Differential Effects of Pirfenidone on Acute Pulmonary Injury and Ensuing Fibrosis in the Hamster Model of Amiodarone-Induced Pulmonary Toxicity

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Pulmonary toxicity, including fibrosis, is a serious adverse effect associated with the antidysrhythmic drug amiodarone (AM). We tested the potential usefulness of pirfenidone against AM-induced pulmonary toxicity in the hamster model. Intratracheal AM administration resulted in pulmonary fibrosis 21 days posttreatment, as evidenced by an increased hydroxyproline content and histological damage. Dietary pirfenidone administration (0.5% w/w in chow), for 3 days prior to and continuously after AM, prevented fibrosis and suppressed elevation of pulmonary transforming growth factor (TGF)-β_1 mRNA content at 7 and 21 days post-AM. Protection against AM-induced lung damage was not observed when supplementation with pirfenidone was delayed until 7 days following AM administration, suggesting that alteration of early events in AM lung toxicity is necessary for the protective effect of pirfenidone. Both AM and bleomycin, another pulmonary fibrogen, caused inflammation 24 h after intratracheal dosing, measured as increased lactate dehydrogenase activity, protein content, and cellular alterations in bronchoalveolar lavage fluid, with the response to AM markedly greater than that to bleomycin. Administration of AM, but not bleomycin, also caused whole lung mitochondrial dysfunction, alveolar macrophage death, and an influx of eosinophils into the lung, of which pirfenidone was able to decrease only the latter. We conclude that: (1) AM induces alveolar macrophage death and severe, acute pulmonary inflammation with associated eosinophilia following intratracheal administration; (2) mitochondrial dysfunction may play an early role in AM pulmonary injury; and (3) pirfenidone decreases AM-induced pulmonary fibrosis in the hamster, probably through suppression of TGF-β_1 gene expression.

Key Words: amiodarone; pirfenidone; lung; inflammation; fibrosis; mitochondria.

Pulmonary fibrosis is a debilitating condition for which there is currently no effective therapy and for which patient prognosis is poor (Cooper, 2000; Gross and Hunninghake, 2001).

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Numerous agents cause lung damage that can progress to fibrosis, although different agents have different mechanisms of initiation of lung tissue damage. It is believed that injury to the epithelium and basement membranes is a requisite step in the etiology of pulmonary fibrosis (Coker and Laurent, 1998; Selman *et al.*, 2001), after which several cell types, including inflammatory and immune cells as well as fibroblasts, migrate to and/or proliferate in areas of injury and release numerous cytokines that lead to further cell recruitment, inflammation, and eventual matrix remodeling. This culminates in an overproduction of collagen and other matrix components characteristic of fibrosis (Coker and Laurent, 1998; Selman *et al.*, 2001). One of the cytokines that is most important in extracellular matrix remodeling, including collagen deposition, is transforming growth factor (TGF)-β_1 (Cooper, 2000; Sime and O'Reilly, 2001). Thus, pharmacological inhibition of TGF-β_1 action may be a valuable strategy for the treatment of fibrosis of the lung and other tissues.

Until recently, treatment of pulmonary fibrosis has been based primarily on anti-inflammatory and immunosuppressive therapies, with very limited success (Selman *et al.*, 2001). As such, new therapeutic strategies are desirable. Pirfenidone is an agent with demonstrated anti-fibrotic activity in several organs in experimental animals, including the lung (Iyer *et al.*, 1995; Kehrer and Margolin, 1997; Mirkovic *et al.*, 2002; Shimizu *et al.*, 1998; Tada *et al.*, 2001), and a phase II clinical study showed pirfenidone to be a promising treatment for idiopathic pulmonary fibrosis (Raghu *et al.*, 1999). Furthermore, an anti-inflammatory action of pirfenidone has been described in the hamster model of bleomycin-induced pulmonary fibrosis and may contribute to its overall anti-fibrotic effect (Iyer *et al.*, 2000). As such, pirfenidone may prove beneficial for a range of fibrotic conditions through both anti-inflammatory and anti-fibrotic mechanisms.

Amiodarone (AM) is an efficacious anti-dysrhythmic agent, the use of which is associated with numerous adverse effects, including pulmonary toxicity that can progress to fibrosis (Mason, 1987; Pollak, 1999). While several mechanisms have been proposed for the initiation and progression of AM-induced pulmonary toxicity (Massey *et al.*, 1995; Reasor and Kacew,

1996), the pathogenesis remains unknown. Considerable evidence suggests that mitochondrial dysfunction plays an initiating role in AM-induced toxicities, including pulmonary toxicity. We observed a disruption of mitochondrial membrane potential prior to ATP depletion and subsequent cell death in isolated lung cells exposed to AM or its metabolite, *N*-desethylamiodarone (DEA) (Bolt *et al.*, 2001), and other *in vitro* studies have shown that AM causes both functional (respiratory and membrane potential) and structural alterations in mitochondria from various tissues (Card *et al.*, 1998; Fromenty *et al.*, 1990; Guerreiro *et al.*, 1986; Yasuda *et al.*, 1996).

The potential for pirfenidone to prevent AM-induced pulmonary fibrosis has not been evaluated. Given the widespread clinical use of AM for treating tachyarrhythmias and for reducing mortality post-myocardial infarction (Nolan *et al.*, 1998), it was of interest to determine if experimental pulmonary fibrosis caused by AM could be attenuated by pirfenidone. Hence, the current study was designed to test whether pirfenidone could prevent AM-induced pulmonary fibrosis in a hamster model. Furthermore, the acute pulmonary effects of intratracheal AM, including alteration of mitochondrial respiratory function and the subsequent inflammatory response, were profiled and compared to those resulting from exposure to bleomycin, an agent that causes pulmonary fibrosis in the hamster following a single intratracheal administration with a time course similar to that observed for AM.

MATERIALS AND METHODS

Chemicals.   Chemicals and reagents were obtained as follows: pirfenidone from Marnac, Inc. (Dallas, TX); sodium pentobarbital from M.T.C. Pharmaceuticals (Mississauga, ON, Canada); ketamine hydrochloride from Rogar/STB Inc. (London, ON, Canada); trans-4-hydroxy-*L*-proline from Aldrich Chemical Co. (Milwaukee, WI); and bleomycin sulphate (Faulding Inc., Montreal, PQ, Canada) from the Kingston General Hospital pharmacy (Kingston, ON, Canada). Unless otherwise stated, all other chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Co. (Oakville, ON, Canada).

Animals and treatments.   All animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care, and experimental protocols were approved by the Queen's University Animal Care Committee. Male golden Syrian hamsters (110–120 g on arrival from Charles River Canada Inc., St. Constant, PQ, Canada) were housed in group plastic cages with chipped hardwood bedding, using a 12 h light/12 h dark cycle, and were allowed to acclimatise for at least one week prior to use. The hamsters were given free access to water and either pulverized rodent laboratory chow #5001 (Purina Mills Inc., St. Louis, MO) or the same chow containing 0.5% pirfenidone (w/w). Hamsters randomly designated for pirfenidone supplementation began the diet either 3 days prior to intratracheal treatment (early pirfenidone group) or 7 days after treatment (late pirfenidone group).

AM was dissolved in distilled H_2O at 60°C and allowed to cool to room temperature before transoral intratracheal instillation as described in Card *et al.* (1999). Following treatment, animals were returned to their respective diets and water *ad libitum* for the duration of the study period, with the exception of the late pirfenidone group, which began the pirfenidone-supplemented diet 7 days after AM treatment.

Bleomycin was dissolved in isotonic saline and administered at a dose of 0.9 unit per animal by transoral intratracheal administration in a similar fashion to

that for AM. This administration protocol has been demonstrated to result in pulmonary fibrosis 21 days postdosing in the hamster (Iyer *et al.*, 1995).

