TNF-α upregulates G_{iα} and G_{qα} protein expression and function in human airway smooth muscle cells

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Hotta, Kunihisa, Charles W. Emala, and Carol A. Hirshman. TNF-α upregulates G_{iα} and G_{qα} protein expression and function in human airway smooth muscle cells. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L405–L411, 1999.—Chronic inflammation is a characteristic feature of asthma. Multiple inflammatory mediators are released within the asthmatic lung, some of which may have detrimental effects on signal transduction pathways in airway smooth muscle. The effects of tumor necrosis factor (TNF)-α on the expression and function of muscarinic receptors and guanine nucleotide-binding protein (G protein) α-subunits were examined in human airway smooth muscle cells. Cultured human airway smooth muscle cells were incubated in serum-free culture medium for 72 h in the presence and absence of 10 ng/ml of TNF-α, after which the cells were lysed and subjected to electrophoresis and G_{iα}, G_{qα}, and G_{αi-3} protein subunits were detected by immunoblot analysis with specific antisera. TNF-α treatment for 72 h significantly increased the expression of G_{iα-2} and G_{qα} proteins and enhanced carbachol (10^{-7} M)-mediated inhibition of adenylyl cyclase activity and inositol phosphate synthesis. These data provide new evidence demonstrating that TNF-α not only increases expression of G_{iα-2} and G_{qα} proteins but also augments the associated signal transduction pathways that would facilitate increased tone of airway smooth muscle.

cytokines; asthma; adenylyl cyclase; inositol phosphate

CHRONIC INFLAMMATION is a characteristic feature of asthma. There is increasing evidence that proinflammatory cytokines, which are released from lung macrophages (12), mast cells (3, 11), eosinophils (6, 10), and epithelial cells (7), play an important role in producing and perpetuating airway inflammation. Several lines of evidence suggest that tumor necrosis factor (TNF)-α is important in the pathogenesis of asthma. First, TNF-α is produced locally in the lung, in close proximity to the airway smooth muscle, and both TNF-α and mRNA for TNF-α are found in the bronchoalveolar lavage fluid and bronchial biopsies of asthmatic patients (4). Second, levels of TNF-α detected in bronchoalveolar lavage fluid are increased in symptomatic compared with asymptomatic asthmatic patients (4), and TNF-α mRNA expression increases in allergic inflammation (30). Third, inhaled TNF-α increases airway responsiveness to serotonin in rodents (16) and to methacholine in normal volunteers (26), whereas the TNF-α receptor antagonist Ro-45-2081 prevents allergen-induced bronchial hyperresponsiveness in guinea pigs (21).

Evidence is now accumulating that proinflammatory cytokines play an important role in regulating airway smooth muscle tone. TNF-α impairs β-adrenoceptor-mediated airway smooth muscle relaxation in guinea pig (29) and rabbit tracheae (14) and enhances thrombin- and bradykinin-induced intracellular Ca^{2+} release and inositol phosphate turnover in cultured human airway smooth muscle cells (2).

A major determinant of airway smooth muscle tone is the concentration of the second messenger cAMP, which is synthesized by the enzyme adenylyl cyclase. Emala et al. (8) previously showed that pretreatment of cultured canine airway smooth muscle cells with TNF-α for 72 h produced a specific impairment in the ability of β-adrenoceptors to stimulate adenylyl cyclase activity, with no decrease in β-adrenoceptor number. These data provide one explanation for the impaired β-adrenoceptor-mediated relaxation seen in previous studies (14, 29). However, adenylyl cyclase is under dual regulation, and an upregulation of the inhibitory pathway is an alternative or additional possibility. TNF-α is known to modulate the expression of guanine nucleotide-binding (G) protein Gi, the G protein that inhibits adenylyl cyclase. Reithmann et al. (20) reported that treatment with TNF-α for 48 h upregulated G_{αi} protein in rat cardiomyocytes, whereas Scherzer et al. (24) reported that treatment with TNF-α for 10 min increased the expression of G_{αi-2} and G_{αi-3} proteins in polymorphonuclear leukocytes.

