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Attenuation of Amiodarone-Induced Pulmonary Fibrosis by Vitamin E Is Associated with Suppression of Transforming Growth Factor-β_1 Gene Expression but Not Prevention of Mitochondrial Dysfunction

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ABSTRACT

Amiodarone (AM) is an efficacious antidysrhythmic agent that can cause numerous adverse effects, including potentially life-threatening pulmonary fibrosis. The current study was undertaken to investigate potential protective mechanisms of vitamin E against AM-induced pulmonary toxicity (AIPT) in the hamster. Three weeks after intratracheal administration of AM (1.83 μmol), increased pulmonary hydroxyproline content and histological damage were observed, indicative of fibrosis. These effects were preceded by increased pulmonary levels of transforming growth factor (TGF)-β_1 mRNA at 1 week post-AM, which remained elevated 3 weeks post-AM. Dietary supplementation with vitamin E resulted in rapid pulmonary accumulation of the vitamin, and prevention of AM-induced increases in TGF-β_1, hydroxyproline, and histological damage. Although dietary supplementation also markedly elevated lung mitochondrial vitamin E content, it did not attenuate AM-induced inhibition of mitochondrial respiration or disruption of mitochondrial membrane potential in vitro, or lung mitochondrial respiratory inhibition resulting from in vivo AM administration. These results suggest that vitamin E reduces the extent of pulmonary damage after AM administration via down-regulating TGF-β_1 overexpression but that it does not modify AM-induced mitochondrial dysfunction, a potential initiating event in AIPT.

Pulmonary toxicity is an adverse effect of great concern in patients on amiodarone (AM) pharmacotherapy (Pollak, 1999). This is primarily due to the potential for development of pulmonary fibrosis, a condition for which there is currently no effective treatment and for which patient prognosis is poor (Phan, 1995). The pathogenesis of AM-induced pulmonary toxicity (AIPT) has not been elucidated and may involve numerous processes (Massey et al., 1995; Reasor and Kacew, 1996). Several in vitro studies have demonstrated deleterious effects of AM on mitochondrial structure and function (Fromenty et al., 1990; Yasuda et al., 1996; Card et al., 1998). Furthermore, AM and its primary metabolite *N*-desethylamiodarone (DEA) cause disruption of mitochondrial membrane potential and decrease cellular ATP levels before cell death in freshly isolated hamster lung cells, with DEA being more potent than AM (Bolt et al., 2001a). Thus, mitochondrial dysfunction induced by AM and DEA may be critical to initiation of AM pulmonary cytotoxicity that precedes fibrosis.

Vitamin E has been reported to decrease AM-induced cytotoxicity in cultured pulmonary (Futamura, 1996) and non-pulmonary cells (Kachel et al., 1990; Ruch et al., 1991), whereas other antioxidant treatments were ineffective. Additionally, cell type-selective protection against AM-induced cytotoxicity by vitamin E in isolated hamster lung cells has been observed (Bolt et al., 2001b). In the in vivo hamster model of AIPT, we showed that dietary vitamin E supplementation substantially reduced the extent of pulmonary collagen deposition and histological damage after intratracheal AM administration (Card et al., 1999). To date, the mechanism(s) of protection of vitamin E against AIPT has not been determined.

Recognized primarily for its free radical scavenging and chain-breaking antioxidant properties, vitamin E has re-

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ABBREVIATIONS: AM, amiodarone; AIPT, AM-induced pulmonary toxicity; DEA, *N*-desethylamiodarone; TGF, transforming growth factor; RCR, respiratory control ratio; ADP:O, ratio of adenosine diphosphate to oxygen.

cently gained attention for exerting several effects that cannot be attributed solely to antioxidant activity (Azzi and Stocker, 2000). Protective effects of vitamin E against mitochondrial damage have been reported (Augustin et al., 1997; Padma and Setty, 1997). Furthermore, in models of fibrosis, vitamin E down-regulates expression of proinflammatory and profibrotic genes (Parola et al., 1992; Chojkier et al., 1998). One of these, transforming growth factor (TGF)-β_1, is a critical mediator of fibrosis (Cooper, 2000; Sime and O'Reilly, 2001). Targeting this cytokine directly, or the steps involved in its activation or signaling, may prove to be an effective therapeutic strategy against fibrosis.

The current study was undertaken to investigate potential protective mechanisms of vitamin E against AIPT in the hamster model. The pulmonary accumulation of vitamin E after extended dietary supplementation was determined, and the effects of this supplementation on AM- and DEA-induced mitochondrial dysfunction and on AM-induced alterations in TGF-β_1 mRNA levels and pulmonary fibrosis were examined.

