Mechanisms underlying TNF-α effects on agonist-mediated calcium homeostasis in human airway smooth muscle cells

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Amrani, Yassine, Vera Krymskaya, Christopher Maki, and Reynold A. Panettieri, J. R. Mechanisms underlying TNF-α effects on agonist-mediated calcium homeostasis in human airway smooth muscle cells. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1020–L1028, 1997.—We have previously shown that tumor necrosis factor (TNF)-α, a cytokine involved in asthma, enhances Ca2+ responsiveness to broncechiorconstrictor agents in cultured human airway smooth muscle (ASM) cells. In the present study, we investigated the potential mechanism(s) by which TNF-α modulates ASM cell responsiveness to such agents. In human ASM cells loaded with fura 2, TNF-α and interleukin (IL)-1β significantly enhanced thrombin- and bradykinin-evoked elevations of intracellular Ca2+. In TNF-α-treated cells, Ca2+ responses to thrombin and bradykinin were 350 ± 14 and 573 ± 93 nM vs. 130 ± 17 and 247 ± 48 nM in nontreated cells, respectively (P < 0.0001). In IL-1β-treated cells, the Ca2+ response to bradykinin was 350 ± 21 vs. 127 ± 12 nM in nontreated cells (P < 0.0001). The time course for TNF-α potentiation of agonist-induced Ca2+ responses requires a minimum of 6 h and was maximum after 12 h of incubation. In addition, cycloheximide, a protein synthesis inhibitor, completely blocked the potentiating effect of TNF-α on Ca2+ signals. We also found that TNF-α significantly enhanced increases in phosphoinositide (PI) accumulation induced by bradykinin. The percentage of change in PI accumulation over control was 115 ± 8 vs 210 ± 15% in control cells vs. 128 ± 10 to 437 ± 92% in TNF-α-treated cells for 3 × 10–9 to 3 × 10–6 M bradykinin. The PI turnover to 10 mM NaF, a direct activator of G proteins, was also found to be enhanced by TNF-α. The percentage of change in PI accumulation over control increased from 280 ± 35% in control cells to 437 ± 92% in TNF-α-treated cells. Taken together, these results show that TNF-α can potently regulate G protein-mediated signal transduction in ASM cells by activating pathways dependent on protein synthesis. Our study demonstrates one potential mechanism underlying the enhanced Ca2+ response to broncechiorconstrictor agents induced by cytokines in human ASM cells.

asthma; bronchial hyperreactivity; cytokines; inflammation; hyperresponsiveness; tumor necrosis factor-α

TUMOR NECROSIS FACTOR (TNF)-α may be an important mediator in airway inflammatory diseases such as asthma and bronchiolitis (20). Increased levels of TNF-α have been found in the bronchoalveolar fluid of symptomatic asthmatic patients (6), and administration of TNF-α to healthy volunteers (40) and animals (21, 42) induces a bronchial hyperreactivity, a characteristic feature of asthma. The role of TNF-α in modulating airway hyperresponsiveness in vivo has recently been shown using Ro-45-2081, a potent TNF receptor (TNFR) antagonist (33). This receptor antagonist prevented allergen-induced bronchial hyperreactivity. These data provide evidence for an implication for TNF-α in asthma. The mechanism(s), however, by which TNF-α induces bronchial hyperreactivity remains unknown.

In isolated airways, TNF-α or interleukin (IL)-1β alters the contractile response of tracheal smooth muscle to cholinergic agonists (30) as well as of tracheal smooth muscle relaxation to β-adrenergic stimulation (13, 44, 45). In cultured human airway smooth muscle (ASM) cells, we have shown that TNF-α significantly enhanced the Ca2+ responsiveness to bronchoconstrictor agents, i.e., bradykinin and carbachol, and induced cell adhesion molecule expression (1, 3, 4, 23). In addition, we showed that TNF-α elicited its cellular effects by activation of the p55 TNFR subtype (TNFRp55) (4). The TNFRp55 has been shown to mediate most effects of TNF-α, such as apoptosis (19, 27), proliferation of fibroblast, and adhesion molecule expression (reviewed in Ref. 39). TNF-α effects mediated by activation of the p75 TNFR subtype (TNFRp75) appear to be more restricted; TNFRp75 is involved in T-cell development, activation (11), and apoptosis (17). Recent studies have revealed several downstream signaling events induced by TNFRp55 activation, namely activation of GTP-binding proteins (46), phosphatidylinositol-specific phospholipase (PL) C (24), and nuclear factor-κB (18). In ASM cells, investigators have recently shown that TNF-α also activates the c-Jun NH2-terminal kinase in rabbit ASM cells (36). However, the downstream signaling events coupling the TNFRRp55 to Ca2+ responses remain unknown.

