Inhibitory Effects of the Dual Endothelin Receptor Blocker Bosentan on Thrombin-Activated Lung Fibroblasts Isolated from Scleroderma Patients

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Abstract

**Background:** Endothelin-1 (ET-1) and thrombin are elevated during lung injury and repair, as seen in scleroderma and other interstitial lung diseases, and each plays important roles in remodeling epithelium, blood vessels and connective tissue. Both factors promote lung myofibroblast differentiation, the hallmark of pulmonary fibrosis. This study was undertaken to investigate whether bosentan, the dual, specific and competitive inhibitor of endothelin, interferes with thrombin signaling in scleroderma lung fibroblasts.

**Methods:** Endothelin-1 secretion was measured by enzyme-linked immunosorbent assay. Lung fibroblast proliferation was studied by DNA Synthesis and Quick Cell Proliferation assays. Expression of α-smooth muscle actin (α-SMA) was analyzed on Western blots. Contractile activity of lung fibroblasts was measured by a collagen gel contraction assay. Lung fibroblast migration was studied by wound-healing "scratch" assay.

**Results:** We show that thrombin significantly induces endothelin-1 expression in human lung fibroblasts. Bosentan significantly decreases α-SMA and collagen gel contraction of human lung fibroblasts stimulated by thrombin, suggesting that thrombin-induced differentiation of fibroblasts to the myofibroblast phenotype is at least in part regulated by endothelin-1. Bosentan decreases thrombin-induced migration of normal and scleroderma lung fibroblasts and inhibits innately increased migration of scleroderma lung fibroblasts suggesting that endogenous endothelin may be in part responsible for enhanced migration of lung fibroblasts in pulmonary fibrosis. We also report that bosentan inhibits thrombin-induced thymidine incorporation and decreases lung fibroblast proliferation.

**Conclusions:** Fibrogenic effects of thrombin in scleroderma lung fibroblasts are mediated in part by endothelin-1. The dual endothelin receptor blocker bosentan restrain profibrotic effects of endogenous and thrombin-induced endothelin-1 in scleroderma lung fibroblasts.

Keywords: Scleroderma lung fibroblast; Endothelin; Thrombin; Bosentan

Abbreviations: ET-1: Endothelin-1; SSc: Systemic Sclerosis; SSc-ILD: Scleroderma Associated Interstitial Lung Disease; ECM: Extra Cellular Matrix; α-SMA: α-Smooth Muscle Actin

Introduction

Bosentan is an endothelin receptor antagonist that inhibits the effects of endothelin-1 (ET-1) and currently is used to treat patients with pulmonary arterial hypertension. Most of ET-1’s cellular effects are mediated by binding to ETA and ETB receptors belonging to the superfamily of seven-transmembrane G-protein-coupled receptors. Numerous studies have identified dysregulated endothelin receptors in various tissues and diseases. For example, ETB receptors are increased over ETA receptors in chronic heart failure and in scleroderma associated fibrotic lung tissue or scleroderma skin fibroblasts [1-3]. Many of the effects of endothelin such as fibrosis, mitogenesis, and inflammation are mediated by both ETA and ETB receptors, but in some models the ETB receptor is the only mediator of endothelin’s actions [1]. Using specific endothelin receptor(s) inhibitors, it has been shown that collagen matrix contraction induced by ET-1 is mediated by the ETA, but not the ETB, receptor [3]. Also, both the ETA and the ETB receptor contribute to lung fibroblast proliferation induced by ET in scleroderma (systemic sclerosis, SSc) patients [1]. It has been shown that when both endothelin receptor subtypes coexist, selective blockade of either the ETA or the ETB receptor is ineffective or insufficient for the inhibition of collagen deposition induced by ET, indicating compensation by the other, un-antagonized ET receptor subtype, when only one receptor is antagonized [2,3]. This so-called “cross-talk” has been shown by the requirement for dual antagonism to inhibit collagen synthesis induced by ET in human fibroblasts. When selective ETA or ETB receptor antagonists are used, this “cross-talk” may have other consequences: it may cause chronic stimulation of the un-antagonized receptor subtype, and actually aggravate fibrosis. This has been shown using a selective ETA receptor antagonist, which had beneficial effects in certain models of fibrosis but worsened cardiac and renal fibrosis [3]. Similar observations have been made with selective ETB receptor blockade, which worsened fibrosis in some cases, but was sufficient to prevent cardiac fibrosis in hypertension and chronic heart failure models [2,3]. Therefore, using the dual ETA/ETB receptor antagonist bosentan has promise in treatment of various connective tissue diseases.

