0026-895X/00/061123-09$3.00/0
MOLECULAR PHARMACOLOGY

MOL 57:1123–1131, 2000 /13020/824350

Post-Transcriptional Regulation of Bradykinin B1 and B2 Receptor Gene Expression in Human Lung Fibroblasts by Tumor Necrosis Factor-α: Modulation by Dexamethasone

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Received October 12, 1999; accepted February 8, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The cellular and molecular mechanisms governing bradykinin B1 and B2 receptor expression and function are poorly understood. We investigated the regulation of both B1 and B2 receptors in human embryonic lung fibroblasts (HEL 299) by the proinflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). TNF-α and IL-1β both induced a rapid and transient increase in B1 and B2 receptor mRNA expression that was maximal by 2 h, accompanied by an increase in B1 and B2 receptor protein, as measured by radioligand binding assay with [^{3}H]des-Arg^{10}-kallidin, and [^{3}H]bradykinin, respectively. The induced B1 receptors were functionally coupled, because the B1 agonist, des-Arg^{10}-kallidin, induced an increase in arachidonic acid release in TNF-α-stimulated cells but not in control cells. The induction of B1 and the up-regulation of B2 receptors by TNF-α was partly mediated through activation of p38 mitogen-activated protein kinase and that of B2 receptor by protein kinase A. TNF-α and IL-1β regulation of both B1 and B2 receptors was inhibited by dexamethasone. When compared with vehicle-treated cells, dexamethasone increased the rate of decline of both B1 and B2 receptor mRNAs. Nuclear run-on experiments demonstrate that the induction of B1 and the up-regulation of B2 receptors as well as the inhibitory effect of dexamethasone are entirely mediated through post-transcriptional mechanisms.

Bradykinin (BK) and the related peptide kallidin (KD or lys-BK) are formed from high and low molecular weight kininogen precursors following the activation of plasma and tissue kallikreins by pathophysiological stimuli such as tissue damage, inflammation, or anoxia (Farmer and Burch, 1992; Hall, 1992). There is considerable evidence to suggest that BK plays a key role in airway inflammation and inflammatory diseases such as asthma and rhinitis (Barnes, 1992; Trifilieff et al., 1993). The biological actions of kinins are mediated via an interaction with constitutive B2 receptors and inducible B1 receptors. These receptors have been defined initially based on pharmacological criteria, and subsequently by molecular cloning (Hess et al., 1992; Menke et al., 1994; Webb et al., 1994) and both receptors belong to the G-protein-coupled receptor family. B2 receptors show a higher affinity for BK and KD, whereas B1 receptors show a higher affinity toward the metabolites [des-Arg^9]BK and [des-Arg^{10}]KD. Most of the biological actions of BK appear to be mediated through the activation of B2 receptors. Studies in different cell types have shown that activation of the B2 receptor leads to a number of intracellular events, including activation of phospholipase C, an increase in intracellular calcium, and activation of phospholipase D and PLA2 with subsequent release of arachidonic acid (Farmer and Burch, 1992).

The B2 receptor is constitutively expressed on most cell types, and this expression may be up-regulated by cytokines, growth factors, and by cAMP-elevating drugs (Bathon et al., 1992; Dixon, 1994; Dixon et al., 1996). By contrast, B1 receptors are not present in tissues under "normal" conditions but are induced during inflammatory insults (Marceau, 1995). The induction of B1 receptors may therefore be of considerable importance in inflammation. Functionally, the induced B1 receptors have been shown to mediate fibroblast proliferation and tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) release from macrophage cell lines (Marceau and Tremblay, 1986; Tiffany and Burch, 1989). Despite the evi-

This work was supported by a Wellcome Trust Career Development Award (to A.J.F.) and by a fellowship from the European Union (to E.-B.H.).

ABBREVIATIONS: BK, bradykinin; KD, kallidin; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal protein kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SSC, sodium chloride/sodium citrate buffer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; HBSS, Hanks' balanced salt solution; [^{3}H]AA, [^{3}H]arachidonic acid.

dence that B1 and B2 receptors are induced or up-regulated by inflammatory insults, little is known of the intracellular or molecular mechanisms regulating their expression.

The aims of the present study were, therefore, to examine the regulation of B1 and B2 receptor expression in cultured human lung fibroblasts by the proinflammatory cytokines, TNF-α and IL-1β, and to elucidate the intracellular signaling pathways leading to receptor regulation, as well as the transcriptional and post-transcriptional mechanisms underlying B1 and B2 receptor gene expression. Finally, the functional significance of B1 and B2 receptor regulation by TNF-α and possible inflammatory relevance were also evaluated.

Materials and Methods

Cell Culture. All tissue culture reagents except Hanks' balanced salt solution (HBSS) and Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, UK) were obtained from Sigma. HEL 299 cells were obtained from the American Type Culture Collection (ATCC code CCL 137; Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/l amphotericin B in 95% air and 5% CO_2 at 37°C. All experiments were performed on cells at passage 9. The medium was replaced every 3 to 4 days, and, on reaching confluence, cells were subcultured by detaching the monolayer with 0.05% trypsin/1 mM EDTA.

Radioligand Binding Studies. All membrane preparation procedures were performed at 4°C. HEL 299 cells were treated with human recombinant TNF-α (R&D System, UK), washed twice with HBSS, and harvested by cell scraping using ice-cold 25 mM TES (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino} ethanesulfonic acid) buffer (pH 6.8), containing 1 mM 1,10-phenanthrolin, 140 μg/ml bacitracin, 10 μM captopril, 1 mM dithiothreitol, 20 μg/ml leupeptin, 100 μg/ml trypsin inhibitor, and 20 μM phenylmethylsulfonyl fluoride. Cells were homogenized, and membranes were pelleted by centrifugation at 40,000*g* for 20 min and resuspended in an appropriate volume of TES buffer containing the cocktail of peptidase inhibitors. [^{3}H]BK and [^{3}H]des-Arg^{10}-KD (NEN, Houslow, UK) saturation curves were carried out at 25°C, using increasing concentrations (0.04 to 4 nM) in a final volume of 0.5 ml of membrane suspension. After an incubation period of 3 h, bound [^{3}H]BK and [^{3}H]des-Arg^{10}-KD were harvested by rapid vacuum filtration through GF/C glass-fiber filters pretreated with 0.3% aqueous polyethyleneimine. Filters were rinsed three times with 4 ml of ice-cold 25 mM Tris-HCl (pH 6.8) and counted in 4 ml of scintillation cocktail. Nonspecific binding was determined in the presence of 10 μM unlabeled agonists. Binding data were analyzed with the nonlinear regression program LIGAND as described previously (Haddad et al., 1994).

