Primary pulmonary hypertension (PPH) is characterized by increased pulmonary arterial pressure and vascular resistance. We and others have observed that inflammatory cytokines and infiltrates are present in the lung tissue, but the significance is uncertain. Treprostinil (TRE), a prostacyclin analogue with extended half-life and chemical stability, has shown promise in the treatment of PPH. We hypothesize that TRE might exert beneficial effects in PPH by antagonizing inflammatory cytokine production in the lung. Here we show that TRE dose-dependently inhibits inflammatory cytokine (tumor necrosis factor-α, interleukin-1β, interleukin-6, and granulocyte macrophage colony-stimulating factor) secretion and gene expression by human alveolar macrophages. TRE blocks NFκB activation, but IκB-α phosphorylation and degradation are unaffected. Moreover, TRE does not affect the formation of the NFκB-DNA complex but blocks nuclear translocation of p65. These results are the first to illustrate the anti-cytokine actions of TRE in down-regulating NFκB, not through its inhibitory component or by direct binding but by blocking nuclear translocation. These data indicate that inflammatory mechanisms may be important in the pathogenesis of PPH and cytokine antagonism by blocking NFκB may contribute to the efficacy of TRE therapy in PPH.

Primary pulmonary hypertension (PPH) is an idiopathic disorder characterized by progressively increasing pulmonary artery pressure and vascular resistance in the absence of secondary causes (1). The pathogenesis of PPH is not clearly understood. Chronic pulmonary hypertension leads to profound structural alterations in affected vessels, commonly referred to as plexiform lesions (2). Although the pathogenesis of PPH is not clearly understood, genetic predisposition coupled with inflammation are implicated in the development of PPH. Inflammatory cells (T- and B-lymphocytes and macrophages) are present in PPH plexiform lesions (3), and several reports describe increased inflammatory cytokines in PPH (4–7). Elevated serum levels of interleukin (IL)-1 and IL-6 have been noted in PPH (4). Increased mRNA levels of macrophage inflammatory protein-1, vascular endothelial growth factor, and RANTES have been detected in PPH lung biopsy specimens (5–7). Our previous observation of enhanced NFκB activity in alveolar macrophages from PPH patients also demonstrates that PPH alveolar macrophages are activated and suggests a role for inflammatory cytokines in PPH (8). Increased urinary excretion of prostaglandin D2 metabolites by PPH patients are also suggestive of macrophage activation (9). These observations suggest that inflammatory cytokines may participate in the pathogenesis of PPH.

Recent studies have demonstrated the therapeutic efficacy of prostacyclin for PPH (10–13). Prostaglandins comprise a family of lipid compounds derived from arachidonic acid. In 1976, Moncada et al. (32) discovered prostaglandin I2 and named it prostacyclin (reviewed in Ref. 14). The potent vasodilator activity and capacity for inhibition of platelet aggregation provide the rationale for prostacyclin therapy in PPH. Indeed, continuous intravenous infusion of prostacyclin improves hemodynamics, exercise tolerance, and survival in PPH patients (10–13). Other properties of prostacyclin may also be important in therapeutic efficacy as suggested by the fact that patients who do not have acute hemodynamic improvement still appear to benefit from chronic therapy (14). Interestingly, several studies have demonstrated the inhibitory effects of prostacyclin on cytokine production by blood mononuclear cells, one of the major sources of cytokines in the lung, is unknown. Treprostinil (TRE) is a more stable benzidine derivative of prostacyclin and has shown success in treating PPH (19). We hypothesized that in addition to its direct hemodynamic effects, TRE might also decrease cytokine production and exert anti-inflammatory effect in the lung. To test this hypothesis, we investigated the effect of TRE on inflammatory cytokine secretion and gene expression in human alveolar macrophages.

MATERIALS AND METHODS

Reagents—Salmonella typhimurium lipopolysaccharide (LPS) was obtained from Sigma and used at 0.5 μg/ml for all experiments. TRE was a gift of the United Therapeutic Corporation (Research Triangle Park, NC).

Alveolar Macrophages—Fiberoptic bronchoscopy with bronchoalveolar lavage was performed as described previously (8). The study population consisted of healthy volunteers 18–65 years of age with no lung disease and on no medication. All of the volunteers provided written informed consent, and the study was approved by the institutional review board of the Cleveland Clinic Foundation. Alveolar macrophages were obtained by adhering cells from bronchoalveolar lavage as described previously (8, 20). Nonadherent cells were removed by washing.
The adherent cell population consisted of greater than 99% macrophages. Alveolar macrophages were cultured overnight prior to in vitro treatment.

