MECHANISMS OF AMIODARONE AND *N*-DESETHYLAMIODARONE TOXICITY IN LUNG CELLS

by

Michael William Bolt

A thesis submitted to the Department of Pharmacology and Toxicology in conformity with the requirements for the degree of Doctor of Philosophy

Queen's University

Kingston, Ontario, Canada

July, 2001

ABSTRACT

Michael W. Bolt: Mechanisms of Amiodarone and *N*-desethylamiodarone toxicity in lung cells. Ph.D. thesis, Department of Pharmacology and Toxicology, Queen's University at Kingston, August 2001.

Amiodarone (AM) is a potent antidysrhythmic agent that causes life-threatening pulmonary fibrosis. Susceptibilities of different hamster lung cell types to AM-induced cytotoxicity were compared. AM (100 and 200 μM)-induced cytotoxicity was significantly greater in hamster alveolar macrophages than in alveolar type II (PT) cells or non-ciliated bronchiolar epithelial (Clara) cells. However, Clara cells were most susceptible to 50 μM AM-induced cytotoxicity.

Disruption of cellular energy homeostasis as an initiating mechanism of AM-induced cell death was investigated. In all enriched, hamster lung cell preparations, AM (100 μM) and *N*-desethylamiodarone (DEA, 50 μM) disrupted mitochondrial membrane potential and decreased intracellular ATP levels prior to cell death. Glucose, niacin, or cyclosporin A did not decrease AM-induced cytotoxicity. In isolated lung mitochondria, DEA was more potent at disrupting function and accumulated to a greater extent than AM. Therefore, AM- and DEA-induced disruption of mitochondrial function may initiate cell death.

Free radical formation and subsequent lipid peroxidation may initiate AM-induced cytotoxicity. Vitamin E (300 μM) decreased 100 μM AM-induced cytotoxicity in hamster macrophages but not in any other enriched lung cell fraction or in any fraction incubated with 50 μM DEA. AM and DEA did not cause

lipid peroxidation in any fraction. Thus, lipid peroxidation does not initiate AM- and DEA-induced cytotoxicity.

AM-induced cytotoxicity in isolated human PT cells and alveolar macrophages was similar to that observed in rodent models. However, PT cells were more susceptible than macrophages to AM-induced cell death. Vitamin E slightly protected against AM-induced cytotoxicity. An HPLC peak that co-elutes with DEA was observed in some PT cells, as well as in human lung microsomes exposed to AM.

Overall, AM and DEA initiate cell death in hamster alveolar macrophages, PT cells and Clara cells by disrupting mitochondrial function and not by inducing lipid peroxidation. Human lung cells can be used to study AM-induced cytotoxicity and biotransformation of AM to DEA may occur in human lung.

Keywords: amiodarone, desethylamiodarone, lung, mitochondria, ATP, vitamin E, lipid peroxidation

QUEEN'S UNIVERSITY AT KINGSTON

SCHOOL OF GRADUATE STUDIES AND RESEARCH

PERMISSION OF CO-AUTHORS

I/we, the undersigned, hereby grant permission to microfilm any material designated as being co-authored by me/us in the thesis copyrighted to the person named below:

Michael Bolt	
Name of copyrighted author	Signature of copyrighted author
Name(s) of co-author(s)	Signatures of co-author(s)
Jeff Card	
Graeme Smith	

DATE: July 13, 2001

CO-AUTHORSHIP

This research was conducted by the candidate Michael W. Bolt, under the supervision of Dr. Thomas E. Massey.

Jeff Card performed oxygen consumption and drug accumulation experiments in isolated whole lung mitochondria (Chapter 3).

Graeme Smith assisted with the human lung cell isolation procedure and with the human lung microsome incubations (Chapter 4).

ACKNOWLEDGEMENTS

I would like to thank my research supervisor, Dr. Tom Massey, for introducing me to the field of toxicology and for his guidance and support during the course of this research. I appreciate all he has done for me and hope to beat him at golf one day.

I would also like to thank Drs. William J. Racz and James F. Brien for their advice on how to approach research problems and for their daily discussions regarding curling or the Detroit Red Wings. Also, I would like to thank all other professors in the Department of Pharmacology and Toxicology for their scientific help or for use of their equipment.

Research wouldn't have been the same without the contributions of all of the personalities within our lab, both past and present (Sophia Ali, Leanne Bedard, Jeff Card, Willie Chung, Dr. Patty Donnelly, Dr. Randy Leeder, Dr. Liz Rafeiro, Graeme Smith, Dr. Rick Stewart and Andrew Tam). You guys made me laugh (and run for cover) when I needed to the most. I also appreciate all the assistance provided by departmental staff (René Roscher, Chris Berga, Bernice Ison, Janet LeSarge, Jane Warns, and Marylin Oosten).

A special thank you goes to my parents, Grandma, sister and brother, all of the McBrides, DeWolfes, Hylands and Smiths and my friends for their support and encouragement.

Finally, I would like to thank the Canadian Institutes of Health Research, The School of Graduate Studies and the Department of Pharmacology and Toxicology at Queen's University for financial support.

Dedicated with love to my parents

TABLE OF CONTENTS

Page

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 2

DIFFERENTIAL SUSCEPTIBILITIES OF ISOLATED HAMSTER LUNG CELL TYPES TO AMIODARONE TOXICITY

CHAPTER 3

DISRUPTION OF MITOCHONDRIAL FUNCTION AND CELLULAR ATP LEVELS BY AMIODARONE AND *N*-DESETHYLAMIODARONE IN THE INITIATION OF AMIODARONE-INDUCED PULMONARY CYTOTOXICITY

CHAPTER 4

EFFECTS OF VITAMIN E ON CYTOTOXICITY OF AMIODARONE AND *N*-DESETHYLAMIODARONE IN ISOLATED HAMSTER LUNG CELLS

CHAPTER 5

TOXICITY OF AMIODARONE AND *N*-DESETHYLAMIODAREONE IN FRESHLY ISOLATED HUMAN LUNG CELLS

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

LIST OF FIGURES

Page

LIST OF TABLES

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AIPT	amiodarone-induced pulmonary toxicity
AM	amiodarone
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AV	atrioventricular
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
^{o}C	degrees celsius
Ca^{2+}	calcium ion
CAMIAT	Canadian Amiodarone Myocardial Infarction Arrhythmia Trial
CCl_4	carbon tetrachloride
CD	cell digest
CHF-STAT	The Survival Trial of Antiarrhythmic Therapy in Congestive Heart Failure
Ci	curie
Clara	non-ciliated bronchiolar epithelial
cm	centimetre(s)
^{51}Cr	chromium

CYP450	cytochrome P450 polysubstrate monooxygenase
DEA	*N*-desethylamiodarone
dH_2O	distilled water
DMSO	dimethylsufloxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E1	elutriation fraction #1
E2	elutriation fraction #2
E3	elutriation fraction #3
E4	elutriation fraction #4
EDTA	ethylenediamine tetraacetic acid
e.g.	exempli gratia (for example)
EGTA	ethylene glycol-bis (β-amino ethyl ether) tetraacetic acid
EMIAT	European Myocardial Infarction Amiodarone Trial
ESR	electron spin resonance
et al.	et alia (and others)
etc.	et cetera (and others especially of the same kind)
FCCP	carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone
g	gram(s)
g	relative centrifugal force
GESICA	Gruppo de Estudo de la Sobrevida en la Insuficiencia Cadiaca en Argentina
G_i protein	inhibitory guanyl-nucleotide binding protein

GSH	glutathione, reduced form
GSSG	glutathione, oxidized from
h	hour(s)
^3H	tritium
HCl	hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazine-2-ehtane sulfonic acid
H_2O	water
H_2O_2	hydrogen peroxide
$HOO^{\bullet}$	hydroperoxyl radical
HpBS	HEPES buffered salt solution
HPLC	high performance liquid chromatography
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
iv	intravenous
JC-1	5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolylcarbocyanine iodide

K^+	potassium ion
L	litre(s)
LDH	lactate dehydrogenase
$LO^•$	lipid alkoxyl radical
$LOO^•$	lipid peroxyl radical
LOOH	lipid hydroperoxide
LPO	lipid peroxidation
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
M	molar
μ	micro
MAC	alveolar macrophages
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre
MOPS	3-[N-morpholino]propane-sulphonic acid
MPT	mitochondrial permeability transition
N	normal
n	number (of experiments)
Na^+	sodium ion
NBT	nitroblue tretazolium
NAD	nicotinamide adenine dinucleotide

NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	nanometre(s)
$^{\bullet}OH$	hydroxyl radical
O_2	oxygen
$O_2^{\bullet-}$	superoxide anion radical
p<	probability less than
PBS	phosphate buffered saline
PG	prostaglandin(s)
pH	negative logarithm of the hydrogen ion concentration
±	plus or minus
PMN	polymorphonuclear leukocyte
PT	Percoll© top fraction
PUFAs	polyunsturated fatty acids
$R^{\bullet}$	radical
RCR	respiratory control ratio
$RO^{\bullet}$	alkoxyl radical
$ROO^{\bullet}$	peroxyl radical
ROS	reactive oxygen species
rpm	revolutions per minute
SD	standard deviation
sec	second(s)
TBAP	tetrabutylammonium dihydrogen phosphate

TGF	transforming growth factor
TNF-α	tumour necrosis factor alpha
Type II	alveolar type II cell(s)
UV	ultraviolet
V	vitamin E (α-tocopherol)
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

GENERAL INTRODUCTION

1.1 STATEMENT OF RESEARCH PROBLEM

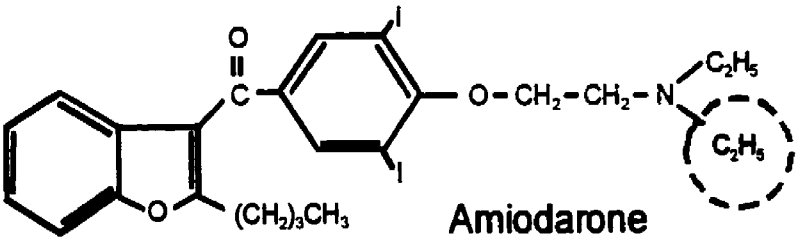
Amiodarone (AM, Cordarone®, Wyeth-Ayerst Laboratories Inc.) is a potent and efficacious antidysrhythmic agent. However, AM has many adverse effects, some of which are life threatening. The adverse effect of greatest clinical concern is pneumonitis, which can progress to pulmonary fibrosis causing mortality. The etiology of AM-induced pulmonary toxicity (AIPT) is not known. However, as reviewed by Massey *et al.* (1995), and Reasor and Kacew (1996a), several possible mechanisms have been proposed. The goal of this thesis research was to investigate the initiating process(es) involved in AIPT. Specifically, susceptible cell types for AM-induced cytotoxicity were identified and the role of AM-induced perturbation of mitochondrial function as an initiating mechanism of cell death was examined within these cells. In addition, the role of lipid peroxidation and possible protection by vitamin E in AM-induced cytotoxicity was investigated. Identification of the mechanism of cell death which initiates AIPT would be of significant clinical value because it may lead to the development of treatment strategies to prevent or counteract AM-induced adverse effects, or to the development of new antidysrhythmic agents which are clinically as effective as AM but do not cause pulmonary fibrosis.

1.2 HISTORY AND THERAPEUTIC USES OF AMIODARONE

AM (2-butyl-3-(3',5'-diiodo-4'-diethylaminoethoxybenzoyl)-benzofuran) (Figure 1.1) was first synthesized in Belgium in 1961, and initially was developed and marketed in Europe as an antianginal agent (Deltour et al., 1970) before it was found to possess antidysrhythmic properties (Charlier et al., 1969; Van Schepdael and Solvay, 1970). In 1985, the United States of America approved oral AM for treating life-threatening ventricular and supraventricular dysrhythmias resistant to other pharmacotherapies, and in 1986, similar AM use was approved in Canada. In 1996, AM was considered to be the most effective agent to control ventricular tachycardia and fibrillation and to maintain sinus rhythm in patients with atrial fibrillation (Singh, 1996). In 1997, intravenously administered AM was approved in the US for use in the short-term management of ventricular tachyarrhythmias, changing the use of AM from "last resort" (Gill et al., 1992) to first line of therapy (Desai et al., 1997). In 1998, AM was the number one prescribed antidysrhythmic agent in the United States (Connolly, 1999).

