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F₂-isoprostanes can mediate bleomycin-induced lung fibrosis

Beatrice Arezzini^{1*}, Daniela Vecchio^{1*}, Cinzia Signorini¹, Blerta Stringa^{1,2} and Concetta Gardi¹

¹*Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy;*

²*Centre for Integrative Biology, University of Trento, Trento, Italy.*

*these two authors equally contributed to this work.

Corresponding author:

Concetta Gardi, Ph.D.

Department of Molecular and Developmental Medicine

Siena University, via A. Moro, I-53100 Siena – Italy

Tel. +39-0577-234002

E-mail: concetta.gardi@unisi.it

Keywords: F₂-Isoprostanes; Bleomycin; Lung Fibrosis; α -SMA; Lung Fibroblast; Oxidative Stress; TP receptor; Myofibroblasts.

Highlights:

- Lung and plasma F₂-IsoPs increase during BLM-induced lung fibrosis
- In BLM animal model, lung F₂-IsoPs amounts are correlated with collagen content
- F₂-IsoPs can activate cultured fibroblasts to myofibroblasts
- F₂-IsoPs induce cell proliferation and collagen synthesis in rat lung fibroblasts
- F₂-IsoPs elicit their fibrogenic effect through the binding to TP receptor

Abbreviations: α -SMA, α -smooth muscle actin; BAL, bronchoalveolar lavage; BHT, butylated hydroxytoluene; BLM, bleomycin; Bmax, maximal binding capacity; DMEM, Dulbecco's modified Eagle's medium; EBC, exhaled breath condensate; F₂-IsoPs, F₂-isoprostanes; FBS, fetal bovine serum; GC/NICI-MS/MS, tandem mass spectrometry; HNE, 4-hydroxy-2,3-nonenal; IPF, idiopathic pulmonary fibrosis; OS, oxidative stress; PGF_{2 α} -d₄, tetradeuterated prostaglandin PGF_{2 α} ; RLF, rat lung fibroblasts; SQ29548 [1S-[1a,2a(Z),3a,4a]]-7-[3-[[2-[(phenylamino) carbonyl]hydrazino] methyl]-7-oxabicyclo[2.2.1] hept-2-yl]-5-heptanoic acid; TCA, trichloroacetic acid; TP receptor, thromboxane A₂ receptor; TxA₂, thromboxane A₂

ABSTRACT

F₂-isoprostanes (F₂-IsoPs) have been considered markers of oxidative stress in various pulmonary diseases, but little is known about their possible role in pulmonary fibrosis. In this study, we have investigated the potential key role of F₂-IsoPs as markers and mediators of bleomycin (BLM)-induced pulmonary fibrosis in rats. During the *in vivo* study, plasma F₂-IsoPs showed a peak at 7 days and remained elevated for the entire experimental period. Lung F₂-IsoP content nearly tripled 7 days following the intratracheal instillation of BLM, and by 28 days, the value increased about fivefold compared to the controls. Collagen deposition correlated with F₂-IsoP content in the lung. Furthermore, from day 21 onwards, lung sections from BLM-treated animals showed α -smooth muscle actin (α -SMA) positive cells, which were mostly evident at 28 days. *In vitro* studies performed in rat lung fibroblasts (RLF) demonstrated that either BLM or F₂-IsoPs stimulated both cell proliferation and collagen synthesis. Moreover, RLF treated with F₂-IsoPs showed a significant increase of α -SMA expression compared to control, indicating that F₂-IsoPs can readily activate fibroblasts to myofibroblasts. Our data demonstrated that F₂-IsoPs can be mediators of key events for the onset and development of lung fibrosis, such as cell proliferation, collagen synthesis and fibroblast activation. Immunocytochemistry analysis, inhibition and binding studies demonstrated the presence of the thromboxane A₂ receptor (TP receptor) on lung fibroblasts and suggested that the observed effects may be elicited through the binding to this receptor. Our data added a new perspective on the role of F₂-IsoPs in lung fibrosis by providing evidence of a profibrotic role for these mediators in the pathogenesis of pulmonary fibrosis.

1. INTRODUCTION

Pulmonary fibrosis is a progressive and often lethal lung disease characterized by excessive fibroblast activation and proliferation, resulting in abnormal accumulation of extracellular matrix. Persistent inflammation and oxidative stress (OS) contribute to exacerbations and disease progression [1,2]. Serum levels of OS markers are significantly higher in patients with idiopathic pulmonary fibrosis (IPF) suggesting that OS is involved in its pathogenesis [3,4]. Despite advances in research and diagnostic approach, the molecular mechanisms of the disease are poorly understood and no effective therapies are currently available. Further investigation of the factors involved in the initiation and progression of pulmonary fibrosis is urgently needed to develop effective treatments against this disease.