ET-1 production is increased in different pathological conditions including pulmonary fibrosis [1-3]. Recently, it was speculated that the elevated levels of endothelin-1 expression in SSc fibroblasts may directly contribute to lung fibrosis by causing the enhanced contractile ability of scleroderma fibroblasts, thereby promoting the formation of scar tissue [4]. Additionally, ET-1 was shown to promote microvascular changes, such as enhanced proliferation of endothelial cells and neovascularization [5].


**Received** December 13, 2011; **Accepted** December 31, 2011; **Published** February 02, 2012

**Citation:** Bogatkevich GS, Akter T, Ludwicka-Bradley A, Silver RM (2012) Inhibitory Effects of the Dual Endothelin Receptor Blocker Bosentan on Thrombin-Activated Lung Fibroblasts Isolated from Scleroderma Patients. Rheumatology 5:1-5. doi: 10.4172/2161-1149.1000005

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The concentration of thrombin is also elevated in idiopathic pulmonary fibrosis (IPF), scleroderma associated interstitial lung disease (SSc-ILD), and bleomycin-induced lung fibrosis [6,7]. Thrombin, besides its importance in thrombosis and hemostasis, also has several important functions in the stimulation of platelets, leukocytes, endothelial cells, smooth muscle cells, and lung fibroblasts [6,8,9]. The multiple effects of thrombin on cells include regulation of proliferation, stimulation and expression of various cytokines, and regulation of extracellular matrix (ECM) [9-11]. Previously, we demonstrated that thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the PAR-1 receptor and via a PKC-dependent pathway [6]. Thrombin is known to induce ET-1 in various cell types [12,13]. Moreover, ET-1 and thrombin have similar pro-fibrogenic effects in lung fibroblasts and endothelial cells [14-16]. We, therefore, postulated that ET-1 may mediate in part the fibrogenic effects of thrombin in scleroderma lung fibroblasts. Here we demonstrate that bosentan reduces pro-fibrotic effects of thrombin in lung fibroblasts isolated from controls and from SSc-ILD patients. Additionally, we show that bosentan inhibits increased migration of SSc lung fibroblast and the proliferation of normal and SSc lung fibroblasts.

**Materials and Methods**

**Cell culture**

Lung fibroblasts were derived from lung tissues obtained at autopsy from SSc patients and from age-, race-, and sex-matched normal subjects who died from nonpulmonary causes. Lung tissue was diced (0.5 x 0.5 mm pieces) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, gentamicin sulfate (50 µg/ml), and amphoterin B (5 µg/ml) at 37°C in 10% CO₂. Medium was changed every three days to remove dead and non-attached cells until fibroblasts reached confluence. Monolayer cultures were maintained in the same medium. Lung fibroblasts were used between second and fourth passages in all experiments.

**Human endothelin-1 immunoassay**

Endothelin-1 secretion was measured in lung fibroblast culture supernatant collected from cells incubated in serum-free medium supplemented with different concentrations of thrombin for 24 hours, using Human Endothelin-1 ELISA kit according to manufacturer's instructions (R & D Systems, Minneapolis, MN). The data were adjusted in accordance with cell counts at the time of sampling, and values are given as ET-1 per milliliter per 10⁶ cells.

**Collagen gel contraction assay**

Collagen lattices were prepared by using type I collagen from rat tail tendon (BD Bioscience, Bedford, MA) adjusted to a final value of 2.5 mg/ml with 0.01% acetic acid. Human lung fibroblasts, in a concentration of 2.5 x 10⁵ cells/ml, were suspended in collagen (1.25 mg/ml of collagen) and aliquoted into 24 well plates (300 µl/well). Collagen lattices were polymerized for 45 minutes in a humidified 10% CO₂ atmosphere at 37°C, and 500 µl/well DMEM containing 10% FCS was added for 4 hours, followed by overnight incubation in serum-free medium. To initiate collagen gel contraction polymerized gels were gently released from the underlying culture dish and then cells were stimulated with 0.5 U/ml of thrombin in serum-free DMEM in the presence or absence of bosentan (1-100 µmol, added 30 minutes prior to the addition of thrombin). To determine the degree of collagen gel contraction, photographs were taken after 2 and 24 hours with a digital camera. Measurement of the diameter of the gel in mm was recorded as the average values of the major and minor axes. Calculation of gel contraction was presented as the difference between diameters of non-released and released gels.