Within the last 10 years, numerous trials have been conducted to assess the prophylactic effectiveness of low doses of AM to prevent mortality post myocardial infarction. Results from these trials are equivocal. For example, the Canadian Amiodarone Myocardial Infarction Arrhythmia Trial (CAMIAT) demonstrated a significant reduction in sudden death and resuscitated cardiac arrest (Cairns et al., 1997), whereas the European Myocardial Infarction Amiodarone Trial (EMIAT) reported insignificant reductions in arrhythmic events

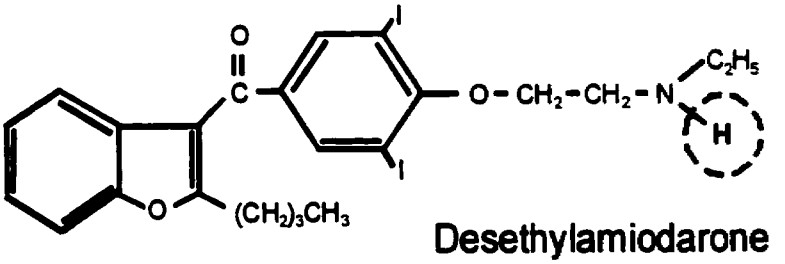
A)



Amiodarone

Hepatic Cytochrome
P450
3A4 and 2C8
1A2, 2C19,2D6

B)



Desethylamiodarone

Figure 1.1 The Chemical structures of A) amiodarone and B) its major metabolite, desethylamiodarone.

and no change in total mortality (Julian et al., 1997). Results combining the data from the four largest trials (CAMIAT, EMIAT, The Survival Trial of Antiarrhythmic Therapy in Congestive Heart Failure (CHF- STAT), and Gruppo de Estudo de la Sobrevida en la Insuficiencia Cadiaca en Argentina (GESICA)) suggested that AM reduced the risk of cardiac arrest or arrhythmic sudden death in patients with left ventricular dysfunction secondary to myocardial infarction and in patients with congestive cardiac failure 20-30%, but total mortality was not significantly reduced (Campbell, 1997). However, meta-analysis of all 13 trials indicated that AM reduces both dysrhythmic (29%) and total death (13%) (Amiodarone Trials Meta- Analysis Investigators, 1997), suggesting that EMIAT, CAMIAT, GESICA, and CHF-STAT trials were not sufficiently large enough to detect AM-induced effects on total mortality.

As of 1999, the American Heart Association added the use of intravenous AM to its recommended resuscitation protocol for treating those suffering from cardiac arrest (Erich, 2000). This was based largely on results from the Amiodarone in Out-of-Hospital Resuscitation of Refractory Sustained Ventricular Tachyarrhythmias trial which demonstrated that in patients suffering from out-of-hospital cardiac arrest, AM recipients were more likely to survive to hospital admission compared to placebo control (Kudenchuk et al., 1999).

1.3 PHARMACODYNAMICS OF AMIODARONE

Although AM has been demonstrated to possess characteristics of all four Vaughan-Williams antidysrhythmic classes, AM is classified as a class III

antidysrhythmic agent. Specifically, AM blocks Na^+ channels (class I), non-competitively antagonizes α- and β-adrenergic receptors (class II), increases action potential duration, thereby prolonging refractoriness and repolarization (class III) and blocks Ca^{2+} channels (class IV) (Rothenberg et al., 1994).

In all cardiac tissues, the efficacy of AM as an antidysrhythmic agent is largely attributed to its ability to prolong action potential duration, which is caused by K^+ channel blockade (Podrid, 1995) and possibly down regulation of K^+ channel gene expression (Kodama et al., 1999). Consequently, membrane excitability and ultimately automaticity of the atrioventricular (AV) node and heart rate are reduced. Contrary to other class III antidysrhythmic agents, AM-induced prolongation of action potential duration persists at higher heart rates, making it especially effective for treating tachycardias (Anderson et al., 1989). However, class III properties alone cannot account for AM's antidysrhythmic effects (Nattel and Talajic, 1988; Podrid, 1995).

AM blocks Na^+ channels, which slows action potential upstroke velocity and ultimately the rate of membrane depolarization and impulse conduction (Mason et al., 1983; Mason et al., 1984). Therefore, Na^+ channel blockers are generally used to treat ventricular dysrhythmias (Nattel and Talajic, 1988). However, unlike other Na^+ channels blockers, AM selectively blocks inactivated Na^+ channels, and for this reason, AM is more effective in depolarized tissue (as in myocardial ischemia), tissue with a brief diastole (as during tachycardia), and tissue with prolonged repolarization (Mason, 1987).

AM increases the conduction time and refractoriness of the AV node via its anti-adrenergic actions and blockade of slow Ca^{2+} channels (Nattel et al., 1987; Podrid, 1995). These actions also contribute to AM's antianginal effects, including dilating coronary and systemic arteries, resulting in increased coronary blood supply and decreased systemic blood pressure and afterload (Singh and Vaughan Williams, 1970).

1.4 PHARMACOKINETICS OF AMIODARONE

The pharmacokinetics of AM are complex and highly variable between patients and are discussed in detail below to assist in understanding the clinical toxicities produced by this drug.

1.4.1 Physicochemical Properties

The AM molecule is amphiphillic, containing both hydrophillic (tertiary amine) and hydrophobic (benzofuran and di-iodinated benzene rings) portions (Figure 1.1) (Bonati et al., 1984). Therefore, it is highly lipophillic and it preferentially accumulates in lipid rich regions of the cell including membranes (Chatelain and Laruel, 1985). AM is highly soluble in chloroform and poorly soluble in water (Singh, 1983) with a pK_a of 6.56.

1.4.2 Absorption of Amiodarone

Following oral administration, absorption of AM from the gastrointestinal tract is slow (Andreasen et al., 1981; Holt et al., 1983), resulting in incomplete

(30-65%) bioavailability (Roden, 1999). Also, variability in AM bioavailability is thought to arise from AM's high lipid solubility and extensive first pass metabolism (Holt et al., 1983; Latini et al., 1984). Peak plasma levels of AM are reached 3-7 h after oral AM administration (Roden, 1993), but steady state levels are not reached until 1-5 months after continuous oral administration (Kerin et al., 1985; Latini et al., 1984; Mostow et al., 1986). The therapeutic plasma concentration of AM has been suggested to range from 1-2.5μg / ml with toxicities occurring at plasma concentrations of 2.5-4 μg/ml (Mahmarian et al., 1994; Pollak et al., 2000; Roden, 1999; Rotmensh et al., 1984). However, considerable interindividual variability exists between plasma AM concentrations and observed therapeutic or toxic effects.

1.4.3 Distribution of Amiodarone

AM distributes extensively throughout the body, with an estimated volume of distribution of approximately 5000 L (Holt et al., 1983). Furthermore, AM is highly (>98%) protein bound (Andreasen et al., 1981; Heger et al., 1984) and very lipid soluble. Examination of autopsy tissue of patients receiving chronic AM therapy demonstrated that AM levels in tissues were 10 to 1000 times greater than plasma concentrations (Plomp et al., 1984; Riva et al., 1982). Furthermore, AM accumulation was the greatest in adipose, lung and liver tissues and to a lesser extent in bone marrow, pancreas, spleen, heart, kidney, skeletal muscle, thyroid, and brain tissues (Brien et al., 1987; Haffajee et al., 1983; Holt et al., 1983). Following chronic therapy, lung AM levels ranged from 20-734 μg (.029-

1.05 μmoles) / g of lung tissue (Broekhuysen et al., 1969; Holt et al., 1983; Nalos et al., 1987; Plomp et al., 1984; Rosen and Rauckman, 1977).

1.4.4 Biotransformation of Amiodarone

The metabolism of AM has not been completely characterized, especially in humans. However, *N*-desethylamiodarone (DEA), a primary metabolite of AM (Figure 1.1), has been found in the plasma and tissues of patients on AM therapy (Flanagan et al., 1982; Gillis and Kates, 1984; Latini et al., 1984) and in experimental animals (Brien et al., 1990; Daniels et al., 1990a). In humans, hepatic CYP3A4, CYP2C8, and CYP1A1 are significantly involved in AM biotransformation to DEA (Ha et al., 1993; Ohyama et al., 2000a; Trivier et al., 1993) with minor contributions of CYP1A2, CYP2C19, and CYP2D6 (Figure 1.1) (Ohyama et al., 2000a). In experimental animals, N-dealkylation of AM to DEA is believed to be catalyzed mainly by hepatic cytochrome P-450 (CYP450) (Blake and Reasor, 1995a; Rafeiro et al., 1990; Young and Mehendale, 1987), especially the subfamily CYP450 3A (Rafeiro et al., 1990). Furthermore, DEA formation occurs in the intestine to a much smaller extent via the flavin containing monooxygenase (Young and Mehendale, 1987). However, AM biotransformation is complicated by the fact that in microsomes from human β-lymphoblastoid cells expressing many different isoforms of CYP450, AM inactivated CYP3A4 and DEA inactivated CYP1A1, CYP1A2, CYP2B6, and CYP2D6 (Ohyama et al., 2000b). Furthermore, in rats, mice and hamsters, a biologically inactive complex CYP450-Fe(II)-AM metabolite complex has been identified in the liver (Larrey et

al., 1986). This suggests that AM can inhibit P450 enzymes involved in its metabolism.

Despite its presence in human lung in relatively high amounts following AM administration to patients (Brien et al., 1987), DEA production was not quantifiable in uninduced hamster and rat lung microsomes (Blake and Reasor, 1995a; Rafeiro et al., 1990; Young and Mehendale, 1987) or in isolated perfused rat lung (Camus and Mehendale, 1986). However, experiments investigating biotransformation of AM to DEA in human lung have not been reported.

DEA exerts pharmacological effects similar to those of the parent drug and was once considered as an antidysrhythmic agent itself (Nattel, 1986). Upon initiation of AM therapy, less DEA is present in the plasma relative to AM (Heger et al., 1983). However, steady state AM and DEA plasma concentrations are comparable (Brien et al., 1987; Holt et al., 1983; Plomp et al., 1984). Furthermore, with the exception of adipose tissue, DEA levels are typically greater than AM in corresponding tissues (Nalos et al., 1987). This phenomenon is explained by the fact that compared to AM, DEA is less bound to plasma proteins and has a different ionization state (pK_a) resulting in increased diffusion of DEA from the plasma into tissues (Plomp et al., 1985).

Although DEA is recognized as a primary metabolite of AM, other metabolites have been identified. In rat liver microsomes, small quantities of an *N*-oxide metabolite of AM were detected (Myung et al., 2000). Furthermore, *N'N'*-didesethylamiodarone has been detected in hamsters (Daniels et al., 1989) and in dogs (Brien et al., 1990) and a deiodinated metabolite has been observed

in rats (Kannan et al., 1989a). Recently, it has been demonstrated that DEA generated by rat liver microsomes undergoes hydroxylation (Ha et al., 2001). Other unidentified metabolites have been detected in serum from AM-treated patients (Staubli et al., 1985), in *in vitro* incubations of AM with rabbit hepatic and gut microsomes (Endoh et al., 2000) and in human hepatic microsomes (Trivier et al., 1993).

1.4.5 Elimination of Amiodarone

Both AM and DEA are eliminated via biliary and fecal routes (Andreasen et al., 1981; Broekhuysen et al., 1969). However, the pathway of elimination and the extent of enterohepatic recirculation is undetermined (Roden, 1999). Although renal excretion of AM is negligible (Anastasiou-Nana et al., 1982; Plomp et al., 1985; Riva et al., 1982), its deiodination is thought to be responsible for the free iodide detected in the urine (Rao et al., 1986).

The plasma elimination half-life of AM following a single dose ranges from 3.2-79.7 h (Latini et al., 1984) and ranges from 14-107 days following long term AM therapy (McKenna et al., 1983). Recently, it has been determined that, after termination of AM therapy when steady-state levels were obtained, AM serum concentrations decreased 25% in three days and 50% by 36 days (Pollak et al., 2000), but that AM was detectable in the plasma for up to nine months (Singh, 1996). This may be explained by the fact that, after drug withdrawal, elimination of AM from the plasma is biphasic. Initial redistribution from its central compartment is rapid and short. However, secondary elimination from poorly

perfused peripheral tissues (such as adipose) is much slower (Freedman and Somberg, 1991; Holt et al., 1983; Mason, 1987). Thus, despite the patient's withdrawal from the drug therapy, toxicities may progress (Martin, 1990a).

