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Endothelin-1 Induces Proliferation of Human Lung Fibroblasts and IL-11 Secretion Through an ET_A Receptor-Dependent Activation of Map Kinases

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Abstract Endothelin-1 (ET-1) is implicated in the fibrotic responses characterizing interstitial lung diseases, as well as in the airway remodeling process occurring in asthma. Within such a context, the aim of our study was to investigate, in primary cultures of normal human lung fibroblasts (NHLFs), the ET-1 receptor subtypes, and the intracellular signal transduction pathways involved in the proliferative effects of this peptide. Therefore, cells were exposed to ET-1 in the presence or absence of an overnight pre-treatment with either ET_A or ET_B selective receptor antagonists. After cell lysis, immunoblotting was performed using monoclonal antibodies against the phosphorylated, active forms of mitogen-activated protein kinases (MAPK). ET-1 induced a significant increase in MAPK phosphorylation pattern, and also stimulated fibroblast proliferation and IL-6/IL-11 release into cell culture supernatants. All these effects were inhibited by the selective ET_A antagonist BQ-123, but not by the specific ET_B antagonist BQ-788. The stimulatory influence of ET-1 on IL-11, but not on IL-6 secretion, was prevented by MAPK inhibitors. Therefore, such results suggest that in human lung fibroblasts ET-1 exerts a profibrogenic action via an ET_A receptor-dependent, MAPK-mediated induction of IL-11 release and cell proliferation. J. Cell. Biochem. 96: 858–868, 2005.

Key words: ET-1; lung fibroblasts; ET-1 receptors; MAPK; IL-6; IL-11

Endothelin-1 (ET-1) is a 21-amino acid peptide which has potent vasoconstrictive, bronchoconstrictive, and mitogenic activities [Uchida et al., 1988; Yanagisawa et al., 1988; MacNulty et al., 1990; Glassberg et al., 1994; D'Agostino et al., 2001]. ET-1 expression is upregulated in fibrotic processes such as those characterizing interstitial lung diseases and airway remodeling in asthma [Hay et al., 1996; Geiser, 2003]. Indeed, these pathological conditions share a common tendency toward increased fibroblast proliferation, myofibroblast differentiation, and collagen synthesis leading to enhanced extracellular matrix deposition. ET-1 is produced by alveolar type II, as well as bronchial epithelial cells, thus being released nearby the adjacent fibroblasts. The lung tissue of patients with idiopathic pulmonary fibrosis is characterized by increased levels of ET-1 mRNA expression, ET-1 immunoreactivity, and ET-1 converting enzyme activity, responsible for catalytic cleavage of biologically inactive precursors (big endothelins) [Geiser, 2003]. ET-1 also appears to contribute to airway remodeling

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in asthma, at least in part by stimulating bronchial fibroblasts to synthesize DNA and proliferate [Dubé et al., 2000]. Furthermore, bronchial epithelial cells exposed to a mechanical stress of similar magnitude to that occurring during bronchoconstriction, release large quantities of ET-1 which can act locally at the focal points of subepithelial fibrosis [Tschumperlin et al., 2003]. Therefore, it is noteworthy that both plasma and bronchoalveolar lavage fluid (BALF) from patients with either pulmonary fibrosis or asthma contain high amounts of ET-1, whose circulating levels can be directly correlated with the severity of lung fibrotic phenotype, as well as with the degree of airway obstruction [Aoki et al., 1994; Cambrey et al., 1994; Vancheeswaran et al., 1994; Redington et al., 1995].

ET-1 exerts its biological actions via stimulation of membrane G protein coupled ET_A and ET_B receptors, which have been cloned, sequenced and, owing to the development of selective antagonists, also pharmacologically characterized [Arai et al., 1990; Sakurai et al., 1990; Hay et al., 1996]. However, only very recently the downstream, intracellular signal transduction pathways responsible for ET-1 mitogenic functions, are becoming to be elucidated. In this regard, a key role may be played by mitogen-activated protein kinases (MAPKs), which operate via phosphorylation cascades responsible for regulation of several substrates, mainly including transcription factors implicated in inflammation, development, cell proliferation, and apoptosis [Widmann et al., 1999; Chang and Karin, 2001]. Three major MAPK subgroups are currently known, activated by dual phosphorylation on tyrosine and threonine residues and named c-Jun N-terminal kinases (JNK), p38, and extracellular signal-regulated kinases (ERK), respectively. In particular, ERK activation is frequently required for mediating cell growth and differentiation induced by various stimuli, also including ET-1 [Whelchel et al., 1997].

Another interesting aspect, related to the biological functions performed in pulmonary fibroblasts by ET-1, concerns its potential effects on the synthesis of cytokines such as interleukins 6 (IL-6) and 11 (IL-11). These are multifunctional molecules secreted by many cells, also including fibroblasts [Elias et al., 1994; Taga, 1997]. IL-6 production is enhanced in patients with chronic respiratory diseases such as asthma, sarcoidosis, and systemic sclerosis with lung involvement [Crestani et al., 1994; Shahar et al., 1996; Elias et al., 1999]. Moreover, experimental overexpression of IL-11 resulted to be associated, in mice, with accumulation of fibroblasts and myofibroblasts, airway subepithelial fibrosis, and bronchial hyperresponsiveness [Zhu et al., 2001]. Therefore, both IL-6 and IL-11 seem to be actively involved in lung connective tissue repair and airway remodeling.