Preparation of Whole Cell Extracts (WCEs)—After overnight incubation, macrophages were treated with LPS ± TRE (2–200 ng/ml) or left untreated for 4 h. TRE did not adversely affect cell viability at any dose tested as measured by trypan blue dye exclusion and cell adherence.

Preparation of Whole Cell Extracts (WCEs)—After overnight incubation, macrophages were treated with LPS ± TRE (2–200 ng/ml) or left untreated for 4 h. TRE did not adversely affect cell viability at any dose tested as measured by trypan blue dye exclusion and cell adherence.

**Fig. 1.** A, TRE inhibits IL-6 secretion in a dose-dependent manner. Each concentration of treprostinil (2–400 ng/ml) was tested on LPS-stimulated alveolar macrophages from 3–5 different individuals ($p < 0.0017$). TRE was tested at 200 and 400 ng/ml on unstimulated alveolar macrophages. B, TRE inhibits LPS-mediated production of the pro-inflammatory cytokines (TNF-α, GM-CSF, IL-1β, and IL-6). Human alveolar macrophages from four different individuals were incubated with LPS ± TRE (200 ng/ml) for 24 h or left untreated ($p = 0.004$). Supernatants were collected, and cytokines were measured by enzyme-linked immunosorbent assay.

**Fig. 2.** A and B, TRE inhibits LPS-induced pro-inflammatory cytokine RNA expression in a time-dependent fashion. Human alveolar macrophages were treated with LPS ± TRE for 4, 8, and 24 h or left untreated. RNase protection assay was performed using custom-made panel and RNase protection kit from BD PharMingen. Quantification was done using StormImager with the software ImageQuant.
Treprostinil Blocks NFκB Nuclear Translocation

Fig. 3. A and B, treprostinil inhibits LPS-mediated NFκB activation. Whole cell extracts were analyzed by electrophoretic mobility shift assay using a 32P-labeled oligonucleotide containing the NFκB consensus sequence and analyzed on a 4% nondenaturing acrylamide gel. Specificity of the band was confirmed by supershift analysis with p50 consensus sequences and analyzed on a 4% nondenaturing acrylamide gel. Expression of p50 was examined by Western blot analysis using anti-p50 antibody and competition with cold oligonucleotide. Quantification was done from three different experiments using the StormImager with ImageQuant analysis.

Fig. 4. Treprostinil does not inhibit LPS-mediated degradation of IκBα. Immunoblotting of whole cell lysate using anti-IκBα shows the disappearance and phosphorylation of IκBα after LPS and LPS + TRE treatment.

Results

TRE Inhibits Inflammatory Cytokine Production Dose-Dependently—The effect of TRE (2–400 ng/ml) on LPS-stimulated production of IL-6 was evaluated by enzyme-linked immunosorbent assay (Fig. 1A). Treatment with LPS alone increased cytokine production, whereas the presence of TRE significantly inhibited LPS-mediated increases in a dose-dependent manner (p = 0.0017). To determine whether this result was cytokine-specific, the effect of TRE (200 ng/ml) on TNF, IL-1, and GM-CSF was tested. All of the three cytokines were inhibited by TRE (Fig. 1B).

TRE Decreases Cytokine mRNA Expression—Treatment with LPS also increased alveolar macrophage mRNA expression of TNF-α, IL-1β, IL-6, and GM-CSF in a time-dependent fashion (Fig. 2A) when compared with unstimulated cells with a peak for TNF at 8 and 24 h for the other cytokines. Simultaneous treatment with TRE (200 ng/ml) inhibited LPS-induced mRNA expression of all four cytokines. Fig. 2B shows the quantification of the same gel by ImageQuant analysis.

TRE Blocks NFκB Activation—Because NFκB is a ubiquitous transcription factor involved in the regulation of all four cytokines studied as well as many others, we next determined...
the effect of TRE on LPS-induced NFκB activation (Fig. 3). LPS treatment increased NFκB activation when compared with unstimulated cells (Fig. 3A). Simultaneous treatment with TRE reduced the formation of the NFκB-DNA complex, suggesting that TRE inhibited NFκB activation. Specificity was confirmed by supershift with p65 and p50 antibodies and competition with cold oligonucleotide. These data were quantified (Fig. 3B by ImageQuant analysis).

