Raf-1 kinase mediates adenylyl cyclase sensitization by TNF-α in human airway smooth muscle cells

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Osawa Y, Yim PD, Xu D, Panettieri RA, Emala CW. Raf-1 kinase mediates adenylyl cyclase sensitization by TNF-α in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 292: L1414–L1421, 2007. First published February 2, 2007; doi:10.1152/ajplung.00123.2006.—Tumor necrosis factor (TNF)-α is a potent inflammatory cytokine implicated in the exacerbation of asthma. Chronic exposure to TNF-α has been reported to induce G protein-coupled receptor desensitization, but adenylyl cyclase sensitization, in airway smooth muscle cells by an unknown mechanism. Cyclic AMP, which is synthesized by adenylyl cyclases in response to G protein-coupled receptor signals, is an important second messenger involved in the regulation of the airway muscle proliferation, migration, and tone. In other cell types, TNF-α receptors transactivate the EGF receptor, which activates raf-1 kinase. Further studies in transfected cells show that raf-1 kinase can phosphorylate and activate some isoforms of adenylyl cyclase. Cultured human airway smooth muscle cells were treated with TNF-α in the presence or absence of inhibitors of prostaglandin signaling, protein kinases, or G proteins. TNF-α caused a significant dose- (1–10 ng/ml) and time-dependent (24 and 48 h) increase in forskolin-stimulated adenylyl cyclase activity, which was abrogated by pretreatment with GW5074 (a raf-1 kinase inhibitor), was partially inhibited by an EGF receptor inhibitor, but was unaffected by pertussis toxin. TNF-α also increased phosphorylation of Ser388 on raf-1 kinase, indicative of activation. IL-1β and EGF sensitization of adenylyl cyclase activity was also sensitive to raf-1 kinase inhibition by GW5074. Taken together, these studies link two signaling pathways not previously characterized in human airway smooth muscle cells: TNF-α transactivation of the EGF receptor, with subsequent raf-1 kinase-mediated activation of adenylyl cyclase.

GW5074: mitogen-activated protein kinase; phosphorylation; immuno blot; cell culture

ASTHMA TYPICALLY INDUCES REVERSIBLE airway obstruction and is orchestrated in part by a complex interplay between many inflammatory cytokines (18, 24, 27). Traditionally, the influx of leukocytes with secretion of cytokines during asthma exacerbations has received the most attention, but multiple cells within the airway respond to, and themselves synthesize, cytokines, including airway smooth muscle (24). Tumor necrosis factor (TNF)-α is one cytokine implicated in the exacerbation of asthma (3, 6, 10), and recent clinical studies raise hope that targeted therapy against TNF-α may improve asthma symptoms (6). Cyclic AMP is an important second messenger involved in the regulation of the contractile state, proliferation (8, 25, 34), migration (19), and cytokine secretory abilities (1) of airway smooth muscle cells. Previous studies in human airway smooth muscle cells have shown that cytokines, such as TNF-α and IL-1β, directly influence cellular cAMP levels by “sensitizing” the activities of adenylyl cyclase enzymes (9, 29), but the intracellular signaling pathways for the modification of adenylyl cyclase activity are unknown.

Cyclic AMP is synthesized by a nine-member family of membrane-associated adenylyl cyclase enzymes. These family members are differentially regulated both acutely and chronically. Acute regulation by phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) or by G protein βγ-subunits has been most extensively characterized. Chronic regulation leading to increased enzyme activity has been most commonly demonstrated following chronic activation of G1-coupled receptors, which, via raf-1 kinase-mediated phosphorylation, leads to increased enzyme activity and has been variably referred to as “sensitization,” “supersensitization,” “cAMP overshoot,” “supersensitivity,” “superactivation,” or “heterologous sensitization” (36, 37).