Preparation of lung tissue.   For determination of indicators of pulmonary fibrosis, each animal was killed by injection of sodium pentobarbital (300 mg/kg i.p.) at 7 or 21 days post-AM treatment, a thoracotomy was performed, and the trachea was exposed and cannulated. The right bronchus was ligated, and the right lung was removed, weighed, frozen in liquid nitrogen, and stored at -80°C until determination of hydroxyproline content or isolation of total RNA. The left lung was inflated with 10% neutral-buffered formalin to a pressure of 20-cm H_2O for 1 h. The trachea was then ligated, and the lung was removed and placed in formalin. Sections from the upper, middle, and lower portions of the lung were dehydrated and embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin for histological evaluation.

Histopathology.   To evaluate morphological damage, a disease index was computed for each animal as described in Card *et al.* (1999), with the evaluator unaware of the animal treatments. The disease index, which quantified septal thickening and cellular infiltration, was calculated as the mean of the values for equal numbers of sections taken from the upper, middle, and lower lung.

Hydroxyproline measurement.   The lung content of hydroxyproline was determined as a biochemical index of fibrosis. Aliquots of frozen right lung tissue (~100 mg) were pulverized in liquid nitrogen and hydrolyzed in 5.0 ml of 6.0 N HCl at 110°C for 72 h. Following neutralization with 2.75 ml of 10-M NaOH, the hydroxyproline content was determined in duplicate by the spectrophotometric method of Lindenschmidt and Witschi (1985).

Preparation of molecular probes.   The TGF-β_1 template was purchased as an *Escherichia coli* plasmid insert (American Type Culture Collection, Manassas, VA). The 18S rRNA DECA probe template was obtained from Ambion Inc. (Austin, TX). Plasmid isolation, insert purification, and probe labeling were performed as described in Card *et al.* (2003).

Total RNA isolation and hybridization analyses.   Total RNA was isolated from aliquots of frozen right lung tissue (~30 mg) with a QIAGEN® RNeasy mini kit as per the manufacturer's instructions (QIAGEN, Valencia, CA). Hybridization analyses for pulmonary TGF-β_1 mRNA and 18S rRNA contents were performed as described in Card *et al.* (2003).

Bronchoalveolar lavage.   For determination of inflammation following AM or bleomycin administration, each animal was killed by injection of sodium pentobarbital (300 mg/kg i.p.), the lungs and trachea were removed en bloc, and two successive 5.0-ml aliquots of warm (37°C) phosphate-buffered saline (PBS, pH 7.4) were infused and slowly withdrawn from the lungs through a cannula inserted into the trachea. Recovered bronchoalveolar lavage fluid (BALF) volumes routinely measured between 8.0- and 9.0-ml. BALF were centrifuged at 1000 × g for 10 min at 4°C, after which the supernatants were removed and saved for determination of lactate dehydrogenase (LDH) activity and protein content. The cell pellets were resuspended in PBS, and total cell counts were determined on a haemocytometer. Concurrently, alveolar macrophage viabilities were assessed by trypan blue exclusion. In addition, BALF cell samples were centrifuged onto microscope slides using a Shandon Cytospin 2 centrifuge (Shandon Southern Products Limited, Runcorn, Cheshire, England), stained with Wright's stain, and differential cell counts determined under a light microscope by counting 300 to 400 cells per animal. Absolute cell numbers were $\log_{10}$ transformed for data analysis and presentation. The LDH activity of the BALF supernatants was determined using a commercially available kit (Sigma LDH procedure No. DG1340-UV), and the BALF protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as the standard.

Isolation of whole lung mitochondria.   Hamsters were killed by injection of sodium pentobarbital (300 mg/kg ip) after 3 days on either the control or pirfenidone-supplemented diet. Lung mitochondria were isolated by differential centrifugation as described by Fisher *et al.* (1973), using a homogenization buffer comprised of 225-mM mannitol, 75-mM sucrose, 2.0-mM EDTA, 5.0-mM MOPS, and 2% (w/v) fatty acid–free BSA (pH 7.2). The solutions were kept ice-cold, and all manipulations were performed on ice or at 4°C. For

each experiment to determine the effect of *in vitro* exposure to AM or bleomycin on mitochondrial respiratory function or membrane potential, mitochondria from four sets of hamster lungs were pooled.

For determination of the effect of *in vivo* treatments on mitochondrial respiratory function, the hamsters were maintained on the control or pirfenidone-supplemented diet for 3 days prior to intratracheal dosing with AM, bleomycin, or distilled H_2O as described above. Whole lung mitochondria were then isolated from the individual hamsters at various times after intratracheal treatment. The protein content of the mitochondrial suspensions was determined by the method of Lowry *et al.* (1951), using BSA as the standard.

Mitochondrial oxygen consumption. Oxygen consumption of the isolated lung mitochondria was measured at 30°C as described in Card *et al.* (1998), using a YSI biological oxygen monitor (model 5300) and a Clark-type polarographic oxygen electrode (model 5301; Yellow Springs Instrument Co. Inc., Yellow Springs, OH). Briefly, mitochondria (1–2-mg protein) were added to 3.0 ml of respiration buffer (145-mM KCl, 5.0-mM KH_2PO_4, 20-mM Tris–HCl, pH 7.2) in a magnetically stirred sample chamber. Respiration supported by complex I (NADH-ubiquinone oxidoreductase) of the electron transport chain was assessed using glutamate (5.0 mM) and malate (5.0 mM) as substrates, whereas succinate (10 mM, in the presence of 3.0-μM rotenone) was used to assess respiration at complex II (succinate-ubiquinone oxidoreductase). State 3 (active) respiration at either complex was induced by adding 0.2-mM ADP to the chamber; when respiration converted to the state 4 (resting) rate upon expenditure of the added ADP, oxygen utilization noticeably declined. At least 2 min following the transition from state 3 to state 4 respiration, the effect of *in vitro* exposure to AM, bleomycin, or the H_2O vehicle (up to 50 μl) on state 4 respiration at complex I or II was examined by adding these drugs individually at 50–400-μM final concentrations. For determination of the effect of *in vivo* treatments on mitochondrial respiratory function, oxygen consumption supported by complexes I and II was determined in mitochondria isolated at various times following intratracheal AM, bleomycin, or distilled H_2O administration, without additional *in vitro* drug addition. The respiratory control ratios (RCRs; ratio of state 3 to state 4 oxygen consumption rates) and ADP:O ratios (ratio of nmol of ADP consumed to nmol of oxygen consumed during state 3 respiration) were calculated as indicators of the integrity of mitochondrial respiratory function as described in Card *et al.* (1998).

Mitochondrial membrane potential. The membrane potential of isolated lung mitochondria was determined as described previously (Card *et al.*, 1998; Fromenty *et al.*, 1990), utilizing the fluorescent properties of safranine dye. Lung mitochondria (1–2 mg protein) were incubated at 30°C for 5 min in 2.7 ml of reaction buffer (0.2-M sucrose, 20-mM KCl, 20-mM HEPES, 0.38-mM EDTA, pH 7.2) in the presence of safranine (10 μM) and rotenone (3.0 μM). The mixture was decanted into a quartz cuvette, and the fluorescence of safranine dye was determined for 2 min using a Perkin-Elmer LS-5B luminescence spectrometer (excitation 510 nm, emission 570 nm, 10-nm slit widths). Succinate was added (10-mM final concentration) as a substrate for state 4 respiration in order to establish a high initial membrane potential and the change in fluorescence monitored. Following stabilization (approximately 2 min), the H_2O vehicle (up to 50 μl) or AM or bleomycin were added (50–400-μM final concentration) and the change in fluorescence monitored for 10 min.

