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MECHANISTIC STUDIES OF AMIODARONE-INDUCED PULMONARY TOXICITY

by

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degree of Doctor of Philosophy

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ABSTRACT

Jeffrey W. Card: Mechanistic studies of amiodarone-induced pulmonary toxicity. Ph.D. thesis, Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, Canada, September 2002.

Amiodarone (AM) is an efficacious antidysrhythmic agent that is associated with numerous adverse effects, including potentially life-threatening pulmonary fibrosis. The current research investigated mitochondrial dysfunction as a potential initiating mechanism of AM-induced pulmonary toxicity (AIPT), and assessed the ability of vitamin E and pirfenidone to alleviate AIPT in a hamster model.

In vitro exposure of isolated hamster lung mitochondria to AM or its primary metabolite, *N*-desethylamiodarone (DEA), resulted in significant drug accumulation coinciding with inhibition of respiratory function and collapse of inner mitochondrial membrane potential. The effects of DEA were more pronounced and / or more rapid in onset than those of AM, and induction of lipid peroxidation was not associated with the mitochondrial effects of either drug. Intratracheal administration of AM to hamsters resulted in inhibition of lung mitochondrial respiratory function as early as 1 hour post-dosing, that persisted for up to 24 hours, while indicators of lung cell injury (lactate dehydrogenase (LDH) activity and total protein content in bronchoalveolar lavage fluid) were elevated early after AM dosing. Thus, mitochondrial dysfunction may play a role in initiating lung cell damage that leads to the fibrotic response to AM in the lung.

Dietary supplementation with vitamin E or pirfenidone was effective at decreasing AM-induced pulmonary fibrosis in the hamster, as assessed by hydroxyproline content and histological disease scoring 21 days post-dosing. Both agents decreased pulmonary over-expression of the pro-fibrotic cytokine transforming growth factor (TGF)-β_1 following AM administration, suggesting a possible mechanism of their protective profiles. However, neither vitamin E nor pirfenidone supplementation was able to prevent AM- and DEA-induced mitochondrial dysfunction *in vitro*, or respiratory inhibition in lung mitochondria isolated following *in vivo* AM administration.

In summary, mitochondrial dysfunction likely plays a role in initiating AM-induced lung cell death that leads to pulmonary fibrosis, and DEA is a more potent and rapid inhibitor of mitochondrial function than AM. Both vitamin E and pirfenidone displayed a protective effect against AIPT in the hamster model, mediated at least in part by their ability to down-regulate TGF-β_1 over-expression following AM treatment while not altering AM- and DEA-induced mitochondrial dysfunction. These agents may prove useful in the treatment of clinical AIPT.

Keywords: amiodarone, *N*-desethylamiodarone, mitochondria, vitamin E, pirfenidone, pulmonary toxicity, fibrosis, TGF-β_1

CO-AUTHORSHIP

This research was conducted by the candidate, Jeffrey W. Card, under the supervision of Drs. Thomas E. Massey and William J. Racz.

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Dedicated with love to my mother and father.

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LIST OF ABBREVIATIONS, SYMBOLS, AND CHEMICAL FORMULAS

ad libitum	to it pleases
ADP	adenosine diphosphate
ADP:O	ADP to oxygen utilization ratio during state 3 respiration
AIPT	amiodarone-induced pulmonary toxicity
AM	amiodarone
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
°C	degrees Celsius
Ca^{2+}	calcium ion
CAMIAT	Canadian Amiodarone Myocardial Infarction Arrhythmia Trial
CCl_4	carbon tetrachloride
cDNA	complementary deoxyribonucleic acid
cm	centimetre(s)
CYP450	cytochrome P450 polysubstrate monooxygenase
dCTP	deoxycytidine triphosphate
DEA	*N*-desethylamiodarone
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid

EMIAT	European Myocardial Infarction Amiodarone Trial
ESR	electron spin resonance
et al.	et alia (and others)
etc.	et cetera (and others specifically of the same kind)
g	gram(s)
g	relative centrifugal force
G_i protein	inhibitory guanyl-nucleotide binding protein
GSSG	glutathione, oxidized form
h	hour(s)
HCl	hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazine-2-ethane sulfonic acid
H_2O	water
H_2O_2	hydrogen peroxide
HOO•	hydroperoxyl radical
HPLC	high performance liquid chromatography
H_2SO_4	sulfuric acid
i.e.	id est (that is)
IL	interleukin
im	intramuscular
in situ	"in place"
in vitro	"within a glass" (outside the living body)
in vivo	within the living body
ip	intraperitoneal

it	intratracheal
IU	international units
K^+	potassium ion
KCl	potassium chloride
kg	kilogram(s)
KOH	potassium hydroxide
L	litre(s)
LDH	lactate dehydrogenase
LOO•	lipid alkoxyl radical
LOOH	lipid hydroperoxide
LPO	lipid peroxidation
M	molar
µg	microgram(s)
µl	microlitre(s)
µm	micron(s) or micrometre(s)
µM	micromolar
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MOPS	3-[N-morpholino]propane-sulphonic acid
MPT	mitochondrial permeability transition

mRNA	messenger ribonucleic acid
N	normal
n	number (of experiments or samples)
Na^+	sodium ion
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	nanometre(s)
•OH	hydroxyl radical
O	oxygen
O_2	molecular oxygen
$•O_2^-$	superoxide anion radical
$p<$	probability less than
^{32}P	radioactive phosphorous isotope 32
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
pH	negative logarithm of the hydrogen ion concentration
±	plus or minus
PUFA	polyunsaturated fatty acid(s)
R•	radical
RCR	respiratory control ratio
RNA	ribonucleic acid
RO•	alkoxyl radical
ROO•	peroxyl radical

ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SSC	sodium chloride / sodium citrate solution
TGF	transforming growth factor
TNF	tumour necrosis factor
Type II	alveolar type II cells
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
X	times
<	less than
>	greater than

Chapter 1

GENERAL INTRODUCTION

1.1 STATEMENT OF RESEARCH PROBLEM

Amiodarone (AM, Cordarone®, Wyeth-Ayerst Laboratories), an iodinated benzofuran derivative, is an efficacious antidysrhythmic agent. Associated with AM usage are numerous adverse effects that have historically limited its clinical use to that of a "last resort" drug, although this restriction has significantly lessened over recent years and it is now commonly prescribed. The adverse effect of greatest concern is AM-induced pulmonary toxicity (AIPT), which can progress to potentially fatal pulmonary fibrosis. Currently, no effective therapy exists for the treatment of pulmonary fibrosis, and the prognosis for patients with this condition is poor. While numerous mechanisms have been proposed to be involved in the initiation and progression of AIPT (reviewed in Massey et al. (1995), and Reasor and Kacew (1996)), the etiology of this condition remains unknown. The purpose of the research described in this thesis was twofold; 1) to study the potential involvement of mitochondrial dysfunction induced by AM and its primary metabolite, *N*-desethylamiodarone, as an initiating mechanism of AIPT; and 2) to determine the ability of two novel anti-fibrotic compounds, vitamin E and pirfenidone, to ameliorate pulmonary fibrosis in the hamster model of AIPT. Elucidating the initiating mechanism(s) of AIPT could lead to the development of other antidysrhythmic agents devoid of the adverse pulmonary effects of AM, or to therapies to combat these effects,

while identification of agents that decrease the pulmonary toxicity of AM in an animal model, and mechanisms associated with this protection, may lead to the development of a clinical treatment for this serious condition.

1.2 HISTORY AND THERAPEUTIC USE OF AMIODARONE

First synthesized and developed in the early 1960's as a coronary vasodilator, AM (Figure 1.1) was originally used in Europe as an antianginal agent (Deltour et al., 1970). Its antidysrhythmic properties were recognized in animal studies several years later (Charlier et al., 1969), and the first reports of clinical antidysrhythmic activity of AM in humans were published shortly thereafter (Van Schepdael and Solvay, 1970; Rosenbaum et al., 1976). The use of oral AM for treatment of life-threatening ventricular and supraventricular dysrhythmias was approved in the United States in 1985, and in Canada in 1986. In 1996, AM was considered the best agent for controlling ventricular tachycardia and fibrillation, and for maintaining sinus rhythm in patients with atrial fibrillation (Singh, 1996). New therapeutic strategies, including lower maintenance dosages, allowed the clinical application of AM to expand beyond that of a "last resort" agent, to use in less ill patients who were less likely to develop complications (Pollak, 1998). In 1997, intravenous AM was approved in the United States for use as first line therapy in short-term management of ventricular tachydysrhythmias (Desai et al., 1997).

Recent studies, such as the Canadian Amiodarone Myocardial Infarction Arrhythmia Trial (Cairns et al., 1997) and the European Myocardial Infarction Amiodarone Trial (Julian et al., 1997), examined the usefulness of AM as a prophylactic agent to prevent mortality following myocardial infarction. Results from these trials

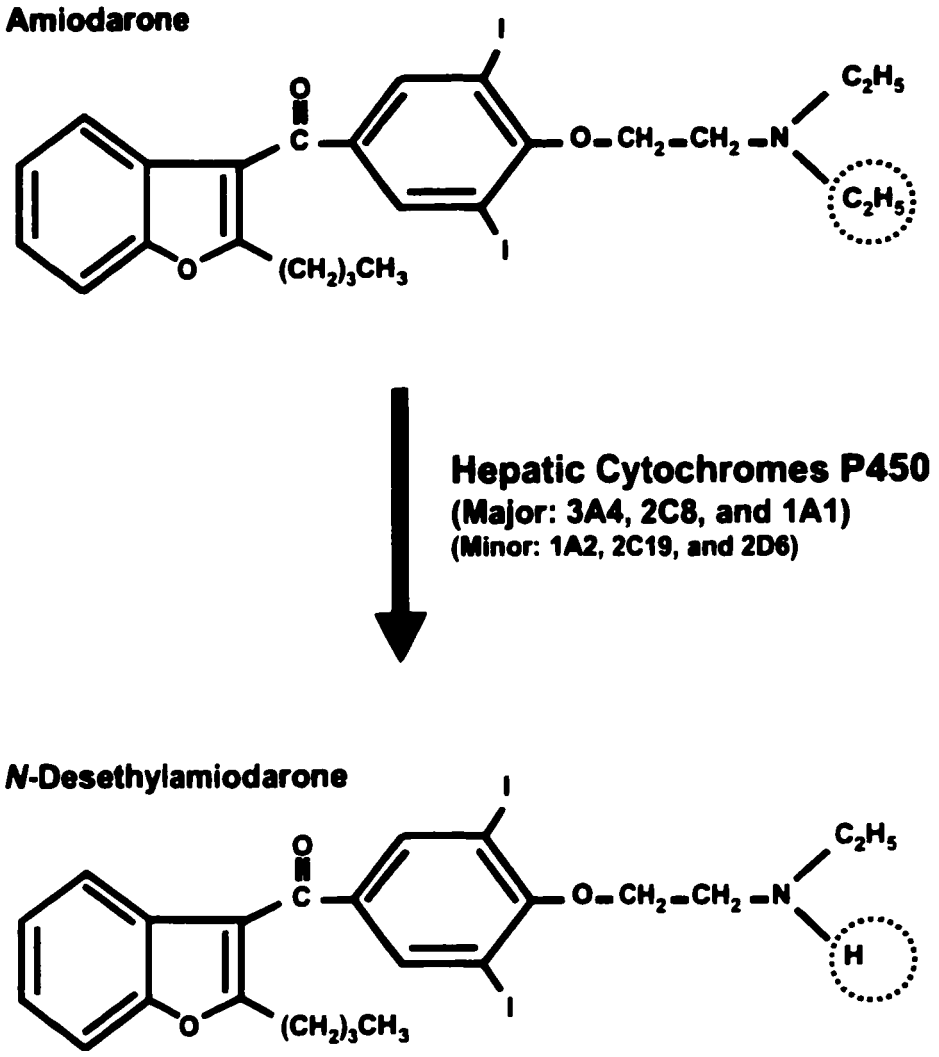


Figure 1.1 Chemical structures of amiodarone and *N*-desethylamiodarone.

were equivocal, but meta-analysis of these and several other trials indicated that AM reduces deaths due to dysrhythmias, suggesting that the individual trials were not large enough to detect an AM effect (Amiodarone Trials Meta- Analysis Investigators, 1997). In 1999, intravenous AM was added to the resuscitation protocol recommended by the American Heart Association for treatment of cardiac arrest (Erich, 2000), thus strengthening its status as an agent with significant potential for prophylactic use against further cardiac complications following myocardial infarction.

1.3 PHARMACODYNAMICS OF AMIODARONE

The effects of AM on the electrophysiology of cardiac tissue is complex, and has not been completely characterized. Historically categorized as a class III antidysrhythmic agent according to the Vaughan-Williams classification system, the broad electrophysiological actions of AM suggest that this may be too simplistic (Singh, 1996). Indeed, AM exerts effects characteristic of all four classes of the Vaughan-Williams system, including Na^+ channel blockade (class I), non-competitive antagonism of adrenergic (α and β) receptors (class II), prolongation of action potential duration via inhibition of K^+ channels (class III), and Ca^{2+} channel blockade (class IV) (Rothenberg et al., 1994). Thus, the antidysrhythmic action of AM is likely due to a combination of several effects on cardiac tissue acting in concert.

A unique characteristic of AM is that, unlike other class III antidysrhythmic agents, it prolongs action potential duration at elevated heart rates, making it an ideal candidate for the management of tachycardias (Nattel and Talajic, 1988). In addition, its selective blockade of inactive Na^+ channels (class I activity) renders it more effective in

depolarized tissue, and ultimately helps to slow the rate of membrane potential depolarization and impulse conduction (Mason et al., 1983). The anti-adrenergic (class II) and Ca^{2+} channel blocking (class IV) activities of AM increase conduction time and refractoriness of the AV node (Nattel et al., 1987), and contribute to the antianginal effect of the drug via dilation of coronary and systemic arteries (Singh and Vaughan Williams, 1970).

1.4 PHARMACOKINETICS OF AMIODARONE

1.4.1 Physicochemical Properties

AM possesses complicated pharmacokinetic properties, and large interindividual differences exist for all aspects of its disposition, including dose-concentration relationships and serum and tissue levels associated with efficacy and toxicity (Podrid, 1995). The unusual pharmacokinetic features of AM have been attributed in part to the amphiphilic nature of the molecule, as it possesses both hydrophobic (benzofuran and di-iodinated benzene) and hydrophilic (tertiary amine) regions (Bonati et al., 1984). As it is highly lipophilic, AM accumulates extensively in lipid-rich membranes and tissues (Chatelain and Laruel, 1985), which contributes significantly to its complicated pharmacokinetic profile.

1.4.2 Absorption and Bioavailability

Absorption of AM from the gastrointestinal tract is slow and incomplete following oral administration (Andreasen et al., 1981; Holt et al., 1983). Peak plasma levels following oral AM are attained within 3 to 7 hours (Roden, 1993), while steady

state plasma levels are not reached until 1 to 5 months of continuous oral administration (Latini et al., 1984; Kerin et al., 1985; Mostow et al., 1986). The bioavailability of oral AM varies considerably, ranging from 20-86% following a single dose (Andreasen et al., 1981; Plomp et al., 1984; Latini et al., 1984). The low aqueous solubility and extensive first-pass metabolism of AM may contribute to its unpredictable bioavailability and resulting plasma concentrations (Holt et al., 1983; Latini et al., 1984). Suggested therapeutic plasma concentrations range from 1.0 to 2.5 μg / ml (1.5 to 3.7 μM), with the risk of toxicities increasing at concentrations above 2.5 μg / ml (Rotmensh et al., 1984; Mahmarian et al., 1994; Pollak et al., 2000).

1.4.3 Distribution

AM is widely distributed throughout the body, and due to extensive (>98%) plasma protein binding (Andreasen et al., 1981; Heger et al., 1983) and high lipophilicity, it has an estimated volume of distribution of 5000 L (Holt et al., 1983). Accumulation of AM occurs in a variety of tissues, often at concentrations several fold greater than in plasma (Plomp et al., 1985; Brien et al., 1987). Following chronic therapy, tissues with the greatest amount of AM upon autopsy analysis include adipose, lung and liver, while other tissues such as bone marrow, pancreas, spleen, heart, kidney, skeletal muscle, thyroid and brain contain lesser amounts (Haffajee et al., 1983; Holt et al., 1983; Brien et al., 1987). Pulmonary AM content following chronic therapy ranges from 20 to 734 μg / g (0.029 to 1.05 μmol / g) lung tissue (Haffajee et al., 1983; Maggioni et al., 1983; Plomp et al., 1985; Brien et al., 1987).

1.4.4 Biotransformation

Metabolism of AM has not been completely characterized. However, considerable evidence suggests that AM is metabolised by the cytochrome P450 (CYP450) enzyme system, primarily in liver (Young and Mehendale, 1987; Blake and Reasor, 1995a). The primary metabolite of AM is *N*-desethylamiodarone (DEA), which has been detected in blood and other tissues in humans (Flanagan et al., 1982; Latini et al., 1984; Brien et al., 1987) and experimental animals (Brien et al., 1990; Daniels et al., 1990). The CYP450 isoforms CYP3A4, CYP2C8, and CYP1A1 have been identified as the major contributors to AM metabolism in humans (Ha et al., 1993; Ohyama et al., 2000a), with CYP1A2, CYP2C19, and CYP2D6 contributing to a minor extent (Ohyama et al., 2000b) (Figure 1.1). Studies in experimental animals also suggest hepatic CYP450 metabolism of AM to DEA (Young and Mehendale, 1987; Rafeiro et al., 1990; Blake and Reasor, 1995a), while a role for intestinal metabolism via flavin-containing monooxygenases has also been proposed (Young and Mehendale, 1987). Interestingly, AM and DEA have been shown to inactivate many isoforms of CYP450 involved in AM metabolism in human β-lymphoblastoid cells expressing CYP450 isoforms (Ohyama et al., 2000b), and AM was shown to form a biologically inactive CYP450-Fe(II)-AM metabolite complex in liver tissue of rats, mice and hamsters (Larrey et al., 1986).

Accumulation of DEA rivals or exceeds that of AM in all tissues of the body, with the exception of adipose tissue. This has been suggested to be a result of DEA being less bound to plasma proteins than AM, and to possess a different ionization state (pK_a), thus allowing for increased diffusion of DEA from plasma to non-adipose tissue stores (Plomp et al., 1985). While pulmonary metabolism of AM has been investigated, DEA was not

detectable in uninduced hamster and rat lung microsomal incubations (Young and Mehendale, 1987; Rafeiro et al., 1990; Blake and Reasor, 1995a), or in isolated and perfused rat lung (Camus and Mehendale, 1986). Recent studies with human whole lung microsomes incubated with AM revealed an apparent AM metabolite that co-eluted with DEA upon HPLC analysis, suggesting that human lung may be capable of AM metabolism (Bolt, 2001). However, definitive identification as DEA could not be performed, and further studies are warranted. As such, pulmonary accumulation of DEA is not likely due to extensive formation within lung, but rather due to sequestration following hepatic metabolism of AM and subsequent release into the systemic circulation.

Other AM metabolites, while quantitatively minor compared to DEA, have been identified. For example, *N,N*-didesethylamiodarone has been detected in hamsters (Daniels et al., 1989) and dogs (Brien et al., 1990), and a deiodinated metabolite has been observed in rats (Kannan et al., 1989). Hydroxylation of DEA generated by rat liver microsomes has recently been reported (Ha et al., 2001), while other unidentified AM metabolites have been detected in serum from AM-treated patients (Staubli et al., 1985), and in *in vitro* incubations of AM with microsomes from rabbit liver and gut (Young and Mehendale, 1986) and human liver (Trivier et al., 1993).

1.4.5 Elimination

Elimination of AM and DEA occurs via the biliary route with eventual fecal excretion (Broekhuysen et al., 1969; Andreasen et al., 1981), although the extent of enterohepatic recirculation is unknown (Roden, 1999). Less than 1% of administered

AM is recovered in urine, suggesting that renal excretion is minimal (Andreasen et al., 1981; Plomp et al., 1985)). However, considerable deiodination of AM occurs, resulting in free iodide being excreted in urine (Rao et al., 1986).

Following a single dose of AM, the plasma elimination half-life ranges from 3.2 to 79.7 hours (Latini et al., 1984), and increases to 14 to 107 days following long-term therapy (McKenna et al., 1983). The plasma elimination profile of AM following discontinuation of therapy is biphasic, consisting of a rapid and short elimination from its central compartment, followed by a slow secondary elimination phase from poorly perfused tissue stores such as adipose (Holt et al., 1983; Freedman and Somberg, 1991). Once steady-state levels of AM have been attained, termination of therapy results in a decrease of serum concentrations by 25% after three days, and by 50% after 36 days (Pollak et al., 2000), with AM still detectable in plasma up to 9 months later (Singh, 1996).

1.5 CLINICAL TOXICITIES OF AMIODARONE

Numerous adverse effects are associated with AM use, spanning a wide range of organs and tissues (Rothenberg et al., 1994). Many of these effects are relatively minor and, although they occur with a frequency of 10 to 100%, can be diminished or eliminated with dose reduction or withdrawal of AM therapy (Vrobel et al., 1989). Such adverse effects include corneal microdeposits, elevation of serum liver enzymes, gastrointestinal disturbances, skin photosensitivity, ataxia and resting tremors. Other less common adverse effects, with incidences of 1 to 10%, are also reversible and include

grey / blue skin discolouration, hypothyroidism, hyperthyroidism, and peripheral neuropathy (Gill et al., 1992; Jafari-Fesharaki and Scheinman, 1998).

More serious complications associated with AM use include hepatotoxicity in the form of cirrhosis or hepatitis (<1% incidence) (Marchlinski, 1987), exacerbation of cardiovascular disturbances such as tachycardia, bradycardia, and congestive heart failure, and pulmonary toxicity (Mason, 1987; Gill et al., 1992). The incidence of AM-induced pulmonary toxicity (AIPT) has been reported to be between 1 and 27% (Kudenchuk et al., 1984; Magro et al., 1988; Adams et al., 1988; Pollak, 1999), with differences in diagnostic criteria responsible for this variation. Of patients with AIPT, a mortality rate of 10 to 23% has been reported (Mason, 1987; Vrobel et al., 1989), making it the adverse effect of greatest clinical concern.

Although the underlying basis of AIPT is unknown, it appears as though the likelihood of developing AIPT may be dependent upon dose and duration of AM therapy. Doses of 400 mg / day or greater have been associated with increased risk of developing pulmonary complications (Magro et al., 1988; Adams et al., 1988), while lower doses result in a lower incidence of pulmonary toxicity (Sunderji et al., 2000). However, the cumulative amount of AM and duration of therapy, rather than daily dose values, may be an important determinant of the risk of developing AIPT (Rakita et al., 1983; Martin and Rosenow, 1988; Adams et al., 1988).

1.6 AMIODARONE-INDUCED PULMONARY TOXICITY (AIPT)

1.6.1 Clinical Presentation of AIPT

Two types of clinical presentations of AIPT have been proposed, namely an acute hypersensitivity type and a subacute / chronic type (Rotmensch et al., 1980). The acute hypersensitivity type of presentation is observed in approximately one third of patients who develop AIPT, is typically associated with fever that may mimic infectious pneumonitis, and has a relatively rapid onset (Fraire et al., 1993). A predominantly alveolar pattern with patchy distribution is routinely observed on chest x-ray in this form of presentation. The more common subacute / chronic type of presentation rarely occurs prior to two months of therapy (Martin and Rosenow, 1988), has an insidious onset, and is rarely observed in patients receiving doses of less than 400 mg / day. Chest x-rays reveal a primarily diffuse interstitial pattern of parenchymal infiltrates (Martin and Rosenow, 1988; Fraire et al., 1993).

Symptoms of AIPT are non-specific, and are often masked by the symptoms of overt cardiac failure (Jessurun et al., 1998). Signs and symptoms associated with AIPT include a non-productive cough, pleuritic chest pain, dyspnea, fever, weakness, and weight loss (Martin and Rosenow, 1988). Clinical diagnosis of AIPT requires the exclusion of other possibilities, together with an assortment of new or worsening symptoms, abnormal findings on chest x-ray, or a decline of carbon monoxide diffusion capacity or total lung capacity (Marchlinski et al., 1982; Magro et al., 1988; Pollak, 1999).

1.6.2 Histopathology of AIPT

Although considerable variation exists between patients presenting with AIPT, the most common features associated with this condition include interstitial and alveolar thickening, cellular infiltration of the interstitium and alveoli consistent with pneumonitis, and fibrosis (Costa-Jussa et al., 1984; Pollak and Sami, 1984; Brien et al., 1987; Myers et al., 1987). Pulmonary fibrosis, characterized by an abnormal deposition of excess collagen and elastin in the lung (Reiser and Last, 1986), can have a pattern of damage that is either diffuse or patchy, and can affect the alveoli, interstitium, or both (Vrobel et al., 1989; Dusman et al., 1990).

Cellular infiltrates in the lung include alveolar macrophages, along with variable numbers of lymphocytes, neutrophils, eosinophils, and plasma cells (van Zandwijk et al., 1983; Myers et al., 1987). Alveolar wall thickening results from fibroblast proliferation and excessive production of matrix components, which can progress to fibrosis. Hyperplasia of alveolar type II cells has also been observed, which is a normal reparative response to type I cell injury (Marchlinski et al., 1982; Brien et al., 1987).

A curious finding in lungs exposed to AM is the presence of "foamy" macrophages within alveoli and interstitial spaces. These cells appear "foamy" due to accumulation of phospholipids within multilamellar inclusion bodies (Marchlinski et al., 1982; Gefter et al., 1983; Myers et al., 1987). However, these cells are present in virtually all patients on AM therapy (Reasor and Kacew, 1991; Fraire et al., 1993), thus precluding their presence as a diagnostic tool for AIPT.

1.6.3 Treatment of AIPT

AIPT presents a difficult clinical problem because the etiology is unknown, there are no standard markers for early detection, and there are no "gold standards" for confirming the diagnosis (Pollak, 1999). Furthermore, treatment options are limited once AIPT has been diagnosed. The first and most frequently used option is to decrease or discontinue AM administration. However, the long elimination half-life of AM may result in persistence of toxicities long after drug withdrawal (Vrobel et al., 1989; Jessurun et al., 1998). Administration of corticosteroids has been used in the management of AIPT in conjunction with decreasing or removing AM therapy (Rakita et al., 1983; Cazzadori et al., 1986), even though use of steroids is indicated only when there is an immunological basis to pulmonary inflammation or disease. As such, their usefulness is questionable, since AIPT may resolve without their use (Suarez et al., 1983; Gibb and Melendez, 1986).

1.7 MECHANISMS OF AIPT

The etiology of AIPT is currently unknown. However, several mechanisms have been proposed to be involved in the initiation of AIPT, and are broadly classified as being either indirect (immunological) or direct toxic mechanisms (Figure 1.2). It should be noted that these mechanisms are not necessarily mutually exclusive, and are discussed here as separate entities for the purpose of clarity.

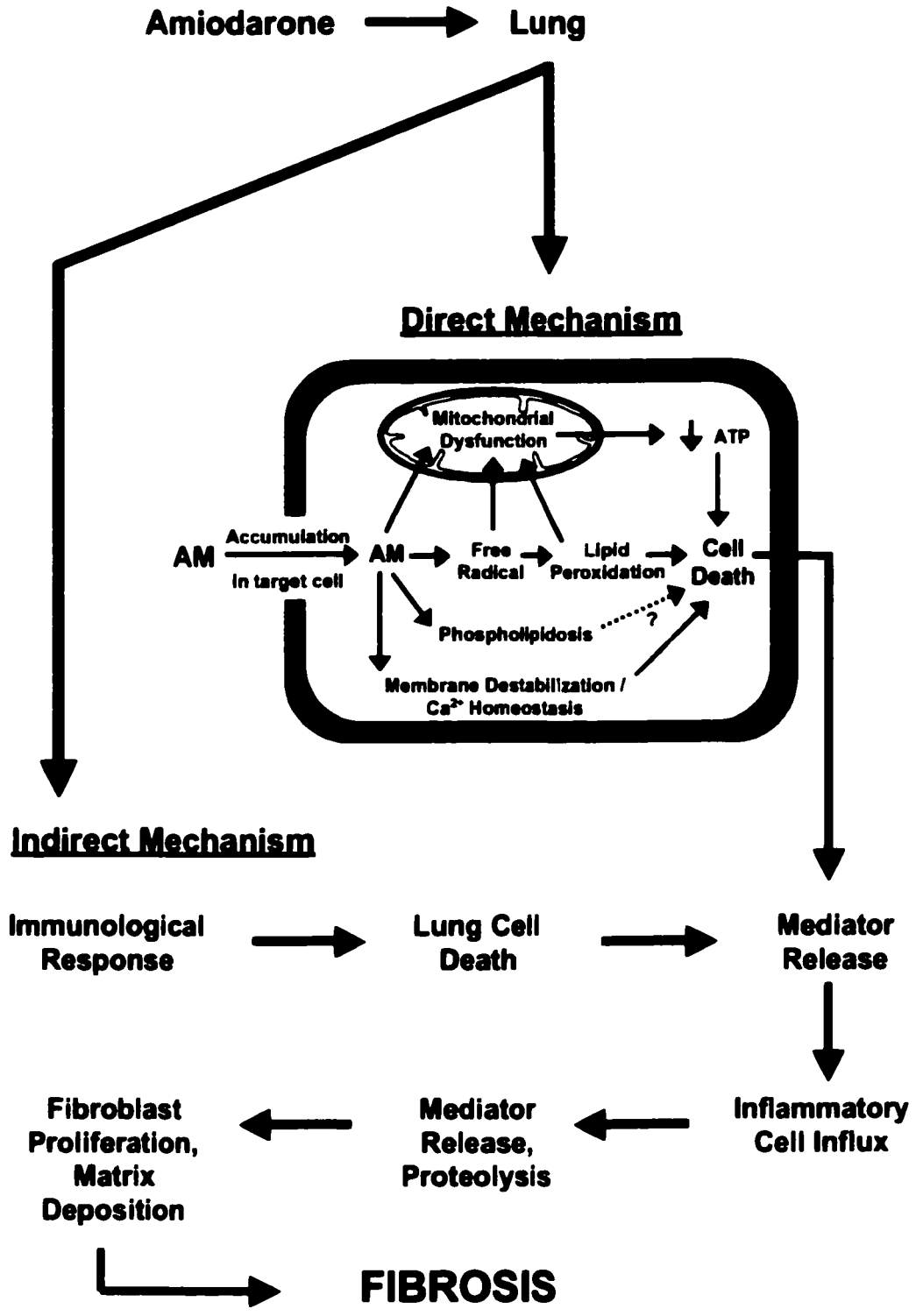


Figure 1.2 Proposed mechanisms of amiodarone-induced pulmonary toxicity (AIPT) (modified from Massey et al., 1995).

1.7.1 Indirect Versus Direct Mechanisms

An indirect mechanism of AIPT implies that an immunological response is crucial to the initiation of lung injury, whereas a direct mechanism would involve an insult to cells of the lung directly by AM or a metabolite. It is likely that AIPT is a multifactorial process, and as such, it is important to differentiate between events that initiate lung injury and those that are a result of such (an) event(s) in order to fully understand the etiology of this condition.

1.7.2 Indirect Mechanisms of AIPT

1.7.2.1 Immunologic Mechanism

An immunologic mechanism of AIPT has been proposed, based on the fact that AIPT occurrence cannot be predicted based on dose or blood levels of AM (Dunn and Glassroth, 1989). However, evidence exists both for the support of an immunologic mechanism initiating AIPT, as well as for rejection of this concept, as discussed below.

Supporting evidence for an immunologic mechanism of AIPT includes: i) the presence of serum anti-AM antibodies that react with lungs of AIPT patients and with AM itself, but not with normal lung or fibrotic lung from patients not using AM (Fan et al., 1987; Pichler et al., 1988); ii) increases in bronchoalveolar lavage fluid (BALF) levels of immunoglobulin in AIPT patients (Sandron et al., 1986), and of BALF cellularity consisting of a variable increase in lymphocytes, neutrophils, eosinophils, and mast cells (Israel-Biet et al., 1987; Coudert et al., 1992); iii) lymphocytosis in BALF (Israel-Biet et al., 1985); iv) secretion of leukocyte inhibitory factor in the presence of

AM (Akoun et al., 1984); and v) alleviation of AIPT by corticosteroid therapy with or without discontinuation of AM (Israel-Biet et al., 1987; McNeil et al., 1992).

Evidence refuting an immunologic mechanism of AIPT includes: i) lack of detection of complement, immunoglobulins, or lymphocytosis in AIPT patients (Adams et al., 1986; Ohar et al., 1992); ii) similar immune responses between patients with AIPT and those without (Pichler et al., 1988; Nicolet-Chatelain et al., 1991; Coudert et al., 1992); and iii) the ability of patients to recover from AIPT by discontinuing AM therapy with or without use of corticosteroids (Gefter et al., 1983; Leech et al., 1984), and a lack of improvement of AIPT with corticosteroid use (Cooper et al., 1986).

The often conflicting evidence surrounding the involvement of an immunologic mechanism in initiating AIPT has led to the suggestion that it may be a secondary response to an initial direct toxic insult to the lung by AM or a metabolite (Israel-Biet et al., 1987; Nicolet-Chatelain et al., 1991).

1.7.2.2 Altered Inflammatory Mediator Release

Mediators released from activated inflammatory cells play a critical role in the activation and proliferation of cells involved in the fibrotic process (Crystal et al., 1991). It is plausible that AM may modify the secretion of such mediators via effects on alveolar macrophages and other cells of the lung. Macrophage activation and cytokine release are important factors in the pulmonary fibrotic response to the anti-neoplastic agent bleomycin (Phan and Kunkel, 1992), and likely play a similar role in AIPT. Indeed, alveolar macrophages secrete a host of molecules involved in inflammation and repair,

including monocyte chemotactic factors (Denholm and Phan, 1989), fibroblast growth factors (Denholm et al., 1989), and cytokines (Phan and Kunkel, 1992).

Tumour necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) are important cytokines released by macrophages that play primary roles in the pathogenesis of pulmonary fibrosis (Ward and Hunninghake, 1998; Coker and Laurent, 1998). Following intratracheal AM administration, increased TNF-α levels have been detected in alveolar macrophages isolated from rats (Reinhart and Gairola, 1997), and in BALF from hamsters (Blake and Reasor, 1995b). In addition, *in vitro* exposure of murine alveolar macrophages to AM resulted in increased TNF-α secretion (Futamura, 1997). Lung levels of TGF-β mRNA and protein were shown to increase early in the fibrotic response to intratracheal AM in rats (Chung et al., 2001), and increased TGF-β has been demonstrated following administration of other pulmonary fibrogens such as bleomycin (Iyer et al., 2000). Other mediators or chemotactic factors whose releases have been found to increase as a result of AM include interleukin-1 (IL-1) (Wilson and Lippmann, 1993; Futamura, 1995; Futamura, 1996a), interleukin-6 (IL-6) (Reasor et al., 1996), and eicosanoids (Zitnik et al., 1992; Futamura, 1997).

