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GLYCOGEN SYNTHASE KINASE-3β INHIBITION ATTENUATES THE DEVELOPMENT OF BLEOMYCIN-INDUCED LUNG INJURY

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Glycogen synthase kinase-3 (GSK-3) is an ubiquitous serine-threonine protein kinase that participates in a multitude of cellular processes and has recently been implicated in the pathophysiology of a number of diseases. The aim of this study is to investigate the effects of TDZD-8, a potent and selective GSK-3β inhibitor, on the development of lung injury caused by administration of bleomycin (BLM). Mice subjected to intra-tracheal administration of BLM developed significant lung injury characterized by marked neutrophil infiltration and tissue edema. An increase in immunoreactivity to nitrotyrosine, iNOS, TNF-α and IL-1β was also observed in the lungs of BLM-treated mice. In contrast, administration of BLM-treated mice with TDZD-8 (1 mg/kg daily) significantly reduced (I) the degree of lung injury, (II) the increase in staining (immunohistochemistry) for myeloperoxidase (MPO), nitrotyrosine, iNOS, TNF-α and IL-1β and (III) the degree of apoptosis, as evaluated by Bax and Bcl-2 immunoreactivity and TUNEL staining. Taken together, these results clearly demonstrate treatment with the GSK-3β inhibitor TDZD-8 reduces the development of lung injury and inflammation induced by BLM in mice.

Idiopathic pulmonary fibrosis is a progressive interstitial lung disease of unknown etiology. The disease, which can cause significant morbidity and mortality (1), most commonly affects middle-aged adults, although infants and children are also affected. It is characterized by the excessive deposition of extracellular matrix in the lung interstitium (2). The pathophysiological features of inflammation and fibrosis are well appreciated but little is known about its etiology and pathogenesis (3). To date, there is no satisfactory treatment of idiopathic pulmonary fibrosis. Various anti-inflammatory agents such as corticosteroids (4), colchicines (5), and cytotoxic agents such as azathioprin (6) and cyclophosphamide (7) have been used alone or in combination to treat the disease. However, less than one-third of patients respond to treatment with corticosteroids and/or cytotoxic therapy. In view of the poor outcomes and

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therapeutic options available in idiopathic pulmonary fibrosis and other fibrotic lung diseases, there is an urgent need for novel therapeutic alternatives (8).

Glycogen synthase kinase (GSK)-3 was first identified in 1980 as an ubiquitous serine-threonine protein kinase involved in glycogen metabolism (9). It has since been implicated in a multitude of cellular processes, ranging from cell membrane-to-nucleus signaling, gene transcription, translation, and cytoskeletal organization to cell cycle progression and survival (10). Two isoforms have been isolated in mammals, GSK-3α and GSK-3β. GSK-3 is constitutively active in cells, although phosphorylation of a specific serine residue (Ser21 in GSK-3α and Ser9 in GSK-3β) located in its N-terminal domain inhibits GSK-3 activity and, hence, reduces its activity to alter cell function (10). A wide variety of extracellular stimuli, including insulin, epidermal growth factor, and fibroblast growth factor, exert their effects by inhibiting GSK-3 activity (11). Unique to GSK-3β is its reported ability to influence the activity of the transcription factor nuclear factor (NF)-κB (12). This concept was based on the findings that GSK-3β knockout mice showed a similar phenotype to mice in which the gene for p65 or IκB kinase 2 (and hence, NF-κB activation) had been deleted (13). In both cases, disruption of the p65 gene and disruption of the murine GSK-3β gene resulted in embryonic lethality caused by severe liver degeneration (13). The regulatory influence of GSK-3β on the activity of NF-κB, which has since been confirmed in a range of systems (14-15), is the basis for the hypothesis that GSK-3β may play a key role in the regulation of the inflammatory response.

Recent *in vivo* studies have demonstrated that TDZD-8 and SB 415286, potent selective inhibitors of GSK-3β, reduced the renal and liver dysfunction caused by both endotoxaemia and administration of endotoxin and peptidoglycan (16). In this study, it was proposed that the protective effect of the GSK-3β inhibitors was due to inhibition of the phosphorylation of Ser536 on p65. In agreement with these findings, it was recently reported that GSK-3β deficient mice challenged with LPS and administered with GSK-3β inhibitor reduced mortality and pro-inflammatory cytokine production (17). In addition, there is also evidence that TDZD-8 reduces the development of colon injury associated with experimental colitis (18) and development of arthritis (19) as well as modulating secondary damage following spinal cord injury (20). Thus, inhibitors of the activity of GSK-3β may be useful in the treatment of a variety of inflammatory diseases.

Here we explore the possible role of GSK-3β in the modulation of pulmonary fibrosis. Intra-tracheal instillation of the anti-tumor agent bleomycin (BLM) is the most commonly used animal model for pulmonary fibrosis (21). Using this well-established mouse model, we investigated the effects of the selective GSK-3β inhibitor TDZD-8 on: (i) lung injury, (ii) infiltration of PMNs in the lung, (iii) staining (immunohistochemistry) for MPO, (iv) iNOS expression and nitration of tyrosine residues in the lung, (v) TNF-α and IL-1β levels, (vi) apoptosis (TUNEL and Bax and Bcl-2 expression), and (vii) weight loss and mortality.