Plasma and lung pirfenidone analyses. Tissue and plasma pirfenidone concentrations were determined using a modification of a previously published method (Mirkovic *et al.*, 2002). Hamsters were killed by injection of sodium pentobarbital (300 mg/kg i.p.) after 3 days on the pirfenidone-supplemented diet. Following blood-sample collection via cardiac puncture, plasma was prepared by centrifuging anticoagulant-treated blood samples at 16,000 × g for 5 min and was kept on ice. The lungs were removed, weighed, and homogenized in 0.9% (w/v) saline solution. Aliquots of plasma or lung homogenates were vigorously mixed with one volume of high-performance liquid chromatography (HPLC) mobile phase (50% (v/v) acetonitrile in distilled H_2O) and centrifuged at 16,000 × g for 1 min. Aliquots of supernatants (25 μl) were used for pirfenidone quantification by reverse-phase HPLC with ultraviolet

detection at 315 nm. The HPLC system consisted of a Shimadzu LC-10AD pump (Shimadzu Scientific Instruments, Columbia, MD) set at a flow rate of 1.0 ml/min, a Supelco Supelcosil 5-μm C_{18} column (25-cm length, 4.6-mm diameter; Sigma, St. Louis, MO), and a Waters Lambda-Max Model 481 LC spectrophotometric detector (Waters, Milford, MA). For each sample, the area of the chromatographic signal at the retention time of pirfenidone was interpolated on a standard curve prepared with known concentrations of authentic pirfenidone dissolved in mobile phase. For these HPLC conditions, pirfenidone had a retention time of 4.17 min.

Statistical analyses. Data are expressed as mean ± standard deviation. Statistical comparisons among treatment groups were performed by randomized-design one- or two-way analysis of variance (ANOVA) followed by the Newman-Keuls post-hoc test for more than two groups, or by an unpaired Student's *t*-test for two groups. The histological disease index data underwent arcsine transformation prior to statistical analysis, as described by Sokal and Rohlf (1973) for percentage data. When necessary, a $\log_{10}$ transformation of data was performed prior to statistical analysis to correct for heterogeneity of variance. In all cases, statistical significance was defined as $p < 0.05$.

RESULTS

Pulmonary Histopathology and Hydroxyproline Content

Normal pulmonary architecture was observed 21 days posttreatment in hamsters maintained on the control or pirfenidone-supplemented diets and administered intratracheal H_2O (Figs. 1A and 1B). Intratracheal AM resulted in cellular infiltration and patchy septal thickening and fibrosis (Fig. 1C) that were prevented by early pirfenidone supplementation (Fig. 1D). Late pirfenidone supplementation following AM treatment resulted in some areas of septal thickening and fibrosis similar to those observed in the AM group maintained on the control diet (Fig. 1E). The administration of AM resulted in an increased disease index value 21 days posttreatment in the control-diet animals (Fig. 2A). This increase was prevented by early pirfenidone supplementation, while late pirfenidone supplementation did not significantly alter the response to AM.

No differences in right lung hydroxyproline content were found among the diet and treatment groups 7 days posttreatment (data not shown); however, a significant increase (~23%) resulted from intratracheal AM administration in the control-diet group at 21 days (Fig. 2B), consistent with previous reports (Card *et al.*, 1999; Rafeiro *et al.*, 1994). This increase was prevented by early, but not by late, pirfenidone supplementation.

Pulmonary TGF-β_1 mRNA Content

Intratracheal AM administration increased right lung TGF-β_1 mRNA content at 7 and 21 days posttreatment by 59 and 340%, respectively (Figs. 3A and 3B), similar to previous findings (Card *et al.*, 2003). Early pirfenidone supplementation prevented the AM-induced elevation of TGF-β_1 mRNA at 7 and 21 days, and late pirfenidone supplementation was partially effective at 21 days (Figs. 3A and 3B). The lower TGF-beta1:18s rRNA ratio in the H_2O-treated groups at 21 days (Fig. 3B) versus 7 days (Fig. 3A) was observed previously

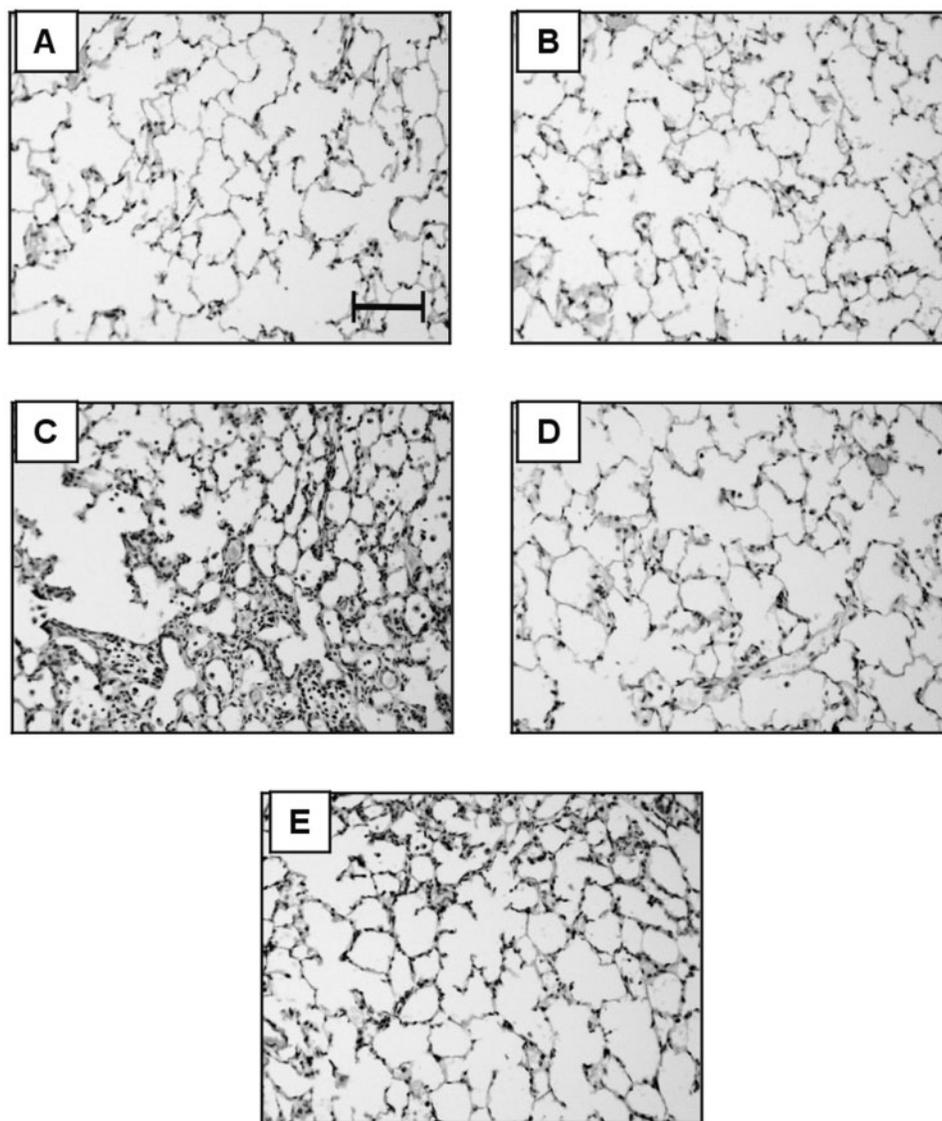**FIG. 1.** Hematoxylin- and eosin-stained hamster lung sections 21 days following intratracheal administration of AM (1.83 μmol) or vehicle (0.1 ml H_2O). Hamster diets were supplemented with pirfenidone (PD; 0.5 % w/w), beginning either 3 days prior to (early) or 7 days following (late) intratracheal treatments, and until animals were sacrificed at 21 days posttreatment. (A) Control-diet hamster treated with H_2O. (B) Early pirfenidone-diet hamster treated with H_2O. (C) Control-diet hamster treated with AM. (D) Early pirfenidone-diet hamster treated with AM. (E) Late pirfenidone-diet hamster treated with AM. The scale bar in (A) represents 100 μm in all micrographs.

(Card *et al*., 2003) and may be attributable to a transient effect of the intratracheal administration procedure itself.