Materials and Methods

Materials. Chemicals and reagents were obtained as follows: DEA was generously donated by Wyeth-Ayerst (Princeton, NJ); 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 Purina Laboratory Rodent Chow no. 5001 enriched with vitamin E (*dl*-α-tocopherol acetate, 500 IU/kg) from Ren's Feed and Supplies Ltd. (Oakville, ON, Canada). Unless otherwise stated, all other chemicals and reagents were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO).

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, QC, Canada) were housed in group plastic cages with chipped hardwood bedding, using a 12-h light/dark cycle. Hamsters were randomly assigned to either the control diet (Purina Laboratory Rodent Chow no. 5001, containing 49 IU of *dl*-α-tocopherol acetate/kg) or the vitamin E-enriched diet (supplemented to 500 IU of *dl*-α-tocopherol acetate/kg), and were maintained on their respective diets and water ad libitum for up to 6 weeks before use.

For intratracheal drug administration, AM was dissolved in distilled H_2O at 60°C and allowed to cool to room temperature before intratracheal instillation as described previously (Card et al., 1999). After treatment, animals were returned to their respective diets and water ad libitum for the remainder of the study period. Although AM administration by the intratracheal route differs from systemic drug delivery during clinical pharmacotherapy, it is the only route of AM administration that has been demonstrated to cause pulmonary toxicity, including fibrosis in experimental animals (Massey et al., 1995). We (Daniels et al., 1989; Card et al., 1999) and others (Cantor et al., 1985; Wang et al., 1992) have shown that intratracheal administration of 1.83 μmol (1.25 mg) of AM hydrochloride to the hamster reliably produces pulmonary toxicity, including patchy interstitial fibrosis and cellular infiltration into the interstitial spaces and alveoli, which morphologically resembles that observed clinically in patients with AIPT.

Preparation of Lung Tissue. At 7 or 21 days postdosing, each animal was killed by injection of sodium pentobarbital (300 mg/kg i.p.), thoracotomy was performed, and the trachea was exposed and cannulated. The right bronchus was ligated, and the right lung 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 of H_2O for 1 h. The trachea was then ligated, and the lung was removed and placed in formalin. Sections from 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 previously (Card et al., 1999), with the evaluator unaware of the animal treatments. The disease index, which quantified septal thickening and cellular infiltration into the interstitial spaces and alveoli, was calculated as the mean of the values for equal numbers of sections taken from upper, middle, and lower lung from each animal.

Hydroxyproline Determination. Lung content of hydroxyproline, an amino acid found almost exclusively in collagen (Lindenschmidt and Witschi, 1985) and routinely used as an indicator of collagen deposition, 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. After neutralization with 2.75 ml of 10 M NaOH, hydroxyproline content was determined in duplicate for each sample 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 plasmid was isolated using a QIAprep Spin miniprep kit (QIAGEN, Valencia, CA) and was subjected to endonuclease restriction digestion with *Bam*HI for 1 h at 37°C. After resolution in a 1% (w/w) agarose gel, the 0.75-kilobase insert was purified with a QIAGEN gel extraction kit. The 18S rRNA DECA probe template was obtained from Ambion (Austin, TX). Radiolabeled cDNA probes (25 ng of template DNA per probe) were generated with [α-^{32}P]dCTP, using a random primer labeling kit (Invitrogen, Burlington, ON, Canada). Unincorporated [α-^{32}P]dCTP was removed using Quickspin Sephadex columns (Roche Applied Science, Laval, QC, Canada).

Total RNA Isolation and Hybridization Analyses. Isolation of total RNA from aliquots of frozen right lung tissue (~30 mg) was carried out using a QIAGEN RNeasy mini kit. Total RNA (10 μg/lane) was electrophoresed through 1% (w/v) agarose/3% (v/v) formaldehyde gels buffered with 50 mM 3-[*N*-morpholino]propanesulfonic acid (pH 7.0) and transferred overnight to Hybond nylon membranes (Amersham Biosciences, Oakville, ON, Canada) via capillary blotting. Membranes were UV cross-linked with a CL-1000 ultraviolet crosslinker (Diamed, Mississauga, ON, Canada), and prehybridization was carried out at 68°C for 1 h in 8.0 ml of QuikHyb hybridization buffer (Stratagene, La Jolla, CA). Denatured, ^{32}P-labeled TGF-β_1 cDNA probe was added (2.4×10^7 counts/min), and hybridization was carried out at 68°C for 4 h. After hybridization, membranes were washed in low- and high-stringency buffers to eliminate nonspecific binding. Hybridized probe was visualized and quantitated using a STORM 820 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Membranes were stripped of bound probe, and the hybridization procedure was repeated with 18S rRNA cDNA probe. The band intensity of TGF-β_1 mRNA was divided by the band intensity of 18S rRNA to correct for variations in the quantity of RNA loaded.

