Synergy between Tumor Necrosis Factor α and Interleukin 1β in Inducing Transcriptional Down-regulation of Muscarinic M2 Receptor Gene Expression

IN INVOLVEMENT OF PROTEIN KINASE A AND CERAMIDE PATHWAYS*

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Stimulation of HEL 299 cells with tumor necrosis factor α (TNF-α) or interleukin 1β (IL-1β) had no effect on M₂ muscarinic receptor expression. However, the combination of these two cytokines markedly down-regulated muscarinic M₂ receptor protein and mRNA expression and uncoupled M₂ receptors from adenylyl cyclase. There was no effect of TNF-α and IL-1β on the m2 muscarinic receptor mRNA stability, and nuclear run-on assays showed reduced m2 receptor gene transcription. Sequential cytokine addition suggests that the synergy involves postreceptor events. Although the cAMP-dependent protein kinase inhibitor H8 provided a significant protection against receptor down-regulation, the protein kinase C inhibitor GF109203X had no effect. The ceramide analog C₂-ceramide (N-acetylsphingosine) was without effect on m2 receptor expression. However, a strong synergistic effect was demonstrated when cells were treated with the combination of C₂-ceramide and TNF-α or IL-1β. TNF-α and/or IL-1β combination also activated the 46- and 55-kDa c-Jun NH₂-terminal protein kinases and to a lesser extent p42 and p44 mitogen-activated protein kinase isofoms. Cycloheximide abolished the TNF-α and IL-1β effect, suggesting that de novo protein synthesis is required for receptor down-regulation. These results suggest that the TNF-α and IL-1β synergize to induce transcriptional down-regulation of the M₂ muscarinic receptor, which seems to be mediated through activation of both ceramide and cAMP-dependent protein kinase pathways. Furthermore, these results suggest that M₂ receptor expression is under the control of a cytokine network.

Inflammation is an important component of the acute and chronic phases of asthma in humans. Cytokines released by immune and inflammatory cells infiltrating the airways are well recognized as key mediators in the orchestration and perpetuation of the chronic inflammation in asthma (1, 2). Many cytokines have been implicated in the pathophysiology of asthma. Of particular interest, tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) have been reported to be increased in asthma (2). They are released on mast cell degranulation, by antigen-stimulated T lymphocytes, or from cytokine-stimulated macrophages or monocytes, although other types can be stimulated to secrete TNF-α and IL-1β (3, 4). These pleiotropic cytokines are critical for the initiation of cytokine cascades that facilitate leukocyte recruitment through the induction of adhesion molecule expression and chemotactic protein (chemokine) production from multiple cell types (5–7).

Of the five cloned muscarinic receptor subtypes, the human lung expresses only three muscarinic receptor subtypes that are differentially distributed in the airways (8). Prejunctional M₂ receptors regulating acetylcholine release from cholinergic nerves have been demonstrated in human airways (9), and in vitro studies suggest that they may be dysfunctional in patients with asthma (10). Alteration of these M₂ autoreceptors on parasympathetic nerves was also demonstrated in the guinea pig after ozone exposure, antigen challenge, and viral infection (11). However, the mechanisms by which the function of these receptors is altered in asthma are unclear. We have previously shown that M₂ receptors can be down-regulated by muscarinic and β₂-adrenergic receptor agonists (12, 13), an effect that was not due to reduced m2 receptor gene transcription. These receptors can also be transcriptionally down-regulated by protein kinase C and by the multifunctional cytokine transforming growth factor β1 (14, 15).

To gain better understanding of the regulation of M₂ receptors, particularly in inflammatory diseases such as asthma, and to identify potential endogenous modulators of M₂ receptor function, we have examined the effect of TNF-α and IL-1β on muscarinic M₂ receptor gene expression in HEL 299 cells.

EXPERIMENTAL PROCEDURES

Cell Culture

HEL 299 cells were obtained from the American Type Culture Collection (Rockville, MD; ATCC code CCL 137) and maintained in culture in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) as described previously (12). Treatments, performed on cells at passage 9, were carried out such that cells could be harvested simultaneously at preconfluence.