**TRE Does Not Affect IκBα Degradation**—The possibility that the antagonistic effect of TRE on LPS-induced NFκB was directed against IκBα was next examined (Fig. 4). Fig. 4A shows a rapid (within 45 min) disappearance of IκBα upon LPS treatment with or without TRE. Similarly, the phosphorylation of IκBα occurred after LPS treatment in the presence or absence of TRE (Fig. 4B). Data suggest that TRE is not targeting IκBα phosphorylation or degradation.

**TRE Prevents Nuclear Translocation of p65**—Because TRE had no apparent effect on IκBα, we investigated whether TRE altered the nuclear translocation of the p65-p50 complex or whether it directly blocked DNA binding. To assess the former mechanism, we employed immunocytochemistry to visualize p65 at different time intervals (30 min, 1, 2, 3, and 4 h) after LPS treatment (Fig. 5, 30-min time point). From 30 min to 4 h after LPS treatment, nuclear staining of p65 was prominent. Simultaneous treatment with TRE prevented p65 nuclear translocation as shown by the reduced numbers of intensely stained nuclei. Image quantification confirmed the reduction of p65 translocation (Fig. 5E). To assess the second mechanism, we exposed whole cell extracts from LPS-treated cells to TRE (200 ng/ml) in vitro. TRE failed to block NFκB activation as determined by electrophoretic mobility shift assay (data not shown). These results strongly suggest that TRE blocks NFκB activation by impeding nuclear translocation of p65 but not by inhibiting cognate binding.

**DISCUSSION**

The potent vasodilator activity and capacity for inhibition of platelet aggregation provide the rationale for prostacyclin use in PPH. Other properties of prostacyclin may also be important in therapeutic efficacy as suggested by the fact that patients who do not have acute hemodynamic improvement still appear to benefit from chronic therapy (14). Furthermore, the association of PPH with inflammatory cytokines has been well documented (4–7). Based on these observations, we hypothesized...
that prostacyclins might effect inflammatory cytokine production. The current findings show for the first time that the prostacyclin analogue TRE effectively reduces human alveolar macrophage inflammatory cytokine secretion by inhibiting NFkB activity and subsequent transcription of cytokine genes. Furthermore, our data show that the inhibition of NFkB activation by TRE is by blockade of p65 nuclear translocation.

The molecular regulation of inflammatory cytokine genes is a complex process in which NFkB constitutes a critical element (22, 23). In quiescent cells, NFkB is located in the cytosol as a heterodimer or homodimer of protein components bound to an inhibitor IκB. The activation of this transcription factor is controlled by sequential phosphorylation, ubiquitination, and proteasome-mediated degradation of IκB, resulting in the migration of the NFkB complex to the nucleus and binding to the promoter region of many cytokine and growth factor genes (23, 24). Most known inhibitors of NFkB activity such as glucocorticosteroids, aspirin, and nitric oxide have been shown to block NFkB activation by interfering with IκB degradation and/or stimulating IκB synthesis (8, 25–27). TRE prevents NFkB activation without affecting IκB-α degradation by interfering with nuclear translocation of the p65/p60 complex. Other reagents, such as caffeic acid phenethyl ester are known to target nuclear translocation of p65 as a mechanism for anti-NFkB activity (28). Several reports have demonstrated that prostacyclin inhibits cytokine secretion from human peripheral blood mononuclear cells and the human monocytic cell lines THP-1 and Mono Mac 6 (15–18,29). Both Luttmann et al. (16) and Crutchley et al. (29) demonstrated that prostacyclin inhibited gene expression, but whether NFkB is involved was not explored. Surprisingly, the prostacyclin analogue iloprost blocked IκB-α degradation in the murine monocyte/macrophage cell line J774 (30). Whether this difference is because of the fact that the human alveolar macrophages are primary cells and/or whether there are species-specific differences remains to be investigated. Furthermore, because cytokines promote smooth muscle cell proliferation, the recent study demonstrating that TRE inhibits proliferation of human pulmonary artery cells in vitro may be attributed to anti-cytokine effects (31). Nevertheless, the actions of TRE make it an effective cytokine inhibitor, and this effect is mediated at least in part by blocking NFkB activation, although other mechanism(s) may also be involved in cytokine regulation by TRE.

The data presented here show that TRE blocks NFkB translocation, thereby potentially inhibiting inflammatory cytokine production. The association of PPH with inflammatory cytokine production has been well documented (4–7). Moreover, our previous studies indicate that PPH alveolar macrophages are endogenously activated as illustrated by activated NFkB (8). Therefore, it is intriguing to speculate that some of the clinical efficacy shown by TRE in PPH may be attributed to down-regulation of inflammatory cytokine production.

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