MATERIALS AND METHODS

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). TDZD-8 was obtained from Calbiochem (Merck Biosciences Ltd, Beeston, Nottingham, U.K.) All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, UK). Mini-osmotic pumps, Model 2001, were obtained from Alzet (Charles River, Milan, Italy).

Male CD mice (25-35 g; Harlan Nossan, 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).

Experimental groups

Mice were randomized into four experimental groups. Sham animals were subjected to the identical surgical procedure, but received intra-tracheal instillation of saline (0.9%) instead of BLM, and were treated with either vehicle (32% dimethylsulfoxide (DMSO) 1 ml/kg/day) or TDZD-8 (1 mg/kg/day) via a mini-osmotic pump. The remaining mice were subjected to BLM-induced lung injury and treated via mini-osmotic pumps with either vehicle (32% DMSO 1 ml/kg/day) or TDZD-8 (1 mg/kg/day).

In the first study, the mice were sacrificed after 7 days for analyses of injury, inflammation and apoptosis, N=10/

group. In the second study, the mice were observed for changes in body weight and survival rate over a period of 15 days, N=20/group.

Mini-osmotic pumps and implantation

Alzet pumps are precision drug administration tools. After a 4-6 h start-up transient, the Model 2001 pumps infuse at a constant rate that lies within a predictable range until all but about 5% of the reservoir contents have been delivered; then the rate falls rapidly to zero. The pumping rate was 1.0 µl/h (± 0.15 µl/h) over a period of 7 days. The reservoir volume was 200 µl.

The Alzet mini-osmotic pump was implanted subcutaneously in the mice. A small incision was made in the skin between the scapulae. Using a hemostat, a small pocket was formed by spreading the subcutaneous connective tissues apart. The pump was inserted into the pocket with the flow moderator pointing away from the incision. The skin incision was closed with sutures.

Induction of lung injury by BLM

Mice received, via a catheter (tracheotomy tube), a single intra-tracheal instillation of saline (0.9%) or saline containing BLM sulfate (0.1U/mouse) at end-expiration in a volume of 100 µl, and the liquid was followed immediately by 300 µl of air to ensure delivery to the distal airways and were killed after 7 days by pentobarbitone overdose.

Measurement of fluid content in lung

The wet lung weight was measured 7 days after BLM administration by careful excision of the lung from adjacent extraneous tissues. The lung was exposed for 48 h at 180°C and the dry weight was measured. Water content was calculated by subtracting dry weight from wet weight.

Histological examination

Lung biopsies were taken 7 days after injection of BLM. Lung biopsies were fixed for 1 week in 10% (w/v) phosphate buffered saline (PBS)-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Sections were then deparaffinized with xylene, stained with hematoxylin and eosin and van Gieson's solution.

All sections were studied using light microscopy (Dialux 22 Leitz, Ziess, Milan, Italy). The severity of fibrosis was semi-quantitatively assessed according to the method proposed by Ashcroft and co-workers (22). Briefly, the grade of lung fibrosis was scored on a scale from 0 to 8 by examining randomly chosen sections, with fields per sample at a magnification of x100. 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.

Immunohistochemical localisation of TNF-α, IL-1β, INOS, nitrotyrosine, MPO, Bax and Bcl-2.

At the end of the experiment, tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 µm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with: 1) anti-TNF-α polyclonal antibody (1:500 in PBS, v/v), or anti-IL-1β polyclonal antibody (1:500 in PBS, v/v); 2) anti-MPO polyclonal antibody (1:500 in PBS, v/v); 3) anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v); 4) anti-iNOS antibody (1:500 in PBS, v/v); 5) anti-Bax antibody (1:500 in PBS, v/v); or 6) anti-Bcl-2 antibody (1:500 in PBS, v/v). Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). In order to confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for TNF-α, IL-1β, MPO, Bax and Bcl-2, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay

TUNEL assay was conducted using a TUNEL detection kit according to the manufacturer's instructions (Apotag, HRP kit DBA, Milano, Italy). Briefly, sections were incubated with 15 µg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections

were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

Myeloperoxidase (MPO) activity

MPO activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (23). MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37° C and was expressed in milliunits per g of wet tissue.

Measurement of cytokines

Lung sections collected 7 days after BLM administration were homogenized as previously described (24) in PBS containing 2 mmol/L of phenyl-methyl sulfonyl fluoride (Sigma Chemical Co., Milan, Italy) and tissue levels of TNFα and IL-1β were evaluated. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation, USA) according to the manufacturer's instructions. All cytokine determinations were performed in duplicate serial dilutions.

Statistical evaluation

All values in the figures and text are expressed as mean ± standard error (s.e.m.) of the mean of *n* observations. For the *in vivo* studies n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissue sections collected from all the animals in each group. Data sets were examined by one- or two-way analysis of variance, and individual group means were then compared with Student's unpaired t test. Statistical analysis for survival data was calculated by Fisher's exact probability test. The Mann-Whitney U test (two-tailed, independent) was used to compare medians of the body weight. A P value of less than 0.05 was considered statistically significant.