Radioligand Binding Studies

All the membrane preparation procedures were performed at 4 °C. Cells were treated with human recombinant TNF-α and/or IL-1β (Promega, Southampton, United Kingdom) washed twice with Hank’s balanced salt solution, harvested by cell scraping using ice-cold Tris buffer (25 mM, pH 7.4), and homogenized with an Ultra-Turax homogenizer. Membranes were pelleted by centrifugation at 40,000 × g for 20 min and resuspended in an appropriate volume of Tris buffer. [3H]N-Methyl-

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1 The abbreviations used are: TNF-α, tumor necrosis factor α; IL, interleukin; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal protein kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.
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scopolamine (DuPont NEN) saturation curves were carried out as described previously (12). Binding data were analyzed with the computerized nonlinear regression program LIGAND.

**Cyclic AMP Measurements**

Following stimulation, cells were washed, and the cAMP-phosphodiesterase inhibitor Org 29241 (30 μM) was added to fresh media for 30 min at 37 °C. From each group of treatments basal levels of cAMP were measured, as well as accumulation following forskolin exposure (100 μM) for 10 min in the presence and absence of carbacarb (100 μM). Cyclic AMP content was measured by radioimmunoassay as described previously (12).

**Northern Blot Analysis**

Following treatments, cells were washed twice with Hank’s balanced salt solution, total RNAs were isolated, and mRNA was prepared using a PolyTract® mRNA isolation kit (Promega) according to the manufacturer’s instructions. Samples of mRNA were size fractionated and blot- ted onto Hybond-N filters (Amersham Corp.) by capillary action using 20 × SSC (1 × SSC = 0.15 mM NaCl, 0.015 mM sodium citrate, pH 7.0). Cloned human m2 muscarinic receptor cDNA (a gift from Dr. N. J. Buckley, National Institute for Medical Research, London, UK) was labeled by random priming using [α-32P]dCTP (3000 Ci/mMole; Amer sham). Prehybridizations and hybridizations were carried out at 42 °C as described previously (15). Following hybridization, blots were washed to a stringency of 0.1 × SSC, 0.1% SDS at 65 °C for 30 min before exposure to Kodak X-OMAT-S film. To account for differences in loading or transfer efficiencies of the mRNA, the blots were hybridized with a 1272-base pair PstI fragment from rat glyceraldehyde-3-phosphate dehydrogenase cDNA. The intensities of the signals were then quantified by laser densitometry (Quantity One software; PDI Imageware Systems, New York, NY).

**Nuclear Run-on Assay**

For the measurement of gene transcription, nuclei were prepared, and in vitro transcription was performed with nuclei (5 × 10⁶) using 300 μCi of [α-32P]UTP as described previously (14). Briefly, labeled RNAs were isolated and added to 2 ml of hybridization solution (50% formamide, 5 × SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 5 × Denhardt’s solution, 50 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, 0.02 μg of poly(A), and 0.02 μg poly(G) RNA). Following 4 h of prehybridization in the above buffer, hybridization was carried out at 42 °C for 72 h to 10 μg of the immobilized plasmid pGEM3Z as a control or to plasmids containing inserts of the m2 muscarinic receptor cDNA. Cytoplasmic proteins were boiled for 5 min subsequent to centrifugation for 15 min in sample buffer (62.5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% SDS, 1 μg/ml RNase A, and 10 units/ml RNase T1) at 37 °C for 30 min and then in buffer B (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 0.4% SDS) to a stringency of 55 °C for 30 min and autoradiographed.

**MAP Kinase Assays**

**Extraction of Cytosolic Proteins**—Following treatments, cells were washed in Hank’s balanced salt solution and then scraped into cold lysis buffer (1% Triton X-100, 1% SDS, 1.5% deoxycholate, 20 mM Tris base, pH 7.4, 150 mM NaCl, 20 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 20 μg/ml leupeptin, 200 μg/ml apro tinin, 10 mM NaF, and 20 mM sodium pyrophosphate). Cytosolic proteins were boiled for 5 min subsequent to centrifugation for 15 min in sample buffer (62.5 mM Tris-HCl, 20% glycerol, 2% SDS, and 10 mM 2-mercaptoethanol) and stored at −70 °C until used for Western blot analysis and “in-gel” phosphorylation assay.

