

# Increased Synthesis and Release of Endothelin-1 during the Initial Phase of Airway Inflammation

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Recently, we have shown a substantial increase in the endothelin-1 (ET-1) concentration in bronchoalveolar fluid (BALF) during an experimental eosinophilic airway inflammation. Moreover, we observed a significant inhibition of the inflammatory response after treatment with an endothelin receptor antagonist. This indicates that ET-1 may have proinflammatory properties and play a key role in eosinophilic inflammations, such as bronchial asthma. Accordingly, we hypothesized that the synthesis and release of ET-1 precedes the inflammatory response, and that the bronchial epithelium is the site of ET-1 synthesis in the lungs. An eosinophilic airway inflammation was induced by intratracheal Sephadex instillation in rats, and the animals were evaluated after 15 min, 30 min, 1, 2, 3, 6, 12, and 48 h. The ET-1 mRNA synthesis, assessed by Northern and slot blot analyses, was significantly increased 15 min after Sephadex challenge, peaking at 30 min with a 4.7-fold increase, before any signs of inflammation in the BALF could be observed. The increased synthesis was mainly located to the bronchial epithelium and macrophages at sites of inflammation as determined by *in situ* hybridization. A significant increase in tissue ET-1 was observed 3 h after provocation, and the recruitment of eosinophils followed a substantial release of ET-1 peptide in BALF peaking at 24 h with a 13-fold increase. Therefore, the rapid ET-1 mRNA synthesis and the considerable increase in the level of ET-1 indicate that this peptide plays an important role in the initiation of an eosinophilic airway inflammation. Finsnes F, Christensen G, Lyberg T, Sejersted OM, Skjønsberg OH. Increased synthesis and release of endothelin-1 during the initial phase of airway inflammation.

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Endothelin-1 (ET-1), originally isolated from cultured endothelial cells, is among the most potent bronchoconstrictors yet described (1, 2). In addition, ET-1 may stimulate mucus secretion (3) and edema formation in airways (4). There have also been indications that ET-1 is involved in inflammatory reactions in the airways (5-9), but the exact role of ET-1 in inflammation is not yet defined. However, a key role for ET-1 in the pathogenesis of bronchial asthma, a predominantly eosinophilic inflammation, has been suggested (8, 10).

We have recently demonstrated a substantial increase in the ET-1 concentration in bronchoalveolar fluid (BALF) during an experimental eosinophilic airway inflammation (9). In rats evaluated between Days 1 and 14 after induced inflammation, we found a 20-fold increase in the ET-1 concentration. Additionally, that was the first study to report an anti-inflammatory effect of endothelin receptor antagonism in airway inflammation (9), indicating that ET-1 could play a pivotal role in the development of an eosinophilic airway inflammation, such as bronchial asthma.

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Because ET-1 may exert proinflammatory properties, we hypothesized that the ET-1 synthesis and release precedes the inflammatory response. If ET-1 really plays a pivotal role in the pathogenesis of airway inflammation, the early events leading to airway inflammation, as measured by the influx of inflammatory cells to the airway lumen, should involve ET-1 in the initial step. We also hypothesized that the bronchial epithelium is the main source of ET-1 synthesis because the bronchial epithelium is the first target to be attacked by inhaled agents and has a large surface area. To test these two hypotheses, we have investigated the very early phase of an experimental eosinophilic airway inflammation in rats following intratracheal Sephadex challenge. Rats have an endogenous hypersensitivity to dextrans such as Sephadex, which results in an eosinophilic inflammatory response (11). We evaluated the animals at 15 min, 30 min, 1, 2, 3, 6, 12, 24, and 48 h after instillation of either Sephadex or phosphate-buffered saline (PBS) intratracheally. The BALF was analyzed for cell counts and concentrations of mature ET-1; additionally, ET-1 was measured in homogenized lung tissues. Northern and slot blot analyses were used to assess the ET-1 messenger RNA (mRNA) in the lungs during airway inflammation. To localize the site of ET-1 synthesis, the *in situ* hybridization technique was employed.

## METHODS

### Experimental Procedure

A total of 213 male Wistar rats age 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 National Institutes of Health (NIH) guidelines.