[^{3}H]Arachidonic Acid Release. Preconfluent cells were incubated with [^{3}H]arachidonic acid ([^{3}H]AA, 0.5 μCi/ml, NEN, UK) for 24 h. After this incubation period, cells were extensively washed and then treated for 4 h with TNF-α (10 ng/ml). After the TNF-α stimulation, cells were stimulated with the B1 (des-Arg^{10}-KD) and B2 (BK) agonists. The supernatant was removed at appropriate time intervals and counted in a liquid scintillation counter. At the end of the experiment, cells were lysed and radioactivity counted. Results were expressed as a fraction of the total [^{3}H]AA released.

Northern Blot Analysis. Cells were washed twice with HBSS, and total RNAs were isolated as previously described (Haddad et al., 1996b). Poly(A)$^+$ RNA was prepared using an mRNA system kit (PolyTract, Promega, Southampton, UK) according to the manufacturer's instructions. Samples of mRNA were size-fractionated on a 1% agarose/formaldehyde gel containing 20 mM morpholinosulfonic acid, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0) and blotted onto Hybond-N filters (Amersham plc, Amersham, UK) by capillary action using 20× SSC (standard saline citrate, 1× SSC, 0.15 mM NaCl, and 0.015 M sodium citrate at pH 7.0).

Prehybridizations and hybridizations were carried out at 42°C in a buffer consisting of 4× SSC, 50% formamide, 50 mM Tris-HCl (pH 7.5), 5× Denhardt's solution, 0.1% SDS, 5 mM EDTA, and 250 μg/ml sonicated denatured salmon sperm DNA. Cloned human B1 and B2 receptor cDNAs (30 to 50 ng) were labeled by random priming using [α-^{32}P]dCTP (Amersham Pharmacia) added to the prehybridization chambers at a final activity of 1 to 2 $\times$ 10^6 cpm/ml, and incubated for 12 to 16 h at 42°C. After hybridization, blots were washed to a stringency of 0.1× SSC/0.1% SDS for 30 min at 60°C and then exposed to Kodak X-OMAT S film at −70°C with intensifying screens for 1 to 7 days. After an appropriate exposure time, blots were stripped in 50% formamide, 10 mM NaH_2PO_4 for 1 h at 65°C before subsequent rehybridization. To account for differences in loading or transfer of the RNA, the blots were hybridized with a 1272-base pair *Pst*I fragment from rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The intensities of the signals were then quantified by laser densitometry (Quantity One Software, PDI, New York, NY).

Nuclear Run-on Assay. For the measurement of gene transcription, nuclei were prepared as previously described (Haddad et al., 1996b). Isolated nuclei were resuspended in Tris-HCl (10 mM, pH 7.4), $MgCl_2$ (5 mM), glycerol (50%), sorbitol (0.5 M), Ficoll (2.5%), spermidine (0.008%), and dithiothreitol (1 mM) and stored at −70°C until use. In vitro transcription was performed with nuclei (5 $\times$ 10^7) incubated for 30 min at 27°C with 300 μCi of [^{32}P]UTP, ATP (0.625 mM), CTP, GTP (0.31 mM), Tris-HCl (40 mM), NH_4Cl (150 mM), $MgCl_2$ (7.5 mM), and RNasin (120 U). DNA digestion was carried out with a 15-min incubation at 27°C with RQ-1 DNase (75 U) and RNasin (40 U) before protein digestion for 3 h at 37°C with proteinase K (1 mg/ml) in buffer containing Tris-HCl (pH 7.4, 10 mM), EDTA (15 mM), SDS (3%), and heparin (3 mg/ml). RNA extraction was then carried out with a phenol, phenol/chloroform (1:1), and a chloroform wash and then precipitated three times with 100% ethanol in the presence of 1.33 M ammonium acetate. The radiolabeled RNAs were dissolved in 100 μl of TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and added to 2 ml of hybridization solution (50% formamide, 5 $\times$ SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 5 $\times$ Denhardt's solution, 50 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, 0.02 μg of poly A, and 0.02 μg of poly G RNA). Following 4-h 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 rat GAPDH cDNA and human B1 and B2 receptor cDNAs. The filters were washed first in buffer A (300 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% SDS, 1 μg/ml RNase A, and 10 U/ml RNase T1) at 37°C for 30 min then in buffer B (10 mM NaCl, 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.

Western Blot Analysis of MAP Kinase Expression. Following treatments, HEL 299 cells were washed in HBSS and 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 aprotinin, 10 mM NaF, and 20 mM sodium pyrophosphate). Cytosolic proteins were boiled for 5 min before 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.

The phosphorylation of p38 mitogen-activated protein kinase (MAPK) was 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 (PAGE) on 10% acrylamide gels and then transferred to nitrocellulose 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 p38 proteins

were run in parallel and served as negative and positive controls for immunodetection of MAPKs. To block nonspecific antibody binding, membranes were incubated for 1 h in blocking buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% (w/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 where nonfat milk was replaced with 5% BSA. Membranes were washed with blocking buffer for 3 × 5 min and incubated with 1/10,000 dilution of alkaline phosphatase-conjugated anti-rabbit secondary antibody, before being washed again. Protein detection was then carried out using CDP-Star chemiluminescent reagent. Membranes were drained from excess developing solution and exposed to Kodak X-OMAT S film. To reprobe membrane, antibodies were stripped using 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris (pH 6.7) at 50°C for 10 min.

Data Analysis. Data were expressed as mean ± S.E. Statistical analysis of data was performed using the nonparametric Mann-Whitney *U* test for stepward comparison. *P* values less than .05 were considered to be significant.

