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Finsnes, Finn, Torstein Lyberg, Geir Christensen, and Ole H. Skjønsberg. Effect of endothelin antagonism on the production of cytokines in eosinophilic airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 280: L659–L665, 2001.—Endothelin (ET)-1 has been launched as an important mediator in bronchial asthma, which is an eosinophilic airway inflammation. However, the interplay between ET-1 and other proinflammatory mediators during the development of airway inflammation has not been elucidated. We wanted to study *1*) whether the production of ET-1 precedes the production of other proinflammatory mediators and *2*) whether ET-1 stimulates the production of these mediators within the airways. These hypotheses were studied during the development of an eosinophilic airway inflammation in rats. The increase in ET-1 mRNA level in lung tissue preceded the increase in mRNA levels of tumor necrosis factor-α, interleukin (IL)-1β, and IL-8. Treatment of the animals with the ET receptor antagonist bosentan resulted in a substantial decrease in the concentrations of tumor necrosis factor-α, IL-4, IL-1β, interferon-γ, and ET-1 in bronchoalveolar lavage fluid. In conclusion, the synthesis of ET-1 as measured by increased mRNA level precedes the synthesis of other proinflammatory cytokines of importance for the development of an eosinophilic airway inflammation, and ET antagonism inhibits the production of these mediators within the airways. Whether treatment with ET antagonists will prove beneficial for patients with eosinophilic airway inflammations like bronchial asthma is not yet known.

asthma

SEVERAL CYTOKINES HAVE BEEN CLAIMED to play key roles in the pathogenesis of bronchial asthma, which is an inflammatory disease dominated by eosinophilic granulocytes. However, no mediator has emerged as being the single most important one. Over the last years, interest has been drawn to the 21-amino acid peptide endothelin (ET)-1 (18), which has proved to be a very potent bronchoconstrictor both in vitro (36) and in patients with asthma (7). In addition, ET-1 has the ability to induce mucus secretion from airway submucosal glands (40) and also to increase vascular leakage, leading to edema (41). These properties are all characteristics of bronchial asthma. Increased levels of ET-1 have been demonstrated in the bronchoalveolar lavage (BAL) fluid (BALF) from patients with asthma (28, 33, 38, 42) and also in experimental models of eosinophilic airway inflammation (1, 13, 14).

Although there are indications of ET-1 being involved in the development of eosinophilic airway inflammation (13, 14, 16), the mechanism responsible for the proinflammatory action of ET-1 and the interplay between ET-1 and other mediators of inflammation have not been elucidated. For development of new therapeutic strategies, it seems of crucial importance to identify the initiating mediator of the inflammatory cascade. Therefore, our first hypothesis was that the generation of ET-1 precedes the release of other proinflammatory mediators. Accordingly, we investigated the production of ET-1 in rat airways in relation to other mediators during the development of an eosinophilic airway inflammation. An eosinophilic inflammation was induced by intratracheal instillation of Sephadex (SDX), a dextran to which rats have an endogenous hypersensitivity (23). We investigated the earliest phases of the inflammatory process to study both the synthesis and release of ET-1, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-8, and interferon (IFN)-γ and also the generation of leukotriene (LT) B_4, which is the most potent chemoattractant of the LTs (15). The concentrations of all these mediators are found to be elevated in patients with bronchial asthma (6, 24, 27, 31, 44). However, it should be acknowledged that other mediators may be involved in the eosinophilic inflammation as well (2).

A finding of ET-1 being generated before the other proinflammatory mediators in vivo would be consistent with ET-1 playing a key role in the development of airway inflammation, possibly by promoting the generation of the other mediators. Our second hypothesis was that ET-1 actually stimulates the production of other proinflammatory mediators within the airways. This hypothesis was studied by evaluating the effect on the concentration of the proinflammatory mediators listed above in BALF after treatment of the rats with the ET-specific receptor antagonist bosentan (8, 9).

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METHODS

Experimental procedure. One hundred twenty-nine male Wistar rats aged 11 wk, with an average weight of 310 g, were used in the study. The experiments were approved by the Norwegian Ethics Committee for Animal Research and performed according to the National Institutes of Health guidelines for the use of experimental animals.