The cellular and molecular mechanisms underlying the contribution of ET-1 to the fibrogenic processes occurring in various respiratory disorders, such as interstitial pulmonary diseases and asthma, need to be further characterized. With this aim we investigated, in primary cultures of human lung fibroblasts, the role of ET-1 in MAPK activation, cell proliferation, and IL-6/IL-11 production; additionally, we tried to define which receptor subtype is responsible for the effects of ET-1 on lung fibroblast proliferation and cytokine synthesis.

MATERIALS AND METHODS

Reagents

ET-1 was purchased from Sigma (St. Louis, MO). Anti-phospho-p38, anti-phospho-ERK1/2, and anti-phospho-JNK monoclonal antibodies were purchased from New England Biolabs (Beverly, MA); anti-(total)-p38, anti-(total)-ERK1/2, and anti-(total)-JNK polyclonal antibodies were provided from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The ERK and p38 inhibitors, named PD98059 and SB203580, respectively, were from Calbiochem (San Diego, CA); the JNK inhibitor SP600125 was from Tocris Cookson, Inc., (Ellisville, MO). The selective ET_A and ET_B receptor antagonists, named BQ-123 and BQ-788, respectively, were provided by Calbiochem (San Diego). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma.

Primary Cultures of Human Lung Fibroblasts

Primary lines of normal human lung fibroblasts (NHLFs) were established by using an outgrowth from explant according to the method described by Jordana et al. [1988] [Vancheri et al. [2000]]. Fibroblasts were obtained from histologically normal areas of surgical specimens taken from patients who underwent either lobectomy or pneumonectomy

for lung cancer. Lung specimens were chopped into pieces less than 1 mm^3 and washed once with phosphate-buffered saline (PBS) and twice with RPMI containing 10% fetal calf serum (FCS), penicillin 100 U/ml, streptomycin 100 μg/ml, and fungizone 25 μg/ml (supplemented RPMI) (Gibco, Paisley, UK); 8–10 pieces of washed specimens were then plated in a 100-mm polystyrene dish (Falcon, Becton-Dickinson, Lincoln Park, NJ), and overlaid with a coverslip held to the dish with sterile vaseline. Ten milliliters of supplemented RPMI were added and the tissue was incubated at 37°C with 5% CO_2. The medium was changed weekly. When a monolayer of fibroblast-like cells covered the bottom of the dish, usually 5–6 weeks later, the explant tissue was removed, and the cells were then trypsinized for 10 min, resuspended in 10 ml of supplemented RPMI, and plated in 100-mm tissue culture dishes. Subsequently, cells were split 1:2 at confluence, usually weekly. Aliquots of cells were frozen and stored in liquid nitrogen. In all experiments, we used cell lines at a passage earlier than the tenth.

Cell Stimulation

NHLFs were incubated for different periods of time (24, 48, and 72 h) with ET-1 (100 nM), in the absence or presence of a mixture of MAPK inhibitors (40 μM PD98059, 20 μM SP600125, and 1 μM SB203580), and also pre-treated or not with one of the ET-1 antagonists (BQ-123 or BQ-788, 300 nM). These pharmacological pretreatments initiated 12 h before cell exposure to ET-1. The solvent used to dissolve ET-1 was utilized as a control. After cell stimulation, fibroblasts were processed for protein extraction and immunoblotting.

Protein Extraction and Immunoblot Analysis

Following stimulation with ET-1, cells were lysed for Western blotting in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM HEPES, pH 7.4, plus PPIM, 25 mM β-glycerophosphate, 1mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Protein extracts were then separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia, Little Chalfont, UK). Immunoblotting was performed using anti-phospho-p38, anti-phospho-ERK1/2, and anti-phospho-JNK monoclonal antibodies. After being "stripped," the membranes were re-probed with polyclonal antibodies against total (phosphorylated and unphosphorylated) p38, ERK1/2, and JNK. Antibody binding was visualized by enhanced chemiluminescence (ECL-Plus; Amersham Pharmacia); intensities of experimental bands were analyzed by computer-assisted densitometry and expressed as arbitrary units (AU; control levels set at 100). These experiments were performed in triplicate.

Cell Viability and Proliferation Assay

Cell viability was assessed by Trypan blue exclusion. To evaluate cell proliferation, we utilized the MTT assay, based on the conversion by mitochondrial dehydrogenases of the substrate containing a tetrazolium ring into blue formazan, detectable spectrophotometrically. The level of blue formazan was then used as an indirect index of cell density. Briefly, after treatment with ET-1, cells were exposed to MTT (5 μg/ml) for 150 min at 37°C. The medium was then removed and cells were solubilized with acidified isopropanol and 2% sodium dodecyl sulfate. After complete solubilization, the formation of blue formazan was evaluated spectrophotometrically with a reference wavelength of 650 nm. All experiments were performed in triplicate.