**Western Blot Analysis**—The phosphorylation of p44 and p42 MAP kinases (ERK1 and ERK2) were identified and quantified by Western blot analysis using a PhosphoPlus™ MAPK antibody kit (New England Biolabs, Hitchin, UK) according to the manufacturer’s recommendations. Protein samples were separated by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels and then transferred to polyvinylidene difluoride membranes (Amersham) for 1 h at 300 mA in transblotting buffer (0.2 M glycine HCl, 25 mM Tris base, and 20% [v/v] methanol). Nonphosphorylated and phosphorylated MAP kinase proteins were run in parallel and served as negative and positive controls for immunodetection of MAP kinases. To block nonspecific antibody binding, membranes were incubated for 1 h in blocking buffer (PBS, pH 7.4, and 0.1% Tween 20) containing 5% [v/v] nonfat dry milk. Membranes were then incubated overnight at 4 °C with the anti-phosphoMAPK polyclonal antibody used at a dilution of 1:1000 in blocking buffer in which nonfat milk was replaced with 5% bovine serum albumin. Membranes were washed with blocking buffer three times for 5 min each, incubated with a 1:1500 dilution of alkaline phosphatase-conjugated anti-rabbit secondary antibody, and washed, and protein detection was carried out using the CDP-Star® chemiluminescent reagent. Membranes were drained from excess developing solution and exposed to Kodak X-OMAT-S film. To reprobe the membrane, antibodies were stripped using 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris, pH 6.7, at 55 °C for 10 min.

**In-gel Phosphorylation Assay**—Cytosolic proteins (10 μg) were size fractionated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel containing 0.5 mg/ml myelin basic protein kinases or glutathione S-transferase-c-Jun (1-135) (Jun kinases). After electrophoresis SDS was removed by three 20-min washes with 20% propan-2-ol in 50 mM Tris-HCl, pH 8.0, before a further 1-h wash with 50 mM Tris-HCl, pH 8.0, and 5 mM 2-mercaptoethanol (buffer A). A protein denaturation step was followed by two washes for 30 min in buffer A containing 6 M guanidine HCl before renaturation by several washes in buffer A containing 0.4% Tween 40 at 4 °C over 18 h. The gel was then incubated in kinase assay buffer (40 mM HEPES, pH 8.0, 5 mM 2-mercaptoethanol, 100 mM MgAc, and 25 μM ATP) containing 25 μCi of [γ-32P]ATP for 1 h. Nonspecific radioactivity was removed by five washes with 5% trichloroacetic acid and 1% sodium pyrophosphate before drying under a vacuum and subsequent exposure to Kodak X-OMAT-S film for an appropriate time.

**RESULTS**

**M2 Receptor Binding**

We first sought to determine whether TNF-α and IL-1β are capable of affecting the expression of M2 muscarinic receptors in cultured human embryonic lung fibroblasts, a cell line that expresses the muscarinic M2 receptor subtype with no evidence of M1, M3, and M4 receptors. M2 muscarinic receptor protein was measured with the nonspecific and hydrophilic muscarinic antagonist [3H]N-methyl-scopolamine. TNF-α or IL-1β alone (10 ng/ml) had no effect on the density of muscarinic binding sites over the time points investigated (data not shown). However, a strong synergistic effect was demonstrated between IL-1β and TNF-α. Indeed, the combination of the two cytokines induced a time-dependent decrease in M2 muscarinic receptor density. This down-regulation was slow, with a 53 ± 5% loss of total receptors after 24 h of cytokine stimulation (Fig. 1). The affinity of [3H]N-methyl-scopolamine for the remaining binding sites was unaltered by the treatment.

**M2 Receptor Coupling**

The muscarinic M2 receptor is coupled to the inhibition of adenylyl cyclase activity via a pertussis toxin-sensitive GTP-binding protein. To assess whether M2 receptor down-regulation was associated with a functional desensitization of the
receptors, cAMP accumulation was measured in intact cells that had been treated with the combination of TNF-α and IL-1β for 24 h. In untreated cells, direct stimulation of adenyl cyclase activity with forskolin resulted in a marked increase (100-fold) in cAMP accumulation, which was significantly inhibited following co-stimulation with the muscarinic agonist carbachol (Fig. 2). However, this inhibition by carbachol was lost after stimulation of the cells with TNF-α and IL-1β for 24 h. The basal level of cAMP was not significantly different between control and cytokine-treated cells.