The animals (n = 6 in each group) were evaluated at 15 and 30 min and 1, 2, 3, 6, 12, 24, and 48 h after induced inflammation. These were compared with control animals (n = 3 in each group) examined at the same time points. An eosinophilic airway inflammation was induced by intratracheal instillation of SDX particles (G-200 Superfine, Pharmacia & Upjohn, Uppsala, Sweden) dissolved in phosphate-buffered saline (PBS) as previously described (14) and compared with that in control animals receiving a similar volume of PBS intratracheally. BAL was performed by instillation of 3 + 2 + 2 ml of PBS in the right stem bronchus distal to the upper lobe, and the procedure was repeated in the main bronchus on the left side. Immediately after the BAL procedure, the lungs were removed and snap frozen in liquid nitrogen for tissue RNA extraction 15 and 30 min and 1, 2, 3, 6, 12, 24, and 48 h after SDX instillation (n = 3 animals at each time point) and 30 min after intratracheal instillation of saline into control animals (n = 3).

Bosentan (Actelion, Allschwil, Switzerland), a nonpeptide compound with mixed antagonist properties for ET_A and ET_B receptors (9), was given intravenously (great saphenous vein) to 12 animals (30 mg/kg body wt) 1 h before intratracheal SDX instillation. Bosentan is a highly specific ET receptor antagonist, which is shown by selectivity screening on 40 different receptors and channels (8, 9). Six animals were evaluated after 3 h. The animals that were evaluated after 24 h (n = 6) received a repeated dose of bosentan 8 h after SDX instillation. In separate control experiments, bosentan treatment of animals (n = 6) receiving saline intratracheally was shown to have no influence on the concentration of either ET-1 (7.43 ± 2.25 pg/ml in bosentan plus saline vs. 2.5 ± 0.41 pg/ml in saline), TNF-α (1 ± 0 vs. 1 ± 0 pg/ml), IL-1β (65.9 ± 16 vs. 85.7 ± 18.9 pg/ml), IL-4 (17.1 ± 5.2 vs. 26.7 ± 6.7 pg/ml), IL-8 (4.5 ± 1.0 vs. 4.1 ± 0.86 pg/ml), IFN-γ (2.8 ± 0.7 vs. 3.7 ± 1.0 pg/ml), or LTB_4 (75.3 ± 22.3 vs. 74.8 ± 13.3 pg/ml) in BALF evaluated 24 h after saline provocation.

Cytokine mRNA analyses. mRNA was extracted from homogenized lung tissue with oligo(dT)-conjugated paramagnetic beads (Dynal, Oslo, Norway) loaded on nylon membranes and hybridized with radiolabeled cDNA probes as previously described in detail (13). The filters were hybridized with glyceraldehyde-3-phosphate dehydrogenase, preproET-1 mRNA (provided by Dr. Takashi Miyauchi), TNF-α mRNA (a gift from Prof. Jack Gauldie), the IL-8 mRNA-like cytokine-induced neutrophil chemoattractant/growth-related protein (CINC/GRO; supplied by Dr. Toshiaki Ohtsuka), IL-1β mRNA, IL-4 mRNA (from Prof. Thomas Blankenstein), and IFN-γ mRNA (provided by Dr. Chris Broeren). Autoradiography of the filters was carried out in a storage phosphorscreen and analyzed by densitometric scanning analysis with the ImageQuant version 3.3 software from Molecular Dynamics (Sunnyvale, CA). To estimate the cytokine mRNA tissue levels, the samples were corrected for any variations by determining the ratio to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Biochemical analysis of BALF. BALF was collected into prechilled tubes containing EDTA and kept on ice until centrifuged at 800 g for 10 min at 4°C. The supernatants from the BALF were immediately stored at −70°C until analyzed. ET-1 was determined with a radioimmunolinked ET 1–21-specific ^{125}I assay system from Amersham Pharmacia Biotech International (Cardiff, UK). Before ET-1 analysis, samples were extracted in duplicate from 2 ml of BALF. The BALF was added to 6.0 ml of 4% acetic acid on Sep-Pak C18 cartridges (Millipore, Milford, MA). The cartridges were prewashed with 10 ml of 86% ethanol and 10 ml of 4% acetic acid. The extract was freeze-dried and dissolved in 500 μl of 0.02 M borate buffer. The ET-1 assay has a limit of detection equivalent to 1.6 pg/ml. For the samples with ET-1 concentrations below this limit, the values were set to 1.6 pg/ml.