IL-6 and IL-11 Release

Cell-free culture supernatants were collected and assayed for IL-6 and IL-11 by enzyme linked immunosorbent assay (ELISA) using a commercially available kit (Peli-Kine kit; Eurogenetics, Hampton, UK; sensitivity limit, 1 pg/ml), accordingly to manufacturer's protocol. These experiments were performed in triplicate.

Expression of ET-1 Receptor Subtype mRNAs

Gene expression for ET-1 receptor subtypes was assessed using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cell cultures using TRI REAGENT (Molecular Research Center, Inc., OH) according to the manufacturer's protocol. mRNA levels relative to both ET_A and ET_B receptors were measured by RT-PCR amplification, performed as previously reported [Galderisi et al., 1999].

Sequences for human mRNAs from the GeneBank (DNASTAR, Inc., WI) were used to design primer pairs for RT-PCR reactions (OLIGO 4.05 software, National Biosciences, Inc., MN). Appropriate regions of HPRT cDNA were used as controls. PCR cycles were adjusted to have linear amplification for all the targets. Each RT-PCR reaction was repeated at least three times. A semi-quantitative analysis of mRNA levels was carried out by the "GEL DOC UV SYSTEM" (Biorad Company, CA).

Statistical Analysis

All data are expressed as mean ± standard error (SEM). Statistical evaluation of the results was performed by analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by unpaired Student's *t*-test. The threshold of statistical significance was set at $P < 0.05$.

RESULTS

Effects of ET-1 on MAPK Phosphorylation

Evaluation of the results obtained in three independent sets of experiments showed that exposure of NHLFs to ET-1 (100 nM) induced a significant increase, with respect to control levels, in the amount of phosphorylated MAPKs, and these effects of ET-1 resulted to be time related. In particular, after 24 h of cell stimulation, the most sensitive MAPK subgroup to the phosphorylating action of ET-1 was p38 (mean increase in densitometry expressed as AU: from 100 to 238) (Figs. 1 and 4), whereas at 48 and 72 h the highest increases in MAPK phosphorylation were observed with regard to JNK (from 100 to 250 AU) (Figs. 2 and 4) and ERK1/2 (from 100 to 335 AU) (Figs. 3 and 4). Therefore, we detected a sequential effect of ET-1 on the three main MAPK subgroups, with p38 being the first enzyme to undergo phosphorylation, followed by JNK and then by ERK1/2. Since the monoclonal antibodies (anti-phospho-p38, anti-phospho-JNK, and anti-phospho-ERK1/2) used in this study specifically recognize the phosphorylated, active forms of MAPKs, the remarkable induction of MAPK phosphorylation can

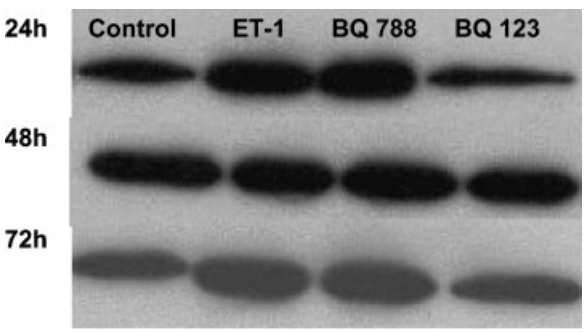


Fig. 1. Time course of p38 phosphorylation. Fibroblasts were stimulated with ET-1 (100 nM), in the presence or absence of either BQ 788 (an ET_B selective antagonist) or BQ 123 (an ET_A selective antagonist). After 24, 48, and 72 h of cell stimulation with ET-1, p38 phosphorylation was evaluated by Western blot analysis, using specific anti-phospho-p38 monoclonal antibodies.

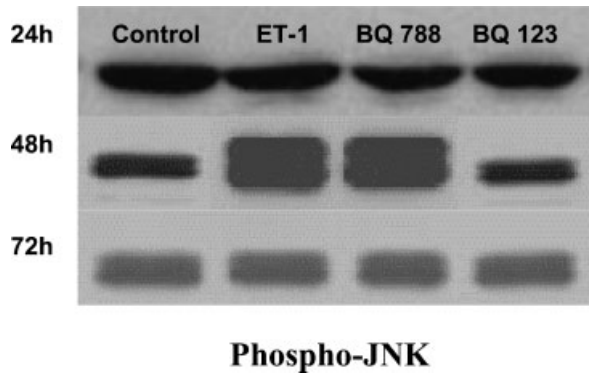


Fig. 2. Time course of JNK phosphorylation. Fibroblasts were stimulated with ET-1 (100 nM), in the presence or absence of either BQ 788 (an ET_B selective antagonist) or BQ 123 (an ET_A selective antagonist). After 24, 48, and 72 h of cell stimulation with ET-1, JNK phosphorylation was evaluated by Western blot analysis, using specific anti-phospho-JNK monoclonal antibodies.