1.5 CLINICAL TOXICITIES OF AMIODARONE

There are many adverse effects associated with AM therapy (Rothenberg et al., 1994), some of which are common (occurring in 10-100% of patients receiving AM) and can be diminished or abolished with dose reduction or cessation of AM therapy (Vrobel et al., 1989). These effects include: skin photosensitivity, ataxia and hand tremors, gastrointestinal disturbances, corneal microdeposits, and elevation of serum liver enzymes. Other AM-induced adverse effects are reversible but less frequent (1-10% incidence) including hypothyroidism, hyperthyroidism, peripheral neuropathy and grey / blue skin discolouration (Gill et al., 1992).

In addition, some adverse effects of AM therapy are less frequent and potentially fatal. They include hepatitis (<1% incidence) (Marchlinski, 1987), exacerbation of coronary disorders (tachycardia, bradycardia, congestive heart failure) and pulmonary toxicity (Gill et al., 1992; Mason, 1987). AM-induced pulmonary toxicity (AIPT) occurs in 1-27% of patients receiving AM (Adams et al., 1988; Kudenchuk et al., 1984; Magro et al., 1988; Mason, 1987; Pollak, 1999; Vrobel et al., 1989). However, of those patients with AIPT, a mortality rate of 10-23% has been observed (Mason, 1987; Vrobel et al., 1989) and therefore, AIPT is the adverse effect of AM with greatest clinical concern.

AIPT incidence appears to be dependent upon dose and duration of AM treatment. Five to 27% of patients receiving greater than 400 mg of AM daily develop AIPT. However, AIPT was observed in only 1.6% of patients receiving daily AM doses of 400 mg or less (Sunderji et al., 2000). Furthermore, the total cumulative amount of AM (rather than the daily dose) may be important for assessing risk of developing AIPT (Adams et al., 1988; Fraire et al., 1993; Martin and Rosenow, 1988; Rakita et al., 1983). In addition, patients with a preexisting lung disease (i.e. abnormal chest X-rays or pulmonary function status prior to AM therapy) have increased risk for developing AIPT (Darmanata et al., 1984; Kudenchuk et al., 1984; Mason, 1987)

1.6 AMIODARONE-INDUCED PULMONARY TOXICITY (AIPT)

1.6.1 Clinical Presentation of AIPT

AIPT clinically presents in humans as either the acute type or (more commonly) as the subacute/chronic form (Rotmensch et al., 1980). The acute hypersensitivity type of AIPT is associated with a more rapid onset, occurs in about one third of patients, is typically associated with fever, and may mimic infectious pneumonitis. Chest X-rays demonstrate a predominantly alveolar pattern with a patchy distribution (usually in the peripheral areas of the lung). The subacute / chronic form has an insidious onset, rarely begins before two months of AM therapy, and is rarely seen in patients receiving less than 400 mg / day. Chest X-rays demonstrate parenchymal infiltrates with primarily a diffuse interstitial pattern (Fraire et al., 1993; Martin and Rosenow, 1988).

Signs and symptoms of AIPT are nonspecific and include a non-productive cough, pleuritic pain, dyspnea, fever, weakness and weight loss (Martin and Rosenow, 1988). Therefore, decreased diffusing lung capacity for carbon monoxide, an elevated erythrocyte sedimentation rate, and appearance of lipid-laden alveolar macrophages in bronchoalveolar lavage fluid (BALF) are also used to diagnose AIPT (Fraire et al., 1993).

1.6.2 Histopathology of AIPT

Although they vary considerably between patients, the most common histopathological features of AIPT include interstitial and alveolar thickening, cellular infiltration of the interstitium / alveoli consistent with pneumonitis, and fibrosis (Brien et al., 1987; Costa-Jussa et al., 1984; Myers et al., 1987; Nalos et al., 1987; Pollak and Sami, 1984; Suarez et al., 1983; van Zandwijk et al., 1983). Pulmonary fibrosis, defined as an excessive deposition of collagen in the lung (Reiser and Last, 1986), can have a pattern of damage that is either patchy or diffuse and may affect the alveoli, the interstitium, or both (Dusman et al., 1990; Vrobel et al., 1989).

Cellular infiltrates include alveolar macrophages, a variable number of lymphocytes and neutrophils and to a lesser extent eosinophils and plasma cells (Myers et al., 1987; van Zandwijk et al., 1983). Fibroblast proliferation and excessive production of collagen and elastin results in alveolar wall thickening which, in some patients, can progress to life threatening fibrosis (Cazzadori et al.,

1986; Dean et al., 1987; Gefter et al., 1983; van Zandwijk et al., 1983). Alveolar type II cell hyperplasia, a normal reparative response to type I cell injury (Brien et al., 1987; Marchlinski et al., 1982; Pollak and Sami, 1984), and hyaline membrane formation (Brien et al., 1987; Cazzadori et al., 1986; Himmel et al., 2000; Myers et al., 1987) have also been observed.

Alveolar spaces and the interstitium contain numerous alveolar macrophages with "foamy" appearance due to the presence of cytoplasmic multilamellar phospholipid inclusion bodies (Gefter et al., 1983; Marchlinski et al., 1982; Myers et al., 1987; Nalos et al., 1987; Pollak and Sami, 1984; Suarez et al., 1983). Cytoplasmic multilamellar phospholipid inclusion bodies have also been observed in endothelial, epithelial and interstitial pneumocytes. However, "foamy" cells can be observed in individuals on AM therapy who do not have AIPT (Fraire et al., 1993).

1.6.3 Treatment of AIPT

Once AIPT has been diagnosed, a limited number of treatments are available. The most frequently used option is to discontinue AM therapy. However, when AM is the best or only antidysrhythmic therapy available, AM doses are lowered to below 400 mg / day (Martin and Rosenow, 1988). Although some symptoms of AIPT may begin to resolve within a few days after discontinuing / reducing AM therapy, the long elimination half-life and tendency for AM to accumulate in the lung can cause symptoms of AIPT to persist for several months (Vrobel et al., 1989). Corticosteroid therapy is indicated when

there is an immunological basis to pulmonary inflammation or disease. However, the benefit of using corticosteroids is uncertain as AIPT can resolve without their use (Gibb and Melendez, 1986; Suarez et al., 1983).

1.7 PROPOSED MECHANSIM OF AIPT

The etiology of AIPT is unknown. However, there are two major classes of mechanisms proposed to initiate AIPT: 1) an indirect (or immunological) mechanism or 2) a direct toxic mechanism (Fig 1.2).

1.7.1 Direct versus Indirect Mechanisms

The stimulation of an immune response in the lung by AM (or DEA) is an indirect mechanism. In contrast, a direct mechanism implies that AM (or DEA) is directly toxic to the cell and may be dependent upon accumulation in the lung. However, it is possible that more than one mechanism may be involved in the development of AIPT (Fraire et al., 1993). Therefore, in order to fully elucidate the mechanism of AIPT, it is critical to distinguish between events which initiate AIPT and those that occur subsequently. A multifactorial mechanism of pulmonary toxicity (i.e. exhibiting both direct and indirect mechanisms) has also been suggested for other drugs (such as bleomycin) which cause lung damage (Martin, 1990b).

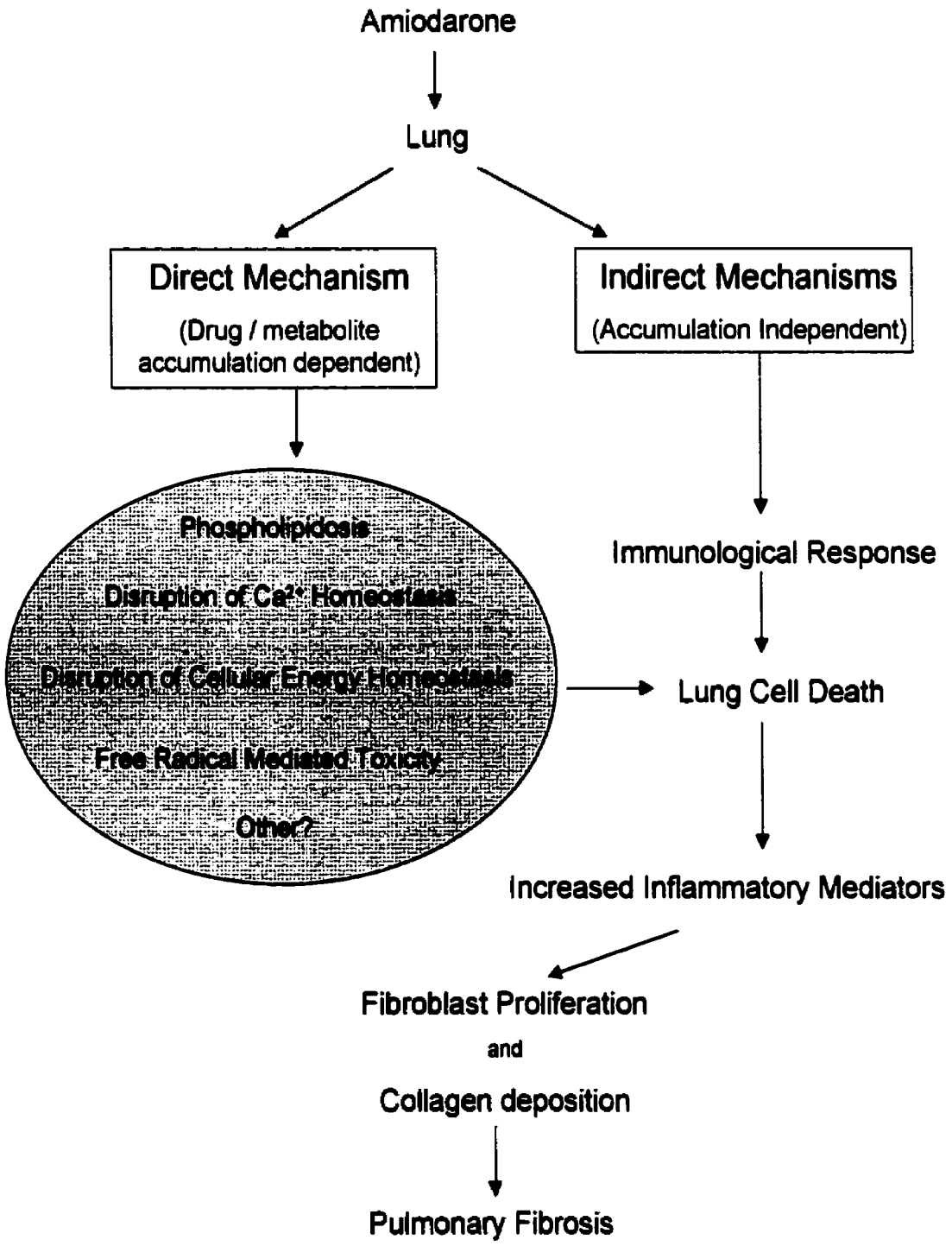


Figure 1.2 Proposed mechanism of AIPT (modified from Massey et al., 1995)

1.7.2 INDIRECT MECHANISM OF AIPT

It has been proposed that an immunological mechanism is responsible for the development of AIPT. This hypothesis was based on the fact that the occurrence of AIPT is not always proportional to AM blood levels or dose (Dunn and Glassroth, 1989). Nevertheless, evidence to support and reject an immunological basis for AIPT exists.

An immunologically mediated mechanism of AIPT is supported by clinical studies which demonstrated that: 1) serum anti-AM antibodies that react with AM and lungs of patient with AIPT but not with normal lung or fibrotic lungs of patients not exposed to AM have been reported (Fan et al., 1987; Pichler et al., 1988); 2) corticosteroids alleviated AIPT with and without AM discontinuation (Isreal-Biet et al., 1987; McNeil et al., 1992; Venet et al., 1984); 3) leukocyte inhibitory factor secretion occurred in the presence of AM (Akoun et al., 1984); 4) lymphoblastic transformation tests and skin and basophil degranulation tests were positive in the presence of AM (Akoun et al., 1984); 5) in patients with AIPT, bronchoalveolar lavage fluid (BALF) demonstrated lymphocytosis (Akoun et al., 1984; Israel-Biet et al., 1985; Venet et al., 1984), an increase in immunoglobulin levels (Sandron et al., 1986), and a variable increase in the number of lymphocytes, neutrophils, eosinophils and mast cells (Akoun et al., 1991; Coudert et al., 1992; Isreal-Biet et al., 1987; Venet et al., 1984).