The aim of the present study was to dissect the mechanisms underlying the enhancement of Ca2+ responsiveness by TNF-α induced by bronchoconstrictor agents. The current study demonstrates that TNF-α markedly enhanced phosphoinositide (PI) accumulation induced by bradykinin and NaF, an agonist that bypasses membrane receptors and directly activates G proteins. Therefore, it is conceivable that the effects of TNF-α on Ca2+ responsiveness may be due to a direct modulatory effect on G protein-mediated signal transduction. We also show that IL-1β, a cytokine that also has been described to alter ASM responsiveness in vitro (37, 45), enhances agonist-induced Ca2+ responses. Taken together, these data suggest that cytokine-induced alteration of Ca2+ homeostasis in ASM cells may represent, in part, a mechanism underlying bronchial hyperreactivity in asthmatic patients.

MATERIALS AND METHODS

Cell culture. The methods of cell culture for human ASM are identical to that previously described (29). Briefly, human
trachea was obtained from lung transplant donors, in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was removed under sterile conditions, and the trachealis muscles were isolated. Through the use of this technique, ~0.5 mg of wet tissue was obtained, minced, centrifuged, and resuspended in 10 ml of buffer containing 0.2 mM CaCl₂, 640 U/ml collagenase, 1 mg/ml soybean trypsin inhibitor, and 10 U/ml elastase. Enzymatic dissociation of the tissue was performed for 90 min in a shaking water bath at 37°C. The cell suspension was filtered through 105-mm Nytex mesh, and the filtrate was washed with equal volumes of cold Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 mg/ml amphotericin B and was replaced every 72 h. Cell counts were obtained in triplicate wells with 0.5% trypsin in 1 mM EDTA solution.

Measurement of cytosolic free Ca²⁺ concentration. ASM cells were plated at low density onto 15-mm coverslips 3–5 days before experiments. All experiments were done with the use of subconfluent cells between the third and fifth passages. Cells were loaded with 2.5 µM fura 2-acetoxymethyl ester [AM; in medium 199 supplemented with 1 mg/ml bovine serum albumin (BSA)] for 1 h at 37°C. After loading, cells were washed with HEPES buffer and replaced with inositol-free medium containing 0.1% BSA (Grand Island, NY). A concentrated solution of 50 µM fura 2-AM was added to the medium and was replaced every 72 h. Cell counts were obtained in triplicate wells with 0.5% trypsin in 1 mM EDTA solution.

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RESULTS

TNF-α and IL-1β potentiate the increase in cytosolic free Ca²⁺ concentration induced by bradykinin in human ASM cells. The effects of TNF-α and IL-1β (10 ng/ml for 24 h) on the increase in cytosolic free Ca²⁺ concentration ([Ca²⁺]i) induced by 1 µM bradykinin are shown in Figs. 1 and 2. As shown in Figs. 1A and 2A, bradykinin induced a typical biphasic increase in [Ca²⁺]i, characterized by a rapid transient peak followed by a sustained elevation of [Ca²⁺]i. Addition of 40 mM KCl during the sustained phase rapidly lowered the sustained elevation of [Ca²⁺]i to baseline. In cells treated with TNF-α or IL-1 for 24 h (Figs. 1B and 2B), both phases of the [Ca²⁺]i increase were potentiated compared with those treated with diluent. These data are summarized as the net values of the peak and sustained increase in [Ca²⁺]i, respectively, obtained in treated versus untreated cells in Figs. 1, C and D, and 2, C and D. In TNF-α-treated cells, the net [Ca²⁺]i increase to bradykinin was 57 ± 93 vs. 247 ± 48 nM in untreated cells (P < 0.0001). In IL-1β-treated cells, the Ca²⁺ response to bradykinin was 350 ± 21 vs. 127 ± 12 nM in untreated cells (P < 0.0001, unpaired Student’s t-test). For the sustained phase of increase in [Ca²⁺]i (Figs. 1D and 2D), there was also a significant difference in cells treated with both cytokines (P < 0.01, unpaired Student’s t-test).