TNF-α was measured with a rat-specific sandwich enzyme-linked immunosorbent assay (ELISA; Factor-Test-X, Genzyme, Cambridge, MA). Obtained values below the detection limit of this assay system (10 pg/ml) were consequently set to 10 pg/ml. IL-1β, IL-4, and IFN-γ were analyzed with rat-specific ELISAs (Cytoscreen, BioSource International), with detection limits of 3, 15, and 13 pg/ml, respectively. IL-8 (CINC/GRO) was measured with Panatest rat IL-8 ELISA (Panapharm Laboratories, Kumamoto, Japan), with a detection limit of 0.8 pg/ml. The LTB_4 concentration in BALF was measured with a competitive enzyme immunoassay kit (ACE, Cayman Chemical), with a detection limit of 4.43 pg/ml. All measurements were performed in duplicate, and values are given as means. For TNF-α, IL-1β, IL-4, IL-8, and IFN-γ assays, the BALF was concentrated tenfold before analysis, and the results are given as picograms per milliliter of unconcentrated BALF. The BALF was concentrated with Minicon cells (Amicon Division, WR Grace, Beverly, MA). For LTB_4 analysis, BALF samples were purified according to the manufacturer's instructions with a C18 reverse-phase cartridge (Sep-Pak, Millipore). Because there were no differences observed in control animals receiving PBS intratracheally at the different time points studied, these were pooled into one group.

Statistical analysis. All values are expressed as means ± SE. Statistical analyses were performed with scientific statistical software (SigmaStat version 2.0, Jandel Scientific, Ekrath, Germany). The groups were compared with Kruskal-Wallis one-way analysis of variance on ranks, followed by a multiple comparison procedure versus control with Dunn's method. A P value of <0.05 was considered significant.

RESULTS

Synthesis of cytokine mRNA in lung tissues. The first increase in cytokine gene expression detected after intratracheal SDX provocation was that of ET-1 (Fig. 1*A*). As early as 15 min after challenge, the lung tissue ET-1 mRNA increased significantly (Fig. 1*A*). At 30 min, the ET-1 mRNA peaked and reached a fourfold increase. Interestingly, this abundant rise in ET-1 mRNA was primarily detected in the very early phase of inflammation, with a decline after 2 h. Compared with ET-1, the synthesis of the other cytokines occurred at a later stage of the inflammatory process. IL-1β and IL-8 mRNA expression did not increase significantly until 12 h after SDX instillation (Fig. 1, *B* and *C*, respectively). IL-1β mRNA was increased approximately fourfold at 12, 24, and 48 h (Fig. 1*B*). IL-8 was elevated fivefold 12 h after induction of airway inflammation and sixfold after 48 h (Fig. 1*C*). No significant increase in TNF-α mRNA was measured until 48 h after provocation, when a twofold increase was