**M₂ Receptor mRNA**

To determine whether the effects of the combination of TNF-α and IL-1β on M₂ muscarinic receptor protein could be extended to mRNA production, expression of the m2 muscarinic receptor gene was evaluated by Northern blotting. Whereas down-regulation of m2 muscarinic receptor mRNA was absent with either TNF-α or IL-1β alone, there was a dramatic and sustained decrease in down-regulation of m2 mRNA when the two cytokines were administered in combination (Fig. 3). The kinetics depicted in Fig. 3B shows that the m2 muscarinic receptor mRNA steadily decreased over time. This effect was apparent after 4 h of stimulation and reached a plateau of 89% of control at 14 h and was stable up to 24 h.

To investigate the mechanism of the synergy between TNF-α and IL-1β, cytokines were added sequentially to HEL 299 cells. Cells were incubated for 2 h with TNF-α. They were then washed and cultured for 6 h with IL-1β and vice versa. Muscarinic m2 receptor mRNA expression was then assessed by Northern blot analysis. Using this protocol, we could not detect any significant down-regulation of m2 receptor mRNA (data not shown), suggesting that the role of IL-1β or TNF-α is not to sensitize HEL 299 cells to the stimulating action of the other cytokine.

**m2 Receptor mRNA Stability and Gene Transcription**

To gain further insight into the mechanism of m2 mRNA down-regulation, we measured the stability of the muscarinic m2 receptor mRNA and the rate of transcription of the muscarinic m2 receptor gene. To measure the half-life of the m2 mRNA, HEL 299 cells were exposed to the combination of TNF-α and IL-1β for 4 h. After this incubation period, cells were washed, and the transcription inhibitor actinomycin D (5 μg/ml) was added. The results indicate that the m2 muscarinic half-life (~3–4 h) was not affected by the combination of TNF-α and IL-1β (data not shown). To measure the influence of the combination of TNF-α and IL-1β on the rate of m2 muscarinic receptor gene transcription, nuclear run-on assays were performed. As shown in Fig. 4, transcription of the m2 gene in cells that had been exposed to TNF-α and IL-1β for 24 h was reduced by 56% compared with vehicle-treated cells. This result indicates that TNF-α- and IL-1β-induced down-regulation of m2 muscarinic mRNA is due to an inhibition of transcription.

**Protein Synthesis and Receptor Down-regulation**

To determine whether synthesis of a protein factor is necessary for TNF-α- and IL-1β-induced m2 mRNA expression, HEL 299 cells were exposed to the translation inhibitor cycloheximide (10 μg/ml). The result depicted in Fig. 5 indicates that although cycloheximide alone had no effect on m2 mRNA expression, it significantly protected against TNF-α- and IL-1β-induced m2 receptor mRNA down-regulation. This result suggests that the synthesis of at least one protein is required for M₂ receptor down-regulation by TNF-α and IL-1β.

**Cell Signaling Pathways**

We attempted to characterize the intracellular signaling pathways that may lead to receptor down-regulation.

**Role of PKC and PKA**—We have previously shown that PKC stimulation with the phorbol ester 4β-phorbol-13,14-dibutyrate induced a down-regulation of M₂ muscarinic receptor protein and mRNA (15). To determine whether this kinase participates in m2 receptor down-regulation, we attempted to block the effect of TNF-α and IL-1β with the specific protein kinase C inhibitor GF109203X. This compound did not antagonize the effect of TNF-α and IL-1β on m2 receptor expression, thereby excluding this kinase in receptor down-regulation (Fig. 6). We also tested the possible involvement of other protein phosphorylation pathways in m2 muscarinic mRNA regulation. In HEL 299 cells IL-1β, but not TNF-α, induced a slight but
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**Fig. 4.** Relative rate of nuclear transcription of the m2 gene following TNF-α and IL-1β treatment. Cells were treated with vehicle (CTRL) or TNF-α and IL-1β for 24 h, and nuclei were collected for nuclear run-on assays. <sup>32</sup>P-labeled mRNA was transcribed in vitro from isolated cell nuclei, and 1.5 × 10<sup>6</sup> cpm of run-on products were hybridized to each blot as described under "Experimental Procedures." The plasmids used were pGEM3Z without any insert (negative control) or containing m2 receptor or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA inserts. Data are the average of two separate experiments and represent the ratios of the absorbances of m2 mRNA. Values are the mean ± S.E. (bars) of four to six independent experiments.