Determination of Vitamin E Content. After 1 to 6 weeks on the control or vitamin E diet, pulmonary vitamin E content was determined by the method of Taylor et al. (1976) as described previously (Card et al., 1999). Lung mitochondrial vitamin E content was determined after 6 weeks on the control or vitamin E diet. Lung mitochondria were isolated from individual hamsters (see below), frozen in liquid nitrogen, and stored at −80°C. Aliquots were analyzed for protein content by the method of Lowry et al. (1951) using bovine serum albumin as the standard, and for total tocopherol content by the method of Taylor et al. (1976).

Isolation of Whole Lung Mitochondria. After 6 weeks on the control or vitamin E diet, hamsters were killed by injection of sodium

pentobarbital (300 mg/kg i.p.). After perfusion in situ with ice-cold 0.9% saline solution, lungs were removed, blotted dry, and weighed. 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 3-[*N*-morpholino]propanesulfonic acid, and 2% (w/v) fatty acid-free bovine serum albumin (pH 7.2). Solutions were kept ice-cold, and all manipulations were performed on ice or at 4°C. To isolate sufficient lung mitochondria for a single experiment to determine the effects of in vitro exposure to AM and DEA, four pairs of hamster lungs were pooled. To determine the effects of in vivo AM administration on lung mitochondrial respiratory function, mitochondria were isolated from individual hamsters 3 h post-treatment. Aliquots of mitochondrial suspensions were used for determination of protein content by the method of Lowry et al. (1951).

Polarographic Measurement of Oxygen Consumption. Oxygen consumption of isolated lung mitochondria was measured at 30°C as described previously (Card et al., 1998). The effect of in vitro exposure to AM or DEA on state 4 respiration supported by complexes I (glutamate dehydrogenase) and II (succinate dehydrogenase) of the mitochondrial electron transport chain was examined by adding these drugs at least 2 min after the total expenditure of 0.2 mM ADP. To determine the effect of in vivo AM administration on oxygen consumption, respiration supported by complexes I and II was monitored in mitochondria isolated 3 h after intratracheal administration of AM, without additional in vitro drug addition. Respiratory control ratios (RCRs) and ADP:O ratios were calculated as indicators of the integrity of mitochondrial respiratory function.

Monitoring Mitochondrial Membrane Potential. Membrane potential of isolated lung mitochondria was determined by safranine fluorescence as described previously (Fromenty et al., 1990; Card et al., 1998). Mitochondria (1–2 mg of protein) were incubated with succinate (10 mM, in the presence of 3.0 μM rotenone) for 5 min at 30°C, to establish a high initial membrane potential. The total change in safranine fluorescence (which is inversely proportional to membrane potential) and the rate of change of fluorescence were determined for 10 min after addition of AM or DEA using a PerkinElmer LS-5B luminescence spectrometer (excitation 510 nm, emission 570 nm, 5-nm slit widths).

Statistical Analyses. Data are expressed as mean ± standard deviation for each experimental group. Statistical comparisons among treatment groups were performed by randomized design one- or two-way analysis of variance followed by Newman-Keuls post hoc test for more than two groups, or by unpaired Student's t test for two groups. Histological disease index data underwent arcsine transformation before statistical analysis, as described by Sokal and Rohlf (1973) for percentage data. In all cases, statistical significance was defined as $p < 0.05$.

Results

Vitamin E Content. The vitamin E-enriched diet increased total lung vitamin E content by 114% after 1 week, and levels remained elevated for the duration of the 6-week treatment period (Fig. 1A). After 6 weeks, lung mitochondrial vitamin E content was increased by 176% in the vitamin E group compared with the control diet group (Fig. 1B).