**DNA synthesis assay**

DNA synthesis assay was performed as previously described [5]. Briefly, human lung fibroblasts were cultured to sub-confluence on 12-well plates in DMEM containing 10% FCS followed by incubation in serum-free medium. Subsequently, cells were stimulated with or without thrombin (0.5 U/ml) for 24 hours in the presence or absence of bosentan (1-100 µmol, added 30 min prior to the addition of thrombin); then 1µCi/ml [3H] thymidine was added for an additional 6 hours incubation. Cells then were washed 3 times with ice-cold PBS and 3 times with ice-cold trichloroacetic acid (5%, w/v), followed by the addition of 250 µl NaOH/SDS (both 0.1% w/v) per well for at least 1 hour at room temperature. Samples were collected into scintillation vials with scintillation liquid (4 ml per small vial, 10 ml per big vial) and counted in a scintillation counter.

**Quick cell proliferation assay**

Quick Cell Proliferation Assay was performed in accordance with manufacturer's instructions (BioVision Research Products, Mountain View, CA). Lung fibroblasts (10⁴/well) were cultured in 96-well plates in a final volume of 100 µl/well DMEM in the absence or presence of thrombin and bosentan. After 24 hours of incubation, 10 µl well tetrazolium salt WST-1 in Electro Coupling Solution (ECS) was added, and cells were incubated for another two hours in standard culture conditions. Plates were then placed for 1 minute on a shaker, and the optical density of each well was determined using a microplate reader set to 450 nm.

**Cell migration wound-healing “scratch” assay**

Lung fibroblasts were cultured to form a monolayer on fibronectin-coated 6-well plates. Cells were serum deprived overnight and then mechanically “wounded” by scraping with a Fisherbrand ready-tip (size 1-200µl). Cell monolayers were washed twice with PBS and treated with or without thrombin and bosentan or cell proliferation inhibitor S-Fluorouracil (Sigma, Saint Louis, MO).

After 6 hours of incubation fibroblasts were washed with PBS and stained with Diff-Quick solution. Pictures were taken at 2.5 X magnification. Migration rate was calculated by counting the cells that cross into the “scratch” (wounded) area and presented as a percentage of total cells on identical “non-scratch” area (100%).

**Preparation of cell extracts and immunoblotting**

Lung fibroblasts on 6-well plates were washed with ice-cold PBS and lysed with ice-cold lysis buffer (10 mM Tris, 10 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, pH = 7.4). Protein concentration was determined by BCA™ protein assay according to the manufacturer's instructions (Pierce, Rockford, IL). For each sample, 40 µg of protein was denatured, subjected to SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with appropriate antibodies.

**Statistical analysis**

Statistical analyses were performed with KaleidaGraph 4.0 (Synergy Software, Reading, PA). All data were analyzed using analysis of variance with Tukey HSD post-hoc testing. The results were considered significant if p<0.05.
Results

Thrombin induces ET-1 expression in human lung fibroblasts

Endothelin-1 has been shown to promote expression of proteins that contribute to the contractile and fibrotic phenotype of myofibroblasts [14,15,17]. Previously we demonstrated that thrombin also contributes to the phenotype of lung myofibroblasts [6,16]. We thus determined whether thrombin induces expression of ET-1 in lung fibroblasts. ET-1 was measured by ELISA in supernatants from normal and scleroderma lung fibroblast cultures stimulated with various concentrations of thrombin. We found that thrombin is a potent inducer of ET-1 secretion: as low as 0.1 U/ml of thrombin induced a significant amount of ET-1. The induction of ET-1 by thrombin was dose-dependent, reaching a 15-fold increase by only 0.5U of thrombin (Figure 1).

Bosentan inhibits thrombin-induced differentiation of lung fibroblasts to myofibroblasts

Previously we reported that thrombin induces α-SMA expression and differentiates normal lung fibroblasts to a myofibroblast phenotype. In agreement with these observations we found that thrombin in a concentration of 0.5U/ml significantly increased α-SMA expression in normal lung fibroblasts within 24 hours of exposure (Figure 2A). Since thrombin enhances endothelin production, which also is implicated in inducting myofibroblasts [3,18], we sought to determine whether the endothelin receptor antagonist bosentan would interfere with thrombin’s effects on lung fibroblasts. We found that bosentan in concentrations of 1 µM, 10 µM, and 100 µM had no effect on α-SMA expression when compared to control (serum-free medium). However, at a concentration of 100 µM bosentan significantly decreased thrombin-induced α-SMA in normal lung fibroblasts suggesting that this effect of thrombin is in part mediated by endothelin (Figure 2A). SSc lung fibroblasts expressed significantly higher amounts of α-SMA compared with normal lung fibroblasts. Thrombin further increased α-SMA expression in SSc lung fibroblasts (from 249 ± 42 to 396 ± 73 densitometry units; p < 0.05). Interestingly, bosentan at a concentration of 100 µM inhibited not only thrombin-induced α-SMA but also the basal level of α-SMA in SSc lung fibroblasts (Figure 2B).

