16,16-Dimethyl Prostaglandin E_2 Efficacy on Prevention and Protection from Bleomycin-Induced Lung Injury and Fibrosis

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In this study, we evaluated the protective effect and therapeutic potential of the prostaglandin E_2 (PGE_2) synthetic analog 16,16-dimethyl-PGE_2 ($dmPGE_2$) in the animal model of pulmonary fibrosis induced by bleomycin. Mice subjected to intratracheal administration of bleomycin (1 mg/kg) received a $dmPGE_2$ dose of 30 μg/kg/day by continuous subcutaneous infusion. Bronchoalveolar lavage (BAL); immunohistochemical analysis for IL-1, TNF-α, and nitrotyrosine; measurement of fluid content in lung; myeloperoxidase activity assay; and lung histology were performed 1 week later. Lung histology and Sircol assay for collagen deposition were performed 3 weeks after treatments. Changes of body weight and survival rate were also evaluated at 1 and 3 weeks. Compared with bleomycin-treated mice, $dmPGE_2$ co-treated mice exhibited a reduced degree of body weight loss and mortality rate as well as of lung damage and inflammation, as shown by the significant reduction of: (*1*) lung infiltration by leukocytes; (*2*) myeloperoxidase activity; (*3*) IL-1, TNF-α, and nitrotyrosine immunostaining; (*4*) lung edema; and (*5*) histologic evidence of lung injury and collagen deposition. In a separate set of experiments, $dmPGE_2$ treatment was started 3 days after bleomycin administration, and the evaluation of lung damage and inflammation was assessed 4 days later. Importantly, delayed administration of $dmPGE_2$ also was able to protect from inflammation and lung injury induced by bleomycin. These results, indicating that $dmPGE_2$ is able to prevent and to reduce bleomycin-induced lung injury through its regulatory and anti-inflammatory properties, encourage further research to find new options for the treatment of pulmonary fibrosis.

Keywords: lung fibrosis; bleomycin; 16,16-dimethyl-PGE_2; mice; IPF

Cyclooxygenase (COX)-derived prostaglandin E_2 (PGE_2) is commonly considered a potent proinflammatory mediator involved in different diseases ranging from periodontitis to rheumatoid arthritis (1, 2). However, PGE_2 plays a very different role in the lung by modulating inflammation and reparative processes within the epithelial mesenchymal trophic unit (3), where it is synthesized mainly by alveolar epithelial cells, and in minor quantities by fibroblasts or migratory monocytes/macrophages. In normal conditions, PGE_2 concentration in the alveolar compartment is much higher than in plasma (4). On the contrary, several studies show that fibrotic diseases such as idiopathic pulmonary fibrosis (IPF), are characterized by reduced COX-2 expression and PGE_2 synthesis in lung fibroblasts (5, 6). Moreover, in the experimental model of pulmonary fibrosis, knocking out COX-2 significantly raises the susceptibility to lung fibrosis (7). Furthermore, no induction of COX-2 in response to multiple stimuli was observed in fibrotic fibroblast (5). However, COX-2 expression is increased in the regenerating metaplastic epithelium of the lung in patients suffering from IPF and other pulmonary disorders (8), while lung PGE_2 levels are increased after bleomycin administration in the animal model of pulmonary fibrosis (9, 10), likely in an attempt to restore the integrity of the alveolar architecture by modulating the reparative process after injury. PGE_2 effects are mediated by four different receptors termed E prostanoid receptors (EPs) (11). In particular, EP2 and EP4 receptors are considered to be responsible for the inhibitory effects of PGE_2 on fibroblast activation and transformation into myofibroblast and on collagen production (12, 13).There is evidence that after bleomycin administration the EP2 expression in pulmonary fibroblasts is significantly altered (14). This might account, at least in the mouse model of pulmonary fibrosis, for the refractoriness of lung fibroblasts to the increased PGE_2 production, thus justifying the insufficient control of reparative processes after bleomycin-induced damage. Intratracheal instillation of the antitumor agent bleomycin is the most commonly used animal model of pulmonary fibrosis, even though this model, with its intrinsic limitations, cannot mimic every aspect of human pulmonary fibrosis (15). However, the model reproduces the iatrogenic pulmonary fibrosis arising as a side effect of the administration of bleomycin as an antitumor drug in humans.

In this study we explored the possibility of overriding bleomycin-induced lung injury and inflammation as well as alterations of physiologic PGE_2 pathways by administering 16, 16-dimethyl-PGE_2 ($dmPGE_2$), a stable synthetic EP receptor agonist (16). We showed protective effects and therapeutic potentialities of this PGE_2 analog in the lung fibroreparative processes.

CLINICAL RELEVANCE

We showed protective effects and therapeutic potentialities of 16,16-dimethyl–prostaglandin E_2 in the lung fibroreparative processes. These results encourage further research to find new options for the treatment of pulmonary fibrosis.

MATERIALS AND METHODS

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK). All other chemicals were of the

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highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter, Liverpool, UK).

Animals

Male CD mice (25–35 g; Harlan Nossan, Correzzana, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Methods

Induction of lung injury by bleomycin. Mice received a single intratracheal instillation of saline (0.9%) or saline containing bleomycin sulfate (1 mg/kg body weight) in a volume of 50 μl and were killed after 7 or 21 days by pentobarbitone overdose.

Experimental Design and Groups

Mice were randomly allocated into the following groups for each experiment. (*1*) BLEO group (n = 10): mice were subjected to bleomycin-induced lung injury. (*2*) SHAM+saline group (n = 5): sham-operated mice group in which saline was administered instead of bleomycin. (3) BLEO+dmPGE$_2$ group (n = 10): same as the BLEO group, but mice were co-treated with dmPGE$_2$ delivered through a subcutaneous implanted Alzet 2002 mini-osmotic pump (Durect Co., Cupertino, CA). This route of administration was preferred because of constant drug delivery. The pump loaded with 200 μl of dmPGE$_2$ solution in PBS (Cayman Chemical, Ann Arbor, MI) had a release rate of 0.5 μl/hour that produced a total amount of 30 μg/kg dmPGE$_2$ daily during the 7 or 21 days of the experimental setup. This dose was previously demonstrated to be successful in other context (17) and in a our pilot study was found to be both innocuous and efficacious. (*4*) SHAM+PGE$_2$ group (n = 5): identical to SHAM+saline group, except for the administration of dmPGE$_2$ delivered as described above. Mice were killed at 7 or 21 days. In a separate set of experiments, the same groups were employed, but dmPGE$_2$ treatment in group 3 was started 3 days after bleomycin administration (n = 10). In this case, animals were killed 4 days later.