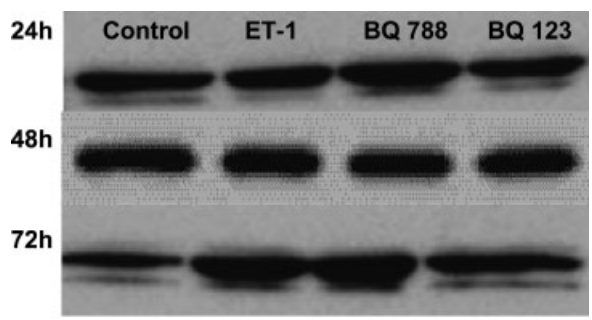


Fig. 3. Time course of ERK1/2 phosphorylation. Fibroblasts were stimulated with ET-1 (100 nM), in the presence or absence of either BQ 788 (an ET_B selective antagonist) or BQ 123 (an ET_A selective antagonist). After 24, 48, and 72 h of cell stimulation with ET-1, ERK1/2 phosphorylation was evaluated by Western blot analysis, using specific anti-phospho-ERK1/2 monoclonal antibodies.

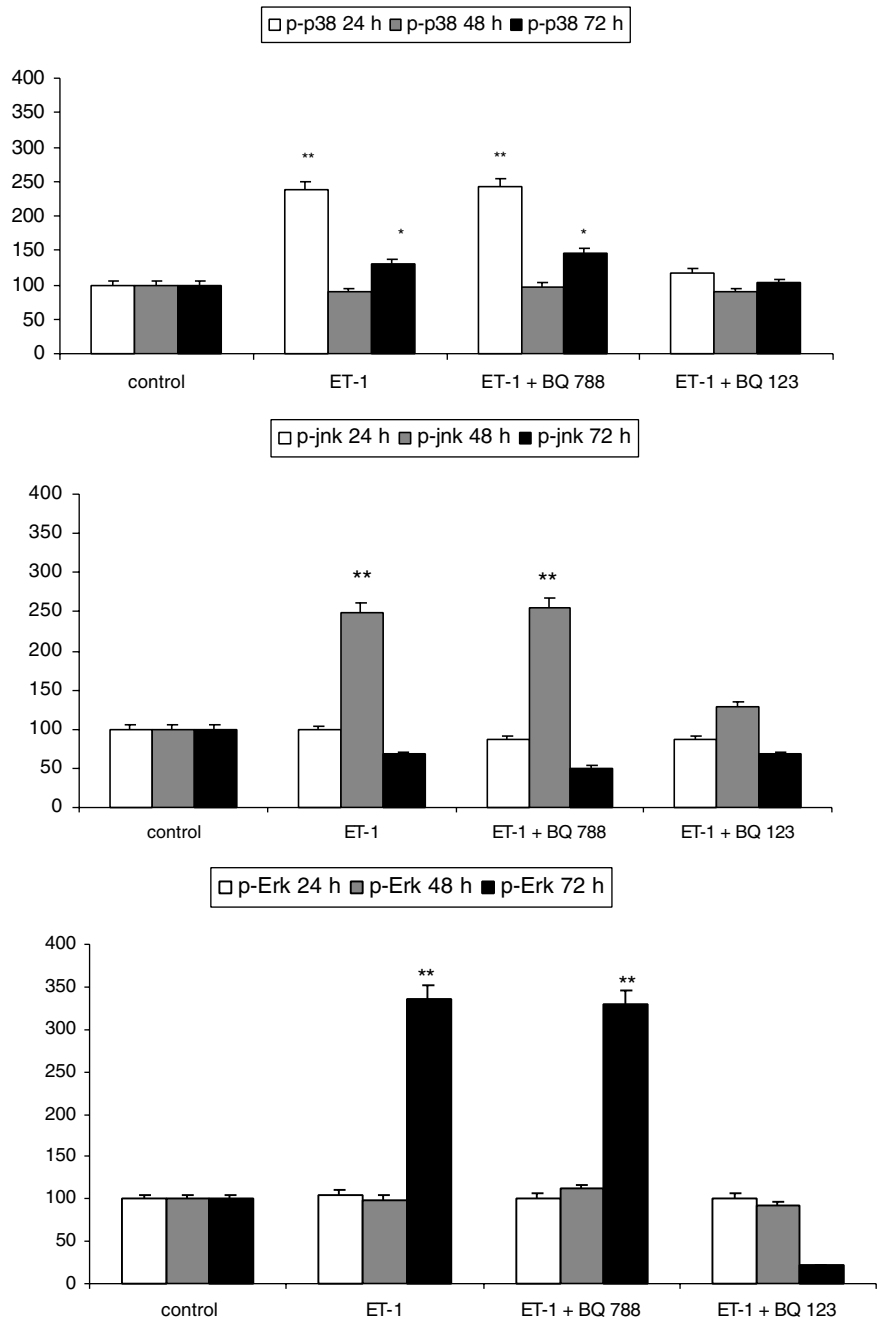


Fig. 4. ET-1-induced phosphorylation of p38, JNK and ERK1/2. Exposure of primary cultures of NHLFs to ET-1 induced a time-related increase in the amount of phosphorylated p38, JNK, and ERK1/2, that was inhibited by BQ 123, but not affected by BQ 788. Densitometric evaluation of Western blots is expressed as arbitrary units (control levels: 100). * $P < 0.05$; ** $P < 0.01$.

be considered as a reliable marker of their highly efficient activation elicited by ET-1. Interestingly, ET-1-stimulated phosphorylation of MAPKs was almost completely inhibited by pre-treatment with the selective ET_A receptor antagonist (BQ-123, 300 nM), whereas it was not affected by the specific ET_B antagonist (BQ-788, 300 nM) (Figs. 1–4).

