or c-Src (76), and further study is needed to reveal the downstream signaling pathways.

**References**


**Author disclosures** are available with the text of this article at www.atsjournals.org.

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