Measurement of Fluid Content in Lung

The wet lung weight was measured after careful excision of extraneous tissues. The lung was exposed for 48 hours at 180°C and the dry weight was measured. Water content was calculated by subtracting dry weight from wet weight.

Bronchoalveolar Lavage

Seven days after treatments, mice were killed and the trachea was immediately cannulated with an intravenous polyethylene catheter (Neo Delta Ven 2; delta Med, Viadana, Italy) equipped with a 24-gauge needle on a 1-ml syringe. Lungs were washed once with 0.5 ml D-PBS (GIBCO, Paisley, UK). In over 95% of the mice, the recovery volume exceeded 0.4 ml. The bronchoalveolar lavage (BAL) fluid was spun at 800 rpm, the supernatant was removed, and pelleted cells were resuspended in PBS. BAL cells were enumerated by counting on a hemocytometer in the presence of the trypan blue stain. Cytospins were prepared from resuspended BAL cells.

Cytospins of BAL cells were made by centrifuging 50,000 cells onto microscope slides using a Shandon Cytospin 3 (Shandon, Astmoore, UK). Slides were allowed to air dry and were then stained with Diff-Quick Stain Set (Diff-Quick; Baxter Scientific, Miami, FL). By using optical microscopy, a total of 400 cells were examined for each sample from randomly chosen fields.

Histologic Examination

Excised lung were taken 7 or 21 days after injection of bleomycin, processed as previously described (18), and stained by hematoxylin and eosin or by the van Gieson method for collagen stain. The severity of fibrosis was semiquantitatively assessed according to the method of Ashcroft and coworkers (19). Briefly, the grade of lung fibrosis was scored on a scale from 0 to 8 by examining randomly chosen fields of the left middle lobe at a magnification of ×100. Criteria for grading lung fibrosis were as follows: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to lung architecture; grade 5, increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of structure and large fibrous areas; grade 8, total fibrous obliteration of fields. Grades 2, 4, and 6 were used as intermediate pictures between the aforementioned criteria. All sections were scored by a single investigator in a blinded fashion.

Soluble Collagen Assay

The total content of lung collagen was measured by performing the Sircol Soluble Collagen Assay (Biocolor, Newtownabbey, Northern Ireland), based on a modification of the sirius red method, following the manufacturer's instructions. Briefly, after mice were killed, their lungs were explanted and homogenized. Samples were then incubated at 4°C for 2 hours and centrifuged at 15,000 × g. Supernatants (20 μl) were diluted five times in lysis buffer, added to 1 ml of Sircol Dye Reagent, and then mixed for 30 minutes at room temperature in a mechanical shaker. The collagen–dye complex was precipitated by centrifugation at 10,000 × g for 10 minutes. The unbound dye solution was then carefully removed. The precipitated complex was resuspended in 1 ml of alkali reagent. The obtained solution was finally placed in a 96-well flat-bottomed plate and evaluated in a plate reader (absorbance = 540 nm). Absolute collagen content was calculated by comparing samples values to a pertinent standard curve.

Immunohistochemical Localization of IL-1β, TNF-α, and Nitrotyrosine

IL-1β and TNF-α were determined by immunohistochemistry as previously described (18). Sections were incubated overnight with anti–IL-1β, anti–TNF-α, or anti–nitrotyrosine (anti–n-tyr) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) polyclonal antibody (1:500 in PBS, vol/vol). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (DBA, Milan, Italy). Controls included buffer alone or nonspecific, purified rabbit IgG. Immunohistochemistry photographs were assessed by densitometry. The assay was performed by using Optilab Graftek software on a Macintosh personal computer.

Myeloperoxidase Activity Assay

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described in lung homogenates (20). The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μMol of peroxide/minute at 37°C and was expressed in milliunits per gram of wet tissue.

Statistical Evaluation

All values in text and figures are expressed as mean ± SEM of N observations for each experiment. At least two separate experiments were performed. For the *in vivo* studies, N represents the total number of animals for each experiment. Results were analyzed by one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons. A P value of less than 0.05 was considered significant. For survival data, statistical analysis was performed by Fisher's exact probability test. For such analysis, a $P < 0.05$ was considered significant.

RESULTS

Cytologic Analysis of BAL Fluid

BAL takes into account infiltrating leukocytes, especially lymphocytes and neutrophils, as well as alveolar macrophages. As shown in Figures 1A and 1C, 1 week after bleomycin administration total cellularity significantly increased in BAL fluid of bleomycin-exposed animals (BLEO), whereas co-treatment as well as delayed treatment with dmPGE$_2$ determined a significant reduction of BAL cellularity when compared with BLEO's one. Differential cell counts (Figures 1B and 1D) showed that