Animals were evaluated at 15 min, 30 min, 1, 2, 3, 6, 12, 24, and 48 h after induced inflammation. An eosinophilic airway inflammation was provoked by intratracheal instillation of Sephadex particles (G-200 Superfine; Pharmacia & Upjohn, Uppsala, Sweden) dissolved in PBS (5.0 mg · ml<sup>-1</sup>) given intratracheally through a cannula in a volume of 1.0 ml · kg<sup>-1</sup> body weight as previously described (9). Control animals receiving PBS (n = 3 in each group) were examined at identical time points. Six animals in each group were used for Northern blot analyses. Additionally, tissue specimens for *in situ* hybridization were obtained at 30 min, 3 h, and 24 h after induced inflammation as well as from control animals at identical time points (n = 3 in each group). During intratracheal challenge with Sephadex or PBS alone (controls), the animals were anesthetized with a mixture of 30/70% O<sub>2</sub>/N<sub>2</sub>O, and 4% halothane (Fluothane; Zeneca, Macclesfield Cheshire, UK). Atropine sulfate (Atropin; Nycomed Pharma, Oslo, Norway), 1.0 ml · kg<sup>-1</sup> body weight, was injected intraperitoneally immediately before the challenge to reduce saliva production. Later, the animals were killed by a mixture of midazolam (Dormicum; Hoffman-La Roche, Basel, Switzerland) and fluanison (Hypnorm; Janssen Pharmaceutica BV, Beerse, Belgium).

Bronchoalveolar lavage was performed as previously described (9), by instillation of 3 + 2 + 2 ml PBS in the right stem bronchus, distal to the upper lobe, and the procedure was repeated on the left side. For determination of ET-1 content in lung tissues as well as Northern and slot blot analyses, animals were anesthetized as described, the lungs immediately removed and frozen in liquid nitrogen. The specimens for *in situ* hybridization were fixed for 8 h in freshly prepared 4% paraformaldehyde, then washed with PBS, and subsequently preserved in PBS with 15% sucrose and 0.01% Na azide until processed.

#### Cell and ET-1 Analysis in BALF and Lung Tissues

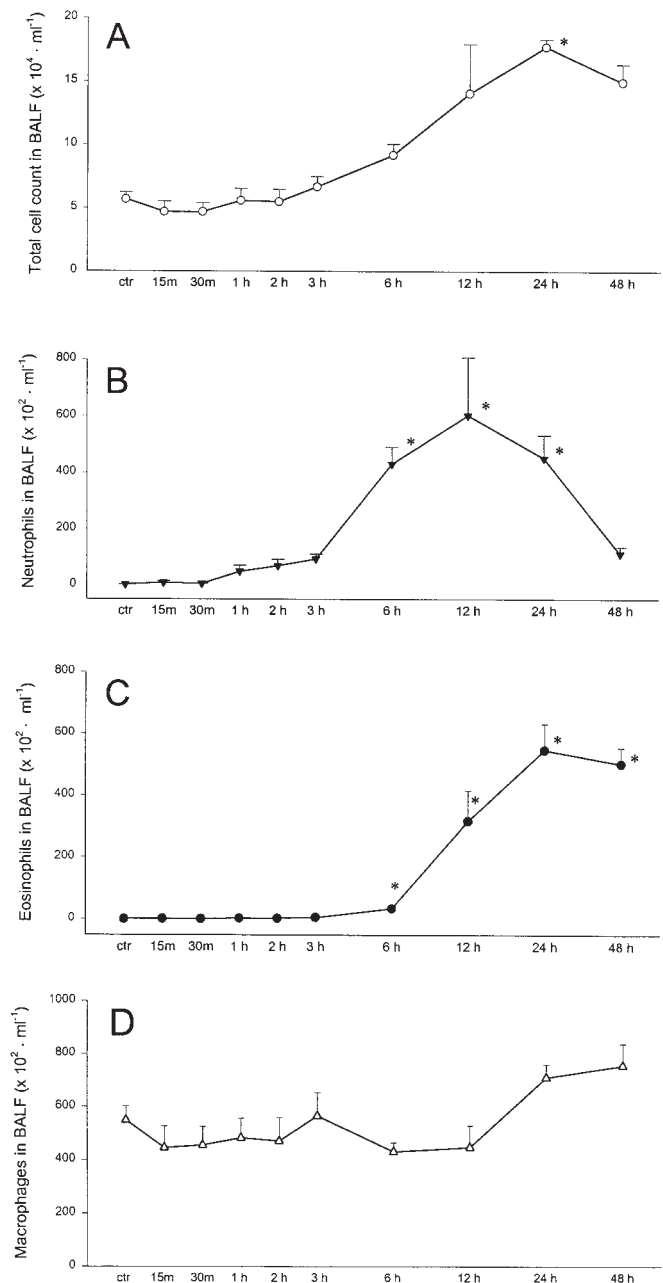
The lavage fluid was collected into prechilled tubes containing EDTA and kept on ice until centrifuged at 800 *g* for 10 min at 4° C. The cell pellet from the BALF was resuspended in PBS, pH 7.4, and the total number of cells was counted in a Bürker hemocytometer. Cytospin preparations were obtained using a cytocentrifuge (Cytospin Shandon, Southern Ltd, Runcorn, UK) operated at 100 *g* for 5 min. The slides were stained using the May-Grünwald-Giemsa staining procedure and at least 400 nonepithelial cells were counted to determine the proportion of pulmonary alveolar macrophages, eosinophils, neutrophils, and lymphocytes. The supernatants from the BALF were immediately stored at -70° C until analyzed. ET-1 was determined using a radioimmuno-linked Endothelin 1-21 specific [<sup>125</sup>I] assay system (RPA 555) from Amersham International (Cardiff, UK). Prior to ET-1 analysis, BALF samples were extracted in duplicate from 2 ml as previously described (9). The ET-1 assay has a limit of detection equivalent to 1.6 pg · ml<sup>-1</sup>. For the samples with ET-1 concentrations below this limit, the values were set to 1.6 pg · ml<sup>-1</sup>. For determination of tissue ET-1, frozen lung tissue samples were cut into small pieces and heated at 95° C in 20 volumes of 1 M acetic acid for 10 min. The samples were subsequently chilled in an ice-water bath, homogenized, and centrifuged for 15 min at 3,200 *g* at 4° C. The supernatants from tissues were lyophilized, and stored at -20° C until analysis by an ET-1-specific ELISA (R&D Systems, Oxon, UK). Total protein content was measured with a colorimetric method, using bicinchoninic acid (BCA) (Pierce b.v., BA oud-Beijerland, Netherlands).