Macrophages exposed to AM may alter lung repair and / or the progression of fibrosis, possibly via secretion of fibroblast-activating factors, which would further promote injury (Wilson et al., 1989; Massey et al., 1995). However, other studies have demonstrated AM to have no effect on macrophage release of IL-1, IL-6, TNF-α, or TGF-β (Zitnik et al., 1992; Reasor et al., 1996). As such, it is likely that alteration of inflammatory mediators by AM is a mechanism by which AIPT progression is advanced,

as opposed to an initiating mechanism of lung injury (Israel-Biet et al., 1987; Nicolet-Chatelain et al., 1991).

1.7.3 Direct Mechanisms of AIPT

1.7.3.1 Phospholipidosis

Phospholipidosis, an accumulation of intracytoplasmic multilamellar inclusion bodies derived from lysosomal phospholipid (Reasor, 1989), is a characteristic feature of AM exposure in a variety of pulmonary and non-pulmonary cell types (Marchlinski et al., 1982; Dake et al., 1985; Riva et al., 1987; Massey et al., 1995). This results from the inhibition of lysosomal phospholipases A_1, A_2, and C by AM (Hostetler et al., 1988; Kodavanti and Mehendale, 1991). In the lung, alveolar macrophages are the primary cell type affected, resulting in a "foamy" appearance attributable to phospholipid accumulation (Reasor et al., 1988).

Phospholipidosis has been observed in numerous animal models following various routes of AM administration (Mazue et al., 1984; Riva et al., 1987; Reasor et al., 1988; Wilson et al., 1991; Wang et al., 1992). Development of phospholipidosis is time and dose dependent, and is reversible upon cessation of drug administration (Reasor et al., 1988; Antonini et al., 1994). A decrease of AM-induced phospholipidosis has been shown after vitamin E, taurine or niacin administration (Wang et al., 1992; Honegger et al., 1995), and DEA is more potent than AM at inhibiting lysosomal phospholipases, and thus at inducing phospholipidosis (Hostetler et al., 1988; Kannan et al., 1990a).

Despite overwhelming evidence that AM causes phospholipidosis, and that phospholipidosis consistently presents in AIPT, unequivocal evidence is lacking to

support the concept that phospholipidosis is a cause of AIPT. Indeed, most patients on AM therapy develop phospholipidosis without developing AIPT. Animal models demonstrate lung phospholipid accumulation without fibrosis following oral administration of AM or DEA, whereas intratracheal drug administration results in significant pulmonary fibrosis with little or no phospholipid accumulation (Blake and Reasor, 1995c). In addition, other cationic amphiphilic drugs, such as imipramine or chlorpromazine, cause phospholipidosis but do not cause fibrosis (Lullmann et al., 1978; Wilson, 1982). As such, phospholipidosis is considered a common adverse effect of AM therapy, but is not likely a direct initiating mechanism of AIPT.

1.7.3.2 Disruption of Intracellular Calcium Homeostasis

Sustained, unregulated increases of intracellular calcium (Ca^{2+}) levels can lead to over-stimulation of Ca^{2+}-dependent degradative enzymes such as endonucleases and proteases that can in turn lead to degradation of cellular constituents and ultimately cell death (Nicotera et al., 1992). Both AM and DEA have been shown to increase intracellular Ca^{2+} levels in a variety of cell types (Powis et al., 1990; Kodavanti et al., 1992; Hageluken et al., 1995), although the mechanisms responsible for this effect are speculative. Evidence in the literature suggests that AM and DEA may increase intracellular Ca^{2+} via: i) destabilization of plasma membranes, leading to altered membrane fluidity and increased permeability to extracellular Ca^{2+} (Chatelain et al., 1985; Honegger et al., 1993; Antunes-Madeira et al., 1995); ii) interaction with calmodulin, thus modifying its Ca^{2+}-binding properties (Vig et al., 1991); iii) activation of G_i (inhibitory guanyl-nucleotide-binding) proteins (Hageluken et al., 1995); and iv)

free radical generation, in turn affecting ion channel transport mechanisms (Himmel et al., 2000). With regard to cellular injury induced by AM and DEA, however, increased intracellular Ca^{2+} levels may be a consequence, rather than an initiator of cytotoxicity.

1.7.3.3 Free Radical Formation

1.7.3.3.1 Free Radicals and Reactive Oxygen Species

A free radical is defined as an atom or molecule that contains one or more unpaired electrons (Bergendi et al., 1999). Free radicals can be anionic, cationic, or neutral in charge, and can be formed as a result of various processes including exposure to ionizing radiation, as normal by-products of xenobiotic metabolism, upon leakage from electron transport, during oxidative burst in activated phagocytes, and during metabolism of fatty acids, among others (Freeman and Crapo, 1982). Free radicals can donate unpaired electrons to molecular oxygen, leading to formation of superoxide ($\bullet O_2^-$), with the possible subsequent formation of more damaging oxygen-centred species such as the hydroxyl radical ($\bullet OH$) and peroxyl ($ROO\bullet$) radicals. These species, and others that contain chemically reactive O_2, are collectively termed reactive oxygen species (ROS) (Halliwell and Gutteridge, 1999a). Free radicals and ROS can attack proteins, carbohydrates, lipids, and nucleic acids, causing metabolic disturbances that can lead to cell death (Davies, 1995). Indeed, free radicals have been implicated in the toxicity of several compounds, including many drugs (Aust et al., 1993). When polyunsaturated fatty acids (PUFA) and cholesterol are targets of free radical attack, lipid peroxidation (LPO) can ensue.

1.7.3.3.2 Lipid Peroxidation

LPO is considered a basic contributing mechanism to the toxicity of a wide variety of chemicals, the consequences of which include membrane damage, enzyme inhibition, release of lysosomal enzymes, and protein-protein cross-linking (Halliwell and Gutteridge, 1999b). LPO is initiated by the abstraction of a hydrogen atom from the side chain of PUFA as a result of free radical attack at a methylene carbon (Figure 1.3). This leads to bond rearrangement of the newly formed carbon-centred lipid radical to form a conjugated diene, which then reacts with O_2 to form a peroxyl radical (ROO•). The peroxyl radical can then react with another peroxyl radical, attack membrane proteins, or abstract a hydrogen atom from an adjacent fatty acid side chain leading to production of another ROO•, thus propagating the chain reaction of LPO (Halliwell and Chirico, 1993; Halliwell and Gutteridge, 1999b). The end result of LPO can be cell and / or subcellular membrane destabilization and ultimately cell death.

Various techniques exist for the measurement of LPO in biological systems, each with their own advantages and disadvantages. Generally, LPO is determined by measuring the loss of PUFA, or by determining the generation of lipid peroxides or secondary peroxidation products such as hydrocarbon gases and carbonyls (Halliwell and Chirico, 1993). Measurement of thiobarbituric acid-reactive substances (TBARS), such as malondialdehyde (MDA), as an indicator of LPO in various experimental systems is a well-established and widely used technique. Although artificial generation of TBARS can occur during sample preparation (Aust, 1994), the use of appropriate control groups during experimental procedures diminishes the likelihood of experimental results being altered as a result of this occurrence.

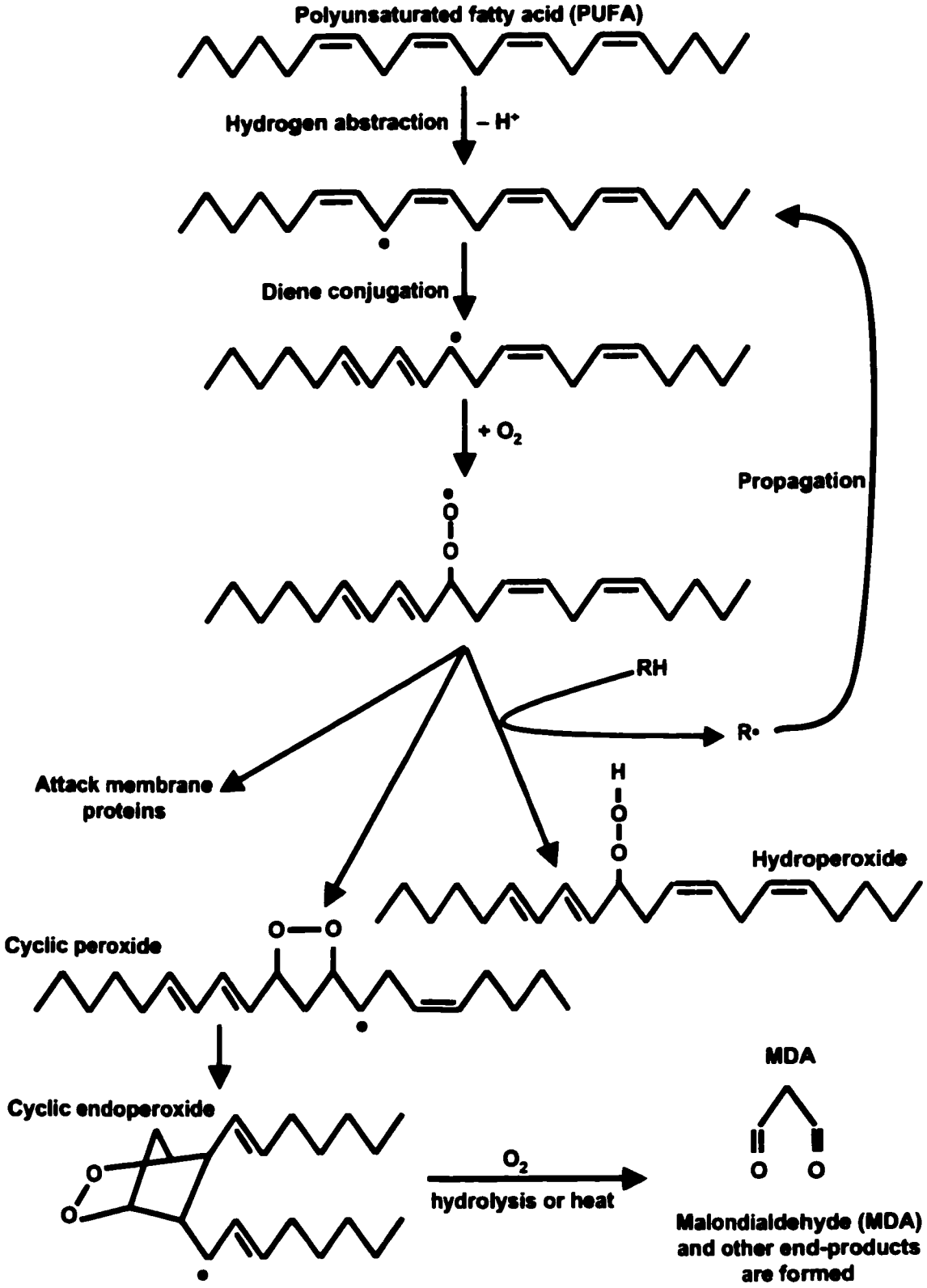


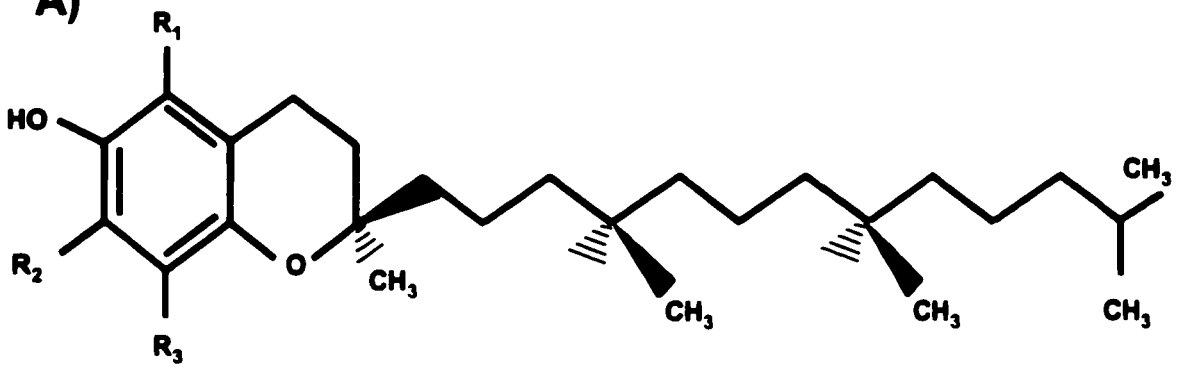
Figure 1.3 Initiation and propagation of lipid peroxidation (LPO). (adapted from Halliwell and Gutteridge, 1999b).

1.7.3.3.3 Vitamin E

Vitamin E is the generic name for the group consisting of four tocopherols and four tocotrienols that display vitamin E antioxidant activity (Herrera and Barbas, 2001). Biologically, α-tocopherol is the most potent of these molecules with regard to antioxidant activity, and accounts for approximately 90% of the vitamin E activity in tissues (Wang and Quinn, 2000). Henceforth, the term vitamin E will refer to α-tocopherol, unless stated otherwise. As a non-enzymatic antioxidant, vitamin E rapidly scavenges lipid peroxyl radicals before they can react with other PUFA side chains or with membrane proteins, effectively halting the propagation of LPO. Despite being present at a relatively low concentration in most cell membranes (i.e. less than 2 mol / mol phospholipid), redox cycling of vitamin E occurs in membranes to regenerate the antioxidant from the tocopheroxyl radical, which itself is very stable due to delocalisation of the unpaired electron about the fully substituted chromanol ring. This redox cycling of vitamin E is mediated by several cell constituents, including vitamin C, vitamin A, glutathione, and coenzyme Q (Wang and Quinn, 2000; Herrera and Barbas, 2001) (Figure 1.4). In some instances vitamin E can act as a pro-oxidant, although this action is dependent on the concentration and structure of reaction partners that are present, and does not appear to occur in the presence of other co-antioxidants such as vitamin C and coenzyme Q (Brigelius-Flohe and Traber, 1999).

Vitamin E has recently been recognised for possessing numerous activities that cannot be attributed to its antioxidant function (Azzi and Stocker, 2000). These include membrane stabilization via formation of complexes with certain membrane lipid components, up-regulation of cytosolic phospholipase A_2 and cyclooxygenase activities

A)



The Tocopherols

	R_1	R_2	R_3
α-tocopherol	CH_3	CH_3	CH_3
β-tocopherol	CH_3	H	CH_3
γ- tocopherol	H	CH_3	CH_3
δ-tocopherol	H	H	CH_3

B)

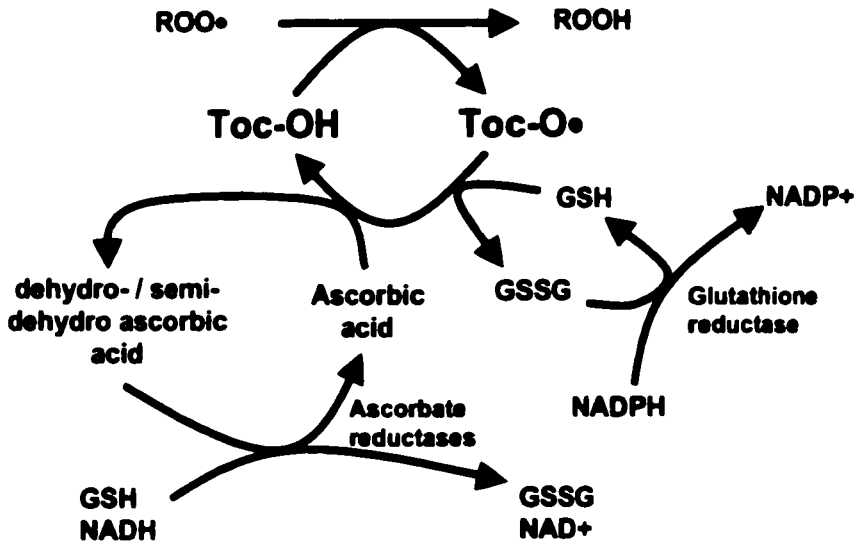


Figure 1.4 A) Chemical structure of the vitamin E tocopherols. (Adapted from van Acker et al., 1993). B) Vitamin E regeneration systems. (Adapted from Chow, 1991). (ROO• = peroxyl radical, ROOH = peroxide, Toc-OH = vitamin E, Toc-O• = tocopheroxyl radical, GSH = reduced glutathione, GSSH = oxidized glutathione).

leading to enhancement of prostacyclin release, inhibition of protein kinase C via a mechanism not involving direct interaction with the enzyme, and modulating effects on the expression of various genes including collagenase and connective tissue growth factor. As such, mechanisms of action of vitamin E in experimental systems must take into account the potential contribution of these non-antioxidant effects.

1.7.3.3.4 Free Radicals, Reactive Oxygen Species, and AIPT

Free radical formation may be involved in the initiation and / or progression of AIPT. The following evidence suggests that oxygen-centred radicals and other ROS might be involved in these processes. Isolated rabbit lungs exposed to AM displayed an elevated concentration of oxidized glutathione and enhanced chemiluminescence, suggestive of superoxide formation (Kennedy et al., 1988). Interestingly, pre-treatment with antioxidant agents prior to AM exposure decreased pulmonary edema in these studies (Kennedy et al., 1988). Furthermore, a recent study used electron spin resonance (ESR) spectroscopy to detect formation of the hydroxyl radical (•OH) in aqueous solutions of AM (Taylor et al., 2001). Taken together, these results suggest a possible role for ROS in the development of AIPT.

Contrary to the above findings, results from other studies suggest that ROS are not involved in AIPT. *In vitro* experiments with various cell systems did not detect formation of ROS in the presence of AM (Leeder et al., 1996; Di Matola et al., 2000). Moreover, cytotoxicity of AM was not prevented by the antioxidants superoxide dismutase, catalase, butylated hydroxytoluene, ascorbic acid and *N*-acetylcysteine in cultured rat hepatocytes and human pulmonary artery endothelial cells (Kachel et al.,

1990; Ruch et al., 1991). In the hamster model of AIPT, the antioxidants butylated hydroxyanisole, diallyl sulfide and *N*-acetylcysteine did not prevent AM-induced pulmonary fibrosis, even though increases of pulmonary oxidized glutathione (GSSG) content and of the activities of superoxide dismutase and glutathione peroxidase (responses indicative of oxidative stress) were observed following AM dosing (Leeder et al., 1994). Thus, the bulk of evidence does not support the action of ROS as being the cause of AIPT.

A role for an AM or metabolite radical in AIPT is supported by several findings. Li and Chignell (1987) discovered that ultraviolet irradiation of AM resulted in formation of a carbon-centred radical that could abstract a hydrogen from linoleic acid and initiate LPO. Meanwhile, Vereckei and colleagues (1993) proposed that a highly reactive aryl radical is released following ^{60}Co-γ and electron pulse radiolysis of AM. Recently, ESR evidence of AM-derived carbon-centred radicals has been produced in an aqueous solution of AM (Taylor et al., 2001) and in isolated hamster lung and liver microsomes incubated with AM (Rafeiro, 1997). These results support a role for the formation of AM or metabolite radicals in the initiation and / or progression of AIPT.

1.7.3.3.5 AM-Induced Lipid Peroxidation

Evidence exists both in favour of and against the notion of lipid peroxidation being involved in AIPT. Following a single intratracheal dose of AM to hamsters, malondialdehyde equivalents (an indication of LPO) were increased in whole lung homogenates (Wang et al., 1992) and in bronchoalveolar lavage fluid (Blake and Reasor, 1995b). In rat liver microsomes, LPO induced by NADPH and Fe^{3+} was greater when

rats were treated with AM (Vereckei et al., 1993), and ultraviolet-irradiated liposomal phospholipid peroxidation was increased by AM in a model membrane system (Sautereau et al., 1992).

In contrast to the above evidence supporting AM-induced LPO, other studies have reported negative results in this regard. Decreased LPO as a result of AM has been reported in rat liver mitochondria (Ribeiro et al., 1997), rat liver microsomes (Rekka et al., 1990), and rat hepatocytes (Ruch et al., 1991). Meanwhile, no change in LPO parameters was found in liposomes of bovine heart mitochondrial lipids (Mansani et al., 1999) and in the sera of rats administered AM (Pre et al., 1991). As such, the involvement of LPO in initiating AIPT is unclear at present.

1.7.3.3.6 Vitamin E and AM-Induced Toxicities

Vitamin E has shown protective effects against AM-induced cytotoxicity in several *in vitro* studies (Kachel et al., 1990; Ruch et al., 1991; Honegger et al., 1995; Scuntaro et al., 1996; Futamura, 1996b), whereas a variety of other antioxidants were ineffective. The fact that vitamin E is lipid soluble, while the ineffective antioxidants that were tested were water soluble, suggests that vitamin E is able to interact with some facet of AM toxicity involving lipid regions of cells or subcellular compartments. As such, scavenging of AM, metabolite or lipid radicals that are inaccessible to water soluble antioxidants might be a mechanism of the protective effect of vitamin E. Other potential factors contributing to the protection offered by vitamin E against AM toxicities may include a decrease of phospholipidosis by prevention of AM-induced inhibition of phospholipase A_2 activity (Honegger et al., 1995; Scuntaro et al., 1996), a decrease of

AM accumulation in cells (Scuntaro et al., 1996), and prevention of AM-induced increases of membrane fluidity (Honegger et al., 1995). The ability of vitamin E to prevent pulmonary toxicity and fibrosis in an animal model has not been ascertained.

1.7.3.4 Disruption of Cellular Energy Homeostasis

Mitochondria are essential for the generation of cellular ATP, accounting for up to 95% of cellular energy production through oxidative phosphorylation (Figure 1.5) (Wallace et al., 1997; Saraste, 1999). As such, mitochondria are targets for initiation of cell dysfunction caused by many compounds. Disruption of mitochondrial function to initiate cellular toxicity can occur by several methods, including inhibition and / or uncoupling of oxidative phosphorylation, generation of free radicals and ROS through electron leakage, and activation of mitochondrial permeability transition (MPT) (Wallace et al., 1997). MPT is characterized by a sudden increase in the permeability of the inner mitochondrial membrane to solutes of molecular mass less than approximately 1500 Da, which leads to membrane depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, and mitochondrial swelling (Lemasters et al., 1998). MPT is involved in the initiation of apoptotic and necrotic forms of cell death, and is regulated through a voltage-dependent channel that is inhibited by cyclosporin A.

Adverse effects of AM on mitochondrial structure and function have been observed. Structural alterations including swelling, disorganized cristae, ruptured inner membranes, and electron lucent-appearance in electron micrographs have been reported in mitochondria following exposure of rat hepatocytes (Gross et al., 1989), human

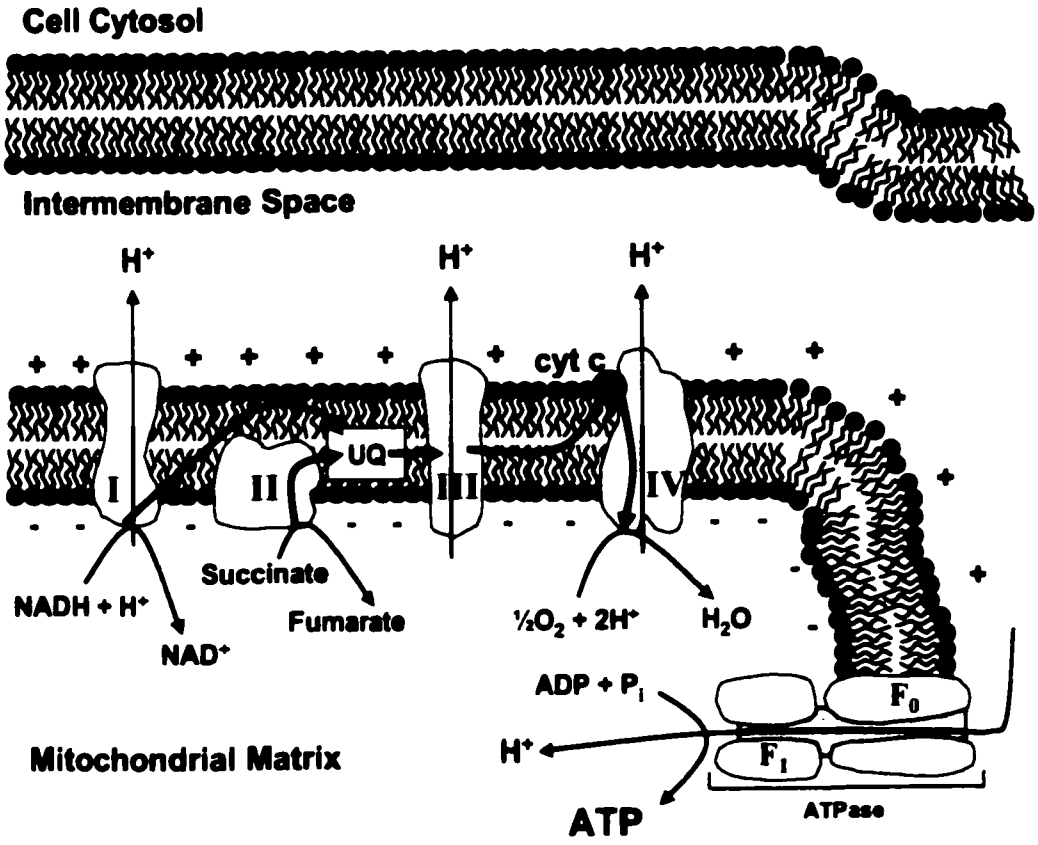


Figure 1.5 The mitochondrial electron transport chain. Electron transfer through the respiratory chain results in the pumping of protons into the intermembrane space, creating a pH and electrochemical gradient across the inner membrane. This results in a proton-motive force that is responsible for the shuttling of protons back into the matrix through ATPase, coinciding with synthesis of ATP.
(UQ = ubiquinone, cyt c = cytochrome c). (Adapted from Lehninger et al., 1993).

lymphocytes (Yasuda et al., 1996), and isolated rat heart mitochondria (Guerreiro et al., 1986) to AM. Furthermore, accumulation of AM in isolated mouse liver mitochondria (Fromenty et al., 1990a) and in lung mitochondria from AM-treated rats (Hostetler et al., 1988) has been reported, indicating a significant affinity of the drug for these organelles.

Functional changes resulting from AM have been documented in mitochondria from various sources. AM inhibited oxygen consumption and β-oxidation of fatty acids, and decreased membrane potential in isolated mouse liver mitochondria (Fromenty et al., 1990a; Fromenty et al., 1990b), and also inhibited oxygen consumption in isolated rat heart mitochondria (Guerreiro et al., 1986). Similarly, electron transport through complex I of the electron transport chain was inhibited by AM in isolated rat liver mitochondria (Ribeiro et al., 1997), as was the activity of mitochondrial ATPase in guinea pig heart mitochondrial preparations (Dzimiri and Almotrefi, 1993). Recently, Bolt and colleagues observed a decrease of mitochondrial membrane potential prior to cellular ATP depletion and ensuing cell death in isolated hamster lung cells exposed to AM (Bolt et al., 2001a). The combined structural and functional effects of AM on mitochondria suggest a potential target for initiating AM toxicities, including AIPT. To date, however, the effect of AM on lung mitochondrial function *in vitro* or *in vivo* in an animal model of AIPT has not been reported.

1.8 PULMONARY FIBROSIS

Fibrosis is a common response to a variety of insults or injuries to the lung that can be caused by numerous agents. The hallmark of pulmonary fibrosis is the deposition of excess collagen and other matrix components, leading to the replacement of functional alveolar capillary units with accumulated connective tissue. Areas of fibrosis with associated "honeycomb" appearance alternate with areas of relatively normal lung tissue, resulting in a patchy appearance. The prognosis for patients with pulmonary fibrosis is poor, with a median survival rate of 4 to 5 years after the onset of symptoms (Ryu et al., 1998), and no therapy tested to date has shown prolongation of survival. As such, delineating the mechanisms involved in the initiation and progression of this condition is the goal of many researchers, in the attempt to identify effective therapeutic interventions.

1.8.1 Pathogenesis of Pulmonary Fibrosis

Pulmonary fibrosis can be idiopathic, or caused by numerous agents that reach the lung by inhalation or systemic routes. Current reasoning suggests that injury to epithelial cells and aberrant wound healing mechanisms result in areas containing fibroblast foci, and that these areas are rich in replicating mesenchymal cells and in extracellular matrix deposition (Gross and Hunninghake, 2001; Selman et al., 2001). Numerous cytokine signalling pathways, often overlapping and compensatory in nature, mediate many of the key events involved in fibrogenesis. Increasing evidence suggests that an imbalance between Type-1 (IFN-γ, IL-2, IL-12, IL-18, and others) and Type-2 (IL-4, IL-5, IL-10, IL-13, and others) cytokine responses to lung injury can lead to fibrosis when the Type-2

response predominates (Sime and O'Reilly, 2001) (Figure 1.6). Alveolar epithelial cells are known to express several pro-fibrotic cytokines during pulmonary fibrosis, including platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β_1 (Antoniades et al., 1990; Khalil et al., 1996), and are likely a key source of these chemical signals during the initiation and progression of fibrosis. Expression of TGF-β_1 has been shown to increase in a temporal and contiguous fashion following induction of lung injury in animal models (Zhang et al., 1995), and over-expression of this cytokine alone in lung can result in fibrosis (Sime et al., 1997). As such, TGF-β_1 is considered a crucial mediator of the pathogenesis of pulmonary fibrosis.

The role of inflammation in pulmonary fibrosis is a topic of much debate. Traditional theory held that chronic inflammation injured the lung, and led to the eventual development of fibrosis (Keogh and Crystal, 1982). Animal models of pulmonary fibrosis caused by bleomycin and AM have revealed that these agents induce an inflammatory response in lung tissue following intratracheal administration that may play a role in the development of fibrosis (Blake and Reasor, 1995b; Iyer et al., 2000). However, other experimental evidence reveals that it is possible to dissociate inflammation from the fibrotic response in animal models of pulmonary fibrosis (Sime et al., 1997; Munger et al., 1999), thus casting doubt on the necessity of inflammation as an initiating factor for the development of fibrosis.

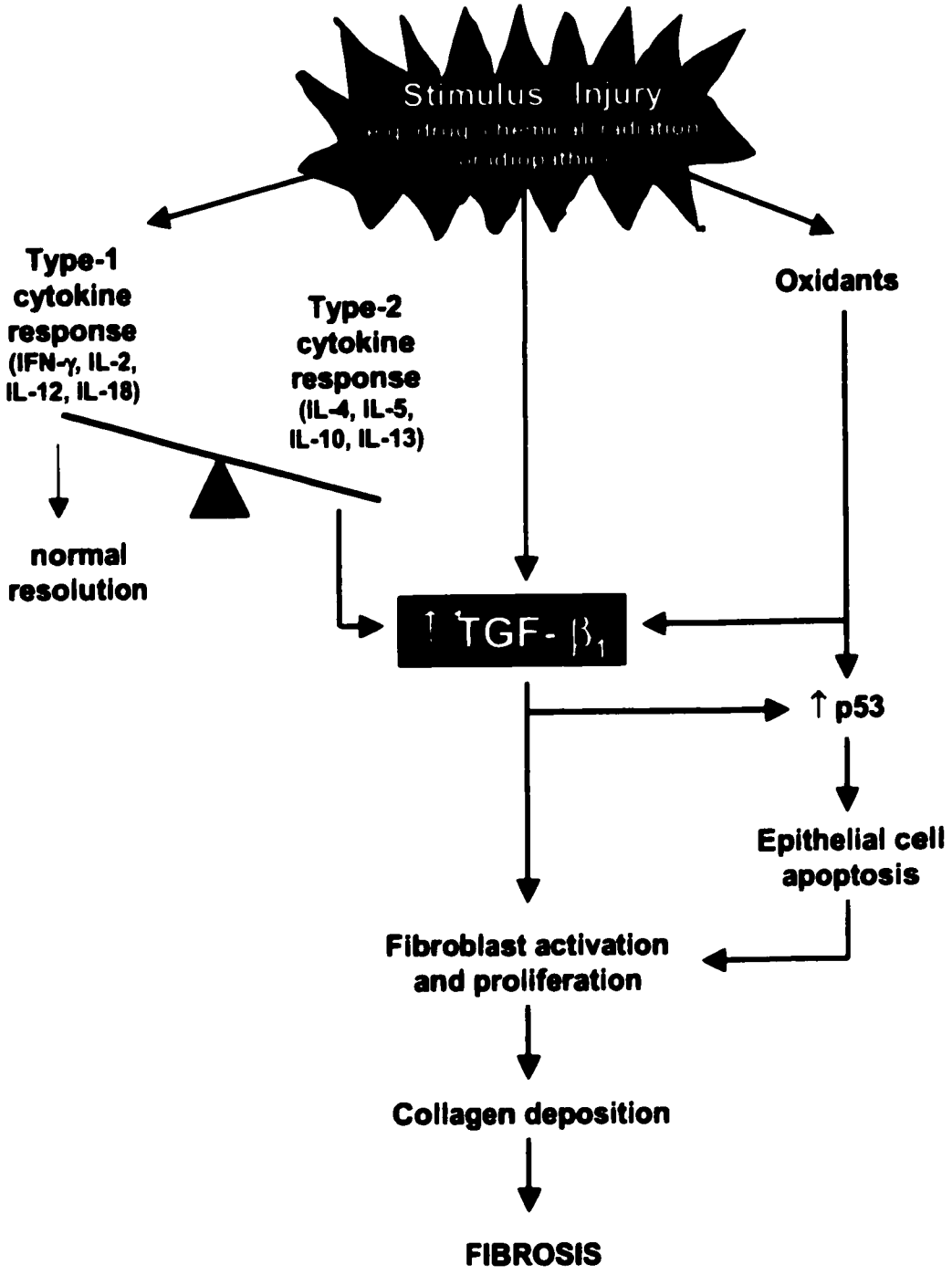


Figure 1.6 Simplified scheme depicting various signaling pathways involved in pulmonary fibrosis, and the suggested central role of TGF-β_1. Adapted from Cooper Jr. (2000) and Sime and O'Reilly (2001).

1.8.2 Treatment of Pulmonary Fibrosis

Currently, there are no effective therapies for pulmonary fibrosis. Conventional treatment has focused on suppressing inflammation, with the hope of preventing further progression of the fibrosis. However, anti-inflammatory and immunosuppressive therapies have met with little success, and are associated with severe side effects (Gross and Hunninghake, 2001; Sime and O'Reilly, 2001). Recent advances in the understanding of the pathogenesis of pulmonary fibrosis have resulted in new treatment approaches aimed at preventing or inhibiting the fibroproliferative response and enhancing normal alveolar re-epithelialization following injury. Agents such as pirfenidone, relaxin, interferon-γ, and others have demonstrated promising anti-fibrotic activity *in vitro* and / or in animal models, and are potentially of value in the clinical treatment of pulmonary fibrosis (Selman et al., 2001). These compounds are currently under investigation, and further animal studies and clinical trials with these and other agents will undoubtedly lead to advances in the prevention and / or treatment of this disease.