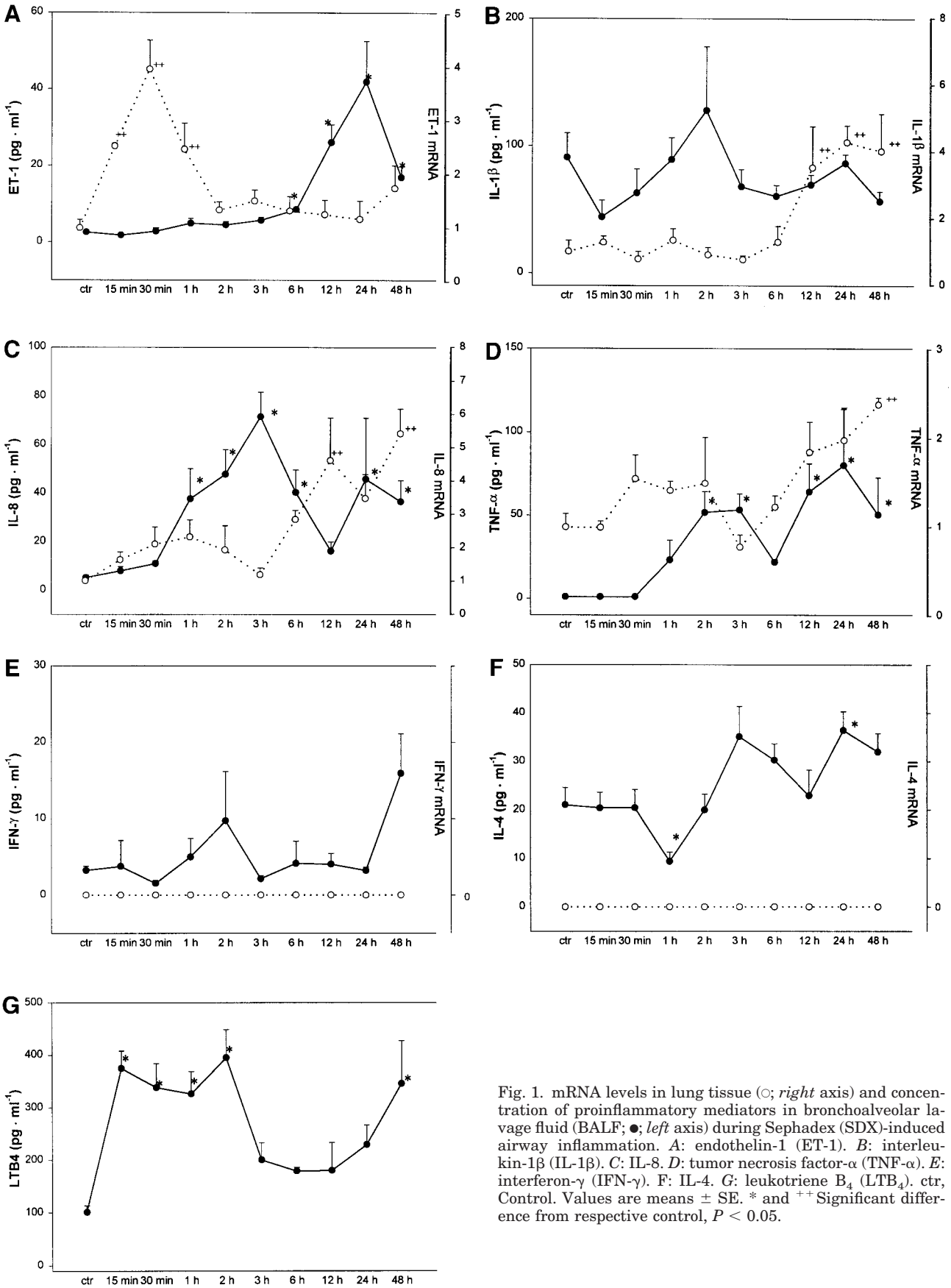


Fig. 1. mRNA levels in lung tissue (○; *right* axis) and concentration of proinflammatory mediators in bronchoalveolar lavage fluid (BALF; ●; *left* axis) during Sephadex (SDX)-induced airway inflammation. *A*: endothelin-1 (ET-1). *B*: interleukin-1β (IL-1β). *C*: IL-8. *D*: tumor necrosis factor-α (TNF-α). *E*: interferon-γ (IFN-γ). F: IL-4. *G*: leukotriene B_4 (LTB_4). ctr, Control. Values are means ± SE. * and $^{++}$Significant difference from respective control, $P < 0.05$.

observed (Fig. 1*D*). With regard to IFN-γ and IL-4, mRNAs for these cytokines were not detectable in the lung tissues during the first 48 h of the eosinophilic inflammation (Fig. 1, *E* and *F*, respectively).

Concentration of proinflammatory mediators in BALF. In BALF, a substantial amount of LTB_4 was found as early as 15 min after induced inflammation (Fig. 1*G*). This high level of LTB_4 was maintained for 2 h. Notably, a second delayed rise of LTB_4 was observed at 48 h (Fig. 1*G*). IL-8 increased significantly 1 h postchallenge (Fig. 1*C*) followed by TNF-α after 2 h (Fig. 1*D*). These cytokines tended to express a biphasic response, with peaks at 3 and 24 h. The concentration of ET-1 was significantly elevated 6 h after SDX provocation, peaking after 24 h with a 35-fold increase (Fig. 1*A*). The increase in IL-4 was not significant until 24 h (Fig. 1*F*). Regarding IL-1β and IFN-γ, the increase in these cytokines did not reach significance during the period studied (Fig. 1, *B* and *E*, respectively). However, the response of these three cytokines (IL-4, IL-1β, and IFN-γ) also indicated a biphasic pattern.