Bleomycin (BLM) is one of the most widely used drugs for inducing lung fibrosis in animals, due to its ability to reproduce a histologic lung pattern similar to that described in patients undergoing chemotherapy [5]. The BLM-induced pulmonary fibrosis was first described as a side-effect of the BLM antineoplastic activity. This complex of glycopeptide antibiotics from *Streptomyces verticillus* has long been used as an antineoplastic drug for different malignancies [6-11]. Since BLM chelates metal ions producing a pseudoenzyme that reacts with oxygen, it is believed that BLM antineoplastic activity is mediated by production of reactive oxygen species (ROS). This in turn leads to single- or double-stranded DNA breaks [12-14]. Genetic susceptibility [15] and the intracellular hydrolase activity [16,17] appear to be involved in BLM toxicity in lung. Moreover, pro-inflammatory cytokines play a relevant role in BLM-induced lung injury [18]. The fibrogenic effect of BLM is considered secondary to OS. The early stage is characterized by the inflammatory response, followed by a chronic stage that leads to extracellular matrix remodeling and collagen deposition [19].

The F₂-isoprostanes (F₂-IsoPs) are the most proximal products of lipid peroxidation from arachidonic acid and are one of the most reliable approaches to evaluate OS *in vivo* in different pathologies [20]. They are considered OS markers of a wide variety of inflammatory lung conditions [21-23]. Elevated levels of F₂-IsoPs have been reported in exhaled breath condensates (EBC), plasma

and urine from patients with asthma [21,24], chronic obstructive pulmonary disease [25,26], cystic fibrosis [27] and in the bronchoalveolar lavage (BAL) fluid from patients with different interstitial lung diseases [28,29].

In addition to being OS markers, F₂-IsoPs also elicit a wide variety of responses in different cell types [20], including lung cells. In the lung, F₂-IsoPs regulate different cellular processes such as contraction of airway smooth muscle [30,31], anion conductance across epithelial cells [32], and stimulate adhesion and function of inflammatory cells [33,34].

We previously demonstrated, in an experimental model of hepatic fibrosis, that F₂-IsoPs are both markers of OS and mediators of fibrotic effects. The addition of F₂-IsoPs to cultured hepatic stellate cells (HSC) greatly stimulated cell proliferation and collagen synthesis, two hallmarks of fibrosis [35]. In the liver, these fibrogenic events appear to be mainly mediated through the activation of thromboxane A₂ (TxA_{2r} or TP receptor) receptors analogous [36,37].

Increasing evidence suggests that fibroblast activation and proliferation may be a critical event in the pathogenesis of pulmonary fibrosis [38,39]. Patients with active fibrosis usually present a greater profusion of fibroblastic *foci*. This is attributed to an increased number of activated fibroblasts, many of which exhibit the characteristics of myofibroblasts [40,41]. These cells show intermediate features between the smooth muscle cell and the fibroblast, and are characterized by the expression of α -smooth muscle actin (α -SMA) [42,43]. The localization of myofibroblasts at sites undergoing active extracellular matrix deposition suggests their important role in the progression of fibrotic lesion.

To date, only few studies have examined the effect of OS on the release of F₂-IsoPs by lung fibroblasts [44-46]. However, no studies performed have investigated the biological effects of F₂-IsoPs on lung fibroblasts, with the exception of our previous *in vitro* work on human lung fibroblasts [46]. The present study was carried out in a rat model of BLM-induced pulmonary fibrosis to better understand the role of F₂-IsoPs in the onset/development of this disease. In particular, we sought to investigate whether F₂-IsoPs produced after BLM injury elicit a biological effect on lung fibroblasts,

acting as mediators of the fibrotic process.

2. MATERIALS AND METHODS

2.1. Animals

Male Sprague Dawley rats weighing 200-250 g (Harlan-Nossan, Correzzana, Italy) were maintained in a temperature- and light-controlled environment, and fed Harlan Global diet and water *ad libitum*. All animal studies were approved by the Siena University Ethic-Deontological Committee.

2.2. In vivo study

2.2.1. Animal model of bleomycin-induced pulmonary fibrosis

Forty-eight rats were randomly divided into 4 groups (n=12 each). Each treatment group included 6 control animals and 6 animals treated with BLM. Anaesthetized rats received a single intratracheal instillation of BLM (Nippon Kayaku, Sanofi Aventis, Milan, Italy), 5 mg/kg body weight in 0.3 ml phosphate buffered saline, as previously described [47]. Control animals received the same volume of sterile saline only. Animals were sacrificed at different time points (7, 14, 21 and 28 days) after BLM injection. Blood and lungs were processed for biochemical and morphological analysis as described in the following sections.

2.2.2. Lung histology and immunohistochemistry for α -SMA

After death, lung tissues were fixed by inflation with a buffered 10 % formalin solution for 24 h and then embedded in paraffin. Tissues were then sectioned, stained with Mallory trichrome for collagen staining and examined for pulmonary fibrosis.