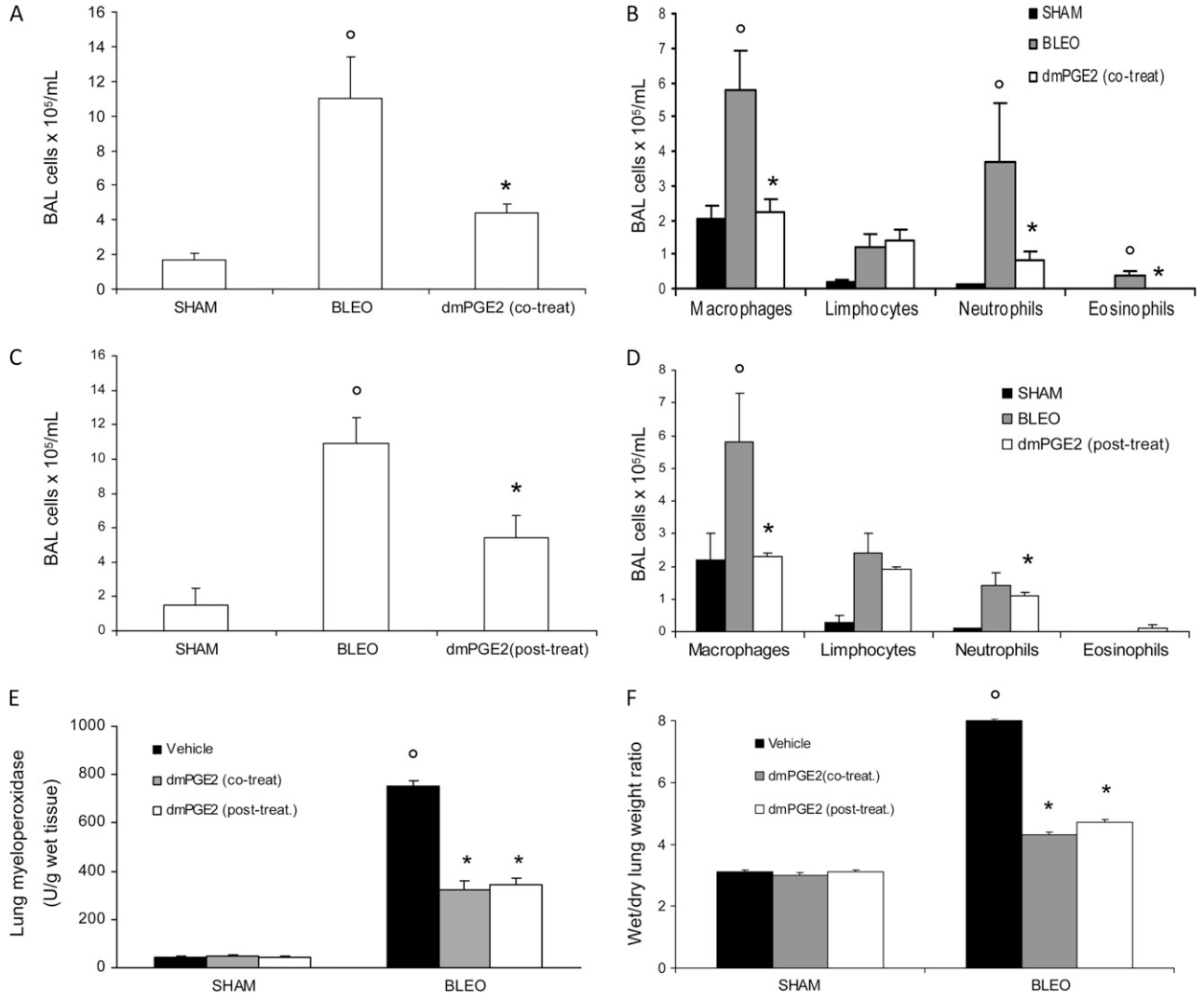

Figure 1. Effects of 16,16-dimethyl-PGE_2 ($dmPGE_2$) administration on bleomycin-induced leukocytes infiltration, myeloperoxidase (MPO) activity, and lung edema after 1 week of bleomycin treatment. Total cellularity in the bronchoalveolar lavage (BAL) fluid of sham-operated (SHAM), bleomycin-treated (BLEO), as well as either (*A*) $dmPGE_2$ co-treated or (*C*) $dmPGE_2$ post-treated mice was evaluated by a hemocytometer in the presence of the trypan blue stain. Differential cell counts (*B* and *D*) in cytospins from the same BAL fluids were obtained by observing with optical microscopy a total of 400 cells in randomly chosen fields of each sample. Graphs report mean values ± SEM of at least five mice for each group in one representative experiment. $^{\circ}P < 0.05$ versus SHAM, $^{*}P < 0.01$ versus BLEO. Results of MPO activity measured (as illustrated in MATERIALS AND METHODS) in mice lung tissues show (*E*) a huge increase of MPO activity in BLEO mice, whereas both $dmPGE_2$ co- and post-administration in BLEO mice significantly reduced the MPO activity. Reported data are means ± SEM from at least five mice for each group in one representative experiment. $^{\circ}P < 0.01$ versus SHAM, $^{*}P < 0.01$ versus BLEO. Similarly, results of lung fluid measurements (as illustrated in MATERIALS AND METHODS) show (*F*) that both $dmPGE_2$ co- and post-administration significantly reduced the lung edema in bleomycin-treated mice. Reported data are means ± SEM from at least five mice for each group in one representative experiment. $^{\circ}P < 0.01$ versus SHAM, $^{*}P < 0.01$ versus BLEO.

in BLEO there was a significant increase of macrophages, lymphocytes, neutrophils, and eosinophils, and instead all cell types, but lymphocytes, were significantly reduced in $dmPGE_2$ co-treated mice, compared with BLEO. Similar results were obtained in the case of the delayed treatment with $dmPGE_2$.

MPO Activity and Lung Edema

The bleomycin-induced inflammatory response after 1 week was also characterized by the huge increase of MPO activity, as an indicator of polymorphonuclear leukocyte (PMN) infiltration and a substantial lung edema, as shown in Figures 1E and 1F, respectively. On the contrary, both $dmPGE_2$ co- and post-administration in BLEO mice significantly reduced both MPO activity and lung fluid content.

IL-1β and TNF-α Immunostaining

As shown in Figures 2B and 2B1, immunohistochemical analysis revealed a positive staining for IL-1β mostly in inflammatory cell infiltrate present in the interstitium and in the airspace (i.e., alveolar macrophages), but also in the vascular zone (i.e., vascular endothelium) in the lungs of BLEO mice after 1 week of treatment. By contrast, a very weak staining for IL-1β was found in the lungs of $dmPGE_2$–co-treated animals as well as of mice that received $dmPGE_2$ delayed treatment (Figures 2C and 2D, respectively). The pertinent densitometric analysis shown in the *upper right panel* of Figure 2 demonstrated this significant inhibition of bleomycin-induced IL-1β overexpression.

Similarly, a strong TNF-α staining of infiltrating cells was evident in the lung of BLEO mice (Figures 3B and 3B1). This