Effect of ET-1 antagonism on proinflammatory mediators in BALF. Treatment with the ET receptor antagonist bosentan effectively inhibited the increase in the TNF-α concentration in BALF at both 3 and 24 h after SDX provocation (Fig. 2*A*). The inhibition was most pronounced at 24 h when the TNF-α level in the bosentan-treated animals was 18% of that in the animals not receiving the ET-1 antagonist. The levels of IL-4, IL-1β, and IFN-γ were also significantly reduced after bosentan treatment after both 3 and 24 h (Fig. 2, *B–D*, respectively). The maximal inhibition of IL-4 was observed after 3 h, with a reduction to 22% of the level in the animals receiving SDX alone (Fig. 2*B*). The maximal inhibition of IL-1β and IFN-γ occurred at 24 h. At this time point, the level of IL-1β was reduced to 27% and the level of IFN-γ to 9% of the cytokine concentrations in BALF from animals that did not receive bosentan. The concentration of ET-1 was unaffected by bosentan treatment at 3 h but was substantially reduced to 19% 24 h after induced inflammation in the animals receiving bosentan (Fig. 2*E*). The IL-8 concentration in BALF, however, was not significantly reduced by bosentan treatment, although a tendency toward a decreased IL-8 level at 24 h was observed (Fig. 2*F*). The level of LTB_4 was not influenced by treating the animals with the ET-1 receptor antagonist (Fig. 2*G*).

DISCUSSION

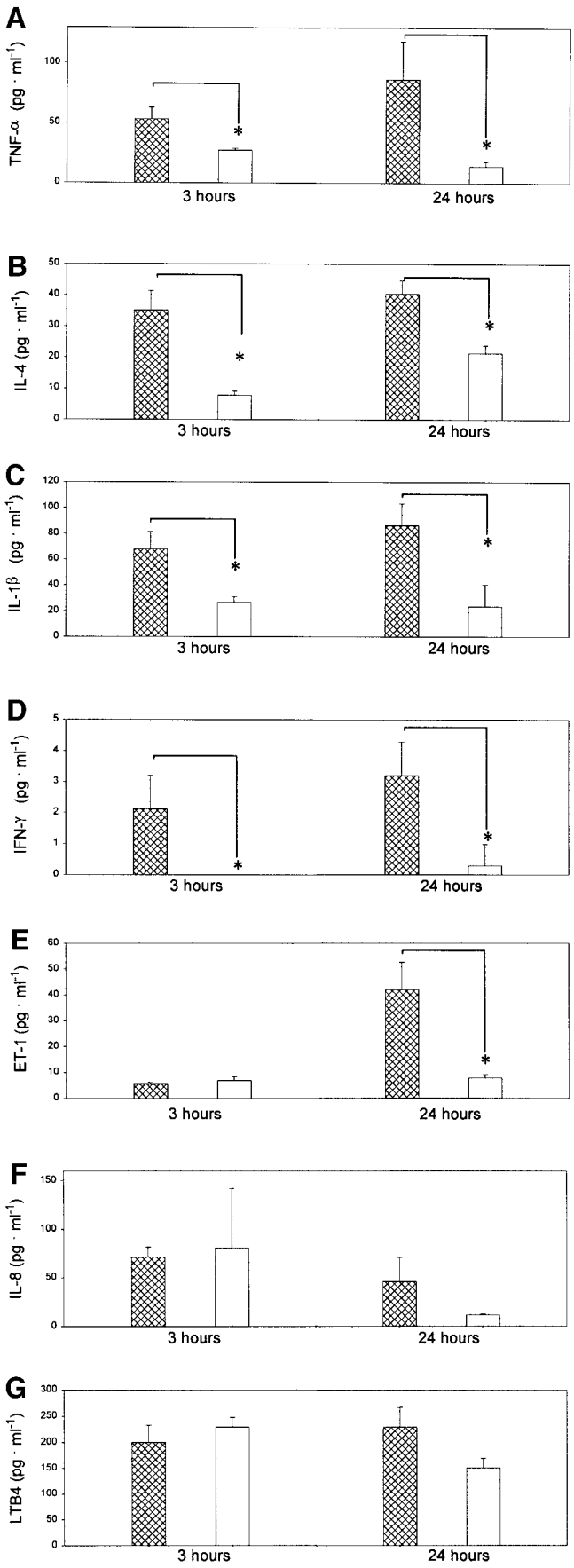
From a therapeutic point of view, it would be desirable to attack the initial factors of the inflammatory cascade to inhibit the inflammatory reaction. Consequently, monitoring the sequential pattern of cytokine expression after an inflammatory stimulus seems important. In this setting, it was intriguing to observe that the increase in ET-1 mRNA levels preceded the expression of the other proinflammatory cytokines. We assessed the expression of ET-1, IL-1β, IL-8, TNF-α, IFN-γ, and IL-4 because all these cytokines have been associated with bronchial asthma and airway inflammation. ET-1 antagonism strongly inhibited the production of some of these cytokines within the airways. This may be interesting with regard to developing new anti-inflammatory treatments.