Some of the lung sections (3-4 μ m) were used for immunohistochemistry. The expression of α -SMA marker was performed on lung sections by using “ α -SMA ImmunoHistology Kit” (IMM-2, Sigma), following manufacturer’s instructions.

2.2.3. Collagen Measurement

Collagen content was determined by hydrolyzing lung samples in 6 N HCl and by measuring

hydroxyproline according to the method of Kivirikko [48]. Collagen content was estimated by multiplying the amount of hydroxyproline by a factor of 7.69. Results are expressed as milligram of collagen per gram of lung (wet weight).

2.2.4. F₂-IsoPs in lungs and plasma

For plasma free F₂-IsoP determination, platelet poor plasma was obtained from heparinized blood by centrifugation at 2400×g and butylated hydroxytoluene (BHT) (final concentration 90 μM) was added to plasma as an antioxidant. Aliquots of plasma were stored under nitrogen at -70°C until analysis (within 2 months). Immediately after thawing, sample acidification (2 ml of acidified water, pH 3) was carried out and plasma (1 ml) was spiked with tetradeuterated prostaglandin PGF_{2α} (PGF_{2α}-d₄) (500 pg in 50 ml of ethanol) as an internal standard. Plasma sample extraction and purification were performed by solid-phase separation steps: an octadecylsilane (C18) cartridge followed by an aminopropyl (NH₂) cartridge [49,50].

For lung total (sum of free and esterified) F₂-IsoP determination, tissue was frozen in dry ice and stored at -80 °C until assay. At the time of the assays, sample was homogenized (10% w/v) in phosphate-buffered saline, pH 7.4. Aqueous KOH (1 mM, 500 μl) was added to an aliquot (1 ml) of lung homogenate. After incubation at 45°C for 45 min, pH was adjusted to 3 by adding HCl (1 mM, 500 μl). Each sample was spiked with PGF_{2α}-d₄ (500 pg), as an internal standard, and ethyl acetate (10 ml) was added to extract total lipids by vortex-mixing and centrifugation at 1,000 g for 5 min at room temperature. The total lipid extract was applied to an NH₂ cartridge and isoprostanes were eluted [50,51].

For both plasma and lung eluted samples, the carboxylic group was derivatized as the pentafluorobenzyl ester whereas the hydroxyl groups were converted to trimethylsilyl ethers. F₂-IsoP determinations were carried out by gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis. The measured ions were *m/z* 299 and *m/z* 303 derived from the [M-181]⁻ precursor ions (*m/z* 569 and *m/z* 573) produced from the derivatized 15-

F_{2t}-IsoP (the most represented isomer for F₂-IsoP measurement) and the PGF_{2α}-d₄, respectively [49].

2.3. In vitro study

2.3.1. Rat Lung Fibroblast (RLF) isolation

Cells were isolated as previously described [52] and modified by our group [53]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics and then seeded at 10⁶ cells/flask (25 cm²). An aliquot of freshly isolated RLF were seeded on coverslips in 24-well plates to evaluate the expression of α-SMA. The remaining cells were incubated at 37°C in 95% air-5% CO₂. Upon reaching confluence, cells were trypsinized, counted, seeded in flasks and used for the following experiments.

2.3.2. Treatment with Bleomycin or F₂-IsoPs

Cells were seeded at a density of 6×10⁴ cells/ml in DMEM supplemented with 10% FBS and allowed to grow to confluence. Twenty-four hours before the treatment, the medium was changed to serum-free DMEM. Cells were then treated with either BLM (0.1–100 mU/ml) or with 15-F_{2t}-IsoP (Cayman Chemical, USA) in the range of concentrations (10⁻¹⁴-10⁻¹² M). A stock solution of 15-F_{2t}-IsoP (1 mg/ml in ethanol) was diluted to a concentration of 10⁻⁵ M and then further diluted to final concentrations with DMEM.

2.3.3. Determination of total F₂-IsoPs in RLF

RLF were seeded in T75 flasks (6×10⁶ cells/flask) and treated with BLM (50 mU/ml) for 24 h. Cells were then harvested and the pellet was suspended again with 90 μM of the antioxidant BHT. Subsequently, samples were sonicated for 30 seconds and then incubated at 45°C for 45 min in presence of 0.5 N KOH for the basic hydrolysis. The samples were then acidified to pH 3 with HCl (final concentration 0.5 N), and spiked with PGF_{2α}-d₄ (500 pg) as internal standard. After extraction with ethyl acetate, the total lipids were applied to a NH₂-cartridge. The final eluates were derivatized to pentafluorobenzyl ester and trimethylsilyl ethers. As for plasma and lung samples, identification

and quantification of the ions m/z 299 and m/z 303 (derived from 15-F_{2t}-IsoP and PGF_{2α}-d₄, respectively) were performed by GC/NICI-MS/MS [54]. Total F₂-IsoPs levels were expressed as pg/10⁶ cells.