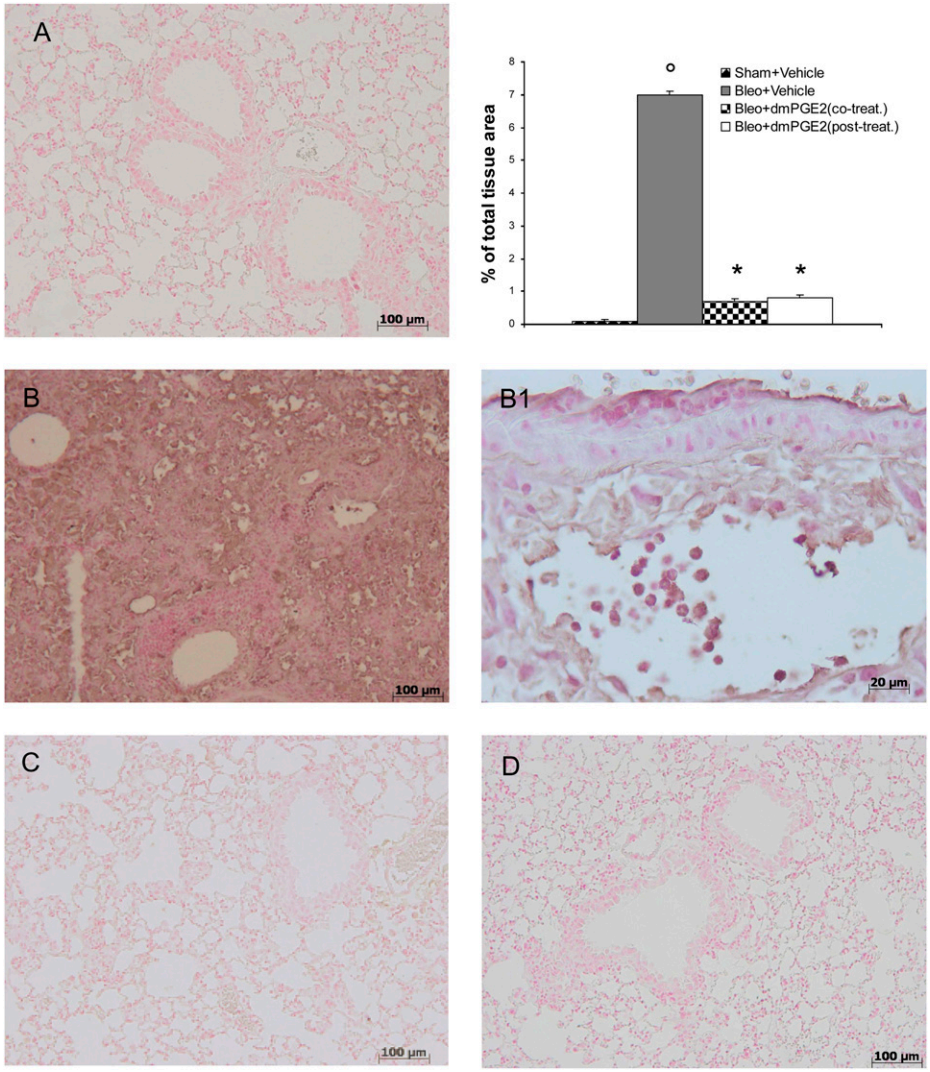

Figure 2. Immunohistochemical localization of IL-1β in lung sections. Effects of dmPGE$_2$ administration on bleomycin-induced IL-1β expression. Immunohistochemistry microphotographs (×150) of lung sections of (*A*) sham-operated mice show no IL-1β staining, whereas in *B* and, in particular, *B1* (×750), you can observe a robust IL-1β staining mostly in inflammatory cell infiltrate present in the interstitium and in the airspace as well as in the vascular zone after 1 week of bleomycin administration. By contrast, you can observe a very weak IL-1β staining in the lung sections of dmPGE$_2$ co-treated animals as well as of mice that received dmPGE$_2$ delayed treatment (*C* and *D*, respectively). Results of the densitometric analysis are shown in the *upper right panel*. As indicated, the *shaded bar* represents values of the bleomycin-treated group, the *checkered bar* represents values of dmPGE$_2$ co-treated animals, and the *open bar* represents values of animals which received the dmPGE$_2$ treatment 3 days after bleomycin administration. Reported data are mean values ± SEM from at least five mice for each group in one representative experiment. °$P < 0.01$ versus sham group, *$P < 0.01$ versus bleomycin-treated group.

effect was significantly abrogated in both dmPGE$_2$ co- and post-treated animals (Figures 3C and 3D, respectively, and Figure 3 *upper right panel*).

N-tyr Immunostaining

MPO employs hydrogen peroxide derived by dismutation of superoxide to produce hypochlorous acid, a compound with relevant antibacterial properties (21). However, superoxide can react with nitric oxide to generate highly reactive metabolites such as peroxynitrite. This compound is able to oxidize proteins, resulting in direct nitration of tyrosine residues. Protein structure and function can be subsequently altered and enzymatic activity affected. Proteins containing n-tyr residues have been previously detected in the lungs of bleomycin-treated animals. As shown in Figure 4, the n-tyr staining in the lung of BLEO animals (Figure 4B and 4B1) after 1 week of treatment was significantly reduced in the case of both dmPGE$_2$ co- and post-treated mice (Figures 4C and 4D, respectively, and Figure 4 *upper right panel*).

Histologic Examination

As shown in Figure 5B, after 7 days of bleomycin treatment histologic examination of the mice lungs revealed: (*1*) an extensive inflammatory infiltration by monocytes/macrophages, neutrophils, lymphocyte, and plasma cells extending through the lung epithelium; (*2*) granulomas in perivascular region; and (*3*) abundant tissue damage. By contrast, dmPGE$_2$ co-treatment prevented both inflammatory response and tissue damage (Figure 5C). Interestingly, delayed treatment with dmPGE$_2$ also was able to abrogate bleomycin-induced inflammatory infiltration and lung damage (Figure 5D). Moreover, as shown in Figure 6, 3 weeks after bleomycin administration the abundant extracellular matrix (ECM) deposition in the lungs of BLEO mice (Figures 6B and 6B1) was substantially reduced in the lungs of dmPGE$_2$ co-treated mice (Figures 6C and 6C1). Accordingly, as shown in the *upper right panel* of Figure 6, histologic scoring of lung fibrosis in BLEO mice after 3 weeks of bleomycin administration show a severe fibrosis reaction, which was significantly reduced in animals co-treated with dmPGE$_2$ ($7{,}0 \pm 0.5$ versus 1.8 ± 0.3, $P < 0.01$).

Collagen Content in the Lung

In accord with histologic data, by measuring the total lung collagen content at 3 weeks after bleomycin treatment we observed a significant reduction of collagen deposition in dmPGE$_2$ co-treated mice compared with BLEO mice, as shown in Table 1.