The SDX model is a well-characterized model, primarily one of eosinophilic inflammation (1, 4, 13, 14, 23). Previously, Finsnes and colleagues (13, 14) have shown that there is a three- to fourfold increase in total BALF cell counts 24 h after SDX provocation. After 3 h, a transient increase in neutrophils occurs (13). The eosinophilic inflammation is initiated 3–6 h after intratracheal SDX provocation and lasts for >2 wk (14). The kinetics of this BALF finding resembles the cellular profile in human asthma, which likewise is characterized by an eosinophilic inflammatory response preceded by a transient increase in neutrophils after local allergen challenge (12). In addition, intravenous SDX injection increases airway hyperreactivity (30, 32, 37, 43), which is another typical feature of asthma, and this airway hyperreactivity may be inhibited by glucocorticosteroids (32, 37, 43).

IL-1β is increased in BALF of symptomatic but not of asymptomatic asthmatic patients (6) and has been implicated in the migration of inflammatory cells. In vitro studies have shown that IL-1β increases ET-1 release from cultured airway epithelial cells (34), and, conversely, IL-1β is found to be produced by ET-1 stimulation of human macrophages (19). The kinetics of gene expression in the present study indicate that IL-1β does not initiate the ET-1 synthesis in this eosinophilic airway inflammation.

Increased levels of IL-8 and increased IL-8 mRNA expression have been found in sputum from asthmatic patients (17), and increased IL-8 immunostaining has also been observed in bronchial epithelium (22). We also observed an increase in this peptide in BALF that appeared before the increase in IL-8 mRNA levels. This may be due to release of prestored IL-8 (45). The second rise in BALF IL-8, on the other hand, corresponded to the increased level of IL-8 mRNA in the lung tissue. IL-8 is an important mediator that promotes the migration of neutrophils (5) and eosinophils (11, 29) from the circulation to the inflammatory site. To our knowledge, no study has examined the relationship between ET-1 and IL-8 expression. Based on the order of appearance, our data indicate that IL-8 is not an initiating stimulus of ET-1 synthesis in eosinophilic airway inflammation.

Over the last years, TNF-α has gained interest as an important mediator in inflammatory disorders such as asthma (26, 46). Clinical studies inhibiting the action of this cytokine are currently performed in patients, and therapeutic intervention in asthma to inhibit TNF-α may prove beneficial. However, it should be noted that the synthesis of TNF-α is preceded by the synthesis of ET-1. Therefore, we suggest that ET-1 may be a stimulus of TNF-α synthesis in airway inflammation, which could provide the basis for an alternative approach to inhibit the generation of TNF-α.



IFN-γ may activate airway epithelial cells to release cytokines and express adhesion molecules but may also inhibit the allergic inflammation (44). However, in the present study, no significant increase in IFN-γ mRNA was detected during the experimental period. IL-4 is important for the increase in eosinophilic granulocytes in allergic inflammation, and increased mRNA levels have been shown in bronchial biopsies from asthmatic patients (24). In our study, however, no IL-4 mRNA could be detected in the lungs by Northern blot analysis. One cannot exclude that a minor mRNA increase in lung tissue, insufficient to be detected by Northern blotting, might still give a detectable rise in peptide levels. However, increased levels of IFN-γ and IL-4 peptides without detectable mRNA may be explained by the fact that these proteins are prestored and might be rapidly released from inflammatory cells recruited from the circulation (3, 39).