ET-1 exerted its effects uniquely on phosphorylation-dependent activation of MAPKs, without affecting their total expression, as demonstrated by the unchanged binding patterns of the anti-(total)MAPK polyclonal antibodies (data not shown). Furthermore, MAPK total expression was not significantly affected by either BQ-123 or BQ-788.

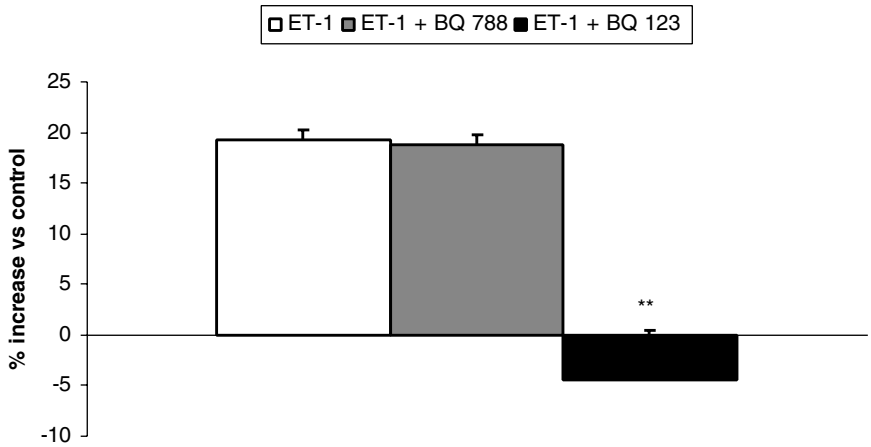


Fig. 5. ET-1-induced cell proliferation. Exposure of primary cultures of NHLFs to ET-1 enhanced the cell proliferation rate, evaluated as percentage of MTT reduction after 72 h of cell stimulation with ET-1. This effect was significantly inhibited by BQ 123, but unaffected by BQ 788. $^{**}P < 0.01$.

Effects of ET-1 on Cell Viability and Proliferation

ET-1 enhanced by almost threefold the number of NHLFs ($P < 0.01$), as detected by total cell count (data not shown). ET-1 also elicited an almost 20% increase in the proliferation rate of our primary cultures of NHLFs, evaluated as percentage of MTT reduction and detected after 72 h of cell stimulation (Fig. 5). This effect was significantly ($P < 0.01$) inhibited by pre-treatment with BQ-123, whereas it was not affected by BQ-788 (Fig. 5).

Effects of ET-1 on the Release of IL-6 and IL-11

ET-1 stimulated significantly the production of both IL-6 and IL-11, as assessed by ELISA in cell free culture supernatants at different time points (Figs. 6 and 7). These effects resulted to be characterized by a time-dependent pattern, starting 2 h after exposure to ET-1 (mean values: from 510 ± 75 to 645 ± 82 pg/ml for IL-6; from 1190 ± 110 to 2209 ± 180 pg/ml for IL-11), and reaching the highest detectable increase after 72 h (from 769 ± 95 to 1290 ± 135 pg/ml for IL-6; from 1850 ± 158 to 4500 ± 229 pg/ml for IL-11).

The effects of ET-1 on IL-6 secretion were significantly inhibited by BQ-123 at 8 h ($P < 0.05$) and 24–48–72 h ($P < 0.01$), but not by either BQ-788 or MAPK inhibitors (Fig. 6). The ET-1-induced release of IL-11 was prevented by MAPK inhibitors, and significantly inhibited by BQ-123 in both presence ($P < 0.05$ at 48 h and $P < 0.01$ at 72 h) and absence ($P < 0.01$ at any time) of MAPK inhibitors, whereas BQ-788 did not elicit any significant change (Fig. 7).

Expression of ET-1 Receptor Subtypes

Because the pharmacological antagonism of the ET_B receptor did not produce any relevant biological effect in our experimental model, we investigated the expression of both ET-1 receptor subtypes. In this regard, RT-PCR demonstrated that both mRNAs encoding ET_A and ET_B receptor subtypes, respectively, are detectable in human normal lung fibroblasts. Surprisingly, ET_B receptor expression resulted to be remarkably higher than ET_A at the mRNA level, as shown by the very different intensities of UV visualization of the two PCR products (Fig. 8).

DISCUSSION

In this study, we show that ET-1 was capable, in primary cultures of NHLFs, of inducing MAPK activation, IL-6/IL-11 production, and cell proliferation. All these effects resulted to be dependent on stimulation of the ET_A receptor, although its expression in our cell cultures was remarkably lower than the ET_B subtype. In particular, ET-1-stimulated MAPK phosphorylation displayed a temporal pattern characterized by a sequential involvement of p38, JNK, and ERK1/2, respectively. Such a phenomenon might be interpreted as a cascade of molecular mechanisms underlying several different biological events, also including an increase in IL-11, but not IL-6 synthesis. In fact, ET-1-induced release of IL-6 into our lung fibroblast culture