Another characteristic of myofibroblasts is their ability to contract collagen gel lattices. Thrombin rapidly contracted collagen gel from ~15 mm in diameter (serum-free medium) to less than 7 mm in diameter within 24 hours (Figure 3A). Bosentan significantly inhibited thrombin-induced contractility in a dose of 100 µM. However, it had no effect on collagen gel contraction when added without thrombin. Similarly, bosentan inhibited thrombin-induced collagen gel contraction in SSc lung fibroblasts (Figure 3B).

Bosentan’s effects on lung fibroblast proliferation

Cells with a myofibroblast phenotype are also characterized by an increase in proliferative capacity. Thrombin is a well known mitogen and has been shown to induce human lung fibroblast proliferation [5,18,19]. We measured the effect of bosentan on thrombin-induced lung fibroblast proliferation using two different methods, [3H] thymidine incorporation and a quick cell proliferation assay. Basal levels of [3H] thymidine incorporation were in a range of between 551 and 924 cpm in normal lung fibroblasts and between 1057 and 2011 cpm in SSc lung fibroblasts. Thrombin induced a 4.1-fold increase in [3H] thymidine incorporation in normal and a 1.9-fold increase in SSc lung fibroblasts (Figure 4). Bosentan in concentrations of 10 µM, 20 µM, and 50 µM did not affect lung fibroblast DNA synthesis; concentrations of 100 µM bosentan decreased [3H] thymidine basal incorporation but the results were statistically not significant. Bosentan in a concentration of 100 µM significantly inhibited thrombin-induced DNA synthesis in normal and SSc lung fibroblasts (Figure 4A and 4B).

Quick cell proliferation assay appears to be a more sensitive method to measure bosentan’s effect on the proliferation of lung fibroblasts. This method is based on cleavage of a tetrazolium salt, WST-1, to formazan by cellular mitochondrial dehydrogenases. Expansion of the number of viable cells results in an increase in the activity of the mitochondrial dehydrogenases leading to an increase in the amount of formazan dye, which can be detected by spectrometry. Basal level of viable cells was equal to 0.38 ± 0.03 optical densities (OD) for normal and 0.59 ± 0.05 OD for SSc lung fibroblasts. Thrombin increased the level of viable cells to 0.63 ± 0.05 OD for normal and 0.79 ± 0.06 OD for SSc fibroblasts (p<0.05) (Figure 5). Bosentan at a concentration of 100 µM significantly decreased thrombin-induced cell proliferation measured by this assay. Moreover, 100 µM bosentan alone significantly inhibited proliferation of lung fibroblasts as well.

Effect of bosentan on lung fibroblast migration

We next studied the effect of bosentan on thrombin-induced migration of lung fibroblasts using a wound healing scratch assay that measures cell migration toward the injured sites. Confluent monolayers of lung fibroblasts were scratched and migrating cells were counted after 6 hours. We observed that normal lung fibroblasts in serum-free medium migrate very slowly with migration rates between 9% and 15%. Bosentan alone at a concentration of 100 µM did not affect normal lung fibroblast migration as compared with control cells. Thrombin significantly increased the migration rate of normal lung fibroblasts by 2.1-fold (Figure 6A). The migration rate of thrombin-induced lung fibroblasts pre-treated with bosentan was reduced by 1.4-fold.

SSc lung fibroblasts migrated more intensively as compared with normal lung fibroblasts. The migration rate of SSc lung fibroblasts reached 32.47 ± 4.72% in 6 hours, which is 2.7-fold higher as compared with normal lung fibroblasts. Thrombin further increased the migration rate of SSc lung fibroblasts to 61.79 ± 11.38. Bosentan significantly inhibited cell migration in both normal and SSc lung fibroblasts (Figure 6B).
**Figure 2: Effect of bosentan on thrombin-induced α-smooth muscle actin (SMA) expression.** Normal (A) and scleroderma (B) lung fibroblasts were serum-deprived overnight, then treated with various concentration of thrombin and/or bosentan for 24 hours. Cell lysate proteins (40µg) were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-SMA antibody. The immunoblots were then stripped and re-blotted with anti-β-actin antibody from Sigma as a loading control. The images were scanned and analyzed with NIH Imaging software. Densitometric analysis of immunoblots from 3 independent experiments is presented. The *asterisk represents statistically significant differences between cells stimulated with thrombin versus cells stimulated with thrombin and bosentan.