Evaluation of Body Weight and Mortality

As shown in Figure 7, the severe lung injury caused by bleomycin administration was associated with a significant loss of body weight and decrease of survival rate (40% of

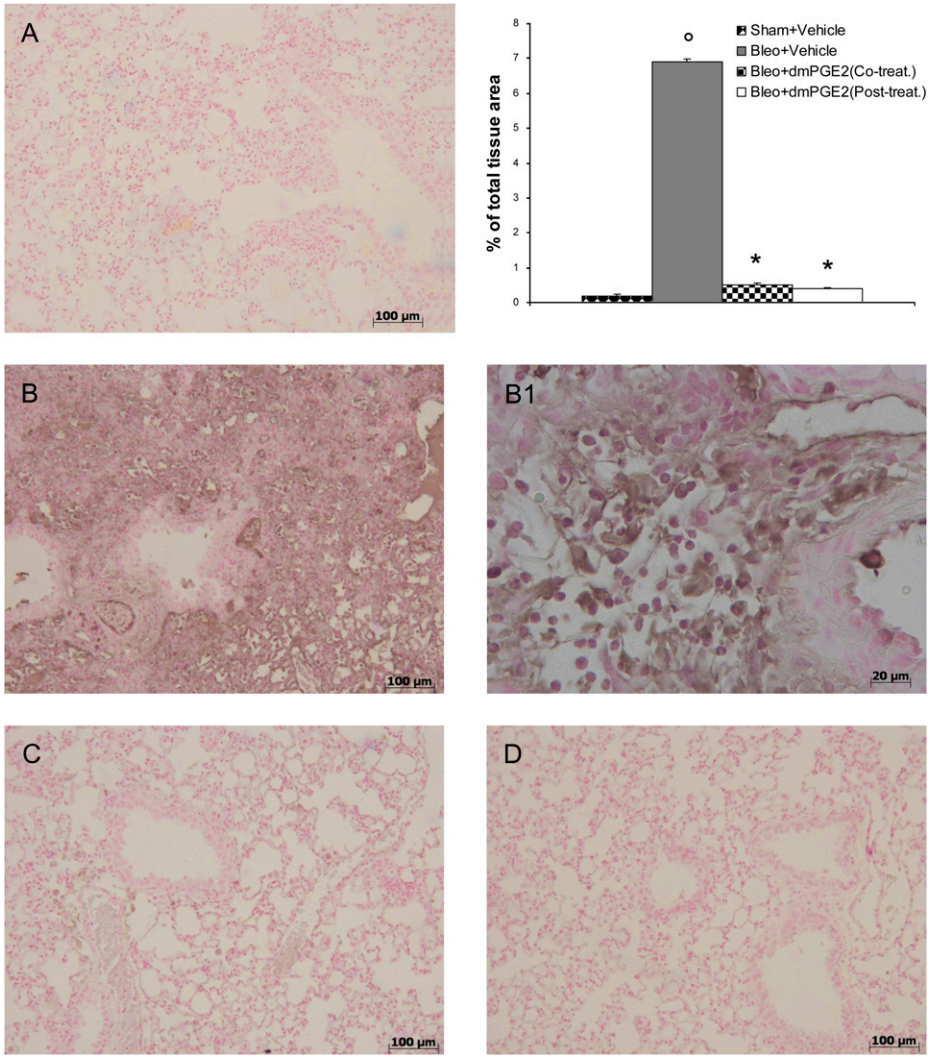

Figure 3. Effects of $dmPGE_2$ administration on TNF-α expression after 1 week of bleomycin treatment. Immunohistochemistry microphotographs of lung sections show that also TNF-α expression, absent in sham-operated mice (*A*), was instead induced mainly in infiltrating inflammatory cells and in bronchial epithelium of bleomycin-treated mice (*B* and in particular *B1*). On the other hand, one can observe a marked reduction of TNF-α immunostaining in lung sections from $dmPGE_2$–treated animals (*C*) as well as from mice which received the delayed $dmPGE_2$ treatment (*D*). Results of the densitometric analysis are shown in the *upper right panel*. As indicated, the *shaded bar* represents values of bleomycin-treated group, the *checkered bar* represents values of $dmPGE_2$ co-treated animals, and the *open bar* represents values of animals that received the $dmPGE_2$ treatment 3 days after bleomycin administration. Reported data are mean values ± SEM from at least five mice for each group in one representative experiment. $^{\circ}P < 0.01$ versus sham group, $^{*}P < 0.01$ versus bleomycin-treated group.

bleomycin-treated animals died within 1 week). On the contrary, $dmPGE_2$ co-administration prevented the loss of body weight. Moreover, none of the $dmPGE_2$ co-treated animals died within the first week, whereas their mortality rate at 3 weeks was significantly lower than that of bleomycin-treated mice. Interestingly, also delayed treatment with $dmPGE_2$ was able to revert the body weight loss and the increased mortality induced by bleomycin.

DISCUSSION

Common pathologic features of interstitial lung diseases including idiopathic pulmonary fibrosis (IPF) comprise fibrosis of the interstitium involving collagen, elastic and smooth muscle elements, architectural remodeling, and chronic inflammation (22). In this respect, cytokines, growth factors, and other relevant proteins have been thoroughly characterized, yet a growing body of evidence also support the role of lipid mediators in the pathogenesis of this condition. In particular, metabolites of arachidonic acid such as prostaglandins and leukotrienes do participate in the control of lung inflammation and reparative processes with apparently opposite effects. While a pathogenic role has been attributed to leukotrienes, compelling evidence shows that COX-2–derived metabolites, and PGE_2 in particular, might exert a protective role in the lung (3), by limiting leukocytes accumulation and activation, as well as collagen synthesis and fibroblast activation (23). By contrast, fibrotic diseases such as IPF, are characterized by reduced COX-2 expression and PGE_2 synthesis in lung fibroblasts (5, 6). Moreover, reduced PGE_2 levels have been found in the bronchoalveolar lavage fluid (24) and in the conditioned medium of IPF-derived alveolar macrophages (25). Similarly, COX-2 expression in lung biopsies of patients with IPF was found reduced, even though some controversial results have been reported on the expression of this crucial enzyme (8, 26, 27). In this context, several interesting observations have been taken in the bleomycin model of lung fibrosis. PGE_2 levels were increased after bleomycin administration in mice (9, 10), likely in an attempt to modulate the reparative process after injury, and COX-2–deficient mice showed an increased susceptibility to bleomycin, depending on PGE_2 levels (9, 28). Interestingly, lipoxygenase-deficient mice were protected by decreased leukotriene levels and increased levels of PGE_2 (29).

In this study, we examined the effects of $dmPGE_2$ administration in the bleomycin mouse model of lung fibrosis to assess therapeutic potentialities of prostanoids in the fibrosis setting. We chose the synthetic PGE_2 analog $dmPGE_2$, characterized by the resistance to prostaglandin 15-dehydrogenase and thus by a prolonged half-life *in vivo* (30), which was previously shown to decrease the collagen formation in fibrotic rat liver (31).