Because TNF-α alters many signaling pathways in airway smooth muscle cells, it is unclear whether the functional effects of chronic TNF-α exposure result from G protein α-subunit upregulation. The correlation between levels of G protein expression and changes in signaling pathways appears to be cell and pathway specific. A stoichiometric study (18) of G proteins suggested that an overabundance of G proteins is available for transmitting cellular signals such that increases or decreases in G protein expression have no effect on downstream signaling. In contrast, multiple studies (1, 17, 20, 23) in a variety of cell types have shown a correlation between changes in G protein expression and changes in the function of downstream signaling pathways.

Although it is known that TNF-α increases the expression of G_{αi} protein in cardiomyocytes and polymorphonuclear leukocytes and impairs β-adrenoceptor-mediated relaxation of airway smooth muscle, it is not known whether this upregulation of the inhibitory pathway of adenylyl cyclase by TNF-α occurs in human airway smooth muscle, whether expression of other G protein α-subunits are altered by TNF-α, and, if so,
whether this upregulation is functionally important in the regulation of airway smooth muscle tone.

Therefore, with the use of cultured human airway smooth muscle cells, the aim of the present study was to evaluate the effects of chronic TNF-α exposure on the expression and function of muscarinic receptors, the G protein α-subunits that couple to these receptors, and the second messengers downstream from these G proteins.

METHODS

Cell culture and TNF-α treatment protocols. Primary cultures of previously characterized (15, 28) human tracheal smooth muscle cells were a kind gift from Dr. Ian Hall (Queens Medical Center, Nottingham, UK). These cells that express functionally coupled M3 muscarinic receptors and low levels of M1 muscarinic receptors were grown in 75-cm2 cell culture flasks containing culture medium (M-199 medium, 100 units/ml of penicillin G, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 10% fetal bovine serum) at 37°C in 5% CO2-95% air. The cells were plated on 24-well plates and incubated until they reached confluence. Subsequently, the confluent cells in 24-well plates were incubated in serum-free M-199 medium for 72 h (chosen because a previous study (8) showed effects on adenylyl cyclase function at this time point) in the presence and absence (control) of 10 ng/ml of human recombinant TNF-α, with the medium and TNF-α being replaced every 24 h.

Immunoblot analysis. Expression of Gαi2, Gαq, and Gαs proteins was determined by immunoblot analysis. At the end of the treatment with TNF-α, the cells were washed three times with serum-free medium, and the cells from each group were lysed in 100 µl of sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol) for immunoblot analysis. The remaining wells from each of the three groups were harvested for cell viability quantification with trypan blue staining. Thirty microliters of each sample were electrophoresed at room temperature through 10% polyacrylamide gels at 80 V. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes overnight at a constant voltage of 20 V at room temperature in a transfer buffer (192 mM glycine, 25 mM Tris, and 10% methanol).

After transfer, the membranes were washed twice in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, and 500 mM NaCl) and were incubated in TBS containing 1% nonfat dry milk at room temperature for 90 min to block nonspecific protein binding. G protein antiserum AS/7, QL, and RM/1 for Gαi2, Gαq, and Gαs proteins at 1:500, 1:250, and 1:1000 dilutions, respectively, in TBS-0.1% Tween 20 (TBS-T) containing 1% nonfat dry milk and 0.2% sodium azide were added, and the PVDF membranes were incubated with gentle rocking at room temperature for 2-16 h. After incubation with the G protein antiserum, the PVDF membranes were washed three times for 10 min each with TBS-T. The PVDF membranes were then incubated for 90 min at room temperature in goat anti-rabbit IgG conjugated to alkaline phosphatase at a 1/10,000 dilution in TBS-T containing 1% nonfat dry milk. After incubation with the goat anti-rabbit IgG, the PVDF membranes were washed three times for 10 min each with TBS-T. The G proteins were detected with enhanced chemiluminescence immunoblotting detection reagents according to the manufacturer’s recommendations (Immunolite II, Bio-Rad, Hercules, CA), with subsequent exposure to autoradiography film. The intensities of the immunoblots were quantified with a scanner coupled to a personal computer with MacBas 2.2 software.