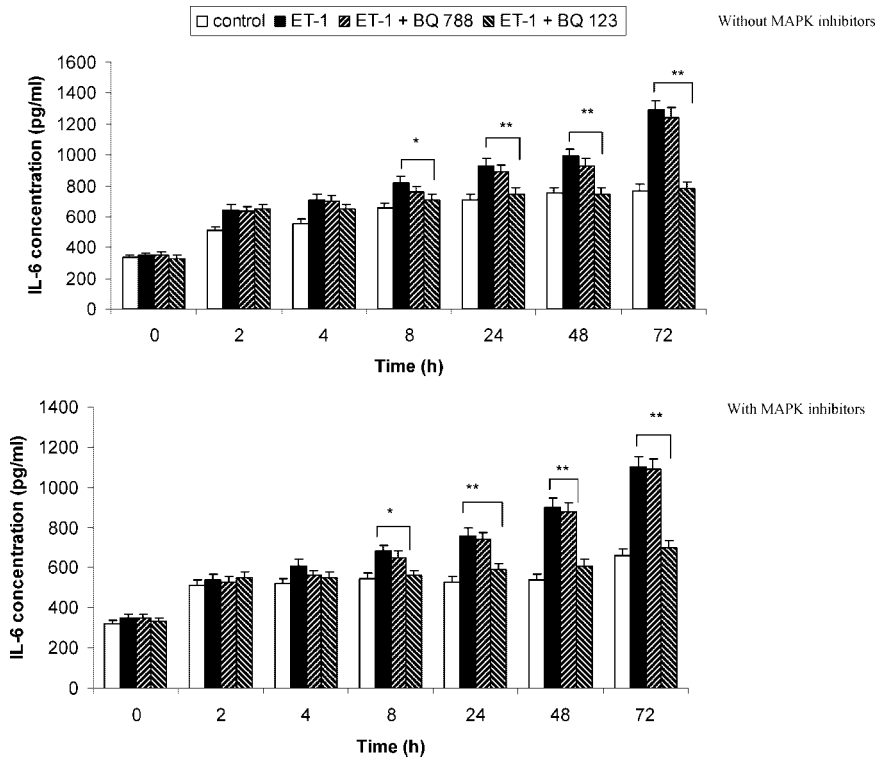


Fig. 6. ET-1-induced release of IL-6. Exposure of NHLFs to ET-1 induced a time-related increase in the release of IL-6 into cell culture supernatants, in both absence (upper panel) and presence (lower panel) of an overnight pre-treatment with a mixture of MAPK inhibitors (PD98059, 40 μM; SP600125, 20 μM; SB203580, 1 mM). This effect was inhibited by by BQ 123, but unaffected by BQ 788. $^{*}P < 0.05$; $^{**}P < 0.01$.

supernatants was not affected by MAPK inhibitors, thereby suggesting the involvement of other signal transduction pathways. However, in other experimental models exemplified by lung fibroblast activation elicited by intercellular contacts with mast cells, Fitzgerald et al. [2004] have shown that IL-6 secretion was p38 dependent. These findings imply that MAPK contribution to cytokine production by pulmonary fibroblasts is strictly stimulus-specific. Referring to our experiments, the cause of the increase in IL-6 control levels within 2 h is not clear, though does not seem to be dependent on MAPK activation; however, it cannot be ruled out that changes in cell-to-cell contacts, occurring within relatively short periods of time, may affect IL-6 release by unknown mechanisms possibly including autocrine loops.

With regard to ET-1-induced ERK phosphorylation, our results evidenced that this event picked at the 72nd h of cell stimulation, after p38 and JNK phosphorylation, thus suggesting a possible activation of late genes involved in cell-cycle regulation. Consistently with this interpretation, Moodley et al. [2003] have recently shown, in lung fibroblasts obtained from both normal subjects and patients with idiopathic pulmonary fibrosis, that IL-11 was capable of eliciting a powerful proliferative effect via ERK1/2 activation, downregulation of the cyclin dependent kinase (cdk) inhibitor $p27^{kip1}$, and the consequent induction of cyclins D_1 and E_1 which are responsible for progression through the cell cycle. Furthermore, in addition to the stimulatory effect on IL-11 synthesis documented by our results, ET-1-mediated MAPK activation is also responsible, according to other recent studies performed by Shi-wen et al. [2004a] in lung fibroblasts, for an increased expression of several matrix-associated genes. The latter include various collagen types, matrix metalloproteinases, and their tissue inhibitors, as well as α and β integrins, basic fibroblast growth factor 2, the TGF-β activator thrombospondin 1, and the profibrotic protein CCN2, also named connective tissue growth factor (CTGF) [Shi-wen et al., 2004a]. Therefore, our data referring to the proliferative effect of ET-1 may be explained by its direct mitogenic action, also associated with the