Sensitization of adenylyl cyclase in airway smooth muscle cells has been reported following chronic exposure to agonists for receptors that couple to G1 (7, 26) cytokines (9, 29) or following rhinovirus exposure (9). In airway smooth muscle cells, all chronic G1-coupled receptor-mediated sensitization of adenylyl cyclase has been blocked by pertussis toxin (PTX) (7, 26), consistent with a G1→raf-1 kinase-mediated sensitization pathway reported in other cell types following chronic opioid receptor activation (13, 35, 36). However, the role for raf-1 kinase in mediating adenylyl cyclase sensitization in airway smooth muscle cells has not been directly demonstrated. Moreover, the kinases potentially involved in cytokine-mediated (i.e., TNF-α, IL-1β) sensitization of adenylyl cyclase in airway smooth muscle cells are unknown.

Understanding the mechanisms of cytokine-induced alterations in signal transduction pathways in human airway smooth muscle is important, as cytokines are an important component of the inflammatory response in asthmatic airways. In some cells, the activation of the TNF-α receptor is known to transactivate the EGF receptor (EGFR), which, in turn, can activate raf-1 kinase (2, 12). In unrelated experiments in transfected human embryonic kidney-293 cells, EGF activation of raf-1 kinase mediates phosphorylation and activation of adenylyl cyclase (5). We questioned whether the cellular signaling mechanism of TNF-α-mediated sensitization of adenylyl cyclase activity in native human airway smooth muscle cells.

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involved the transactivation of the EGFR, resulting in raf-1 kinase-mediated adenylyl cyclase sensitization.

METHODS

Materials. Cells were cultured in SmGM-2 smooth muscle medium (Cambrex, Walkersville, MD). Trypan blue stain 0.4% solution was obtained from Gibco (Grand Island, NY). [α-32P]ATP (800 CI/mmol) and [3H]cAMP (32 Ci/mmol) were obtained from MP Biomedicals (Irvine, CA). Recombinant human TNF-α, PD98059, the EGFR/erbB-2 inhibitor [4-(4-benzyloxyanilino)-6,7-dimethoxyquinazoline, 4557W], and the monoclonal antibody (MAb) directed against the EGFR (Mab225) were obtained from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cell culture. Primary cultures of human airway smooth muscle cells were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings, as previously described (28). The cells were grown to confluence on 24-well or 6-well plates in culture medium (SmGM-2, supplemented with 5% FBS, 5 µg/ml insulin, 1 ng/ml human fibroblast growth factor, 500 pg/ml human EGF, 30 µg/ml gentamicin, and 15 ng/ml amphotericin B, Cambrex) at 37°C in 5% CO2-95% air. In all studies, EGF was omitted from the media at least 10 days before the beginning of treatments. Media containing serum and growth factors were changed twice weekly, but not changed for the 72 h immediately preceding 48-h treatments with TNF-α (72 h of media conditioning before 48-h TNF treatments).

Treatment protocols. Preliminary studies revealed an enhanced effect of TNF-α on forskolin-stimulated adenylyl cyclase sensitization in 72-h conditioned media. Therefore, in all studies, cell culture media (supplemented SmGM-2 devoid of EGF) were not changed for 72 h before the beginning of treatment with 10 ng/ml TNF-α. Preliminary experiments were performed to determine the optimum time and dose effect of TNF-α treatments. Forskolin-stimulated adenylyl cyclase activity was measured after 7, 24, or 48 h of TNF-α with redosing of TNF-α at 24 h for the 48-h time point. Subsequently, the dose effect (0.01–10 ng/ml) TNF-α was assayed for 48 h. Based on these preliminary studies, all subsequent TNF-α incubations were at 10 ng/ml for 48 h with redosing of TNF-α at 24 h.

In some studies, an inhibitor of prostaglandin synthesis, indomethacin (10 µM), was added 30 min before the beginning of 48-h TNF-α treatments. These inhibitors were also redosed at 24 h. In some studies, cells were pretreated with PTX 4 h before the beginning of 48-h TNF-α treatments.