Mitochondrial Respiratory Function and Membrane Potential. Calculated RCRs and ADP:O ratios for complex I- and II-supported respiration did not differ between mitochondria isolated from the control and vitamin E diet groups. The RCR values for control and vitamin E diet groups were 2.94 ± 0.47 and 2.84 ± 0.42 for complex I and 1.51 ± 0.11 and 1.52 ± 0.14 for complex II. The ADP:O ratios for control and vitamin E groups were 4.37 ± 0.45 and 4.12 ± 0.32 for complex I and 2.18 ± 0.46 and 2.46 ± 0.86 for complex II. No

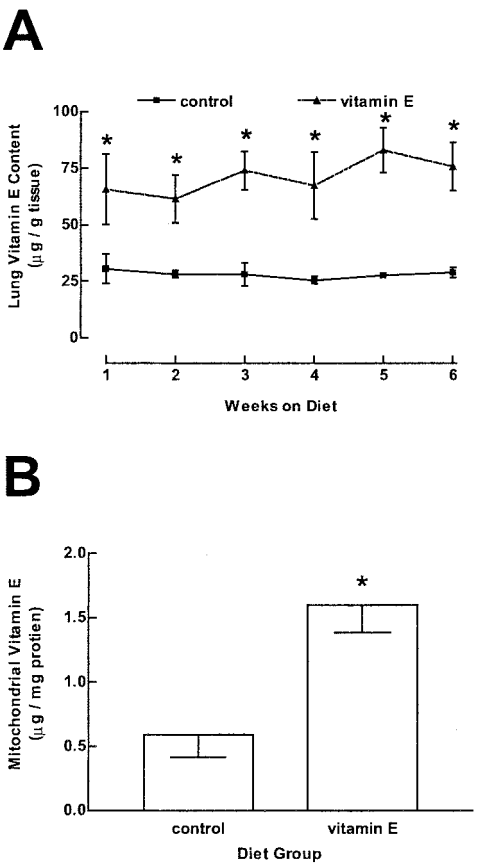

Fig. 1. Vitamin E content in hamster lungs (A) and lung mitochondria (B) after dietary supplementation. A, significant difference (*) from control diet group, $p < 0.05$ (n = 3/data point). B, significant difference (*) from control diet group, $p < 0.05$ (n = 3/group).

significant differences were found between the diet groups for baseline state 4 oxygen consumption rates (i.e., before drug additions were made; data not shown). Mitochondria from both diet groups demonstrated tight coupling at complex I (RCR > 2.50), but not at complex II (RCR ≤ 1.50), similar to our previous report (Card et al., 1998).

When added directly to isolated lung mitochondria, AM and DEA (≥100 μM) significantly inhibited state 4 respiration supported by complex I (by 65 to 95%) and complex II (by 65 to 100%) (Fig. 2). Maintenance on the vitamin E-enriched diet did not alter these effects of AM and DEA. Effects of AM and DEA on state 3 respiration could not be determined, because preincubation of lung mitochondria with these drugs at all concentrations tested (50–400 μM) resulted in complete abolition of oxygen consumption in response to the addition of respiratory substrates. Mitochondrial membrane potential was decreased to a similar maximal extent by AM and DEA (Fig. 3A), although the rate of decrease was much more rapid for higher concentrations of DEA (Fig. 3B). Exposure to drug vehicle (distilled H_2O) did not affect membrane potential (data not shown), and neither the maximal decreases nor the rates of decrease caused by AM and DEA were affected by dietary vitamin E supplementation.

Intratracheal AM administration resulted in inhibition of state 3 respiration supported by complex I at 3 h post-treatment, reflected by decreased RCR and increased ADP:O ratio (Fig. 4, A and B), indicative of uncoupling of respiration. Respiration supported by complex II was not affected by AM

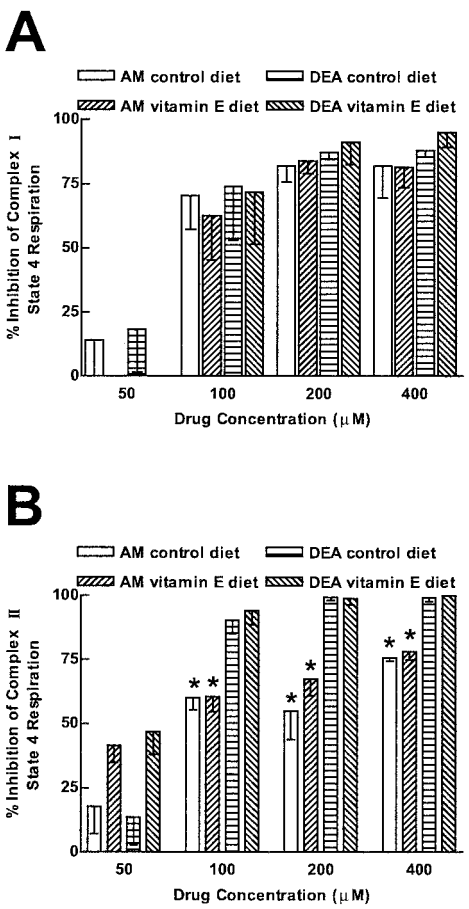

Fig. 2. Effect of AM or DEA on state 4 (resting) oxygen consumption supported by complex I (A) and complex II (B) in isolated lung mitochondria from control and vitamin E-supplemented hamsters after 6 weeks on the specified diets. Significant difference (*) from DEA groups within the same drug concentration ($p < 0.05$; $n = 4$/data point).