Because there were no time-dependent effects of intratracheal PBS instillation, these data were pooled in one group, herein referred to as controls.

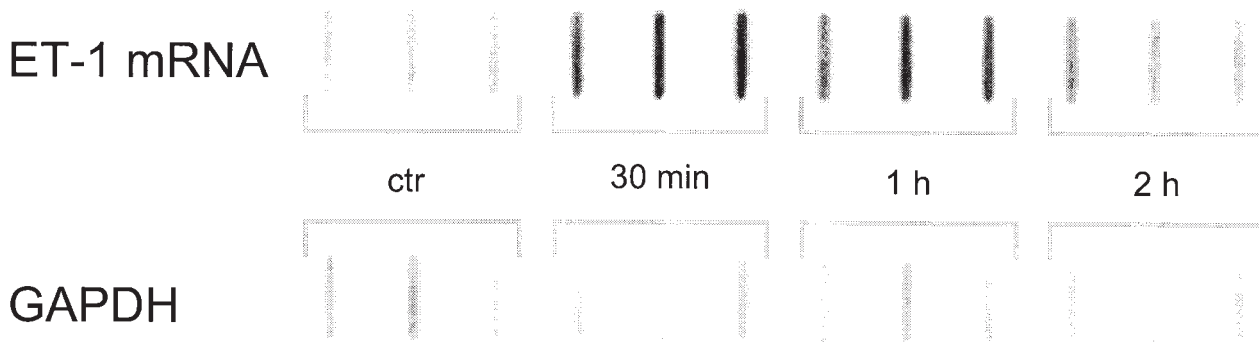
#### Northern and Slot Blot Analysis of ET-1 mRNA

Messenger RNA was extracted from homogenized lung tissue using oligo-deoxythymidine (dT)-conjugated paramagnetic beads according to the manufacturer's instructions (Dynal A/S, Oslo, Norway). The poly A<sup>+</sup> RNA was denatured in a solution containing 60% formamide, 7.2% formaldehyde, 24 mM HEPES, 6 mM sodium acetate, and 1.2 mM EDTA, subsequently size-fractionated on a formaldehyde-agarose gel using 15 µg poly A<sup>+</sup> RNA per lane, transferred to a Bio-

trans nylon membrane (ICN Biomedicals Inc., OH) by capillary blotting, and hybridized with radiolabeled complementary DNA (cDNA) probes for prepro-ET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For slot blot analyses, 0.5, 1.0, and 2.0 µg poly A<sup>+</sup> RNA was loaded onto the nylon membrane of a Minifold II (Schleicher & Schuell, Dassel, Germany), respectively. The nylon membranes were prehybridized at 42° C for 3 h in a solution containing 5 × standard saline citrate (SSC), 5 × Denhardt's solution, and 0.1% sodium dodecyl sulfate (SDS) and were then hybridized with <sup>32</sup>P-labeled cDNA probes in the same solution at 42° C for 17 h. The filters were finally washed twice in 2 × SSC with 0.1% SDS at room temperature for 5 × 5 min before washing twice with 0.1 × SSC at 60° C for 15 min



**Figure 1.** (A) Total cell count in BALF ( $\times 10^4 \cdot \text{ml}^{-1}$ ). (B–D) Absolute number of neutrophils, eosinophils, and macrophages in control animals (n = 27), and in animals receiving intratracheal Sephadex (n = 6 in each group), evaluated at 15 min, 30 min, 1, 2, 3, 6, 12, 24, and 48 h after the challenge (\*p < 0.05 compared with controls). Values are given as means  $\pm$  SEM.