2.3.4. DNA synthesis

For the evaluation of cell proliferation, ³H-thymidine incorporation was measured, according to Boscoboinik et al. [55] in 24-well plates. Sub-confluent cells were incubated with either BLM or 15-F_{2t}-IsoP for 24 h in serum-free DMEM. Six hours before the measurements, 10 μCi/ml of ³H-thymidine were added. Cell layers were then washed, fixed for 20 min with ice-cold 5% trichloroacetic acid (TCA), and solubilized in 0.4 ml of 0.1 M NaOH/ 2% Na₂CO₃. Samples were mixed with 8 ml of Ultima Gold (Packard) and counted for radioactivity in a Packard 2100 TriCarb activity liquid scintillation analyzer. Results are expressed as ³H-thymidine incorporation (dpm) per well.

2.3.5. Collagen synthesis

To evaluate the effect of BLM or F₂-IsoPs on collagen metabolism, RLF were seeded on 12-well plates and grown to visual confluence. Medium was changed to serum-free DMEM for 24 h to allow the cells to become relatively quiescent. In addition to DMEM, 11.5 μg/ml L-proline, 50 μg/ml ascorbic acid, 50 μg/ml β-aminopropionitrile were added. After incubation, cells were treated either with BLM or 15-F_{2t}-IsoP (which is currently used as biomarker of *in vivo* F₂-IsoP formation) for 48 h. Collagen synthesis was assessed as previously reported [46]. Briefly, 16 h before the end of the 48 h treatment, 10 μCi/ml of ³H-proline (Amersham International; specific activity 23 Ci/mmol) were added to each well. Media was harvested for determination of ³H-proline incorporation into collagen and non-collagen proteins following the collagenase digestion method, by using highly purified bacterial collagenase (Calbiochem Cat. no. 234134, 250 IU). Incorporation of radioactivity into collagen and non-collagen proteins was determined following precipitation with TCA. Collagen-incorporated radioactivity was recovered in the TCA-soluble fraction, while non-collagen

radioactivity was recovered from TCA precipitate. Percentage collagen synthesis was estimated as previously reported [56].

2.3.6. Expression of α -SMA in Rat Lung Fibroblasts

Freshly isolated cells were grown on coverslips and α -SMA expression was evaluated by “ α -SMA ImmunoHistology Kit” (IMM-2, Sigma) at III, V and VIII day, after treatment with 15-F_{2t}-IsoP. Cells were fixed with cold acetone at 4°C for 5 min to block endogenous peroxidases, washed twice with PBS and incubated with 0.5% BSA in PBS for 30 min. Cells were incubated with the primary monoclonal antibody raised against α -SMA for 1 h and then with the appropriate biotinylated secondary antibody for 20 min. At the end of incubation, Peroxidase reagent was added for 20 min. Following PBS washes, cells were incubated with Substrate Reagent for 5 min, washed with water and counterstaining with 0.2% Fast-Green in water for 8 min. At last, coverslips were washed with water, fixed on glass and observed on microscope.

2.4. Identification of TP receptor on RLF

2.4.1. Immunoblot analysis for TP receptor

Western Blots were performed in both freshly isolated and activated RLF. An aliquot of freshly isolated cells was resuspended in the lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 10 mM EGTA, 10 mM iodoacetamide, 0.02% sodium azide and 0.01% PMSF) and placed on ice for 30 min. Cells were homogenized, sonicated and centrifuged for 45 min at 40000 g at 4°C. In parallel, activated RLF were seeded and allowed to reach confluence. Cells were then collected from the culture plates, resuspended in the lysis buffer and treated as described above for freshly isolated cells. Pellets from both cell preparations were collected and resuspended in the lysis buffer. An aliquot of membrane protein (30 μ g) was loaded in each lane, separated electrophoretically in 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Immunoblot was performed using a polyclonal antibody raised against human TP receptor (1:1000) (Cayman Chemical, Ann Arbor, USA). Secondary goat anti-rabbit IgG antibody (BioRad, Hercules, CA, USA) (1:15000) was added to the membrane.

Immunodetected proteins were visualized using ECL assay kit (Amersham Biosciences), following the manufacturer's recommended protocol.

2.4.2. Binding Studies

Cells were seeded on 12-well cell plates as 6×10^4 cells/ml and used when they reached confluence. After washing twice with PBS, cells were incubated with a specific TxA_2r antagonist, ^3H -SQ29548 (Perkin Elmer, Boston, MA, USA; specific activity 48.2 Ci/mmol), in the concentration range of 1.25–20 nM for 2.5 h at 4°C , as previously described [36]. The reaction was terminated by four washes with ice-cold PBS to remove unbound ligand. Cells were dissolved in 1 ml 1N NaOH followed by neutralization with 1 ml 1N HCl. Radioactivity was counted in a Packard 2100 TriCarb liquid scintillation analyzer. Specific binding was calculated as the difference between binding measured in the absence and presence of unlabeled SQ29548 (10 μM). The maximum number of binding sites (Bmax) and the dissociation constant (Kd) were calculated by nonlinear regression analysis of the saturation curve performed by one-site curve fit.