To define the time course by which TNF-α modulates bradykinin-induced cytosolic Ca²⁺ responses, cells were treated for 4, 6, 8, and 12 h with TNF-α, and then the degree of stimulation (percent change in [Ca²⁺]i) was compared with those that were treated with diluent alone. As shown in Fig. 3, the potentiating effects of TNF-α on bradykinin-induced cytosolic Ca²⁺ peak reached a maximal level after 12 h of pretreatment and was sustained over the subsequent time points (n = 60–100 cells/time point, mean ± SE). In nontreated cells, no significant change was observed in the bradykinin-induced Ca²⁺ transient (Fig. 3). These data suggest that cytokines directly modulate Ca²⁺ homeostasis in a time-dependent manner in human ASM cells.

TNF-α and IL-1 also augment the increase in [Ca²⁺]i induced by thrombin. We have previously shown that thrombin increased [Ca²⁺]i in ASM cells (29). We next examined whether TNF-α also alters Ca²⁺ responses induced by thrombin. As shown in Fig. 4, A and B, the biphasic increase in [Ca²⁺]i induced by 1 IU/ml thrombin was greater in TNF-α-treated cells compared with control cells. The net [Ca²⁺] increases to thrombin were 350 ± 14 vs. 130 ± 17 nM in nontreated cells for the peak and 110 ± 7 vs. 150 ± 9 nM for the sustained phase in nontreated cells (P < 0.0001, statistical significance using unpaired Student’s t-test). Similar results

Drugs. Bradykinin and fura 2-AM were purchased from Sigma Chemical (St. Louis, MO). Dulbecco’s modified Eagle’s medium-F-12, fetal calf serum, trypsin, and antibiotics (penicillin and streptomycin) were obtained from Gibco-BRL (Grand Island, NY). α-Thrombin, IL-1, and TNF-α were purchased from Boehringer Mannheim (Indianapolis, IN) and Calbiochem (La Jolla, CA).
were also obtained when cells were pretreated with IL-1 (data not shown). Because cytokines modulated both bradykinin- and thrombin-evoked Ca\(^{2+}\) responses and because our previous studies showed that TNF-\(\alpha\) has no effect on cell surface expression of agonist receptors (3), these data suggest that cytokines modulate Ca\(^{2+}\) responses downstream from the contractile agonist receptor.

Effect of TNF-\(\alpha\) on bradykinin- and NaF-stimulated PI formation. To further dissect the effect of TNF-\(\alpha\) on Ca\(^{2+}\) responses, we postulated that TNF-\(\alpha\) augmented bradykinin-induced PI formation. ASM cells prelabeled with \(^{3}\text{H}\)inositol for 48 h were then incubated with or without 10 ng/ml TNF-\(\alpha\) for 24 h and were stimulated with bradykinin. Figure 5 shows the time course of the inositol monophosphate (IP\(_1\)) generation to bradykinin. In our study, \(^{3}\text{H}\)IP\(_1\) was used as a measure of PI turnover by this method, but similar results were also obtained when measuring \(^{3}\text{H}\)inositol 1,4,5-trisphosphate (\(^{3}\text{H}\)IP\(_3\)) accumulation (data not shown). Addition of \(10^{-6}\) M bradykinin to cultured ASM cells stimulated a rapid accumulation of \(^{3}\text{H}\)IP\(_3\). This rise was
observed as early as 15 s and increased until 30 min. Treatment of cells with TNF-α for 24 h markedly increased bradykinin-induced IP$_1$ formation. The percentage of change over basal level (basal counts/min (cpm) were 159 ± 18) after 5, 10, 20, and 30 min of stimulation with bradykinin was 590 ± 86, 1,324 ± 130, 3,134 ± 268, and 4,361 ± 488%, respectively, in TNF-α-treated cells compared with 273 ± 22, 736 ± 138, 1,406 ± 47, and 2,305 ± 341%, respectively, in nontreated cells. When cells were pretreated with TNF-α, bradykinin-stimulated PI accumulation was significantly enhanced, and the percentage of change over control was 128 ± 10, 217 ± 24, 388 ± 75, and 437 ±
pretreatment of ASM cells with TNF-α significantly augmented transmembrane signaling events downstream from the receptor at either the level of G protein coupling or a more distal site in the signal transduction pathway.