Evidence against an immunological basis for AIPT include the following: 1) complement, immunoglobulins or lymphocytosis were not detected in patients

with AIPT (Adams et al., 1986; Ohar et al., 1992); 2) immune responses of patients with AIPT did not differ from those of patients without (Coudert et al., 1992; Nicolet-Chatelain et al., 1991; Pichler et al., 1988); 3) corticosteroids did not ameliorate AIPT (Cooper et al., 1986) and symptoms of AIPT were alleviated without corticosteroids (Gefter et al., 1983; Leech et al., 1984). Because of this evidence against an immunological mechanism, some investigators have suggested that the immune response may result from cell death caused by direct drug toxicity (Isreal-Biet et al., 1987; Nicolet-Chatelain et al., 1991).

1.7.2.1 Altered Release of Inflammatory Mediators

It is generally accepted that, upon stimulation, inflammatory cells release mediators such as cytokines and growth factors that can cause the proliferation of cells involved in the development of fibrosis (Crystal et al., 1991). Therefore, several *in vivo* and *in vitro* experiments have investigated whether AM influences the release of these mediators.

Analysis of BALF from hamsters (Blake and Reasor, 1995b) and rats (Reinhart and Gairola, 1997) exposed to AM *in vivo* demonstrated increased chemiluminescence, and release of tumour necrosis factor alpha (TNF-α), indicative of activation of macrophages or neutrophils or inflammation. Furthermore, AM increased interleukin 6 (IL-6) and TNF-α release from lipopolysaccharide (LPS)-stimulated rat alveolar macrophages (Reasor et al., 1996a). In Fisher 344 rats, AM caused transforming growth factor beta (TGF-β) mRNA and protein levels to increase much earlier than the onset of AM-induced

pulmonary fibrosis (Chung et al., 2001). Alveolar macrophages exposed to AM *in vitro* demonstrated increased release of IL-1, TNF-α, prostaglandins (PG) E_2, and phorbol myristate acetate-stimulated release of superoxide (Futamura, 1995; Futamura, 1996a; Futamura, 1997; Zitnik et al., 1992). Therefore, altered inflammatory mediator release by AM without cell death (especially by macrophages) was suggested by others as a possible mechanism for AM-induced lung damage.

However, a direct link between AM-induced increased release of inflammatory mediators without cell death and initiation of AIPT has not been established. Furthermore, in other experiments, AM had no effect on macrophage phagocytosis, or the release of IL-1, TNF-α, or IL-6, leukotriene B_4 (LTB_4), TGF-β or superoxide (Reasor et al., 1996a; Reinhart and Gairola, 1997; Zitnik et al., 1992). Therefore, it is believed that, although AM-induced alterations of mediator release may advance the progression of AIPT, it is not considered to be a mechanism that initiates AIPT (Isreal-Biet et al., 1987; Nicolet-Chatelain et al., 1991).

1.7.3 DIRECT MECHANISMS OF AIPT

In the direct mechanism of AIPT, it is believed that AM and DEA are directly toxic to lung cells. Similar to other models of chemically induced pulmonary fibrosis, prolonged and / or pronounced AM-induced cytotoxic insult to the airway epithelium could initiate the release of inflammatory mediators, influx of inflammatory cells, fibroblast proliferation, and collagen deposition (Sheppard

and Harrison, 1992) (Figure 1.2). There is both *in vivo* and *in vitro* evidence to support that AM is directly toxic to lung cells. In a rat model of AIPT, cytotoxicity occurs *in vivo* shortly after exposure to AM (Taylor et al., 2001), and in the hamster, early *in vivo* alveolar type II cell proliferation consistent with epithelial cell loss has been demonstrated (Cantor et al., 1987).

The strongest evidence for a direct mechanism of AM toxicity is obtained from *in vitro* studies that demonstrate concentration-dependent cytotoxicity. AM has been shown to be directly cytotoxic to a variety of pulmonary cell types such as hamster and rat alveolar macrophages (Bolt et al., 1998; Leeder et al., 1996; Ogle and Reasor, 1990), alveolar type II cells (Bargout et al., 2000; Bolt et al., 1998; Wilson and Lippmann, 1996b), non-ciliated bronchiolar epithelial (Clara) cells (Bolt et al., 1998), rat fibroblasts (Wilson and Lippmann, 1996b), and bovine and human pulmonary artery endothelial cells (Kachel et al., 1990; Martin and Howard, 1985). Furthermore, AM has also been shown to be cytotoxic to non-pulmonary cells including rat hepatocytes (Gross et al., 1989; Ruch et al., 1991), human umbilical cord vein endothelial cells (Baudin et al., 1996), and human thyrocytes (Beddows et al., 1989). In many experiments, lower concentrations of DEA caused cytotoxicity similar in magnitude to that of AM (Bargout et al., 2000; Beddows et al., 1989; Gross et al., 1989; Matola et al., 2000; Ogle and Reasor, 1990; Ruch et al., 1991; Wilson and Lippmann, 1990). Therefore, DEA is likely to play a role in the etiology of AIPT.

There are two major types of cell death, necrosis and apoptosis (Cohen, 1993; Ellis et al., 1991). The type of cell death caused by AM which initiates

pulmonary fibrosis is not clearly defined and may be related to the concentration of AM. Recently, AM and DEA have been demonstrated to cause apoptosis in cultured thyroid (TAD-2) and endometrial carcinoma (HeLa) cells (Matola et al., 2000), and in cultured rat and human (A549) alveolar type II cells (Bargout et al., 2000). However, in the same study by Bargout et al. (2000), slightly higher concentrations of AM were demonstrated to cause necrosis. Nevertheless, it is generally accepted that inflammatory responses are activated by necrotic cell death (Cohen, 1993; Ellis et al., 1991).

The mechanism of AM-induced cell death which initiates AIPT could involve one or a combination of: 1) phospholipidosis; 2) disruption of Ca^{2+} homeostasis; 3) free radical production; or 4) perturbation of cellular energy homeostasis.

1.7.3.1 Phospholipidosis

Phospholipidosis is characterized by the accumulation of excessive amounts of intracytoplasmic multilamellar inclusion bodies derived from lysosomal phospholipid (Reasor, 1989). Although it has been reported in different lung cell types (Colgan et al., 1984; Heath et al., 1985; Marchlinski et al., 1982; Riva et al., 1987), AM-induced phospholipidosis is most commonly observed in alveolar macrophages (Costa-Jussa et al., 1984; Heath et al., 1985; Hostetler et al., 1986; Padmavathy et al., 1993; Reasor et al., 1988). However, phospholipidosis can also occur in non-pulmonary tissue where AM has

demonstrated toxicity (i.e. liver, skin, peripheral nerves and cornea) (D'Amico et al., 1981; Dake et al., 1985; Honegger et al., 1995; Poucell et al., 1984).

As a result of its potent inhibition of lysosomal phospholipases A_1, A_2, (Heath et al., 1985; Hostetler et al., 1986; Reasor et al., 1996b), and C (Kodavanti and Mehendale, 1991), AM causes phospholipidosis following various routes of administration (such as oral, ip, or it) and in a variety of animal models (hamster, rat, mouse, dog) (Mazue et al., 1984; Reasor et al., 1988; Riva et al., 1987; Wang et al., 1992; Wilson et al., 1991). Furthermore, the development of phospholipidosis is time and dose dependent (Reasor et al., 1988), is reversible upon cessation of AM administration (Antonini et al., 1994; Mazue et al., 1984; Reasor et al., 1988) and is decreased by vitamin E, taurine or niacin (Honegger et al., 1995; Wang et al., 1992). In addition, DEA is more potent than AM at inhibiting lysosomal phospholipases and ultimately, inducing phospholipidosis (Hostetler et al., 1988; Kannan et al., 1990a).

However, evidence suggests that phospholipidosis is not pivotal to the initiation of AIPT. Despite the fact that phospholipidosis is observed in virtually all patients receiving AM therapy, most patients do not develop AIPT. Similarly, hamsters administered AM or DEA orally, demonstrate increased lung phospholipid accumulation without displaying pulmonary fibrosis. In contrast, hamsters receiving AM or DEA intratracheally, exhibit significant pulmonary fibrosis with only minimal phospholipid accumulation (Blake and Reasor, 1995c). Also, other cationic amphiphillic drugs (such as imipramine or chlorpromazine) cause phospholipidosis but do not cause pulmonary fibrosis (Lullmann et al.,

1978; Wilson, 1982). For these reasons, phospholipidosis is considered a common adverse effect of AM therapy, but an unlikely initiator of AIPT.

1.7.3.2 Disruption of Ca^{2+} Homeostasis

Sustained elevations of intracellular Ca^{2+} can lead to the overstimulation of Ca^{2+}-dependent degradative enzymes such as proteases and endonucleases and ultimately the breakdown of cell constituents and cell death (Nicotera et al., 1992). AM and DEA have been demonstrated to increase intracellular Ca^{2+} levels in a variety of cell types (Hageluken et al., 1995; Kodavanti et al., 1992; Powis et al., 1990; Roussel et al., 1995). For example, in cultured human pulmonary artery endothelial cells, AM caused an influx of extracellular Ca^{2+} across the plasma membrane (Powis et al., 1990), and the influx coincided with the onset of cytotoxicity. In cultured bovine aortic endothelial cells, DEA increased cytoplasmic Ca^{2+} due to increased Ca^{2+} release from the endoplasmic reticulum and mitochondria and prevented cell proliferation (Himmel et al., 2000).

The mechanism of AM- or DEA-induced increases in intracellular Ca^{2+} remains to be elucidated, but evidence in the literature suggests that the following processes may be involved: 1) AM destabilizes plasma membranes (Antunes-Madeira et al., 1995; Chatelain et al., 1985; Dzimiri and Almotrefi, 1993; Kikuta et al., 1989; Osowole et al., 1992), altering membrane fluidity (Antunes-Madeira et al., 1995; Honegger et al., 1993) and causing an increased permeability to extracellular Ca^{2+} (Powis et al., 1990); 2) AM and DEA interact with calmodulin to modify its Ca^{2+} binding properties (Vig et al., 1991); 3) AM

activates G_i proteins (inhibitory guanyl-nucleotide-binding protein) resulting in an increase in cytosolic Ca^{2+} (Hageluken et al., 1995).; 4) protective effects of ascorbic acid suggest that DEA causes free radical generation which in turn, affects ion channel transport mechanisms and increases intracellular Ca^{2+} (Himmel et al., 2000). However, increases in intracellular Ca^{2+} may be a consequence of cellular injury rather than an initiating mechanism. Nevertheless, effects of AM and DEA on the regulation of cellular ions including Ca^{2+} has not been adequately addressed in the lung and therefore may be deserving of further investigation.

1.7.3.3 Free Radical Formation

1.7.3.3.1 Free Radicals and Reactive Oxygen Species

A free radical is defined as any chemical species that contains one or more unpaired electron and is capable of independent existence (Halliwell and Gutteridge, 1999a). It is formed upon exposure to ionizing radiation, as by products of normal xenobiotic metabolism, during oxidative burst in activated phagocytes, upon leakage from electron transport, or during metabolism of fatty acids (Aust et al., 1993; Freeman and Crapo, 1982). A free radical can donate their unpaired electron to oxygen to form oxygen radicals such as superoxide ($O_2^{\bullet}$), hydroxyl ($^{\bullet}OH$), or peroxyl ($ROO\bullet$) radicals and, along with non-radicals that contain chemically reactive O_2, are commonly referred to as reactive oxygen species (ROS) (Halliwell and Gutteridge, 1999b). Free radicals and ROS can attack lipids, proteins, nucleic acids and carbohydrates, causing metabolic

disturbances and ultimately cell death (Davies, 1995). When polyunsaturated fatty acids (PUFAs) or membrane cholesterol are sites for free radical attack, lipid peroxidation (LPO) results (Figure 1.3).

1.7.3.3.2 Lipid Peroxidation

LPO is initiated when a free radical attacks a methylene carbon within the side chain of a PUFA, resulting in the abstraction of a hydrogen atom and leaving behind a carbon-centred lipid radical (Figure 1.3). The carbon-centred lipid radical undergoes bond rearrangement to form a conjugated diene, which reacts with O_2 to form a peroxyl radical (ROO•). Peroxyl radicals can react with each other, attack membrane proteins, or abstract hydrogen from an adjacent fatty acid side chain to produce another ROO• which can propagate the chain reaction of LPO (Halliwell and Chirico, 1993; Halliwell and Gutteridge, 1999c; Moore and Roberts II, 1998; Rimbach et al., 1999). The end result is cell membrane destabilization and ultimately cell death.