2.4.3. Colocalization of α -SMA and TP receptor on lung fibroblasts

Fibroblasts were grown on coverslips and fixed with cold acetone at 4°C for 5 min. The colocalization of α -SMA and TP receptor on RLF was performed by a first block of nonspecific binding with 3% BSA in PBS for 1 h, followed by incubation with primary monoclonal mouse antibody anti- α -SMA (Sigma) (1:100) for 1 h. After washing with PBS, cells were incubated for 1 h with TRITC-conjugated anti-mouse IgG (Sigma, St Louis, MO, USA) (1:200). A second block for nonspecific immune-binding sites was performed with 3% BSA, and cells were incubated for 1 h with rabbit polyclonal antibody raised against human TP receptor (1:100 in 0.3% BSA/PBS). After 1 h incubation with FITC-conjugated anti-rabbit IgG (Sigma, St Louis, MO, USA) (1:200), the immunofluorescence was analyzed by a Nikon Eclipse TE300 microscope.

2.5. Inhibition experiments

To investigate whether the fibrogenic effects of F₂-IsoP in RLF are mediated by TP binding, experiments on DNA and collagen synthesis were carried out in the presence of SQ29548 (Cayman Chemical, Ann Arbor, USA), a specific TP antagonist. Subconfluent cells were made quiescent by incubation in serum-free DMEM and then treated with 15-F_{2t}-IsoP (10⁻¹³M) in the presence or absence of SQ29548, in a 1:10 molar ratio. DNA and collagen synthesis were estimated as reported under previous paragraphs (2.3.4. DNA synthesis and 2.3.5. Collagen synthesis).

2.6. Statistical Analyses

The results of each experimental *in vitro* condition were determined from the mean of triplicate experiments. For the *in vivo* studies, 6 animals per group were used unless noted otherwise. An independent sample two-tailed Student's t-test was used to assess the significance between the two groups. Statistical analysis was carried out by analysis of variance followed by appropriate post hoc tests including multiple comparison tests. Nonlinear regression analysis and statistical analysis were performed using commercially available software (Prism 4.0; GraphPad Software Inc., San Diego, CA, USA). All data are expressed as means ± standard deviation (SD). The value of $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. In vivo study

3.1.1. BLM-induced lung fibrosis

Administration of BLM was associated with an increase in collagen, in particular in perialveolar areas. By day 7, tissues showed progressive morphological changes and a regional interstitial fibrosis which became more pronounced at 28 days (Fig. 1). Control lungs from animals instilled with saline did not demonstrate any pathologic changes at any of the time points.

In contrast to earlier time points (and control lungs), after 21 days, lung sections showed α -SMA positive cells, of which the majority were evident at day 28 (Fig. 2). The expression of α -SMA demonstrates the population of cells being composed of myofibroblasts (fibroblastic *foci*). This feature was absent at 7 days and in controls.

Similarly, biochemical evaluation of hydroxyproline showed a progressive increase in lung collagen content in BLM-treated rats ($10.25 \pm 0.41 - 34.24 \pm 0.62$ mg/g tissue) when compared to saline treated rats ($7.34 \pm 0.35 - 16.27 \pm 0.65$ mg/g) (Fig. 3A). In comparison to saline treated rats, the BLM treatment also induced significant progressive increases in total lung F₂-IsoPs (Fig. 3B). Along the experimental time (0-28 days), a significant positive correlation between lung collagen content and the amount of F₂-IsoPs in the lung was observed ($r = 0.9274$, $P < 0.001$).

As depicted in Fig. 3C, plasma F₂-IsoP levels showed a significant increase compared to controls, with a peak at 7 days after treatment (+107%) and remained elevated for the entire experimental period (about 60%).

3.2. In vitro study

3.2.1. Effect of BLM on Lung Fibroblasts

RLF incubated with different BLM concentrations (0.1-100 μ g/ml) showed increased levels of cell proliferation (Fig 4A) and collagen synthesis (Fig. 4B). In particular, treatment with 50 μ g/ml of BLM induced the highest response in cell proliferation and collagen synthesis. Although, both parameters

decreased at 100 $\mu\text{g/ml}$ of BLM concentration, they remained higher than the controls.

Moreover, the same concentration of BLM (50 $\mu\text{g/ml}$) induced a 3-fold increase in the levels of F₂-IsoPs released in culture media (Fig. 5).