Because agonist-stimulated tracheal smooth muscle contraction is dependent on PLC activation and because generation of IP₃ evokes increases in [Ca²⁺] (8), we next examined whether TNF-α modulates PI turnover induced by bradykinin. Bradykinin-induced PI formation was significantly enhanced in ASM cells that were pretreated with TNF-α. Augmented Ca²⁺ responses induced by TNF-α may be directly due to enhanced PI metabolism or potentially to inhibition of specific phosphatases. Similar results have been reported using human epidermoid carcinoma cells. In these cells, TNF-α treatment enhanced PI turnover generated by bradykinin (34). Interestingly, the authors also found a decrease in cell surface expression of bradykinin receptors. This suggests that TNF-α may enhance coupling of agonist receptors to downstream signaling events and that PLC activity may be altered by TNF-α. Similarly, the increased PI turnover induced by NaF, which has been described to stimulate inositol phospholipid turnover, was seen downstream from the receptor and possibly at the level of the G protein. Our data are consistent with two previous studies that showed a direct modulatory effect of TNF-α on G protein-mediated signal transduction. In rat cardiomyocytes and in human leukocytes, TNF-α significantly enhanced isoproteosin-mediated G protein activation of adenyl cyclase (32) and N-formylmethionyl-leucyl-phenylalanine-stimulated G protein activation (22), respectively. The mechanism by which TNF-α enhanced transmembrane signaling remains unknown. The NaF data suggest that treatment of cells with TNF-α increases receptor coupling at a level downstream from the receptor located at the level of either the G protein or PLC. Because TNF-α has been shown to directly activate G proteins in osteoblast cells (46), it is also possible that TNF-α regulates the activity...
of G protein by enhancing, for example, the formation of the \( \alpha \beta \gamma \)-complex upon stimulation by an agonist. In this manner, the transduction through the G proteins could be increased. Alternatively, TNF-\( \alpha \) may increase the quantity of G protein involved in the receptor coupling. It has already been shown that this cytokine stimulates de novo synthesis of \( G_\alpha \) in some cells (14, 22, 32, 35). Interestingly, this increase in \( G_\alpha \) protein induced by TNF-\( \alpha \) was associated with an alteration in cell responsiveness to subsequent receptor stimulation coupled to the G protein (22, 32, 35). Recently, cytokines have also been reported to increase \( G_1 \) expression in rabbit ASM cells (14). It is unlikely, however, that this mechanism can account for the observed effects of TNF-\( \alpha \) on thrombin-induced Ca\(^{2+} \) mobilization because the thrombin receptor that evokes Ca\(^{2+} \) transients in human ASM is coupled to a non-pertussis toxin-sensitive G protein (28, 29). The effect of TNF-\( \alpha \) may be located at the level of PLC activation. TNF-\( \alpha \) may directly stimulate or "prime" ASM cells for increased PLC through the activation of PLA\(_2\). In some cell types, activation of the TNFRp55 receptor, which mediates TNF-\( \alpha \) effects in ASM cells (4), also stimulates neutral sphingomyelinase-induced PLA\(_2\) activity (5). The arach-
idonate pathway may represent another pathway that mediates TNF-α effects on cell activation. To support this notion, others reported that treatment of rat ASM cells with either TNF-α or IL-1β increased the expression of inducible cyclooxygenase and PLA2 (41). Effects of both cytokines were also inhibited by treating ASM cells with either dexamethasone or cycloheximide. In human bronchial ASM cells, the mitogenic effect of TNF-α was also completely abolished by dexamethasone and aspirin (38). Other investigators who used Swiss 3T3 fibroblasts (7) and osteoblast-like cell lines (46) have reported that TNF-α effects on signal transduction were inhibited by aspirin. Clearly, more studies are needed to dissect whether cytokines can directly modulate G protein expression or PLA2 activation. These two potential mechanisms may not be mutually

Fig. 5. TNF-α enhances bradykinin-induced phosphoinositide (PI) turnover. Time-dependent inositol monophosphate generation in response to bradykinin in TNF-α-treated (●) and TNF-α-untreated (○) airway smooth muscle (ASM) cells. Values are means ± SE from 5 separate experiments performed in quadruplicate (*P < 0.01, statistical significance using analysis of variance (ANOVA)).