LPO is determined by measuring the loss of PUFAs, or by measuring the amount of lipid peroxides or secondary peroxidation products such as carbonyls or hydrocarbon gases (Halliwell and Chirico, 1993). Therefore, there are various methods used to measure LPO, each having distinct advantages and disadvantages. One measurable class of LPO products is the isoprostanes. Membrane-bound arachidonic acid is normally metabolized by cyclooxygenase to form prostaglandins (PG). However, when attacked *in situ* by free radicals in

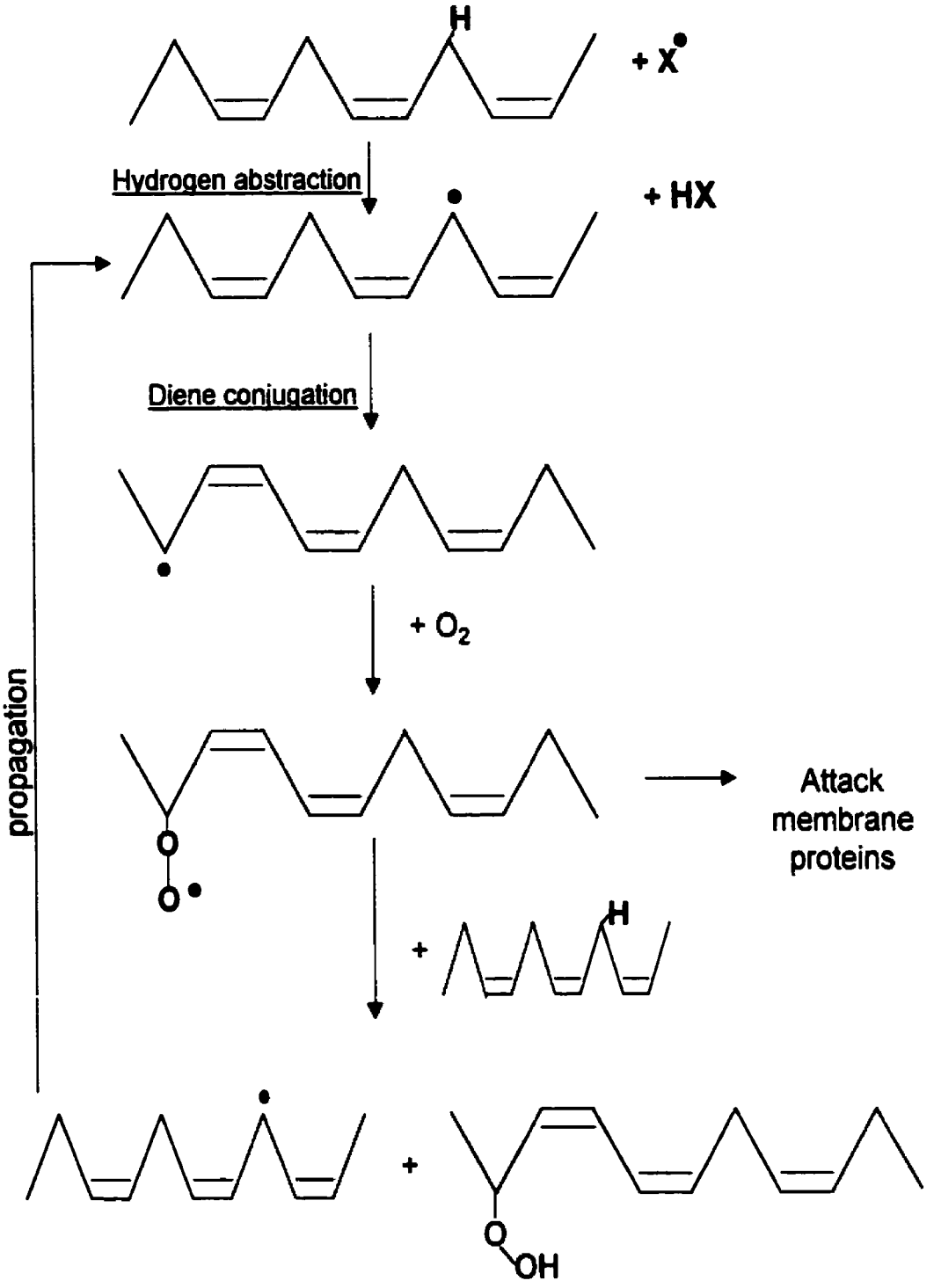


Figure 1.3 Lipid Peroxidation. (Modified from Halliwell and Gutteridge, 1999)

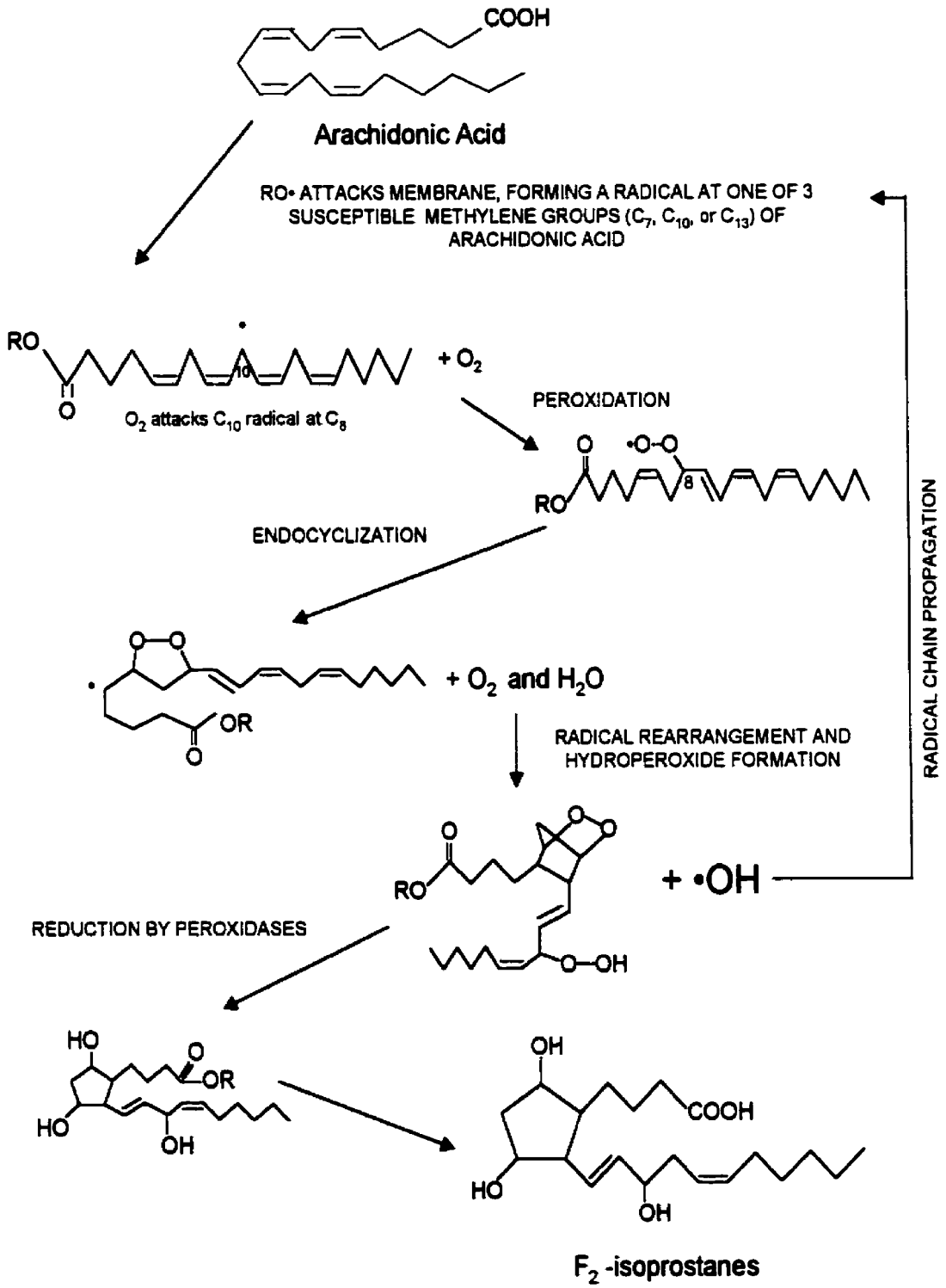


Figure 1.4. Formation of Isoprostanes

phospholipids, stereoisomers of $PGF_{2\alpha}$ called isoprostanes are formed (Figure 1.4). In contrast with cyclooxygenase-derived $PGF_{2\alpha}$, the side chains of isoprostanes are predominantly oriented *cis* in relation to the prostane ring (Morrow and Roberts, 1997). Isoprostane levels increase under conditions of oxidative stress (e.g. in plasma and urine of cigarette smokers), in BALF of rats exposed to elevated O_2, and in animals treated with CCl_4 (Johnston and Kroening, 1998; Pratico, 1999).

1.7.3.3.3 Vitamin E

Vitamin E (α-tocopherol) is a lipid soluble radical scavenging agent and antioxidant. An antioxidant is defined as any substance that significantly delays or prevents the oxidation of an oxidizable substrate (Halliwell and Gutteridge, 1999d). Because vitamin E scavenges lipid peroxyl radicals much faster than these radicals can react with adjacent PUFA side chains or with membrane proteins (Halliwell and Gutteridge, 1999d), it prevents the propagation of free radical reactions (such as LPO) (Brigelius-Flohe and Traber, 1999; Burlakova et al., 1998). However, in some situations, vitamin E can reduce Fe^{3+} to Fe^{2+}, and Fe^{2+} initiates lipid peroxidation via Fenton chemistry (Halliwell and Gutteridge, 1999d; Witting et al., 1998). Thus vitamin E can also act as a pro-oxidant (Figure 1.5).

Vitamin E also possesses functions other than as a radical scavenger. Vitamin E can stabilize membranes (Urano et al., 1988), decrease

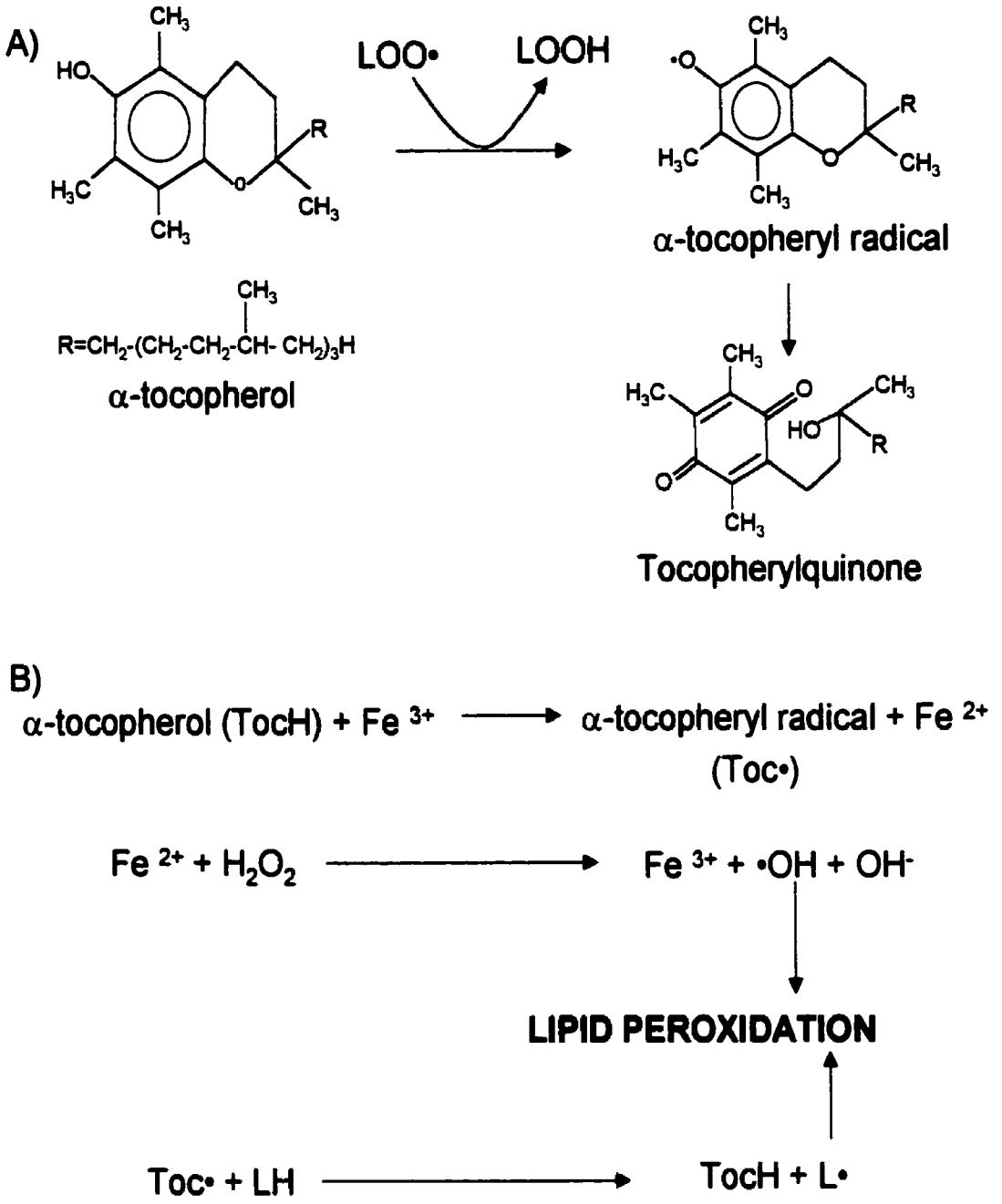


Figure 1.5. Vitamin E (α-tocopherol) acts as:
A) an antioxidant and B) a prooxidant

collagen gene expression (Card et al., 2001), up-regulate cytosolic phospholipase A_2 activity, and decrease protein kinase C activity (Brigelius-Flohe and Traber, 1999). Therefore, when assessing protective effects of vitamin E, one must consider that its mechanism of action may not always be by scavenging radicals.