Pulmonary Inflammation

Given that pirfenidone decreased AM-induced pulmonary fibrosis when supplementation was begun prior to AM administration, but not if started 7 days after AM, the effect of pirfenidone on acute AM-induced pulmonary inflammation was investigated. Bleomycin was used as a comparative pulmonary toxicant in this experiment, since pirfenidone has established anti-inflammatory and anti-fibrotic effects against bleomycin in this model (Iyer *et al*., 1995, 1999, 2000). Analysis of BALF 24 h posttreatment revealed a significant increase of LDH activity ($\sim$7-fold) and protein content ($\sim$85-fold) as a result of AM administration, whereas bleomycin caused no

increase in LDH activity and only a slight increase in protein content ($\sim$4-fold) (Table 1); neither measurement was significantly altered by maintenance on the pirfenidone diet. The total BALF cell number was dramatically increased 24 h following AM (524.8 $\pm$ 10.8 $\times$ 10^7 versus 8.3 $\pm$ 0.3 $\times$ 10^7 in vehicle-treated animals) and was increased to a much lesser extent by bleomycin (34.7 $\pm$ 0.2 $\times$ 10^7; Table 1). Differential cell counts revealed $\sim$950-fold (380.2 $\pm$ 9.5 $\times$ 10^7) and $\sim$54-fold (21.8 $\pm$ 0.3 $\times$ 10^7) increases in the neutrophil numbers following treatment with AM or bleomycin, respectively, versus vehicle-treated animals (0.4 $\pm$ 0.1 $\times$ 10^7) and $\sim$151-fold increase in eosinophil numbers following AM treatment (15.1 $\pm$ 0.6 $\times$ 10^7 versus 0.1 $\pm$ 0.1 $\times$ 10^7 in vehicle-treated animals). Of the alterations of BALF cell number resulting from AM or bleomycin treatment, only the AM-
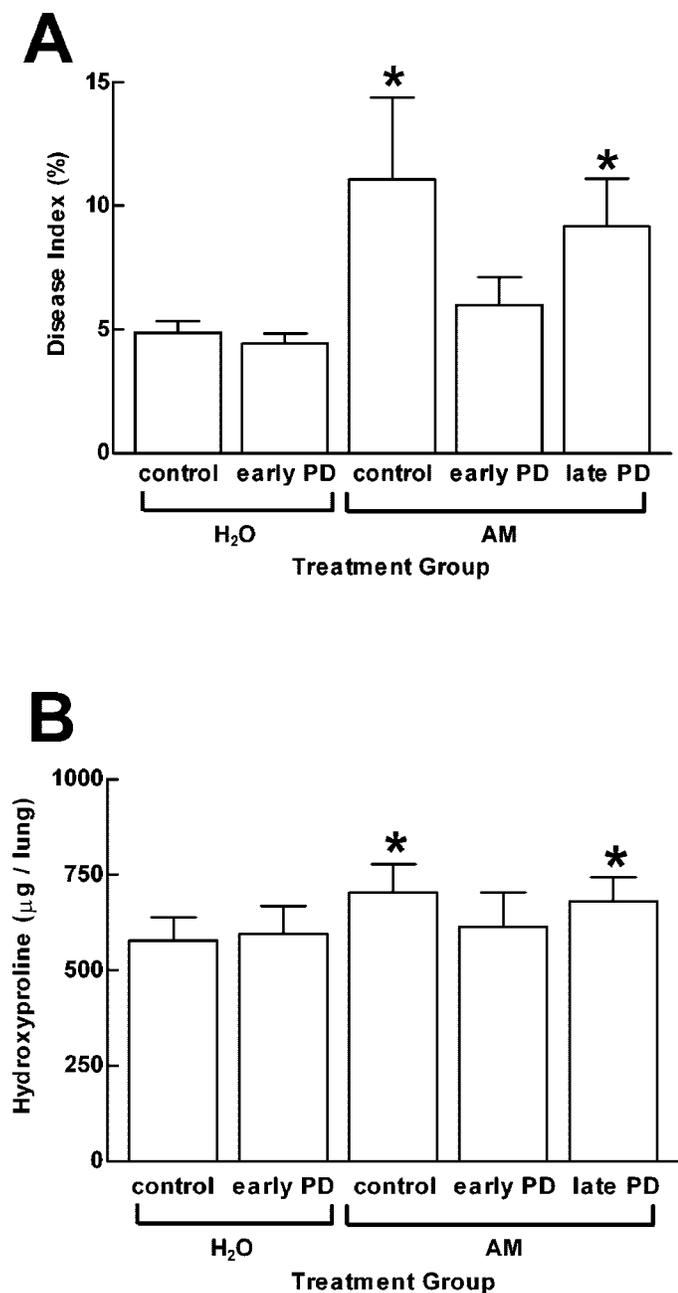
FIG. 2. Histological disease index values (A) and right lung hydroxyproline content (B) 21 days following intratracheal administration of AM (1.83 μmol) or vehicle (0.1-ml H_2O). Treatment groups are as described in the legend to Figure 1. *Significant difference from control + H_2O, early PD + H_2O, and early PD + AM groups, $p < 0.05$ ($n = 6$–8).

induced eosinophilia was significantly attenuated by maintenance on the pirfenidone diet ($3.7 \pm 0.1 \times 10^7$; Table 1). Alveolar macrophage viability was decreased at the 24-h time point by AM but not by bleomycin (Table 1), and it was not prevented by dietary pirfenidone supplementation.

Since a pronounced inflammatory response to AM was observed at 24 h, LDH activity, protein content, and inflamma-

tory cell influx were determined at selected earlier time points. At 30 min after AM treatment, increases of both LDH activity ($\sim$2-fold) and protein content ($\sim$6-fold) were observed that increased to $\sim$5- and $\sim$35-fold, respectively, at the 3-h time point (Figs. 4A and 4B). As occurred at the 24-h time point, a considerable increase of BALF total cell number (8.55×10^7 versus 3.45×10^7 for control) and a decrease of alveolar macrophage viability (58.8 ± 5.2 % versus 91.3 ± 3.7 % for control) were observed at 30 min post-AM treatment.
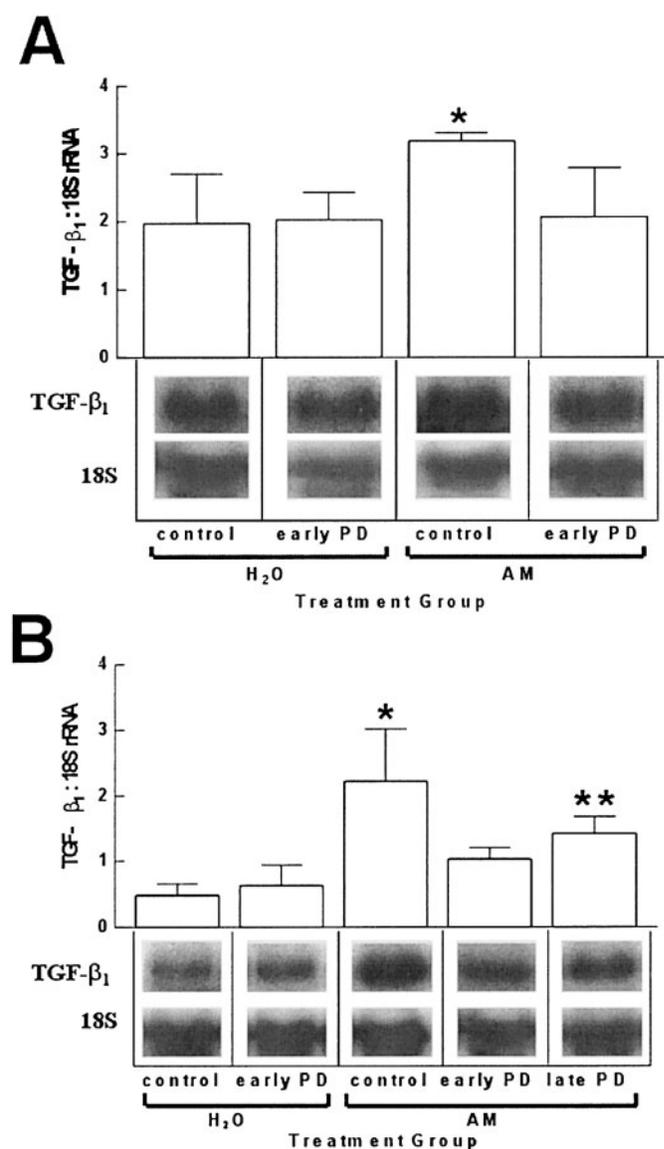

FIG. 3. Pulmonary TGF-β_1 mRNA content following intratracheal administration of AM (1.83 μmol) or vehicle (0.1-ml H_2O). Treatment groups are as described in the legend to Figure 1. (A) 7 days postdosing ($n = 3$–6). (B) 21 days postdosing ($n = 4$–6). *Significant difference from all other groups, $p < 0.05$. **Significant difference from all groups except early PD + AM, $p < 0.05$.