By immunohistochemical analysis, we investigated the effects on expression of TNF-α and IL-1β, two proinflammatory

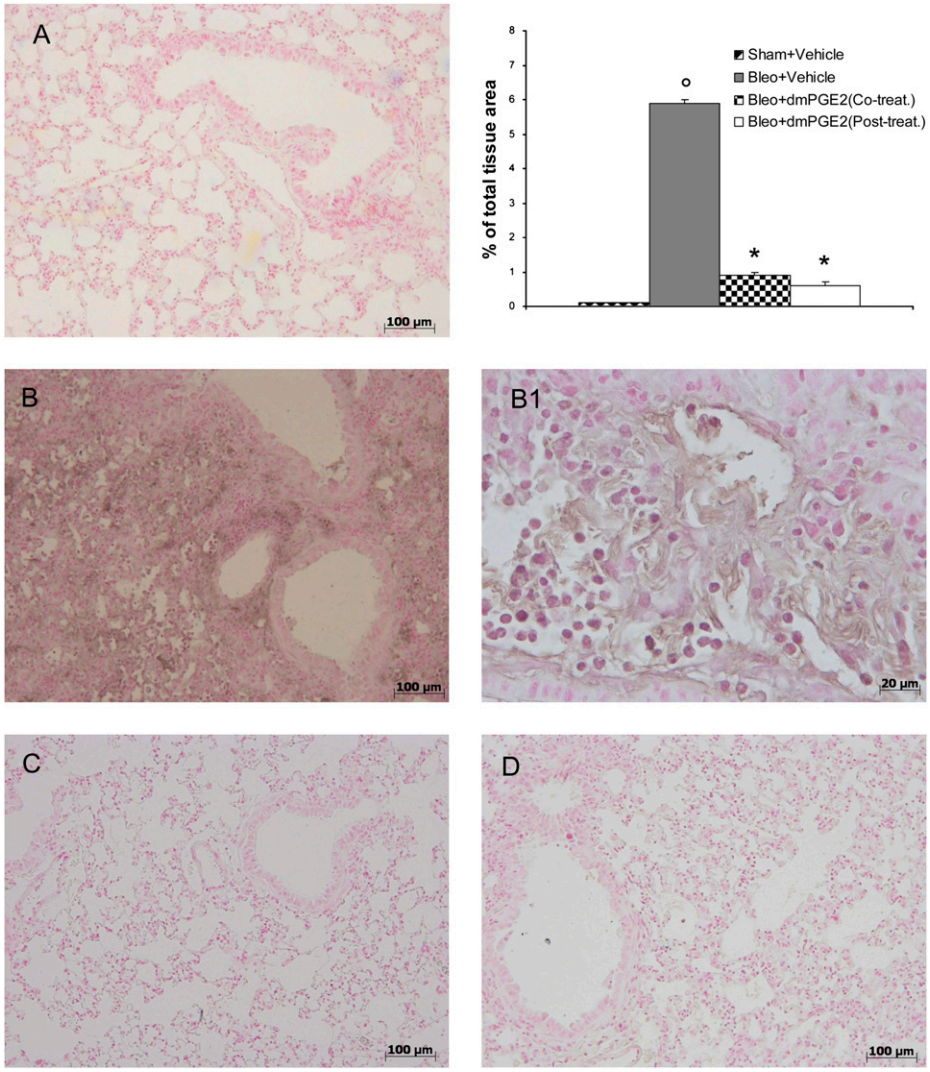

Figure 4. Effects of dmPGE$_2$ administration on oxidative damage in lungs after 1 week of bleomycin treatment. The presence of n-tyrosine (n-tyr) residues in lung proteins accounts for an excessive activity of MPO with consequent oxidative tissue damage. No detection of n-tyr residues is observable in immunohistochemistry microphotographs of lung sections of sham-operated mice (*A*), whereas in microphotographs of bleomycin-treated mice one can see a strong n-tyr staining mainly in inflammatory infiltrate present in the interstitium but also in the alveolar pneumocyte layer (*B* and in particular *B1*). By contrast, a faint n-tyr staining is observable in the lungs of dmPGE$_2$ co-treated mice (*C*) as well as of animals that received delayed dmPGE$_2$ treatment (*D*). Results of the densitometric analysis are shown in the *upper right panel*. As indicated, the *shaded bar* represents values of the bleomycin-treated group, the *checkered bar* represents values of dmPGE$_2$ co-treated animals, and the *open bar* represents values of animals that received the dmPGE$_2$ treatment 3 days after bleomycin administration. Reported data are mean values $\pm$ SEM from at least five mice for each group in one representative experiment. $^{\circ}P <$ 0.01 versus sham group, $^{*}P < 0.01$ versus bleomycin-treated group.