**Fig. 5.** Effect of the translation inhibitor cycloheximide on TNF-α and IL-1β-induced down-regulation of m2 muscarinic receptor mRNAs. Cells were treated for 14 h with vehicle (CTRL), TNF-α and IL-1β, or cycloheximide (CYCLO.) or pretreated for 1 h with cycloheximide (10 μg/ml) before incubation with TNF-α and IL-1β. Messenger RNA was then isolated and evaluated for m2 muscarinic receptor expression by Northern blot analysis. Values are the mean ± S.E. (bars) of four to six independent experiments, *p < 0.005 compared with vehicle-treated cells; #, p < 0.005 compared with TNF-α and IL-1β-treated cells.

**Fig. 6.** Effect of PKC and PKA inhibitors on m2 muscarinic receptor expression. Cells were preincubated for 1 h with the PKC or PKA inhibitors GF109203X (1 μM; GF) and H8 (20 μM), respectively, and then stimulated with the combination of TNF-α and IL-1β (both at 10 ng/ml) for 14 h. Muscarinic m2 receptor mRNA expression was then assessed by Northern blot analysis. Values are the mean ± S.E. (bars) of five to seven independent experiments. *, p < 0.05 compared with TNF-α and IL-1β stimulation.

**Fig. 7.** Effect of C2-ceramide on the level of m2 muscarinic receptor expression. Cells were treated with the cell-permeable analog of natural ceramide C2-ceramide (10 μM) either alone for the time indicated or in combination with TNF-α or IL-1β (both at 10 ng/ml) for 24 h. Top panel, representative Northern blot. Bottom panel, mean densitometric measurements of the Northern blot data. Values are the mean ± S.E. (bars) of four to six independent experiments. *, p < 0.01 compared with vehicle-treated cells.

significant increase in the level of intracellular cAMP (data not shown), which raises the possibility that PKA might be involved. Indeed, using the PKA inhibitor H-8, we have demonstrated a significant inhibition of TNF-α and IL-1β-induced m2 receptor down-regulation (Fig. 6).

Role of Ceramide Pathways—Several reports recently implicated the lipid second messenger ceramide in TNF-α and IL-1β signaling pathways (16–18). We tested this hypothesis using the cell-permeable analog of ceramide, C2-ceramide (N-acetyl-sphingosine). As shown in Fig. 7, C2-ceramide did not affect the steady-state levels of m2 mRNA. However, when C2-ceramide was co-incubated with either TNF-α or IL-1β for 24 h, a marked down-regulation was achieved (Fig. 7) implicating this pathway in m2 receptor down-regulation by TNF-α and IL-1β.

Effect of TNF-α and IL-β on MAP Kinase Activation—The possibility that MAP kinases might be involved in the TNF-α and IL-1β signal transduction mechanisms was also investigated. Using a polyclonal antibody that recognizes tyrosine 204-phosphorylated MAP kinase p44<sub>MAPK</sub> and p42<sub>MAPK</sub> isoforms (ERK1 and ERK2, respectively), we showed that cell treatment with the combination of TNF-α and IL-1β stimulates the phosphorylation of both p42<sub>MAPK</sub> and p44<sub>MAPK</sub>, which was maximal around 10–30 min following cytokine exposure and resolved by 60 min (Fig. 8, A and B). A similar profile was observed when the cells were treated with ceramide or individual cytokines (data not shown). We have also confirmed the activation of ERK1 and ERK2 by an in-gel phosphorylation assay using myelin basic protein as the substrate, suggesting therefore the involvement of this pathway in TNF-α and IL-1β.
Gels as described under "Experimental Procedures." The blot shown is the result of receptor phosphorylation. The effect of TNF-α could be accounted for by internalization of the receptor as demonstrated at the mRNA level. The down-regulation of m2 receptor mRNA preceded that of the receptor protein, suggesting that the decrease in the receptor protein is due to a decrease in the rate of receptor synthesis as a consequence of the decrease in the steady-state levels of its mRNA. Functional desensitization of M2 muscarinic receptors was also assessed in TNF-α- and IL-1β-treated cells. In control cells, cAMP generation with forskolin was significantly inhibited by co-stimulation with carbachol. However, this inhibitory effect of carbachol was lost in cells exposed to a combination of these two cytokines for 24 h, suggesting that M2 receptor down-regulation is accompanied by desensitization of the muscarinic response. Forskolin-induced cAMP accumulation was not significantly different between control and cytokine-treated cells, suggesting that the functional desensitization does not occur at the level of adenyl cyclase activity.