1.8.3 Animal Models of Pulmonary Fibrosis

Animal models of pulmonary fibrosis have been extensively employed in order to elucidate mechanisms of this disease and to determine potential therapeutic interventions. Because the nature of the initiating lung injury is known in the majority of these models (i.e. the damaging drug or agent is known), and usually results from a single, non-persistent insult to the lung, the ensuing fibrosis is temporal in nature and generally resolves within a given period of time ranging from weeks to months (Phan, 1995). This

is in contrast with the clinical situation, whereby the etiology of the fibrotic response to repeated injury is often unknown, and fibrosis is usually not detected until well into the progression of the disease. Identification of a candidate mediator as being causally related to the etiology of pulmonary fibrosis requires that several criteria be fulfilled (Zeldin, 2002). These include: i) that the mediator is produced during the disease; ii) that the mediator is capable of producing the disease; and iii) that interventions that reduce levels of the mediator should protect against development of the disease. Studies in animals describing mediators that comply with these criteria have provided a wealth of information regarding the role of various cytokines, growth factors, signalling molecules, cellular and subcellular targets, and other events in the etiology of pulmonary fibrosis, and have given insight into numerous potential therapeutic strategies to combat this disease.

1.9 RESEARCH HYPOTHESES AND OBJECTIVES

Pulmonary toxicity, including potentially fatal fibrosis, is a serious adverse effect associated with use of the antidysrhythmic drug AM. Elucidation of the initiating mechanism(s) of AIPT could lead to the development of antidysrhythmic agents devoid of the adverse pulmonary effects of AM, or to therapies to combat these effects. Likewise, identification of agents that decrease the pulmonary toxicity of AM in an animal model, and of mechanisms associated with this protection, may lead to the development of preventive and / or treatment strategies for the clinical setting. Furthermore, such findings would expand our current knowledge of chemical-induced pulmonary fibrosis.

The overall objectives of this research were to investigate the potential involvement of mitochondrial dysfunction as an initiating mechanism of AIPT and fibrosis, and to determine the protective capacity of two novel anti-fibrotic agents, vitamin E and pirfenidone, in an animal model of AIPT. Based on the preceding background information, research hypotheses were formulated, and specific objectives were adopted in order to test these hypotheses, as outlined below.

Hypothesis 1:

Hamster lung mitochondrial function is disrupted by AM and DEA.

Objective 1a:

To determine and compare the effects of *in vitro* exposure to AM and DEA on oxygen consumption, membrane potential, lipid peroxidation, and drug accumulation in isolated hamster lung mitochondria.

Objective 1b:

To determine the effect of intratracheal AM administration on oxygen consumption in hamster lung mitochondria isolated at various times post-dosing.

Hypothesis 2:

Dietary vitamin E supplementation decreases AIPT in an *in vivo* hamster model.

Objective 2a:

To determine the effect of dietary vitamin E supplementation on pulmonary injury following intratracheal AM administration, as assessed by histological damage and hydroxyproline content.

Objective 2b:

To determine the effect of dietary vitamin E supplementation on lung mitochondrial dysfunction induced by *in vitro* exposure to AM and DEA, and by *in vivo* AM administration.

Objective 2c:

To determine the effect of dietary vitamin E supplementation on pulmonary TGF-β_1 gene expression following *in vivo* AM administration.

Hypothesis 3:

Administration of pirfenidone via the diet decreases AIPT in a hamster model.

Objective 3a:

To determine the effect of pirfenidone, administered via the diet, on pulmonary injury following intratracheal AM dosing, as assessed by histological damage and hydroxyproline content.

Objective 3b:

To determine the effect of pirfenidone, administered via the diet, on lung mitochondrial dysfunction induced by *in vitro* exposure to AM and DEA, and by *in vivo* AM administration.

Objective 3c:

To determine the effect of pirfenidone, administered via the diet, on pulmonary TGF-β_1 gene expression following *in vivo* AM administration.

Chapter 2

DISRUPTION OF HAMSTER LUNG MITOCHONDRIAL FUNCTION *IN VITRO* BY AMIODARONE AND *N*-DESETHYLAMIODARONE

2.1 INTRODUCTION

Although several processes are likely involved in the pathogenesis of amiodarone (AM)-induced pulmonary toxicity (AIPT) (Massey et al., 1995; Reasor and Kacew, 1996), a definitive initiating mechanism has not been identified. Characterization of such an event could lead to the design of preventive and / or treatment regimens against AIPT, and could aid in the development of agents devoid of the adverse pulmonary effects of AM. Additionally, it would contribute to the current knowledge of chemical-induced pulmonary toxicity and fibrosis, and potentially shed light on the initiating mechanisms of lung injury caused by other pulmonary fibrogens.

Mitochondria account for up to 95% of cellular energy production, making them ideal targets for initiation of cytotoxicity induced by numerous compounds (Wallace et al., 1997). The mitochondrion has been identified as a prospective target for initiation of AIPT, as both structural and functional alterations resulting from AM exposure have been documented, although primarily in mitochondria from non-pulmonary tissue. Structurally, mitochondria in rat hepatocytes (Gross et al., 1989) and human lymphocytes (Yasuda et al., 1996), as well as isolated rat heart mitochondria (Guerreiro et al., 1986), displayed abnormalities following *in vitro* exposure to AM that included swelling,

disorganized cristae, ruptured inner membranes, and / or electron-lucent appearance in electron micrographs. Functionally, *in vitro* AM exposure has been shown to inhibit respiration in mitochondria isolated from mouse liver (Fromenty et al., 1990a) and rat heart (Guerreiro et al., 1986), and to inhibit electron transfer at complex I of the respiratory chain in isolated rat liver mitochondria (Ribeiro et al., 1997). Furthermore, ATPase activity in guinea pig heart mitochondrial preparations was inhibited by AM (Dzimiri and Almotrefi, 1993), as was the β-oxidation of fatty acids in isolated mouse liver mitochondria (Fromenty et al., 1990b). A recent study using freshly isolated hamster lung cells showed a collapse of mitochondrial membrane potential by AM prior to cellular ATP depletion and ensuing cell death (Bolt et al., 2001a), thus providing the first evidence of a potential causal role for mitochondrial dysfunction in AM-induced pulmonary cytotoxicity.

The primary metabolite of AM, *N*-desethylamiodarone (DEA), possesses antidysrhythmic properties similar to those of AM (Abdollah et al., 1989), but is considerably more cytotoxic and fibrogenic than AM in experimental systems. For example, DEA elicited greater toxic effects than AM on rat alveolar macrophages (Ogle and Reasor, 1990) and on other, non-pulmonary cell types, including human thyrocytes (Beddows et al., 1989) and rat hepatocytes (Gross et al., 1989; Ruch et al., 1991). In the hamster model of AIPT, pulmonary fibrosis induced by DEA was found to be greater in severity and duration than that induced by AM (Daniels et al., 1989). Moreover, pulmonary accumulation of DEA is extensive following AM treatment in humans and animals, often reaching a level greater than that of AM (Adams et al., 1985; Brien et al.,

1987; Daniels et al., 1989; Wilson and Lippmann, 1990). As such, DEA is a potentially toxic metabolite of AM, and may play a significant role in the etiology of AIPT.

Lipid peroxidation (LPO) is recognised as a basic contributing mechanism to the toxicity of numerous chemicals (Halliwell and Gutteridge, 1999b), but its role in AIPT is unclear. Indicators of LPO have been detected in hamster lung tissue (Wang et al., 1992) and bronchoalveolar lavage fluid (BALF) (Blake and Reasor, 1995b) following intratracheal AM administration. Conversely, *in vitro* AM has been shown to decrease LPO in isolated rat liver mitochondria (Ribeiro et al., 1997), microsomes (Rekka et al., 1990), and hepatocytes (Ruch et al., 1991). Further still, other studies have revealed a neutral effect of AM on LPO (Pre et al., 1991; Mansani et al., 1999). The mitochondrion could be a target organelle for initiation of LPO by an AM or metabolite radical formed as a result of electron leakage from the respiratory chain (Rafeiro, 1997). To date, however, the effects of AM and DEA on lung mitochondrial LPO have not been determined.

The purpose of this study was to undertake an evaluation of the *in vitro* lung mitochondrial effects of AM and DEA as they may pertain to initiation of AIPT. Studies were performed with isolated hamster lung mitochondria to determine and compare the accumulation of AM and DEA *in vitro*, and the effects of exposure to these drugs on respiratory function, membrane potential, and LPO.

2.2 MATERIALS AND METHODS

2.2.1 Chemical Sources

Chemicals and reagents were obtained from suppliers as follows: amiodarone hydrochloride, rotenone (95-98%), adenosine 5'-diphosphate (ADP, free acid), β-nicotinamide adenine dinucleotide phosphate (β-NADPH, reduced form, tetrasodium salt), L-glutamate (monosodium salt), ethylenediamine tetraacetic acid (EDTA, disodium salt, dihydrate), succinate (disodium salt, hexahydrate), D-mannitol, 3-[N-morpholino]propanesulfonic acid (MOPS), bovine serum albumin (BSA), fatty acid-free BSA, paraquat dichloride (methyl viologen), and safranine-O dye from Sigma Chemical Co. (St. Louis, MO, USA); carbon tetrachloride (CCl_4) from Fisher Scientific (Nepean, ON, Canada); sodium pentobarbital from M.T.C. Pharmaceuticals (Mississauga, ON, Canada); *N*-desethylamiodarone hydrochloride was generously donated by Wyeth-Ayerst Research (Princeton, NJ, USA). All other chemicals were of analytical grade, and were purchased from standard commercial suppliers.

2.2.2 Animals

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-hour light / 12-hour dark cycle, and were allowed to acclimatise for at least 1 week prior to use. Hamsters were

given free access to water and rodent laboratory chow #5001 (Purina Mills Inc., St. Louis, MO, USA).

2.2.3 Isolation of Whole Lung Mitochondria

For determination of the effects of *in vitro* exposure to AM and DEA on lung mitochondrial oxygen consumption, membrane potential, lipid peroxidation, and drug accumulation, hamsters were killed by injection of sodium pentobarbital (300 mg / kg ip). Following perfusion *in situ* via the pulmonary artery 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 an homogenization buffer comprised of 225 mM mannitol, 75 mM sucrose, 2 mM EDTA, 5.0 mM MOPS, and 2% (w/v) fatty acid-free BSA (pH 7.2). Solutions were kept ice-cold, and all manipulations were performed on ice or at 4°C. Lungs were minced with scissors and homogenized (4 ml buffer / g tissue) using a motorized Potter-Elvehjem® teflon pestle and glass tube. The homogenate was centrifuged at 1 300 x *g* for 5 minutes, and the supernatant collected and centrifuged at 13 000 x *g* for 10 minutes to harvest the mitochondrial pellet. The pellet was washed with approximately half the original volume of homogenization buffer (excluding MOPS and BSA) and centrifuged (13 000 x *g* for 10 minutes), and this process was repeated a second time. The final pellet was gently resuspended by manual homogenization in 0.35 ml of homogenization buffer and kept on ice until use. In order to isolate sufficient lung mitochondria for a single *in vitro* experiment, four pairs of hamster lungs were pooled. Aliquots of mitochondrial

suspensions were saved and used for determination of protein content by the method of Lowry *et al.* (1951), using BSA as the standard.

2.2.4 Polarographic Measurement of Mitochondrial Oxygen Consumption

Oxygen consumption of isolated lung mitochondria was measured at 30°C with a YSI Biological Oxygen Monitor (model 5300) and a Clark-type polarographic oxygen electrode (model 5301; Yellow Springs Instrument Co. Inc., Yellow Springs, OH). Mitochondria (1-2 mg protein) were added to 3 ml of respiration buffer (145 mM KCl, 5 mM KH_2PO_4, 20 mM Tris-HCl, pH 7.2) in a magnetically stirred sample chamber. Respiration supported by complex I of the respiratory chain was assessed using glutamate (5 mM) and malate (5 mM), whereas succinate (10 mM, in the presence of 3 μM rotenone) was used to assess respiration at complex II. To examine the effect of *in vitro* exposure to AM or DEA on state 4 respiration supported by complexes I and II of the electron transport chain, these drugs were added individually to 50-400 μM final concentrations at least 2 minutes following the total expenditure of 0.2 mM ADP. Respiratory control ratios (RCRs) and ADP:O ratios were calculated as indicators of the integrity of mitochondrial respiratory function (Estabrook, 1967; Nedergaard and Cannon, 1979).

2.2.5 Determination of Mitochondrial Membrane Potential

Membrane potential of isolated lung mitochondria was determined at 30°C as described previously (Fromenty et al., 1990a), utilizing the fluorescent properties of safranine dye (Akerman and Wikstrom, 1976). Lung mitochondria (1-2 mg protein;

pooled from 4 hamsters) were incubated at 30°C for 5 minutes in 2.7 ml of reaction buffer (0.2 M sucrose, 20 mM KCl, 20 mM HEPES, and 0.38 mM EDTA, pH 7.2) in the presence of safranine (10 μM) and rotenone (3 μM). The mixture was decanted into a quartz cuvette, and the fluorescence of safranine dye determined for 2 minutes using a Perkin-Elmer LS-5B luminescence spectrometer (excitation 510 nm, emission 570 nm, 5 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 minutes), AM or DEA were added (50-400 μM final concentration), and the change in fluorescence monitored for 10 minutes.

2.2.6 Determination of Mitochondrial Lipid Peroxidation

Lipid peroxidation in isolated lung mitochondria was assessed by the generation of thiobarbituric acid-reactive substances (TBARS). Mitochondria (1-2 mg protein; pooled from 4 hamsters) were incubated at 37°C in a shaking water bath in 2 ml total volume of respiration buffer (145 mM KCl, 5 mM KH_2PO_4, 20 mM Tris-HCl, pH 7.2). AM or DEA were added to a final concentration of 50-400 μM, while CCl_4 or paraquat (used as positive controls) were added to 400 μM final concentrations, in the presence of 1.0 mM NADPH. Initial experiments determined that NADPH did not alter the generation of TBARS in incubations performed with AM or DEA (results not shown), but that it was required for CCl_4 and paraquat to induce significant TBARS generation. Thus NADPH was not included in the incubations performed with AM or DEA. Additionally, none of the compounds tested altered TBARS generation in incubations

lacking mitochondria (results not shown). At selected time intervals after drug addition (15, 30, and 60 minutes), 0.5 ml aliquots were removed and assayed for TBARS by the method of Beuge and Aust (1978). Briefly, the 0.5 ml aliquots were mixed with 1.0 ml of an aqueous solution containing 0.25 M HCl, 26 mM thiobarbituric acid, and 0.9 M trichloroacetic acid. Samples were then placed in a 75°C water bath for 15 minutes, after which time they were removed and placed on ice for 5 minutes. Following cooling, samples were centrifuged at 1 000 x *g* for 10 minutes at room temperature in order to pellet the precipitated protein. Supernatant aliquots (0.5 ml) were removed and absorbance of the chromophore determined at a wavelength of 535 nm using a Beckman DU-7 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). Results were expressed as nmol TBARS / mg protein.

2.2.7 Determination of Mitochondrial Drug Accumulation

Accumulation of AM and DEA in isolated lung mitochondria was determined following incubation with 400 μM AM or DEA at 30°C. Mitochondria (1-2 mg protein; pooled from 4 hamsters) were incubated in 1.5 ml of respiratory buffer for 5 minutes prior to addition of AM or DEA. Following incubation for 0.5 or 3 minutes, samples were centrifuged at 4 000 x *g* for 1 minute to pellet any precipitated drug. Aliquots (250 μl) of supernatants were used for analysis of drug levels by high performance liquid chromatography (HPLC; (Brien et al., 1987; Bolt et al., 1998)), and for protein content by the method of Lowry *et al.* (1951). Because of limited solubility of AM and DEA in the respiratory buffer, concurrent incubations with AM or DEA in the absence of mitochondrial protein controlled for drug precipitation into the buffer (background drug).

To measure drug levels, 250 µl aliquots were centrifuged (5 minutes at 13 000 x *g*) and the resulting mitochondrial pellets were washed, mixed with 250 µl of respiration buffer, and centrifuged again. The supernatants were discarded, and the pellets stored at -20°C for up to 1 month. On the day of analysis, 500 µl of HPLC mobile phase (5% (v/v) acetic acid: acetonitrile, 20:80 (v/v)) was added to the individual pellets, which were mixed vigorously and centrifuged at 13 000 x *g* for 5 minutes. Supernatants were removed and filtered using 0.45 µm syringe filters (Millex-HV syringe-driven filter units; Millipore, Bedford, MA, USA). Fifty µl of these filtrates were analyzed by reverse phase HPLC with UV detection (Brien et al., 1987), using authentic AM and DEA to construct standard curves. Background drug levels in the buffer were subtracted from the drug concentrations measured in the presence of mitochondrial protein.

2.2.8 Statistical Analyses

Data are expressed as mean ± standard deviation for each experimental group. Statistical comparisons amongst treatment groups were performed by randomized one- or two-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test for more than two groups, or by unpaired or paired Student's *t*-test for two groups, as appropriate. Where required, $\log_{10}$ transformation of data was performed to correct for heterogeneity of variance prior to statistical analysis. In all cases, statistical significance was defined as $p<0.05$.

2.3 RESULTS

2.3.1 *In Vitro* Mitochondrial Oxygen Consumption

Pooled hamster lung mitochondria isolated for examination of the effects of *in vitro* exposure to AM and DEA on oxygen consumption had RCR values of 2.90 ± 0.43 and 1.52 ± 0.10 for complexes I and II, respectively, and ADP:O ratios of 4.27 ± 0.42 and 2.20 ± 0.43 for complexes I and II, respectively. Tight coupling of respiration was observed at complex I (RCR > 2.50), while poor coupling occurred at complex II (RCR ≤ 1.50). The reason for this discrepancy is unknown, but may be a result of damage and degradation during mitochondrial preparation, due in part to the high content of lipids and lysosomal enzymes in lung tissue (Fisher et al., 1973; Spear and Lumeng, 1978).

Baseline state 4 respiratory rates (i.e. before drug addition) were 31.8 ± 9.8 and 79.6 ± 22.3 nmol O / min / mg protein for complexes I and II, respectively. Addition of AM or DEA significantly inhibited state 4 respiration supported by complexes I and II at concentrations of 100 μM and greater (Figure 2.1), with DEA causing a greater inhibition than AM at complex II. This inhibition was preceded by a brief stimulatory phase that lasted from 2 to 5 minutes as a result of AM exposure (Figure 2.2). Conversely, a stimulatory phase was not observed following DEA exposure, but rather the inhibition of oxygen consumption occurred almost immediately upon drug addition. Addition of drug vehicle (distilled H_2O) did not alter oxygen consumption. Pre-incubation of mitochondria with either AM or DEA resulted in complete lack of state 3 oxygen consumption in response to the addition of respiratory substrates.

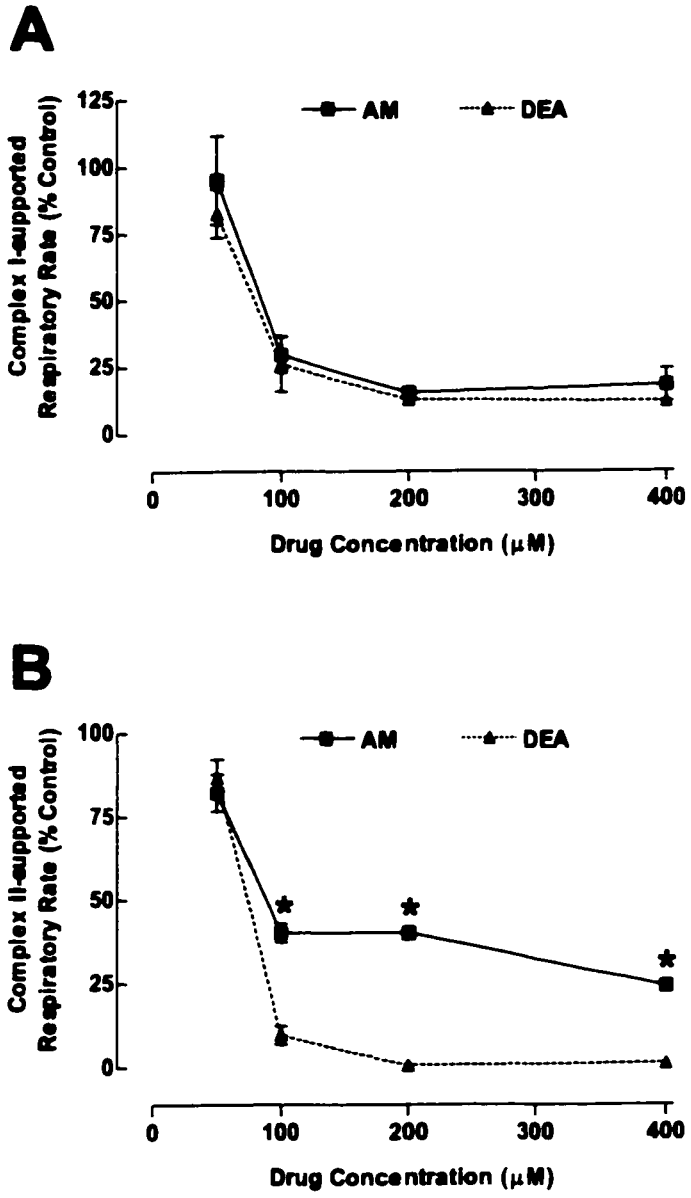


Figure 2.1 Effects of *in vitro* exposure to AM and DEA on state 4 (resting) oxygen consumption rates supported by A) complex I, and B) complex II of the electron transport chain in isolated hamster lung mitochondria. * significant difference from equimolar concentrations of DEA, $p<0.05$ (n=3-4).

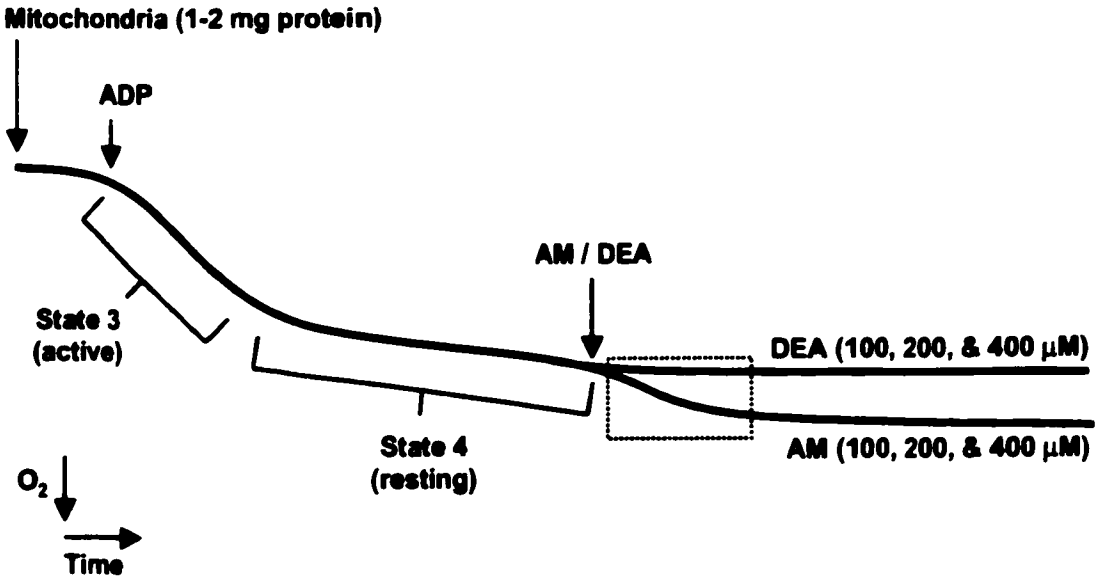


Figure 2.2 Representative lung mitochondrial oxygen consumption tracing, depicting the effects of AM and DEA on complex I-supported state 4 respiration. Note the immediate inhibition of respiration as a result of DEA addition, compared to the initial stimulation followed by secondary inhibition as a result of AM (dashed box). Similar effects were observed for respiration supported by complex II as a result of addition of AM or DEA.

2.3.2 *In Vitro* Mitochondrial Membrane Potential

A significant decrease of membrane potential (as indicated by increased safranine dye fluorescence) was observed following addition of AM or DEA to isolated lung mitochondria (Figure 2.3A), with higher drug concentrations resulting in greater fluorescence changes. While the total change in fluorescence was similar for equimolar concentrations of AM and DEA, the rate of change of fluorescence was significantly greater for DEA at concentrations of 200 and 400 μM (Figure 2.3B).

2.3.3 *In Vitro* Mitochondrial Lipid Peroxidation

Generation of TBARS in isolated lung mitochondria was not altered by AM or DEA in incubations lasting up to 60 minutes (Figure 2.4), while the positive controls CCl_4 and paraquat significantly increased TBARS generation at this time point.

2.3.4 *In Vitro* Mitochondrial Drug Accumulation

Significantly greater amounts of DEA were measured in isolated lung mitochondria following incubation with AM or DEA for 0.5 or 3 minutes (Figure 2.5). No differences were found between the time points with regard to AM or DEA levels.

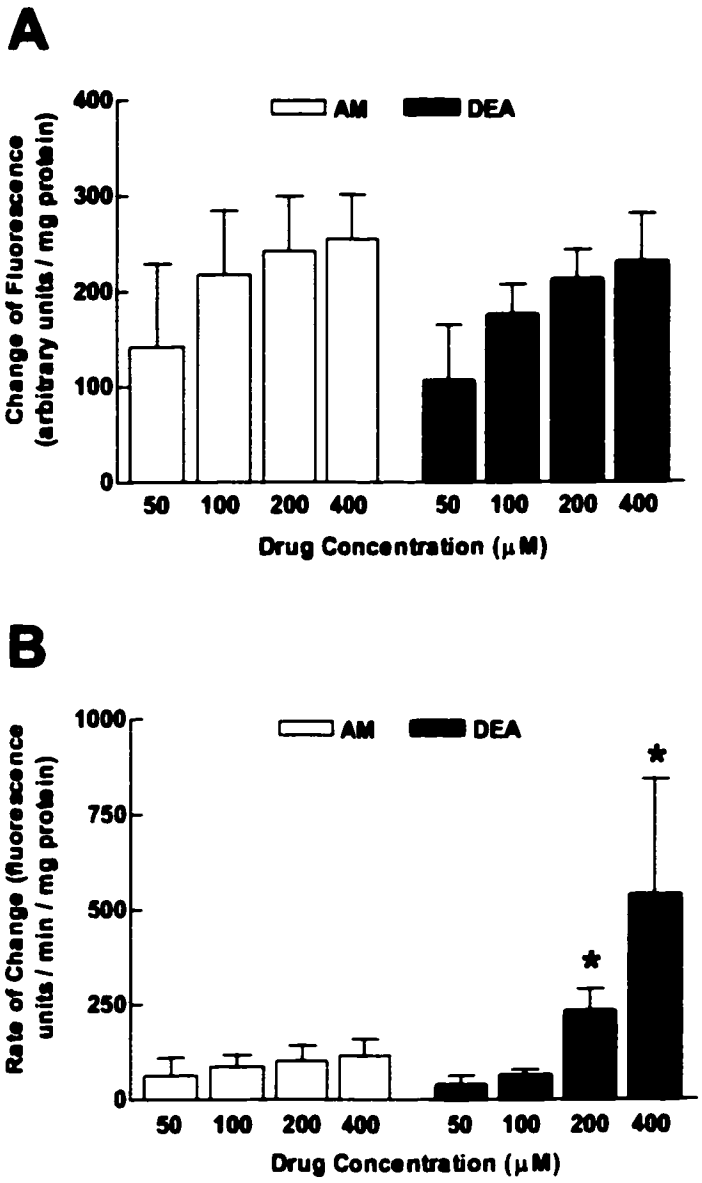


Figure 2.3 Effects of *in vitro* exposure to AM and DEA on membrane potential of isolated hamster lung mitochondria, as measured by safranine dye fluorescence. A) Total change of fluorescence during 10 minute incubation. B) Initial rate of change of fluorescence during 10 minute incubation. * significant difference from equimolar concentrations of AM and from lower concentrations of DEA, $p<0.05$ (n=3-4).

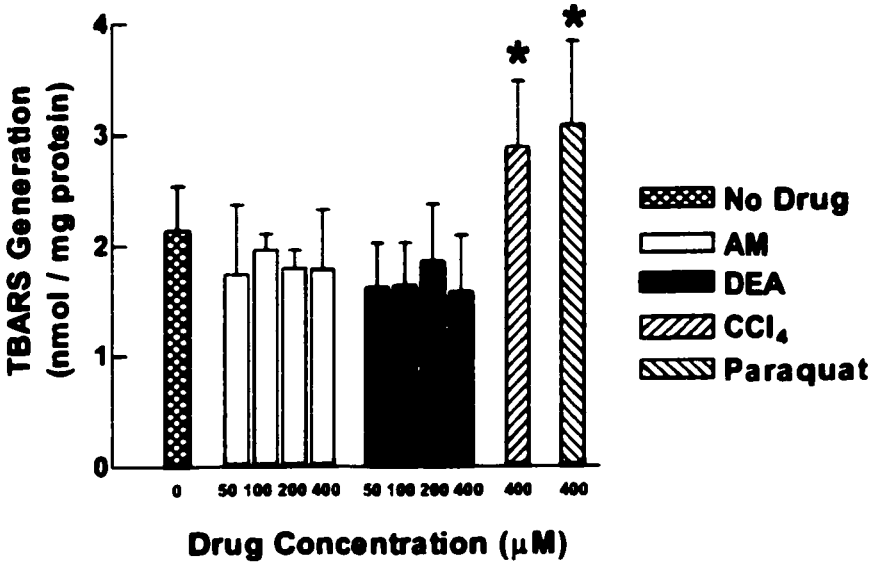


Figure 2.4 Effects of *in vitro* exposure to AM and DEA on lipid peroxidation (measured as thiobarbituric acid-reactive substance (TBARS) production) in isolated hamster lung mitochondria following 60 minutes of incubation.
* significant difference from control (no drug) and from all AM and DEA groups, $p<0.05$ (n=4).

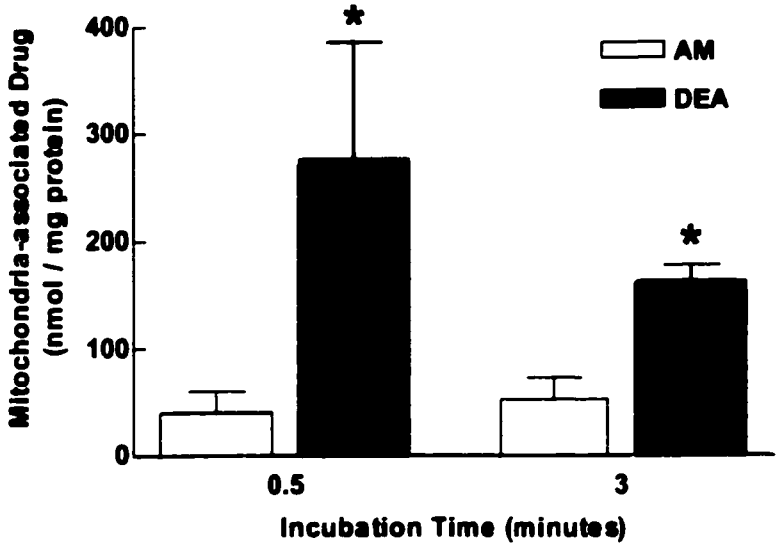


Figure 2.5 AM and DEA levels in isolated hamster lung mitochondria following *in vitro* incubation. * significant difference from AM at the same time point, $p<0.05$ (n=3-4).

2.4 DISCUSSION

The mitochondrion is recognised as a target for the initiation of cytotoxicity caused by numerous agents (Wallace et al., 1997), and may play a similar role in the pathogenesis of AIPT. Considerable evidence suggests that AM disrupts the structure and function of mitochondria from various sources, although the majority of experiments to date have been performed using non-pulmonary cell types or mitochondria isolated from non-pulmonary tissue. As such, the current study was undertaken to thoroughly investigate the effects of *in vitro* exposure to AM and its primary metabolite, DEA, on lung mitochondrial function including oxygen consumption, membrane potential, and lipid peroxidation, and to determine the *in vitro* mitochondrial accumulation of these drugs.

Incubation of isolated whole lung mitochondria with AM or DEA resulted in significant inhibition of state 4 respiration supported by complexes I and II of the electron transport chain. Specifically, decreases in oxygen consumption resulted from the addition of concentrations of AM or DEA of at least 100 μM, while 50 μM AM and DEA had less prominent and inconsistent effects on respiration. The drug concentrations used were chosen based on the reported values for human lung content following AM therapy (Plomp et al., 1984; Brien et al., 1987). Although both drugs decreased oxygen consumption at complexes I and II, notable differences were observed with regard to their effects. In particular, the inhibitory effect of DEA was much more rapid than that of AM, occurring immediately upon addition of the drug to the incubation chamber. In contrast, AM inhibited respiration after a lag period as long as 5 minutes following addition, during which time oxygen consumption was initially stimulated. This effect of AM is

consistent with that observed in mouse liver mitochondria (Fromenty et al., 1990a), and is believed to result from the rapid accumulation of protonated AM within the mitochondrial matrix, leading to release of protons and stimulation of respiration via an uncoupling effect. This is followed by a secondary inhibition of respiratory complex function as a result of the continued accumulation of the drug within the organelle (Fromenty et al., 1990a). Respiration supported by complex II appeared to be affected more by DEA than by AM, as the rate of oxygen consumption was decreased to a greater extent by the metabolite. The reason(s) for this difference is (are) unknown, but may be related to an as-of-yet unidentified complex-specific effect of the metabolite. The fact that complex II was not tightly coupled in any of the mitochondrial preparations (i.e. RCR $\leq$ 1.50) may also have contributed to this difference.

The effects of AM and DEA on mitochondrial membrane potential also demonstrated qualitative differences. While the absolute change in membrane potential was not differentially affected by AM and DEA, the rate at which the drugs decreased membrane potential was dissimilar. Higher concentrations of DEA (200 and 400 μM) decreased membrane potential much more rapidly than did lower concentrations, as well as more rapidly than equimolar concentrations of AM.

In light of the observed temporal differences between the effects of AM and DEA on mitochondrial oxygen consumption and the rate of decrease of membrane potential, the extent of *in vitro* accumulation of the drugs in lung mitochondria was determined. DEA was found to accumulate to a greater extent than AM after both 0.5 and 3 minutes of incubation. Interestingly, the effects of DEA on oxygen consumption and membrane potential were found to occur in less than 0.5 minutes, consistent with the more rapid

uptake of the metabolite into mitochondria. This difference in drug accumulation may explain, at least partially, the differential effects of AM and DEA on respiration and membrane potential, but the reason(s) for the difference in accumulation and the ensuing mitochondrial effects of the two drugs remain(s) unknown. Regardless, these results are consistent with those from other *in vitro* studies demonstrating a greater and / or more rapid toxic effect of DEA compared to AM in isolated cell systems (Gross et al., 1989; Beddows et al., 1989; Ogle and Reasor, 1990; Ruch et al., 1991) and in the hamster model of AIPT (Daniels et al., 1989).