**Figure 3: Effect of bosentan on collagen gel contraction.** Normal (A) and scleroderma (B) lung fibroblasts were cultured in the presence of 1.5mg/ml collagen in 24-well plates, followed by 24 hours incubation in serum-free medium with or without thrombin 0.5U/ml and with or without bosentan in indicated concentrations. The degree of collagen gel contraction was calculated as the difference between diameters of wells and those of contracted gels. The experiments were performed four times and mean values ± SD are presented. The *asterisk represents statistically significant differences between cells stimulated with thrombin versus cells stimulated with thrombin and bosentan.

**Figure 4: DNA synthesis regulated by thrombin and bosentan in normal (A) and scleroderma (B) lung fibroblasts.** Cells were stimulated with/throughout thrombin and/or bosentan for 30 hours, and [3H] thymidine incorporation was measured as detailed under Materials and Methods. The experiments were performed three times and mean values ± SD are presented. The *asterisk represents statistically significant differences between cells stimulated with thrombin versus cells stimulated with thrombin and bosentan.
reduced both the basal and thrombin-induced migration of SSc lung fibroblasts to $21.63 \pm 3.92\%$ and $37.26 \pm 10.03\%$, respectively.

**Discussion**

The conceptual process of fibrogenesis in various interstitial lung diseases including SSc-ILD involves microvascular and tissue injury associated with release of fibrogenic factors, induction of myofibroblasts and deposition of ECM. The net result may be severe lung dysfunction and death from respiratory failure. Studies from our laboratory suggest that the serine protease thrombin contributes to the development of SSc-ILD by activation of microvascular endothelial cells and by inducing the differentiation of lung fibroblasts to myofibroblasts [6,19-21]. In the early stages of SSc-ILD we observed that the formation of new vessels occurs and coincides with the appearance of myofibroblasts [22]. These results suggest an interaction of endothelial cells with myofibroblasts, which may be crucial in the development of pulmonary fibrosis as seen in SSc-ILD and other ILDs.

It is now well accepted that thrombin induces endothelin, and levels of both are elevated in SSc [13], as well as in other pathological conditions of vascular endothelium [4]. Endothelin has been also reported to be an important endogenous factor promoting induction of myofibroblasts [4]. Previously, we demonstrated that thrombin can induce normal lung fibroblasts to assume a myofibroblast phenotype

![Figure 5: Effect of bosentan on thrombin-induced lung fibroblasts proliferation.](image)

![Figure 6: Effect of bosentan and thrombin on lung fibroblast migration.](image)
Cell migration is a fundamental cellular process for normal development and homeostasis of tissue and organ. Fibroblasts of normal stroma are relatively stationary despite the absence of architectural boundaries such as basement membranes. Explant culture is selective for migratory fibroblasts and can be used as a model in vitro to study the migration of myofibroblasts in vivo to the foci of lung fibrosis. Using a wound-healing scratch assay we demonstrate herein that bosentan significantly decreased migration of SSc lung fibroblasts and thrombin-inhibited migration of normal and SSc lung fibroblasts. These data suggest that migration of SSc lung fibroblasts toward injury and thrombin-inhibited migration of normal lung fibroblasts have a similar mechanism that is regulated by endogenous endothelin. However, the possible mechanism linking ET-1 to the development of fibrosis is not yet clear. Recently, Bellissai et al. [24] reported that bosentan decreases proinflammatory interleukins in SSc patients. Kambas et al. [25] demonstrated that ET-1 promotes fibrosis in vitro in a bronchopulmonary dysplasia model by activating the extrinsic coagulation cascade indicating that ET-1 signaling contributes to fibrosis by upregulating a tissue factor / thrombin amplification loop.

Bosentan was employed in two randomized clinical trials, one in IPF (BUILD-1) and another in SSc-ILD (BUILD-2) [26,27]. Unfortunately, neither trial demonstrated efficacy of bosentan, although there did appear to be a trend in favor of bosentan delaying the time to death or disease progression in patients with biopsy-proven usual interstitial pneumonia [26]. A smaller, more recent study of nine SSc-ILD patients also failed to demonstrate improvement in lung function or imaging after two years of bosentan treatment [27]. In addition to patient selection, other factors such as primary endpoint selection might also have influenced the results. Based on our in vitro studies, as well as animal studies showing reduced lung fibrosis with endothelin receptor antagonist therapy [28], further studies are warranted to examine a potential role for such therapy in patients with SSC-ILD and other ILDs.

Acknowledgements

The authors would like to thank C. Beth Singleton for her excellent technical work.

Grants

This work was supported in part by a career award from National Institutes of Health K01AR051052 (to GSB) and by Actelion (to RMS).

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Rheumatology

Lung Involvement in Scleroderma

ISSN: 2161-1149 Rheumatology, an open access journal