Results

BK B1 and B2 Receptor Binding Studies

B1 receptors labeled with the B1-selective agonist [^{3}H]des-Arg^{10}-KD were not detected in untreated cells (Fig. 1). However, treatment with TNFα (10 ng/ml) for 4 h resulted in a marked and transient induction of B1 receptors (*B*max = 465 ± 41 fmol/mg protein). Single-site analysis of the saturation data yielded a K_d value of 0.12 ± 0.03 nM. B2 receptor binding sites measured with [^{3}H]BK were present in untreated cells (*B*max = 490 ± 73 fmol/mg protein), and the number of sites were significantly up-regulated after 4 h of TNFα stimulation (939 ± 114 fmol/mg protein). The increase in B2 receptor expression was transient and returned to basal levels 8 h following TNF-α stimulation (Fig. 1). The up-regulation of B2 receptors was not accompanied by any change in the affinity of [^{3}H]BK for these binding sites (K_d = 0.72 ± 0.17 nM).

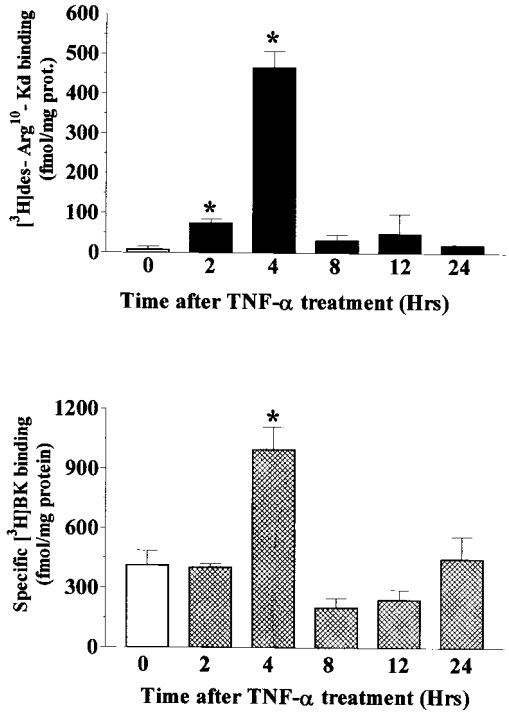

Fig. 1. Time course of TNF-α effect on B1 and B2 receptor protein densities in HEL 299 cells. HEL 299 cells were stimulated with TNF-α (10 ng/ml) for the time indicated. Receptor densities were measured in cell homogenates using the B1-selective ([^{3}H]des-Arg^{10}-KD, top) and the B2-selective ([^{3}H]BK, bottom) agonists. There were very few B1 receptors in naive cells, but this was significantly increased by TNF-α with a maximal effect at 4 h. B2 receptors were present in naive cells and significantly increased at 4 h after stimulation. *$P < .05$, compared to time 0. Data shown are the mean ± S.E. of four to eight separate experiments performed in triplicate.

BK B1 and B2 Receptor mRNA Expression

Changes in BK receptor expression were also evident at the mRNA level. Northern blot analysis using human B1 and B2 receptor cDNA demonstrated that in untreated cells there was expression of B2 and very low levels of B1 receptor mRNAs. After treatment with TNF-α, expression of mRNA for both receptors increased, peaking at 2 to 4 h post-treatment and declining toward basal levels thereafter (Fig. 2). A similar profile of B1 and B2 receptor expression was also

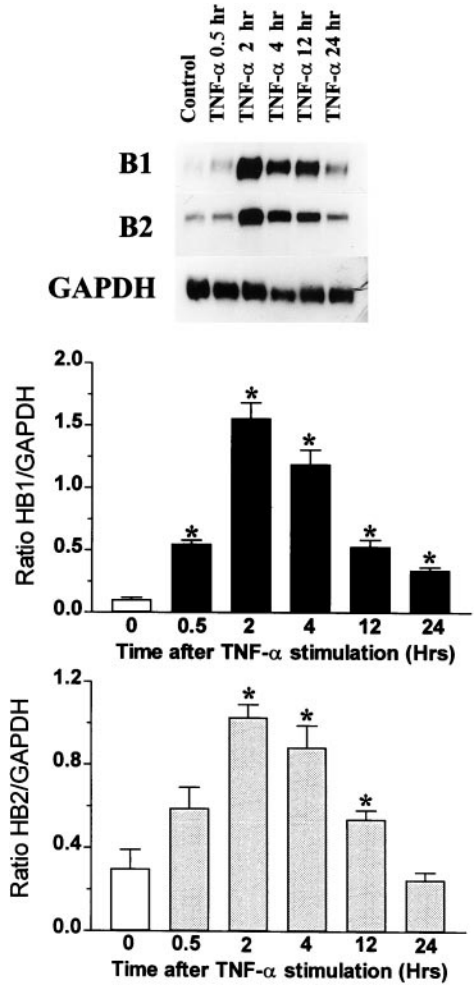

Fig. 2. Effect of TNF-α on the steady-state levels of human B1 and B2 receptor mRNAs. Preconfluent cells were treated with TNF-α (10 ng/ml) for the times indicated. Cells were then harvested, and mRNA was extracted and assessed for B1 and B2 mRNA expression using Northern blot analysis. Top, a representative Northern blot autoradiogram with the B1 and B2 receptor mRNAs. Bottom panels, the mean densitometric measurements of the Northern blot data showing B1 and B2 receptor mRNA levels expressed as a ratio to the housekeeping gene, rat GAPDH, used as an internal standard to correct for differences in RNA loading or transfer efficiencies. Values are the mean ± S.E. of three to six separate experiments. *$P < .05$, compared with vehicle-treated cells.

observed following IL-1β (10 ng/ml) stimulation (data not shown).

AA Accumulation after BK Receptor Induction

To determine whether the induced B1 and the up-regulated B2 receptors were functionally coupled, we measured the accumulation of [^{3}H]AA after cell stimulation with TNF-α. In untreated cells, there was no significant increase in [^{3}H]AA release in response to the B1 agonist des-Arg^{10}-KD. However, when the B1 receptors were induced by TNF-α stimulation, the B1 agonist elicited a significant increase in [^{3}H]AA accumulation (Fig. 3). In control cells, BK induced an increase in [^{3}H]AA release, which was not significantly different in TNF-α stimulated cells (Fig. 3). The increase in [^{3}H]AA release in TNF-α-treated cells by des-Arg^{10}-KD was inhibited by the selective B1 antagonist des-Arg^{10}-[HOE 140] but not by the B2 antagonist HOE 140 (data not shown).