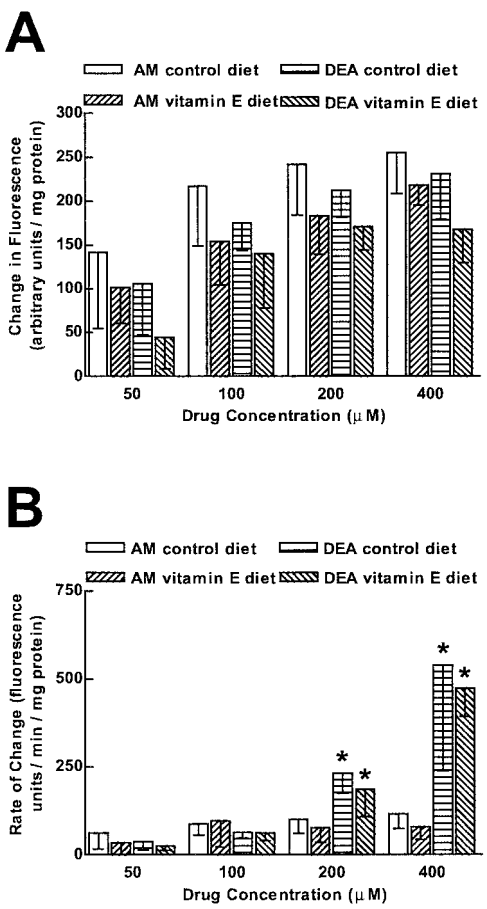

Fig. 3. Effect of AM or DEA on maximal change in membrane potential, obtained after 10 min of incubation (A), and rate of change of membrane potential (B) in isolated lung mitochondria from control and vitamin E-supplemented hamsters after 6 weeks on the specified diets. A, no differences were found between diet groups for equimolar concentrations of AM or DEA, or between equimolar concentrations of AM and DEA within the same diet groups. B, significant difference (*) from equimolar concentrations of AM and from lower concentrations of DEA within respective diet groups, $p < 0.05$ ($n = 3$–5/data point).

treatment (data not shown), although a relatively low RCR (<1.50) was found for vehicle-treated animals, suggesting that an uncoupling effect may not have been observed at complex II due to weak initial coupling at this complex. Maintenance on the vitamin E diet did not alter the lung mitochondrial respiratory effects caused by intratracheal AM administration (Fig. 4, A and B).

Pulmonary Histopathology and Hydroxyproline Content. Intratracheal AM resulted in increased histopathological damage 21 days post-treatment, as indicated by cellular infiltration and thickening of the interstitial spaces (Figs. 5 and 6A), and this damage was prevented by dietary vitamin E supplementation. Intratracheal AM did not alter hydroxyproline levels at 7 days (data not shown), but did result in a significant (23%) increase at 21 days (Fig. 6B). This increase was prevented by dietary vitamin E supplementation.

Pulmonary TGF-β_1 Gene Expression. Northern blot analysis revealed 61 and 300% increases in the level of TGF-β_1 mRNA resulting from AM at 7 and 21 days post-treatment, respectively (Fig. 7, A and B). Dietary supplementation with vitamin E prevented the AM-induced increase of TGF-β_1 mRNA at both time points.

Discussion

Considerable evidence both in vitro and in vivo suggests that vitamin E may be beneficial against the adverse effects of AM (Kachel et al., 1990; Ruch et al., 1991; Futamura, 1996; Card et al., 1999). In the current study, pulmonary accumulation of vitamin E after initiation of supplementation was rapid, and total lung levels were more than doubled compared with those of the control diet group after 1 week. Interestingly, continued supplementation for a total of 6 weeks did not result in further accumulation of vitamin E, indicating that maximal pulmonary content is attained rapidly after initiation of supplementation. Hamsters administered AM intratracheally after 6 weeks of vitamin E supplementation were protected against increases in pulmonary hydroxyproline content and histological damage indicative of fibrosis 21 days post-treatment.