An interesting finding in this report was the synergistic action of TNF-α and IL-1β to induce down-regulation of M2 muscarinic receptors, which is a previously undescribed effect of these cytokines on muscarinic receptor expression. This setting may reflect the situation in vivo more closely, when both cytokines are likely to be present together in the inflamed airways. Synergism between TNF-α and IL-1β or interferon γ has been observed in several biological responses, including nitric oxide synthase induction (22), cytokine production such as IL-6 (23) and IL-8 (24), and adhesion molecule expression (25, 26). The mechanism of such synergy appears to vary according to the experimental model and may involve induction of TNF-α receptors (27, 28). In our experiments, there was no synergy when HEL 299 cells were preincubated with TNF-α and followed by stimulation with IL-1β. This indicated that up-regulation of IL-1β receptor expression by TNF-α is not responsible for the synergy we observed. Similarly, preincubation of HEL 299 cells with IL-1β did not sensitize them to the stimulating effect of TNF-α. Furthermore, HEL 299 cells constitutively express TNF-α receptors, and the level of expression is not up-regulated by IL-1β (data not shown). It is therefore likely that the synergy observed between TNF-α and IL-1β on m2 muscarinic receptor expression involves postreceptor events.

To characterize the intracellular signaling pathways leading to receptor down-regulation, we have investigated the involvement of PKA and PKC in this process. We have previously reported that PKC stimulation induces transcriptional down-regulation of M2 muscarinic receptors with a kinetic profile similar to that described here for the combination of TNF-α and IL-1β (15). To determine whether this kinase participates in m2 receptor down-regulation, we attempted to block the effect of TNF-α and IL-1β with the specific protein kinase C inhibitor GP190203X. This compound, at a concentration that was effective in inhibiting PKC-induced down-regulation of M2 muscarinic receptor protein and mRNA (15), did not antagonize the effect of TNF-α and IL-1β. We were thus unable to detect any apparent involvement of PKC in TNF-α and IL-1β-induced m2 muscarinic receptor down-regulation. In HEL 299 cells, IL-1β but not TNF-α induced a significant increase in intracellular cAMP, in agreement with data obtained in several cell systems (29–31). We therefore attempted to interfere with the cAMP-dependent protein kinase pathway using the kinase inhibitor H-8. This inhibitor provided slight but significant protection against TNF-α- and IL-1β-induced M2 receptor mRNA down-regulation, suggesting that PKA is involved, at least in part, in receptor modulation by TNF-α and IL-1β. This finding is in agreement with data showing that the PKA signal transduction pathway is important in TNF-α and IL-1β induction of IL-6 mRNA in human fibroblasts (32).
In view of the pleiotropic nature of TNF-α and IL-1β, it is not surprising that several alternative pathways can be activated by these cytokines. One potential phosphorylation pathway is represented by the lipid second messenger ceramide (16). IL-1β and TNF-α rapidly increase the cellular content of ceramide produced following the hydrolysis of sphingomyelin by two types of sphingomyelinases, a membrane-associated neutral and an endosomal acidic sphingomyelinase (17, 18, 33). In several cell systems, ceramide mediates several TNF-α or IL-1β-mediated processes, such as IL-6 (34) and cyclooxygenase-2 gene up-regulation (35). Treatment of HEL 299 cells with natural ceramide, did not affect the steady-state levels of m2 muscarinic receptor mRNA over the time course investigated, in an manner analogous to TNF-α and IL-1β alone. However, the combination of Ceramide C2-ceramide either with TNF-α or IL-1β markedly down-regulated m2 receptor mRNA expression after 24 h of treatment to a extent comparable to that produced by the combination of the two cytokines. These results are consistent with a role for the ceramide pathway in m2 receptor down-regulation induced by the combination of TNF-α and IL-1β.