RESULTS

Effects of TDZD-8 on BLM-induced lung injury

When compared to lung sections taken from sham animals (Fig. 1 A, A1, D), histological examination of lung sections taken from mice treated with BLM revealed significant tissue damage characterized by extensive inflammatory infiltration by neutrophils, lymphocytes and plasma cells extending through the lung epithelial, fibrosis and granulomas in the perivascular region (Fig. 1 B, B1, D). Furthermore, administration of BLM elicited an acute inflammatory response characterized by the accumulation of fluid (edema) in lung tissues (Fig. 2a). Administration of TDZD-8 significantly attenuated the histological signs of BLM-induced lung injury (Fig .1 C, C1, D), as well as edema formation (Fig. 2a).

*TDZD-8 modulates production and expression of TNF-*A *and IL-1β after BLM administration*

To test whether TDZD-8 may modulate the inflammatory process through regulation of the secretion of cytokines, the lung levels of the pro-inflammatory cytokines TNF-α and IL-1β were analyzed. A substantial increase in TNF-α and IL-1β formation was observed in lung samples taken from mice 7 days after BLM administration (Fig. 2b and c, respectively). In contrast, there was a significant inhibition of TNF-α and IL-1β in animals administered BLM and treated with TDZD-8 (Fig. 2b and c, respectively). In addition, tissue sections obtained from BLM-treated animals demonstrate positive staining for TNF-α (Fig. 3 B) and IL-1-β (Fig. 3 B1), mainly localized in the infiltrated inflammatory cells in damaged tissues. In BLM mice treated with TDZD-8, the staining for TNF-α (Fig. 3 C) and IL-1-β (Fig. 3 C1) was visibly and significantly reduced when compared with BLM mice treated with vehicle. In the lungs of sham animals no positive staining was observed for TNF-α (Fig. 3 A) or IL-1-β (Fig. 3 A1).

Effects of TDZD-8 on BLM-induced iNOS expression and nitrotyrosine formation

No positive staining for iNOS was observed in the lung tissues obtained from sham animals (Fig. 3D). Immunohistochemical analysis of lung sections obtained from BLM-treated mice revealed positive staining for iNOS (Fig. 3E). In contrast, no staining for iNOS was found in the lungs of BLM-treated mice that had been treated with TDZD-8 (1 mg/kg/day) (Fig. 3F). Immunohistochemical analysis of lung sections obtained from mice treated with BLM