Pulmonary expression of TGF-β_1, a major regulator of extracellular matrix components, including collagens, is up-regulated in several rodent models of pulmonary fibrosis, including a rat model of AM-induced pulmonary fibrosis (Yi et al., 1996; Iyer et al., 1999; Chung et al., 2001). In the present study, significant up-regulation of TGF-β_1 mRNA expression was observed 7 and 21 days after AM treatment, whereas hydroxyproline content was not elevated until 21 days, supporting a central role for this cytokine in the tissue

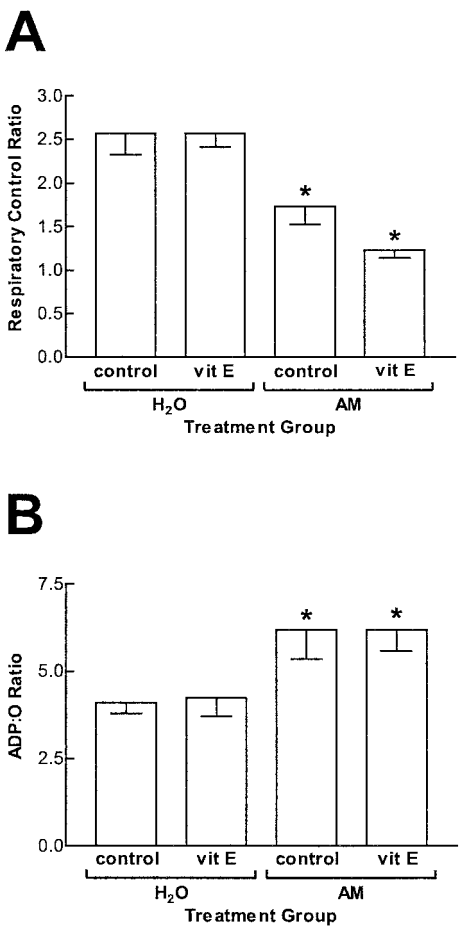

Fig. 4. Effect of intratracheal AM administration on RCRs (A) and ADP:O ratios (B) in lung mitochondria 3 h post-treatment. A, significant difference (*) from H_2O-treated groups, $p < 0.05$ ($n = 3$/data point). B, significant difference (*) from H_2O-treated groups, $p < 0.05$ ($n = 3$/data point).

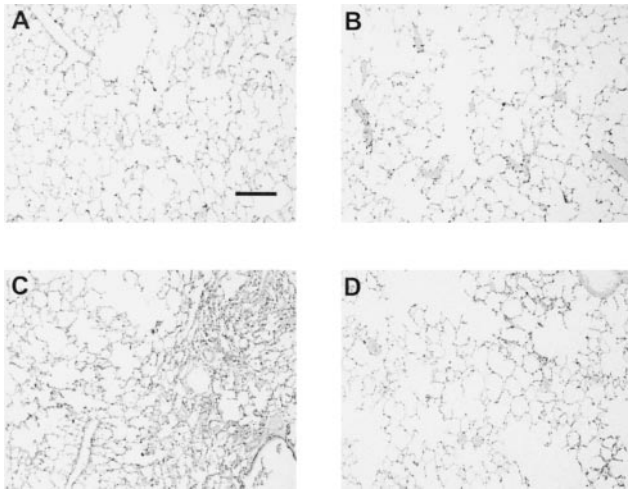

Fig. 5. Photomicrographs of hamster lungs 21 days after intratracheal administration of AM or distilled H_2O with or without dietary vitamin E supplementation for 6 weeks before and continuously after treatment. A, control diet + H_2O. B, vitamin E diet + H_2O. C, control diet + AM. D, vitamin E diet + AM. Slides were stained with hematoxylin and eosin, and the column represents 100 μm in all cases.