To ensure that the band intensities that we measured fell within the linear range of the autoradiographic film response, a series of control exposures was performed. Increasing quantities of cellular protein were subjected to immunoblot analysis as described above with the primary antibody that recognizes Gαi protein. The relative band intensities obtained were plotted against the increasing amount of cellular protein analyzed. This yielded a standard curve of band intensities, with a linear range against which band intensities from control and TNF-α-treated cells were compared. All immunoblot band intensities of Gαi2, Gαq, or Gαs protein that were analyzed for TNF-α-induced changes fell within the linear response range of the film.

To determine whether TNF-α altered the growth of cultured human airway smooth muscle cells and therefore the amount of protein applied to each lane of the gel, immunoblot measurements were corrected for cell number. The arbitrary intensities of the immunoblots were log transformed.

Adenylyl cyclase assays. Adenylyl cyclase activity was measured by the quantification of the synthesis of [α-32P]cAMP from [α-32P]ATP. Assays were performed in 24-well plates. The cells were washed three times with serum-free medium after TNF-α treatment. The cells were immediately lysed in 100 µl of lysis buffer (10 mM HEPES, pH 8.0, 2 mM EDTA, and 100 µM phenylmethylsulfonyl fluoride) for 45 min at 37°C. Adenylyl cyclase assays were performed for 20 min at 30°C in a total volume of 150 µl composed of 100 µl of lysed cells and 50 µl of assay buffer [final concentration: 0.5 mM 3-isobutyl-1-methylxanthine; 50 mM HEPES, pH 8.0; 50 mM NaCl; 0.4 mM EGTA; 1 mM cAMP; 7 mM MgCl2; 0.1 mM ATP; 7 mM creatine phosphate; 50 units/ml of creatine phosphokinase; 0.1 mg/ml of BSA; 10 µCi/ml of [α-32P]ATP (specific activity 800 Ci/mmol), and the indicated effectors]. Preliminary experiments confirmed the linearity of adenylyl cyclase activity at the protein concentrations and incubation times used. The reactions were terminated by the addition of 150 µl of stop buffer [50 mM HEPES, pH 7.5, 2 mM ATP, 0.5 mM cAMP; 2% SDS, and 1 µCi/ml of [3H]cAMP (specific activity 25 Ci/mmol)]. [α-32P]cAMP was recovered by sequential column chromatography (22). Recovery rates of columns were 75-90%. To investigate whether increases in G protein α-subunit expression were reflected in adenylyl cyclase activity, we examined the inhibitory effect of carbachol (10-7 M) on forskolin (10-5 M)-stimulated adenylyl cyclase activity in control and TNF-α-treated cells. Adenylyl cyclase activity was corrected for cell number and is expressed as picomoles of cAMP per milligram of protein per 20 min. Adenylyl cyclase activity in response to carbachol is expressed as the percent inhibition of forskolin-stimulated adenylyl cyclase activity.