Protein Synthesis and Receptor Down-Regulation

To determine whether synthesis of a protein factor was necessary for cytokine-induced up-regulation of B1 and B2 receptor mRNA, HEL 299 cells were exposed to the translation inhibitor cycloheximide (10 μg/ml). The results depicted in Fig. 4 show that cell stimulation with cycloheximide resulted in an induction of B1 and an up-regulation of B2 receptor mRNAs with a time course similar to that seen with TNF-α and IL-1β. There was no synergy between TNF-α and cycloheximide on the levels of B1 and B2 receptor mRNA expression. This result indicates that inhibition of the synthesis of at least one protein may be required for induction of B1 and up-regulation of B2 receptors.

Intracellular Pathways Leading to Receptor Regulation

Role of the Ceramide Pathway. Several reports have implicated the lipid second messenger ceramide in TNF-α and IL-1β signaling pathways (Kolesnick and Golde, 1994). However, we found no evidence for a role of ceramide in the up-regulation of B1 and B2 receptor. Indeed, cell treatment with the cell-permeable analog of ceramide, *N*-acetylsphingosine (C2-ceramide), did not affect the steady-state levels of B1 and B2 receptor mRNA (data not shown). Similarly, we found no synergy between C2-ceramide and TNF-α or IL-1β on receptor mRNA up-regulation (data not shown).

Role of PKC and PKA. To determine whether the classical second messenger kinases, namely, protein kinase A (PKA) and protein kinase C (PKC) participate in B1 and B2 receptor induction and up-regulation, respectively, we examined the effect of TNF-α with the selective inhibitors of PKC (GF109203X) and PKA (H-8). The PKC inhibitor GF109203X

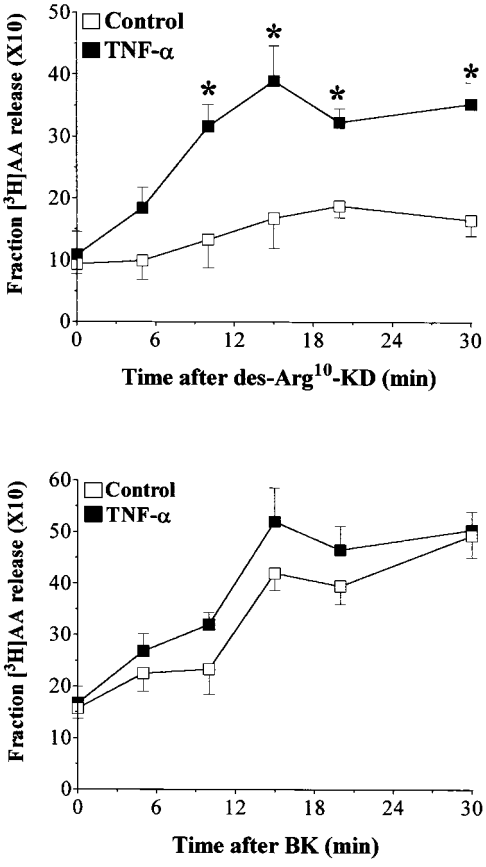

Fig. 3. Effect of TNF-α treatment on [^{3}H]AA following stimulation with the B1 (des-Arg^{10}-KD) or the B2 (BK) agonists in HEL 299 cells. Cells were incubated with [^{3}H]AA (0.5 μCi/ml) for 24 h and then treated for 4 h with either vehicle (control) or TNF-α (10 ng/ml). After this incubation period, cells were extensively washed and then stimulated for the time indicated with the B1 and B2 agonists, des-Arg^{10}-KD (1 μM) and BK (1 μM), respectively. Data shown are the mean ± S.E. for three separate experiments performed in duplicate. *$P < .05$, compared with control values at corresponding times.

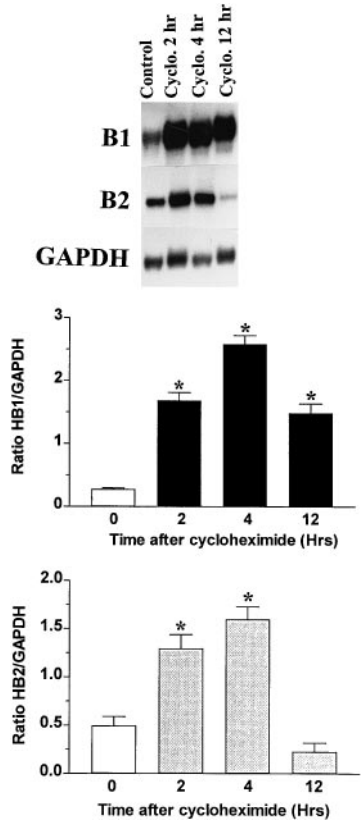

Fig. 4. Effect of cycloheximide on BK B1 and B2 receptor mRNA expression. HEL 299 cells were treated with vehicle or cycloheximide (10 μg/ml) for the times indicated. Cells were then washed and mRNA extracted and assayed for B1 and B2 receptor mRNA expression by Northern blot analysis. The top panel shows the B1 and B2 mRNAs expression, together with the GAPDH mRNA used as an internal standard. The bottom panels demonstrate the time course of the mean ratio of B1 and B2 mRNA to GAPDH mRNA. Data shown are the mean ± S.E. of four separate experiments. *$P < .05$, compared with time 0.

(5 μM) did not antagonize the effect of TNF-α on B1 and B2 receptor expression, thereby excluding this pathway in receptor regulation (Fig. 5). Pretreatment of HEL299 cells with the PKA inhibitor H-8 (30 μM) did not inhibit TNF-α induction of B1 receptor but did provide a significant protection against B2 receptor up-regulation (Fig. 5). These results suggest that PKA is involved in B2 but not B1 receptor regulation by TNF-α.