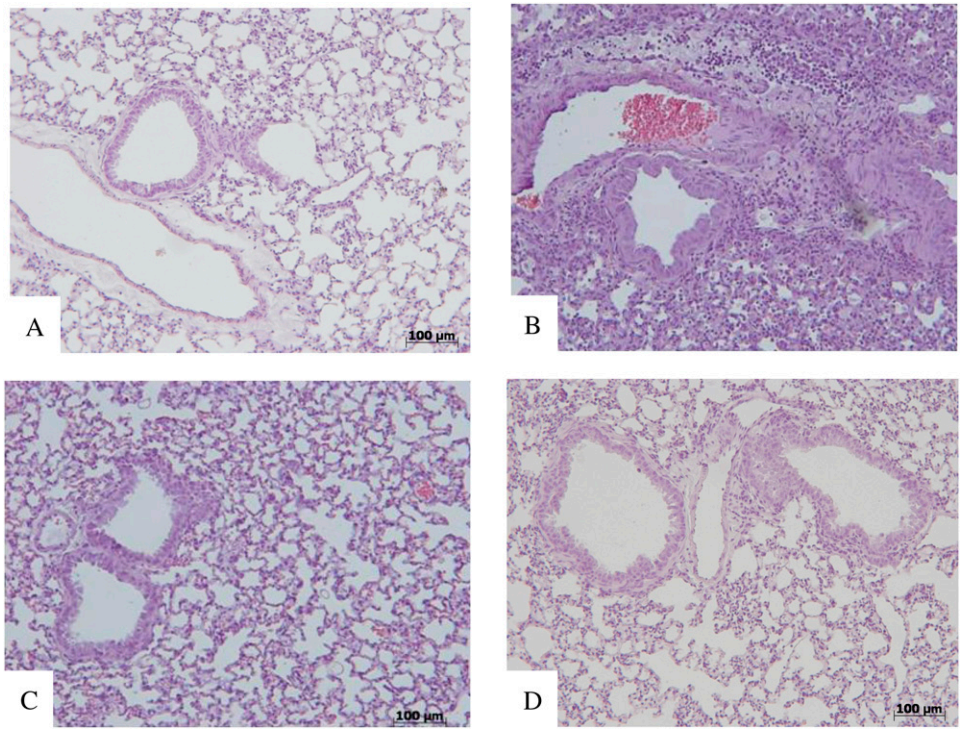

Figure 5. Effect of dmPGE$_2$ on bleomycin-induced inflammatory infiltration and lung histology. At 1 week after treatments, microphotographs ($\times$150) of hematoxylin/eosin-stained lung sections of (*A*) sham-operated animals, (*B*) BLEO (bleomycin-treated mice), (*C*) dmPGE$_2$ co-treated BLEO mice, and (*D*) BLEO mice treated with dmPGE$_2$ 3 days after bleomycin administration show that the robust leukocyte infiltration induced by bleomycin administration was quite abrogated by both dmPGE$_2$ co- and post-treatment. On the other hand, the initial damage to lung architecture observable in sections of BLEO mice was absent in dmPGE$_2$-treated mice.

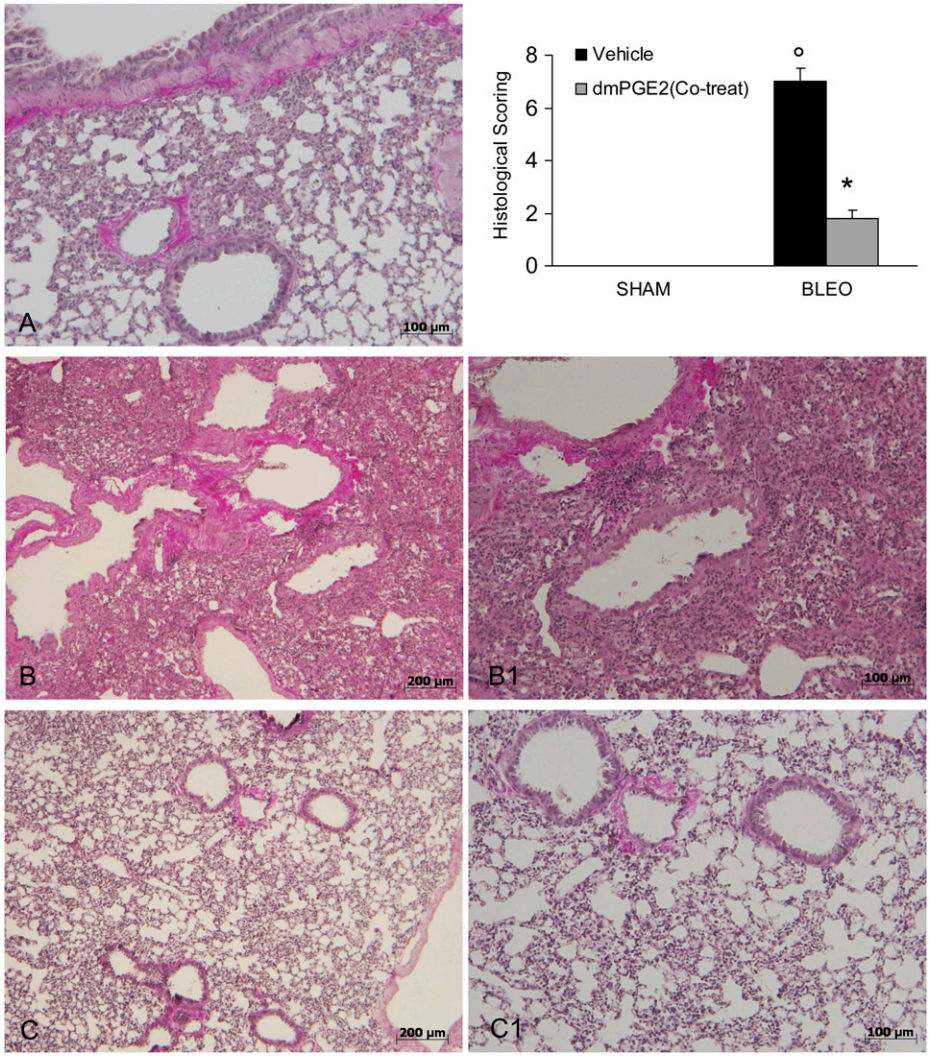

Figure 6. Effect of $dmPGE_2$ administration on bleomycin-induced matrix deposition and fibrosis. At 3 weeks after treatments, lung sections were subjected to the van Gieson stain for collagen. This stain shows collagen in *purple*. Microphotographs (×150) of sections from (*A*) sham-operated animals, (*B* and *B1*) BLEO (bleomycin-treated mice), and (*C* and *C1*) $dmPGE_2$ co-treated animals show that abundant presence of collagen, alveolar thickening, and severe distortion of lung structures observable in lung sections from BLEO was substantially reduced in $dmPGE_2$-treated mice. Lung fibrosis as evaluated by Ashcroft criteria is reported in the *upper right panel*. As indicated, the *solid bar* represents values of bleomycin control group (BLEO), and the *shaded bar* represents values of $dmPGE_2$ co-treated animals. Data are means ± SEM from at least three evaluations of five mice for each group. $^{\circ}P < 0.01$ versus SHAM, $^{*}P < 0.01$ versus BLEO.

cytokines considered to play a fundamental role in fibrotic process and especially in lung fibrotic process observed in response to bleomycin. We show here that both $dmPGE_2$ co-treatment and delayed treatment were able to almost abrogate the strong induction of TNF-α expression in lung infiltrating cells observed in bleomycin-treated mice. It is worth noting that TNF-α can up-regulate the expression of the well-recognized profibrotic cytokine TGF-β1 (32), and that its blockade with either anti–TNF-α antibodies or TNF-α antagonists can inhibit fibrosis (33). We have previously shown that fibroblast-derived PGE_2 is able to down-regulate the TNF-α release from activated monocytes, through the inhibition of the NF-κB activity (34), and that this anti-inflammatory effect is reduced in lung fibroblast derived from patients with IPF (5).

TABLE 1. TOTAL LUNG COLLAGEN DETERMINED BY THE SIRCOL ASSAY AT 3 WK AFTER TREATMENTS

	SHAM	BLEO	BLEO+ $dmPGE_2$
Collagen, μg	3.46 ± 0.96	17.49 ± 3.73	10.47 ± 4.57

Definition of abbreviations: BLEO, bleomycin-treated mice; $dmPGE_2$, 16,16-dimethyl-PGE_2; SHAM, sham-operated mice.