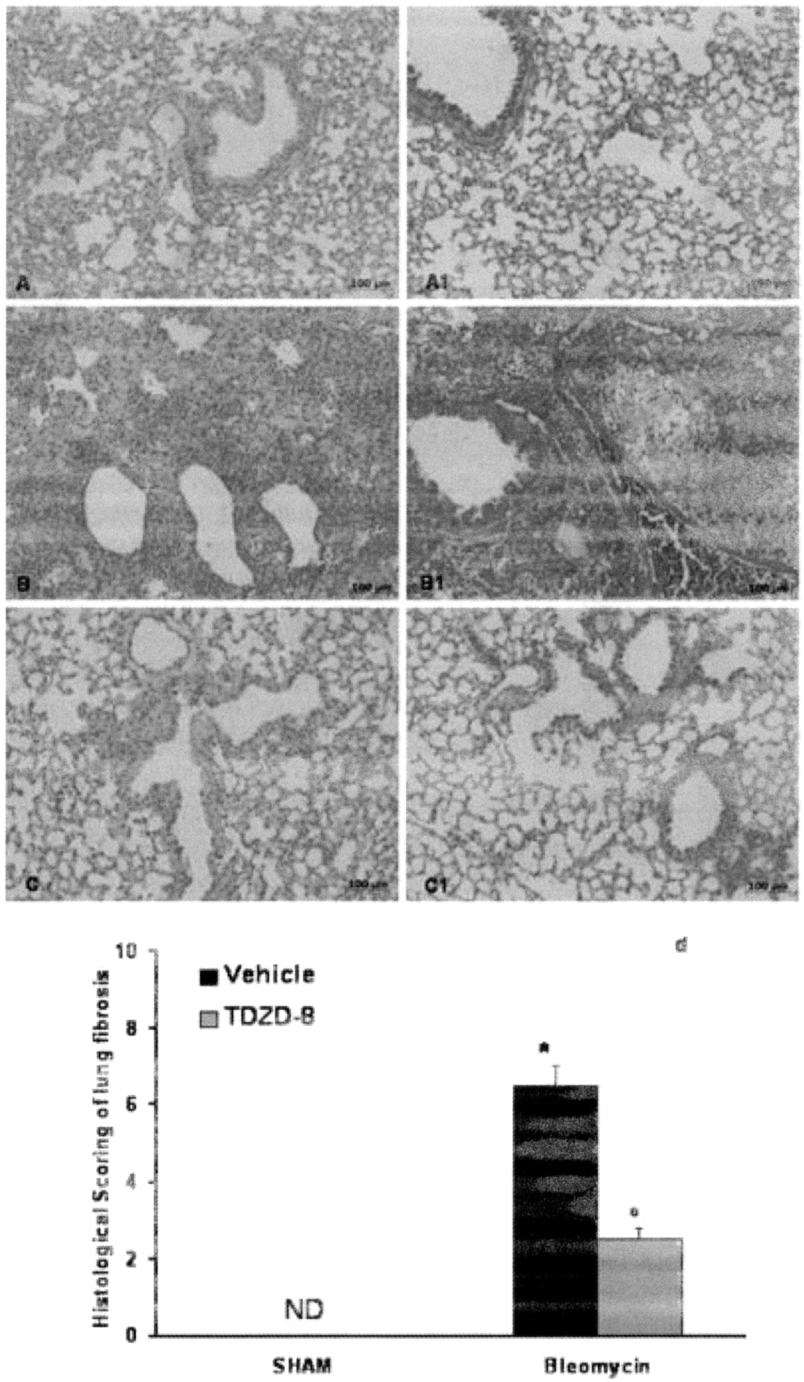

Fig. 1. *Effect of TDZD-8 on histological alterations of lung tissue 7 days after bleomycin-induced injury. Lung sections stained with haematoxylin and eosin (a to c) or trichrome (a1 to c1) taken from sham animals (a, a1) or mice treated with bleomycin and treated with either vehicle (32% DMSO; b, b1) or TDZD-8 (1 mg/kg/day; c, c1). Sections from sham animals revealed normal lung architecture (a, a1) whereas mice exposed to bleomycin exhibited significant lung injury characterized by extensive inflammation with inflammatory cell infiltration and fibrosis (b) and dense areas of collagen (b1). Treatment with TDZD-8 attenuated the disturbances in morphology associated with bleomycin administration (c, c1, d). Original magnification: x 250. The figure is representative of at least three experiments performed on different days. The macroscopic and histological scoring (d) was performed by two independent observers. Data are expressed as means ± SEM from n=10 mice for each group. *P<0.01 versus sham group. °P<0.01 versus bleomycin.*

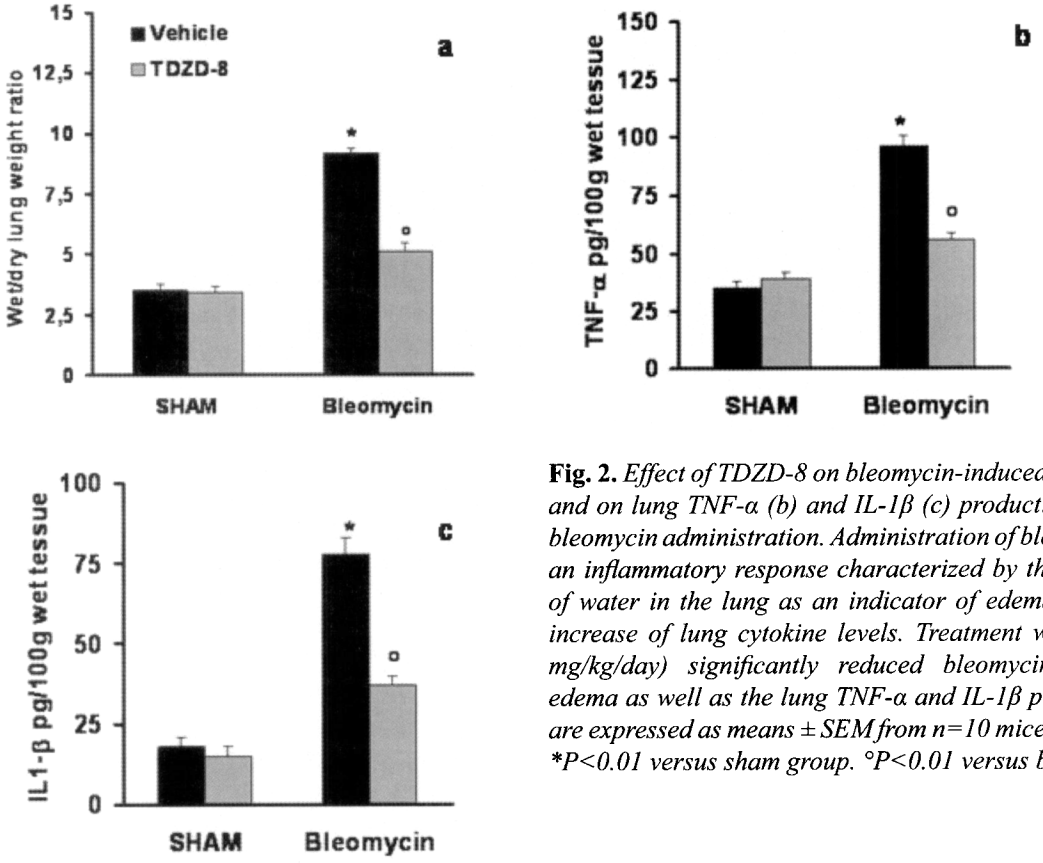

Fig. 2. *Effect of TDZD-8 on bleomycin-induced lung edema (a) and on lung TNF-α (b) and IL-1β (c) production 7 days after bleomycin administration. Administration of bleomycin elicited an inflammatory response characterized by the accumulation of water in the lung as an indicator of edema as well as an increase of lung cytokine levels. Treatment with TDZD-8 (1 mg/kg/day) significantly reduced bleomycin-induced lung edema as well as the lung TNF-α and IL-1β production. Data are expressed as means ± SEM from n=10 mice for each group. *P<0.01 versus sham group. °P<0.01 versus bleomycin.*

also revealed positive staining for nitrotyrosine (Fig. 3E1). In BLM mice treated with TDZD-8, positive staining for nitrotyrosine was significantly reduced (Fig. 3F1). In the lungs of sham animals no positive staining was observed for nitrotyrosine (Fig. 3D1).