TABLE 1

BALF Measurements following Intratracheal Administration of Water, Amiodarone, or Bleomycin to Hamsters Fed a Diet Supplemented with or without Pirfenidone[a]

Tx	Diet	LDH(U/l)	Protein (μg/ml)	Total cell # ($\log_{10}$)	Differential cell # ($\log_{10}$)			Macrophage viability (%)
					Macrophages	Neutrophils	Eosinophils	
H$_2$O	Control	20.3 ± 0.6	81.4 ± 10.6	7.92 ± 0.32	7.56 ± 0.01	6.56 ± 0.48	6.06 ± 0.41	96.5 ± 0.7
	PD	22.5 ± 2.1	99.6 ± 6.9	7.74 ± 0.02	7.65 ± 0.03	5.90 ± 0.40	5.76 ± 0.09	97.0 ± 0.2
AM	Control	137.2 ± 39.8[b]	6924 ± 1609[b]	9.72 ± 0.20[b]	7.39 ± 0.29	9.58 ± 0.24[b]	8.18 ± 0.32[b]	72.4 ± 4.8[b]
	PD	155.5 ± 89.2[b]	4110 ± 2583[b]	9.67 ± 0.20[b]	7.50 ± 0.06	9.50 ± 0.27[a]	7.57 ± 0.22[b,c]	69.7 ± 2.4[b]
BLEO	Control	28.3 ± 2.6	363.3 ± 128.6[d]	8.54 ± 0.06[d]	7.46 ± 0.08	8.34 ± 0.12	[d]6.46 ± 0.05	92.5 ± 1.3
	PD	38.1 ± 11.0	259.2 ± 59.0[d]	8.65 ± 0.23[d]	7.52 ± 0.04	8.51 ± 0.24[d]	6.57 ± 0.20	93.8 ± 1.6

Note. When necessary, data underwent $\log_{10}$ or arcsine transformation prior to analysis ($n = 3$ per data point).

[a]Measurements were made 24 h following intratracheal administration of water (H$_2$O; 0.1 ml), amiodarone (AM; 1.83 μmol), or bleomycin (BLEO; 0.9 units) to hamsters fed a diet supplemented with or without pirfenidone (PD; 0.5% w/w in chow) for 3 days prior to intratracheal treatments.

[b]Significant difference from H$_2$O and BLEO treatments within same diet group, $p < 0.05$.

[c]Significant difference from corresponding AM-treated control diet group, $p < 0.05$.

[d]Significant difference from H$_2$O treatments within same diet group, $p < 0.05$.

Pulmonary Mitochondrial Dysfunction

Lung mitochondria, isolated and pooled from animals maintained on the control or pirfenidone diet, were used to determine the effect of pirfenidone administration on basal mitochondrial respiratory function and membrane potential, as well as for analysis of functional responses following *in vitro* exposure to AM or bleomycin. Oxygen consumption supported by complexes I and II of the electron transport chain was assessed prior to the addition of the test agents, and no differences between diet treatments were found in RCRs or ADP:O ratios during these assessments: At complex I, the RCRs were 2.90 ± 0.24 and 3.12 ± 0.20, and the ADP:O ratios were 4.26 ± 0.10 and 4.47 ± 0.45 for the control- and pirfenidone-diet groups, respectively; at complex II, the RCRs were 1.52 ± 0.04 and 1.40 ± 0.08, and the ADP:O ratios were 2.20 ± 0.14 and 2.19 ± 0.11 for the control- and pirfenidone-diet groups, respectively. Thus, dietary pirfenidone administration did not alter basal lung mitochondrial respiratory function as assessed by oxygen consumption supported by complexes I or II of the electron transport chain. *In vitro* exposure of isolated lung mitochondria to AM resulted in significant inhibition of oxygen consumption, with concentrations ≥100 μM causing at least 75 and 50% inhibition at complexes I and II, respectively (Figs. 5A and 5B). AM also substantially decreased mitochondrial membrane potential (Fig. 5C), while neither parameter of mitochondrial function was affected by *in vitro* exposure to the drug vehicle (distilled H$_2$O; data not shown) or to bleomycin (400 μM). The effects of AM were not significantly altered by dietary supplementation with pirfenidone for 3 days prior to the isolation of lung mitochondria (Figs. 5A–C).

Intratracheal AM administration caused significant inhibition of state 3 respiration at complex I 3 h posttreatment, resulting in decreased RCR and increased ADP:O values compared to the vehicle-treated controls (Fig. 6). Respiration supported by complex II was not affected up to 3 h following AM (Fig. 7). However, respiratory function at both complexes I and II was inhibited by AM at 24 h (Figs. 6 and 7). Maintenance on the pirfenidone diet for 3 days prior to intratracheal AM treatment and subsequent isolation of lung mitochondria 3 h later, the time point when respiratory dysfunction was first observed, did not alter the adverse respiratory effects of AM (Table 2). Intratracheal bleomycin administration did not alter lung mitochondrial respiratory function up to 24 h posttreatment (data not shown).

Plasma and Lung Pirfenidone Concentrations

Following 3 days of dietary administration, the plasma pirfenidone concentration was 2.04 ± 1.81 μg/ml ($n = 3$), consistent with previous reports (Mirkovic *et al.*, 2002), and the pirfenidone concentration in whole lung homogenate was 4.73 ± 0.77 μg/g tissue ($n = 3$).