3.2.2. Effect of 15-F_{2t}-IsoP on Fibroblast Proliferation and Collagen Synthesis

To investigate whether F₂-IsoPs have a biological effect on RLF, we studied the effect of 15-F_{2t}-IsoP (the most representative F₂-IsoP isomer) on two main hallmarks of fibrosis. The addition of 15-F_{2t}-IsoP (10^{-12} - 10^{-14} M) to cultured activated pulmonary fibroblasts induced an increase in both DNA (+74%) and collagen synthesis (+35%) (Fig. 6A and B). The most active concentration for both parameters was 10^{-13} M.

3.2.3. Effect of F₂-IsoPs on Lung Fibroblast Activation

To determine whether F₂-IsoPs play a role in RLF activation, α -SMA expression was evaluated. After 3 days of culture, there was no evidence of α -SMA expression in control or treated cells. However, after five days of treatment with 15-F_{2t}-IsoP, α -SMA expression was more evident in RLF compared to control. After 8 days of treatment, the expression of α -SMA in treated cells had increased approximately 2-fold compared to control cells. These results demonstrate that F₂-IsoPs can more readily activate cultured RLF to myofibroblasts (Fig. 7).

3.3. Identification and localization of TP receptor in Lung Fibroblasts

3.3.1. Immunoblot and binding studies

Immunoblot analysis of membrane proteins from activated RLF revealed the presence of a single immunoreactive band of 55 kD corresponding to TP receptor (Fig. 8, lane B). Molecular weight of this band corresponds to a TP receptor previously found in activated HSC [36]. No band was detected in freshly isolated RLF (Fig. 8, lane A).

Binding studies were carried out to characterize the population of TP receptors on lung fibroblasts.

Analysis of the saturation curve by nonlinear regression indicated that $^3\text{H-SQ29548}$ binds to a single class of receptors on RLF, with a dissociation constant (K_d) of 1.22 ± 0.11 nM and a maximal binding capacity (B_{max}) of 5.28 ± 0.01 fmol/well (Fig. 9).

This result is very similar to that presented in our previous work on activated HSC ($K_d = 1.42 \pm 0.33$ nM; $B_{\text{max}} = 5.91 \pm 0.36$ fmol/well) (36).

3.3.2. Colocalization studies

Colocalization studies carried out with immunofluorescence confirmed the presence of TP receptor on activated lung fibroblasts. In fact, 3 days after cell isolation, both control and $\text{F}_2\text{-IsoPs}$ -treated fibroblasts were negative for the analyzed markers. From the fifth day onward, the TP receptor (evidenced in green) is present in both control and treated fibroblasts (Fig. 10). However, cells treated with $\text{F}_2\text{-IsoPs}$ displayed a higher expression of TP compared to the matched controls. Cells expressing only $\alpha\text{-SMA}$ appeared red (red arrow), while when $\alpha\text{-SMA}$ colocalizes with TP the cells appeared yellow-orange (merge, white arrow). Fig. 10 shows that the presence of TP is paralleled by the expression of $\alpha\text{-SMA}$, which is more evident in RLF treated with $\text{F}_2\text{-IsoPs}$. At day 8, all fibroblasts showed co-expression of both markers (yellow-orange) (Fig. 10).

3.3.3. Inhibition experiments

Inhibition experiments were carried out to evaluate whether the fibrogenic effects induced by $\text{F}_2\text{-IsoPs}$ in RLF are elicited through the binding to TP receptor. Cells were incubated in the presence of both $15\text{-F}_2\text{-IsoP}$ and the specific TP antagonist SQ29548, in a molar ratio of 1:10, and DNA and collagen synthesis were measured. As shown in Figure 11 (A and B), the stimulatory effect of $\text{F}_2\text{-IsoPs}$ on both parameters were inhibited by SQ29548 co-incubation. These results are in agreement with our binding studies demonstrating that RLF express a population of receptors that specifically bind to the TP antagonist $^3\text{H-SQ29548}$. This further strengthens our hypothesis of TP receptor involvement in $\text{F}_2\text{-IsoPs}$ -evoked responses in RLF.

4. DISCUSSION

Although the precise mechanisms of pulmonary fibrosis are not fully understood, disruption of the oxidant/antioxidant balance in the lung is thought to play an important role in many of the processes leading to inflammation and fibrosis [57,58]. F₂-IsoPs belongs to a class of metabolites produced during OS in different pathologies, including pulmonary diseases.

Elevated levels of F₂-IsoPs were found in EBC of patients with cystic or interstitial pulmonary fibrosis [27,59], as well as in serum and bronchoalveolar lavage in IPF [29].

The experimental induction of pulmonary fibrosis by means of BLM is a well-described model in which OS is known to contribute to pathology [5,60,61]. The role of ROS in the pathogenesis of BLM-induced lung injury is largely known, and the absence of ROS production results in protection of the animals against BLM-induced pulmonary fibrosis [60,62-64]. Furthermore, BLM is known to cause an increase in 4-hydroxy-2,3-nonenal (HNE), one of the major bioactive markers of lipid peroxidation [65,66]. HNE is able to induce TGFβ1 expression, which is crucial for the pathogenesis of this disease [67].