Due to the equivocal experimental evidence regarding the role of LPO in AM-induced toxicities, the effect of AM and DEA on lung mitochondrial LPO was investigated in incubations up to 1 hour in duration. The generation of TBARS was not altered by AM or DEA during the course of the incubations, while CCl_4 and paraquat served as positive controls that readily increased TBARS generation. Thus, despite manifesting more prominent effects on respiration and membrane potential than those of its parent compound, DEA did not induce mitochondrial LPO, consistent with the findings for AM. Therefore it appears that LPO is neither a cause, nor a consequence of AM- or DEA-induced mitochondrial dysfunction.

Rapid *in vitro* accumulation of AM and DEA in lung mitochondria was observed in the current study, and AM was shown to accumulate in rat lung mitochondria following 3 days of administration (Hostetler et al., 1988). Mitochondria have been shown to play an important role in the pulmonary accumulation of other basic drugs such as imipramine, chlorphentermine, and quinine. Studies in isolated rat lung mitochondria demonstrated that basic amine drugs reach a maximum accumulation in this organelle

after 2.5 minutes (Hori et al., 1987), and accumulation of basic drugs was highest in the mitochondrial fraction in the isolated and perfused rat lung (Yoshida et al., 1987). However, none of the basic drugs tested are known to cause pulmonary fibrosis, suggesting that mitochondrial accumulation and subsequent functional impairment leading to cell death and fibrosis may be a characteristic response to AM in lung tissue.

The mechanism by which AM disrupts mitochondrial function is unclear. Inhibition of respiratory function by AM was observed in the present study, consistent with results from experiments using mitochondria isolated from mouse liver (Fromenty et al., 1990a) and rat heart (Guerreiro et al., 1986). Disruption of oxidative phosphorylation is a known consequence of induction of mitochondrial permeability transition (MPT), a phenomenon now recognised to be important in both necrosis and apoptosis (Lemasters et al., 1998). However, the involvement of MPT in AM-induced mitochondrial dysfunction and cytotoxicity has only recently been examined. In freshly isolated hamster lung cells, the MPT inhibitor cyclosporin A (Bernardi, 1996) was ineffective at decreasing AM-induced cytotoxicity that was preceded by collapse of mitochondrial membrane potential and cellular ATP depletion (Bolt et al., 2001a). Other reports have demonstrated AM-induced apoptosis in thyroid and non-thyroid cell lines (Di Matola et al., 2000) as well as in rat and human alveolar epithelial cells *in vitro* (Bargout et al., 2000). While it is possible that apoptosis may play a role in AM-induced pulmonary cytotoxicity leading to fibrosis, the role of MPT in this scenario is unclear, and studies examining these events in an *in vivo* animal model have not been reported.

Free radical formation from AM or DEA may be involved in the mitochondrial effects of these drugs. Incubation of hamster liver or lung microsomes with AM or DEA

was shown to result in generation of free radical species, tentatively identified as carbon-centred phenyl-type radicals not of lipid origin (Rafeiro, 1997). It was proposed that electron leakage from the cytochrome P450 enzyme system was responsible for formation of these radical species, although dependence of radical formation on NADPH was not established. Furthermore, the fact that free radicals were formed in incubations containing boiled microsomes suggests that other, non-enzymatic processes such as Fe^{3+}-catalyzed radical formation might be involved. Leakage of electrons from the mitochondrial respiratory chain could also result in generation of AM or metabolite free radicals, leading to enzyme inhibition through direct binding to and modification of the protein-lipid complexes. The extensive presence of iron-sulphur electron transfer proteins in the mitochondrial respiratory chain provides another potential means of mitochondrial free radical formation through Fe^{3+}-mediated effects. However, these possibilities have not been studied to date.

In conclusion, the present work reveals that *in vitro* exposure to AM and DEA induces adverse effects on lung mitochondrial function, including inhibition of respiratory function and a decrease of membrane potential, without stimulating LPO. The effects of DEA on isolated lung mitochondria were more prominent and / or more rapid than those of AM, suggesting a significant role for this metabolite in AM-induced mitochondrial dysfunction. Given the importance of mitochondria for energy production in almost all cell types (Wallace et al., 1997), and the wealth of literature describing adverse mitochondrial effects of AM, these results suggest that mitochondrial dysfunction induced by AM and / or DEA may play a significant role in AIPT. Studies

focusing on prevention or attenuation of mitochondrial dysfunction may lead to the design of novel therapeutic interventions for the treatment of this condition.

Chapter 3

EFFECTS OF DIETARY VITAMIN E SUPPLEMENTATION ON PULMONARY MORPHOLOGY AND COLLAGEN DEPOSITION IN AMIODARONE- AND VEHICLE-TREATED HAMSTERS

3.1 INTRODUCTION

Investigations utilising intratracheal administration of amiodarone (AM) to the hamster as a model of AM-induced pulmonary toxicity (AIPT) are well documented (Cantor et al., 1984; Daniels et al., 1989; Rafeiro et al., 1994; Blake and Reasor, 1995c; Leeder et al., 1996). In this model, AM treatment consistently leads to histopathological findings characteristic of clinical AIPT in humans (Cantor et al., 1984; Daniels et al., 1989; Rafeiro et al., 1994), including inflammation and the development of patchy interstitial fibrosis. However, reports of the prevention or reversal of AIPT *in vivo* are sparse. In one study, the pulmonary fibrosis induced by intratracheal AM was partially attenuated by dietary pretreatment with taurine and / or niacin (Wang et al., 1992).

The lipid-soluble antioxidant, vitamin E, has been shown to decrease or prevent AM cytotoxicity in isolated rat hepatocytes (Ruch et al., 1991) and cultured human pulmonary artery endothelial cells (Kachel et al., 1990), whereas other antioxidants such as *N*-acetylcysteine (NAC), butylated hydroxytoluene (BHT), catalase, and superoxide dismutase, were ineffective. In addition, drug accumulation and phospholipidosis in

cultured human skin fibroblasts exposed to AM were decreased by vitamin E (Honegger et al., 1995; Scuntaro et al., 1996).

Alterations of some of AM's pulmonary effects *in vivo* and *in situ* also have been observed with vitamin E. Several cell types of the lung, and in particular alveolar macrophages, develop phospholipid-containing lamellar inclusion bodies as a result of inhibition of lysosomal phospholipases A_1 and A_2 by AM (Hostetler et al., 1988; Martin et al., 1989). Whether there is a direct link between AM-induced phospholipidosis and fibrosis has not been established. Nonetheless, vitamin E administration to rats partially alleviated AM-induced pulmonary phospholipidosis (Kannan et al., 1990b). In addition, acute lung injury in ventilated and perfused rabbit lungs exposed to AM was decreased by prior treatment with dietary vitamin E (Kennedy et al., 1988).

Although data are available indicating that vitamin E has the potential to decrease AM-induced phospholipidosis and acute cytotoxicity, the possibility of vitamin E preventing the clinically limiting and life-threatening AM-induced lung fibrosis *in vivo* has not been tested. Thus, the present study was undertaken to investigate the effect of dietary supplementation with vitamin E on the development of lung fibrosis following intratracheal instillation of AM to the hamster.

3.2 MATERIALS AND METHODS

3.2.1 Chemical Sources

Chemicals were obtained from suppliers as follows: amiodarone hydrochloride, vitamin E (d,l-α-tocopherol acetate), chloramine-T (*N*-chloro-*p*-toluenesulfonamide sodium salt), Ehrlich's reagent (*p*-dimethylaminobenzaldehyde), sodium thiosulfate,

alanine, and 10% formalin from Sigma Chemical Co. (St. Louis, MO, USA); Purina Laboratory Rodent Chow #5001 enriched with vitamin E (d,l-α-tocopherol acetate, 500 IU / kg) from Ren's Feed & Supply Ltd. (Oakville, ON, Canada); trans-4-hydroxy-L-proline from Aldrich Chemical Co. (Milwaukee, WI, USA); sodium pentobarbital from M.T.C. Pharmaceuticals (Mississauga, ON, Canada); ketamine hydrochloride from Rogar / STB Inc. (London, ON, Canada). All other chemicals were reagent grade, and were obtained from common commercial suppliers.

3.2.2 Animals and Treatments

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-hour light, 12-hour dark cycle. Upon arrival at the Queen's University animal care facility, animals were randomly assigned to either the control diet (Purina Laboratory Rodent Chow # 5001, containing 49 IU d,l-α-tocopherol acetate / kg) or the vitamin E-enriched diet, and were maintained on their respective diets and water *ad libitum* for 6 weeks prior to analysis of tissue vitamin E content or intratracheal dosing. Routine observations for signs of infection and stress were performed, with none detected. Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care, and the experimental protocols were approved by the Queen's University Animal Care Committee.

Intratracheal administration of AM or H_2O was carried out essentially as described previously (Rafeiro et al., 1994; Leeder et al., 1996). AM was dissolved in distilled H_2O at 60°C, and was allowed to cool to room temperature before instillation.

Hamsters were lightly anaesthetised with ketamine hydrochloride (80-100 mg / kg) injected intramuscularly in the hind leg, and a single dose of AM (1.83 μmol) or an equivalent volume of vehicle (0.1 ml distilled H_2O) was administered by transoral intratracheal instillation using a 22-gauge stainless-steel catheter fitted with a piece of polyethylene tubing. Injection of 0.7 ml of air immediately following AM or vehicle administration facilitated deposition into the lungs. Following treatment, animals were returned to their respective diets and water *ad libitum* for the duration of the experiment. For experiments examining pulmonary fibrosis, there were four treatment groups: control diet + vehicle, control diet + AM, vitamin E diet + vehicle, and vitamin E diet + AM.

3.2.3 Determination of Lung Vitamin E Content

After 6 weeks on the specified diets, each hamster was killed by injection of sodium pentobarbital (300 mg / kg ip), its chest cavity was exposed, and the lungs were perfused *in situ* with 0.9% saline via the pulmonary artery. Lungs were removed, blotted dry, weighed, and homogenized in 5 X volume of 0.9% saline with a Brinkmann Polytron® (Brinkmann Instruments Inc., Westbury, NY). Aliquots of homogenate were analysed for total tocopherol content by the method of Taylor *et al.* (1976), using d,l-α-tocopherol acetate to construct a standard curve. Duplicate aliquots (0.75 ml) of homogenates were mixed with 0.25 ml of 25% (w/v) ascorbic acid and 0.5 ml of absolute ethanol in glass centrifuge tubes, and placed in a 70°C water bath for 5 minutes. Following addition of 0.5 ml of 10 N KOH, tubes were incubated for 30 minutes at 70°C. Tubes were removed and allowed to cool to room temperature before adding 2.0 ml of

hexane and mixing well for 1 minute, followed by centrifugation at 4 000 x *g* for 10 minutes at room temperature. The top hexane layer was removed with a pasteur pipette, placed in clean glass centrifuge tubes, and 0.3 ml of 60% H_2SO_4 was added and mixed vigorously for 30 seconds. Tubes were centrifuged again (4 000 x *g* for 10 minutes) and the top hexane layer transferred to clean glass test tubes. Fluorescence of the samples was determined with a Perkin-Elmer LS-5B luminescence spectrometer (excitation 286 nm, emission 330 nm, 5 nm slit widths). A standard curve was treated in the same manner as the samples by diluting a stock solution of d,l-α-tocopherol acetate (20 μg / ml absolute ethanol) to lower concentrations and mixing 0.5 ml aliquots of absolute ethanol containing various concentrations of d,l-α-tocopherol acetate with 0.25 ml of 25% ascorbic acid and 0.75 ml H_2O (in place of homogenate samples).

3.2.4 Preparation of Lung Tissue for Histology and Hydroxyproline Analysis

At 21 days post-dosing, each animal was killed by injection of sodium pentobarbital (300 mg / kg ip). Thoracotomy was performed, and the trachea exposed and cannulated. The right bronchus was ligated, and the right lung removed, weighed, frozen in liquid nitrogen, and stored at -20°C until determination of hydroxyproline content within four weeks. The left lung was inflated with 10% neutral buffered formalin to a pressure of 20 cm H_2O for 1 hour. The trachea was then ligated, and the lung removed and placed in formalin for at least 48 hours. Sections (approximately 2-3 mm^3 in size) from upper, middle and lower portions of the lung were dehydrated and embedded in paraffin, and 5 μm sections cut and stained with hematoxylin and eosin for histological evaluation.

3.2.5 Histopathology and Determination of Histological Disease Index

Fixed lung sections were dehydrated with a Fisher Tissuematon® by the following sequential steps for 1 hour each: 70% (v/v) aqueous ethanol; 80% (v/v) aqueous ethanol; 2 sequential series of 95% (v/v) aqueous ethanol; 3 sequential series of absolute ethanol; 1:1 (v/v) absolute ethanol : toluene; 2 sequential series of toluene; and 2 sequential series of TissuePrep® paraffin wax (Fisher Scientific, Nepean, ON, Canada) at 62°C. Lung sections were then embedded in paraffin blocks using a Tissue Tek II® embedding centre, and blocks were allowed to cool. Five micrometer-thick sections were cut on a microtome and placed on microscope slides. Tissues were then de-paraffinized and hydrated as follows: xylene, 5 minutes; xylene, 2 minutes; absolute ethanol, 2 minutes; 95% (v/v) aqueous ethanol, 1 minute; 70% (v/v) aqueous ethanol, 1 minute; 50% (v/v) aqueous ethanol, 1 minute; distilled H_2O, 1 minute. Tissues were then stained with hematoxylin solution (192 ml Harris' hematoxylin plus 8 ml glacial acetic acid, filtered immediately prior to use) for 2 minutes, rinsed with running tap H_2O for 20 minutes, and counterstained with 1% (w/v) alcoholic eosin for 2 minutes. Tissues were then dehydrated as follows: 50% (v/v) aqueous ethanol, 1 minute; 70% (v/v) aqueous ethanol, 1 minute; 95% (v/v) aqueous ethanol, 1 minute; absolute ethanol, 1 minute; and 2 changes in xylene of 2 minutes each. Tissue sections were allowed to air dry, and coverslips were mounted on slides with Permount® medium (Fisher Scientific, Nepean, ON, Canada).

To evaluate morphological damage, a disease index was computed for each animal (Cantor et al., 1984; Daniels et al., 1989; Leeder et al., 1996) with the evaluator unaware of the animal treatments. Whole stained lung sections were scanned at a

magnification of 200 X with an eyepiece grid consisting of 100 equal sized squares. The percentage of squares containing disease (defined as cellular infiltration of the alveolar space or interstitium, thickening of the interstitium, or fibrosis) represented the disease index. Squares containing more than one contributor to the disease index were only counted once. The disease index was calculated as the mean of the values for equal numbers of sections taken from upper, middle and lower lung. The mean number of squares examined per lung section was 4877 ± 1267 (mean ± SD), and the total number of squares examined per animal was 14638 ± 1954.

3.2.6 Hydroxyproline Determination

Lung content of hydroxyproline was determined as a biochemical index of fibrosis. The right lung was pulverized in liquid nitrogen, and hydrolyzed in 5.0 ml of 6.0 M HCl at 110°C for 72 h. Following neutralization with 2.75 ml of 10 M NaOH and adjustment of the volume to 10 ml with distilled H_2O, hydroxyproline content was determined in duplicate for each sample by the spectrophotometric method of Lindenschmidt and Witschi (1985). Tubes were allowed to cool, mixed well and centrifuged at 1 000 x *g* for 5 minutes at room temperature. Duplicate aliquots of 0.2 ml were removed from each sample and added to 2.3 ml of borate-alanine buffer (1 part of a 1:9 (v/v) aqueous dilution of alanine solution (0.112 M alanine in distilled H_2O, pH adjusted to 8.7 with 5 N KOH) plus 2 parts of a 1:9 (v/v) aqueous solution of borate buffer (1 M boric acid, 3 M KCl in distilled H_2O, pH adjusted to 8.7 with solid KOH). Tubes were saturated with excess solid KCl, mixed well and oxidized by addition of 0.6 ml of freshly prepared 0.2 M chloramine-T. Tube contents were mixed and allowed to

stand at room temperature for 30 minutes. Oxidation was stopped by addition of 2.0 ml of 3.6 M sodium thiosulfate and the tubes were mixed. Toluene (3.0 ml) was added, and tubes were tightly capped and placed in boiling water for 30 minutes. After cooling to room temperature, tubes were shaken vigorously by hand (approximately 100 times) and centrifuged at 1 000 x *g* for 5 minutes. An aliquot of the upper toluene phase (1.5 ml) was removed and added to a clean tube containing 0.6 ml Ehrlich's reagent and mixed. Ehrlich's reagent was prepared by adding 13.7 ml concentrated sulfuric acid to 200 ml absolute ethanol on ice; in another beaker, 130 g of *p*-dimethylaminobenzaldehyde was added to 200 ml absolute ethanol, and the acidified ethanol mixture was then slowly added to this beaker while stirring. After 30 minutes, absorbance of the chromophore was determined at a wavelength of 560 nm using a Beckman DU-7 spectrophotometer. Blanks consisted of tubes treated as described, with the exception that 0.2 ml distilled H_2O was used in place of diluted neutralized hydrolysate. A standard curve was prepared using trans-4-hydroxy-L-proline in 2.5 ml borate-alanine buffer. The amount of hydroxyproline was expressed as μg hydroxyproline / right lung.

3.2.7 Statistical Analyses

Data are expressed as the mean ± standard deviation for each experimental group. Statistical comparisons amongst treatment groups for the histological disease index, body weight, right lung wet weight and right lung hydroxyproline content data were conducted by randomized-design, two-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test. Histological disease index data underwent arcsine transformation prior to statistical analysis, as described by Sokal and Rohlf (1973) for percentage data.

Comparison of lung vitamin E content was conducted by Student's unpaired *t*-test. In all cases, statistical significance was defined as $p<0.05$.

3.3 RESULTS

3.3.1 Gross Observations

The technique of intratracheal instillation resulted in 3 deaths; 2 animals in the control diet + AM group and 1 in the vitamin E diet + vehicle group, all within 1 hour of AM or vehicle administration. One animal of the remaining seven in the control diet + AM group died on day 15 following AM administration; histological analysis of the lungs was not possible due to the time of day of the death. These deaths resulted in the following treatment group distributions: control diet + vehicle (n=6), control diet + AM (n=6), vitamin E diet + vehicle (n=6), and vitamin E diet + AM (n=8). In addition, at 21 days after AM treatment, 1 animal of the remaining 6 in the control diet group that received AM had an apical lobe of the right lung that was severely haemorrhagic. The right lung as a whole weighed twice that of any other lung in this diet and treatment group. We have not encountered such an effect in our previous use of this experimental protocol (Daniels et al., 1989; Leeder et al., 1994; Rafeiro et al., 1994; Leeder et al., 1996). Whether this observation was due to a pre-existing condition in the hamster is unknown. However, this response was considered anomalous, and the lung in question was excluded from further analysis.

3.3.2 Lung Vitamin E Content

The vitamin E-enriched diet increased lung vitamin E content by 234% (38.8 versus 11.6 μg vitamin E / g tissue for the control diet, $p<0.05$) after 6 weeks. This diet did not alter the body weight gain of hamsters relative to those on the control diet during the 6 weeks prior to and during the 3 weeks (21 days) post-intratracheal administration of AM or vehicle (Table 3.1).

3.3.3 Right Lung Wet Weights and Histopathology

Twenty-one days post-intratracheal administration, right lung wet weight was decreased in the vitamin E + vehicle group ($p<0.05$) compared to all other treatment groups (Table 3.1). No other difference in lung weight was found.

Lungs from hamsters on the control diet that received vehicle demonstrated normal lung architecture (Figure 3.1A), and were similar in appearance to those from the vitamin E diet + vehicle group (Figure 3.1B). In contrast, lungs from the control diet + AM group demonstrated areas of patchy fibrosis, with cellular infiltration and thickening of the interstitium, often near airways and blood vessels (see arrow, Figure 3.1C). The vitamin E enriched diet decreased the AM-induced damage to the lungs so that their appearance was similar to control (Figure 3.1D).

The lung disease index values for upper, middle, and lower sections of lungs within treatment groups were not significantly different ($p>0.05$). Disease index values for the control diet + vehicle animals were similar to those found previously and, as expected, were elevated ($p<0.05$) in the control diet + AM group by 42% (Figure 3.2) (Daniels et al., 1989; Leeder et al., 1996). In contrast, the disease index values for the

Table 3.1

Effects of dietary vitamin E supplementation and intratracheal amiodarone (AM) administration on hamster body and right lung wet weights.

			Body weight (g)	
			Experimental time	
Diet	**Treatment**	**Right lung wet weight (g)**	**6 weeks**	**9 weeks**
Control			166.2 ± 13.0 (15)	--
	H_2O	0.37 ± 0.04 (6)	--	162.5 ± 12.9 (6)
	AM	0.41 ± 0.04 (5)	--	182.1 ± 11.1 (6)
Vitamin E			159.6 ± 10.5 (15)	--
	H_2O	0.32 ± 0.03* (6)	--	166.9 ± 12.1 (6)
	AM	0.40 ± 0.04 (8)	--	171.6 ± 12.9 (8)

* significant difference from all other groups, $p<0.05$.

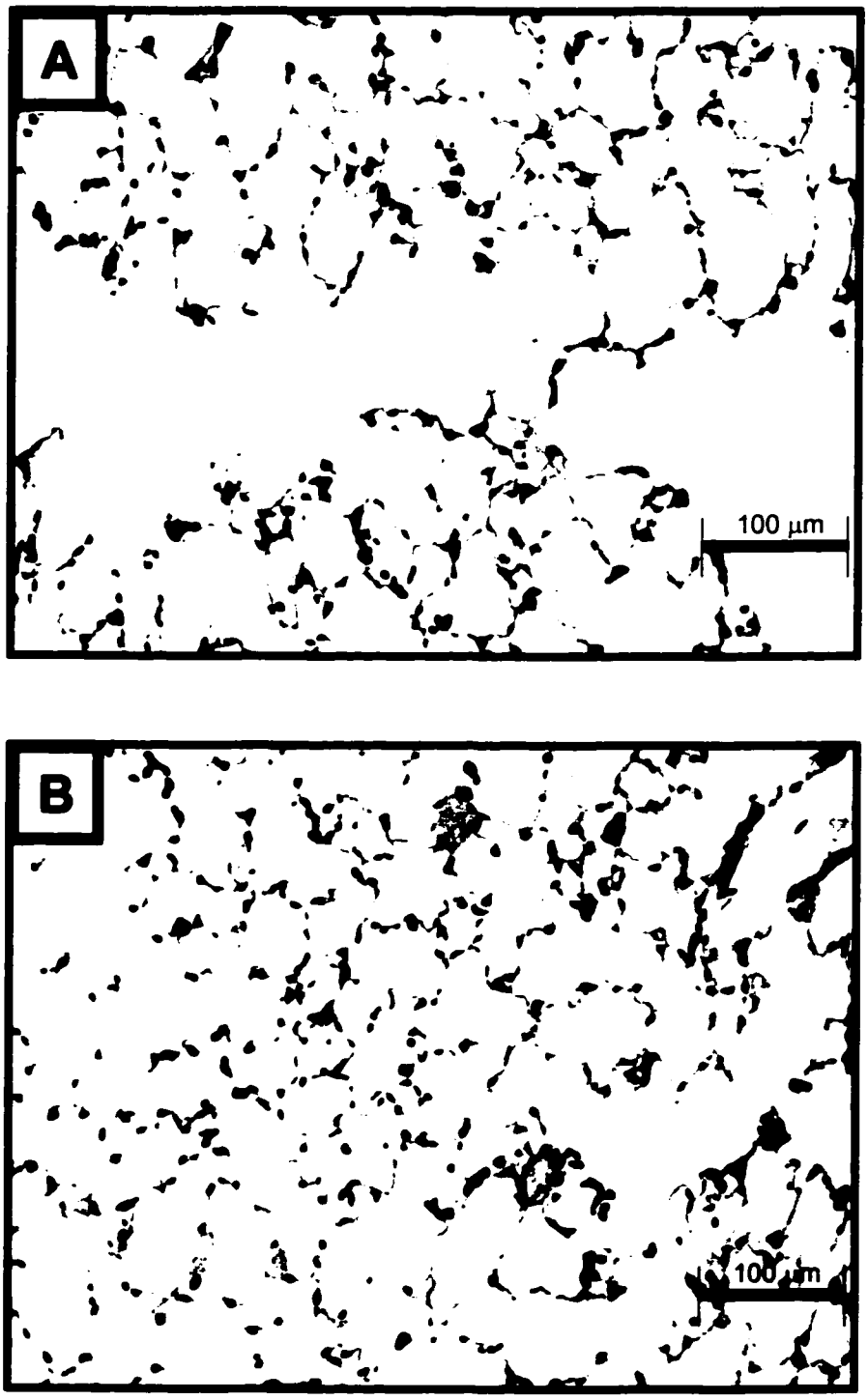


Figure 3.1 Representative light photomicrographs (H&E stain) of hamster lungs 21 days following intratracheal administration of amiodarone (AM) or distilled H_2O with or without dietary vitamin E supplementation for 6 weeks prior to and continuously after treatment. (A) Control diet + H_2O. (B) Vitamin E diet + H_2O. (C) Control diet + AM. (D) Vitamin E diet + AM.

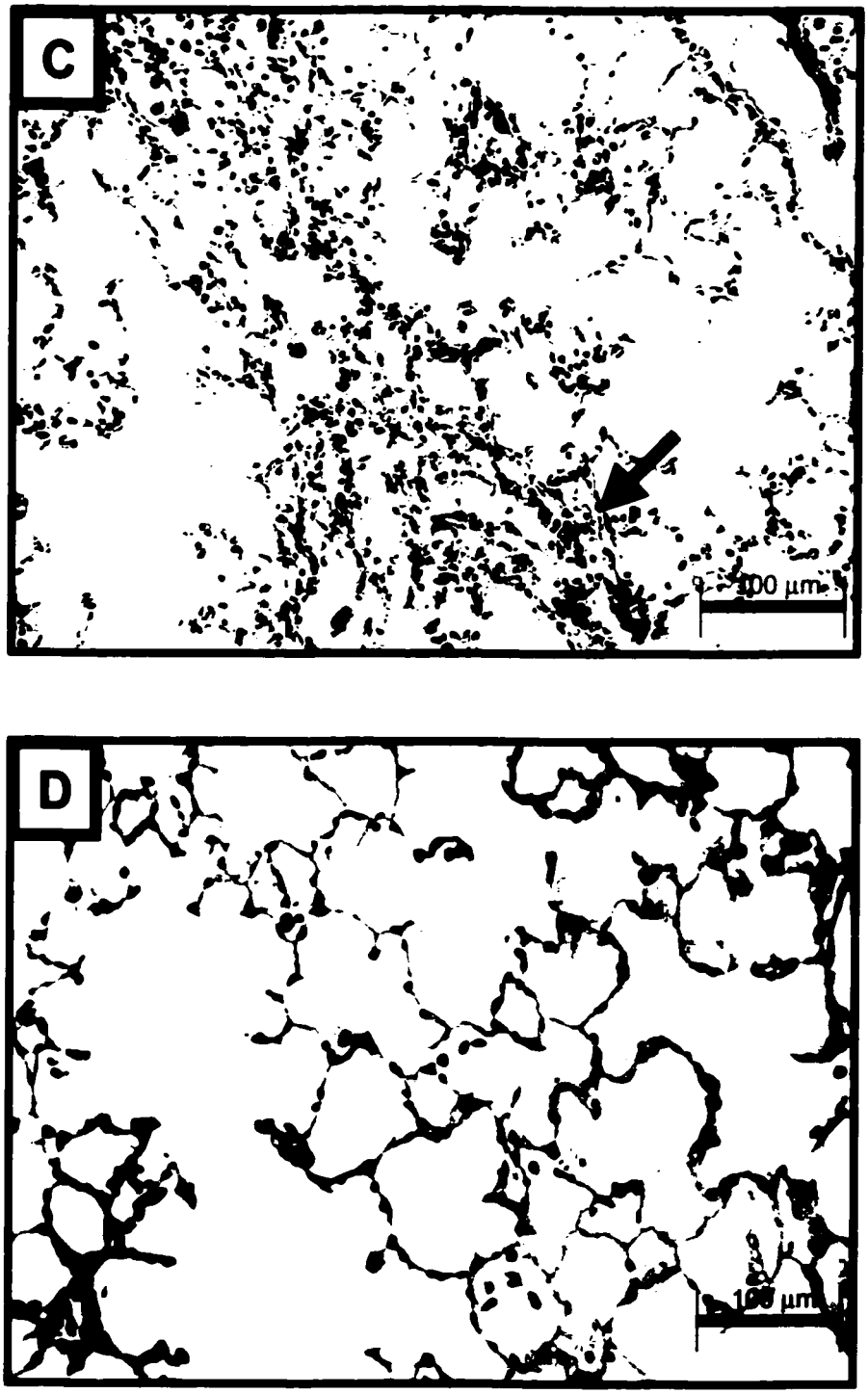


Figure 3.1 Continued.

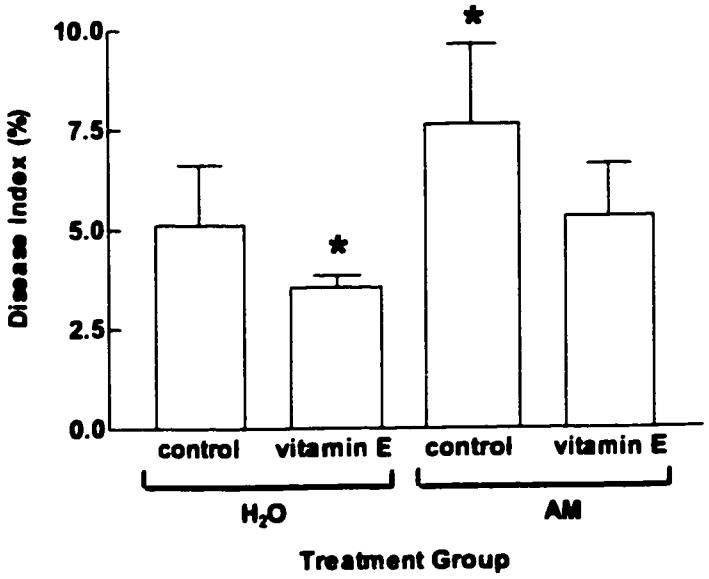


Figure 3.2 Disease index values of hamster lungs 21 days following intratracheal administration of AM (1.83 μmol) or H_2O (0.1 ml) with or without dietary vitamin E supplementation for 6 weeks prior to, and continuously after dosing.
* significant difference from all other groups, $p<0.05$ (n=6-8).

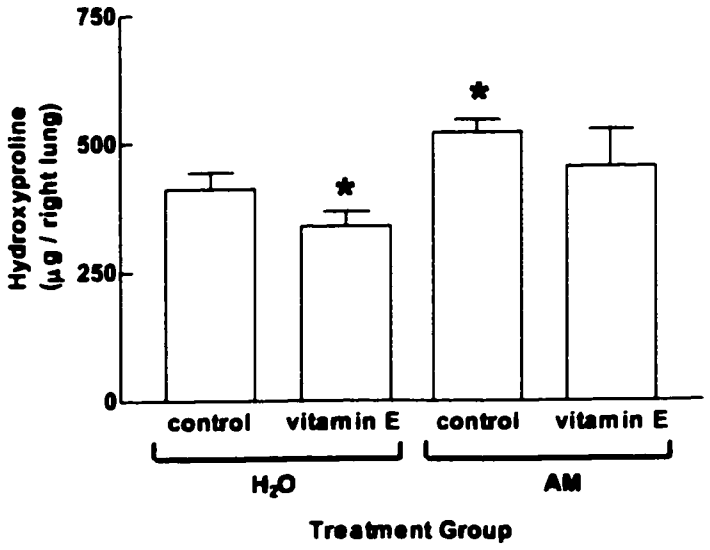


Figure 3.3 Hamster right lung hydroxyproline content 21 days following intratracheal administration of AM (1.83 µmol) or H_2O (0.1 ml) with or without dietary vitamin E supplementation for 6 weeks prior to, and continuously after dosing. * significant difference from all other groups, $p<0.05$ (n=5-7).

vitamin E diet + AM animals were decreased ($p<0.05$) reiative to the control diet + AM group, and were not different from the control diet + vehicle group. However, the disease index data for lungs of hamsters exposed to the vitamin E diet + vehicle were lower ($p<0.05$) than those receiving the control diet + vehicle. This was attributed to decreased interstitial thickening in animals on the vitamin E diet.

3.3.4 Lung Hydroxyproline Content

The lung hydroxyproline data of the four treatment groups demonstrated the same rank-order pattern as the disease index data: control diet + AM > vitamin E diet + AM = control diet + vehicle > vitamin E diet + vehicle ($p<0.05$) (Figure 3.3).

3.4 DISCUSSION

The present study documented morphological and biochemical alterations indicative of pulmonary fibrosis following intratracheal instillation of AM to hamsters that received a dietary supplementation of vitamin E. Analysis of lungs was done at 21 days post dosing because fibrosis due to intratracheal AM adminstration is maximal at that time (Daniels et al., 1989). The increased lung content of vitamin E at the time of AM administration ensured that the target tissue was well supplemented with this antioxidant at the time of drug exposure. The pulmonary vitamin E content in the control diet group at the time of AM administration was similar to that reported for normal human lung (Redlich et al., 1996).

Experimental data regarding the protective effect of vitamin E against chemical-induced lung injury are equivocal. Vitamin E has been shown to protect against

paraquat- and bleomycin-induced pulmonary injury in rats (Suntres and Shek, 1995; Suntres and Shek, 1997), whereas it did not inhibit lung pressure-volume changes produced by bleomycin in hamsters (Sato et al., 1988). Variations in the administration protocols and experimental endpoints have made it difficult to ascertain the protective effects of vitamin E. Results from *in vitro* investigations indicate that vitamin E can prevent or decrease AM-induced cytotoxicity in a number of different cell types (Kachel et al., 1990; Ruch et al., 1991; Honegger et al., 1995). In the present study, we found that dietary supplementation with vitamin E was effective at decreasing pulmonary fibrosis induced by AM *in vivo*. Both the lung disease index and hydroxyproline content (indicative of collagen deposition) data of hamsters given the vitamin E-enriched diet were near control values at 21 days following AM administration. Both of these indicators of lung injury have been previously correlated with interstitial collagen deposition via trichrome staining (Daniels et al., 1989; Rafeiro et al., 1994). This decrease in pulmonary injury is in contrast to the paradoxical delayed enhancement of pulmonary toxicity that short-term steroid treatment can invoke 2-3 weeks following induction of lung injury by fibrogens (Kehrer and Witschi, 1981; Kehrer et al., 1984). The vitamin E administration regimen employed in the present study did not augment AM toxicity at the time of maximal AM-induced pulmonary injury, but rather resulted in a marked reduction.