DISCUSSION

This study demonstrates that dietary administration of pirfenidone prevents AM-induced pulmonary fibrosis in the hamster. Pirfenidone prevented AM-induced up-regulation of pulmonary TGF-β_1 gene expression, both several days following the toxic insult and at the time of maximal fibrosis. Pirfenidone similarly down-regulated TGF-β_1 expression and lessened experimental bleomycin-induced lung inflammation and fibrosis, effects attributed at least in part to decreased TGF-β_1 gene transcription (Iyer *et al.*, 1999, 2000). TGF-β_1 exerts numerous effects on the progression of fibrosis, including induction of extracellular matrix protein synthesis, inhibition of collagen degradation through induction of protease inhibitors, and stimulation of fibroblast mitogenesis and chemotaxis (Kelley, 1993). As such, attenuating the effects of TGF-β_1 in lung tissue
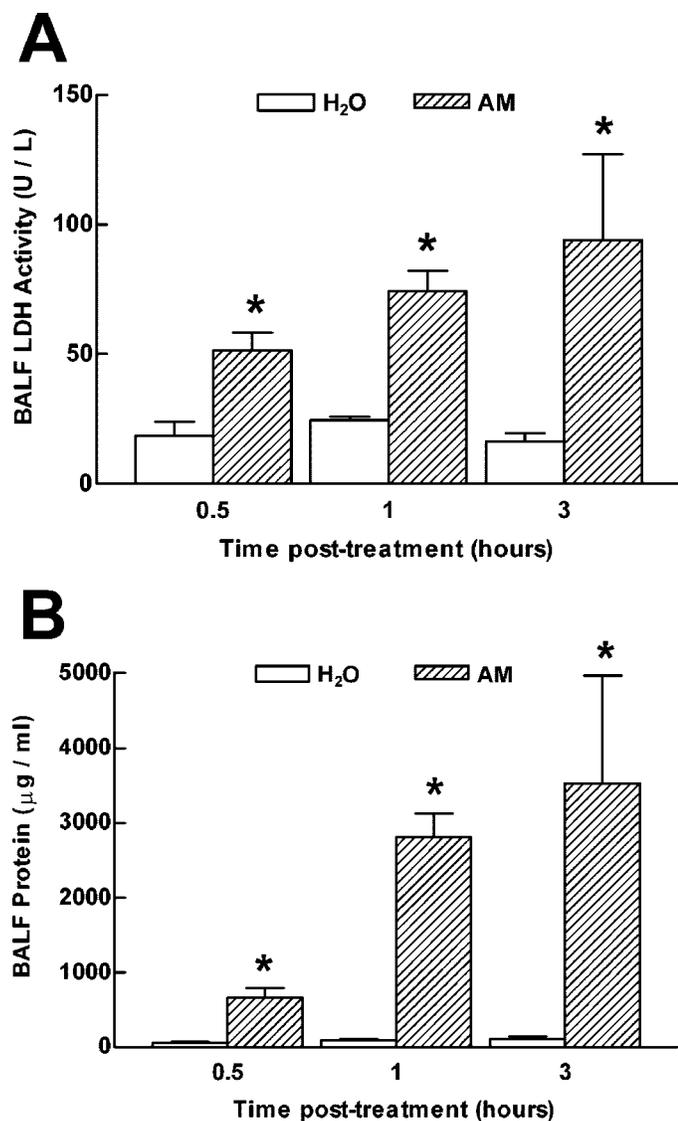
FIG. 4. Effects of intratracheal administration of H_2O (0.1 ml) or AM (1.83 μmol) on LDH activity (A) and protein content (B) in BALF collected from individual hamsters at various times after dosing. *Significant difference from the H_2O-treated group at the same time point, $p < 0.05$ ($n = 3–4$).

patchy interstitial fibrosis is similar in the two situations (Massey *et al.*, 1995) and cannot be achieved by extended systemic AM administration to the hamster (Blake and Reasor, 1995b; Leeder *et al.*, 1994). In the current study, the lack of pirfenidone's anti-fibrotic effectiveness when dietary administration was begun 7 days after AM indicates that maximum anti-fibrotic efficacy requires the presence of pirfenidone during the early stages of remodeling following AM-induced lung damage.

Analysis of BALF 24 h after AM administration revealed a severe inflammatory response, characterized by infiltration of neutrophils, eosinophils, and other cell types along with increases in LDH activity and protein content, events that are likely indicative of airway and/or alveolar cell damage. With the exception of the influx of eosinophils, pirfenidone had no effect on these inflammatory parameters. While it is not possible to rule out effects on other deleterious early events that were not identified in the present study, it is likely that pirfenidone's anti-fibrotic activity occurs largely due to the attenuation of events downstream from the initial cell damage and the acute inflammatory phase of lung injury. The magnitude of the inflammatory response to AM at 24 h was much greater than that to a dose of bleomycin that has been shown to cause experimental pulmonary fibrosis in the hamster with a time course similar to that caused by AM (i.e., maximal fibrosis reached at 21 days postdosing) (Iyer *et al.*, 1995). Indeed, in a study by Iyer *et al.* (2000), the influx of BALF inflammatory cells in response to intratracheal bleomycin in hamsters was shown to peak at a level 2-fold greater than in control animals 3 days post-bleomycin, considerably later and to a much lesser extent than what was observed 24 h post-AM in the current study, where a ~62-fold increase versus the control animals occurred. These results indicate that there are substantial temporal and quantitative differences between the inflammatory responses to intratracheal administration of these two pulmonary fibrogens.

Differences of particular interest between the AM- and bleomycin-induced inflammatory responses in the present study include the increase in BALF eosinophil number and the considerable decrease in alveolar macrophage viability at 24 h post-AM dosing that were not observed following bleomycin, and of which pirfenidone was able to decrease only the former. An increased eosinophil number in BALF has been reported following intratracheal AM administration to the hamster, a response suggested to be evidence of an immunological component of AM-induced pulmonary toxicity (Blake and Reasor, 1995a). That pirfenidone suppressed the influx of eosinophils by ~75% indicates a possible contributing mechanism to its protective effect. Examination of BALF at earlier time points revealed an intense inflammatory response 30 min following AM administration, with decreased alveolar macrophage viability. Although we have demonstrated that alveolar macrophages accumulate considerable amounts of AM and are particularly susceptible to AM cytotoxicity *in vitro* (Bolt *et al.*,
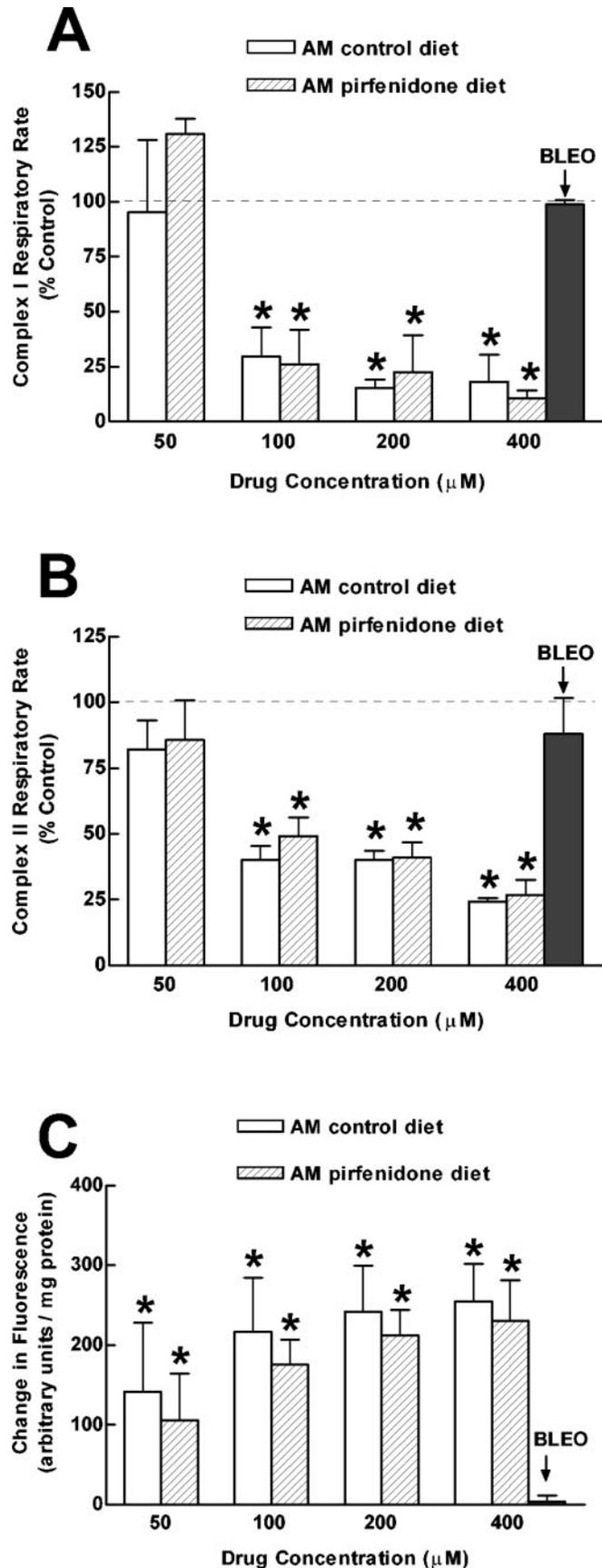
following AM administration is likely a critical component of the anti-fibrotic effect of pirfenidone.