**Figure 2.** Slot blot probed with radiolabeled prepro-ET-1 cDNA probe. Each lane represents RNA extracted from homogenized lung tissue harvested from individual animals during the initial phase of inflammation, compared with three control samples.

and then subjected to autoradiography. The prepro-ET-1 cDNA used as a probe in the present study was generously provided by Dr. Takashi Miyauchi, and is described elsewhere (12). The same membranes were rehybridized with a GAPDH cDNA probe as internal control which allows correction for any variations in loading of the blots with RNA.

Autoradiography of the filters was carried out in a storage phosphor screen and analyzed by densitometric scanning analysis using ImageQuant Version 3.3 software (Molecular Dynamics Lab., Sunnyvale, CA). To estimate the ET-1 mRNA tissue levels, the ET-1 mRNA to GAPDH ratios were determined in each sample.

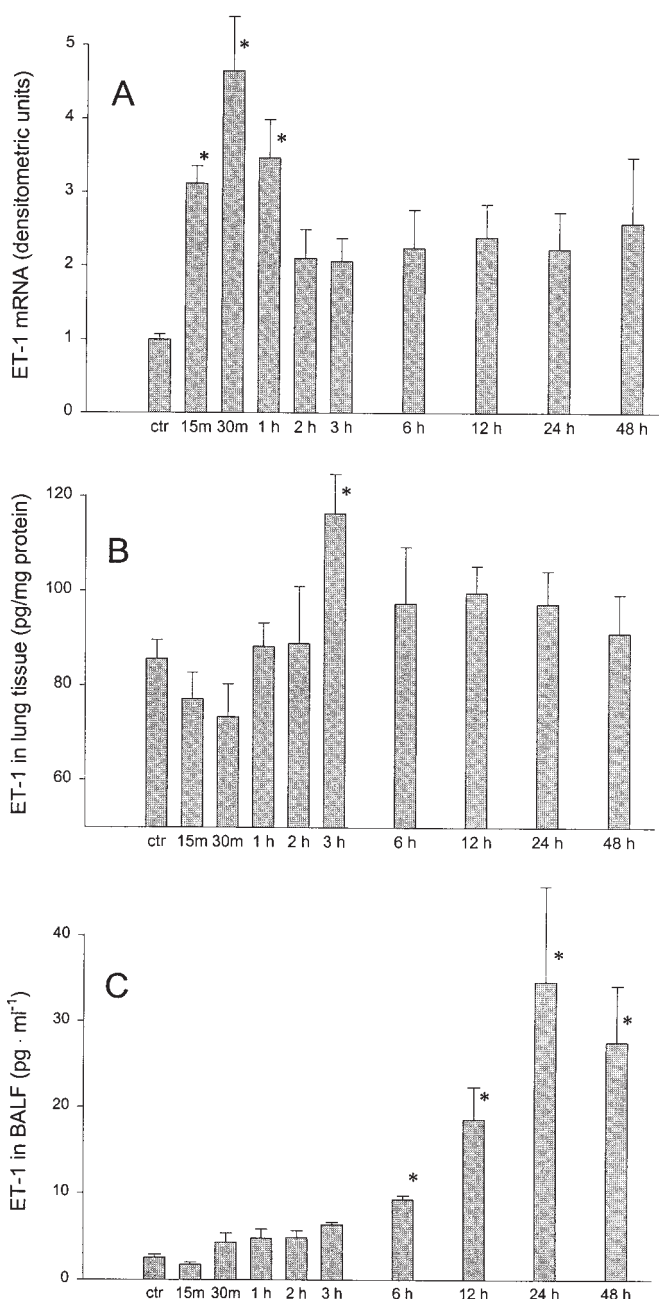
**In situ Hybridization**

Cryostat sections (10 μm thick) from paraformaldehyde-fixed tissues were hybridized with <sup>33</sup>S-labeled ET-1 complementary RNA probe for 14 h at 42° C (1 × 10<sup>6</sup> counts/min per section) as described by Gi-aid and coworkers (13). Sections were subsequently washed in sodium citrate, and unhybridized probe was removed by incubation in a solution of ribonuclease A (RNase A). The sections were dried, dipped in autoradiographic emulsion (Amersham International plc, Buckinghamshire, UK), and exposed for 7 to 14 d at 4° C. Consecutive sections were stained with hematoxylin for histological evaluation of *in situ* hybridization results. Sections from Sephadex-treated airways and controls were hybridized with a probe with a sequence identical to that of the coding ET-1 mRNA (sense probe), or treated with RNase A solution before hybridization with the ET-1 complementary RNA probe.