However, little information is available on the role of F₂-IsoPs in pulmonary fibrosis. Only few studies carried out in animal models of BLM-induced lung fibrosis reported a local increase of F₂-IsoPs in lung tissue [58,68,69], and these observations were limited to 3-8 days after BLM treatment.

In the present work, we demonstrated that lung F₂-IsoP values progressively increased up to 28 days in animals receiving BLM, and positively correlated with lung collagen content. We also showed that F₂-IsoPs rapidly increased in plasma following administration of BLM, reaching values 2 times those of controls by day 7. This increase was associated with the development of fibrosis, as evaluated histologically and biochemically. Our results suggest that plasma levels of IsoPs may be considered as an indicator of oxidant-induced lung damage during BLM-induced pulmonary fibrosis.

Although F₂-IsoPs are widely recognized to evoke a variety of biological responses in airway and pulmonary vascular smooth muscle cells [31,32], their effect on lung fibroblasts and pulmonary

fibrosis is largely unstudied. Fibroblasts are the most common connective tissue cells. Under precise stimulatory signals, fibroblasts either transform to myofibroblasts or proliferate, resulting in vascular occlusion or areas of fibroblastic *foci*. Fibroblastic *foci* are thought to be the sites of active collagen and elastic synthesis with excessive deposition of extracellular matrix components [70-72].

In previous studies, we demonstrated that BLM increased cell proliferation and collagen synthesis in cultured human lung fibroblasts [46]. In our present work, similar data was obtained on RLF. Of interest, when these cells were treated with BLM, a marked release of F₂-IsoPs in the medium was observed. This demonstrated that fibroblasts may be a source of F₂-IsoPs in OS-induced lung injury. With the aim to clarify the possible role of F₂-IsoPs as mediators of fibrotic events, RLF were directly treated with 15-F_{2t}-IsoP. This resulted in a significant increase of cell proliferation and collagen synthesis, further confirming our previous data obtained in hepatic stellate cells [35,73,74].

A large body of literature has demonstrated that pulmonary fibrosis is often characterized by an increase in the number and volume of cells expressing α -SMA and procollagen mRNA, referred as myofibroblasts [75,76]. These cells have been found in IPF and in experimental BLM-induced lung fibrosis and are considered to be the main source of collagen hyperproduction. However, the stimulus and the underlying processes by which fibroblasts differentiate into myofibroblasts are still debated.

We found these fibroblastic *foci* in lung sections of rats treated with BLM and demonstrated *in vitro* that F₂-IsoPs are able to activate fibroblasts to myofibroblasts (as evaluated by α -SMA expression). Our results indicate that F₂-IsoPs may be involved both in the activation of fibroblasts and in the stimulation of cell proliferation and collagen synthesis, both key events in the fibrotic process.

Although the mechanism through which F₂-IsoPs stimulate fibroblasts is unknown, some studies demonstrated that in different cell types they act through receptors analogous to those for thromboxane A₂ (TxA₂) (TP receptors) [36,77,78] We previously reported that HSC express high-affinity receptors for TxA₂ ligands on their surface, and that fibrogenic effects in the liver were

elicited by F₂-IsoPs through these receptors, which set the MAPK and IP3 pathways into motion [36,37,73].

In this work, we showed that TP receptor is also present on RLF and colocalizes with α -SMA. To our knowledge, this is the first study demonstrating the presence of the TP receptor on RLF. Binding studies demonstrated that RLF present a single class of receptors that specifically bind to the TxA_{2r} antagonist ³H-SQ29548, showing an affinity of ligand binding (K_d) and a density of receptors (B_{max}) very similar to those previously found in HSC [36]. Finally, our inhibition studies indicated that the fibrogenic response induced by F₂-IsoPs in RLF is elicited through the binding to TP receptor. Our present results, combined with those previously obtained in HSC [37], contributed to the hypothesis that F₂-IsoPs trigger a shared fibrogenetic mechanism in fibroblasts and fibroblast-like cells.

Further studies are needed to elucidate which pathways are activated by these compounds in lung fibrosis.

Conclusion

This study indicates that F₂-IsoPs are *in vivo* markers of OS during pulmonary fibrosis and also serve as key regulators of fibrogenesis by activating fibroblasts to myofibroblasts and by inducing cell proliferation and collagen synthesis. These effects are mediated by the TP receptor which we found to be expressed by RLF. Together, our results may have important implications in the development of more effective therapies for lung fibrosis.

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Conflict of Interests The authors declare that there is no conflict of interests.