Preincubation with the kinase inhibitors specific for PKC (100 nM GF19203X), mitogen-activated protein (MAP) kinase (MEK) (50 µM PD98059), p38 MAP kinase (MAPK) (10 µM SB203580), tyrosine kinases (20 µM genistein), phosphatidylinositol 3-kinase (500 nM wortmannin), or raf-1 kinase (10 µM GW5074) were initially attempted for 30 min before the beginning of the 48-h TNF-α treatment. However, cell toxicity was evident with all kinase inhibitors during this prolonged incubation time, except for GW5074 (data not shown). Therefore, for relevant time point comparisons, all kinase inhibitors (GF19203X, PD98059, SB203580, genistein, wortmannin, GW5074) were added directly to the wells (to achieve final concentrations of 50 µM GW5074, 100 µM wortmannin, and 100 µM genistein) that functionally occurred with concentrations of TNF-α ranging from 1 to 10 ng/ml, with a peak effect at 10 ng/ml (441 ± 86% of control).

Cell viability. Cell viability following TNF-α (10 ng/ml) exposure for 48 h in the presence or absence of GW5074 (10 µM) was measured in triplicate by Trypan blue dye exclusion. After incubation for 5 min with 0.2% Trypan blue in PBS, 300 cells in each group were counted, and the cells that internalized the dye were considered nonviable.

Statistics. Statistical analysis was performed using repeated-measures of ANOVA, followed by Bonferroni posttest comparison using Prism 4.0 software (GraphPad, San Diego, CA). Data are presented as means ± SEM; P < 0.05 was considered significant.

RESULTS

Adenylyl cyclase activity following TNF-α treatment of human airway smooth muscle cells. TNF-α demonstrated both a concentration-dependent and time-dependent sensitization of forskolin-stimulated adenylyl cyclase activity. Significant sensitization of forskolin-stimulated adenylyl cyclase activity was achieved at 24 h of TNF-α (10 ng/ml) treatment (Fig. 1A). Sensitization of forskolin-stimulated adenylyl cyclase activity occurred with concentrations of TNF-α ranging from 1 to 10 ng/ml, with a peak effect at 10 ng/ml (441 ± 86% of control).
PKC, and Raf-1 kinase, are known to affect adenylyl cyclase sensitization. Smooth muscle cells were pretreated with PTX (inactivates Gi/o proteins by ADP-ribosylation). PTX pretreatment had no effect on the TNF-α-induced sensitization of adenylyl cyclase in these human cultured airway smooth muscle cells (Fig. 3). These results suggest that sensitization of adenylyl cyclase activity by TNF-α is not mediated by prostaglandin release in these cells.

Effect of Giα on TNF-α-induced adenylyl cyclase sensitization. Adenylyl cyclase sensitization has classically been described following the chronic activation of Giα-coupled receptors (37). To determine whether TNF-α-induced sensitization of adenylyl cyclase involved Gi proteins, human airway smooth muscle cells were pretreated with PTX (inactivates Gi/o proteins by ADP-ribosylation). PTX pretreatment had no effect on the TNF-α-induced sensitization of adenylyl cyclase in these human cultured airway smooth muscle cells (Fig. 3).

Effect of kinase inhibitors on TNF-α-induced adenylyl cyclase sensitization. Multiple protein kinases, notably PKA, PKC, and Raf-1 kinase, are known to affect adenylyl cyclase activity via subtype-specific phosphorylation (11, 17, 33). Raf-1 kinase has been reported to mediate the sensitization of adenylyl cyclase in a PTX-sensitive manner following chronic Giα activation (17). Additionally, cytokines such as TNF-α are known to activate a myriad of intracellular kinase pathways (4, 22). Therefore, we questioned whether pretreatment with specific kinase inhibitors could attenuate TNF-α-induced adenylyl cyclase sensitization. Inhibitors of PKC, MEK, p38 MAPK, tyrosine kinases, phosphatidylinositol 3-kinase, or Raf-1 kinase (GF109203X, PD98059, genistein, wortmannin, GW5074, respectively) were added during the final 1 h (Fig. 4A) or the final 6 h (Fig. 4B) of the 48-h TNF-α incubation.