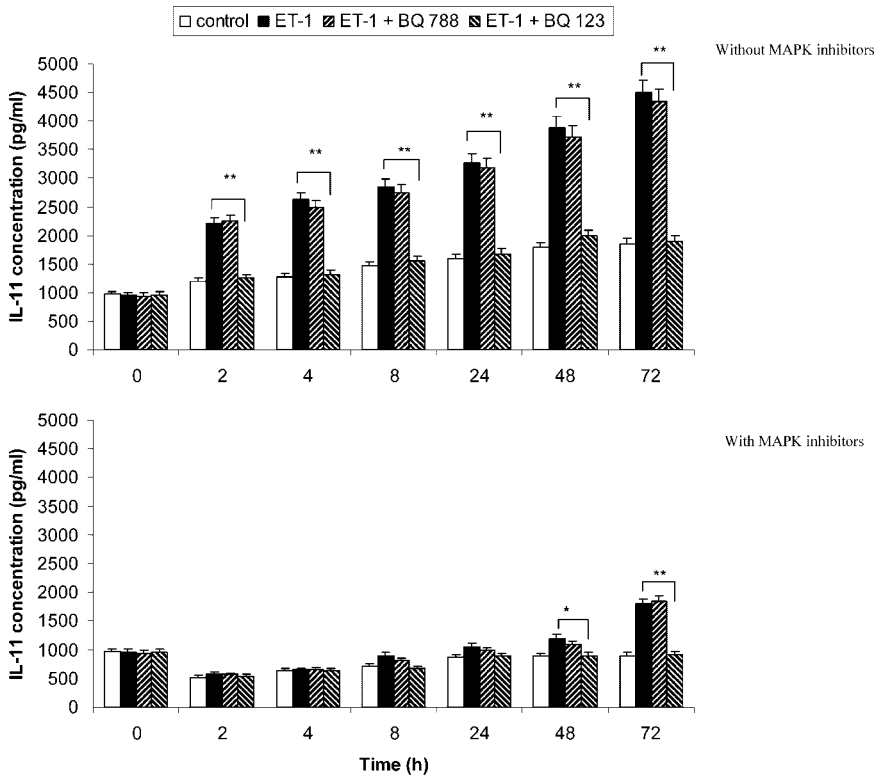
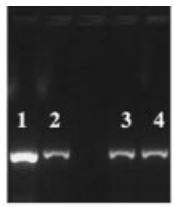


Fig. 7. ET-1-induced release of IL-11. Exposure of NHLFs to ET-1 induced a time-related increase in the release of IL-11 into cell culture supernatants (upper panel), which was prevented by an overnight pre-treatment with a mixture of MAPK inhibitors (lower panel). BQ 123, but not BQ 788, exerted a significant inhibitory effect on IL-11 secretion, in both absence (upper panel) and presence (lower panel) of the overnight pre-treatment with MAPK inhibitors. $^{*}P < 0.05$; $^{**}P < 0.01$.

induction of IL-11 and other fibroblast growth factors. Overall, these MAPK-dependent molecular mechanisms significantly contribute to the fibrogenic role of ET-1, which has also been demonstrated in animal models of interstitial lung diseases, including transgenic mice over-expressing ET-1 and rats developing bleomycin-induced pulmonary fibrosis [Mutsaers et al., 1998; Hocher et al., 2000].

Interestingly, all the biological actions of ET-1 presented in the current report and including MAPK-activation, IL-6/IL-11 release, and cell proliferation, resulted to be due to stimulation of the ET_A receptor subtype. In fact, these effects were suppressed by an ET_A selective antagonist, without being affected by the blockade of the ET_B receptor. Such results suggest that, although our RT-PCR experiments showed that in NHLFs the ET_B receptor subtype is much more expressed than the ET_A, the latter is responsible for the MAPK-mediated effects induced by ET-1 in these cells. To our knowledge, the molecular components connecting the ET_A receptor to the MAPK phosphorylation cascades are currently not known. However, distinct features have been identified by Stannard et al. [2003] within the structures of the two ET-1 receptors expressed by human lung fibroblasts, with regard to their phosphorylation and palmitoylation sites, which are more numerous in the ET_A subtype. This structural diversity could perhaps provide the basis for a parallel stimulation of different signal transduction systems, thus possibly explaining the specific role of the ET_A receptor in these cells. The pathophysiologic relevance of the ET_A receptor present in lung fibroblasts is also confirmed by very recent studies showing that this receptor, but not the ET_B subtype, mediates the ET-1-induced expression of many proteins such as α-smooth muscle actin, ezrin, moesin, and paxillin that contribute to the acquisition of a myofibroblast phenotype [Shi-wen et al., 2004b]. This effect is due to an ET_A-dependent activation of the phosphoinositide 3-kinase/Akt signaling pathway [Shi-wen et al., 2004b]. On the other hand, the functional predominance of the ET_A receptor, though this



	ET_B	ET_A
mRNA level	5000±120	2017±65

Fig. 8. Expression pattern of ET-1 receptor subtypes. RT-PCR showed that, in NHLFs, ET_B receptor expression (**lane 1**) was remarkably higher than ET_A (**lane 2**) at the mRNA level. mRNA levels were measured by a GEL DOC instrument and normalized with respect to HPRT (**lanes 3** and **4** for ET_B and ET_A, respectively), which was chosen as an internal control. Each experiment was repeated at least three times. Variations in gene expression are given as arbitrary units.

subtype may constitute the minor proportion of the ET-1 receptors expressed in lung tissues, appears to involve also other cell types throughout the respiratory system. For instance, with regard to the pro-inflammatory and remodeling responses stimulated in the airways by ET-1, it is the activation of the ET_A receptor subtype that causes an enhanced prostanoid secretion from bronchial epithelial cells, as well as the potentiation of tracheal smooth muscle cell growth [Panettieri et al., 1996; Takimoto et al., 1996].