Effects of TDZD-8 on neutrophil infiltration

The above histological pattern of lung injury appeared to be correlated with an influx of leukocytes into the lung tissue. Therefore, we investigated the role of TDZD-8 on neutrophil infiltration by measurement of the activity of MPO. No positive staining for MPO was found in lung tissues obtained from sham animals (Fig. 4A). In contrast, positive staining for MPO was observed along the bronchial epithelium and in inflammatory cells in tissue sections taken from BLM-treated mice (Fig. 4B). No staining for MPO was found in the lungs of mice administered BLM and treated with TDZD-8 (1 mg/kg/day) (Fig 4C). Indeed, the significant increase in MPO activity observed in mice administered BLM was abolished in animals treated with TDZD-8 (1 mg/kg/day) (Fig. 4D).

Effects of TDZD-8 on apoptosis in lung tissues after BLM-induced lung injury

To investigate whether acute lung inflammation was associated with cell death by apoptosis we measured TUNEL-like staining in lung tissues. At 7 days after BLM administration, lung tissues demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 4 F, F1,F2). In contrast, no apoptotic cells or fragments were observed in tissues obtained from BLM-mice treated with TDZD-8 (1 mg/kg/day) (Fig. 4G). Similarly, no apoptotic cells were observed in the lungs of sham-treated mice (Fig. 4E).

Immunohistochemistry for Bax and Bcl-2

Lung samples were also collected 7 days after BLM administration for immunohistological staining for Bax and Bcl-2. Lung sections taken