1.7.3.3.4 Free Radical Involvement in AIPT

Free radical formation may initiate AIPT. Isolated rabbit lungs exposed to AM demonstrated elevated levels of oxidized glutathione and luminol-enhanced chemiluminescence, effects consistent with superoxide formation (Kennedy et al., 1988). Also, the hydroxyl radical, a suggested product of superoxide anion and hydrogen peroxide, was detected in an aqueous solution of AM by electron spin resonance (ESR) spectroscopy (Taylor et al., 2001). Furthermore, rabbits treated with antioxidant agents prior to AM exposure had decreased AM-induced pulmonary edema relative to AM controls (Kennedy et al., 1988). Although the relationship between edema and pulmonary fibrosis is unclear, these results suggested a potential involvement of ROS in AM toxicity in the lung.

However, other experiments have suggested that ROS are not involved in AIPT. Although a single intratracheal dose of AM to hamsters increased both pulmonary oxidized glutathione (GSSG) and other antioxidant enzyme activities (e.g. superoxide dismutase, glutathione peroxidase) (Leeder et al., 1994; Wang et al., 1992), antioxidant agents, including butylated hydroxyanisole, diallyl sulfide, and *N*-acetylcysteine, did not prevent AM-induced pulmonary fibrosis

(Leeder et al., 1994). *In vitro* evidence also suggested that ROS are not involved in AIPT. Alveolar macrophages and thyroid cell lines (TAD-2 cells) incubated with AM did not produce ROS, as measured by 2',7',-dichlorofluorescin or luminol dependent chemiluminescence (Leeder et al., 1996; Matola et al., 2000; Reasor et al., 1996b). Furthermore, antioxidants such as superoxide dismutase, catalase, butylated hydroxytoluene and *N*-acetylcysteine did not alter AM-induced cytotoxicity in cultured rat hepatocytes or pulmonary artery endothelial cells (Kachel et al., 1990; Ruch et al., 1991). Therefore, the majority of evidence suggests that ROS are not involved in initiating AIPT.

Alternatively, an AM radical may be responsible for initiating AIPT. While investigating AM-induced skin photosensitivity, Li and Chignell (1987) demonstrated that ultraviolet (UV) irradiation of AM produces a carbon-centered radical. Furthermore, this radical was capable of abstracting hydrogen from linoleic acid, thereby initiating LPO. Similarly, Vereckei *et al.,* (1993) proposed that, upon stationary ^{60}Co-γ radiolysis and pulse radiolysis, AM is deiodinated producing a highly reactive aryl radical. Moreover, ESR experiments demonstrated that an AM-derived carbon-centred radical is produced in an aqueous solution of AM (Taylor et al., 2001) and in hamster liver and lung microsomes (Rafeiro, 1997). This provides evidence that an AM-based radical can be generated in biological tissues and therefore, the generation of an AM radical *in vivo* may initiate AIPT.

1.7.3.3.5 AM-Induced Lipid Peroxidation

Results from experiments investigating the involvement of LPO in AM- and DEA-induced toxicity are equivocal. Following a single intratracheal dose of AM, malondialdehyde equivalents (an index of LPO) were increased in hamster whole lung homogenate (Wang et al., 1992) and in hamster bronchoalveolar lavage (Blake and Reasor, 1995b). Furthermore, NADPH and Fe^{3+} induced LPO in rat liver microsomes was greater in AM-treated rats compared to controls (Vereckei et al., 1993). Also, UV-irradiated phospholipid peroxidation in liposomes is increased by AM (Sautereau et al., 1992).

In contrast, AM decreased LPO in rat liver microsomes (Rekka et al., 1990), rat hepatocytes (Ruch et al., 1991) and rat liver mitochondria (Ribeiro et al., 1997), and had no effect on LPO in liposomes of bovine heart mitochondrial lipids (Mansani et al., 1999), hamster liver and lung mitochondria (Card et al., 1998), and in the serum of rats administered AM (Pre et al., 1991). However, the involvement of LPO as an initiating mechanism of AM-induced pulmonary cytotoxicity has not been investigated and warrants further investigation.

1.7.3.3.6 Vitamin E and AM-Induced Toxicity

Vitamin E decreases AM- and DEA-induced toxicity both *in vivo* (Card et al., 1999a) and *in vitro* (Futamura, 1996b; Honegger et al., 1995; Kachel et al., 1990; Ruch et al., 1991; Scuntaro et al., 1996), while a variety of other antioxidants do not (Honegger et al., 1995; Kachel et al., 1990; Ruch et al., 1991). The mechanism of vitamin E-induced protection against AM-induced

cytotoxicity is not known. As a lipid soluble antioxidant, vitamin E can scavenge phospholipid-associated radicals unreachable by water soluble antioxidants such as ascorbic acid, ethanol, butylated hydroxyanisole, diallyl sulfide, N-acetylcysteine, thereby decreasing lipid peroxidation. Alternatively, vitamin E has been demonstrated to decrease phospholipidosis by up-regulating cytosolic phospholipase A_2 activity (Honegger et al., 1995; Kannan et al., 1990b; Scuntaro et al., 1996). Protective effects of vitamin E against AM-induced cytotoxicity could potentially also be attributed to the ability of vitamin E to decrease: 1) AM accumulation (Scuntaro et al., 1996); 2) AM-induced increases in membrane fluidity (Honegger et al., 1995); and 3) AM-induced increases in collagen mRNA expression (Card et al., 2001). Therefore, more experiments are necessary to fully elucidate the mechanism by which vitamin E decreases AM-induced toxicity.

1.7.3.4 Disruption of Cellular Energy Homeostasis

Mitochondria are essential for the generation of cellular ATP. The synthesis of ATP occurs via coupling the transfer of electrons through the respiratory chain to ADP phosphorylation (Saraste, 1999) (Figure 1.6). Depending on the tissue type and metabolic state, cells generate up to 95% of their energy via oxidative phosphorylation in the mitochondria (Wallace et al., 1997). However, mitochondria are targets for chemically-induced toxicity whereby interference of mitochondrial respiration results in depolarization of mitochondrial membrane potential, inhibition of ATP synthesis and ultimately cell death. Mitochondrial mediated toxicity can occur in numerous ways, including:

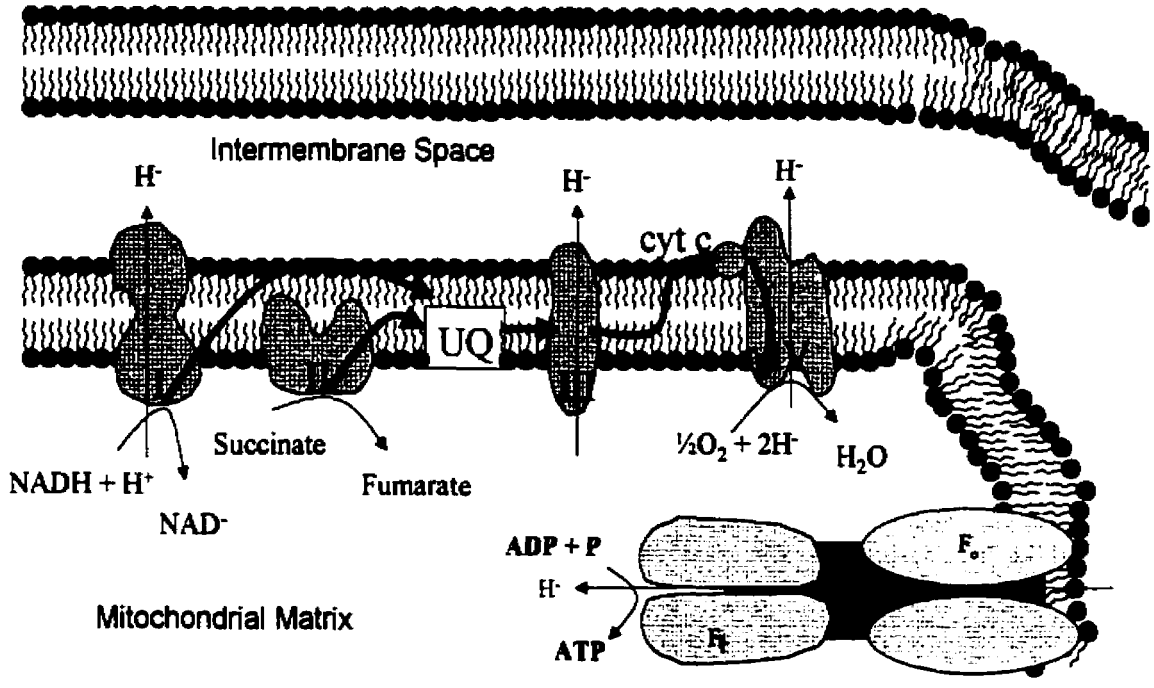


Figure 1.6 The electron transport chain. Electron transfer through the respiratory chain leads to the pumping of protons from the matrix to the cytosolic side of the inner mitochondrial membrane. The pH gradient and membrane potential constitute a proton-motive force that is used to synthesize ATP. (UQ=ubiquinone, cyt c=cytochrome c (Modified from Lehninger et al, 1993)

1) uncoupling of oxidative phosphorylation (e.g. carbonyl cyanide (*p*-trifluoromethoxy)-phenylhydrazone (FCCP)); 2) leakage of electrons to produce free radicals (e.g. rotenone); or 3) activation of mitochondrial permeability transition (MPT) (e.g. 1-methyl-4-phenylpyridinium (MPP^+) (Turrens, 1997; Wallace et al., 1997). MPT, a phenomenon recognized to be important in both necrosis and apoptosis for some agents, is a sudden increase in permeability of the inner mitochondrial membrane, resulting in membrane depolarization, uncoupling of oxidative phosphorylation, mitochondrial swelling, and release of intramitochondrial ions (Lemasters et al., 1998). MPT is regulated by a voltage dependent channel that is inhibited by micromolar concentrations of cyclosporin A, so cyclosporin A protects against cell death involving MPT-activating substances or events (Fujii et al., 1994; Imberti et al., 1990; Imberti et al., 1992; Nazareth et al., 1991; Snyder et al., 1992).

AM has been shown to cause alterations to the mitochondria. AM accumulated in mouse liver mitochondria *in vitro* (Fromenty et al., 1990a) and *in vivo* (Pirovino et al., 1988), and in lung mitochondria of AM treated rats (Hostetler et al., 1988). Furthermore, AM causes structural changes to the mitochondria in a variety of cell types. Mitochondria from rat hepatocytes (Gross et al., 1989), human lymphocytes (Yasuda et al., 1996) and rat heart (Guerreiro et al., 1986a) were either swollen, had disorganized cristae, demonstrated rupturing of the inner mitochondrial membrane, or appeared electron-lucent in electron micrographs when compared to non-treated controls. Moreover, AM caused the

release of cytochrome c from the mitochondria of cultured thyroid cells (Matola et al., 2000).