In the present study, hamsters on the vitamin E enriched diet that received vehicle (i.e. distilled water) intratracheally had lower lung disease index and hydroxyproline content values than their counterparts on the control diet. This finding suggests that vitamin E altered cellularity and connective tissue deposition and / or degradation even in

control animals. Conceivably, this may have occurred because vitamin E prevented damage in response to intratracheal distilled water administration, or it may represent intrinsic pulmonary effects of vitamin E. Indeed, the effects of vitamin E supplementation on indicators of pulmonary damage in the control animals suggest that a component of the protective effect of this antioxidant against AM-induced pulmonary injury is not specific to AM. This is especially evident when considering the hydroxyproline content data, where vitamin E decreased basal levels of lung hydroxyproline. Whether the effects of vitamin E in AM- and vehicle-treated hamsters resulted from down-regulating the expression of procollagen genes, enhancing the degradation of mature collagen fibres, directly scavenging AM or lipid free radical species, decreasing cytotoxicity in target lung cells, or through a combination of these and other mechanisms, is not known at present. Regardless, the pharmacological actions of vitamin E are multi-faceted, and delineating the precise mechanism(s) of the vitamin E-induced decrease in AIPT will require further experimentation.

Vitamin E is considered to be the major non-enzymatic chain-breaking antioxidant in cellular lipid structures (Burton et al., 1983; van Acker et al., 1993), and possesses membrane-stabilizing properties in addition to its antioxidant function (Diplock, 1982; Urano et al., 1988). Dietary deficiency of vitamin E enhances the toxicity of several chemicals, and supplementation lessens the detrimental effects of some agents (Chow, 1991). In addition to its antioxidant properties, this lipid-soluble compound also affects membrane fluidity (Lucy, 1972; Urano et al., 1988), phospholipase activity (Tran and Chan, 1988), the uptake and / or metabolism of certain drugs (Statham et al., 1985), and expression of procollagen I mRNA *in vitro* (Houglum et

al., 1991) and *in vivo* (Pietrangelo et al., 1995). The effect of vitamin E on the uptake and accumulation of AM in cultured cells is not clearly understood. For example, vitamin E-induced protection of human pulmonary artery endothelial cells against AM toxicity was not attributable to inhibition of drug uptake (Kachel et al., 1990), whereas prevention of drug accumulation was cited as the mechanism by which vitamin E protected cultured human skin fibroblasts against AM-induced cytotoxicity (Honegger et al., 1995). Despite having no effect on serum, liver, or lung AM concentrations, consumption of a vitamin E enriched diet partially decreased the pulmonary phospholipid content of AM-treated rats (Kannan et al., 1990b). Similarly, phospholipidosis in cultured human skin fibroblasts exposed to AM and other cationic amphiphilic drugs was decreased by vitamin E (Honegger et al., 1995; Scuntaro et al., 1996). Although the mechanism by which vitamin E decreases phospholipidosis may involve reversal of AM-induced inhibition of phospholipases, the relationship that this has to the reduction of AM-induced pulmonary fibrosis is unclear.

It is not known whether long-acting oxygen radical scavengers such as dimethylthiourea (DMTU) might offer protection against AM-induced pulmonary injury. However, recent *in vivo* and *in vitro* evidence from our laboratory, and those of other investigators, does not support the notion of reactive oxygen species playing a role in AIPT (Kachel et al., 1990; Ruch et al., 1991; Leeder et al., 1996). While the protection offered by vitamin E against the development of AIPT may not be due to scavenging reactive oxygen species, the possibility of vitamin E scavenging lipid or drug radicals cannot be ruled out. Formation of a carbon-centred radical upon UV irradiation of AM has been reported (Li and Chignell, 1987), and it has been proposed that there is

formation of a highly reactive AM aryl radical upon chemical reduction of the drug by a hydrated electron or an organic radical (Vereckei et al., 1993). Scavenging of these radicals may be an important mechanism by which vitamin E decreases AM-induced toxicity *in vivo* and *in vitro*.

In conclusion, dietary vitamin E supplementation decreased histopathological and biochemical indices associated with pulmonary injury in both amiodarone- and vehicle-treated hamsters, indicating that the protective effects of vitamin E are not entirely specific to AM. However, this is the first study to demonstrate that vitamin E can decrease AM-induced pulmonary fibrosis in an *in vivo* animal model. The clinical relevance of these findings may be substantial, considering the relative safety of oral vitamin E in both humans and other mammalian species (Bendich and Machlin, 1988), the widespread clinical use of AM, and the need for efficacious therapies for fibrotic diseases (Franklin, 1995).

Chapter 4

ATTENUATION OF AMIODARONE-INDUCED PULMONARY FIBROSIS BY VITAMIN E IS ASSOCIATED WITH SUPPRESSION OF TGF-β_1 GENE EXPRESSION BUT NOT PREVENTION OF MITOCHONDRIAL DYSFUNCTION

4.1 INTRODUCTION

Vitamin E has been reported to decrease amiodarone (AM)-induced cytotoxicity in cultured pulmonary (Futamura, 1996b) 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 AM-induced pulmonary toxicity (AIPT), we showed that dietary vitamin E supplementation reduced the extent of pulmonary collagen deposition and histological damage following intratracheal AM administration (Chapter 3; Card et al., 1999). To date, the mechanism(s) of protection of vitamin E against AIPT have not been determined.

Recognized primarily for its free radical scavenging and chain-breaking antioxidant properties, vitamin E has recently 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 pro-inflammatory and pro-fibrotic genes (Parola et al., 1992; Chojkier et al., 1998; Chan et al., 1998). One of these, transforming growth factor (TGF)-β_1, is a critical mediator of fibrosis (Cooper, Jr., 2000; Sime and O'Reilly, 2001). Targeting this cytokine directly, or the steps involved in its activation or signalling, may prove to be a valuable therapeutic strategy against fibrosis of several tissues.

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 following 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.

4.2 MATERIALS AND METHODS

4.2.1 Chemical Sources

Chemicals and reagents were obtained as follows: *N*-desethylamiodarone hydrochloride (DEA) was generously donated by Wyeth-Ayerst Research (Princeton, NJ, USA); 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, USA); Purina Laboratory Rodent Chow #5001 enriched with vitamin E (d,l-α-tocopherol acetate, 500 IU / kg) from Ren's Feed & Supplies Ltd. (Oakville, ON, Canada); vitamin E (d,l-α-tocopherol acetate), chloramine-T (*N*-chloro-*p*-toluenesulfonamide, sodium salt), Ehrlich's reagent (*p*-

dimethylaminobenzaldehyde), sodium thiosulfate, alanine, and 10% buffered neutral formalin from Sigma Chemical Co. (St. Louis, MO, USA). Unless otherwise stated, all other chemicals and reagents were of analytical grade and were obtained from standard commercial suppliers.

4.2.2 Animals and Treatments

Animal care, diet assignments, and intratracheal dosing were carried out as described in section 3.2.2.

To examine the effects of dietary vitamin E supplementation on pulmonary fibrosis and TGF-β_1 gene expression following AM administration, animals were sacrificed 7 or 21 days following intratracheal dosing and lung tissue processed as described in section 3.2.4.

To examine the effects of dietary vitamin E supplementation on mitochondrial dysfunction induced by *in vitro* exposure to AM and DEA, lung mitochondria were isolated as described in section 4.2.6 from hamsters that were maintained on the vitamin E-supplemented diet for 6 weeks, and used for determination of oxygen consumption and membrane potential. To examine the effects of dietary vitamin E supplementation on lung mitochondrial respiratory function following AM administration, animals were sacrificed 3 hours following intratracheal dosing and lung mitochondria isolated as described in section 4.2.6.

4.2.3 Preparation of Lung Tissue

4.2.3.1 Lung Vitamin E Analysis

Lung vitamin E content was determined following 1 to 6 weeks on the control or vitamin E diet as described in section 3.2.3.

For lung mitochondrial vitamin E determination, hamsters were killed by injection of sodium pentobartital (300 mg / kg ip) after 6 weeks on the control or vitamin E diet. Lung mitochondria were isolated from individual hamsters as described in section 2.2.3, snap-frozen in liquid nitrogen, and stored at -80°C. Aliquots were analyzed for protein content by the method of Lowry *et al.* (1951), and for total tocopherol content as described in section 3.2.3, using scaled down proportions in order to accommodate smaller mitochondrial sample volumes.

4.2.3.2 Histopathology and Determination of Histological Disease Index

Histopathology and determination of histological disease indices were performed as described in section 3.2.5.

4.2.3.3 Hydroxyproline Determination

Lung content of hydroxyproline was determined as described in section 3.2.6.

4.2.4 Preparation of Molecular Probes

The TGF-β_1 template was purchased as an *Escherichia coli* plasmid insert (American Type Culture Collection, Manassas, VA, USA). Following bacterial culturing via standard microbiological techniques, the plasmid was isolated using a QIAprep Spin

miniprep kit (QIAGEN®, Chatsworth, CA, USA). Following endonuclease restriction digestion of the plasmid with BamHI for 1 hour at 37°C, the 0.75 kb insert was visualized via electrophoresis for 1 hour at 120 V on a 1% (w/v) agarose gel containing 0.25 μg / ml ethidium bromide. The insert was extracted and purified with a QIAGEN® gel extraction kit, and quantified by spectrophotometric measurement of UV absorption at 260 nm. The 18S rRNA DECA probe template was obtained from Ambion Inc. (Austin, TX, USA).

Radiolabeled cDNA probes (25 ng of template DNA per probe) were generated with α-^{32}P-dCTP, using a random primer labelling kit (GIBCO BRL Life Technologies, Burlington, ON, Canada) as per the manufacturer's instructions. Unincorporated α-^{32}P-dCTP was removed using Quickspin Sephadex columns (Roche Biomedicals, Laval, PQ, Canada). Radiolabeling efficiency was determined in an aliquot of the purified probes using a Beckman LS 6500 scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA).

4.2.5 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. RNA was quantified by spectrophotometric measurement of UV absorption at a wavelength of 260 nm (1 optical density unit = 40 μg / ml RNA). Total RNA (10 μg / lane) was combined with loading buffer (10% (v/v) formamide, 12% (v/v) formaldehyde, 50 mM MOPS (pH 7.0), 5% (v/v) glycerol, and 0.5 μg / ml ethidium bromide) to a total volume of 40 μl. This mixture was electrophoresed through 1% (w/v) agarose / 3% (v/v) formaldehyde gels buffered with 50 mM MOPS (pH 7.0) at 120 V (Hoefer® PS 500X DC Power Supply,

Biorad, Mississauga, ON, Canada) for 2 hours in a running buffer of 50 mM MOPS (pH 7.0). Gels were washed twice for 15 minutes each in distilled H_2O, followed by 2 washes for 20 minutes each in 10X SSC buffer (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0). RNA was 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).

Prehybridization of membranes was carried out at 68°C for 1 hour in 8.0 ml of QuikHyb® hybridization buffer (Stratagene, La Jolla, CA, USA) using a rotating hybridization incubator (Model 2000 Micro Hybridization incubator, Robbins Scientific, Sunnyvale, CA, USA). Membranes were rinsed in water and allowed to air dry prior to prehybridization in order to remove salts. A ^{32}P-labeled TGF-β_1 cDNA probe was denatured by boiling in water for 5 minutes, and was then added to the hybridization tube (3.0×10^6 cpm / ml QuikHyb® buffer), and hybridization was carried out at 68°C for 4 hours.

After hybridization, membranes were washed in low stringency buffer (2X SSC and 0.1% (w/v) SDS) at room temperature for 2 periods of 15 minutes each, followed by 2 periods of 30 minutes each in low stringency buffer at 55°C to remove non-specifically bound cDNA probe from membranes. Hybridized probe was visualized and quantitated using a STORM 820 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Membranes were stripped of bound probe by rinsing in water and then immersing in boiling high stringency buffer (0.1X SSC and 0.1% (w/v) SDS) for 1 minute. Following this, membranes were rinsed in water, allowed to air dry, and the hybridization procedure

was repeated with a ^{32}P-labeled 18S rRNA cDNA probe. After hybridization with the 18S probe, membranes were washed in low stringency buffer at room temperature for 2 periods of 15 minutes each, followed by 2 periods of 30 minutes each in high stringency buffer at 68°C to remove non-specifically bound cDNA probe from membranes. 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. The level of TGF-β_1 mRNA was normalized to that of 18S rRNA due to the reported finding of increased expression of other housekeeping genes such as β-actin and GAPDH following induction of lung injury in the hamster (Raghow et al., 1989; Gurujeyalakshmi et al., 1996).

4.2.6 Isolation of Whole Lung Mitochondria

To determine the effect of dietary vitamin E supplementation on lung mitochondrial respiratory inhibition and membrane potential disruption resulting from *in vitro* exposure to AM and DEA, hamsters were maintained on the control or vitamin E-supplemented diet for 6 weeks after which time lung mitochondria were isolated as described in section 2.2.3. To determine the effect of dietary vitamin E supplementation on lung mitochondrial respiratory inhibition resulting from intratracheal AM administration, lung mitochondria were isolated from individual hamsters 3 hours post-dosing. Aliquots of mitochondrial suspensions were saved and used for determination of protein content by the method of Lowry *et al.* (1951), using bovine serum albumin (BSA) as the standard.

4.2.7 Polarographic Measurement of Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption was measured as described in section 2.2.4.

4.2.8 Monitoring Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined as described in section 2.2.5.

4.2.9 Statistical Analyses

Data are expressed as mean ± standard deviation for each experimental group. Statistical comparisons amongst treatment groups were performed via randomized one- or two-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test for more than two groups, or via paired or unpaired Student's *t*-test for two groups, as appropriate. Histological disease index data underwent arcsine transformation prior to statistical analysis, as described by Sokal and Rohlf (1973) for percentage data. Where required, $\log_{10}$ transformation of data was performed to correct for heterogeneity of variance prior to statistical analysis. In all cases, statistical significance was defined as $p<0.05$.

4.3 RESULTS

4.3.1 Whole Lung and Mitochondrial Vitamin E Content

The vitamin E-enriched diet increased total lung vitamin E content by 114% after 1 week (65.6 versus 30.6 μg / g lung tissue for the control diet), and levels remained elevated for the duration of the 6 week treatment period (Figure 4.1A). Accumulation of vitamin E reached a plateau following the first week of supplementation, as the content

did not increase significantly thereafter. After 6 weeks, the amount of vitamin E in lung mitochondria was markedly increased in vitamin E-supplemented hamsters compared to hamsters fed the control diet (Figure 4.1B).

4.3.2 Pulmonary Histopathology and Disease Index

Representative micrographs from the various diet and treatment groups 21 days following intratracheal dosing are shown in Figure 4.2, and calculated disease index values are summarized in Figure 4.3. Intratracheal AM dosing resulted in increased histopathological damage 21 days post-dosing, as evidenced by patchy areas of fibrosis and interstitial thickening with occasional cellular infiltration of the interstitial space and alveoli (Figure 4.2C). This damage was most often observed near airways and blood vessels. Maintenance on the vitamin E-supplemented diet prior to and following AM dosing prevented these histopathological changes (Figure 4.2D). Calculated disease index values for the control diet group dosed with AM were elevated 147% compared to the control diet group dosed with vehicle (Figure 4.3). Maintenance on the vitamin E diet prevented the AM-induced increase in calculated disease index value.

4.3.3 Right Lung Wet Weights and Hydroxyproline Content

Right lung wet weights were significantly increased by AM at 21 days post-dosing, but not at 7 days post-dosing (Figure 4.4). However, lung weight to body weight ratios were increased by AM at both 7 and 21 days post-dosing (Figure 4.5). Maintenance on the vitamin E-supplemented diet did not prevent the increase in lung

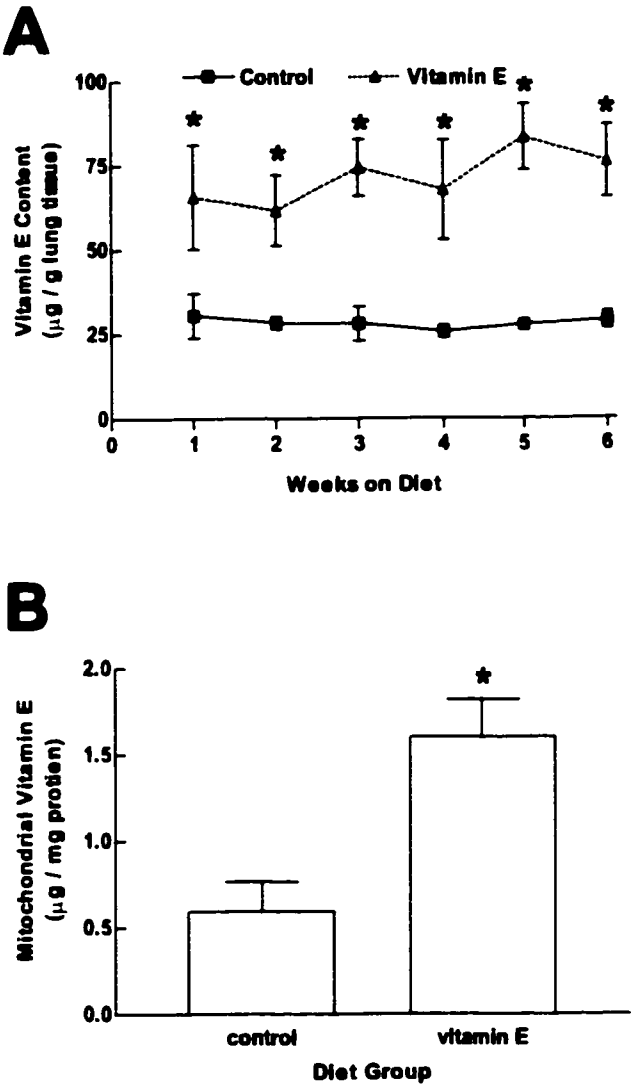


Figure 4.1 Vitamin E content of A) hamster lungs following 1 to 6 weeks of dietary supplementation, and B) hamster lung mitochondria following 6 weeks of dietary supplementation. * significant difference from control diet group, $p<0.05$ (n=3-4).

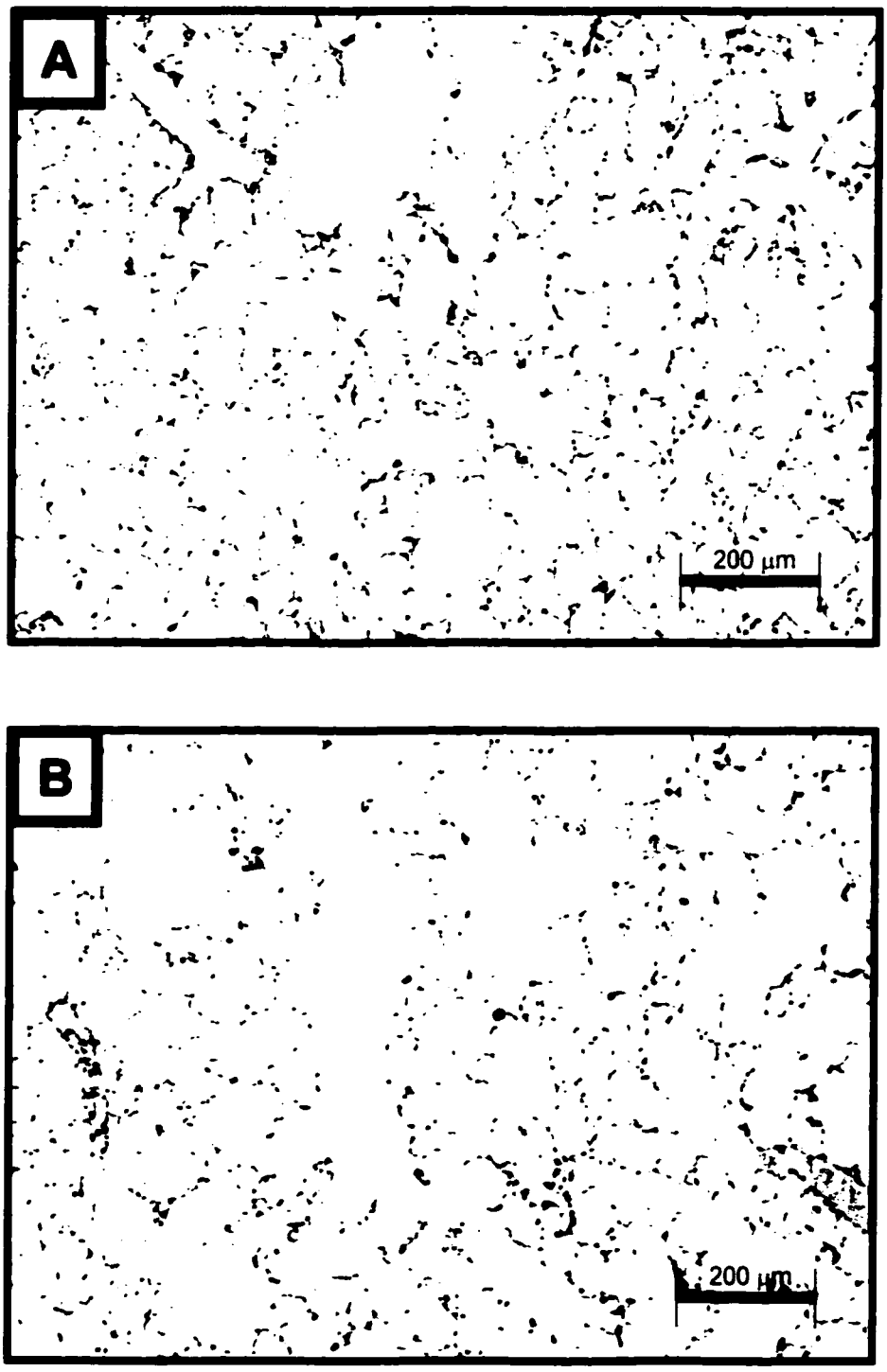


Figure 4.2 Representative light photomicrographs (H&E stain) of hamster lungs 21 days following intratracheal administration of amiodarone (AM) or distilled H_2O with or without dietary vitamin E supplementation for 6 weeks prior to, and continuously after treatment. (A) Control diet + H_2O. (B) Vitamin E diet + H_2O. (C) Control diet + AM. (D) Vitamin E diet + AM.

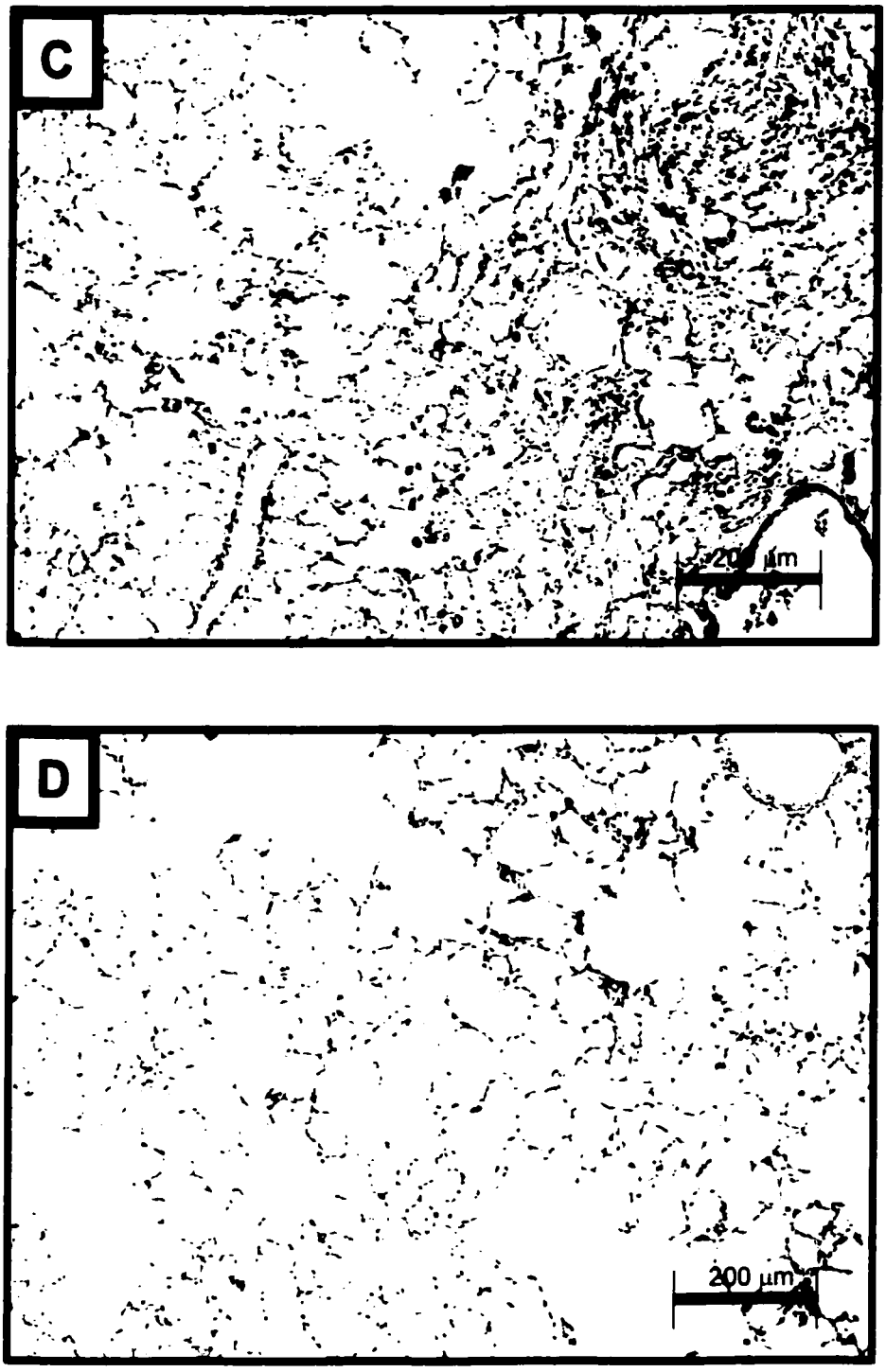


Figure 4.2 Continued.

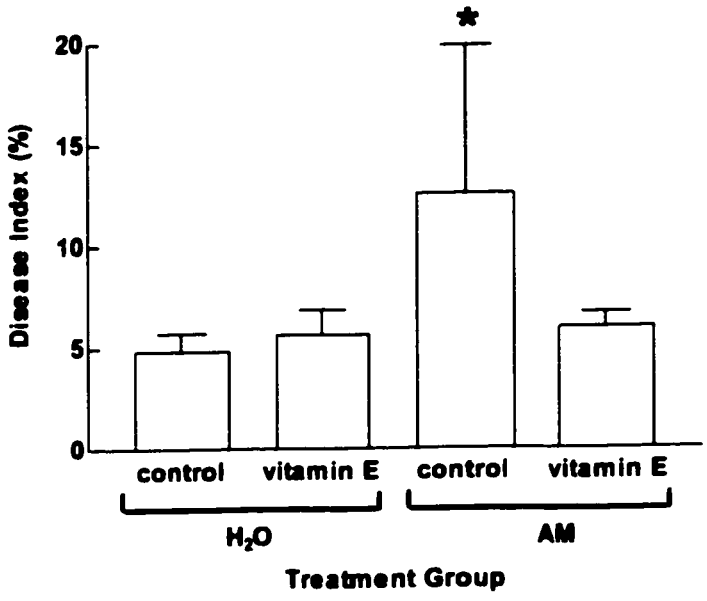


Figure 4.3 Disease index values of hamster lungs 21 days following intratracheal administration of AM (1.83 μmol) or H_2O (0.1 ml) with or without dietary vitamin E supplementation for 6 weeks prior to, and continuously after dosing. * significant difference from all other groups, $p<0.05$ (n=5-8).

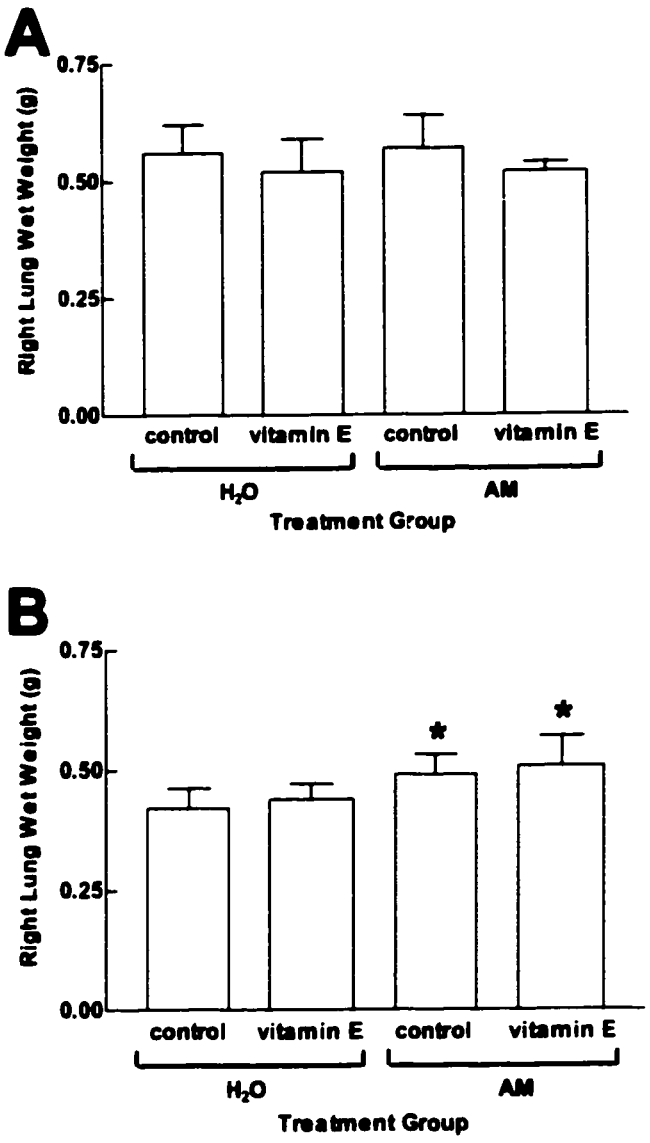


Figure 4.4 Hamster right lung wet weights A) 7 days, and B) 21 days following intratracheal administration of AM (1.83 μmol) or H_2O (0.1 ml) with or without dietary vitamin E supplementation for 6 weeks prior to, and continuously after dosing. * significant difference from the H_2O-treated groups, $p<0.05$ (n=3-7).

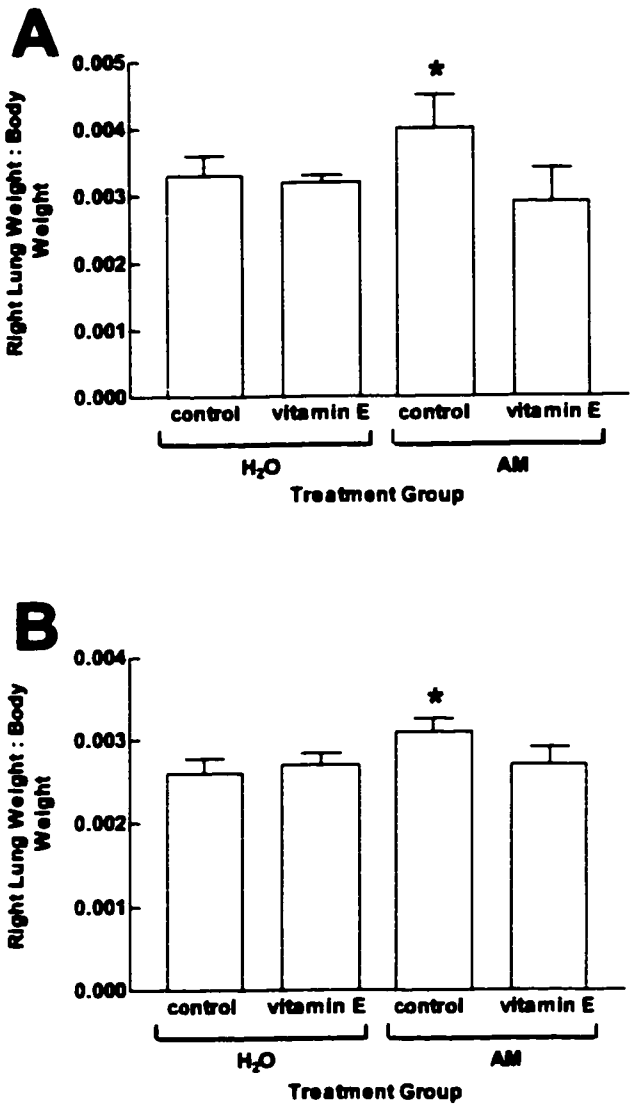


Figure 4.5 Hamster right lung wet weight to body weight ratios A) 7 days, and B) 21 days following intratracheal administration of AM (1.83 μmol) or H_2O (0.1 ml) with or without dietary vitamin E supplementation for 6 weeks prior to, and continuously after dosing. * significant difference from all other groups, $p<0.05$ (n=3-7).

weight at 21 days post-dosing, but did prevent the AM-induced increase in lung weight to body weight ratios at both 7 and 21 days post-dosing (Figure 4.5).

Right lung hydroxyproline content was determined at 7 and 21 days post-dosing as a biochemical index of fibrosis. Intratracheal AM administration did not alter hydroxyproline levels at 7 days post-dosing (Figure 4.6A). At 21 days post-dosing, a significant increase (24%) in the control diet group dosed with AM was observed compared to the control diet group dosed with vehicle (Figure 4.6B). Maintenance on the vitamin E diet prevented the AM-induced increase of hydroxyproline content measured at 21 days post-dosing.

4.3.4 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 dosing at 7 and 21 days, respectively (Figure 4.7A and B). Dietary supplementation with vitamin E prevented the AM-induced increase of TGF-β_1 mRNA at both 7 and 21 days, without altering expression in vehicle-treated hamster lungs.

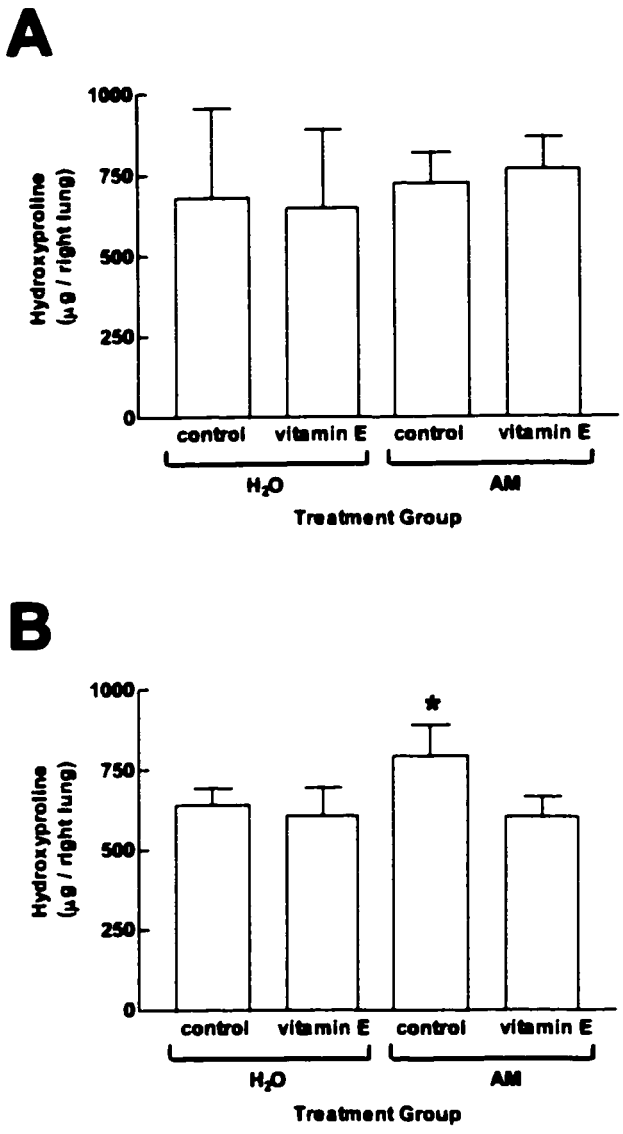


Figure 4.6 Hamster right lung hydroxyproline content A) 7 days, and B) 21 days following intratracheal administration of AM (1.83 µmol) or H_2O (0.1 ml) with or without dietary vitamin E supplementation for 6 weeks prior to, and continuously after dosing. * significant difference from the H_2O-treated groups, $p<0.05$ (n=3-8).