A further downstream signaling event known to be triggered by TNF-α and IL-1β is activation of the MAP kinase cascade, which comprises the ERKs and the JNKs (36, 37). We investigated whether ERK1 (p44 MAPK) and ERK2 (p42 MAPK) isoforms could be activated by TNF-α and IL-1β in HEL 299 cells. Cell exposure to the combination of these cytokines phosphorylated and activated both the p42 and p44 MAP kinase isoforms in a manner similar to that achieved when the cells were stimulated with ceramide or individual cytokines. This result suggests that the activation of ERK may be involved in the signal transduction mechanisms triggered by TNF-α and IL-1β in HEL 299 cells, in agreement with published data (38, 39). However, the finding that TNF-α and IL-1β alone or in combination also activated JNK1 and JNK2 to a greater extent to that seen with ERK isoforms suggests that the JNK pathway may be preferentially activated by cytokines. These results are in agreement with previous observations showing that the ERK module is primarily activated by mitogenic stimuli, whereas JNKs are mainly activated by ceramide, cellular stress such as UV irradiation, and cytokines such as TNF-α and IL-1β (19–21, 40–42). However, the absence of synergy between IL-1β and TNF-α at the level of ERK and JNK activation suggests that activation of MAP kinases is necessary but not sufficient to cause muscarinic m2 receptor down-regulation.

Two mechanisms could account for TNF-α and IL-1β induced m2 muscarinic receptor mRNA down-regulation: a decrease in transcription of the gene or an accelerated degradation of the mRNA, i.e. a decrease in mRNA stability. TNF-α and IL-1β had no effect on the m2 muscarinic receptor mRNA half-life measured with the transcription inhibitor actinomycin D, suggesting that the down-regulation of M2 muscarinic receptors is not due to a posttranscriptional modification of the m2 mRNA. Direct measurements of the transcription of the m2 gene suggest that there is a basal level of transcription of this gene, which can significantly be reduced by the combination of TNF-α and IL-1β. This result agrees with the transcriptional down-regulation of this receptor subtype with another multifunctional cytokine, transforming growth factor β1 (14). It should be noted that although many effects of transforming growth factor β1 (including up-regulation of extracellular matrix proteins and down-regulation of collagenase and transin/stromelysin genes) oppose those of TNF-α (43), it also shares many other effects with TNF-α and IL-1β, including up-regulation of cyclooxygenases 1 and 2 and phospholipase A2 and down-regulation of β2-adrenergic receptor expression (44–46).

We have also shown, using the translation inhibitor cycloheximide, that the synergism between TNF-α and IL-1β for m2 receptor mRNA down-regulation is not a primary event, since it does require intermediate expression of unknown proteins. TNF-α and IL-1β are known to induce the expression and release of several chemokines, including IL-8, in different cell types (47). Therefore, we have addressed the question of whether IL-8 could be part of an autocrine stimulation of HEL 299 cells to induce down-regulation by TNF-α and IL-1β. HEL 299 cells cultured with TNF-α and IL-1β for 24 h in the presence of anti-IL-8-neutralizing antibody did not provide any significant protection against m2 receptor mRNA down-regulation (data not shown). Moreover, we did not observe any synergy when cells were treated with the combination of TNF-α and IL-8 or IL-1β and IL-8 (data not shown). These results exclude any contribution of IL-8 to receptor down-regulation by TNF-α and IL-1β. The possibility, however, of the involvement of other “secondary” cytokines in muscarinic m2 receptor down-regulation is not excluded. Alternatively, the observed synergy might also be at the level of the transcription-regulating proteins. Indeed, it has been shown that the regulation of the expression of several genes can involve cooperativity between different transcription factors (48, 49). The promoter sequence of the m2 gene has not yet been characterized, so the potential role of transcription factors in regulating its expression is not yet known.

In summary, we have demonstrated that TNF-α and IL-1β synergize to induce down-regulation of M2 muscarinic receptor protein and mRNA, which was associated with functional desensitization of the receptor protein. The M2 receptor mRNA down-regulation appeared to be mediated through a reduction in the rate of m2 receptor gene transcription, which may be dependent on the transcription and translation of unknown protein factor(s). Moreover, a role of PKA and ceramide pathways in M2 receptor regulation is suggested. Furthermore, our demonstration of the reduced gene transcription and function of M2 muscarinic receptors provides a mechanistic explanation of previous reports indicating altered function of these receptors in asthma. Our results also suggest that the expression of this receptor subtype may be under the control of a cytokine network.

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