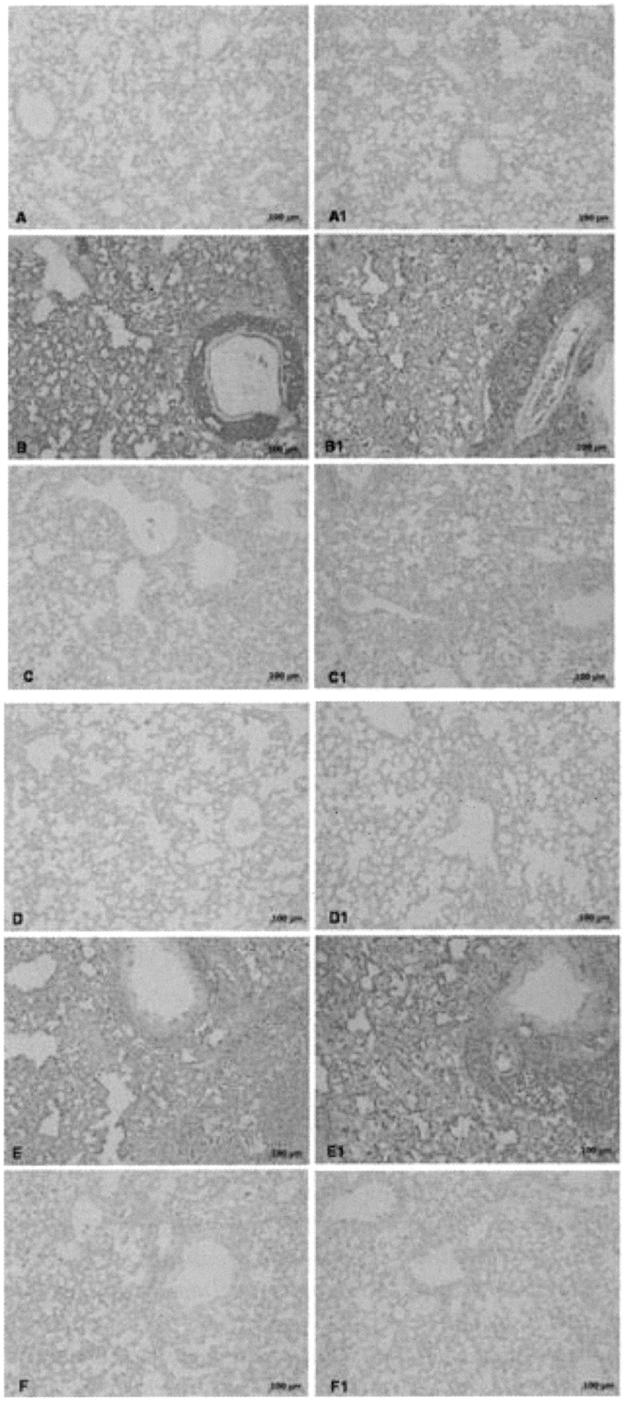

Fig. 3. *Immunohistochemical localization of TNF-α, IL-1β, iNOS and nitrotyrosine in the lung. No positive staining for TNF-α (a) or IL1-β (a1) was observed in lung sections taken from sham mice. Lung sections taken from mice administered bleomycin and treated with vehicle showed positive staining for TNF-α (b) and IL-1β (b1), localized mainly in inflammatory cells in the injured areas of tissue. The degree of positive staining for TNF-α (c) and IL-1-β (c1) was markedly reduced in tissue sections obtained from bleomycin mice treated with TDZD-8 (1 mg/kg/day). In addition, no positive staining for iNOS (d) or nitrotyrosine (d1) was observed in lung sections taken from sham mice. Lung sections taken from mice administered bleomycin and treated with vehicle showed positive staining for iNOS (e) and nitrotyrosine (e1), localized mainly in inflammatory cells in the injured areas of tissue. The degree of positive staining for iNOS (f) and nitrotyrosine (f1) was markedly reduced in tissue sections obtained from bleomycin mice treated with TDZD-8 (1 mg/kg/day). The figure is representative of at least 3 experiments performed on different days.*

from sham mice did not stain for Bax (Fig. 5 A) whereas the tissue obtained from BLM-treated mice exhibited positive staining for Bax (Fig. 5 B). TDZD-8 treatment reduced the degree of positive staining for Bax in mice subjected to BLM-induced lung injury (Fig. 5 C).

Lung tissue extracted from the sham mice demonstrated positive staining for Bcl-2 (Fig. 5A1), while in BLM-treated mice staining for Bcl-2 was absent (Fig. 5 B1). TDZD-8 treatment significantly attenuated the loss of positive staining for Bcl-2 in the lung of mice subjected to BLM-induced injury (Fig. 5 C1).

Effects of TDZD-8 *on changes in body weight and survival rate*

In BLM-treated mice, the severe lung injury caused by BLM administration was associated with a significant loss in body weight (Fig. 5D). The survival of animals was monitored for 15 days. BLM-treated mice, developed severe lung injury and over 70% of these animals died within 15 days of BLM administration (Fig. 5E). Treatment of mice with TDZD-8 resulted in a significant decrease in body weight (Fig. 5D) as well as mortality rate (Fig. 5E).

DISCUSSION

The inhibition of GSK-3β activity is believed to be widely beneficial, for example in the treatment of neurodegenerative diseases, diabetes type II, bipolar disorders, stroke, cancer, sepsis, and chronic inflammatory disease. In this report we demonstrate that pharmacological inhibition of GSK-3β exerts