Inositol phosphate assays. [H]inositol phosphate formation was measured with a modification of the method of Wedegaertner et al. (27). Assays were performed on confluent cells in 24-well plates. The medium was replaced with [inositol-free Dulbecco’s modified Eagle’s medium (DMEM) containing 10 µCi/ml myo-[3H]inositol (specific activity 20 Ci/mmol) in the presence and absence of TNF-α on the day before the assay, 48 h after the beginning of TNF-α treatment. Inositol phosphate accumulation was measured in control or TNF-α-treated cells under basal (unstimulated) and bradykinin-stimulated (0.1 µM) conditions. On the day of assay, the cells were washed with DMEM containing 10 mM LiCl three times and were incubated in 270 µl of the same buffer for 15 min at 37°C. Thirty microliters of agonist or vehicle were then added, and the samples were incubated for 30 min at 37°C. The reaction was terminated by the addition of 330 µl of cold...
methanol. Then 660 µl of chloroform were added, and the samples were transferred to an Eppendorf tube. The phases of the samples were separated by centrifugation at 820 g for 10 min at 4°C. Four hundred microliters of the upper aqueous phase were transferred to a new glass tube. Three hundred microliters of cold 50 mM formic acid and one hundred microliters of 3% ammonium hydroxide were added. Total [3H]inositol phosphates were finally separated from free myo-[3H]inositol by chromatography on Dowex AG1-X columns. The 850-µl samples were loaded onto preequilibrated columns, and then 1 ml of 50 mM NH4OH was added. These 1.85-ml fractions represented free inositol ([3H]inositol). After a wash with 5 ml of 40 mM ammonium formate-0.2 M formic acid, the [3H]inositol phosphate fraction was eluted with 5 ml of 40 mM ammonium formate-0.2 M formic acid twice and 2 ml of 4 M ammonium formate-0.1 M formic acid. Eluted radioactivity was counted after addition of the scintillation cocktail.

Saturation radioligand binding. To determine whether TNF-α treatment for 72 h altered the number of muscarinic receptors, the total muscarinic-receptor number was quantified in human airway smooth muscle cells in the presence and absence of TNF-α, after which the cells were harvested from 75-cm² flasks and incubated in a saturating concentration (1 nM) of [3H]QNB (specific activity 47 Ci/mmol; specific activity 47 Ci/mmol) twice and 2 µM atropine. Binding assays were terminated by filtration over GF/B glass fiber filters followed by three washes with 5 ml of cold normal saline (0.9% NaCl). The filters were then counted in 5 ml of scintillation cocktail. Total muscarinic-receptor numbers were determined from the number of specifically bound counts. Protein assays were performed on sample aliquots, and muscarinic-receptor numbers are expressed as femtomoles of muscarinic receptor per milligram of protein.

Trypan bluestaining for cell count. Because treatment with TNF-α for 72 h may affect cell growth, the immunoblot measurements and adenylyl cyclase activity were corrected for cell number. Cells receiving either TNF-α or vehicle for 72 h were washed with serum-free medium three times, after which the cells were detached with the use of 100 µl of trypsin. Then 50 µl of M-199 medium containing 10% BSA were added, and the cells were stained with 50 µl of trypan blue. Five minutes after the cells were stained, an aliquot (5 × 10⁴ - 10⁵) was counted with a hemocytometer.

Protein determination. Protein content was assayed with the Pierce Chemical (Rockford, IL) bicinchoninic acid protein assay reagent with BSA as a standard (25).

Materials. The primary cultures of human airway smooth muscle cells used in this study have been extensively characterized (15, 28) and were a kind gift from Dr. Ian Hall (Queens Medical Center, Nottingham, UK). G protein antisera (AS/7, QL, and RM/1), [α-32P]ATP (specific activity 800 Ci/mmol), [3H]cAMP (specific activity 25 Ci/mmol), and myo-[3H]inositol (specific activity 20 Ci/mmol) were obtained from NEN (Boston, MA). Goat anti-rabbit IgG was obtained from Bio-Rad (Hercules, CA). PVDF membranes were obtained from Millipore (Bedford, MA). [3H]QNB (specific activity 47 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Cell culture reagents were obtained from Gibco BRL (Grand Island, NY). All other reagents were obtained from Sigma (St. Louis, MO).

Statistics. All data are means ± SE. All data were analyzed by two-tailed paired Student’s t-test. The null hypothesis was rejected when P < 0.05.