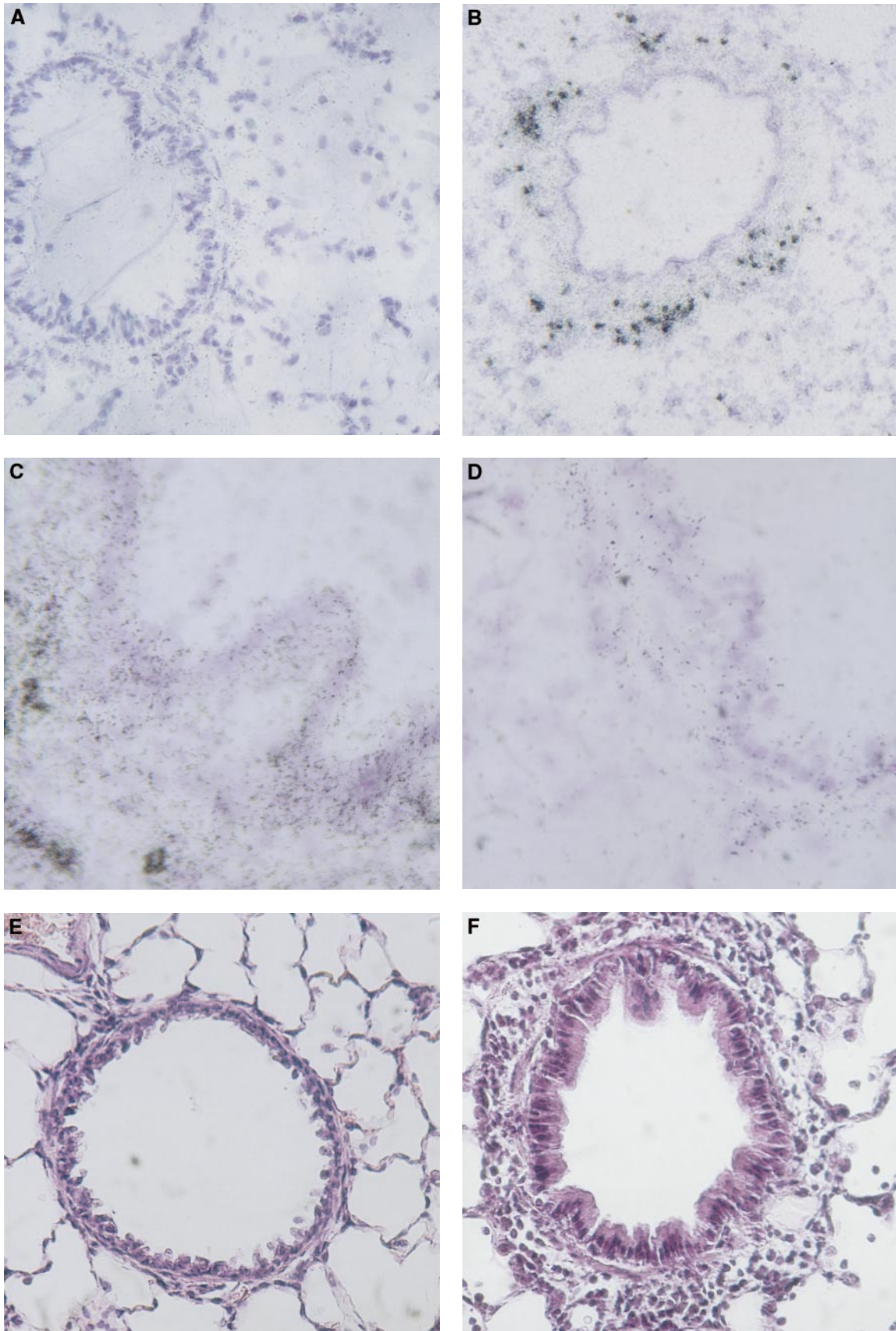
**Statistical Analysis**

All values are expressed as means ± SEM. Statistical analyses were performed using scientific statistical software (SigmaStat version 2.0; Jandel Scientific GmbH, Ekrath, Germany). The groups were compared using Kruskal-Wallis test followed by Dunn's test for multiple comparisons. A p value of less than 0.05 was considered statistically significant.