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LEGEND TO FIGURES

Fig. 1. Effect of bleomycin (BLM) on lung histological changes. Rats were treated with a single intratracheal instillation of saline or BLM and the lungs were harvested for Trichrome staining at different time points after treatment, as described in the Materials and Methods. Shown is representative histology from rats in BLM-treated group. Histological signs of fibrosis are evident since day 7 and progressively increase until 28 days. Original magnification, 100X.

Fig. 2. Immunohistochemical analysis of lung sections from rats treated with BLM. Cells positive to α -SMA are evident after 21 and 28 days of treatment with BLM, while are absent at 7 days. Original magnification, 200X.

Fig. 3. Lung collagen content and F₂-IsoP levels in rats after chronic intoxication with BLM. Rats were treated with BLM and the parameters were evaluated after 7, 14, 21 and 28 days. (A) Lung collagen content was assayed as reported in Materials and Methods and calculated by multiplying the hydroxyproline content for the conversion factor, 7.69. Results are expressed in mg of collagen/g rat lung. F₂-IsoP levels in lung (B) and plasma (C) are reported as pg/mg of tissue protein and pg/ml plasma, respectively. Data are expressed as means \pm SD from 3 controls and 5 BLM-treated animals. * P <0.05, ** P <0.01, *** P <0.001 vs. respective control.

Fig. 4. Effect of BLM on RLF proliferation (A) and collagen synthesis (B). RLF were isolated and treated with BLM at different concentrations. (A) Cell proliferation was evaluated after 24 h by measuring ³H-thymidine incorporation in RLF. Results are expressed as percentage of untreated controls. (B) Collagen content was evaluated after 48 h by measuring the ratio of ³H-proline incorporation into collagen and non-collagen proteins. Results (collagen/total protein) are presented as percentage of untreated controls. Data are means \pm SD of 3 experiments. * P <0.05 vs. Control, ** P <0.01 vs. Control.

Fig. 5. Effect of BLM on F₂-IsoP release in RLF. To assess the effect of BLM in RLF, F₂-IsoP levels were evaluated after 24 h of treatment with 50 μ g/ml of BLM. Compared to controls, BLM-treated RLF showed increased levels of F₂-IsoPs. Data are expressed as means \pm SD of 3 experiments. ** P < 0.01 vs. Control.

Fig. 6. Effect of 15-F_{2t}-IsoP on proliferation (A) and collagen synthesis (B) in RLF. Cells were

treated with increasing concentrations of 15-F_{2t}-IsoP as described in Materials and Methods. (A) DNA synthesis was evaluated as ³H-thymidine incorporation. (B) Collagen synthesis was evaluated as a ratio of ³H-proline incorporation into collagen and non-collagen proteins. Results are presented as percentage of untreated controls. Data are expressed as means ± SD of 3 experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. Control.

Fig. 7. Effect of F₂-IsoPs on freshly isolated RLF. Cells were isolated and treated with 10⁻¹³M 15-F_{2t}-IsoP as described in Materials and Methods. Fibroblast activation was examined immunocytochemically by evaluating α-SMA expression at 3, 5 and 8 days of culture. Original magnification, 100X.

Fig. 8 Western blotting of TP receptor in RLF. Proteins were extracted from membrane lysates of both freshly isolated and activated cells. Immunoblot analysis was performed with a polyclonal antibody raised against TP receptor (1:1000). The figure shows a membrane protein of 55 kDa corresponding to TP in activated RLF (lane B). No band was detected in freshly isolated cells (lane A). Blots were reprobed with β-actin to assess equal loading.

Fig 9. Scatchard plotting of ³H-SQ29548 binding on RLF. Cells were incubated with ³H-SQ29548 (1.25–20 nM). Specific binding was calculated as the difference between binding measured in the absence and presence of unlabeled 10 mM SQ29548. Each point was run in quadruplicate. Data are the means ± SD of three experiments.

Fig. 10. Colocalization of TP receptor and α-SMA in RLF. Activated cells expressed both TP receptor (green labelled) and α-SMA (red labelled). Merged images (yellow-orange) show the degree of colocalization of both markers. Nuclei were stained with DAPI reagent. Original magnification, 400X.

Fig. 11. Effect of 15-F_{2t}-IsoP and SQ on proliferation (A) and collagen synthesis (B) in RLF. Cells were treated with 15-F_{2t}-IsoP (10⁻¹³M) and the specific TP antagonist SQ29548, in a molar ratio of 1:10, as described in Materials and Methods. (A) DNA synthesis was evaluated as ³H-thymidine incorporation. (B) Collagen synthesis was evaluated as a ratio of ³H-proline incorporation into collagen and non-collagen proteins. Results are presented as percentage of untreated controls. Data are expressed as means ± SD of 3 experiments. **P*<0.05 vs. Control; #*P*<0.05 vs ISO; ###*P*<0.001 vs ISO; C, Control; SQ, SQ29548; ISO, 15-F_{2t}-IsoP.