RESULTS

Immunoblot analysis. Immunoblot analysis was performed to investigate the alterations in the expression of G protein α-subunits after 72 h of TNF-α treatment (Fig. 1A). Relative to control cells, TNF-α treatment significantly increased the expression of Gsα by 107% [−log(intensities of immunoblots) = 4.12 ± 0.173 and 4.41 ± 0.154 for control and TNF-α-treated cells, respectively; P = 0.001; n = 7 independent experiments] and Giα protein by 39% [−log(intensities of immunoblots) = 4.05 ± 0.201 and 4.18 ± 0.218 for control and TNF-α-treated cells, respectively; P = 0.02; n = 6 independent experiments; Fig. 1B]. In contrast, the expression of Gqα protein was not significantly different between control and TNF-α-treated cells [−log(intensities of immunoblots) = 4.15 ± 0.0475 and 4.18 ± 0.0447 for control and TNF-α-treated cells, respectively; P = 0.218] (10 ng/ml of TNF-α treatment; Cells incubated in presence and absence of 10 ng/ml of TNF-α for 72 h were subjected to SDS-PAGE through 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with G protein antisera (AS/7, QL, and RM/1 for Gi2, Giα, and Gsα, respectively). G protein α-subunits were detected on autoradiography films after exposure with goat anti-rabbit IgG and enhanced chemiluminescence immunoblot detection reagents. TNF-α increased expression of Gi2 and Giα but not of Gqα. B: quantitative measurements of expression of G protein α-subunits in human airway smooth muscle cells after TNF-α treatment. Intensities of immunoblots for G protein α-subunits were determined by densitometry and corrected for cell number. Data are means ± SE. TNF-α increased expression of Gi2α by 107% (n = 7 experiments) and Gqα by 39% (n = 4 experiments) without significant alteration of expression of Gqα (n = 4 experiments). *P < 0.05 compared with control.
respectively; \( P = 0.56; n = 4 \) independent experiments; Fig. 1B). Intensities of the immunoblots were corrected for cell numbers.

Comparison of immunoblot band intensities is only accurate when multiple factors relative to electrophoresis conditions and film exposure are controlled for. All immunoblot comparisons were made with control and TNF-\( \alpha \)-treated samples that were subjected to analysis on the same gels to control for potential variation in electrophoresis conditions. Moreover, a standard curve of band intensities was made to ensure that all measured band intensities fell within the linear range of autoradiographic film responsiveness (Fig. 2A). Increasing quantities of cellular protein generated a linear increase in band intensity over a limited range (Fig. 2B). All bands analyzed in this study fell within this linear range. The intensity of the single lightest and single darkest bands analyzed in this study is indicated in Fig. 2.

Adenylyl cyclase assays. To investigate whether TNF-\( \alpha \)-induced increased expression of G\(_{\alpha i-2}\) protein altered inhibitory effects on adenylyl cyclase, the effect of carbachol (which activates muscarinic receptors that couple to G\(_{\alpha i-2}\) protein, inhibiting adenylyl cyclase) on forskolin (10\(^{-5}\) M)-stimulated adenylyl cyclase activity was examined in control and TNF-\( \alpha \)-treated cells.

Forskolin (10\(^{-5}\) M)-stimulated adenylyl cyclase activity was 37 ± 4.7 and 54 ± 8.7 pmol cAMP·mg protein\(^{-1}\)·20 min\(^{-1}\) for control and TNF-\( \alpha \)-treated cells, respectively (\( P = 0.09; n = 5 \) independent experiments performed in duplicate). In control cells, carbachol (0.1–1.0 \( \mu \)M) significantly inhibited forskolin-stimulated adenylyl cyclase activity (Fig. 3). Seventy-two hours of TNF-\( \alpha \) pretreatment resulted in significantly greater adenylyl cyclase inhibition by carbachol (10\(^{-7}\) M; Fig. 4). Inhibition of forskolin-stimulated adenylyl cyclase activity by 10\(^{-7}\) M carbachol was 1.9 ± 4.8 and 31 ± 9.3% for control and TNF-\( \alpha \)-treated cells, respectively (\( P = 0.036; n = 5 \) independent experiments performed in duplicate).