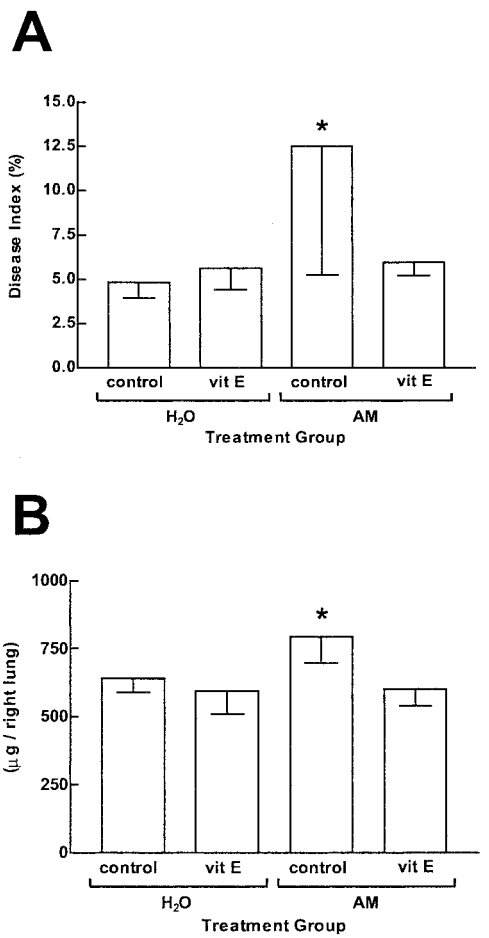

Fig. 6. Effect of intratracheal AM administration on histological disease index values (A) and hydroxyproline content (B) of hamster lungs 21 days after treatment, with or without dietary vitamin E supplementation for 6 weeks before and continuously after treatment. Significant difference (*) from all other groups, $p < 0.05$ ($n = 4$–7/data point).

remodeling that occurs during the course of AM-induced pulmonary fibrosis in the hamster. TGF-β_1 up-regulation after AM administration was suppressed by vitamin E at both time points examined, as were the increases in pulmonary hydroxyproline content and histological damage at 21 days, effects consistent with prevention of TGF-β_1 overexpression after AM administration being an essential component of the protective effect of vitamin E.

Although a role for oxidative stress in the development of AIPT has been proposed, considerable evidence refutes the involvement of reactive oxygen species (Kachel et al., 1990; Ruch et al., 1991; Leeder et al., 1994). Nonetheless, the protective effect of vitamin E against AM toxicities in vitro and in vivo suggests a role for free radical generation in the etiology of AIPT. Furthermore, Wang et al. (1992) reported increased malondialdehyde levels at the time of maximal fibrosis in the hamster model of AIPT, although a causal role for lipid peroxidation in the fibrotic response to AM administration was not established. End products of lipid peroxidation such as 4-hydroxy-2,3-nonenol up-regulate TGF-β_1 gene expression (Leonarduzzi et al., 1997), and vitamin E decreases TGF-β_1 gene expression in other models of fibrosis (Parola et al., 1992). Lipid peroxidation resulting from AM treatment may cause increased TGF-β_1 gene expression, and hence the effectiveness of vitamin E against AIPT may be

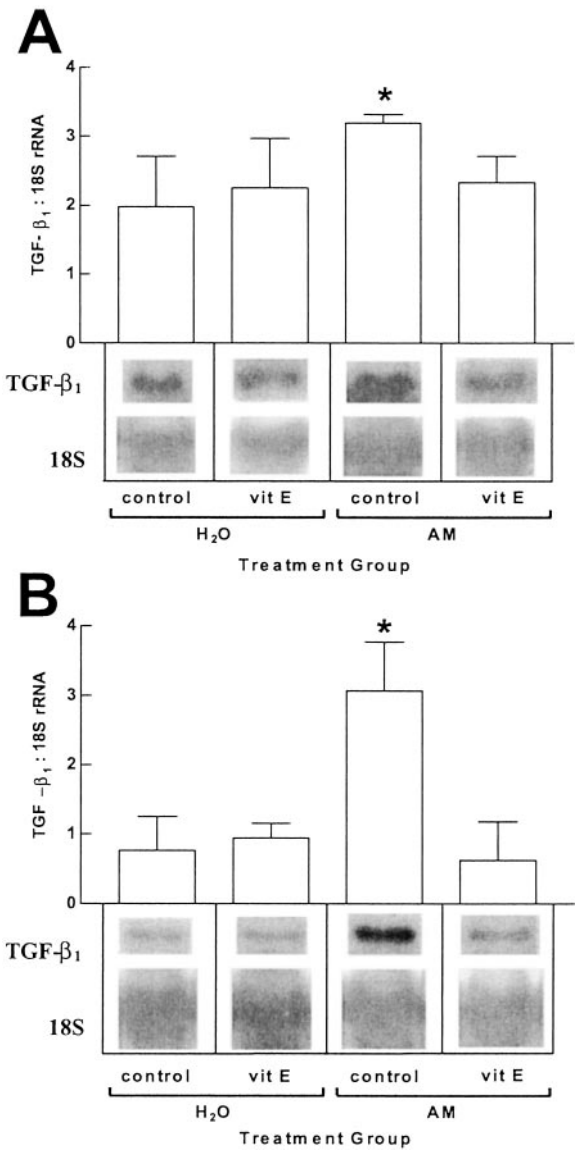

Fig. 7. Pulmonary TGF-β_1 mRNA content 7 days (A) and 21 days (B) after intratracheal administration of AM or distilled H_2O with or without dietary vitamin E supplementation for 6 weeks before and continuously after treatment. Significant difference (*) from all other groups, $p < 0.05$ (n = 3–7/data point).

due to decreasing or preventing lipid peroxidation product effects. However, we did not find evidence of lipid peroxidation in isolated hamster lung mitochondria or whole cells exposed to AM in vitro (Card et al., 1998; Bolt et al., 2001a). Alternatively, down-regulation of TGF-β_1 gene expression by vitamin E could be mediated through the recently described tocopherol-associated protein (Yamauchi et al., 2001).