The addition of the Raf-1 kinase inhibitor GW5074 (10 μM) during the final 1 h (Fig. 4A) or the final 6 h (Fig. 4B) of the 48-h TNF-α treatment period attenuated the TNF-α-induced sensitization of adenylyl cyclase activity. This is in contrast to the lack of effect of all other kinase inhibitors tested at these 1- and 6-h time points (Fig. 4). Addition of the kinase inhibitors alone for either 1 or 6 h had no effect on forskolin-stimulated adenylyl cyclase activity (data not shown). In separate studies, the Raf-1 kinase inhibitor GW5074 (10 μM) was added 30 min before 48 h of TNF-α, which completely reversed the TNF-α-induced sensitization of adenylyl cyclase activity (Fig. 5A). GW5074 alone for 48.5 h had no effect on forskolin-stimulated adenylyl cyclase activity. GW5074 pretreatment also blocked

![Fig. 1. A: time course of 10 μM forskolin-stimulated adenylyl cyclase (AC) activity in cultured human airway smooth muscle cells. Cells were treated with tumor necrosis factor (TNF)-α (10 ng/ml) for the indicated times. B: 10 μM forskolin-stimulated AC activity in cultured human airway smooth muscle cells treated with TNF-α at indicated concentrations for 24 h. Data represent means ± SE; n = 4. Within each experiment, values were determined in triplicate. ***P < 0.001 compared with control.]

![Fig. 2. 10 μM forskolin-stimulated AC activity in cultured human airway smooth muscle cells following 48-h TNF-α (10 ng/ml) in the presence or absence of indomethacin (Ind) (10 μM 30 min before TNF-α). Data represent means ± SE; n = 3. Within each experiment, values were determined in triplicate. ***P < 0.001 compared with control.]

![Fig. 3. 10 μM forskolin-stimulated AC activity in cultured human airway smooth muscle cells following 48-h TNF-α (10 ng/ml) treatment in the presence or absence of pertussis toxin (PTX) (100 ng/ml for 4 h before 48-h TNF-α treatment). Data represent means ± SE; n = 5. Within each experiment, values were determined in triplicate. ***P < 0.001 compared with control.]

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adenylyl cyclase sensitization induced by 48-h exposure to IL-1β (Fig. 5B) and EGF (Fig. 5C).

Effect of Raf-1 kinase inhibitor on cell viabilities. Pretreatment with 10 μM GW5074 for 48.5 h did not affect cell viabilities of the cells treated with TNF-α, as detected by Trypan blue exclusion. Viabilities of human airway smooth muscle cells treated with vehicle, 48-h TNF-α (10 ng/ml), or 48-h TNF-α plus 48.5-h GW5074 (10 μM) were 81.2 ± 4.4, 78.4 ± 4.1, or 78.0 ± 2.6%, respectively (P > 0.05, n = 5). These results suggest that TNF-α-induced adenylyl cyclase sensitization is mediated by raf-1 kinase.

Effect of TNF-α on phosphorylation of raf-1 kinase. Phosphorylation of Ser338 on the raf-1 kinase protein is indicative of kinase activation, which, in turn, is known to sensitize certain isoforms of adenylyl cyclase (15, 16). Therefore, we directly measured phosphorylation of Ser338 on raf-1 kinase following TNF-α treatment for 24 and 48 h in human airway smooth muscle cells. Twenty-four or 48 h of TNF-α treatment significantly increased phosphorylation of Ser338 on raf-1 kinase (Fig. 6, A and B), with no change in total raf-1 kinase. In separate experiments, pretreatment of cells with an inhibitor of the EGFR before 24 h of TNF-α treatment significantly attenuated the phosphorylation of raf-1 kinase (Fig. 6C).