Saturation radioligand binding. To determine whether the enhanced inhibition of forskolin-stimulated adenylyl cyclase activity by carbachol seen after TNF-\( \alpha \) treatment was due to an increase in muscarinic-receptor number, total muscarinic-receptor numbers were measured by saturation radioligand binding. TNF-\( \alpha \) treatment significantly decreased, not increased, the muscarinic-receptor number (1,104 ± 236 and 548 ± 230 fmol/mg protein for control and TNF-\( \alpha \)-treated cells, respectively).

Fig. 2. Confirmation of quantification of immunoblot band intensities by densitometry. A: increasing quantities of cellular protein were subjected to immunoblot analysis on same gel with primary antibody QL directed against G\(_{\alpha i-2}\). 6×, 4×, 2×, 1×, and 0.6×, relative amounts of protein added to gel. B: band intensities were measured by densitometry and plotted against relative amount of cellular protein. This determined range of band intensities that had a linear relationship to amount of cellular protein analyzed. Band intensities that were used to quantitate G\(_{\alpha} \) protein expression before and after TNF-\( \alpha \) treatment were then compared with this control linear plot. Darkest and lightest bands used in analysis of G\(_{\alpha} \) protein expression were within linear range of band intensity.

Fig. 3. Adenylyl cyclase activity in response to carbachol in control cultured human airway smooth muscle cells under forskolin (10\(^{-5}\) M)-stimulated condition. Adenylyl cyclase assay was performed on 24-well plates after 72-h incubation without serum. Data are means ± SE; \( n = 5 \) experiments. Carbachol (1–10 \( \mu \)M) significantly inhibited forskolin-stimulated adenylyl cyclase activity in control cells.

Fig. 4. Effect of TNF-\( \alpha \) on adenylyl cyclase activity in cultured human airway smooth muscle cells. Data are means ± SE; \( n = 5 \) experiments. Pretreatment with TNF-\( \alpha \) for 72 h significantly enhanced carbachol (10\(^{-7}\) M)-induced inhibition of forskolin-stimulated adenylyl cyclase activity. *\( P < 0.05 \) compared with control.
treated cells, respectively; Fig. 5; P = 0.0001; n = 3 independent experiments performed in triplicate).

Inositol phosphate assays. To investigate whether the increase in the expression of $G_{q\alpha}$ results in an enhancement of downstream inositol phosphate synthesis, the accumulation of total inositol phosphate was examined in control and TNF-$\alpha$-treated cells. TNF-$\alpha$ pretreatment significantly increased the synthesis of inositol phosphate under basal conditions. Total inositol phosphate accumulation was $2,835 \pm 520$ and $4,095 \pm 563$ dpm for control and TNF-$\alpha$-treated cells, respectively (P = 0.012; n = 4 independent experiments performed in duplicate; Fig. 6, left). This represented a $148 \pm 10.8\%$ increase in basal inositol phosphate accumulation in cells treated with TNF-$\alpha$ relative to control cells. TNF-$\alpha$ treatment also resulted in a marked augmentation in inositol phosphate accumulation in response to 0.1 $\mu$M bradykinin ($22,919 \pm 5,307$ and $33,256 \pm 7,442$ dpm for control and TNF-$\alpha$-treated cells, respectively; P = 0.024; n = 4 independent experiments performed in duplicate; Fig. 6, right). This represented a $148 \pm 7.5\%$ increase in bradykinin-stimulated inositol phosphate accumulation relative to control cells.