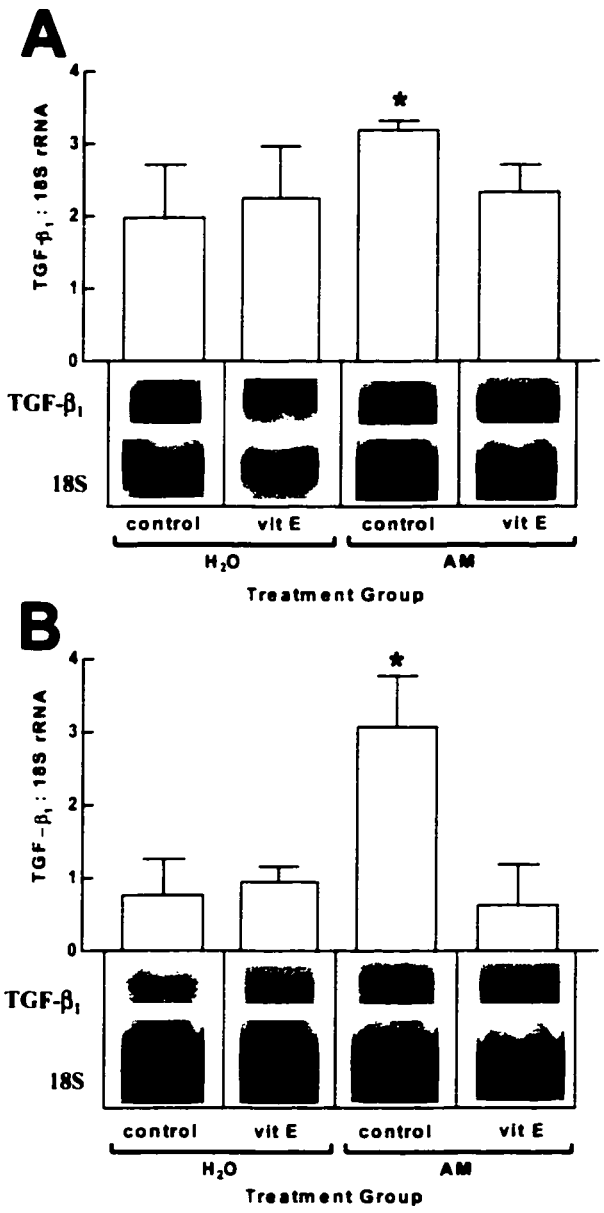


Figure 4.7 Hamster right lung TGF-β_1 mRNA content A) 7 days, and B) 21 days following intratracheal administration of AM (1.83 µmol) or H_2O (0.1 ml) with or without dietary vitamin E supplementation for 6 weeks prior to, and continuously after dosing. * significant difference from all other groups, $p<0.05$ (n=3-7).

4.3.5 *In Vitro* Mitochondrial Oxygen Consumption

Respiratory rates and calculated respiratory control ratios (RCRs) and ADP:O ratios for complex I- and II-supported respiration in lung mitochondria isolated for determination of the *in vitro* respiratory effects of AM and DEA are summarized in Table 4.1. Although a trend toward lower respiratory rates in the vitamin E group was observed, no significant differences were found between the diet groups for any of these parameters. AM and DEA demonstrated significant *in vitro* inhibitory effects on state 4 respiration supported by complexes I and II in isolated lung mitochondria (Figure 4.8), as was observed in Chapter 2. Maintenance on the vitamin E-enriched diet for 6 weeks prior to mitochondrial isolation and analysis did not alter the adverse respiratory effects of AM and DEA.

4.3.6 *In Vitro* Mitochondrial Membrane Potential

Mitochondrial membrane potential (as indicated by increased safranine dye fluorescence) was decreased by AM and DEA to a similar maximal extent (Figure 4.9A), although the rate of decrease was much more rapid for higher concentrations of DEA (Figure 4.9B). Neither measurement was affected by vitamin E supplementation for 6 weeks prior to mitochondrial isolation and analysis.

Table 4.1

Respiratory parameters of isolated hamster lung mitochondria prior to *in vitro* addition of AM or DEA for determination of effects on state 4 respiration. Hamsters were maintained on the control or vitamin E-enriched diet for 6 weeks prior to isolation of lung mitochondria.

Respiratory Complex	Diet group	*n*	Respiratory Rate (nmol O / min / mg protein) State 3	State 4	RCR	ADP:O
I	Control	4	97.3 ± 29.3	31.8 ± 3.5	2.90 ± 0.24	4.26 ± 0.10
	Vitamin E	4	62.9 ± 9.1	22.9 ± 2.5	2.82 ± 0.25	4.18 ± 0.08
II	Control	4	119.6 ± 36.5	79.5 ± 11.3	1.52 ± 0.04	2.20 ± 0.14
	Vitamin E	4	89.9 ± 9.9	59.2 ± 3.0	1.53 ± 0.04	2.32 ± 0.37

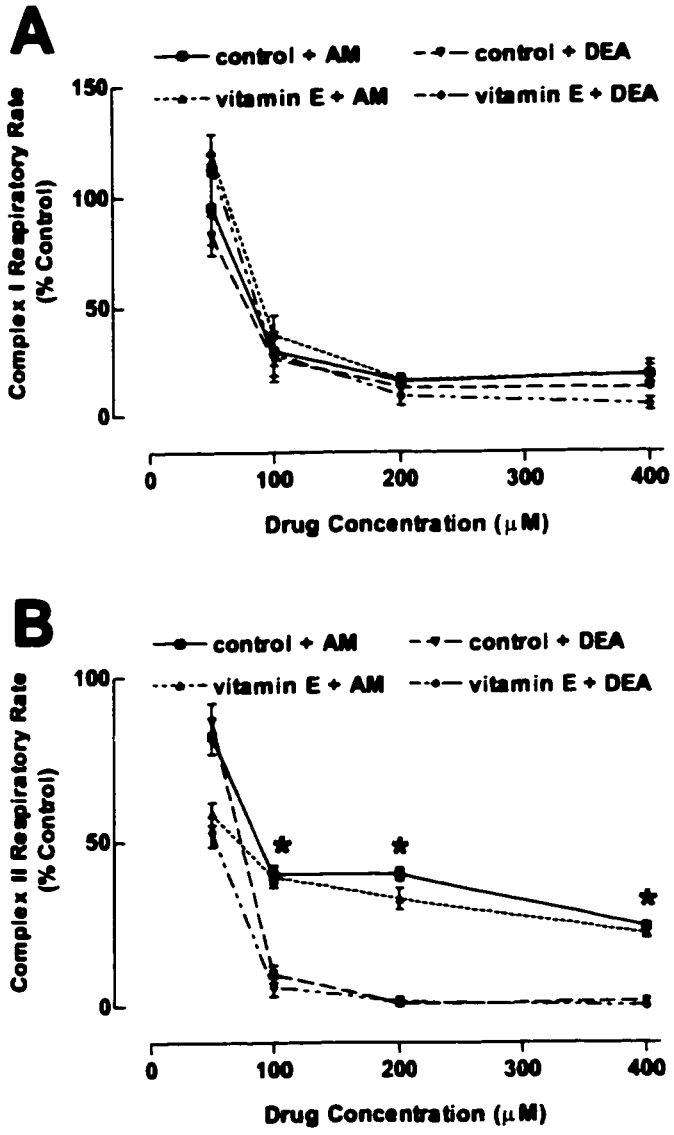


Figure 4.8 Effects of *in vitro* exposure to AM and DEA on state 4 (resting) oxygen consumption rates supported by A) complex I, and B) complex II of the electron transport chain in isolated hamster lung mitochondria with or without dietary supplementation with vitamin E prior to isolation and analysis.
* significant difference from equimolar concentrations of DEA, $p<0.05$ (n=4).

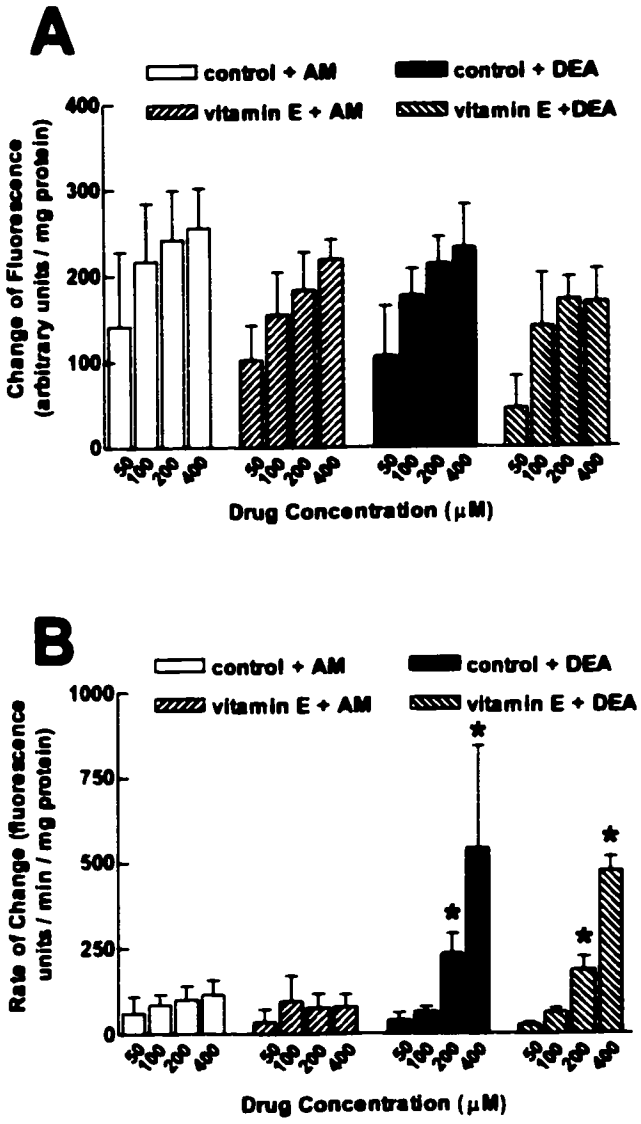


Figure 4.9 Effects of *in vitro* exposure to AM and DEA on A) total change of membrane potential, and B) rate of change of membrane potential of isolated hamster lung mitochondria, as measured by safranine dye fluorescence. Hamsters were maintained on the control or vitamin E supplemented diet for 6 weeks prior to isolation of mitochondria. * significant difference from equimolar concentrations of AM and from lower concentrations of DEA, $p<0.05$ (n=3-4).

4.3.7 Intratracheal Dosing and Mitochondrial Respiratory Function

The effects of intratracheal administration of AM or vehicle on respiratory parameters in lung mitochondria isolated 3 hours post-dosing are summarized in Table 4.2. Intratracheal AM administration resulted in inhibition of state 3 respiration supported by complex I at 3 hours post-dosing, reflected by a decrease of the calculated RCR and an increase of the calculated ADP:O ratio (Table 4.2), indicative of uncoupling of respiration. Maintenance on the vitamin E diet for 6 weeks prior to dosing did not alter the adverse lung mitochondrial respiratory effects caused by intratracheal AM administration at complex I. Respiration supported by complex II was not affected by AM at 3 hours post-dosing (Table 4.2). A relatively low RCR (< 1.50) was found at complex II in mitochondria from vehicle-treated animals, however, suggesting that an uncoupling effect of AM was not observed due to weak initial coupling at this complex.

Table 4.2

Respiratory parameters of lung mitochondria isolated 3 hours following intratracheal dosing with AM (1.83 μmol) or H_2O (0.1 ml). Hamsters were maintained on the control or vitamin E-enriched diet for 6 weeks prior to experimentation.

			Complex I-supported Respiratory Rates (nmol O / min / mg protein)			
Diet	**Treatment**	***n***	**State 3**	**State 4**	**RCR**	**ADP:O**
Control	H_2O	4	54.8 ± 12.5	21.7 ± 5.9	2.57 ± 0.24	4.11 ± 0.32
Vitamin E	H_2O	3	43.5 ± 13.0	17.0 ± 5.6	2.57 ± 0.16	4.25 ± 0.52
Control	AM	4	24.2 ± 16.2*	13.9 ± 7.4	1.73 ± 0.20*	6.19 ± 0.82*
Vitamin E	AM	3	8.5 ± 2.0*	7.0 ± 2.1	1.23 ± 0.09*	6.20 ± 0.60*

			Complex II-supported Respiratory Rates (nmol O / min / mg protein)			
Diet	**Treatment**	***n***	**State 3**	**State 4**	**RCR**	**ADP:O**
Control	H_2O	4	75.6 ± 9.6	58.0 ± 10.0	1.54 ± 0.42	1.40 ± 0.52
Vitamin E	H_2O	3	68.4 ± 23.6	55.0 ± 20.1	1.25 ± 0.06	1.62 ± 0.47
Control	AM	4	56.8 ± 15.7	45.3 ± 13.6	1.37 ± 0.24	1.38 ± 0.22
Vitamin E	AM	3	30.8 ± 11.1*	26.2 ± 7.8	1.12 ± 0.08	1.71 ± 1.03

* significant difference from respective H_2O-treated animals within the same diet group, $p<0.05$.

4.4 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, 1996b; Card et al., 1999). In the current study, pulmonary accumulation of vitamin E following initiation of supplementation was rapid, and total lung levels were more than doubled compared to 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 following 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 (Bienkowski and Gotkin, 1995), 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 following AM treatment, whereas hydroxyproline content was not elevated until 21 days, supporting a central role for this cytokine in the tissue remodelling that occurs during the course of AM-induced pulmonary fibrosis in the hamster. TGF-β_1 up-regulation following 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 over-expression

following AM administration being an essential component of the protective effect of vitamin E.

While 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., 1996). 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 and colleagues reported increased malondialdehyde levels at the time of maximal fibrosis in the hamster model of AIPT (Wang et al., 1992), 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; Chan et al., 1998). 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 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* (Chapter 2; 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 (Chapter 2; Guerreiro et al., 1986; Fromenty et al., 1990a; 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 hours after intratracheal administration of AM is consistent with the timecourse 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 prior to 3 hours post-treatment (data not shown). Thus, this time point was chosen to investigate the effect of vitamin E on mitochondrial dysfunction following AM administration. Comparative effects of an equimolar dose of DEA on mitochondrial function could not be determined, as close to 100% mortality occurred in hamsters within 3 hours following 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 following *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, 1996b) involves prevention of AM-induced mitochondrial dysfunction is unknown. While 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 pro-fibrotic 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 byproducts detected following *in vitro* exposure of mitochondria to AM (Chapter 2; 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 following dietary supplementation, 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; Goldschlager et al., 2000). Up-regulation of TGF-β_1 gene expression was observed in AM-treated hamsters prior to, and at the time of maximal lung injury, and this effect was ablated by dietary vitamin E supplementation. However, elevated mitochondrial vitamin E content following supplementation did not prevent AM- and DEA-induced mitochondrial dysfunction, a potential initiating event in AIPT.

Chapter 5

PIRFENIDONE ATTENUATES AMIODARONE-INDUCED PULMONARY FIBROSIS IN THE HAMSTER

5.1 INTRODUCTION

Numerous agents cause lung damage that can progress to fibrosis, although different agents have different mechanisms of initiation of lung tissue damage (Phan, 1995). It is believed that injury to the epithelium and basement membranes is necessary for the development of pulmonary fibrosis (Coker and Laurent, 1998; Selman et al., 2001). Considerable evidence suggests that mitochondrial dysfunction plays an initiating role in amiodarone (AM)-induced toxicities, including AM-induced pulmonary toxicity (AIPT) (Chapter 2). Disruption of mitochondrial membrane potential prior to ATP depletion and subsequent cell death was observed in isolated lung cells exposed to AM or its primary metabolite, *N*-desethylamiodarone (DEA) (Bolt et al., 2001a), and other *in vitro* studies have shown that AM causes both functional and structural alterations in mitochondria from various tissues (Guerreiro et al., 1986; Fromenty et al., 1990a; Yasuda et al., 1996; Card et al., 1998; Chapter 2). As such, the mitochondrion is a potential target organelle for initiation of AM-induced pulmonary cytotoxicity that occurs prior to inflammation and fibrosis.

Following cytotoxicity, 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 remodelling. This culminates in overproduction of collagen and other matrix components characteristic of fibrosis (Coker and Laurent, 1998; Selman et al., 2001). One of the cytokines most important in extracellular matrix remodelling, including collagen deposition, is transforming growth factor (TGF)-β_1 (Sime et al., 1997; Cooper, Jr., 2000; Sime and O'Reilly, 2001). Thus, inhibition of TGF-β_1 action may be a valuable strategy for treatment of fibrosis of the lung and other tissues.

Treatment of pulmonary fibrosis has traditionally been based on anti-inflammatory and immunosuppressive therapies, with very limited success (Selman et al., 2001). As such, new therapeutic strategies are desirable. Pirfenidone has demonstrated anti-fibrotic activity in several organs, including lung (Iyer et al., 1995; Shimizu et al., 1998; Tada et al., 2001; Mirkovic et al., 2002). Beneficial outcomes in rodent models of bleomycin- and cyclophosphamide-induced pulmonary fibrosis have been reported (Kehrer and Margolin, 1997; Iyer et al., 1998), and a recently completed phase II clinical study showed pirfenidone to be a promising treatment for idiopathic pulmonary fibrosis (Raghu et al., 1999).

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 (Singh, 1996; Nolan, Jr. 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 tested whether pirfenidone could prevent AM-induced disruption of pulmonary mitochondrial function and pulmonary fibrosis in a hamster model.

5.2 MATERIALS AND METHODS

5.2.1 Chemical Sources

Chemicals and reagents were obtained as follows: Pirfenidone was generously donated by Marnac, Inc. (Dallas, Texas, USA); *N*-desethylamiodarone hydrochloride (DEA) was generously donated by Wyeth-Ayerst Research (Princeton, NJ, USA); 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, USA); chloramine-T (*N*-chloro-*p*-toluenesulfonamide, sodium salt), Ehrlich's reagent (*p*-dimethylaminobenzaldehyde), sodium thiosulfate, alanine, and 10% buffered neutral formalin from Sigma Chemical Co. (St. Louis, MO, USA). Unless otherwise stated, all other chemicals and reagents were of analytical grade and were obtained from standard commercial suppliers.

5.2.2 Animals and Treatments

Animals were obtained and cared for as described in section 2.2.2. Hamsters were given free access to water and either pulverized rodent laboratory chow #5001 (Purina Mills Inc., St. Louis, Missouri, USA) 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).

Intratracheal administration of AM or H_2O was carried out as described in section 3.2.2. Following treatment, hamsters 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.

5.2.3 Preparation of Lung Tissue

At 7 or 21 days post-treatment, each animal was killed by injection of sodium pentobarbital (300 mg / kg ip) and lung tissue processed as described in section 3.2.4.

5.2.4 Histopathology and Determination of Histological Disease Index

Histopathology and determination of histological disease indices were performed as described in section 3.2.5.

5.2.5 Hydroxyproline Determination

Lung content of hydroxyproline was determined as described in section 3.2.6.

5.2.6 Preparation of Molecular Probes

Molecular probes were prepared as described in section 4.2.4.

5.2.7 Total RNA Isolation and Hybridization Analyses

Isolation of total RNA and hybridization analyses were performed as described in section 4.2.5.

5.2.8 Isolation of Whole Lung Mitochondria

To determine the effect of dietary pirfenidone supplementation on lung mitochondrial respiratory inhibition and membrane potential disruption resulting from *in vitro* exposure to AM and DEA, hamsters were maintained on the control or pirfenidone-supplemented diet for 3 days, after which time lung mitochondria were isolated as described in section 2.2.3. To determine the effect of dietary pirfenidone supplementation on lung mitochondrial respiratory inhibition resulting from intratracheal AM administration, lung mitochondria were isolated from individual hamsters 3 hours post-dosing. Aliquots of mitochondrial suspensions were saved and used for determination of protein content by the method of Lowry *et al.* (1951), using bovine serum albumin (BSA) as the standard.

5.2.9 Polarographic Measurement of Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption was measured as described in section 2.2.4.

5.2.10 Monitoring Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined as described in section 2.2.5.

5.2.11 Plasma and Lung Pirfenidone Analyses

Quantitative pirfenidone analysis was conducted using a modification of a previously published method (Mirkovic et al., 2002). Hamsters were killed by injection of sodium pentobarbital (300 mg / kg ip) 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 x g for 5 minutes, and was kept on ice. Lungs were removed, weighed, and homogenized in 0.9% saline solution. Aliquots of plasma or lung homogenates were vigorously mixed with 1 volume of high performance liquid chromatography (HPLC) mobile phase (50% acetonitrile in distilled H_2O) and centrifuged at 16 000 x g for 1 minute. 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, USA) 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, USA), and a Waters Lambda-Max Model 481 LC spectrophotometric detector (Waters, Milford, MA, USA). 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 minutes and 10 seconds.

5.2.12 Statistical Analyses

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

5.3 RESULTS

5.3.1 Pulmonary Histopathology and Disease Index

Normal pulmonary architecture was observed 21 days post-treatment in hamsters maintained on the control or pirfenidone-supplemented diets and administered intratracheal H_2O (Figure 5.1A and B). Intratracheal AM resulted in patchy interstitial thickening and fibrosis (Figure 5.1C) that was prevented by early pirfenidone supplementation (Figure 5.1D). Late pirfenidone supplementation following AM treatment resulted in some areas of interstitial thickening and fibrosis similar to those observed in the AM group maintained on the control diet (Figure 5.1E). AM resulted in an increased disease index value 21 days post-treatment in control diet animals (Figure 5.2). Early pirfenidone supplementation prevented this increase, while late pirfenidone supplementation was ineffective.

5.3.2 Right Lung Wet Weights and Hydroxyproline Content

Right lung wet weights were not altered by AM at 7 days post-dosing, but were significantly increased at 21 days post-dosing (Figure 5.3). Lung weight to body weight ratios were increased by AM at both 7 and 21 days post-dosing (Figure 5.4). Early pirfenidone supplementation prevented the increase in lung weight at 21 days-post dosing, while late pirfenidone supplementation was ineffective (Figure 5.3). Similarly, the AM-induced increase in lung weight to body weight ratio at both 7 and 21 days post-dosing was prevented by early, but not by late pirfenidone supplementation (Figure 5.4).

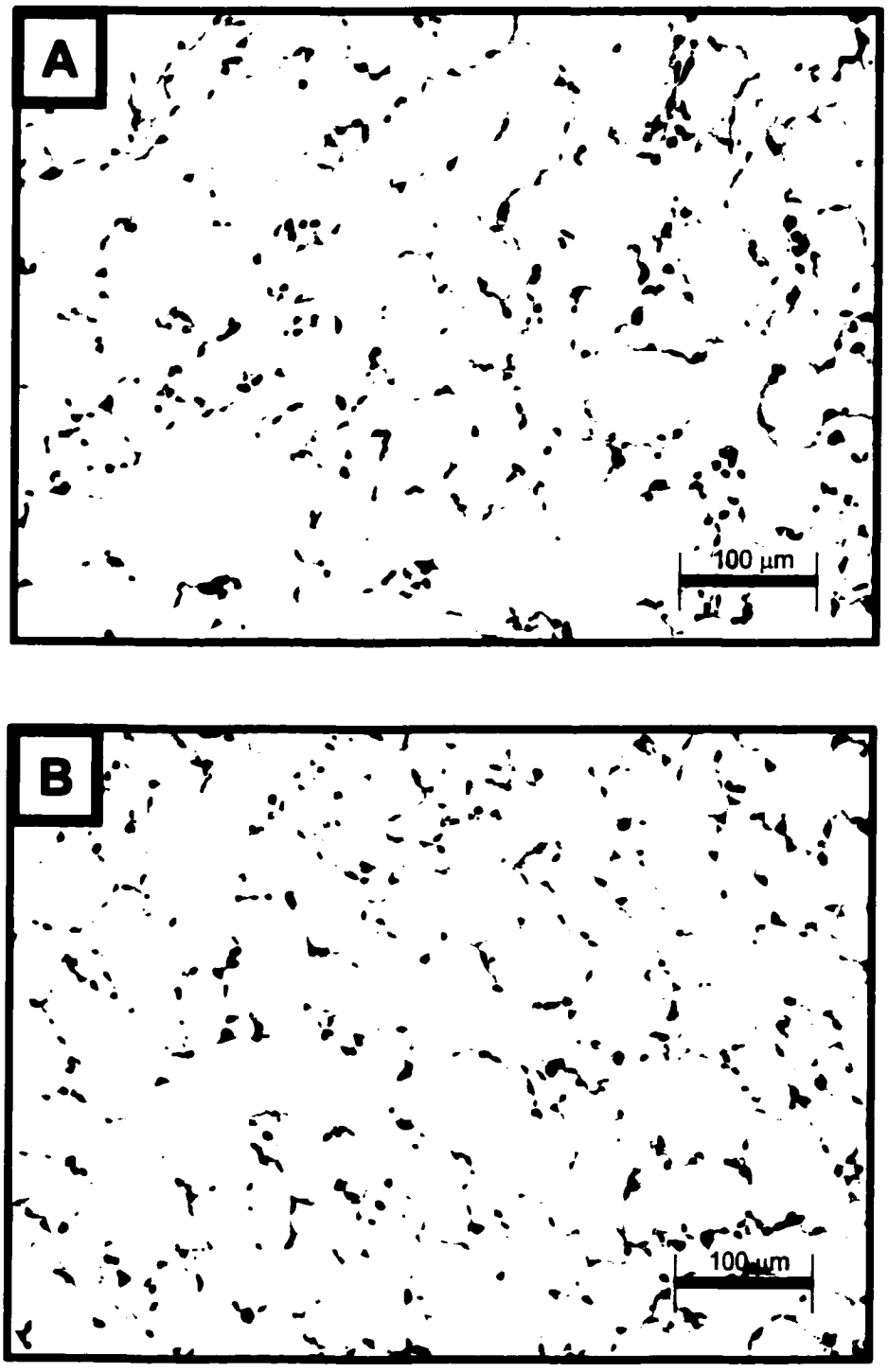


Figure 5.1 Representative light photomicrographs (H&E stain) of hamster lungs 21 days following intratracheal administration of amiodarone (AM) or distilled H_2O with or without dietary pirfenidone supplementation . (A) Control diet + H_2O. (B) Pirfenidone diet + H_2O. (C) Control diet + AM. (D) Pirfenidone diet + AM. (E) Late pirfenidone diet + AM.

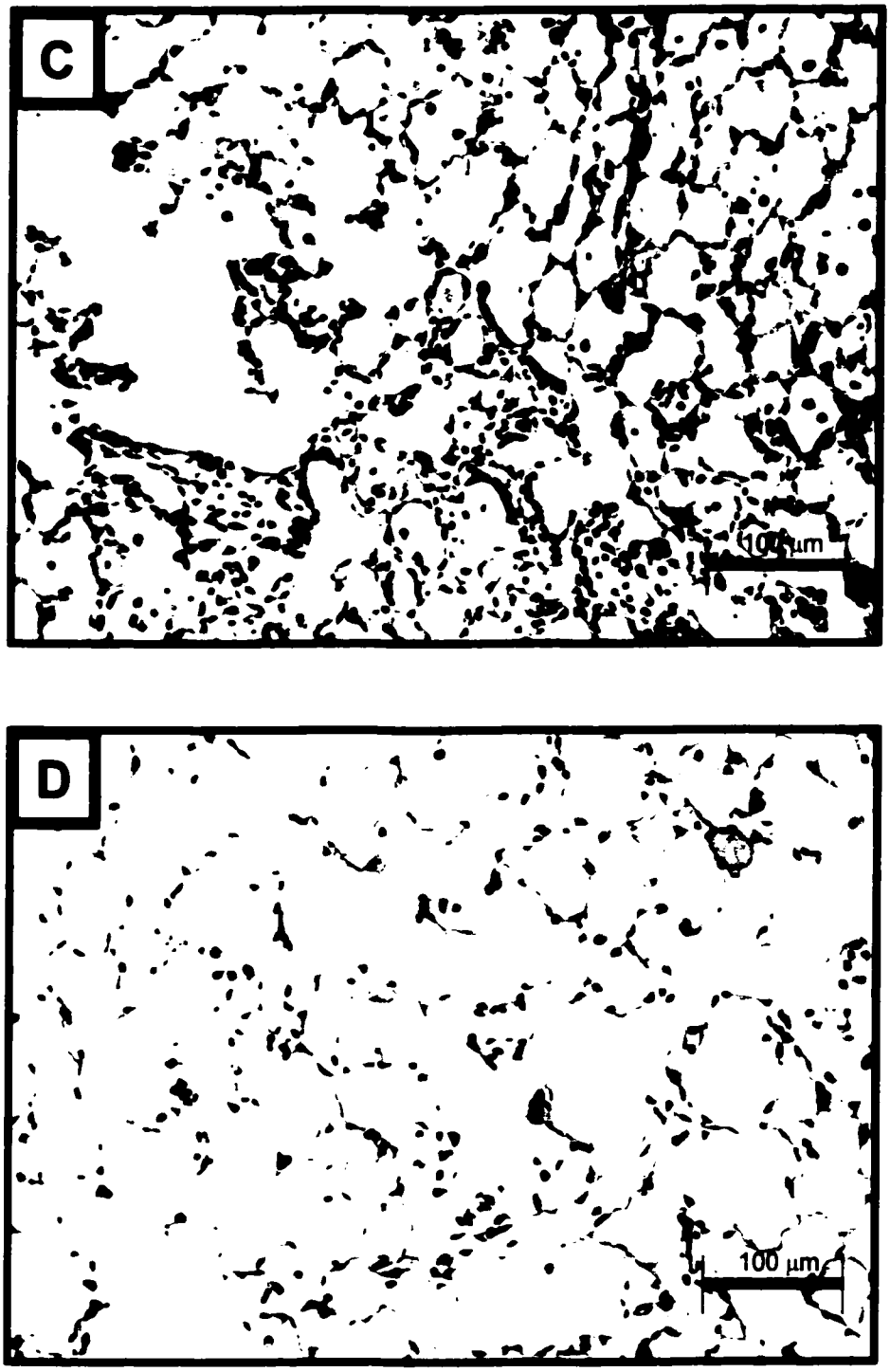


Figure 5.1 Continued.

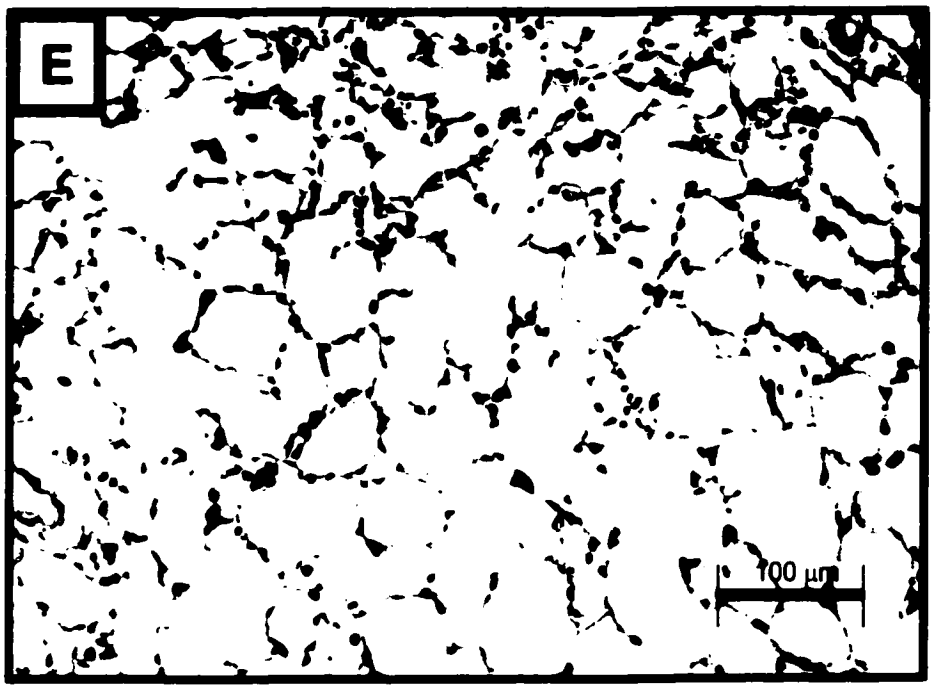


Figure 5.1 Continued.

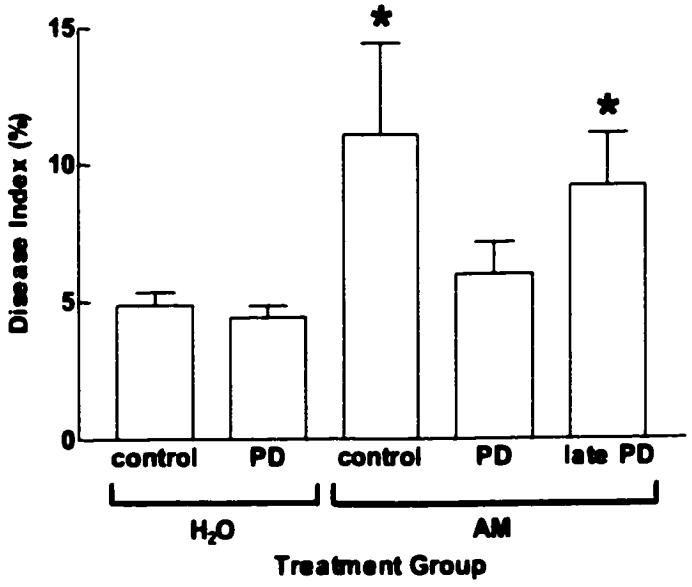


Figure 5.2 Disease index values of hamster lungs 21 days following intratracheal administration of AM (1.83 μmol) or H_2O (0.1 ml) with or without dietary pirfenidone (PD) supplementation. * significant difference from the H_2O-treated groups and from the PD + AM group, $p<0.05$ (n=6-8).