Effect of EGFR activation on TNF-α-induced adenylyl cyclase sensitization. EGFR transactivation has been reported to mediate TNF-α effects in various cell types (2, 12). To investigate the potential role of EGFR signaling in the adenylyl cyclase sensitization induced by TNF-α, adenylyl cyclase activity was measured in the presence or absence of a specific inhibitor of EGFR/erbB-2 tyrosine kinase or using a MAb directed against the EGFR. Pretreatment with the EGFR inhibitor partially blocked TNF-α effect on adenylyl cyclase activity (Fig. 7A). The EGFR inhibitor also inhibited EGF-induced adenylyl cyclase sensitization induced by 48-h exposure to IL-1β (Fig. 5B) and EGF (Fig. 5C).
adenylyl cyclase sensitization, indicating that this agent successfully inhibited the EGF signaling pathway (Fig. 7B). These results suggest that TNF-α-mediated transactivation of the EGFR could be at least one mechanism of TNF-α-induced adenylyl cyclase sensitization. In contrast, pretreatment with 2 μg/ml of EGFR antibody (MAb225) did not block the increase of forskolin-stimulated adenylyl cyclase activity induced by TNF-α, whereas MAb225 completely reversed EGF-induced sensitization of adenylyl cyclase (Fig. 8).

**DISCUSSION**

This study illustrates for the first time in native human airway smooth muscle cells that a mechanism of TNF-α-induced adenylyl cyclase sensitization involves the transactivation of the EGFR and activation of raf-1 kinase. These studies link together two cellular signaling pathways not previously identified or linked in these cells: 1) TNF-α-induced transactivation of the EGFR with raf-1 kinase activation, and 2) raf-1 kinase-mediated sensitization of adenylyl cyclase.

Sensitization of adenylyl cyclase activity in human airway smooth muscle cells has previously been demonstrated in response to cytokines (9, 29), rhinovirus (9), and chronic activation of Gi-coupled receptors (7). Sensitization of adenylyl cyclase activity by chronic activation of Gi-coupled receptors was inhibited by PTX (7), consistent with a pathway shown in other cells Gi → raf-1 kinase → adenylyl cyclase; however, a total adenylyl cyclase protein expression in cells left untreated vs. 48-h TNF-α treated (Fig. 9).

**Effect of TNF-α on total protein expression of adenylyl cyclase isoforms.** To determine whether an increased expression of adenylyl cyclase proteins could account for TNF-α-induced adenylyl cyclase sensitization, we performed immunoblot analysis of cultured human airway smooth muscle cells exposed to TNF-α for 48 h using an antibody against common sequence of all cloned mammalian membrane-associated adenylyl cyclase isoforms. There was no significant change in total adenylyl cyclase protein expression in cells left untreated or 48-h TNF-α treated (Fig. 9).

**Fig. 6.** A: Western blot analysis of phospho-Raf-1 or total Raf-1 expression in cultured human airway smooth muscle cells. Cells were treated with TNF-α (10 ng/ml) for the indicated times. A representative of three experiments is shown. B: relative band intensities of phospho-/total Raf-1 from three separate immunoblots. The effect of 24-h and 48-h treatment with TNF-α (10 ng/ml) on Raf-1 phosphorylation is given as percentage of control (n = 5). C: relative band intensities of phospho-/total Raf-1 indicating the effect of pretreatment with an EGFR inhibitor (EGFR) inhibitor (10 μM, 30 min) before 24-h treatment with TNF-α (10 ng/ml; n = 3). Values are means ± SE. *P < 0.05, **P < 0.01 compared with control. #P < 0.05 compared with 24-h TNF-α.

**Fig. 7.** 10 μM forskolin-stimulated AC activity in cultured human airway smooth muscle cells following 48-h TNF-α (10 ng/ml, n = 6; A) or EGF (1 ng/ml, n = 4; B) treatment in the presence or absence of EGFR/erbB-2 inhibitor (inh) (10 μM for 30 min before each 48-h treatment). Within each experiment, values were determined in triplicate. Data represent means ± SE. *P < 0.05, ***P < 0.001 compared with control. ##P < 0.01 compared with TNF or EGF.
role for raf-1 kinase in adenylyl cyclase sensitization in human airway smooth muscle cells has not been shown. In contrast, sensitization of adenylyl cyclase activity in response to IL-1β was not PTX sensitive (9), in agreement with the present study using TNF-α.