Cell count. Treatment with TNF-$\alpha$ for 72 h resulted in a small decrease in total airway smooth muscle cell numbers ($23,000 \pm 4,000$/well and $19,000 \pm 3,500$/well for control and TNF-$\alpha$-treated cells, respectively; P < 0.0001; n = 12 independent experiments performed in duplicate)

DISCUSSION

In the present study, treatment of cultured human airway smooth muscle cells with TNF-$\alpha$ for 72 h increased the expression of $G_{q\alpha1}$ and $G_{q\alpha3}$ proteins, with no effect on the expression of $G_{q\alpha}$ protein. Moreover, TNF-$\alpha$ treatment enhanced carbachol-induced inhibition of adenylyl cyclase activity and enhanced basal and bradykinin-induced levels of inositol phosphate synthesis. These functional effects of TNF-$\alpha$ were associated with decreased numbers of muscarinic receptors, suggesting that the TNF-$\alpha$-induced increase in $G_{q\alpha1}$ and $G_{q\alpha3}$ subunits resulted in increased activity of their respective downstream second messenger pathways.

The results of the present study are consistent with those of previous studies demonstrating increases in the expression of G protein $\alpha$-subunits by TNF-$\alpha$ in other cell types. TNF-$\alpha$ increased $G_{q\alpha1}$ subunit expression in cultured rat cardiomyocytes (20) and in human polymorphonuclear leukocytes (24). Although Amrani et al. (2) did not measure G protein $\alpha$-subunit expression, they reported enhanced thrombin- and bradykinin-induced intracellular Ca$^{2+}$ release and enhanced inositol phosphate turnover in response to NaF in cultured human airway smooth muscle cells treated with TNF-$\alpha$ for 24 h. An increase in $G_{q\alpha}$ subunit expression, which was observed in the present study, would be consistent with their findings. The findings of the present study are also consistent with those of Hakonarson et al. (14), who reported that pretreatment of rabbit tracheal smooth muscle for 24 h with the proinflammatory cytokine interleukin-1$\beta$ impaired relaxation and enhanced $G_{q\alpha1}$ and $G_{q\alpha3}$ subunit expression. Although isoproterenol-stimulated cAMP generation was decreased in the cytokine-pretreated tissue, $G_i$ protein-activated inhibition of cAMP was not directly measured.

Adenylyl cyclase is under the dual regulation of a stimulatory $G_s$ protein and an inhibitory $G_i$ protein. Increases in the expression of $G_{q\alpha3}$ protein with no change in $G_{q\alpha}$ protein would enhance the inhibitory pathway, leading to lower levels of adenylyl cyclase activity. In the present study, enhanced adenylyl cyclase inhibition by carbachol was seen in TNF-$\alpha$-treated cells. This enhanced inhibition of forskolin-stimulated adenylyl cyclase activity by TNF-$\alpha$ could not be accounted for by an increase in muscarinic-receptor number because we measured a decrease, not an increase, in muscarinic-receptor number after TNF-$\alpha$ pretreatment. This indicates that the enhanced inhibition of forskolin-stimulated adenylyl cyclase activity by carbachol in the TNF-$\alpha$-treated cells must be due to increased expression of the $G_{q\alpha3}$ subunit, resulting in enhanced downstream cellular function.
In contrast to our study, TNF-α did not decrease the expression of M₂ muscarinic-receptor protein in HEL 299 cells, although a combination of TNF-α or interleukin-1β led to downregulation of M₂ muscarinic receptors in these cells (13). The difference between their report and our study most likely is due to the cell type and/or species studied. Our finding of enhanced carbachol inhibition of adenyl cyclase with decreased numbers of muscarinic receptors suggests that the expression of the Gᵢ protein rather than the expression of the muscarinic receptor plays a more important role in the ultimate inhibition of adenyl cyclase. Indeed, a previous study (23) reported that TNF-α enhanced a bradykinin-mediated increase in phosphatidylinositol turnover despite a decrease in bradykinin-receptor number.