Airway and alveolar cell damage occur early after intratracheal AM administration to rats (Taylor *et al.*, 2001), and intense inflammation and alveolar type II cell hyperplasia are observed within 48 h in hamsters (Blake and Reasor, 1995a; Cantor *et al.*, 1984; Daniels *et al.*, 1989), suggesting that lung cell death and inflammation caused by intratracheal AM initiate a cascade of events culminating in fibroblast proliferation, excess deposition of extracellular matrix components, and fibrosis. Although the route of exposure differs between the intratracheal hamster model and systemic delivery to the lung as occurs in humans with AM lung toxicity, the resulting

1998, 2001), to our knowledge this is the first report of AM-induced alveolar macrophage cytotoxicity *in vivo*. Alveolar macrophages are important regulators of lung homeostasis and of the response to and resolution of lung injury induced by a variety of stimuli (Haley *et al*., 1991), and their damage may be an important factor in the initiation of AM-induced pulmonary toxicity.

Inhibition of mitochondrial function has been postulated to be a common feature in the early stages of fibroproliferative disorders (Oury *et al*., 2001) and is a mechanism by which numerous compounds can initiate cytotoxicity. Disruption of mitochondrial respiratory function and/or membrane potential can significantly impair the energy production of a given cell or tissue, rendering it susceptible to further damage. We have demonstrated that mitochondrial membrane potential and cellular ATP content are decreased early during the course of AM cytotoxicity in freshly isolated hamster lung cells (Bolt *et al*., 2001), suggesting a role for mitochondrial dysfunction in initiating the lung damage that leads to AM-induced pulmonary fibrosis. AM-induced structural and functional alterations of mitochondria have also been observed in other experimental systems (Card *et al*., 1998; Fromenty *et al*., 1990; Guerreiro *et al*., 1986; Yasuda *et al*., 1996), further supporting mitochondrial dysfunction as a basis for AM toxicities.

As observed previously (Bolt *et al*., 2001; Card *et al*., 1998), the present study showed that *in vitro* exposure to AM inhibited whole lung mitochondrial respiratory function and decreased mitochondrial membrane potential, effects that were not prevented by dietary pirfenidone supplementation. Furthermore, mitochondrial function was not impaired by *in vitro* exposure to bleomycin at a concentration equivalent to the highest AM concentration examined (i.e., 400 μM), suggesting that, unlike AM, bleomycin does not have acute adverse effects on lung mitochondrial function.

Lung mitochondrial respiratory function was also impaired following *in vivo* AM administration, although this effect was observed subsequent to the initiation of AM-induced cell death, as indicated by increased BALF LDH activity and protein content, and was not prevented by dietary pirfenidone. Interestingly, the time course of mitochondrial respiratory inhibition was consistent with the time course of AM-induced disruption of mitochondrial membrane potential and decreased cellular ATP content prior to cell death in freshly isolated hamster lung cells (Bolt *et al*., 2001). While the increased BALF protein content measured in the current study was likely

FIG. 5. Effects of dietary pirfenidone administration on the *in vitro* effects of exposure to AM or BLEO on isolated lung mitochondrial function. (A) State 4 oxygen consumption supported by complex I (expressed as % control, i.e., response to H_2O vehicle). (B) State 4 oxygen consumption supported by complex II (expressed as % control, i.e., response to H_2O vehicle). (C) Membrane potential assessed by safranine fluorescence (H_2O vehicle did not alter fluorescence). *Significant difference relative to response to control (H_2O vehicle), $p < 0.05$ ($n = 3$–4).
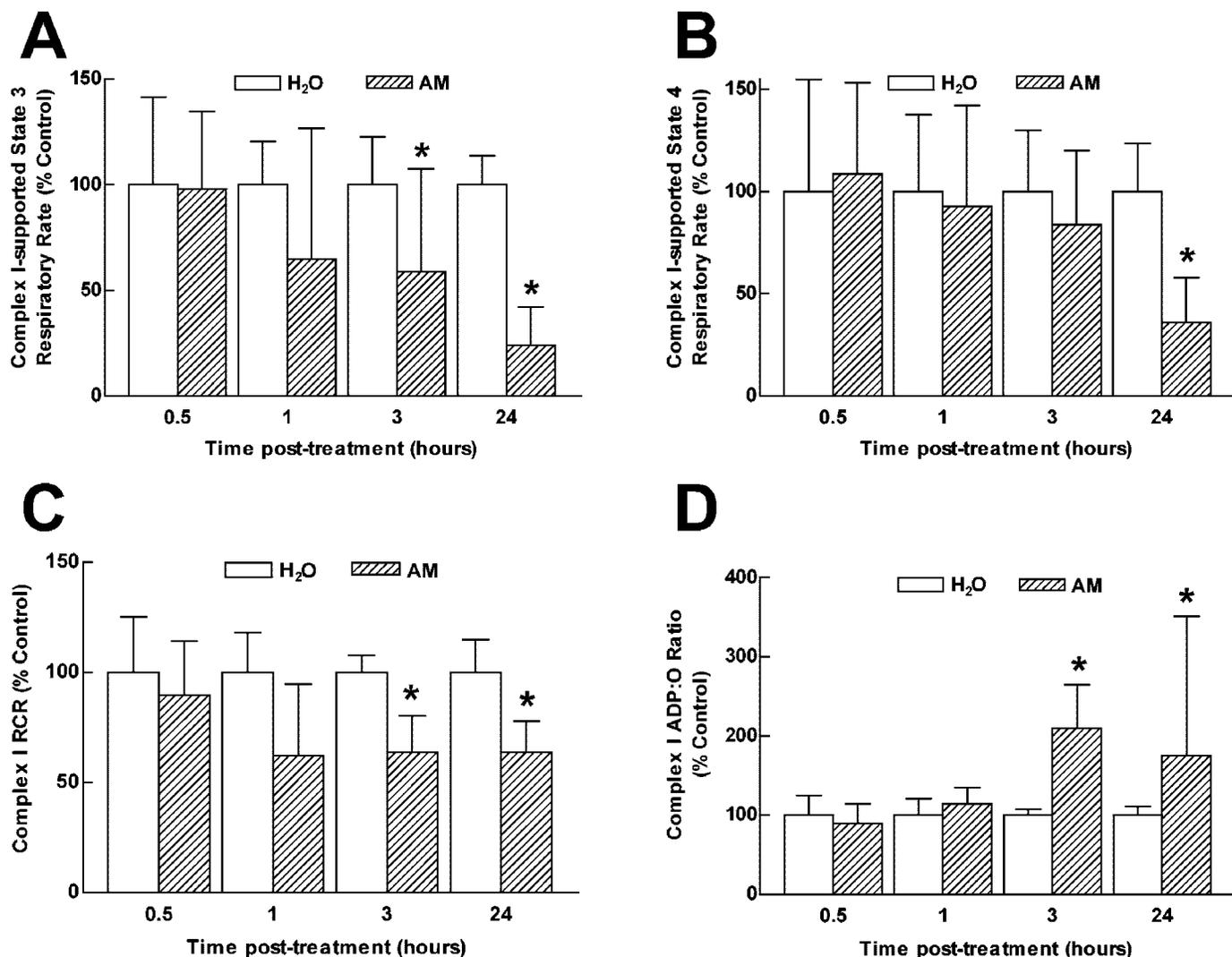
FIG. 6. Effects of intratracheal administration of H_2O (0.1 ml) or AM (1.83 μmol) on complex I supported respiration in lung mitochondria isolated from individual hamsters at various times after dosing. (A) State 3 (active) respiration. (B) State 4 (resting) respiration. (C) Respiratory control ratios (RCR). (D) ADP:O ratios. *Significant difference from the H_2O-treated group at the same time point, $p < 0.05$ ($n = 3$–4).

due to damage to the alveolar–capillary barrier, resulting in leakage of blood-borne protein into the airspaces, the source(s) of the increased BALF LDH activity that was detected at the early time points following intratracheal AM is unknown. Thus, it is possible that a certain population of epithelial cells (and/or alveolar macrophages) was initially damaged as a result of the immediate exposure to a high concentration of AM following intratracheal administration. This could have resulted in rapid cell membrane destabilization unrelated (or related) to mitochondrial effects and leakage of LDH that was detected very early following AM dosing. The subsequent redistribution and accumulation of AM within a larger population of cells might then have resulted in the whole lung mitochondrial dysfunction profile and further increases of BALF LDH activity that were observed at later time points. The trend toward higher BALF LDH activity at later time

points, which could have resulted from cell damage or death due at least in part to the mitochondrial dysfunction that was observed, supports this possibility. Alternatively, mitochondrial dysfunction in a specific cell population or subset of cells that comprise a small percentage of the whole lung cell population could have occurred very early after intratracheal AM, but it would have been undetectable by analysis of whole lung mitochondrial function.