Multiple previous studies have investigated the mechanisms of cytokine-induced G protein-coupled receptor (GPCR)-induced desensitization vs. adenylyl cyclase sensitization (9, 23, 29, 31). Pascaul et al. (29) demonstrated that concomitant treatment of human airway smooth muscle cells with IL-1β and TNF-α or EGF for 18 h potentiated the induction of COX-2 and PGE2 and caused hyporesponsiveness of the GPCR for isoproterenol and prostaglandin E2. Conversely, 18-h treatment of human airway smooth muscle cells with TNF-α, IL-1β, or EGF alone induced sensitization of adenylyl cyclase via a PGE2-independent pathway (29). However, the underlying mechanisms of TNF-α-induced sensitization of adenylyl cyclase were not well understood. Our present study agrees with these findings in that TNF-α-induced sensitization of forskolin-stimulated adenylyl cyclase activity was not affected by pretreatment with indomethacin and thus not dependent on prostaglandin synthesis. These previous studies have shown that cytokines typically decrease β-adrenoceptor and prostaglandin receptor stimulation of adenylyl cyclase, while simultaneously increasing the direct (i.e., basal or forskolin-stimulated) activity of adenylyl cyclase (9, 23, 29, 31). Thus the sensitization of adenylyl cyclase has been proposed to be a compensatory mechanism for the decreased ability of receptor pathways to stimulate adenylyl cyclase.

Sensitization of adenylyl cyclase has been most widely described after chronic stimulation of a Gi-protein coupled receptor. Classically following a chronic exposure to an agonist that acutely inhibits adenylyl cyclase activity, subsequent stimulation of adenylyl cyclase activity results in “overshoot” or supersensitization of adenylyl cyclase activity. This Gi-protein required sensitization of adenylyl cyclase is PTX sensitive. Therefore, in the present study, we questioned whether Gi proteins could be intermediates in the TNF-α-induced sensitization of adenylyl cyclase, but pretreatment with PTX failed to reverse the effect of TNF-α. Therefore, the cellular mechanisms of adenylyl cyclase sensitization by chronic Gi activation vs. TNF-α exposure are different.

Although the regulation of adenylyl cyclase activity is primarily by G protein or calcium-calmodulin interactions, alternate mechanisms involving regulation of the phosphorylation state of the adenylyl cyclase isoforms have also been identified. Because isoforms of adenylyl cyclases are known to be differentially regulated by multiple protein kinases, including PKA (33), PKC (11), raf-1 kinase (17, 36), and tyrosine kinases (32), we examined the effects of various protein kinase inhibitors. In the present study, we demonstrated that TNF-α treatment increased the phosphorylation of Ser338 on raf-1 kinase, which is indicative of enzyme activation (16). Raf-1 is the key protein kinase in the MAPK signal transduction cascade. Recently, it was shown that acute TNF-α-mediated enhancement of p42/44 MAPK phosphorylation in canine tracheal smooth muscle cells involved the activation of the Ras/Raf-1 pathway (16), consistent with the present study. In the present study, a MEK inhibitor failed to inhibit TNF-α-induced adenylyl cyclase sensitization, suggesting that raf-1-mediated sensitization of adenylyl cyclase does not require activation of the MAPK cascade. Although raf-1 is known to classically function upstream as an initiator of the Ras/Raf/ERK signaling cascade, raf-1 has been shown to directly activate adenylyl cyclase, independent of activation of the rest of the MAPK cascade (32), consistent with the present study.

We found that only GW074, a selective raf-1 kinase inhibitor, could reverse the TNF-α-induced sensitization of adenylyl cyclase. Interestingly, addition of this inhibitor during the last 1 h or the last 6 h of the 48-h TNF exposure was sufficient to block the sensitization of adenylyl cyclase activity (Fig. 4). This would suggest that the raf-1 kinase-mediated activation of adenylyl cyclase is a reversible event and requires ongoing raf-1 kinase activation to maintain adenylyl cyclase in a phosphorylated and activated state.