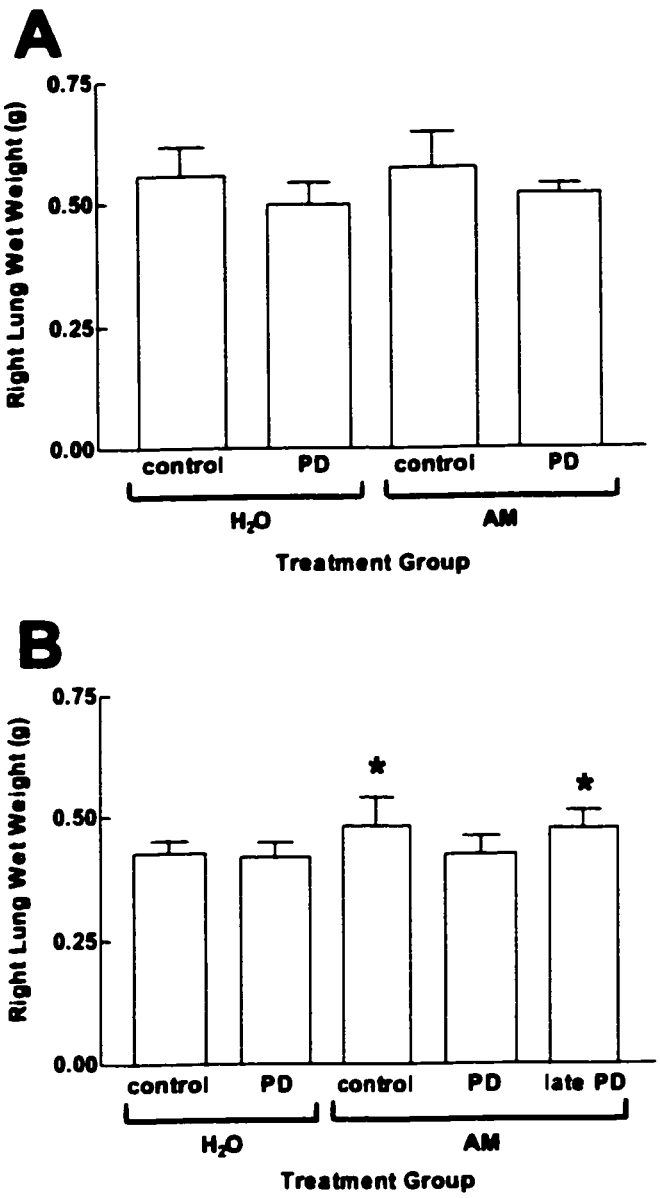


Figure 5.3 Hamster right lung wet weights A) 7 days, and B) 21 days following intratracheal administration of AM (1.83 μmol) or H_2O (0.1 ml) with or without dietary pirfenidone (PD) supplementation. * significant difference from the H_2O-treated groups and from the PD + AM group, $p<0.05$ (n=4-9).

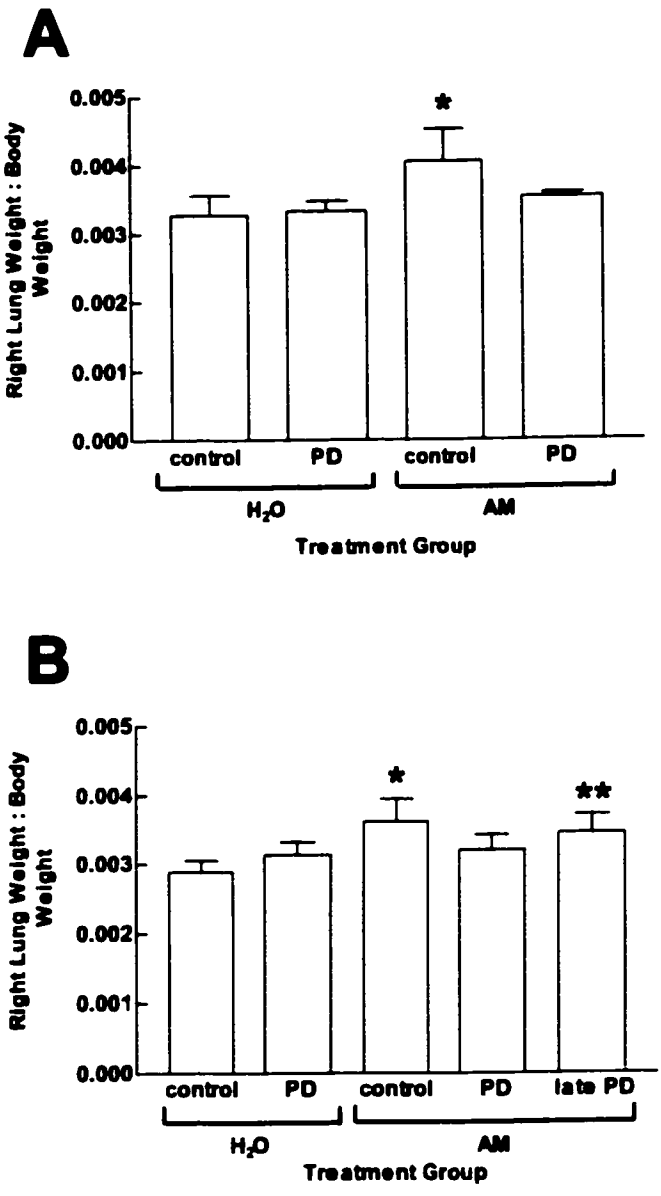


Figure 5.4 Hamster right lung wet weight to body weight ratios A) 7 days, and B) 21 days following intratracheal administration of AM (1.83 μmol) or H_2O (0.1 ml) with or without dietary pirfenidone (PD) supplementation. * significant difference from the H_2O-treated groups and from the PD + AM group; ** significant difference from the H_2O-treated groups, $p<0.05$ (n=3-7).

No differences in right lung hydroxyproline content were found among the diet and treatment groups 7 days post-treatment (Figure 5.5A); however, a significant increase (~25%) resulted from intratracheal AM administration in the control diet group at 21 days (Figure 5.5B), consistent with previous reports (Rafeiro et al., 1994; Card et al., 1999). This increase was prevented by early pirfenidone supplementation, while late pirfenidone supplementation was ineffective.

5.3.3 Pulmonary TGF-β_1 Gene Expression

Intratracheal AM administration increased right lung TGF-β_1 mRNA content at 7 and 21 days post-treatment by 59 and 340%, respectively (Figure 5.6). 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 (Figure 5.6).

5.3.4 *In Vitro* Mitochondrial Oxygen Consumption

Respiratory rates and calculated respiratory control ratios (RCRs) and ADP:O ratios for complex I- and II-supported respiration in lung mitochondria isolated for determination of the *in vitro* respiratory effects of AM and DEA are summarized in Table 5.1. Similar to the results of Chapters 2 and 4, respiratory function was well coupled at complex I but not at complex II. *In vitro* exposure of isolated lung mitochondria to AM or DEA resulted in significant inhibition of oxygen consumption, with concentrations $\geq$ 100 μM resulting in at least 75 and 50% inhibition at complexes I and II, respectively (Figure 5.7). The effects of AM and DEA were not affected by pirfenidone supplementation for 3 days prior to isolation of lung mitochondria.

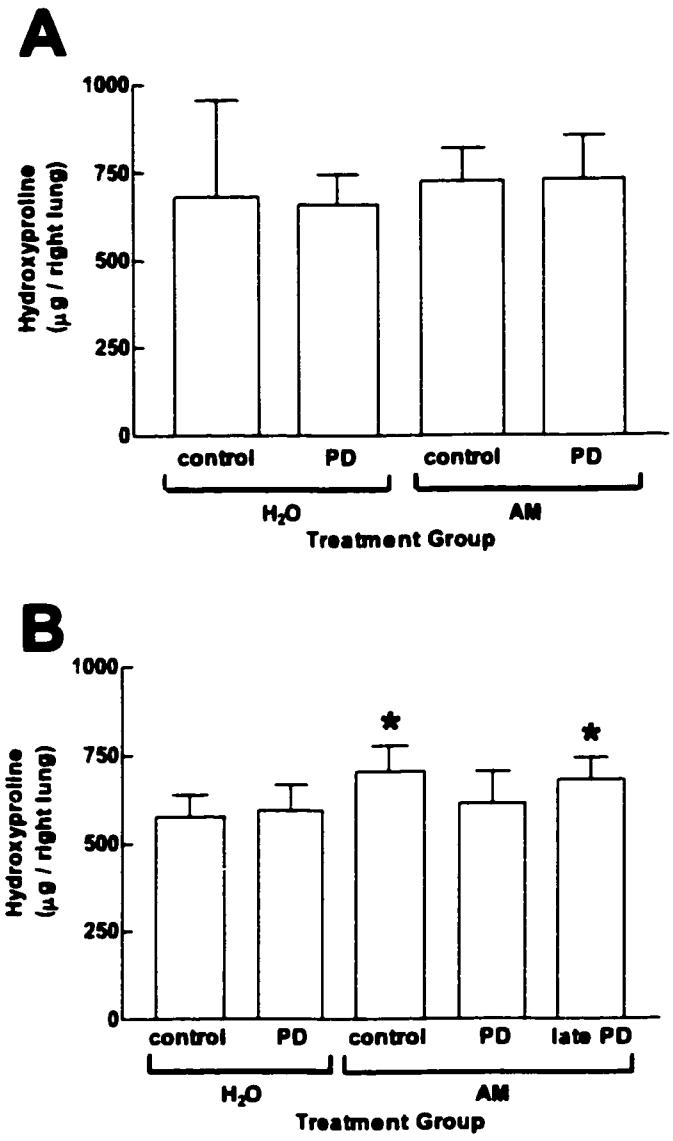


Figure 5.5 Hamster right lung hydroxyproline content A) 7 days, and B) 21 days following intratracheal administration of AM (1.83 μmol) or H_2O (0.1 ml) with or without dietary pirfenidone (PD) supplementation. * significant difference from the H_2O-treated groups and from the PD + AM group, $p<0.05$ (n=3-8).

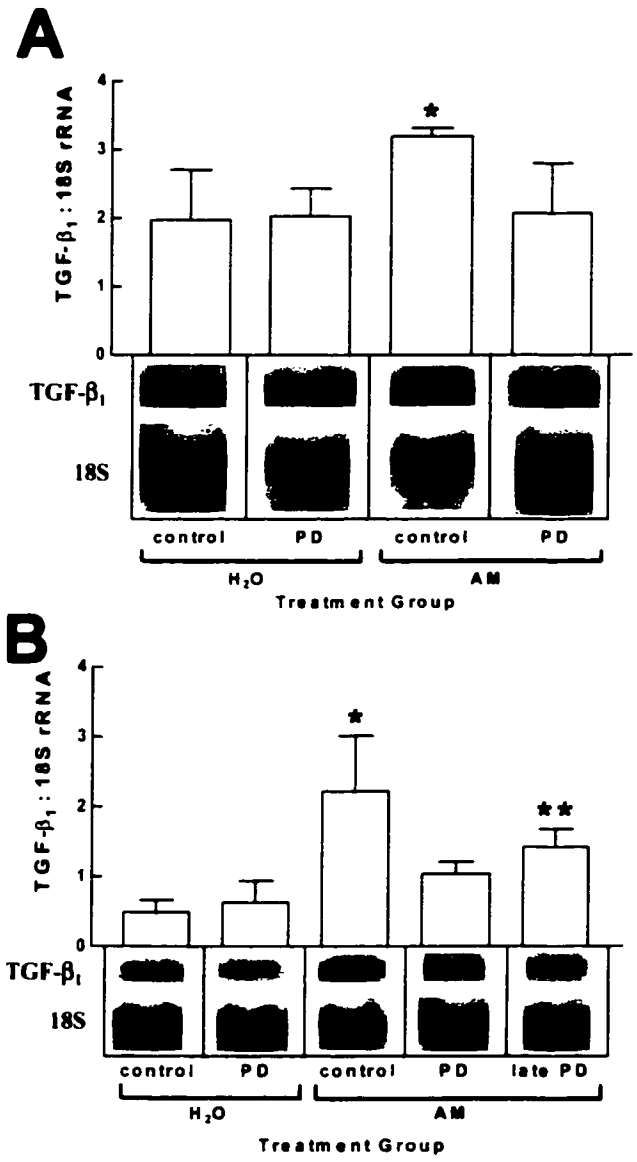


Figure 5.6 Hamster right lung TGF-β_1 mRNA content A) 7 days, and B) 21 days following intratracheal administration of AM (1.83 µmol) or H_2O (0.1 ml) with or without dietary pirfenidone supplementation. * significant difference from all other groups; ** significant difference from all groups except PD + AM, $p<0.05$ (n=3-6).

Table 5.1

Respiratory parameters of isolated hamster lung mitochondria prior to *in vitro* addition of AM or DEA for determination of effects on state 4 respiration. Hamsters were maintained on the control or pirfenidone-supplemented diet for 3 days prior to isolation of lung mitochondria.

Respiratory Complex	Diet group	*n*	Respiratory Rate (nmol O / min / mg protein) State 3	State 4	RCR	ADP:O
I	Control	4	97.3 ± 29.3	31.8 ± 3.5	2.90 ± 0.24	4.26 ± 0.10
	Pirfenidone	4	68.4 ± 18.4	22.7 ± 2.9	3.12 ± 0.20	4.47 ± 0.45
II	Control	4	119.6 ± 36.5	79.5 ± 11.3	1.52 ± 0.04	2.20 ± 0.14
	Pirfenidone	4	85.4 ± 14.5	60.8 ± 2.8	1.40 ± 0.08	2.19 ± 0.11

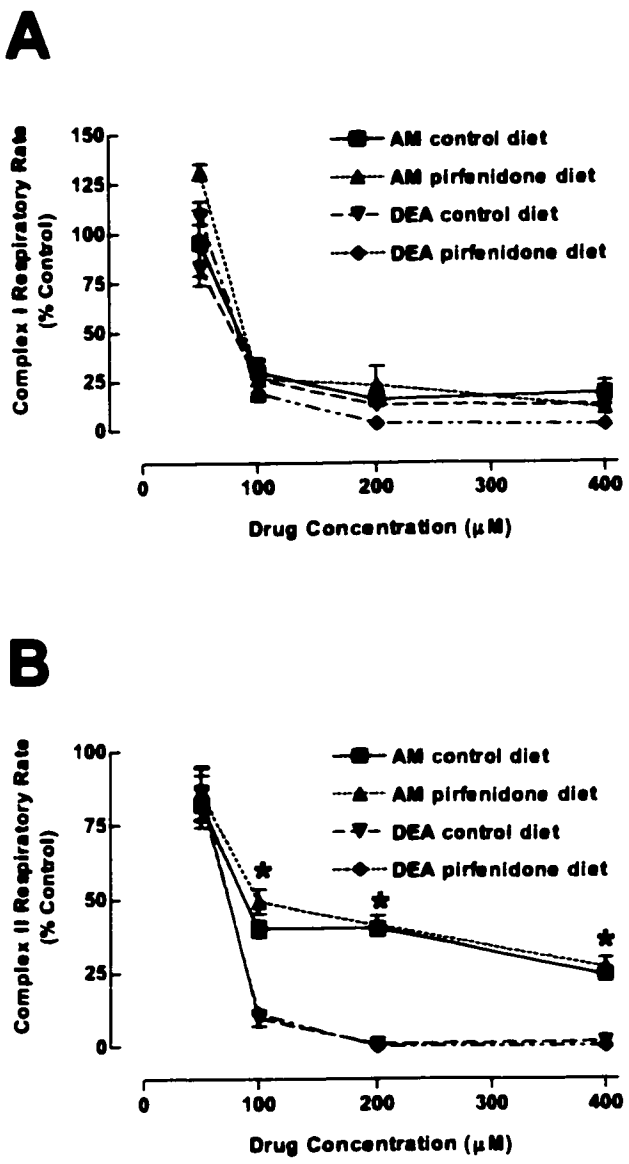


Figure 5.7 Effects of *in vitro* exposure to AM and DEA on state 4 (resting) oxygen consumption rates supported by A) complex I, and B) complex II of the electron transport chain in isolated hamster lung mitochondria with or without dietary pirfenidone supplementation prior to isolation and analysis.
* significant difference from equimolar concentrations of DEA, $p<0.05$ (n=3-4).

5.3.5 *In Vitro* Mitochondrial Membrane Potential

AM and DEA exposure substantially decreased membrane potential of isolated lung mitochondria (Figure 5.8). Despite decreasing mitochondrial membrane potential to similar maximal extents (Figure 5.8A), the rate of the DEA-induced decrease was significantly greater than that induced by AM (Figure 5.8B). Neither parameter of mitochondrial function was affected by exposure to drug vehicle (distilled H_2O). The effects of AM and DEA were not affected by pirfenidone supplementation for 3 days prior to isolation of lung mitochondria.

5.3.6 Intratracheal Dosing and Mitochondrial Respiratory Function

In mitochondria isolated 3 hours after intratracheal AM treatment, inhibition of state 3 respiration was observed at complex I, resulting in decreased RCR and increased ADP:O values compared to vehicle-treated controls (Table 5.2). Respiration supported by complex II was not affected 3 hours following AM, although the relatively weak coupling of respiration at this complex (RCR $\leq$ 1.50) even in mitochondria isolated from vehicle-treated hamsters could have interfered with observation of an inhibitory effect of AM. Maintenance on the pirfenidone diet for 3 days prior to intratracheal treatment and subsequent isolation of lung mitochondria did not alter the adverse respiratory effects of AM (Table 5.2).

5.3.7 Plasma and Lung Pirfenidone Content

Following 3 days of dietary supplementation, plasma pirfenidone concentration was 2.04 $\pm$ 1.81 μg / ml (n=3), consistent with previous reports (Mirkovic et al., 2002),

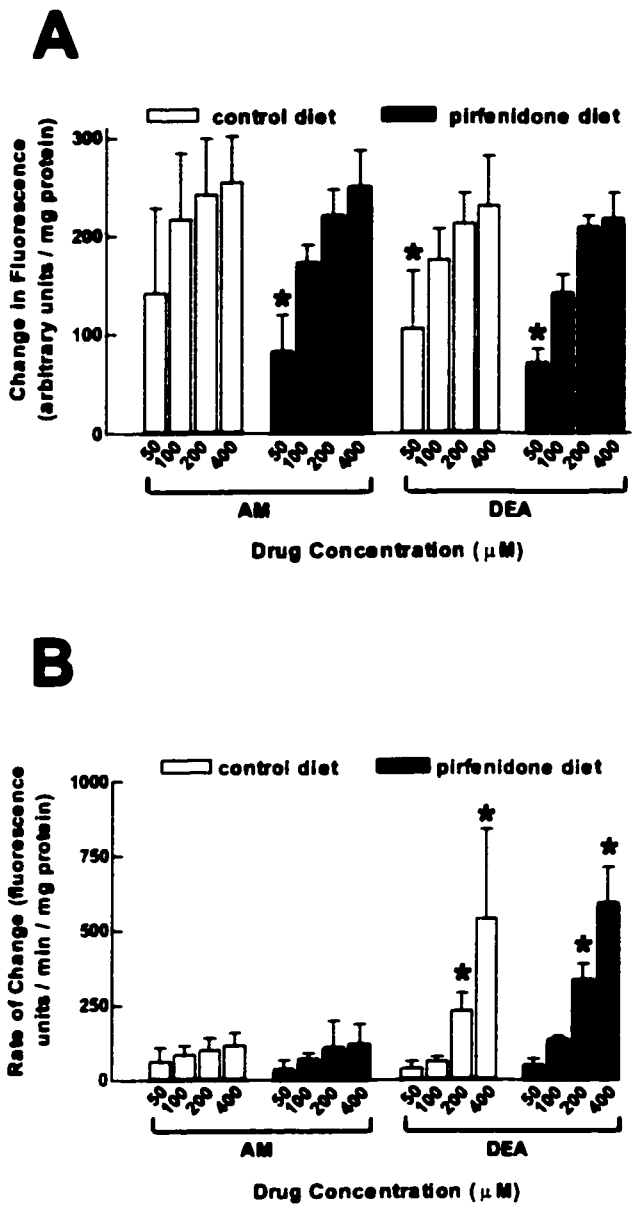


Figure 5.8 Effects of *in vitro* exposure to AM and DEA on A) total change of membrane potential, and B) rate of change of membrane potential of isolated hamster lung mitochondria, as measured by safranine dye fluorescence, with or without dietary pirfenidone supplementation. A) * significant difference from higher concentrations within the same diet and treatment group, $p<0.05$ (n=3-4). B) * significant difference from equimolar concentrations of AM and from lower concentrations of DEA within respective diet groups, $p<0.05$ (n=3-4).

Table 5.2

Respiratory parameters of lung mitochondria isolated 3 hours following intratracheal dosing with AM (1.83 μmol) or H_2O (0.1 ml). Hamsters were maintained on the control or pirfenidone-supplemented diet for 3 days prior to experimentation.

			Complex I-supported Respiratory Rates (nmol O / min / mg protein)			
Diet	**Treatment**	***n***	**State 3**	**State 4**	**RCR**	**ADP:O**
Control	H_2O	4	54.8 ± 12.5	21.7 ± 5.9	2.57 ± 0.24	4.11 ± 0.32
Pirfenidone	H_2O	3	60.6 ± 16.7	21.8 ± 4.9	2.76 ± 0.15	3.55 ± 0.44
Control	AM	4	24.2 ± 16.2*	13.9 ± 7.4	1.73 ± 0.20*	6.19 ± 0.82*
Pirfenidone	AM	3	28.4 ± 15.4*	15.3 ± 3.7	1.77 ± 0.51*	5.73 ± 1.34*

			Complex II-supported Respiratory Rates (nmol O / min / mg protein)			
Diet	**Treatment**	***n***	**State 3**	**State 4**	**RCR**	**ADP:O**
Control	H_2O	4	75.6 ± 9.6	58.0 ± 10.0	1.54 ± 0.42	1.40 ± 0.52
Pirfenidone	H_2O	3	86.4 ± 17.3	63.6 ± 13.5	1.36 ± 0.06	1.50 ± 0.14
Control	AM	4	56.8 ± 15.7	45.3 ± 13.6	1.37 ± 0.24	1.38 ± 0.22
Pirfenidone	AM	3	67.8 ± 18.2	51.0 ± 6.1	1.31 ± 0.21	1.59 ± 0.24

* significant difference from respective H_2O-treated animals within the same diet group, $p<0.05$.

and pirfenidone concentration in whole lung homogenate was 4.73 ± 0.77 μg / g tissue (n=3).

5.4 DISCUSSION

This study demonstrates that pirfenidone, given as a dietary supplement, prevents AM-induced pulmonary fibrosis in the hamster. Pirfenidone prevented AM-induced up-regulation of TGF-β_1 expression, both several days following the toxic insult and at the time of maximal fibrosis, suggesting that attenuation of TGF-β_1 expression is central to pirfenidone's anti-fibrotic activity. Pirfenidone similarly down-regulated pulmonary TGF-β_1 expression and attenuated experimental bleomycin-induced lung inflammation and fibrosis, effects attributed at least in part to decreased TGF-β_1 gene transcription (Iyer et al., 1999; Iyer et al., 2000). The lack of anti-fibrotic effectiveness of pirfenidone when dietary supplementation was started 7 days after induction of lung injury by AM indicates that maximum anti-fibrotic efficacy requires the presence of pirfenidone during the early stages of lung remodelling following AM-induced lung damage.

Inhibition of mitochondrial function has been postulated to be a common feature in the early stages of fibroproliferative disorders (Oury et al., 2001). We have demonstrated that mitochondrial membrane potential and cellular ATP levels are decreased early during the course of AM cytotoxicity in isolated lung cells (Bolt et al., 2001a), 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

(Guerreiro et al., 1986; Fromenty et al., 1990a; Yasuda et al., 1996; Card et al., 1998; Chapter 2), further supporting mitochondrial dysfunction as a basis for AM toxicities.

DEA is considerably more cytotoxic than AM (Beddows et al., 1989; Bolt et al., 2001a), and accumulates in lung tissues of patients on AM pharmacotherapy (Brien et al., 1987), suggesting that DEA contributes to AM lung toxicity. As anticipated, both AM and DEA inhibited respiratory function supported by mitochondrial complexes I and II, and decreased membrane potential of isolated lung mitochondria, with DEA displaying more pronounced and / or rapid effects than AM. We have previously observed similar relatively rapid mitochondrial inhibition (Chapter 2) and cytotoxicity of DEA compared to AM in freshly isolated lung cells (Bolt et al., 2001a), effects that may be related to more extensive accumulation of DEA in mitochondria. Intratracheal AM administration inhibited complex I-supported state 3 respiration 3 hours post-treatment, resulting in decreased RCR and increased ADP:O ratios, indicative of uncoupling of respiratory function. This time point was chosen because preliminary experiments revealed no significant effect of AM on respiratory function prior to 3 hours following *in vivo* administration. Furthermore, this timecourse is consistent with that observed for AM-induced disruption of mitochondrial membrane potential and decreased cellular ATP content prior to cell death in isolated hamster lung cells (Bolt et al., 2001a). Efforts to determine the effect of *in vivo* administration of an equimolar dose of DEA on mitochondrial function were unsuccessful, as close to 100% mortality occurred in hamsters within hours following intratracheal treatment, despite the use of various vehicles and delivery volumes. Should DEA inhibit mitochondrial respiratory function *in vivo*, it is unlikely that pirfenidone would diminish this effect, given that DEA is more

potent and rapid-acting than AM *in vitro*, and pirfenidone did not decrease the adverse mitochondrial effects observed *in vitro* or following *in vivo* AM treatment. The failure of pirfenidone to attenuate mitochondrial disruption caused by AM and DEA *in vitro* and by *in vivo* AM administration, despite being measurable in lung tissue and displaying anti-fibrotic activity, indicates that prevention of mitochondrial dysfunction is not involved in its protective action in the hamster model.

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 hours in hamsters (Cantor et al., 1984; Daniels et al., 1989; Blake and Reasor, 1995b). Thus, lung cell death caused by intratracheal AM appears to initiate a cascade of events culminating in fibroblast proliferation and excess collagen deposition. Although the route of exposure differs between the hamster model and systemic delivery to the lung as occurs in humans with AM lung toxicity, the resulting patchy interstitial fibrosis is similar in the two situations (Massey et al., 1995).

In summary, pirfenidone exhibits anti-fibrotic activity in a hamster model of AM-induced pulmonary fibrosis. Inhibition of relatively early processes in AM lung injury contributes to its anti-fibrotic activity, but prevention of AM-induced mitochondrial dysfunction is not a mechanism of pirfenidone action. Rather, attenuation of AM-induced over-expression of the potent pro-fibrotic cytokine TGF-β_1 is probably pivotal to the anti-fibrotic activity of pirfenidone in this model. Considering the widespread clinical use of AM (Nolan, Jr. et al., 1998) and the fact that lung fibrosis is a serious adverse effect of AM (Pollak, 1999), the possibility that pirfenidone will prove useful in the clinical treatment of AM-induced pulmonary fibrosis deserves further attention.

Chapter 6

EFFECT OF INTRATRACHEAL AMIODARONE ADMINISTRATION ON HAMSTER LUNG MITOCHONDRIAL RESPIRATORY FUNCTION

6.1 INTRODUCTION

While numerous processes have been proposed to be involved in the pathogenesis of amiodarone (AM)-induced pulmonary toxicity (AIPT) (Massey et al., 1995; Reasor and Kacew, 1996), the etiology of this condition is currently unknown. Considerable evidence suggests that the mitochondrion is a target organelle for initiation of AM-induced cytotoxicities, as AM-induced structural and functional impairments have been documented in mitochondria from various non-pulmonary sources (Guerreiro et al., 1986; Gross et al., 1989; Fromenty et al., 1990a; Yasuda et al., 1996). More recently, we have observed adverse functional effects of AM and its major metabolite, *N*-desethylamiodarone (DEA), in isolated hamster lung mitochondria (Chapter 2; Card et al., 1998) and in mitochondria of isolated hamster lung cells prior to AM-induced cell death (Bolt et al., 2001a). However, the effect of AM on lung mitochondrial function *in vivo* has not been investigated.

The purpose of this study was to assess the respiratory function of hamster lung mitochondria following intratracheal administration of AM under conditions known to result in pulmonary fibrosis.

6.2 MATERIALS AND METHODS

6.2.1 Chemical Sources

Chemicals and reagents were obtained from suppliers as follows: amiodarone hydrochloride, rotenone (95-98%), adenosine 5'-diphosphate (ADP, free acid), β-nicotinamide adenine dinucleotide phosphate (β-NADPH, reduced form, tetrasodium salt), L-glutamate (monosodium salt), ethylenediamine tetraacetic acid (EDTA, disodium salt, dihydrate), succinate (disodium salt, hexahydrate), D-mannitol, 3-[N-morpholino]propanesulfonic acid (MOPS), bovine serum albumin (BSA), and fatty acid-free BSA from Sigma Chemical Co. (St. Louis, MO, USA); sodium pentobarbital from M.T.C. Pharmaceuticals (Mississauga, ON, Canada); ketamine hydrochloride from Rogar / STB Inc. (London, ON, Canada). All other chemicals were of analytical grade, and were purchased from standard commercial suppliers.

6.2.2 Animals and Treatments

Animals were obtained and cared for as described in section 2.2.2. Intratracheal administration of AM or H_2O was carried out as described in section 3.2.2. For determination of lung mitochondrial respiratory effects and general lung injury resulting from intratracheal AM dosing, mitochondria were isolated or bronchoalveolar lavage fluid (BALF) was collected from individual hamsters following intratracheal dosing, as described below.

6.2.3 Isolation of Whole Lung Mitochondria

Whole lung mitochondria were isolated as described in section 2.2.3, from individual hamsters at various times (0.5 to 24 hours) after intratracheal administration of AM or vehicle.

6.2.4 Polarographic Measurement of Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption was measured as described in section 2.2.4.

6.2.5 Bronchoalveolar Lavage

Lactate dehydrogenase (LDH) activity and protein content were assessed in BALF as indicators of lung cell injury following intratracheal AM or vehicle administration. Hamsters were killed by injection of sodium pentobarbital (300 mg / kg ip) at various times after intratracheal dosing, the tracheas exposed and cannulated, and the trachea and lungs removed and kept on ice. Lungs were lavaged 3 times each with 5 ml of phosphate-buffered saline (PBS, pH 7.4, 37°C), and the BALF collected and centrifuged at 1 000 x *g* for 10 minutes at 4°C. Aliquots of the supernatants were assayed for lactate dehydrogenase (LDH) activity using a commercially available kit (Sigma LDH procedure No. DG1340-UV), and for protein content by the method of Lowry *et al.* (1951) using BSA as the standard.

6.2.6 Statistical Analyses

Data are expressed as mean ± standard deviation for each experimental group. Statistical comparisons amongst treatment groups were performed via randomized one-

or two-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test for more than two groups, or via unpaired Student's *t*-test for two groups. Where required, $\log_{10}$ transformation of data was performed to correct for heterogeneity of variance prior to statistical analysis. In all cases, statistical significance was defined as $p<0.05$.

6.3 RESULTS

6.3.1 Intratracheal Dosing and Mitochondrial Respiratory Function

State 3 respiratory rates for mitochondria isolated at various times after intratracheal dosing with distilled H_2O were 58.4 ± 16.0 and 84.7 ± 18.8 nmol O / min / mg protein for complexes I and II, respectively, and state 4 respiratory rates were 21.4 ± 7.3 and 67.2 ± 17.1 nmol O / min / mg protein for complexes I and II, respectively. Calculated ADP:O ratios were 4.46 ± 0.62 and 1.75 ± 0.48 for complexes I and II, respectively.

Complex I-supported state 3 respiration was significantly decreased 3 hours following AM treatment, resulting in a significant decrease of the calculated RCR in comparison to the H_2O-treated group (Figure 6.1). This inhibitory effect was maintained through the 6 and 24 hour time points, when both state 3 and state 4 respiratory rates were decreased as a result of AM administration (Figure 6.1). Complex II-supported respiration was significantly inhibited by AM at 6 and 24 hours post-administration, when both state 3 and state 4 respiratory rates were decreased (Figure 6.2).

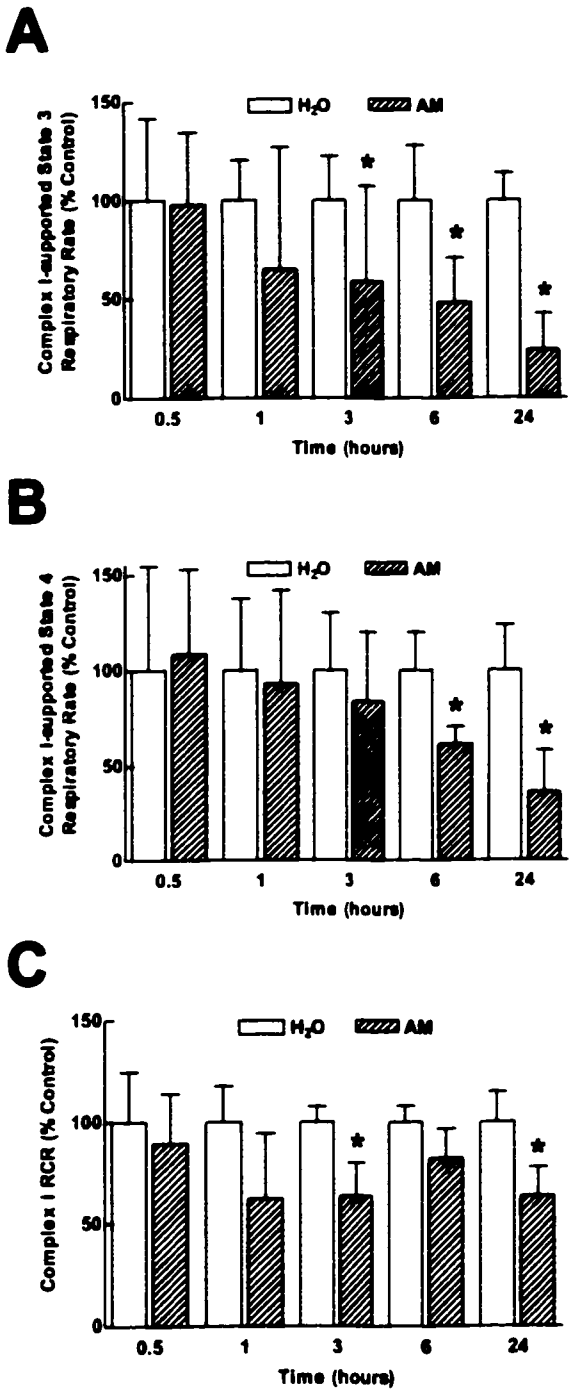


Figure 6.1 Effects of intratracheal administration of H_2O (0.1 ml) or AM (1.83 μmol) on complex I-supported respiration in lung mitochondria isolated from individual hamsters at various times after dosing. A) state 3 (active) respiration; B) state 4 (resting) respiration; C) respiratory control ratios (RCR). * significant difference from H_2O group at the same time point, $p<0.05$ (n=3-4).