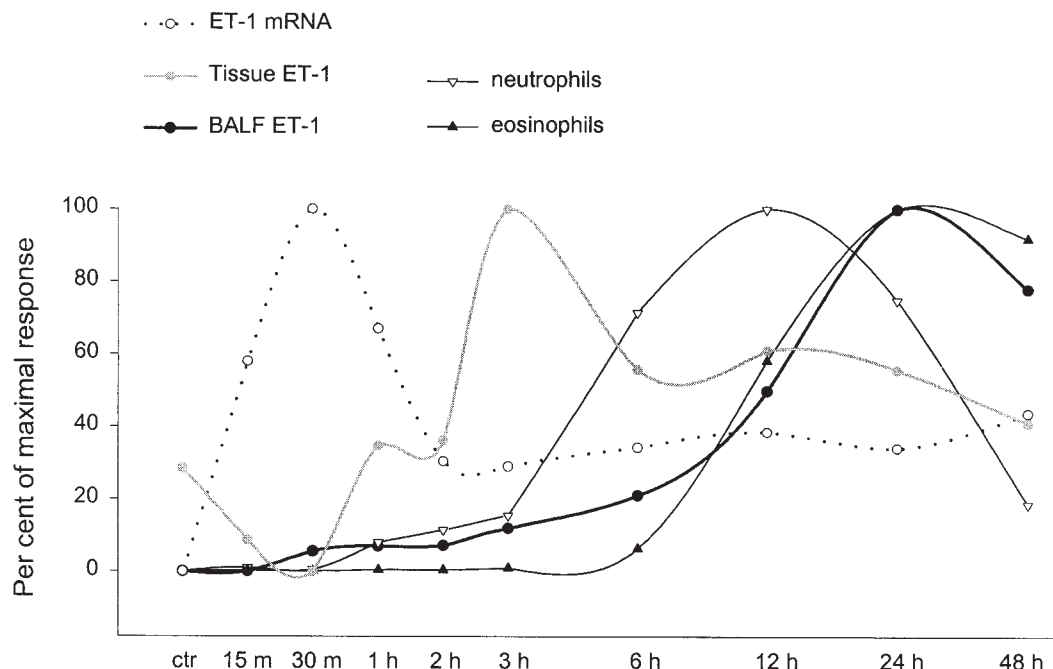
**Figure 3.** (Panel A) Effect of Sephadex-induced airway inflammation on ET-1 mRNA levels in lung tissue. Slot blot analyses were individually normalized to allow for variation in RNA loading using <sup>32</sup>P-labeled GAPDH. Each bar represents six animals as assessed by densitometric analyses, using arbitrary units when control samples were set to the value 1.0. (Panels B and C) The ET-1 concentrations in lung tissues (B) and BALF (C) in control rats (n = 27) and in animals after Sephadex challenge, evaluated at 15 min, 30 min, 1 h, 2, 3, 6, 12, 24, and 48 h (n = 6 in each group). \*p < 0.05 compared with controls. Values are given as means ± SEM.







**Figure 4.** Localization of ET-1 mRNA by radioactive *in situ* hybridization visible by silver grains. Normal lung tissue is presented in *panel A*, Sephadex-challenged tissue in *panel B*. *Panels C* and *D* represent a bronchial epithelial lining after Sephadex instillation (*C*) and in a control animal (*D*). For comparison a H&E-stained section of control specimen is shown in *panel E*. *Panel F* demonstrates a section from lungs removed 24 h after Sephadex challenge, showing peribronchial inflammation. (Original magnifications: *A*, *E*, and *F*:  $\times 100$ , *B*:  $\times 40$ ; *C* and *D*:  $\times 160$ .)



**Figure 5.** The kinetics of ET-1 synthesis, ET-1 release in tissue and BALF, as well as the recruitment of neutrophils and eosinophils into BALF in control rats ( $n = 27$ ) and in animals after Sephadex challenge, evaluated at 15 min, 30 min, 1, 2, 3, 6, 12, 24, and 48 h ( $n = 6$  in each group).