Stimulation of Gₐq protein results in increased inositol phosphate synthesis and intracellular Ca²⁺. TNF-α treatment increased the expression of the Gₐq subunit and enhanced basal and bradykinin-induced inositol phosphate synthesis in the present study, which indicates that an increased level of Gₐq protein resulted in enhanced downstream second messenger function. This is consistent with a study in human A-431 cells in which TNF-α decreased bradykinin-receptor numbers yet still resulted in increased inositol phosphate accumulation in response to bradykinin (23). The increased basal inositol phosphate accumulation after TNF-α seen in the present study, which correlates with increased Gₐq protein, is also consistent with increased basal inositol phosphate accumulation seen in a cell line overexpressing a constitutively active mutant of Gₐq protein (5). Furthermore, our data are also consistent with a previous study (2) in which TNF-α enhanced inositol phosphate turnover in response to NaF (which activates G proteins at a site distal to a receptor), suggesting an upregulation of Gₐq protein and function by TNF-α.

Previous studies (15, 28) in the cultured human airway smooth muscle cells used in the present study have shown that carbachol produced only small increases in inositol phosphate synthesis because M₃ muscarinic-receptor expression is downregulated when compared with M₃ muscarinic-receptor expression in freshly dispersed airway smooth muscle cells or muscle tissue. Although we did not examine the effect of carbachol on inositol phosphate synthesis in the present study, it is possible that TNF-α could enhance carbachol-stimulated inositol phosphate synthesis in vivo by upregulating the expression of Gₐq protein.

Several studies (16, 21, 29) have shown that TNF-α contributes to airway hyperresponsiveness. We demonstrated upregulation of Gₐ₁₂ and Gₐq subunit expression, enhanced carbachol-induced inhibition of adenyl cyclase activity and enhanced synthesis of inositol phosphate after TNF-α pretreatment. The enhanced carbachol-induced inhibition of adenyl cyclase activity and inositol phosphate synthesis are consistent with a functional role for TNF-α in asthma. These results suggest that the upregulation of Gₐ₁₂ and Gₐq subunit expression could be one of the molecular mechanisms by which TNF-α contributes to airway hyperresponsive-ness. It is also possible, but less likely, that the increased function of the downstream signaling pathways seen in the present study was not due to an increase in G protein expression but to a TNF-α-induced change in an other protein(s) important to the function of these pathways.

A previous study (8) in cultured canine airway smooth muscle cells showed that TNF-α treatment for 72 h, but not for 24 h, impaired β-adrenergoreceptor-mediated airway smooth muscle relaxation, which is why we chose 72 h of treatment for investigating the effect of TNF-α on the expression of G protein α-subunits. The present study did not evaluate the time course for TNF-α effects on Gₐα and Gqα protein expression. Other investigators have reported that TNF-α increased expression of G protein α-subunits in human polymorphonuclear leukocytes after 10 min of exposure (24) and in rat cardiomyocytes after 48 h of exposure (20). The mechanism of the acute upregulation of Gᵢ protein is likely to be very different from that seen in the present study. It is also possible that the upregulation of Gᵢ and Gq proteins may start earlier than after 72 h of treatment in cultured human airway smooth muscle cells.

Controversy exists over the ability of changes in G protein expression to affect the function of downstream signaling pathways. A study (18) of the stoichiometry of G₃ protein signaling suggests that this protein may be present in great excess of receptors with which it couples. In this setting, severalfold changes in G₃ protein expression would be expected to have no effect on downstream signaling pathways. In contrast, multiple functional studies (1, 17, 19, 20, 23) in a variety of cell types suggest that changes in G protein amounts correlate with functional changes in signaling pathways. These differences may be due to variable expression of protein components of signaling pathways in different cells or under different conditions.

In summary, treatment with TNF-α for 72 h increased the expression of Gᵢ₁₂ and Gqα but not of Gₐα proteins in cultured human airway smooth muscle cells. This increased expression of G protein α-subunits increased the activity of their respective downstream second messenger pathways, which could potentially lead to increases in airway smooth muscle tone. These findings suggest that increased expression of Gᵢ₁₂ and Gqα proteins are additional mechanisms by which TNF-α could contribute to airway hyperresponsiveness in asthma.

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