AM also causes functional changes to the mitochondria *in vitro*. AM decreases complex I- and complex II-supported oxygen consumption in mouse liver (Fromenty et al., 1990a) and hamster liver and lung mitochondria (Card et al., 1998). This suggests that, in mitochondria, AM inhibits the transfer of electrons from complex I of the electron transport chain to complex II, thereby uncoupling electron transport to ADP phosphorylation (Card et al., 1998; Fromenty et al., 1990a). AM also inhibited the transfer of electrons at complex I of rat liver mitochondria (Ribeiro et al., 1997). Other effects of AM detrimental to mitochondria have been reported. AM decreases mitochondrial ATP formation, β-oxidation and membrane potential in isolated mouse liver mitochondria (Fromenty et al., 1990b; Fromenty et al., 1990a), and decreases membrane potential in hamster liver and lung mitochondria (Card et al., 1998). Furthermore, AM inhibited ATP synthase in guinea pig heart mitochondria (Dzimiri and Almotrefi, 1993).

Niacin partially attenuates AIPT in hamsters (Wang et al., 1992). O'Neil and Giri (1994) later hypothesized that niacin, a precursor for NAD, stimulates ATP synthesis via glycolysis, thereby protecting against AM-induced toxicity. Similarly, glucose, a substrate that feeds into glycolysis, decreases AM-induced reductions in ATP content and cytotoxicity in human lymphocytes (Fromenty et al., 1993).

Although a clear-cut causal relationship between AM-induced mitochondrial disruption and cell death has not been established, evidence supports the hypothesis that AM-induced perturbations of cellular energy production may be responsible for cell death that initiates AIPT.

1.8 CELL TYPES IN THE LUNG

The lung has over 40 different types of cells. However, alveolar macrophages, alveolar type II cells and non-ciliated bronchiolar epithelial (Clara) cells are common targets for xenobiotic-induced toxicity. Furthermore, the relative susceptibilities of these cell types to AM-induced cytotoxicity have not been investigated. Identification of a particularly susceptible cell type for AM-induced cytotoxicity could lead to the elucidation of the etiology of AIPT.

1.8.1 Alveolar Type II Cells

Type II alveolar epithelial cells have complex structure, and uniquely contain lamellar bodies comprised of surfactant phospholipids surrounded by a membrane (Mason and Williams, 1991). Two major functions of alveolar type II cells are: 1) synthesis and secretion of surface-active material; and 2) maintenance of alveolar epithelium by their ability to proliferate and differentiate into alveolar type I cells. Compared to most other cell types in the lung, alveolar type II cells are enriched with cytochrome P-450 enzymes (Arinc, 1993; Domin et al., 1986; Lag et al., 1996; Martin et al., 1993; Willey et al., 1996) and therefore

are important sites for biotransformation of xenobiotics. They are also common targets for bioactivated toxicants (Plopper and Hyde, 1992).

AM causes alveolar type II cell proliferation in hamsters (Miaskiewicz et al., 1987), and humans (Riva et al., 1987). Furthermore, alveolar type II cells incubated with AM demonstrate phospholipid lamellar inclusion bodies independent of surfactant stores (Costa-Jussa et al., 1984). Therefore, experiments investigating possible mechanisms of AM-induced pulmonary cytotoxicity *in vitro* should involve alveolar type II cells.

1.8.2 Non-Ciliated Bronchiolar Epithelial (Clara) Cells

In humans, Clara cells are scarce, comprising less than 1 % of human lung cells. They contain small amounts of endoplasmic reticulum and mitochondria (Plopper et al., 1991). In contrast, Clara cells from the majority of mammalian species (including hamsters) are much more frequent, and contain an abundance of smooth endoplasmic reticulum and mitochondria (Plopper et al., 1991). Generally speaking, rodent Clara cells have approximately twice as much CYP450 content as alveolar type II cells and at least 5 times that of macrophages (Forman and Thomas, 1992). Therefore, one of the primary functions of Clara cells is xenobiotic biotransformation (Plopper et al., 1991).

AM-induced effects in Clara cells have not been investigated. However, because rodent Clara cells are common targets for toxicity mediated by other pulmonary toxicants (Plopper et al., 1991), AM-induced toxicity *in vitro* should be determined in Clara cells.

1.8.3 Alveolar Macrophages

Alveolar macrophages are the most abundant nonparenchymal cells in the lung. Rodent and uninduced human alveolar macrophages have relatively low CYP450 enzyme activity (Arinc, 1993; Domin et al., 1986; Lag et al., 1996; Martin et al., 1993; Willey et al., 1996) and when activated (either by nonspecific phagocytosis of particles or by specific ligands such as immunoglobin-coated bacteria), release oxidants, enzymes and cytokines (Crystal, 1991). Alveolar macrophages play a pivotal role in maintaining normal lung function and structure by: 1) scavenging particles, 2) killing microorganisms, 3) recruiting and activating other inflammatory cells, 4) maintaining and repairing lung parenchyma, and 5) surveilling against neoplasia (Crystal, 1991). However, loss of control of macrophage activation and secretions (such as hydroxyl radicals, TNF-α or TGF-β) can injure normal lung.

AM is directly cytotoxic to alveolar macrophages (Bolt et al., 1998; Leeder et al., 1996) and in some cases, alters their release of inflammatory mediators including: IL-1 and TNF-α (Futamura, 1995; Futamura, 1996a; Futamura, 1997; Reasor et al., 1996a). Furthermore, alveolar macrophages develop phospholipidosis upon expose to AM (Costa-Jussa et al., 1984; Heath et al., 1985; Hostetler et al., 1986; Padmavathy et al., 1993; Reasor et al., 1988; Reasor et al., 1996a). Therefore, alveolar macrophages are targets for AM-induced toxicity *in vitro*.

1.9 RATIONALE, HYPOTHESES AND OBJECTIVES

AM is an efficacious and commonly used antidysrhythmic agent that can cause potentially fatal pulmonary toxicity. Elucidation of the mechanism(s) of cell death which initiates AIPT would be beneficial because it may facilitate: 1) the development of new antidysrhythmic drugs that do not cause pulmonary fibrosis; 2) the development of a clinical therapy to counteract AM's adverse effects; and 3) a better understanding of the pathogenesis of chemically-induced pulmonary fibrosis.

The overall objective of this research was to investigate disruption of mitochondrial function and free radical generation as mechanisms of AM-induced cell death. Hence, specific hypotheses and objectives were designed.

Identification and Comparison of Susceptibilities of Different Potential Target Lung Cell Types to AM-Induced Cytotoxicity

In animal models of AIPT, lung cell death occurs early relative to the onset of pulmonary fibrosis (Taylor et al., 2001). Furthermore, AM-induced cytotoxicity has been demonstrated in different cell types from different species (Leeder et al., 1996; Reasor et al., 1990; Wilson and Lippmann, 1990; Wilson and Lippmann, 1996b). However, the relative susceptibilities of different lung cell types to AM-induced cytotoxicity has not been investigated. Identification of a particularly susceptible cell type to AM-induced cytotoxicity may provide information about the mechanism of cell death that initiates AIPT.

Hypothesis 1

Different lung cell types have different susceptibilities to AM-induced cytotoxicity.

Specific Objective 1

To compare *in vitro* the susceptibility of hamster alveolar macrophages, alveolar type II cells and Clara cells to AM-induced cytotoxicity.

Disruption of Cellular Energy Homeostasis as an Initiating Mechanism of AM and DEA-Induced Cytotoxicity

AM has been shown to cause both structural (Gross et al., 1989; Guerreiro et al., 1986a; Yasuda et al., 1996) and functional disruptions (Card et al., 1998; Fromenty et al., 1990a) in isolated mitochondria. Furthermore, AM-induced toxicity was decreased *in vivo* by niacin (Wang et al., 1992) and *in vitro* by glucose (Fromenty et al., 1993). To invoke a role for mitochondrial disruption as causal in AM and DEA cytotoxicity, it is necessary to demonstrate that mitochondrial disruption occurs early during AM or DEA exposure, and prior to cell death.

Hypothesis 2A

AM or DEA disrupts mitochondrial function and decreases ATP levels prior to cell death in suseptible lung cell types.

Specific Objective 2A-1

To investigate the *in vitro* effects of AM and DEA on mitochondrial membrane potential and cellular ATP levels prior to cell death in intact hamster alveolar macrophages, alveolar type II cells and Clara cells.

Specific Objective 2A-2

To compare the relative susceptibilities of hamster alveolar macrophages, alveolar type II cells and Clara cells to DEA-induced cytotoxicity *in vitro*.

Specific Objective 2A-3

To investigate effects of DEA on mitochondrial membrane potential and cellular ATP levels prior to cell death in intact hamster alveolar macrophages, alveolar type II cells and Clara cells.

Hypothesis 2B

Agents that prevent AM-induced disruption of mitochondrial function or depletion of ATP levels, decrease AM-induced cytotoxicity.

Specific Objective 2B

To determine, *in vitro*, effects of cyclosporin A, glucose or niacin on AM-induced cytotoxicity in hamsters.

Investigation of the Role of Free Radical Mediated Toxicity as a Mechanism of AM and DEA-Induced Cytotoxicity

An aryl radical of AM has been reported in both biological systems and in non-biological solutions (Li and Chignell, 1987; Rafeiro, 1997; Taylor et al., 2001; Vereckei et al., 1993). Furthermore, free radicals can initiate lipid peroxidation and cause cell death (Halliwell and Chirico, 1993; Halliwell and Gutteridge, 1999c; Moore and Roberts II, 1998; Rimbach et al., 1999). Vitamin E decreases

AM-induced toxicity both *in vivo* (Card et al., 1999a) and *in vitro*, at least in some cell types (Futamura, 1996b; Honegger et al., 1995; Kachel et al., 1990; Ruch et al., 1991; Scuntaro et al., 1996). This evidence suggests that free radical formation and subsequent lipid peroxidation may initiate AM and DEA-induced cell death.

Hypothesis 3A

Vitamin E decreases AM-and DEA-induced cytotoxicity and lipid peroxidation in freshly isolated hamster lung cells.

Specific Objective 3A-1

To investigate the effect of vitamin E on AM-induced and DEA-induced cytotoxicity in hamster alveolar macrophages, alveolar type II cells and Clara cells *in vitro*.

Specific Objective 3A-2

To investigate effects of AM, DEA, and vitamin E on lipid peroxidation in hamster alveolar macrophages, alveolar type II cells and Clara cells prior to AM- and DEA-induced cell death.

AM-Induced Cytotoxicity in Freshly Isolated Human Lung Cells.

Rodent models demonstrate AM toxicity (Bolt et al., 1998; Card et al., 1998; Daniels et al., 1989; Ogle and Reasor, 1990; Padma and Setty, 1997; Rafeiro et al., 1990; Reinhart and Gairola, 1997; Taylor et al., 2000) similar to that produced

in humans. However, an *in vitro* system that assesses events occurring in freshly isolated human lung cells has not been described.

Hypothesis 4

AM-induced cytotoxicity occurs in freshly isolated human lung cells and is decreased by vitamin E.

Specific Objective 4

i) To investigate the effects of AM on viability of freshly isolated human alveolar macrophages and alveolar type II cells *in vitro*.

ii) To investigate whether vitamin E decreases AM-induced cytotoxicity in human lung cells *in vitro*.

CHAPTER 2

DIFFERENTIAL SUSCEPTIBILITIES OF ISOLATED HAMSTER LUNG CELL TYPES TO AMIODARONE TOXICITY

2.1 INTRODUCTION

Amiodarone (AM), an iodinated benzofuran derivative, is a potent and efficacious antidysrhythmic agent. However, numerous adverse effects are associated with its use (Mason, 1987; Wilson and Podrid, 1991). When AM is administered in relatively high doses for treating severe dysrhythmias, the most serious and clinically limiting side effect is the development of life-threatening amiodarone-induced pulmonary fibrosis (Martin and Rosenow, 1988; Pitcher, 1992). Furthermore, AM-induced pulmonary toxicity (AIPT) is both potentially lethal and the most frequent adverse effect during low dose administration of AM to post-myocardial infarction patients at risk for ventricular arrhythmias (Julian et al., 1997). Although the critical step for initiation of AM-induced pulmonary toxicity remains unknown, several mechanisms have been proposed, including: phospholipidosis, free radical formation, an immunologically-mediated response, perturbation of cellular energy production, and disruption of calcium ion homeostasis (reviewed by Massey *et al.*, (1995)).