Taken together, our data reported in the present article strongly suggest that the exposure to ET-1 of primary cultures of NHLFs results in stimulation of the ET_A receptor subtype, leading to a sequential activation of p38, JNK, and ERK1/2 MAPKs, respectively. These events are in turn responsible for a significant increase in IL-11 production and cell proliferation (Fig. 9). We believe that such findings add a further piece of experimental evidence to the complex scenario of the cellular and molecular mechanisms underlying the pathogenic function of ET-1 in pulmonary fibrosis and airway remodeling. Indeed, in fibrotic processes affecting the lung parenchyma and the tracheo-bronchial tree, ET-1 is upregulated and acts in concert with several other pro-fibrogenic molecules including TGF-β, platelet derived growth factor, basic fibroblast growth factors, insulin-like growth factor-1, and IL-11 itself [Zhang et al., 1999; Holgate et al., 2000; Geiser, 2003; Knight and Holgate, 2003; Vignola et al., 2003]. In particular, the synergistic interactions involving these mediators are crucial for implementing the tissue

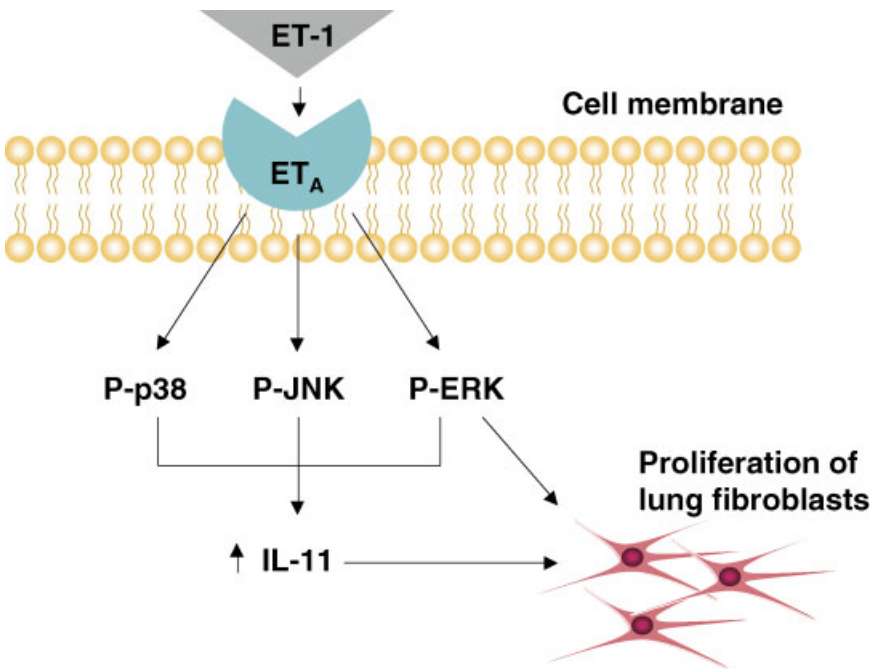


Fig. 9. Schematic diagram of ET-1 effects in NHLFs. ET-1 stimulates ET_A receptors thus promoting MAPK activation, IL-11 secretion, and cell proliferation. The latter effect may be induced by ET-1 both directly and via the increased IL-11 synthesis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

repair response leading to fibroblast proliferation, myofibroblast differentiation, and collagen synthesis. Within this context, a pivotal role is played by MAPKs, whose intervention in the cross-talk between ET-1 and the other fibrogenic factors takes place at multiple levels. In fact, these signal-transducing enzymes are implicated in both synthesis and biological activity of the above-mentioned molecules. Of course, many aspects still remain to be further investigated. For example, more work is needed to elucidate the biochemical modalities whereby ET_A receptor stimulation triggers MAPK phosphorylation cascades. Moreover, the functional connections operating within the intricate network of MAPK-regulated transcription factors and genes involved in proliferation and secretory activity of lung fibroblasts, also require to be fully clarified. In any case, there is no doubt that MAPKs represent a central point of convergence for multiple signaling pathways coordinating and integrating the bi-directional interplay between lung fibroblasts and pulmonary/airway epithelial cells, which represent a rich source of fibroblast growth factors. However, the MAPK-independent, intracellular systems responsible for the ET_A-mediated stimulation of IL-6 release from human pulmonary fibroblasts, should also be elucidated.

In conclusion our findings underscore the role, in ET-1-induced lung fibroblast proliferation, of ET_A receptor subtype, MAP kinase signal transduction pathways, and IL-11 synthesis. Although data obtained in lung fibroblasts should not be directly extrapolated to bronchial fibroblasts, it can be reasonably assumed that these two cell populations share a similar biological behavior in response to ET-1 and other stimuli. Therefore, the molecular mechanisms underlying the above-mentioned ET-1 effects probably contribute to the fibrotic environment of both lung parenchyma and airway wall, thereby representing potential pharmacological targets for treatment of interstitial pulmonary diseases and chronic asthma.

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