Role of MAPK Pathways. MAPK represents an expanding family of proteins located in three fairly distinct protein phosphorylation cascade. They may be activated by a number of stimuli and, on activation, translocate to the nucleus where they are known to phosphorylate a number of transcription factors, including members of the activating transcription factors family and *c-jun* (Karin, 1998). The three MAPK cascades elucidated so far are the extracellular signal-regulated kinase (ERK), NH_2-terminal c-Jun kinase (JNK) and the p38 MAPK cascades (Karin, 1998). Using an "in gel" phosphorylation assay with myelin basic protein and GST-c-Jun as substrates, we have previously shown that both ERK and JNK modules are activated by TNF-α in HEL 299 cells (Haddad et al., 1996b). The activation was maximal around 10 to 30 min following cytokine exposure and resolved by 60 min. We have now extended these observations to the p38 MAPK. Using a phospho-specific polyclonal antibody that recognizes Tyr^{182}-phosphorylated p38 MAPK, we showed that cell treatment with TNF-α induces the phosphorylation of p38, which was apparent after 10 min of stimulation and maintained up to 60 min (Fig. 6). This result indicates therefore the possible involvement of these pathways in TNF-α signaling and regulation of BK receptor expression. This was further substantiated using the p38 kinase inhibitor, SB 203580 (10 μM). This compound provided significant protection against TNF-α induction of B1 receptors and up-regulation of B2 receptors, whereas the MAP kinase kinase inhibitor PD 098059 (30 μM) was without effect (Fig. 5).

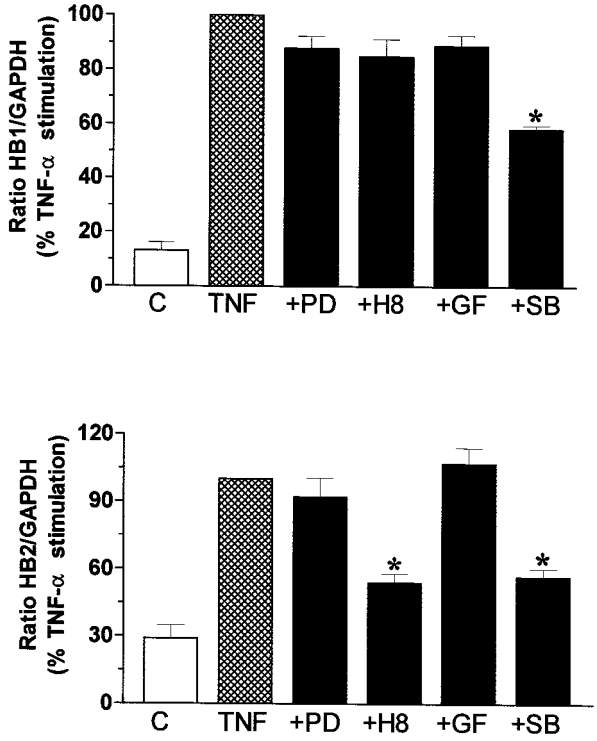

Fig. 5. Effects of PKA, PKC, and MAPK (ERK and p38) inhibitors on the expression of BK B1 and B2 receptor mRNAs induced by exposure to TNF-α. HEL 299 cells were treated with vehicle (C), TNF-α (10 ng/ml each), or pretreated for 1 h with the PKA (H-8, 30 μM), PKC (GF109203X, 5 μM), ERK (PD 098059, 30 μM), and p38 MAPK (SB 203580, 10 μM) inhibitors either alone or in combination with TNF-α for 2 h. Steady-state levels of B1 and B2 receptor mRNA were determined by Northern blotting. There was a significant inhibition of the B1 and B2 receptor expression by SB 203580, whereas H-8 only inhibited the BK B2 mRNA expression. Data shown are the mean ± S.E. of three to four separate experiments. *$P < .05$, compared with TNF-α treated cells.

Regulation of Receptor Expression by Dexamethasone

Glucocorticoids are potent inhibitors of the transcription of many proteins. We have investigated whether the synthetic glucocorticoid, dexamethasone, could inhibit the TNF-α- and IL-1β-induced B1 and B2 receptor expression. As illustrated in Fig. 7, dexamethasone (1 μM) pretreatment provided significant protection against the induction of B1 and the up-regulation of B2 receptor mRNA by TNF-α and IL-1β. In untreated cells, dexamethasone alone significantly reduced B2 receptor mRNA but had no significant effect on B1 receptor expression.

Effect of TNF-α and/or Dexamethasone on B1 and B2 Receptor mRNA Stability and Gene Transcription

The mechanism by which dexamethasone inhibits TNF-α-induced BK receptor expression was further explored by measuring the receptor mRNA half-life and the rate of the receptor gene transcription. In order to measure receptor mRNA half-life, HEL 299 cells were exposed to TNF-α for 2 h to stimulate the expression of B1 and B2 receptor mRNAs. Cells were then washed, and the vehicle, the transcription inhibitor actinomycin D (5 μg/ml), or the combination of actinomycin D and dexamethasone (1 μM) were added. The half-life of B1 and B2 receptor mRNA in this experimental setting was determined to be greater than 4 h (Fig. 8). In vehicle-treated cells, mRNA for B1 and B2 receptors declined very slowly over the time investigated. However, the rate of decay of B1 and B2 mRNAs was markedly enhanced by treatment with dexamethasone. Because little or no B1 mRNA expression was observed in control cells, mRNA half-life could not be determined and compared with the half-life determined in cytokine-treated cells.

To measure the influence of TNF-α and/or dexamethasone on the rate of B1 and B2 receptor gene transcription, nuclear run-on assays were performed (Fig. 9). The rate of the B1 and B2 receptor gene transcription (measured by densitometric scanning of the autoradiograms) was normalized to that of

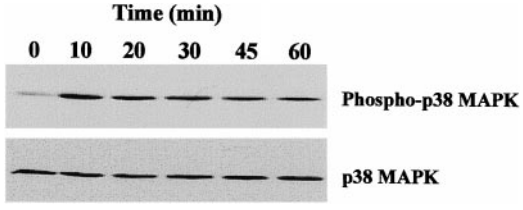

Fig. 6. TNF-α-induced phosphorylation of p38 MAPK in HEL 299 cells. The phosphorylation of p38 MAPK was identified and quantified by Western blot analysis. Cells were treated with TNF-α for the times indicated. After SDS-PAGE and electroblotting onto polyvinylidene difluoride membranes, blots were incubated with a phospho-specific polyclonal antibody that recognizes Tyr^{182}-phosphorylated p38 MAPK as described under *Materials and Methods*. The membrane was stripped and reprobed with a p38 antibody to verify protein loading. The blot shown is representative of three separate experiments.

the housekeeping gene GAPDH. In untreated cells, there was a basal rate of B1 and B2 receptor gene transcription that was not significantly different in cells stimulated with TNF-α and dexamethasone either alone or in combination (Fig. 9). Unlike nuclear RNA data, cytoplasmic RNA extracted from the same samples shows a modulatory effect of both TNF-α and dexamethasone on BK receptor gene expression (Fig. 9). Similar data were also obtained using IL-1β as the stimulus (data not shown).