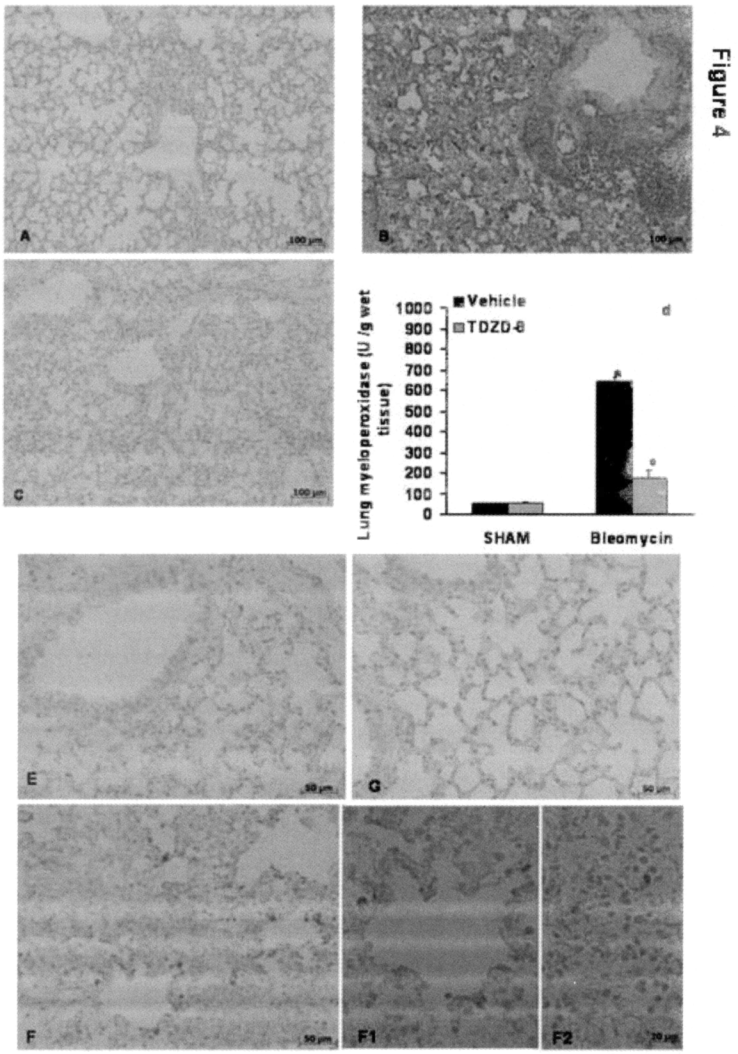

Figure 4

Fig. 4. *Effects of TDZD-8 on myeloperoxidase (MPO) formation and activity in the lung and on bleomycin-induced apoptosis as measured by TUNEL-like staining. No positive staining for MPO (a) was found in the lung sections taken from sham mice. Lung sections taken from mice administered bleomycin and treated with vehicle showed positive staining for MPO (b), mainly localized in the inflammatory cells. No positive staining for MPO was observed in tissue sections taken from bleomycin mice treated with TDZD-8 (1 mg/kg/day) (c). In addition, MPO activity was significantly increased in the lungs of bleomycin-treated mice in comparison with the sham-operated mice (d). Treatment with TDZD-8 significantly reduced the bleomycin-induced increase in MPO activity (d). In addition, positive Tunnel staining was observed in lung sections taken from bleomycin mice pre-treated with vehicle (f, f1, f2). In contrast, tissues obtained from bleomycin mice pre-treated with 1 mg//kg/day TDZD-8 demonstrated no apoptotic cells or fragments (g). Almost no apoptotic cells were observed in lungs of sham mice (e). The figure is representative of at least 3 experiments performed on different days. Data are expressed as means ± SEM from n=10 mice for each group. *P<0.01 versus sham group. °P<0.01 versus bleomycin.*

beneficial effects in a mouse model of BLM-induced lung injury.

In this study we provide evidence that treatment with TDZD-8 attenuates BLM-induced: (i) weight loss and mortality; (ii) lung injury; (iii) infiltration of the lung with PMNs; (iv) positive staining (immunohistochemistry) for MPO; (v) iNOS expression and nitration of tyrosine residues in the lung; (vi) increase in TNF-α and IL-1β levels; and (vii) apoptosis (TUNEL and Bax and Bcl-

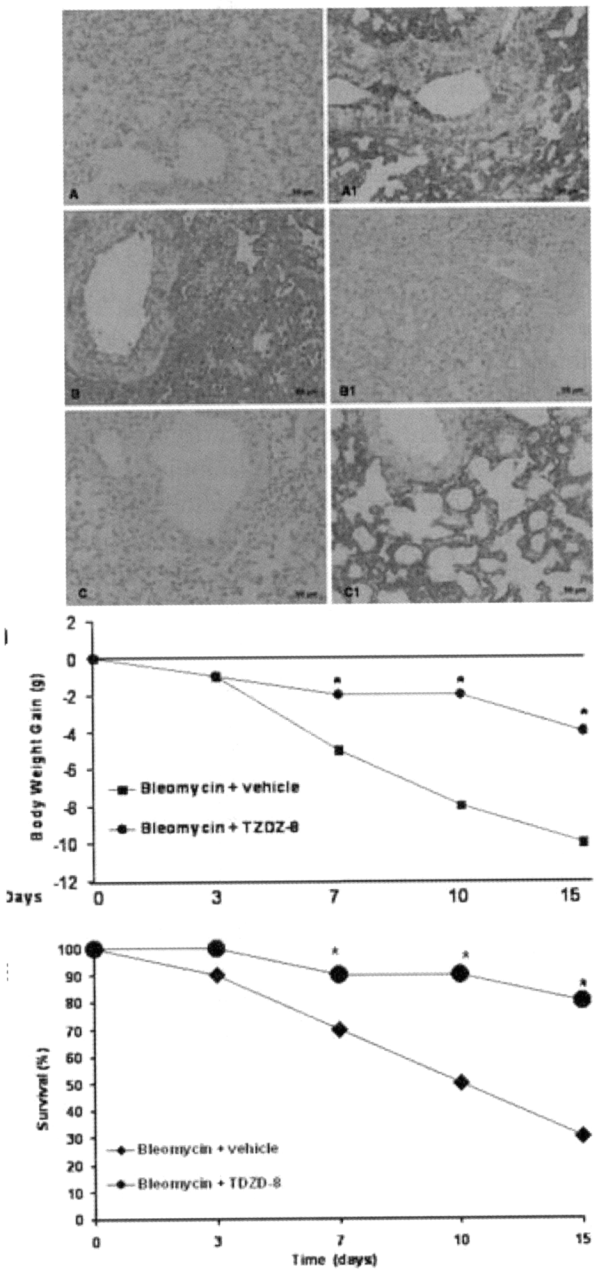

Fig. 5. *Effect of TDZD-8 on bleomycin-induced Bax and Bcl-2 expression in the lung and on body weight and bleomycin-induced mortality. No positive staining for Bax was observed in lung sections taken from sham mice (a). Lung sections taken from bleomycin mice treated with vehicle showed positive staining for Bax localized mainly in the inflammatory cells (b). The degree of positive staining for Bax was markedly reduced in the lungs of mice administered bleomycin and treated with 1 mg/kg/day TDZD-8 (c). Positive staining for Bcl-2 was observed in lung sections taken from sham mice (a1). The degree of positive staining for Bcl-2 was markedly reduced in lung sections obtained from bleomycin-mice treated with vehicle (b1). Treatment with TDZD-8 1 mg/kg/day significantly attenuated the reduction in Bcl-2 expression caused by bleomycin (c1). In addition, body weight was recorded immediately before bleomycin administration and daily for the entire experimental period. Administration of bleomycin caused a significant fall in body weight (d) and increase in mortality rate (e). Treatment with TDZD-8 (1 mg/kg/day), resulted in a significant decrease in body weight (d) and mortality rate (e) in mice administered bleomycin. The figure is representative of at least 3 experiments performed on different days. Data are expressed as means ± SEM from n=10 mice for each group. *P<0.01 versus sham group. °P<0.01 versus bleomycin.*

2 expression). All of these findings support the view that TDZD-8 attenuates the degree of lung inflammation in mice.