A significant increase in the ET-1 peptide was noted 6 h after SDX provocation. The delayed release of ET-1 into BALF compared with the early increase in ET-1 mRNA is probably due to the fact that ET-1 is secreted abluminally, i.e., from the tissue side of the bronchial epithelial cells (35), which is a main source of ET-1 production in this model (13). This is supported by the previous finding by Finsnes et al. (13) that the concentration of ET-1 in lung tissue increases before the ET-1 concentration in BALF. In fact, the peak level of ET-1 in lung tissue occurred 3 h postchallenge, whereas the highest level of ET-1 in BALF was observed after 24 h (13).

The concentration of IL-8 and TNF-α in BALF both indicated a biphasic pattern, with peaks 3 and 24 h after SDX provocation. This is an interesting observation that might correspond to the early and late phases of an allergic reaction. The first peak of IL-8 and TNF-α release appeared before the increase in mRNA. Therefore, this increase in peptide concentration might be caused by the release of prestored cytokines. The second rise in cytokine concentration coincided with the increase in mRNA levels. A tendency toward a biphasic pattern was also observed with regard to the other cytokines studied. The BALF concentration of the chemotactic mediator LTB_4 also displayed two peaks, one very early, ranging from 15 min to 2 h, and one as late as 48 h postchallenge. ET-1 was the only mediator in which BALF concentration showed no tendency toward a biphasic pattern.

Bosentan is a highly specific ET antagonist and has not been shown to directly affect other mediators (8, 9). This is in accordance with the results of our control experiments. According to the literature, one would have expected an increase in ET-1 levels after bosentan treatment due to replacement of the peptide from its receptors (21, 25). However, in these studies, ET-1 was measured in plasma and not in BALF, which, as

Fig. 2. Effect of bosentan treatment on proinflammatory mediators in BALF 3 and 24 h after SDX provocation. *A*: TNF-α. *B*: IL-4. *C*: IL-1β. *D*: IFN-γ. *E*: ET-1. *F*: IL-8. *G*: LTB_4. Values are given as means ± SE; $n = 6$ rats/group. *Significant difference from control animals not receiving bosentan, $P < 0.05$.

Finsnes et al. (14) have shown previously, is not directly comparable. Despite this difference, we observed a tendency toward higher BALF ET-1 levels in bosentan-treated control animals and also in SDX-treated animals 3 h postchallenge. Twenty-four hours postchallenge, however, the potential increase in ET-1 levels was probably masked by the substantial increase in BALF ET-1, corresponding to the pronounced inflammatory response.

Treatment of the animals with the ET receptor antagonist bosentan resulted in a substantial decrease in BALF concentration of several proinflammatory mediators considered to be of importance in bronchial asthma, i.e., TNF-α, IL-4, IL-1β, IFN-γ, and ET-1. This observation, which has not previously been described, provides insight into the mechanism of bosentan as an inhibitor of the inflammatory reaction (14). Besides having a direct chemotactic effect (10, 20), our results indicate that ET-1 also stimulates the generation of other proinflammatory mediators in vivo. However, we cannot exclude that this effect is indirect, i.e., due to the inhibition of cell influx that is seen after bosentan treatment (14). In any case, inhibition of ET resulted in a pronounced decrease in the concentration of these potent proinflammatory mediators within the airways.

Based on these data, we suggest the following hypothesis for the role of ET-1 in the development of an eosinophilic airway inflammation. The very early expression of ET-1 mRNA may be explained by the fact that ET-1 is synthesized by macrophages and the bronchial epithelium, cells that are the first to be attacked by inhaled agents (13). Subsequently, ET-1 may act as a chemoattractant (10, 20) and also stimulate the generation of other proinflammatory mediators, both pathways leading to influx of inflammatory cells.

In summary, the synthesis of ET-1 precedes the synthesis of other proinflammatory cytokines of importance for the development of an eosinophilic airway inflammation, and ET antagonism inhibits the production of these mediators within the airways. Whether treatment with ET antagonists will prove beneficial for patients with eosinophilic airway inflammation like bronchial asthma is not yet known.

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