Discussion

Our main finding is that TNF-α induction of B1 and up-regulation of B2 receptors and the inhibition of these receptors by glucocorticosteroids in the nonimmortalized HEL 299 cells are mediated entirely through post-transcriptional mechanisms. We have also provided insights into the cellular pathways leading to B1 and B2 receptor regulation by TNF-α by demonstrating the involvement of the p38 MAPK pathway for both B1 and B2 receptor induction, whereas PKA was partially involved in the up-regulation of B2 receptors.

This is the first evidence showing that TNF-α has the ability to regulate both B1 and B2 receptor protein and mRNA in human lung fibroblasts. The effect of TNF-α on B1 and B2 receptor protein expression was also demonstrated at the mRNA level and shared by the pleiotropic cytokine IL-1β. The induction of B1 and the up-regulation of B2 receptor mRNA preceded that of receptor protein, suggesting that the increase in receptor protein may be due to an increase in the rate of receptor synthesis as a consequence of the increase in the steady-state levels of its mRNA. The kinetics of B1 receptor induction by TNF-α in HEL 299 cells agrees with data obtained in IMR-90 fibroblasts challenged with IL-1β (Zhou et al., 1998). The ability of TNF-α and IL-1β to up-regulate the protein as well as the mRNA levels of B2 receptors in HEL 299 cells is shared by other cytokines such as platelet-derived growth factor in arterial smooth muscle and IL-1β in human synovial cells (Bathon et al., 1992; Dixon et al., 1996).

We investigated the functional consequence of B1 and B2 receptor up-regulation by examining AA release. Following B1 receptor induction with TNF-α, des-Arg^{10}-KD elicited an increase in [^{3}H]AA release from HEL 299 cells, which was inhibited by the selective B1 antagonist des-Arg^{10}-[HOE 140] but not by the selective B2 antagonist HOE 140. This indicated that the induced B1 receptors are functionally coupled. In untreated cells the B1 agonist was without effect. Induced B1 receptors have also been shown to mediate fibroblast

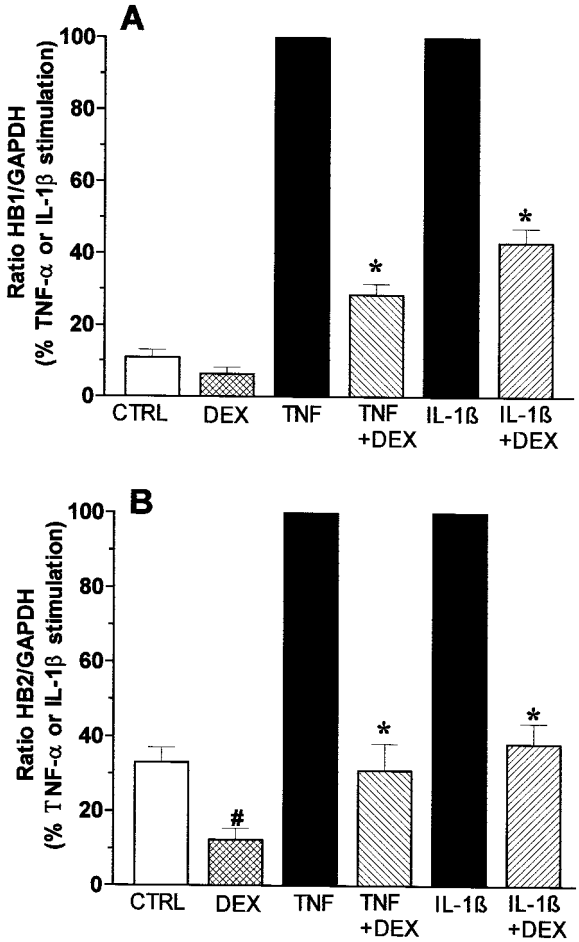

Fig. 7. Effect of dexamethasone pretreatment on B1 (A) and B2 (B) receptor mRNA expression induced by TNF-α or IL-1β. HEL 299 cells were treated with vehicle (control), TNF-α or IL-1β (10 ng/ml each), or pretreated for 1 h with dexamethasone (1 μM) alone or in the presence of TNF-α or IL-1β for 2 h. B1 and B2 receptor mRNA expression was assayed by Northern analysis. Dexamethasone inhibited the induced expression of B1 and B2 receptor mRNA and also the basal expression of B2 mRNA. Data shown as mean ± S.E. of three to four separate experiments. *$P < .05$, compared with corresponding control.

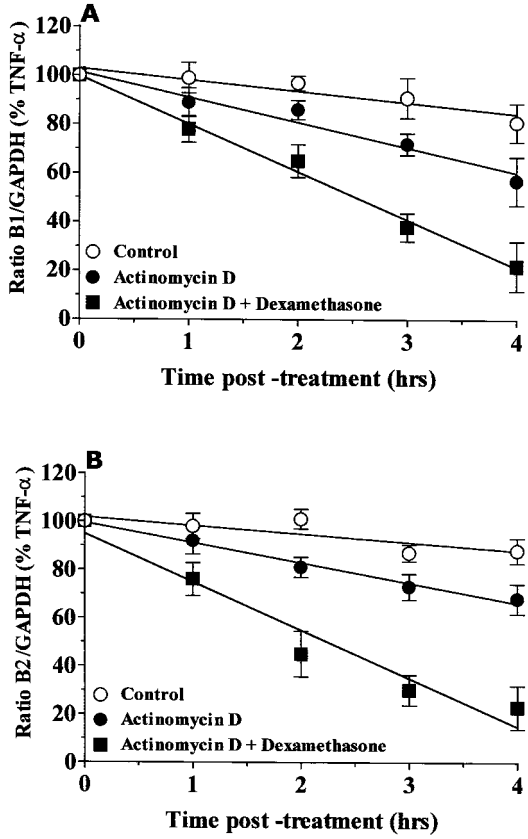

Fig. 8. Effect of actinomycin D and dexamethasone on the steady-state levels of B1 (A) and B2 (B) receptor mRNAs. Cells were incubated with TNF-α for 2 h and then exposed to vehicle, actinomycin D (5 μg/ml), and the combination of actinomycin D and dexamethasone (1 μM). Cells were harvested at the indicated times, and mRNA was isolated and assayed for B1 and B2 receptor expression by Northern blotting. The graphs show the mean time course of the decline in B1 and B2 mRNA levels expressed as percentage at time 0 (end of TNF-α exposure). Results are mean ± S.E. from four to six experiments.