In summary, pirfenidone exhibits anti-fibrotic activity in the hamster model of AM-induced pulmonary fibrosis. Inhibition of relatively early processes in AM lung injury likely contributes to its anti-fibrotic effect, but prevention of AM-induced mitochondrial dysfunction, alveolar macrophage death, and acute neutrophilic inflammation do not appear to be mechanisms of pirfenidone action. Rather, pirfenidone-induced decreased eosinophilia following AM administration may be a
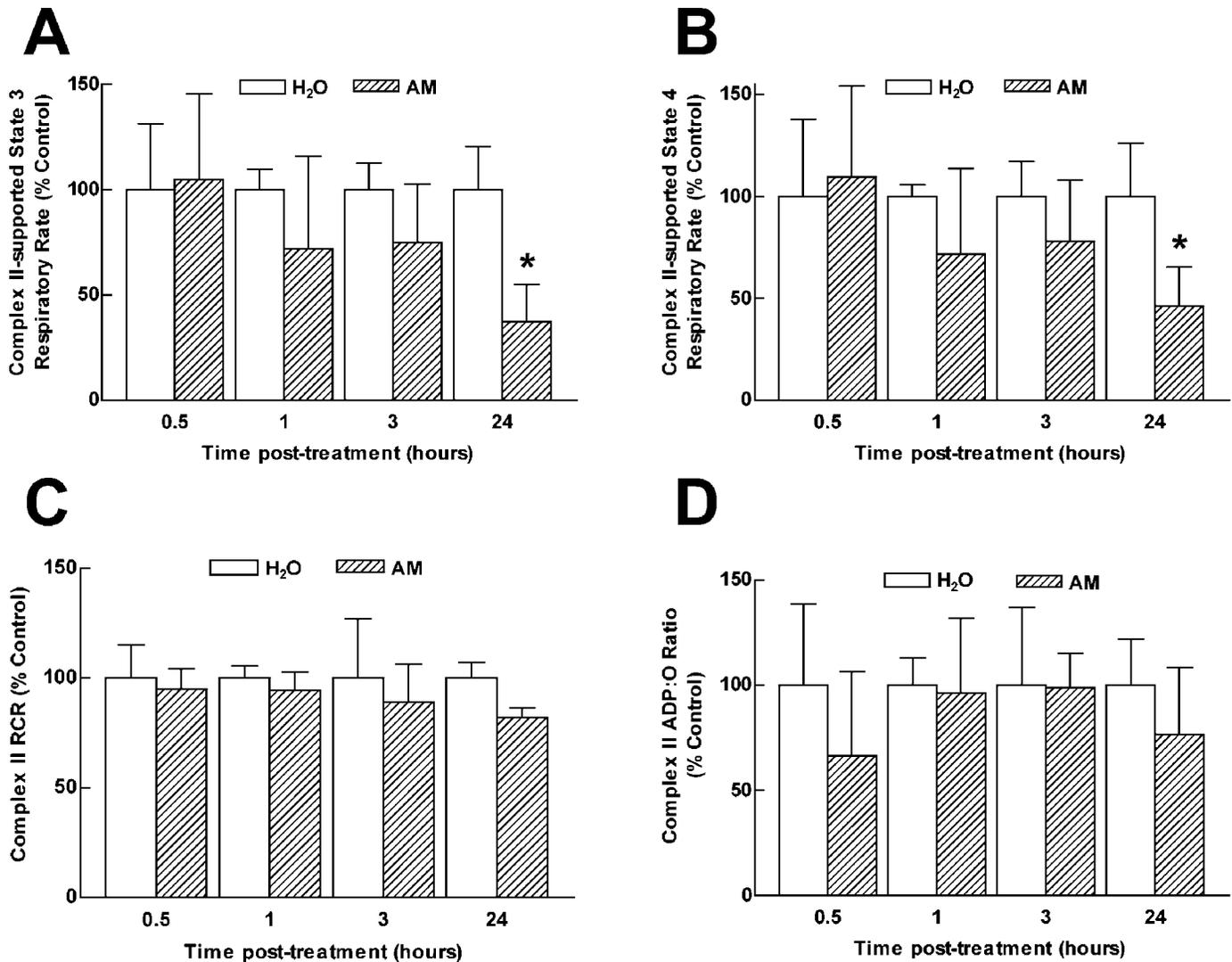
FIG. 7. Effects of intratracheal administration of H_2O (0.1 ml) or AM (1.83 μmol) on complex II supported respiration in lung mitochondria isolated from individual hamsters at various times after dosing. (A) State 3 (active) respiration. (B) State 4 (resting) respiration. (C) Respiratory control ratios (RCR). (D) ADP:O ratios. *Significant difference from the H_2O-treated group at the same time point, $p < 0.05$ ($n = 3$–4).

TABLE 2
Respiratory Parameters of Lung Mitochondria[a]

Diet	Tx	Complex I supported respiration rate (nmol O/min/mg protein)				Complex II supported respiration rate (nmol O/min/mg protein)			
		State 3	State 4	RCR	ADP:O	State 3	State 4	RCR	ADP:O
Control	H_2O	54.8 ± 12.5	21.7 ± 5.9	2.57 ± 0.25	4.11 ± 0.32	75.6 ± 9.6	58.0 ± 10.0	1.54 ± 0.41	1.40 ± 0.52
PD	H_2O	60.6 ± 16.7	21.8 ± 4.9	2.76 ± 0.15	3.55 ± 0.44	86.4 ± 17.3	63.6 ± 13.5	1.36 ± 0.06	1.50 ± 0.14
Control	AM	24.2 ± 16.2^b	13.9 ± 7.4	1.73 ± 0.20^b	6.18 ± 0.82^b	56.8 ± 15.7	45.3 ± 13.6	1.37 ± 0.24	1.38 ± 0.22
PD	AM	28.4 ± 15.5^b	15.3 ± 3.7	1.77 ± 0.51^b	5.73 ± 1.33^b	67.8 ± 10.5	51.0 ± 6.1	1.31 ± 0.21	1.59 ± 0.24

[a]Mitochondria were isolated 3 h following intratracheal administration of water (H_2O; 0.1 ml) or amiodarone (AM; 1.83 μmol) from hamsters that were maintained on a control or pirfenidone (PD)-supplemented (0.5% w/w in chow) diet for 3 days prior to intratracheal treatments.

[b]Significant difference from both H_2O-treated groups, $p < 0.05$ (n=3–4 per data point).

contributing factor, while attenuation of AM-induced overexpression of the potent pro-fibrotic cytokine TGF-β_1 is likely central to the anti-fibrotic activity of pirfenidone in this model. Considering the widespread clinical use of AM (Nolan *et al.*, 1998) and the fact that lung fibrosis is a serious adverse effect associated with its use (Pollak, 1999), the possibility that pirfenidone will prove useful in the clinical treatment of AM-induced pulmonary fibrosis deserves further attention. Studies focusing on prevention or attenuation of mitochondrial dysfunction may also lead to the design of novel therapeutic interventions for the prevention of this condition.

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