Fig. 6. Concentration-dependent inositol monophosphate generation induced by bradykinin. Concentration-dependent inositol monophosphate generation in response to bradykinin in TNF-α-treated (●) and TNF-α-untreated (○) ASM cells. Values are expressed as means ± SE from 6 separate experiments performed in quadruplicate (*P < 0.01, statistical significance using ANOVA).

Fig. 7. NaF, a direct activator of G proteins, evoked inositol monophosphate generation that was enhanced by TNF-α. Cells prelabeled with [3H]inositol were preincubated with TNF-α for 24 h and then were stimulated with 10 mM NaF for 15 min. Values are expressed as means ± SE from 6 separate experiments performed in quadruplicate (*P < 0.01, statistical significance using ANOVA).

Fig. 8. Cycloheximide (CHX) inhibits TNF-α-mediated potentiation of agonist-induced Ca2+ responses. Cells preincubated with cycloheximide (1 and 10 µM) for 15 min were treated with TNF-α for 24 h. Ca2+ responses to 1 µM bradykinin were then studied as described in MATERIALS AND METHODS. Values are expressed as means ± SE from 5 separate experiments (*P < 0.01 and **P < 0.001 compared with cells exposed to TNF-α alone, statistical significance using Student's t-test).
exclusive because cytokines can alter multiple signaling pathways.

Another possibility by which TNF-α potentiated Ca\(^{2+}\) responses is by affecting the Ca\(^{2+}\)- pools activated by the bronchoconstrictor agents. We have shown that the response to thapsigargin, which directly releases Ca\(^{2+}\) from the internal stores, was potentiated by pretreating cells with TNF-α (2, 4). Based on these data, we conclude that, in addition to the receptor coupling, TNF-α could also affect the intracellular stores of Ca\(^{2+}\). Because the sustained phase of elevation of Ca\(^{2+}\) induced by bradykinin and thrombin was also potentiated by TNF-α and IL-1β, it could be possible that both cytokines enhanced Ca\(^{2+}\) influx in our cells. This may result from a direct effect on Ca\(^{2+}\) channels as reported for both cytokines in rat vascular smooth muscle cells (43). In a previous report, we have also shown that, in human ASM cells, the Ca\(^{2+}\) influx pathway was modulated by the filling state of the internal store of Ca\(^{2+}\) (2). These data are consistent with the capacitive model described by Putney (31). The increased Ca\(^{2+}\) influx observed after TNF-α treatment may, therefore, simply be a consequence of the increased magnitude of Ca\(^{2+}\) depletion from the IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores because both phases of elevation of Ca\(^{2+}\), namely the transient and sustained phases, were shown to be linked (2).

In the current study, we also show that ASM cells treated with IL-1β had enhanced agonist-evoked Ca\(^{2+}\) responses to agonists compared with those treated with diluent alone. The ability of IL-1β to mimic the effect of TNF-α on Ca\(^{2+}\) responses may have important implications for understanding the mechanisms that regulate ASM hyperreactivity in asthma. High levels of IL-1β and TNF-α have been detected in the airways of patients with symptomatic asthma (6). Moreover, these cytokines induce bronchial hyperreactivity to agonists either in vivo (40) or in vitro in tracheal and bronchial ASM preparations (30, 37). Because Ca\(^{2+}\) is known to play an important role in the regulation of contractile responses to agonists (reviewed in Ref. 8), our data suggest that cytokine-mediated alteration in Ca\(^{2+}\) homeostasis may represent one mechanism underlying bronchial hyperreactivity in asthma. Because both cytokines were also found to stimulate proliferation of ASM cells (4, 10), the subsequent ASM hyperplasia and hypertrophy may, in part, contribute to bronchial hyperreactivity via an enhanced contractile response or, alternatively, via a decrease in luminal caliber (reviewed in Ref. 28). Additional studies are needed to clarify the precise mechanism(s) by which cytokines alter ASM cell function before new therapeutic interventions can be developed to abrogate these effects.

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