Evidence of a role for GSK-3β in the regulation of lung injury is of special interest as several transcription factors that play a key role in lung inflammation serve as substrates for GSK-3β. Among these is the transcription factor NF-κB, whose function is strikingly altered by GSK-3β (13-14). NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation. These include the genes for TNF-α, IL-1β, iNOS and COX-2, to name but a few (25).

We report in the present study that inhibition of GSK-3β reduces the biosynthesis and/or effects of the pro-inflammatory cytokines TNF-α and IL-1. There is good evidence that TNF-α and IL-1β are involved in the pathogenesis of lung fibrosis; these cytokines are present in lung tissues and can be detected immunohistochemically in the inflamed tissues (26). Direct evidence that TNF-α and IL-1β play a role in the pathogenesis of experimental lung injury has been demonstrated in animal models in which inhibition of these cytokines has been shown to delay the onset of experimental lung injury, suppress inflammation, and ameliorate lung destruction that corresponds to the anti-inflammatory response (27). Here we confirm that the model of lung injury used in this study leads to a substantial increase in the levels of TNF-α and IL-1 in the lung. These findings,

therefore, suggest that the administration of TDZD-8 reduces the activation and subsequent expression of pro-inflammatory genes.

TDZD-8 attenuated BLM-induced expression of iNOS. This inhibition of the expression of iNOS by TDZD-8 may also be attributed to inhibition of the activation of NF-κB mediated by GSK-3β. Furthermore, the tissue damage induced by BLM administration was associated with intense immunostaining for nitrotyrosine, suggesting alterations in the tissue had occurred due to the formation of highly reactive nitrogen-derivatives. Recent evidence indicates that BLM is a well-known cause of intracellular oxidative stress (28), and indeed several findings in this study suggest extracellular oxidative stress plays a role in the pathogenesis of BLM-induced lung injury. We clearly demonstrate that inhibition of GSK-3β using TDZD-8 prevents the induction of iNOS and the formation of peroxynitrite.

Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation "footprint" of peroxynitrite (29). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of MPO with hydrogen peroxide can lead to the formation of nitrotyrosine (30). Increased nitrotyrosine staining is considered, therefore, as an indication of "increased nitrosative stress" rather than a specific marker of the peroxynitrite generation.

We report here that treatment with TDZD-8 significantly reduces the increase in leukocyte infiltration, as assessed by the specific granulocyte enzyme MPO, as well as the lung tissue damage. Neutrophils recruited into the tissue can contribute to tissue destruction by the production of reactive oxygen metabolites, granule enzymes, and cytokines that further amplify the inflammatory response due to their effects on macrophages and lymphocytes (31).

GSK-3β has been found to participate in a remarkable number of signaling pathways, which, based on the findings reported here, include the ultimate decision between cell death and survival (32). Other studies suggest that adequate inactivation of GSK-3β is critical for cell survival, as excessive GSK-3β resulted in a massive activation of caspase-3 following heat shock (33). We have demonstrated that treatment with TDZD-8 attenuates the degree of apoptosis in the lung, measured by TUNEL detection kit. It is known that pathways which inhibit GSK-3β activity, such as PI-3K or Wnt signaling, often lead to induction of the NF-kB cell survival pathway (34). Indeed, GSK-3β is a major target of Akt/PKB (35) which is activated by the PI-3K mediated signaling pathway. Cellular factors implicated in the regulation of astrocyte apoptosis include PI-3K pathway. Inhibition of this signaling cascade has been shown to lead to cell death in several paradigms, and this has been attributed, at least in part, to the reduction in activity of PI-3K's major physiologic target, Akt. A decrease in Akt activity results in the transduction of several pro-apoptotic signals including sequestration of Bcl-2 and enhanced activation of an Akt substrate, GSK-3β. We identified pro-apoptotic transcriptional changes, including up-regulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2, using immunohistochemical staining. We demonstrate that treatment with TDZD-8 in lung injury attenuates apoptotic cell death following BLM administration, suggesting that protection from apoptosis may be a prerequisite for anti-inflammatory approaches. In particular, we demonstrate that treatment with TDZD-8 reduces the signal for Bax, while it increases the expression of Bcl-2. This means that TDZD-8 by inhibiting NF-κB prevents loss of the anti-apoptotic pathway and reduces pro-apoptotic pathway activation, although the mechanism remains unclear.

Taken together, the results of the present study enhance our understanding of the role of GSK-3β in the pathophysiology of lung inflammation. Our results suggest that inhibitors of the activity of GSK-3β may be useful in the therapy of inflammation.

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