proliferation, and TNF-α and IL-1β release from macrophage cell lines (Marceau and Tremblay, 1986; Tiffany and Burch, 1989). In HEL 299 cells, stimulation of [^{3}H]AA labeled cells with BK also resulted in the release of incorporated radiolabel, which was not significantly different between control and TNF-α-treated cells. This response was significantly inhibited by HOE-140 but not by des-Arg^{10}-[HOE 140], suggesting that the release is mediated by B2 receptor activation. This result further suggests that either the [^{3}H]AA release was already maximal or that the up-regulated B2 receptors may subserve other functions such as cytokine release or fibroblast proliferation. BK also synergizes with TNF-α to induce enhanced prostaglandin synthesis in Swiss 3T3 fibroblasts and IL-1β and IL-6 production from human gingival fibroblasts (Burch and Tiffany, 1989; Yucel-Lindberg et al., 1995; Modeer et al., 1998).

To determine whether ongoing protein synthesis is required for receptor regulation by TNF-α, we assessed the effect of the translation inhibitor cycloheximide on the steady-state levels of B1 and B2 receptor mRNAs. We found that cycloheximide alone was able to induce B1 and up-regulate B2 receptors with kinetics similar to TNF-α and IL-1β effects. There was no evidence for a synergy between cycloheximide and TNF-α or IL-1β (data not shown). These results suggest that not only is the synthesis of intermediary proteins not required for B1 receptor induction and B2 receptor up-regulation, but also that protein synthesis is actually inhibitory to B1 and B2 receptor expression. Furthermore, cycloheximide-promoted increase in B1 receptor mRNA was still clearly apparent at 12 h, whereas the increase in B2 receptor mRNA expression has declined toward basal levels at this time point. This Differential effect of cycloheximide on the kinetics of B1 and B2 receptor expression may be related to differences in the nature of protein(s) involved in the regulation of B1/B2 receptor expression. Our data on B2 receptor expression agrees with those obtained in rat arterial smooth muscle where cycloheximide superinduced both basal and PDGF-stimulated B2 receptor mRNA levels (Dixon et al., 1996). Cycloheximide has also been shown to up-regulate B1 receptors in IMR-90 fibroblasts through B1 mRNA stabilization (Zhou et al., 1998). The inducing effect of cycloheximide on B1 receptor expression has also been observed in vivo (DeBlois et al., 1991).

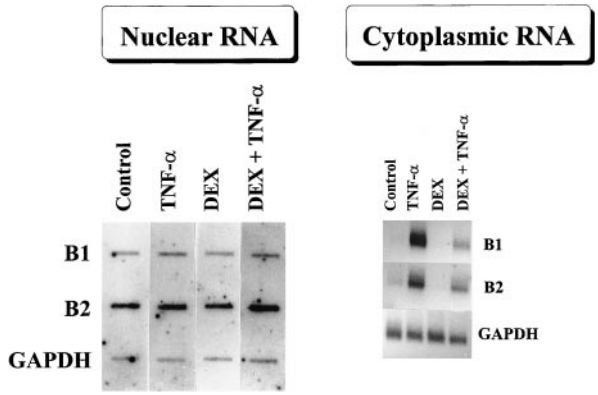

Fig. 9. Relative rate of nuclear transcription of the human BK B1 and B2 genes following TNF-α treatments. HEL 299 cells were treated with vehicle (control) or TNF-α (10 ng/ml) or were pretreated for 1 h with dexamethasone (1 μM) alone or in the presence of TNF-α for 2 h, and nuclei were collected for nuclear run-on assays. ^{32}P-Labeled mRNA was transcribed in vitro from isolated cell nuclei and 1.5×10^6 cpm of run-on products were hybridized to membranes containing plasmids with inserts for GAPDH or for the human B1 and B2 receptor cDNAs. Cytoplasmic mRNA from the same samples were also extracted and assayed for B1 and B2 receptor mRNA expression using Northern blot analysis. The blots shown are representative of three separate experiments performed in duplicate.

To gain further insight into the cellular mechanism leading to B1 receptor induction and B2 receptor up-regulation, we investigated the contribution of cellular kinases in these processes. Previous studies have shown that stimulation of PKC induces an up-regulation of B1 and B2 receptors in WI-38 and IMR-90 fibroblasts (Dalemar et al., 1996; Zhou et al., 1998). We found that the specific PKC inhibitor GF109203X, at a concentration that was effective in inhibiting PKC-induced down-regulation of M_2 muscarinic receptor protein and mRNA in HEL 299 cells (Rousell et al., 1995), did not antagonize the effect of TNF-α on the B1 and B2 receptor mRNA levels ruling out any involvement of this kinase in the TNF-α effect. However, cell treatment with the PKA inhibitor H-8 inhibited TNFα-induced B2 receptor mRNA up-regulation, although having no affect on B1 receptor induction, in agreement with a recent finding in lung fibroblasts (Zhou et al., 1998). Thus, differential signaling pathways activated by TNF-α are involved in B1 receptor induction and B2 receptor up-regulation. By contrast, we found no involvement of the ceramide pathway on the TNF-α-induced regulation of B1 and B2 receptor expression despite its reported mediation of several TNF-α- or IL-1β-mediated processes (Kolesnick and Golde, 1994).