Mitochondrial dysfunction is a well documented effect of AM in several experimental systems, with both structural and functional alterations being reported (Fromenty et al., 1990; Yasuda et al., 1996; Card et al., 1998). Furthermore, we observed a temporal relationship between AM- and DEA-induced disruptions of mitochondrial membrane potential, cellular ATP depletion, and ensuing cytotoxicity in freshly isolated hamster lung cells (Bolt et al., 2001a). As such, mitochondrial dysfunction is a candidate mechanism for initiating AM-induced cytotoxicity that elicits the fibrotic response in the lung. The mitochondrial effects of DEA were more pronounced and/or more rapid than those of AM, consistent with the greater cytotoxic and fibrogenic potency of DEA relative to AM (Daniels et al., 1989; Bolt et al., 2001a). The observation of inhibition of mitochondrial respiratory function 3 h after intratracheal administration of AM is consistent with the time course observed for mitochondrial dysfunction in AM-induced pulmonary cytotoxicity in vitro (Bolt et al., 2001a) and supports the proposed role of mitochondrial dysfunction in the early stages of AIPT. In preliminary studies, intratracheal AM administration did not alter lung mitochondrial function before 3 h post-treatment (data not shown). Thus, this time point was chosen to investigate the effect of vitamin E on mitochondrial dysfunction after AM administration. Comparative effects of an equimolar dose of DEA on mitochondrial function could not be determined, because close to 100% mortality occurred in hamsters within 3 h after intratracheal administration, despite the use of various vehicles and delivery volumes. However, given the lack of effect of vitamin E on mitochondrial dysfunction induced by AM after in vivo administration and by in vitro exposure to AM and DEA, it is unlikely that potential mitochondrial effects induced by DEA administration would be prevented by vitamin E.

Although vitamin E accumulates in mitochondria (Bjorneboe et al., 1990) and protects against toxicant-induced functional damage to mitochondria in other experimental systems (Augustin et al., 1997; Padma and Setty, 1997), the present results indicate that prevention of mitochondrial dysfunction is not likely a mechanism by which vitamin E protects against AIPT. Whether the protective profile of vitamin E in isolated cells (Kachel et al., 1990; Ruch et al., 1991; Futamura, 1996) involves prevention of AM-induced mitochondrial dysfunction is unknown. Although other events contributing to cell injury may occur in concert with or subsequent to mitochondrial dysfunction during AM cytotoxicity (Massey et al., 1995), the evidence for mitochondrial dysfunction as a key initiating event suggests that targeting this occurrence might prove beneficial against AM-induced cytotoxicity in several cell types. Thus, given the current data, the protection offered by vitamin E in vitro and in vivo may be the result of several effects, including decreased cellular AM accumulation, membrane stabilization, altered profibrotic gene expression, and free radical scavenging. The lack of effect of vitamin E on AM- and DEA-induced mitochondrial dysfunction in the present study may be related to its distribution within mitochondria, reported to be primarily within the outer membrane (Lang et al., 1986; Thomas et al., 1989) and therefore not closely associated with the respiratory chain complexes on the inner membrane. It is likely that direct interaction with the respiratory chain complexes, rather than secondary effects due to another event such as lipid peroxidation, is responsible for the adverse mitochondrial effects of AM and DEA, given their rapidity and the lack of lipid peroxidation by-products detected after in vitro exposure of mitochondria to AM (Card et al., 1998). Increasing inner membrane vitamin E content (Smith et al., 1999) might allow for enhanced interaction with AM and DEA, or with radical species produced from them, to decrease respiratory complex inhibition.

In conclusion, the present study reveals that vitamin E is rapidly accumulated in lung tissue after dietary supplemen-

tation and confirms that an increased level of this antioxidant in lung can prevent AM-induced pulmonary fibrosis, an adverse effect of clinical concern (Pollak, 1999). Up-regulation of TGF-β_1 gene expression was observed in AM-treated hamsters before, and at the time of maximal lung injury, and this effect was ablated by dietary vitamin E supplementation. However, elevated mitochondrial vitamin E content after supplementation did not prevent AM- and DEA-induced mitochondrial dysfunction, a potential initiating event in AIPT.

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