## RESULTS

### Cell Profile in BALF after Induced Inflammation

A slight increase in the total cell count in BALF was observed 6 h after intratracheal Sephadex instillation (Figure 1A). From this time point, the total cell count rose further, peaking at 24 h with a threefold increase. At 48 h a decline in total cell count was noted, compared with 24 h. The number of neutrophils in BALF increased from  $2.3 \cdot 10^2 \cdot \text{ml}^{-1}$  to  $48.5 \cdot 10^2 \cdot \text{ml}^{-1}$  1 h after intratracheal Sephadex challenge (Figure 1B), representing 0.4% and 8.1% of the total cell count, respectively. The number of neutrophils continued to increase until 6 h, when 46.2% of the BALF cells were neutrophils, and thereafter decreased to 7.6% at 48 h. The increase in eosinophilic granulocytes was delayed compared with the neutrophils (Figure 1C). At 6 h the fraction of eosinophils was 3.5% as compared with control level of 0.1%. At 12 h the fraction had increased to 22.1% and peaked at 33.4% 48 h after Sephadex instillation. The absolute number of macrophages increased insignificantly during the period studied (Figure 1D).

### Endothelin Synthesis and Release in the Airways

Northern and slot blot analysis revealed the presence of ET-1 mRNA synthesis in homogenized lung and airway tissue in all rats studied. Northern blot analysis showed a single band of  $\sim 2.3$  kb, demonstrating the specificity of the probe. Slot blot analysis revealed a marked upregulation of ET-1 mRNA during the very early phase of inflammation (Figure 2). Fifteen minutes after Sephadex instillation, the expression of ET-1 mRNA was significantly increased compared with control animals. The highest signal was observed 30 min after challenge, when a 4.7-fold increase was measured (Figure 3A). At 1 h the ET-1 mRNA levels were still increased, but declined rapidly, being on average 2-fold higher throughout the period studied until 48 h (NS).

*In situ* hybridization analyses of control animals revealed ET-1 mRNA signals in the airway epithelium and smooth muscle of the bronchi and blood vessels, as well as in single alveolar macrophages (Figure 4A). After Sephadex challenge, there was a stronger expression of ET-1 mRNA in macrophages at the inflammatory sites, i.e., peribronchially as well as in close connection to the Sephadex beads (Figure 4B). Macrophages in the alveoli of regions with no histological signs of inflammation were not different from controls. In addition, an increase in signals from the bronchial epithelium (Figure 4C) and smooth muscle was observed, but the difference between Sephadex and control animals was not as obvious as the marked changes related to the macrophages.

The tissue levels of ET-1 peptide increased significantly at 3 h compared with the values measured in control samples, 15 min and 30 min after Sephadex challenge (Figure 3B). In BALF a small increase in the concentrations of ET-1 was observed during the first 3 h after induced inflammation (Figure 3C). After 6 h a threefold increase occurred, and the highest concentration was measured at 24 h. Comparing the time course of the increase in ET-1 concentrations in BALF with the course of neutrophils and eosinophils, the increase in ET-1 peptide paralleled the increase in neutrophils until 3 h after which the neutrophils rose more rapidly than ET-1 (Figure 5). The increase in the number of eosinophils followed the increase in ET-1 peptide (Figure 5).

## DISCUSSION

The present study demonstrates a very early and marked increase in ET-1 mRNA synthesis and ET-1 peptide release during the initial phase of an experimental eosinophilic airway inflammation. The increased synthesis was mainly located to macrophages at sites of inflammation, in addition to the bronchial epithelium and smooth muscle. The marked ET-1 mRNA synthesis and tissue ET-1 peptide preceded the influx of in-

flammatory cells to the airway lumen. The development of the eosinophilic airway inflammation followed the release of ET-1 after a transient increase in neutrophils.

The ET-1 mRNA synthesis in the lung increased significantly as early as 15 min after Sephadex challenge and peaked at 30 min with a 4.7-fold increase. To our knowledge, this represents the most rapid increase in gene expression *in vivo* of any cytokine or mediator of relevance to asthma so far reported. There are few studies on ET-1 synthesis in airways *in vivo*, however a relatively rapid induction of ET-1 mRNA has been demonstrated by other investigators as well, employing a model of hypoxic exposure of rat lungs (14). In that work, there was a tendency toward increased ET-1 mRNA transcript levels after 24 h of hypoxic exposure, but 48 h of hypoxia was needed to demonstrate a twofold increase (14). *In vitro*, ET-1 synthesis has been reported in pulmonary endothelial cells (15) and in airway epithelial cells (16) upon exposure to proinflammatory stimuli like lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF- $\alpha$ ), and interleukin-1 (IL-1) (15, 16). In our study, the very rapid ET-1 mRNA synthesis in the lungs occurred before any signs of inflammation could be observed in BALF. Neither have any histological changes been noted at this stage during previous work with this model in our laboratory.