A further downstream signaling event known to be triggered by cytokines such as TNF-α and IL-1β is the activation of the MAPK cascade, which comprises the ERK, JNK, and the p38 pathways (Karin, 1998). Despite the activation of the ERK module of the MAPK by TNF-α in HEL299 cells (Haddad and Rousell, 1998), the MAPK kinase inhibitor PD 098059, used at a concentration that inhibited ERK activation by platelet-derived growth factor (Rousell et al., 1997), did not affect the TNF-α effect on B1 and B2 receptor expression. This result is similar to that of a previous report on B1 receptor expression stimulated by IL-1β in IMR-90 fibroblasts (Zhou et al., 1998). Because the p38 kinases mediate several cellular effects of TNF-α, including IL-6 synthesis (Beyaert et al., 1996), we attempted to interfere with this pathway with the selective p38 MAPK inhibitor SB 203580 (Cuenda et al., 1995). This compound significantly inhibited TNF-α induction of B1 and up-regulation of B2 receptors. This is in agreement with the inhibition by SB 203580 of both spontaneous and IL-β-stimulated up-regulation of contractile response to des-Arg^9-BK (mediated by B1 receptor stimulation) in rabbit aortic rings (Larrivee et al., 1998). SB 203580 produced partial inhibition of TNF-α effects, indicating that additional mechanisms such as JNK pathways are involved. However, there are several isoforms of p38 kinases, all of which are not inhibited by the current p38 kinase inhibitors, including SB 203580 (Kumar et al., 1997; Wang et al., 1997). The contribution of the JNK pathway to BK receptor regulation by TNF-α was not investigated due to the lack of selective inhibitors.

Glucocorticoids are among the most potent and widely used anti-inflammatory drugs (Barnes, 1996). We have used the synthetic glucocorticoid dexamethasone to assess the effect of glucocorticoids on TNF-α- and IL-1β-induced B1 and B2 receptor gene expression in HEL 299 cells. Although dexameth-

asone had no effect on its own, it potently inhibited both TNF-α and IL-1β induction of B1 receptors. Similarly we have found that dexamethasone also inhibited both basal and TNF-α- and IL-1β-stimulated B2 receptor expression. These results suggest that the inhibition of basal as well as stimulated BK B1 and B2 receptor expression by glucocorticoids may contribute to their potent anti-inflammatory properties.

Several mechanisms could conceivably account for the increased expression of B1 and B2 receptors by TNF-α and the inhibitory effect of dexamethasone: an increase in the transcription rate of the gene, a decrease in the degradation of the mRNA, i.e., an increase in mRNA stability, or a combination of these two processes. The half-life studies suggest that the inhibitory effect of dexamethasone on TNF-α regulation of B1 and B2 receptor expression is partly achieved through mRNA destabilization. This was further substantiated using a nuclear run-on assay that showed first that TNF-α induction of B1 and up-regulation of B2 receptor mRNA is not caused by increased transcription of the B1 and B2 receptor genes and second that dexamethasone protection is mediated through post-transcriptional mechanisms. With regard to B1 receptor expression, it was shown in IMR-90 fibroblasts where there is a sizeable basal expression of B1 receptors, that IL-1β up-regulates B1 receptor mRNA through increased transcription of the B1 receptor gene (Schanstra et al., 1998; Zhou et al., 1998). The differences between our data and those obtained in IMR-90 cells could be attributed to the fact that the regulation of gene transcription is often cell-type and/or stimulus-dependent. Nuclear run-on experiments conducted with IL-1β as the stimulus show that, like TNF-α, IL-1β had no effect on B1 and B2 receptor gene transcription. These data suggest that, in HEL 299 cells, the post-transcriptional regulation of both B1 and B2 receptor gene expression is not specific for TNF-α as it is shared with IL-1β. This is not surprising because several signaling pathways are shared by both TNF-α and IL-1β. Further experiments are needed to more fully address the stimulus and cell specificity of the regulation of BK receptor gene transcription.

The mechanism of TNF-α and IL-1β regulation of B1 as well as B2 receptor gene transcription reported here is novel. For these cytokines to increase the B1 and B2 receptor mRNA levels solely via mRNA stabilization, it must be true that B1 and B2 receptor gene transcription is constitutive in unstimulated cells. Consistent with this view, we have observed basal transcription of both genes. We postulate that the level expression of mRNA does not normally culminate in active protein due to rapid degradation of mRNA, via a mechanism involving a highly labile protein(s). This was substantiated using cycloheximide where, by blocking synthesis of this labile protein(s), we can trigger the accumulation of B1 and B2 receptor mRNA.

Regulated degradation of several mRNAs in mammalian cells has been shown to depend on specific mRNA sequences or secondary structures termed stem-loop-destabilizing elements (Caput et al., 1986; Brown et al., 1996). These specific sequences, particularly the octanucleotide UUAUUUAU and a more limited portion of this sequence AUUUA present in the 3′-untranslated regions of several mRNA-encoding lymphokines and cytokines have been shown to act as instability determinants governing a rapid turnover of mRNAs (Caput et al., 1986; Brown et al., 1996). It is interesting to note that a mRNA-destabilizing element, AUUUA, has been reported to exist in the 3′- untranslated region of the B1 receptor, suggesting that this sequence may a have a role in post-transcriptional control of B1 receptor gene expression. The conserved UUAUUUAU octanucleotide may contribute to a binding site for a labile protein factor responsible for mRNA destabilization. The labile protein could be a ribonuclease itself or an mRNA-binding protein that enhances access of a site to ribonuclease action. Indeed, several mRNA-binding proteins have been identified and characterized and shown to serve as a signal for targeting mRNA that encode many cytokines, oncoproteins, and G-protein-coupled receptors for degradation (Katz et al., 1994; Nakamaki et al., 1995; DeMaria and Brewer, 1996). However, the significance of these AUUUA-binding proteins to mRNA stabilization has not been explored.

In summary, we have shown that both B1 and B2 receptors are induced and up-regulated, respectively, by the pleiotropic cytokine TNF-α through post-transcriptional mechanisms. We have also demonstrated that p38 MAPK and/or PKA are involved in the TNF-α signal transduction pathways in HEL 299 cells. Furthermore, the glucocorticoid dexamethasone inhibited the expression of these receptors through post-transcriptional mechanisms. Our results also indicate that BK receptors are activated during inflammation and such mechanism may be relevant for the manifestation of acute and chronic inflammatory processes where TNF-α and IL-1β production are the predominant mediators.

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