Reported data are means ± SEM from at least five mice for each group in one representative experiment.

Moreover, in the present study we show that bleomicyn-induced IL-1β overexpression was almost abrogated in $dmPGE_2$-treated mice. This is a noteworthy finding considering that IL-1β is known to act synergistically with TNF-α in the pathogenesis of different fibrotic processes (35), and that its inhibition has been shown to prevent lung fibrosis caused by bleomycin (36) while its transient overexpression can induce lung injury and pulmonary fibrosis in the late stages of the same experimental setting (37).

Furthermore, by cytologic analysis of BAL fluid, measurement of lung edema, MPO activity assay, and immunohistochemical analysis for nitrotyrosine, the $dmPGE_2$ effects on other relevant inflammatory parameters were also evaluated in this study.

Results demonstrate that in our experimental model, $dmPGE_2$ administration was able to prevent and strongly protect from the inflammatory response induced by intratracheal administration of bleomycin. Furthermore, as assessed by Ashcroft histologic scoring of fibrosis and collagen Sircol assay, we show that the bleomycin-induced fibrotic process, in terms of abundant extracellular matrix deposition in the lung and disruption of the alveolar architecture, was significantly reduced in $dmPGE_2$-treated mice, indicating that $dmPGE_2$ administration effectively prevented the bleomycin-induced lung damage and its subsequent altered repair as well as fibrotic process. These pleiotropic effects of $dmPGE_2$ are probably mediated by

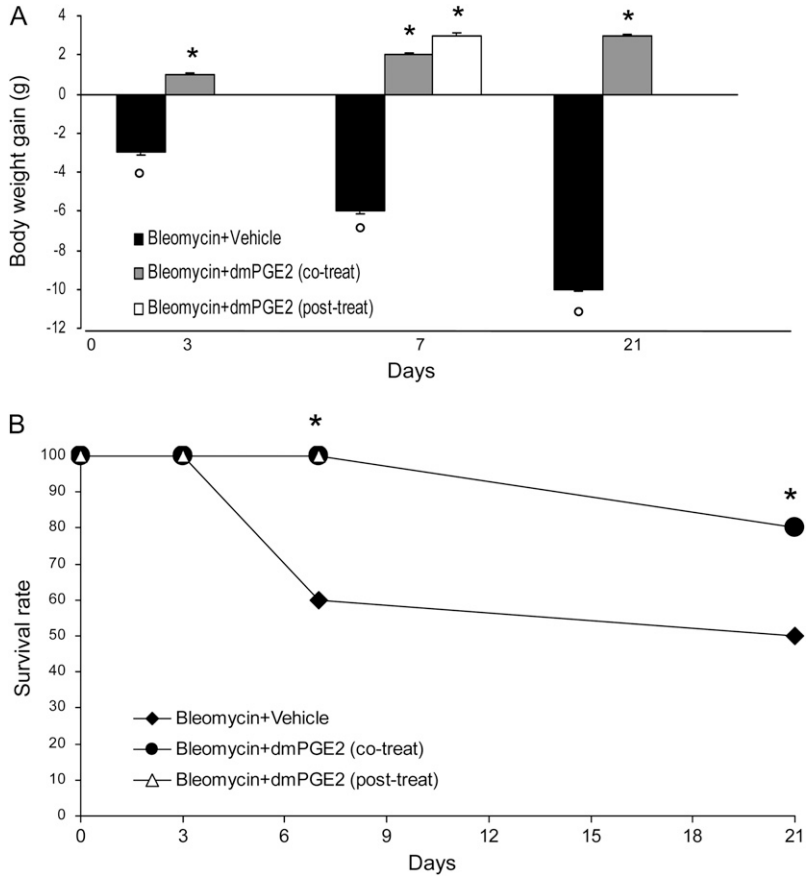

Figure 7. Effect of dmPGE$_2$ administration on mice body weight and survival rate. (*A*) Body weight gain (g) with respect to the control. As indicated, the *solid bar* represents values of the bleomycin-treated group, the *shaded bar* represents values of dmPGE$_2$ co-treated animals, and the *open bar* represents values of animals that received the dmPGE$_2$ treatment 3 days after bleomycin administration. Data are mean values ± SEM from at least five mice for each group in one representative experiment. °$P < 0.01$ versus sham group, *$P < 0.01$ versus bleomycin-treated group. (*B*) Survival rates. As indicated, *solid squares* represent values of the bleomycin group, *solid circles* represent values of dmPGE$_2$ co-treated animals, and *open triangles* represent values of animals that received the dmPGE$_2$ treatment 3 days after bleomycin administration. Data are means ± SEM from 10 mice for each group in one representative experiment. *$P < 0.01$ versus bleomycin.

different EPs in different cell types such as fibroblasts, macrophages, and epithelial cells. Since dmPGE$_2$ is a nonspecific EP receptor agonist, being EP2, EP3, and EP4 responsive (38, 39), and bearing in mind that EP2 and EP4 receptors are considered to be responsible for the inhibitory effects of PGE$_2$ on fibroblast activation and transformation into myofibroblast and on collagen production (12, 13), we can speculate that dmPGE$_2$ mechanistically acts via both these receptors.

On the other hand, there is evidence that after bleomycin administration the EP2 expression in pulmonary fibroblast is significantly altered (14). This fact may explain, at least in the mouse model, the refractoriness of lung fibroblasts to the increased PGE$_2$ production as well as the insufficient control of the reparative process after the lung injury. Further experiments in mice with knockouts for specific EP could help to elucidate mechanisms by which dmPGE$_2$ reverse the bleomycin-induced alterations of physiologic PGE$_2$ pathways.

Last but not least, we report here that dmPGE$_2$ treatment prevents and reverts loss of body weight caused by bleomycin administration, as well as significantly enhances survival rate of bleomycin-treated mice.

In summary, we provide the first evidence for the beneficial effects of exogenous administration of dmPGE$_2$ in bleomycin-induced lung damage. In fact, inflammation, acute lung injury, and fibrosis associated with the intratracheal administration of bleomycin were not only prevented by dmPGE$_2$ co-treatment but also significantly reduced by dmPGE$_2$ delayed treatment. Our data further support the thought about the PGE$_2$ protective role in fibroproliferative disorders of the lung. Further investigation on PGE$_2$ analogs available for human use and on their potential role in regulation of reparative processes in the lung might disclose new therapeutic options for the treatment of pulmonary fibrosis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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