*In situ* hybridization analyses showed an abundant expression of ET-1 mRNA located to macrophages at inflammatory sites, i.e., peribronchially and surrounding Sephadex beads, as well as surface epithelium and smooth muscle. This indicates that activated macrophages may be partly responsible for increased ET-1 synthesis, although the absolute number of macrophages increased insignificantly during the first 48 h. ET-1 synthesis by macrophages has previously been demonstrated (17). Because we recently reported increased ET-like immunostaining in the bronchial epithelium during Sephadex-induced airway inflammation (9), demonstrating the presence of ET-1 peptide, we anticipated ET-1 mRNA signals located to the epithelium. Increased ET-1 mRNA synthesis in the bronchial epithelium was confirmed, but the most striking differences between Sephadex and control animals were located to macrophages and not to the epithelial lining. Other investigators have found increased ET-1 mRNA expression in the airway epithelium of patients with the inflammatory disease cryptogenic fibrosing alveolitis (13). In bronchial asthma as well, increased ET-1 immunoreactivity has been demonstrated in the epithelial cells (18). Due to the large surface of the bronchial epithelial lining, it is likely that even a minor increase in ET-1 mRNA synthesis would contribute significantly to the increased production of ET-1 in the lungs. This synthesis of ET-1 by both epithelial cells and macrophages is consistent with the finding of a very rapid induction of ET-1 mRNA synthesis, because these cells are among the first to be stimulated by inhaled agents.

The release of ET-1 peptide in lung tissue was significantly increased as early as 3 h after Sephadex provocation. In comparison, the ET-1 release into BALF was somewhat delayed. It is believed that the secretion of ET-1 from the endothelium is mainly abluminal (19, 20). There is only one study addressing ET-1 release from epithelial cells. This recently published study demonstrates that more than 90% of the ET-1 peptide release from cultured airway epithelial cells occurs toward the submucosal side (21). This is in line with the present *in vivo* study demonstrating a more rapid increase in the ET-1 peptide concentration in lung tissue than in BALF. We believe that the ET-1 peptide response in lung tissue and BALF is mainly due to a *de novo* synthesis within lung tissue. This is supported both by increased transcription signals, increased immunostaining in inflamed airway epithelium and macro-

phages (9), and the fact that the plasma levels of ET-1 remain unchanged during airway inflammation (9).

The marked synthesis of ET-1 mRNA and the increase in tissue ET-1 preceded the influx of inflammatory cells into the airways. The ET-1 peptide concentration in BALF was twofold increased at 2 h, but the increase did not reach statistical significance until 6 h, coinciding with the increase in eosinophils. Taking into account the close relationship between the kinetics of ET-1 peptide and the time-dependent recruitment of inflammatory cells, particularly eosinophils, chemotactic properties of ET-1 seem probable. This interpretation is in agreement with other investigations, demonstrating that ET-1 may promote cell adhesion and migration of leukocytes into the alveoli (22). In addition, we have recently shown that treatment with an endothelin receptor antagonist inhibited the influx of inflammatory cells to the airways in the rat model employed in the present study (9). Whether ET-1 has chemotactic properties per se or acts through the release of other cytokines is not known. *In vitro*, ET-1 has been shown to stimulate the release of metabolites of the arachidonic acid cascade (23), as well as the cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 (24–26), all substances with chemotactic properties. Thus, both direct chemotactic properties of ET-1 and an interaction between ET-1 and other mediators could possibly initiate both the transient neutrophilic cell influx and the subsequent profound eosinophilic response, a BALF cell pattern similar to what is seen in patients with asthma exacerbation (27) and allergen-induced acute asthma (28). Because ET-1 is found to be increased in these conditions (6, 29), a pathogenic role of ET-1 is possible. The mechanism underlying neutrophilia as a prelude to the recruitment of eosinophils is not clear. Recently it has been reported that a leukotriene B<sub>4</sub> antagonist in asthmatics inhibits the early neutrophilic influx, but no physiological benefit could be measured (30). This questions the functional role of the neutrophils in the pathophysiology of allergen-induced asthma (30).

In conclusion, the present study demonstrates a very early increase in ET-1 mRNA synthesis during the initial phase of an experimental eosinophilic airway inflammation. This increase preceded the influx of inflammatory cells into the airway lumen and was mainly located to macrophages at sites of inflammation, in addition to the bronchial epithelium. Due to the rapid ET-1 mRNA synthesis and ET-1 peptide release, taken together with our previous findings of an inhibitory effect on cell influx of ET-receptor antagonism, this study is consistent with a hypothesis of ET-1 playing an important role in the initiation of an eosinophilic airway inflammation. Whether ET-1 plays a significant role in the pathogenesis of bronchial asthma is an intriguing, but as yet unresolved question.

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