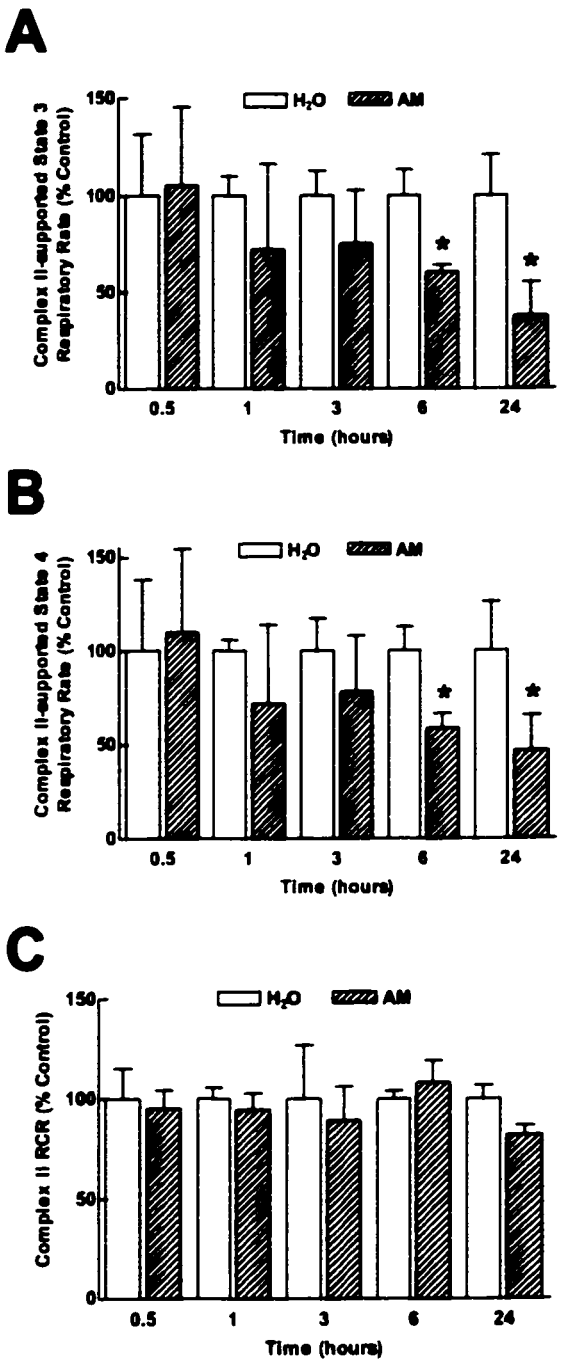


Figure 6.2 Effects of intratracheal administration of H_2O (0.1 ml) or AM (1.83 μmol) on complex II-supported respiration in lung mitochondria isolated from individual hamsters at various times after dosing. A) state 3 (active) respiration; B) state 4 (resting) respiration; C) respiratory control ratios (RCR). * significant difference from H_2O group at the same time point, $p<0.05$ (n=3-4).

Calculated ADP:O ratios, which are based on the amount of oxygen consumed during state 3 (ADP-stimulated) respiration, were inconsistent among time points in response to AM at complex I, and were not altered at complex II at any time point examined (Figure 6.3). A diagrammatic representation of the effects of intratracheal AM administration on lung mitochondrial oxygen consumption 6 hours post-dosing is depicted in Figure 6.4. At 6 hours, both complex I- and complex II-supported state 3 and state 4 oxygen consumption rates were significantly decreased following AM administration, as indicated by the shallow slopes in comparison to consumption rates in lung mitochondria isolated from H_2O-treated hamsters. Although state 3 respiration was decreased at both complexes at 6 hours post-AM, the ADP:O ratios were not altered in comparison to mitochondria from H_2O-treated hamsters because the same amount of oxygen was consumed, but over a longer period of time (time does not factor into the ADP:O ratio, but does factor into the respiratory rate).

6.3.2 Lung Weights and BALF LDH Activity and Protein Content

At all time points examined, lung weights and lung weight to body weight ratios were increased following intratracheal AM dosing (Figure 6.5). As indicators of lung cell injury following intratracheal AM administration, LDH activity and protein content were determined in BALF (Figure 6.6). LDH activity was increased by AM treatment at 0.5 hours post-dosing, and remained elevated compared to the vehicle-treated control group at 1 and 3 hours post-dosing. Similarly, BALF protein content was increased by AM at all time points examined.

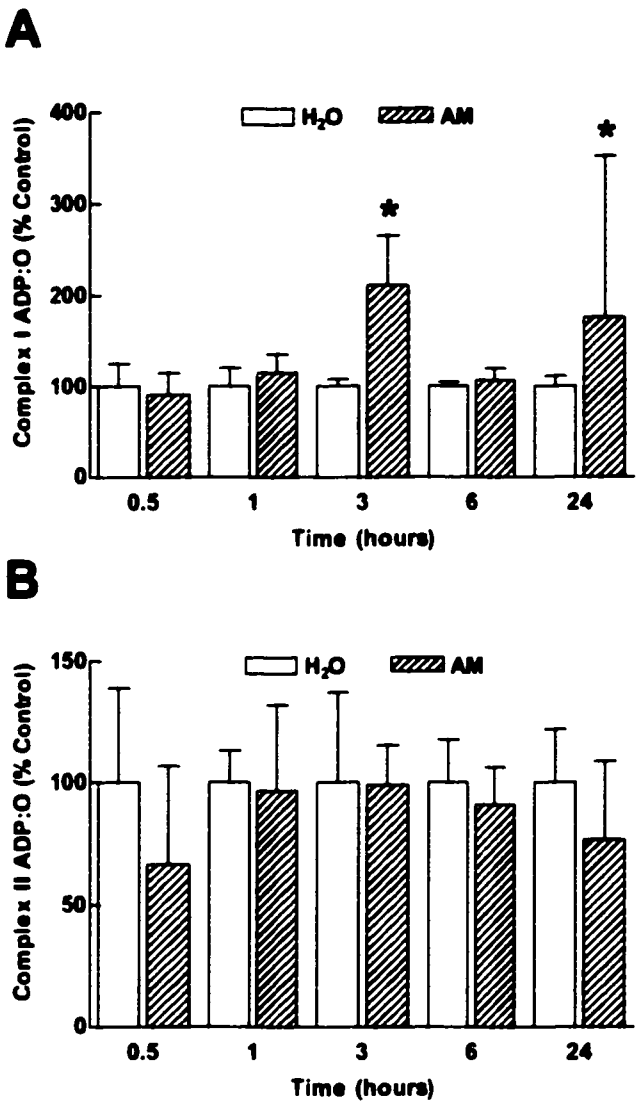


Figure 6.3 Effects of intratracheal administration of H_2O (0.1 ml) or AM (1.83 μmol) on ADP:O ratios at A) complex I, and B) complex II in lung mitochondria isolated from individual hamsters at various times after dosing. * significant difference from H_2O group at the same time point, $p<0.05$ (n=3-4).

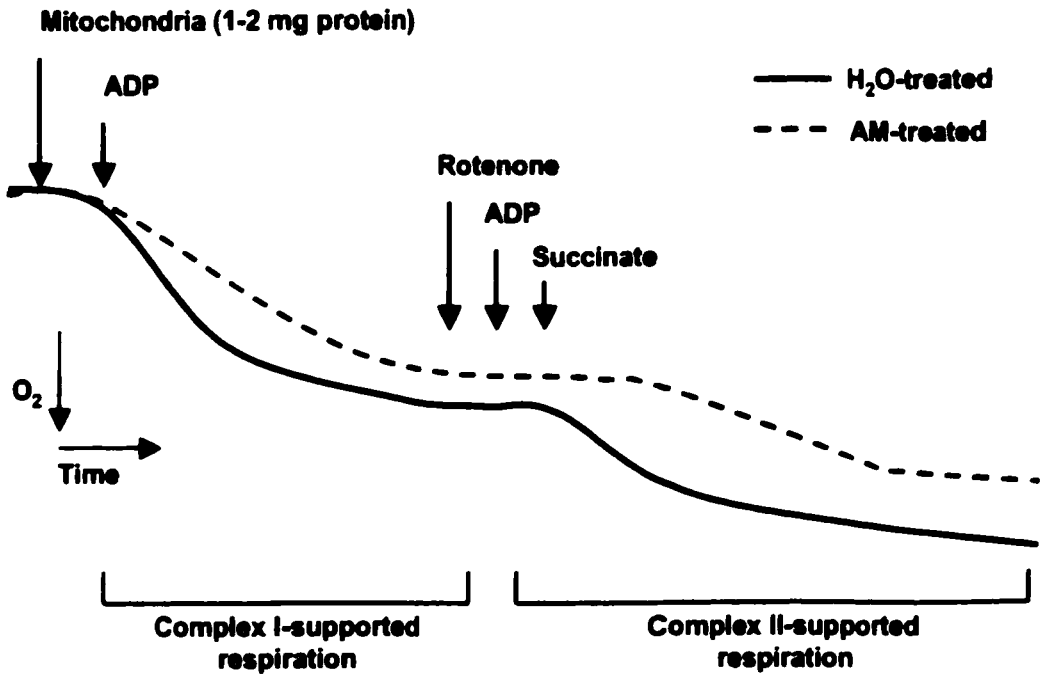


Figure 6.4 Representative lung mitochondrial oxygen consumption tracings, depicting the effects of intratracheal H_2O or AM administration on complex I- and II-supported respiration. These tracings are representative of effects that were observed when lung mitochondria were isolated 6 hours after H_2O or AM dosing.

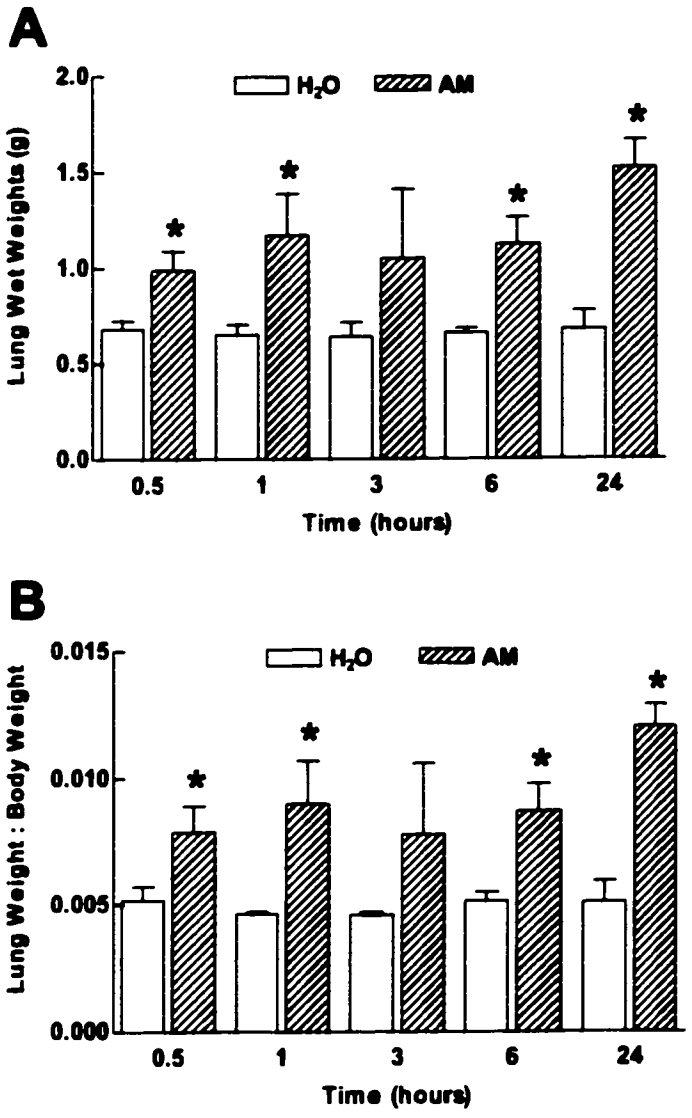


Figure 6.5 Effects of intratracheal administration of H_2O (0.1 ml) or AM (1.83 μmol) on A) lung wet weights, and B) lung weight to body weight ratios in individual hamsters at various times after dosing. * significant difference from H_2O group at the same time point, $p<0.05$ (n=3-4).

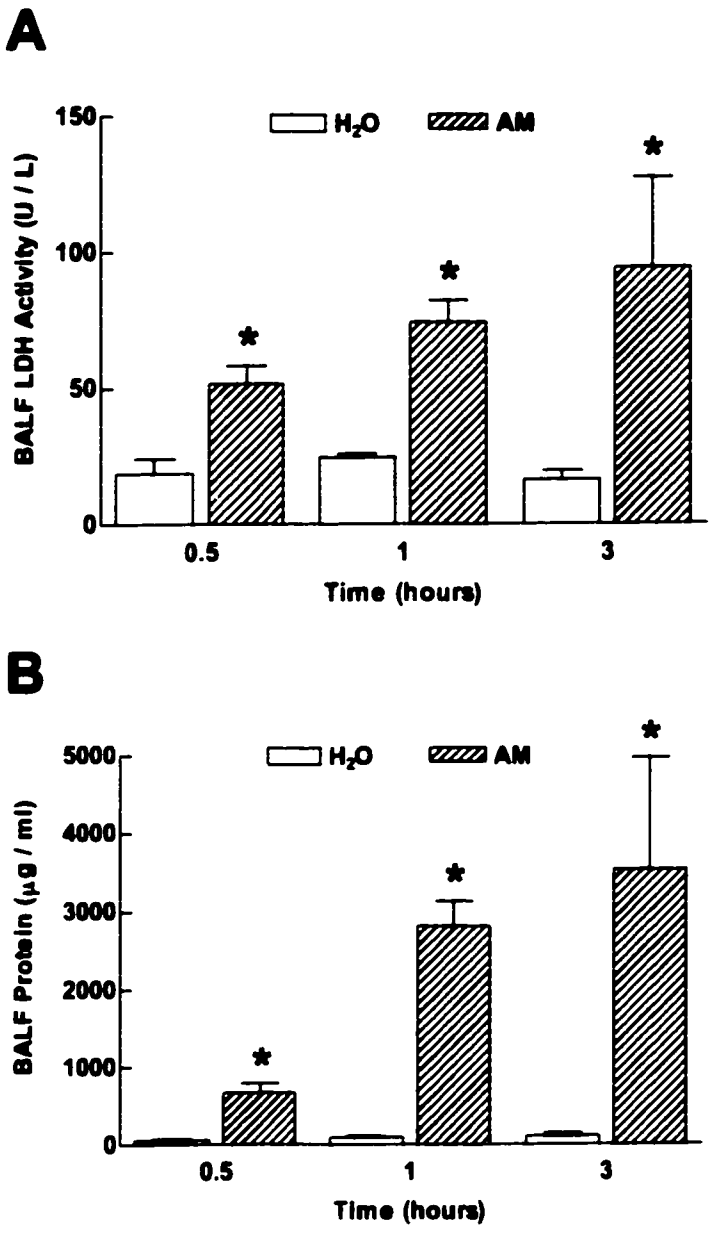


Figure 6.6 Effects of intratracheal administration of H_2O (0.1 ml) or AM (1.83 µmol) on A) LDH activity, and B) protein content in BALF collected from individual hamsters at various times after dosing. * significant difference from H_2O group at the same time point, $p<0.05$ (n=3-4).

6.4 DISCUSSION

In an attempt to characterize a role for mitochondrial dysfunction *in vivo* in the early stages of AM-induced lung injury, respiratory function supported by complexes I and II was determined in hamster lung mitochondria isolated at various times after intratracheal administration of AM or vehicle (distilled H_2O). Inhibition of state 3 respiration supported by complex I was observed as early as 1 hour after AM dosing, but did not reach statistical significance until the 3 hour time point. This effect continued through to 6 and 24 hours post-dosing, at which times both state 3 and state 4 respiratory rates were decreased. Respiration supported by complex II was also inhibited 1 and 3 hours post-dosing, but did not reach statistical significance until the 6 hour time point, when both state 3 and state 4 respiratory rates were decreased. An explanation for the difference between the effects on complexes I and II is not readily apparent, but may be due in part to the weak coupling at this complex (RCR $\leq$ 1.50) even in mitochondria isolated from vehicle-treated hamsters (Chapters 2, 4, and 5). Given the importance of mitochondria for cellular energy production (Wallace et al., 1997), AM-induced impairment of mitochondrial respiratory function could lead to decreased cellular ATP content and cell death due to an inability to maintain cellular homeostasis, as occurs in isolated lung cells exposed to AM (Bolt et al., 2001a). In addition, decreased energy production could render lung cells more susceptible to further injury and / or death due to decreased capacity to deal with other adverse intracellular actions of AM, such as free radical production or membrane destabilisation.

Efforts to determine the effect of *in vivo* administration of an equimolar (i.e. 1.83 μmol) dose of *N*-desethylamiodarone (DEA) on mitochondrial function were

unsuccessful, as mortality approached 100% within 3 hours of intratracheal treatment, despite the use of various vehicles and delivery volumes in an attempt to reduce the mortality. This may be related to a more rapid and / or severe effect of DEA on mitochondrial function and pulmonary cytotoxicity, as was observed *in vitro* in isolated lung mitochondria (Chapter 2) and lung cells (Bolt et al., 2001a). Although speculative, it is also reasonable to assume that other events resulting from DEA administration (such as a more intense inflammatory reaction and / or a greater degree of cytotoxicity than that induced by AM) contributed to the severe pulmonary congestion that was observed upon post-mortem examination, and hence to the impairment of gas exchange that likely caused these deaths.

Indicators of general lung cell injury, including lung weights, lung weight to body weight ratios, and BALF protein content and LDH activity, were all increased early after AM dosing and remained elevated at every time point examined. In particular, BALF protein content and LDH activity were significantly increased 0.5 hours after AM dosing, whereas inhibition of lung mitochondrial respiratory function was observed at 1 hour and reached statistical significance at 3 hours and time points thereafter. This might suggest that lung toxicity was initiated prior to mitochondrial dysfunction, as assessed in mitochondria isolated from whole lung. However, since the source(s) of BALF LDH and protein has not been identified, it is possible that a certain population of epithelial cells was initially damaged as a result of immediate exposure to a high concentration of AM following intratracheal administration. This could have resulted in rapid cell membrane destabilisation unrelated to mitochondrial effects, and leakage of LDH and protein that was detected in BALF very early following AM dosing. 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 and protein that were observed at later time points. The trend toward higher BALF LDH activity and protein content at later time points, that could have resulted from cell death due at least in part to the observed mitochondrial dysfunction that was observed, supports this possibility.

In summary, this is the first reported demonstration of lung mitochondrial dysfunction induced by AM or any other pulmonary fibrogen in an *in vivo* animal model. A limitation of this model is the apparent non-specific lung cell injury that occurred as a result of the route of drug administration that would not likely be observed in humans following systemic administration. Identification of the cell types damaged as a result of intratracheal AM administration would help to clarify the roles of mitochondrial dysfunction and cell death in initiating AIPT in this model. Regardless, the timecourse of mitochondrial effects resulting from AM administration is consistent with that observed in freshly isolated hamster lung cells prior to overt cell death following *in vitro* exposure to AM (Bolt et al., 2001a). As such, although other factors may be involved, it is likely that mitochondrial dysfunction contributes to lung cell death induced by intratracheal AM administration, and further investigations of this event as an initiating mechanism of AIPT are warranted.

Chapter 7

GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 GENERAL DISCUSSION

A simplified scheme of the events proposed to be involved in AIPT is presented in Figure 7.1, with the research described in this thesis highlighted in blue colour.

The mitochondrion is a putative target for initiation of AM-induced toxicities, including AIPT. Furthermore, a role for DEA in AIPT has been suggested, given that it is more cytotoxic and fibrogenic than AM in experimental systems. In the present study, isolated hamster lung mitochondria were adversely affected by AM and DEA, as inhibition of oxygen consumption and collapse of membrane potential were observed within minutes of *in vitro* exposure to the drugs (Chapter 2; Figure 7.1). The effects of DEA were more rapid and / or more severe than those of AM, and coincided with the more rapid and extensive accumulation of the metabolite in mitochondria. Neither AM nor DEA induced lipid peroxidation in isolated mitochondria, however, thus ruling out this process as a cause or consequence of AM- and DEA-induced mitochondrial dysfunction. Although intracellular AM and DEA concentrations in the lung following AM pharmacotherapy have not been reported, the concentration range of AM and DEA employed in the present *in vitro* experiments is comparable to that found in whole human lung tissue following AM pharmacotherapy (Plomp et al., 1984; Brien et al., 1987). Thus, it can be reasonably predicted that the effects of AM and DEA on isolated lung

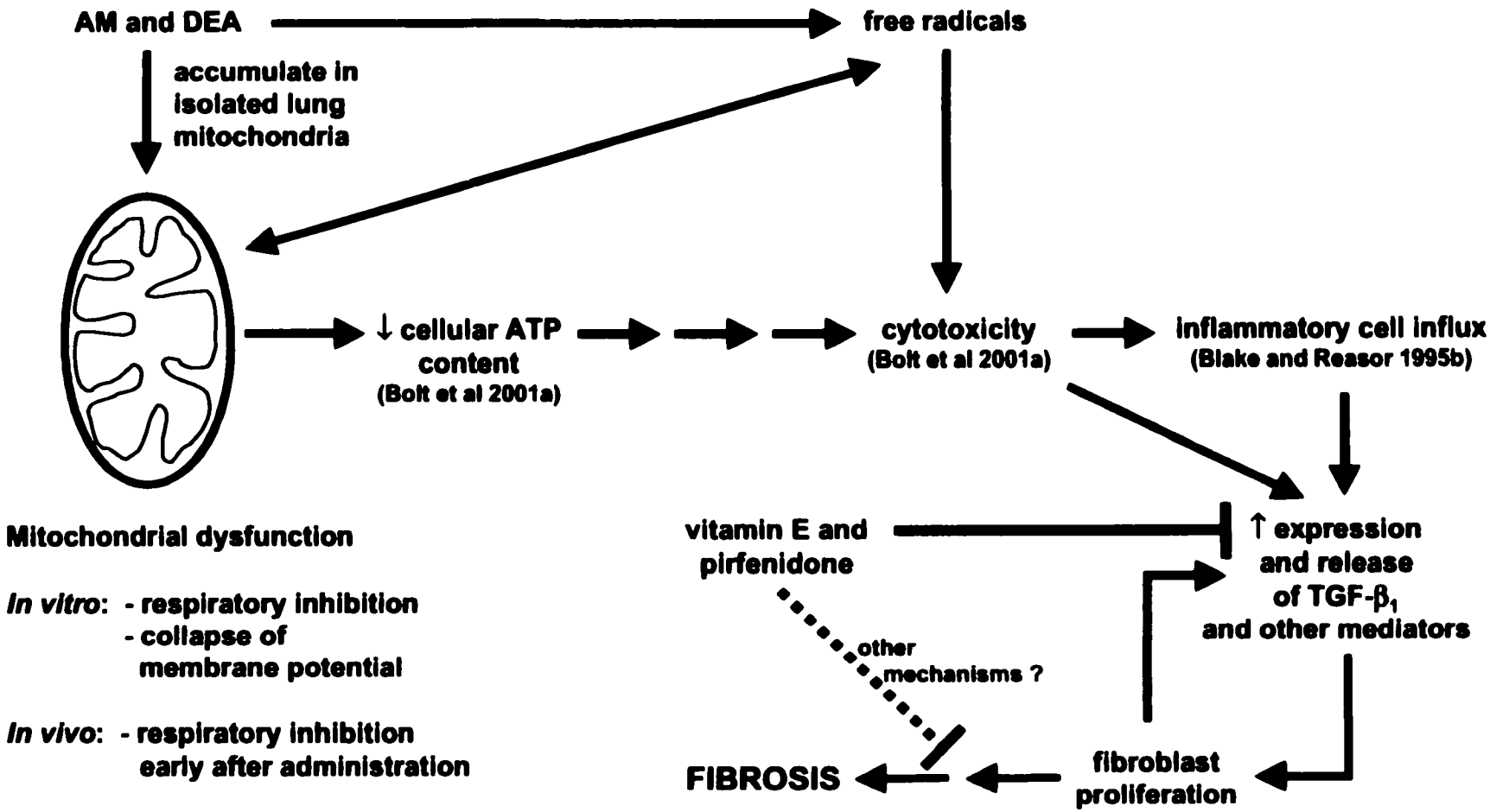


Figure 7.1 Summary of results from the studies outlined in the present thesis and their proposed involvement in the initiation, progression, and prevention of AIPT.

mitochondrial function that were observed here may also occur *in vivo* as a result of exposure to similar intracellular drug concentrations. Furthermore, these *in vitro* results concur with other findings in our laboratory that demonstrated a disruption of mitochondrial membrane potential and depletion of cellular ATP levels prior to overt cell death in freshly isolated hamster lung cells exposed to AM and DEA (Bolt et al., 2001a), and support the proposed contribution of DEA to the etiology of AIPT.

In the first study to examine the potential for vitamin E to prevent AIPT in an *in vivo* animal model, dietary supplementation with this antioxidant resulted in rapid pulmonary accumulation, and prevented AM-induced pulmonary fibrosis in the hamster as indicated by hydroxyproline content and histological damage (Chapters 3 and 4; Figure 7.1). Associated with this protective effect was prevention of AM-induced up-regulation of pulmonary TGF-β_1 gene expression. However, despite increasing lung mitochondrial vitamin E content, dietary supplementation was ineffective at preventing AM- and DEA-induced mitochondrial dysfunction. As such, the protective effect of vitamin E is likely mediated through its ability to down-regulate TGF-β_1 gene expression following AM administration, while not affecting AM- and DEA-induced mitochondrial dysfunction. The potential clinical relevance of these findings is substantial; should vitamin E be shown to not alter the antidysrhythmic efficacy of AM, a clinical study of the effect of vitamin E on the incidence and / or severity AIPT could be of significance.

Pirfenidone was assessed for its anti-fibrotic potential in the hamster model of AIPT (Chapter 5). Provided in the diet, pirfenidone administration for 3 days prior to, and continuously after AM administration prevented AM-induced pulmonary fibrosis. Similar to vitamin E, pirfenidone prevented the AM-induced increase of pulmonary TGF-

β_1 gene expression that preceded and coincided with fibrosis (Figure 7.1). Interestingly, delaying initiation of pirfenidone supplementation until 1 week after AM dosing was not as effective at decreasing pulmonary fibrosis. This suggests that effects on an early or initiating event of AIPT were part of the protective profile of pirfenidone, which may have included modulation of early TGF-β_1-mediated effects crucial to the fibrotic response to AM. Lung mitochondrial dysfunction induced by *in vitro* AM and DEA, or by *in vivo* AM administration, was not altered by maintenance on the pirfenidone diet for 3 days prior to mitochondrial analyses. Thus, pirfenidone prevents TGF-β_1 over-expression and offers complete protection against AIPT when given prior to and continuously after AM administration, but it does not alter AM- and DEA-induced mitochondrial dysfunction, an event that likely plays an early / initiating role in AIPT.

Intratracheal administration of AM (1.83 μmol) results in a pulmonary drug concentration of approximately 10 μg / g lung tissue (Daniels et al., 1989), while levels achieved following clinical pharmacotherapy by the systemic route range from 20 to 734 μg / g (Plomp et al., 1985; Brien et al., 1987). Thus, intratracheal drug administration provides rapid exposure of susceptible lung epithelial cells to a relatively high concentration of AM, as might occur following clinical AM treatment. Following intratracheal administration of AM to hamsters, impairment of lung mitochondrial respiratory function was observed at complexes I and II of the electron transport chain (Chapter 6). Respiratory inhibition at complex I was observed in mitochondria isolated as early as 1 hour and reached statistical significance 3 hours after intratracheal AM dosing, while significant adverse effects at complex II were observed after 6 hours. Respiration supported by both complexes remained inhibited for up to 24 hours post-

dosing, suggesting a severe and prolonged effect of AM on lung mitochondrial respiratory function early after administration. Indicators of lung cell injury, including BALF protein content and LDH activity, were increased as early as 0.5 hours following AM treatment, signifying disruption of the alveolar-capillary barrier and cell damage. These results are in contrast to studies performed with isolated lung cells, whereby *in vitro* exposure to AM induced adverse mitochondrial effects prior to cytotoxicity (Bolt et al., 2001a). This suggests that processes other than mitochondrial dysfunction may contribute to AM-induced lung cell injury following administration via the intratracheal route, and represents a limitation of using the *in vivo* intratracheal model to study mitochondrial dysfunction as an initiating mechanism of AIPT. Regardless, this was the first demonstration of AM-induced lung mitochondrial dysfunction *in vivo*, and coupled with considerable *in vitro* evidence, the current data suggest that AM-induced mitochondrial dysfunction likely plays a role in initiating lung cell death that leads to AIPT.

In conclusion, these experiments revealed that lung mitochondria are adversely affected by both AM and DEA *in vitro*, and that DEA has more rapid and / or extensive effects on mitochondrial function. As such, it is proposed that DEA makes a significant contribution to AM-induced mitochondrial dysfunction and initiation of AIPT. Furthermore, respiratory inhibition in lung mitochondria isolated following *in vivo* AM administration was observed for the first time, although the role of this occurrence in initiating lung injury *in vivo* is unclear at present. Taken together, these results suggest that the mitochondrion is a probable target for initiating AIPT, and that strategies aimed at reducing mitochondrial dysfunction may prove valuable in the prevention or treatment

of AIPT. Vitamin E and pirfenidone both protected against AIPT in the hamster, at least partially through prevention of AM-induced up-regulation of the potent pro-fibrotic cytokine TGF-β_1. However, neither compound was able to prevent AM- and DEA-induced lung mitochondrial dysfunction. The promising results with vitamin E and pirfenidone in the hamster model suggest that these agents may be beneficial against clinical AIPT.

7.2 FUTURE DIRECTIONS

7.2.1 Further Characterization of AM-induced Mitochondrial Dysfunction

The mitochondrion has been identified as a target for initiation of AIPT, based on *in vitro* experiments with isolated lung cells and mitochondria. Currently, the *in vivo* effects of AM on lung mitochondrial structure and function, and the relationship of these effects to the development of AIPT, are unclear. In order to elucidate whether mitochondrial dysfunction is a requisite event in AIPT, it would be of value to determine whether lung mitochondrial function is compromised following AM administration to hamsters via a route that does not lead to fibrosis of the lung despite resulting in significant drug accumulation within the organ (for example, repeated oral dosing or ip injections). If pronounced mitochondrial dysfunction occurs following such administration, then the causal relationship of this event to AIPT and fibrosis must be questioned. Should substantial mitochondrial dysfunction not occur following such administration, a more thorough investigation of this event following intratracheal AM administration including timing, cell types involved, and detailed determination of

adverse respiratory and membrane potential effects, would help clarify its role in the etiology of AIPT.

7.2.2 Free Radical Generation by AM and DEA

Free radical formation from AM or its metabolites has been suggested to be involved in AIPT. The protective effect of vitamin E against AIPT in the hamster model, and against AM-induced cytotoxicity in other experimental systems, supports a free radical-mediated mechanism of AIPT. Carbon-centred radicals were shown to be formed from AM and DEA in incubations with hamster liver and lung microsomes (Rafeiro, 1997), providing the first evidence of free radical generation from AM in biological tissue. However, the structural identity of these radicals and the mechanism(s) of their formation are unknown at present. Identification of the free radical(s) formed from AM and DEA, and determination of the mechanism(s) of their formation, would help clarify their role in AIPT. Electron leakage from systems such as the cytochrome P450 monooxygenase system and the mitochondrial electron transport chain could contribute to their formation. As such, an investigation of the contribution of cytochromes P450 to the generation of AM and DEA radicals in microsomal incubations, using general or specific P450 inhibitors to block enzyme activity, would aid in this regard. Additionally, determination of potential free radical formation in mitochondria would aid in determining the role (and possible mechanism(s)) of free radical generation in AM-induced mitochondrial dysfunction and AIPT. A novel idea would be to determine whether free radicals are present in hamster lung tissue following *in vivo* AM administration, thus potentially resolving their involvement in AIPT in an animal model.

A limitation of this approach, however, is that if free radicals are formed in small quantities in lung tissue, they may be difficult to detect via traditional spin-trapping methods. Determination of the structure of trapped free radicals formed *in vitro* and *in vivo* could be performed via chromatographic separation and mass spectral analysis. Such information would be invaluable in elucidating the role of free radicals in AIPT.

7.2.3 Development of a Mouse Model of AIPT

Historically, the animal model used for studying AIPT has been the hamster, and only recently has a rat model been developed (Reinhart et al., 1996; Chung et al., 2001). However, development of a mouse model of AIPT is desirable, in order to take advantage of the advent of newer technologies and research techniques such as DNA microarrays and transgenic animals. These tools would allow for examination of numerous cellular and subcellular processes that could shed light on mechanisms of AIPT and potential therapeutic interventions. However, the majority of DNA mircoarrays have been developed for use with mouse or human tissue samples, cells, etc., and most transgenic models developed to date have been mice. As such, development of a mouse model, likely using the fibrosis-sensitive C57BL/6 strain, would allow these powerful research tools to be effectively employed in the study of AIPT. It would also be of value to attempt to develop a mouse model of AIPT that avoids the intratracheal dosing route (for example, by employing repeated ip injections or dietary treatment) in order to circumvent possible non-specific injurious pulmonary effects resulting from this means of drug administration.

7.2.4 Role of Apoptosis in AIPT

AM-induced apoptosis has been demonstrated in human thyroid and non-thyroid cell lines (Di Matola et al., 2000) and in rat and human alveolar epithelial cells *in vitro* (Bargout et al., 2000). Interestingly, prevention of *in vivo* alveolar epithelial cell apoptosis was associated with prevention of bleomycin-induced pulmonary fibrosis in rats (Wang et al., 2000). A role for apoptosis in AIPT, however, has not been thoroughly investigated. In a recent study (Bolt et al., 2001a), AM-induced cytotoxicity in freshly isolated hamster lung cells was not prevented by cyclosporin A, a potent inhibitor of mitochondrial permeability transition (MPT) that is involved in both apoptotic and necrotic cell death (Lemasters et al., 1998). Recognizing that ATP is critical for implementation of apoptotic mechanisms, it is likely that cell death in these experiments occurred via necrosis rather than apoptosis due to the extent of AM accumulation and mitochondrial dysfunction that led to cellular ATP depletion. However, accumulation of lower amounts of drug in lung cells *in vivo* following acute or chronic AM dosing may result in apoptotic lung cell death and ensuing fibrosis via mechanisms involving TGF-β_1, p53, and other cellular signalling pathways (Cooper, Jr., 2000). Determination of apoptotic lung cell death *in vivo* following AM dosing in an animal model would aid in elucidating a role for this type of cell death in AIPT. Additionally, studies in freshly isolated lung cells using lower concentrations of AM (or DEA) to avoid necrotic cell death would enable determination of apoptotic events such as cytochrome c release from mitochondria into the cytosol, DNA fragmentation, and activation of caspases known to be involved in apoptosis. Should apoptosis prove to be involved in the pathogenesis of

AIPT, steps involved in this process of cell death could be targeted in an attempt to reduce lung damage resulting from AM administration.

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