In the present study, exposure to IL-1β and EGF also sensitized adenylyl cyclase in a GW074-sensitive manner, suggesting that sensitization of adenylyl cyclase induced by these cytokines and growth factor may share a common signaling pathway involving raf-1 activation. Activation of EGFR is known to stimulate raf-1 and subsequently potentiate adenylyl cyclase type VI (AC6) activation in AC6-transfected human embryonic kidney-293 cells (5), which is consistent with the present study. Recently, TNF-α has been shown to induce EGFR transactivation, which, in turn, mediates cell proliferation and cell survival in various cell lines (2, 12). In the present study, an EGFR-selective inhibitor partially blocked TNF-α-
induced sensitization of adenylyl cyclase and phosphorylation of Raf-1 kinase. This suggests that transactivation of the EGFR is at least partly responsible for the effect of TNF-α on adenylyl cyclase sensitization. Transactivation of the EGFR is known to be induced by various stimuli, such as GPCR ligands, receptor tyrosine kinase agonists, cytokines, and cell adhesion elements (20). This involves both ligand-independent intracellular signaling mechanisms and metalloprotease-mediated release of the EGF-like ligands (20). Transactivation of the EGFR by TNF-α-induced metalloprotease processing of transforming growth factor-α has been demonstrated in hepatocytes and mammary epithelial cells (2, 12). Therefore, in the present study, we questioned whether certain EGF-like ligands could be intermediates in the TNF-α-induced sensitization of adenylyl cyclase. However, pretreatment with a MAβ against the EGFR failed to reverse the effect of TNF-α, while completely blocking the effect of EGF on adenylyl cyclase activity. Taken together, these results suggest that a component of TNF-α-induced sensitization of adenylyl cyclase activity involves transactivation of the EGFR, leading to Raf-1 kinase phosphorylation and activation, but that ligand occupancy of the EGFR is not required for Raf-1 kinase activation in human airway smooth muscle cells.

Although PKC or tyrosine kinases are known to be upstream of Raf-1 kinase and modulate its activation in some cells (14, 32), inhibitors of these kinases (GF109203X or genistein, respectively) failed to inhibit TNF-α-induced adenylyl cyclase sensitization. Moreover, the failure of inhibition of PKC, MEK, p38 MAPK, PI3 kinase, or tyrosine kinases inhibit TNF-α-induced adenylyl cyclase sensitization suggests that these kinases neither were directly responsible for altering adenylyl cyclase activity nor were these kinases acting upstream to activate Raf-1 kinase. However, because the catalytic activity of Raf-1 kinase is modulated by multiple independent mechanisms, it is possible that blocking an individual signaling pathway to Raf-1 kinase may be insufficient to block its activation. For example, Varga et al. (36) reported that only simultaneous inhibition of both tyrosine kinase and PKC obliterated chronic opioid agonist-induced sensitization of adenylyl cyclase.

Recently, adenylyl cyclase II, V, VI, but not I, have been reported to be phosphorylated by Raf-1 (17) and directly sensitize adenylyl cyclase (32). We previously found that adenylyl cyclase V and AC6 are the major adenylyl cyclase isoforms expressed in cultured human airway smooth muscle cells (38). Thus the TNF-α-induced sensitization of adenylyl cyclase via Raf-1-dependent pathway likely involves these adenylyl cyclase isoforms. In the present study, treatment with TNF-α did not affect total protein expression of adenylyl cyclase isoforms, indicating that TNF-α-induced sensitization is mediated by activation, but not increased protein expression of adenylyl cyclase isoforms.

In summary, the present study demonstrates that chronic exposure to the inflammatory cytokine TNF-α induces G protein-dependent sensitization of adenylyl cyclase in cultured human airway smooth muscle cells. This TNF-α effect is at least partly attributable to transactivation of the EGFR, which in turn activates and phosphorylates Raf-1 kinase, which sensitizes adenylyl cyclase activity. A better understanding of the cellular signaling mechanisms underlying regulation of adenylyl cyclase activity under conditions of chronic cytokine exposure should improve therapies directed at airway smooth muscle tone in asthma.

GRANTS

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