The lung is highly heterogeneous in terms of cell types (Sorokin, 1970), and different cell types react differently to AM (Wilson and Lippmann, 1996b). However, the relative susceptibilities of lung cell types to AM-induced cytotoxicity have not been addressed. Identification of (a) particularly susceptible cell type(s) to AM could provide valuable information for elucidation of the mechanism of AM-induced pulmonary toxicity. Cells that are potential targets for AM include: 1) alveolar macrophages, which become hypertrophic and develop intracytoplasmic phospholipid-containing lamellar-inclusion bodies following AM administration (Martin and Standing, 1988; Riva et al., 1987); 2) alveolar type II cells, which

display hyperplasia (Riva et al., 1987) and also develop lamellar inclusion bodies independent of surfactant storage upon exposure to AM (Costa-Jussa et al., 1984); 3) non-ciliated bronchiolar epithelial (Clara) cells, which contain appreciable amounts of cytochrome P450 and other metabolizing enzymes and therefore are important sites of xenobiotic biotransformation (Plopper et al., 1991). All of these cell types are common targets for xenobiotic cytotoxicity (Massey, 1989).

The purpose of this study was to compare *in vitro* the susceptibilities of different lung cell types to AM cytotoxicity. The male Golden Syrian hamster was utilized as a source of cells because it has been used as an *in vivo* model for AM-induced pulmonary fibrosis, which closely resembles clinical AIPT (Cantor et al., 1984; Daniels et al., 1989; Rafeiro et al., 1994). AM-induced cytotoxicity was compared among unseparated cells, and preparations enriched with alveolar macrophages, alveolar type II cells, or Clara cells.

2.2 METHODS AND MATERIALS

2.2.1 Chemicals

Chemicals were obtained from the following suppliers: amiodarone HCl from Ceres Chemical Co. Inc, White Plains NY; DNAse type I, trypsin inhibitor, heparin sulphate, protease type I, antibiotic-antimycotic (containing penicillin, streptomycin, and amphotericin B), ethylene glycol-bis-(aminoethyl ether)-N,N,N´,N´-tetraacetic acid (EGTA) and RPMI 1640 media from Sigma Chemical Co., St. Louis, MO.; heat-inactivated newborn calf serum from Gibco Laboratories, Grand Island, NY; Percoll® from Pharmacia Biotech Inc., Baie d'Urfe, PQ, Canada. 4-(2-hydroxyethyl)-1-piperazine-N´-2-ethanesulfonic acid (HEPES) from Boehringer Mannheim, Laval, PQ. All other reagents were of analytical grade and were obtained from standard commercial sources.

2.2.2 Animals

Male golden Syrian hamsters (140-160 g) were obtained from Charles River Canada Inc., St. Constant, PQ. Animals were housed at room temperature on a 12-hr light / 12-hr dark schedule (with lights on at 7 am) for a minimum of one week prior to experimentation. Hamsters were fed Purina rodent chow and water *ad libitum*, and were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care

2.2.3 Determination of Susceptible Cell Types

The procedure followed for the isolation of hamster lung cells was adapted from a previously published method (Myles et al., 1989) as described below. Twelve hamsters were used for each cell isolation.

2.2.3.1 Isolation of Alveolar Macrophages

Each hamster was injected with heparin sodium (2000 units in 0.5 ml saline) ip 45 min prior to deep anaesthetization with sodium pentobarbital (approximately 300 mg/kg ip). The lungs and trachea were exposed, and a small incision was made in the trachea to insert a polyethylene cannula (O.D. 1.57 mm) with a flared end, which was secured with a suture. A 5 ml syringe containing approximately 4 ml of air was attached to the cannula and used to inflate and deflate the lungs, to facilitate lung perfusion. Lungs were perfused via the pulmonary artery with 5 ml of 25 mM HEPES buffered salt solution (HpBS 37°C, pH 7.4 (Devereux and Fouts, 1981)), while simultaneously inflating / deflating the lungs with the tracheal cannula. The lungs were removed and lavaged 5 times with 5 ml of 37°C HpBS and 3 times with 5 ml of ice-cold HpBS. The bronchoalveolar lavage fluid (BALF) was centrifuged at 500 x *g* for 10 min at

4°C. The supernatant was discarded, and the macrophage-containing cell pellet was resuspended in 1 ml HpBS containing DNAse (20 mg / ml) and stored on ice in 50 ml of RPMI 1640 media containing antibiotic-antimycotic (1.0 ml / L media) during the isolation of other cell types.

2.2.3.2 Isolation and Enrichment of Alveolar Type II and Clara Cells

Alveolar type II cells and Clara cells were released by filling the lavaged lungs via the trachea with a 0.15% protease type 1 solution containing EGTA (0.14 mg / ml), followed by incubation in a shaking water bath for 10 min at 37°C. After incubation, the digested lungs were minced using a McIlwain tissue chopper, followed by chopping with scissors. The minced tissue was then degassed, filtered through gauze, and 103 and 42 μ nylon mesh, centrifuged at 500 x *g* for 10 min and resuspended in 10 ml of elutriation buffer. Initial enrichment from the suspension of isolated unseparated cells (referred to as the cell digest) was done in a Beckman JE-6B centrifugal elutriation system with a standard separation chamber, according to the settings listed in Table 2.1. Further enrichment of alveolar type II cells was accomplished by layering cells from the third elutriation fraction (E3) onto 28% (v/v) Percoll® in RPMI 1640 and centrifuging at 800 x *g* for 20 min with the centrifuge brake off. Cell viabilities and numbers were assessed on a haemacytometer by light microscopy using 0.5% trypan blue dye exclusion. Alveolar type II cells and Clara cells were identified using modified Papanicolaou (Kikkawa and Yoneda, 1974) (Figure 2.1) and nitroblue tetrazolium (Devereux and Fouts, 1980) (Figure 2.2) staining, respectively.

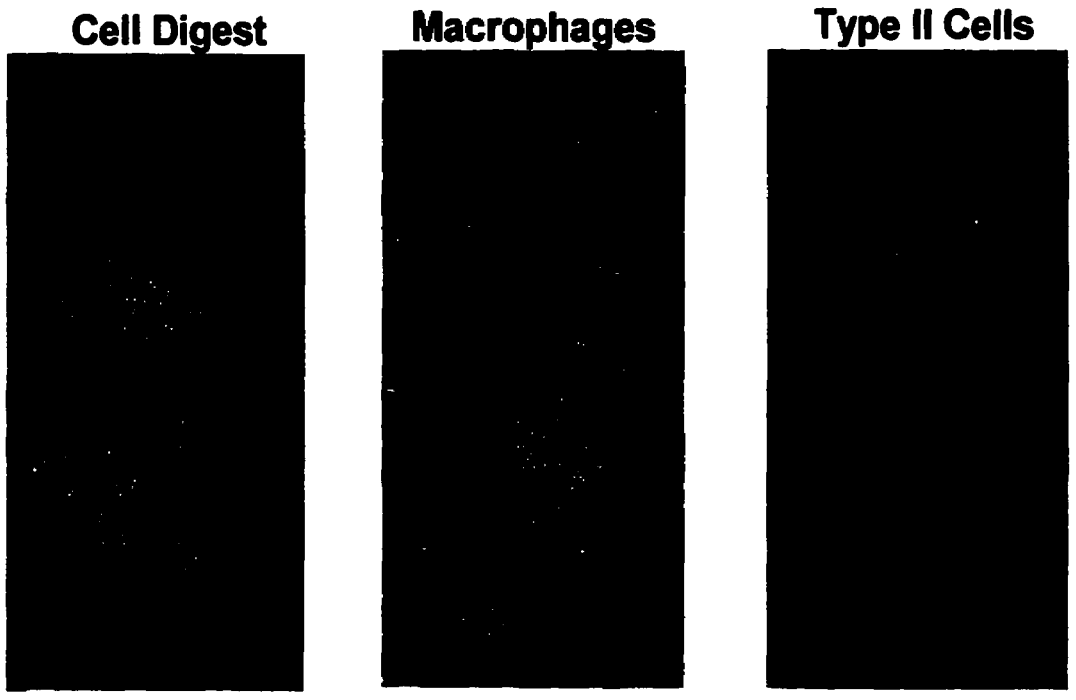


Figure 2.1. Cell digest, and fractions enriched with alveolar macrophages and alveolar type II cells stained with the Papanicalaou stain (200 x magnification). Arrows indicate alveolar type II cells.

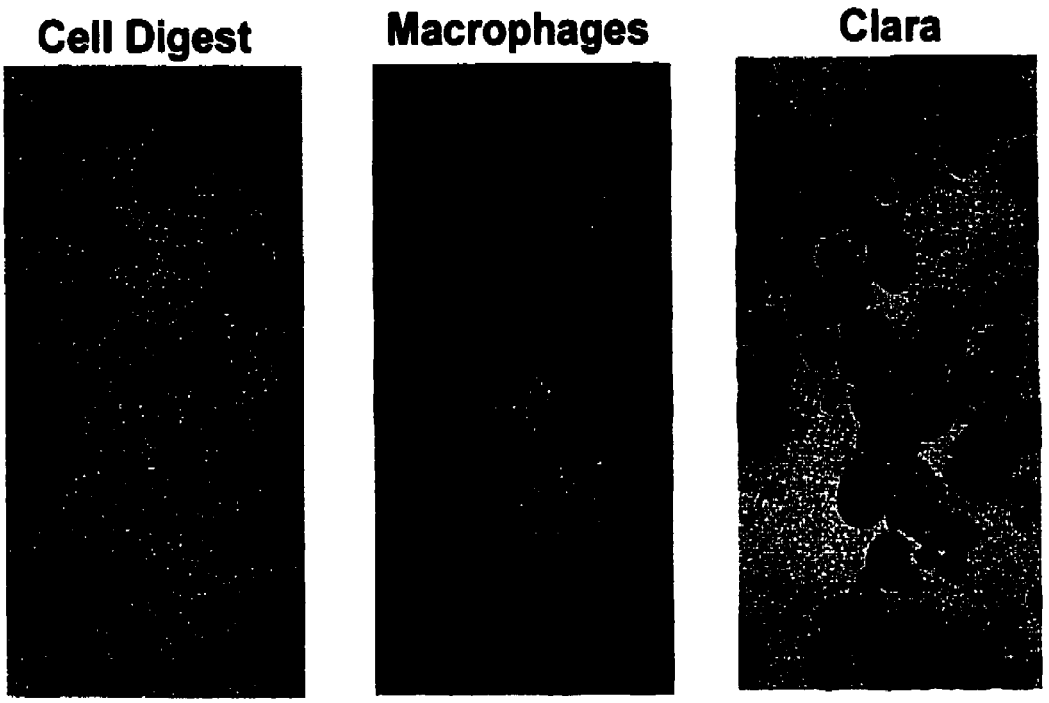


Figure 2.2. Cell digest and fractions enriched with alveolar macrophages and Clara cells stained with nitroblue tetrazolium (200 x magnification). Arrows indicate Clara cells.

2.2.3.3 Incubation of Isolated Cells

Following enrichment, cells were resuspended to 8 x 10^6 cells / ml in RPMI 1640 medium supplemented with 10% heat-inactivated newborn calf serum and antibiotic-antimycotic (1.0 ml / L). Cells were incubated (6.4 x 10^5 cells / well) in flat-bottomed, irradiated, non-adhesive, polystyrene Nunc 96 micro well plates (Gibco Canadian Life Technologies, Burlington, ON) with vehicle (0.1% ethanol) or AM (50, 100, or 200 μM) under 95% air: 5% CO_2 at 37°C. These concentrations of AM were selected because they were representative of post mortem concentrations of AM in the lungs of patients who had been treated with AM. Cell viability was measured by 0.5% trypan blue dye exclusion at 0, 12, 24 and 36 hours.

2.2.4 Quantification of AM in Lung Cells

The amount of AM within cells exposed to 50 or 100 μM AM was measured at 24 hr, prior to extensive cytotoxicity. The cell suspension was centrifuged at 1000 x *g* for 3 min, washed with an equal volume of supplemented RPMI 1640 medium, and centrifuged (3 min at 1000 *x g*) again. A cotton swab was used to remove any excess liquid from inside of the tube. The resulting pellet was stored at -20 °C until HPLC analysis.

The amount of AM within the cell pellet was measured as described by Rafeiro *et al.* (1990), with modifications. Briefly, the cell pellet was thawed, and 100 μl of mobile phase (acetonitrile:5% aqueous acetic acid, 8:2 (v/v) adjusted to pH 5.9 with ammonium hydroxide) were added. The pellets were mixed for 1 min and centrifuged at 16000 x *g* for 3 min. Supernatants were analysed by reverse-phase HPLC with UV-visible spectrophotometric detection at 254 nm (Brien et al., 1983; Brien et al., 1987), and the amount of AM was determined by

interpolation of the peak area of the chromatographic signal on an aqueous AM standard curve that was prepared for each day of analysis.

2.2.5 Expression of Cell Viability and Statistical Analysis

During incubation, viability of different cell types decreased to varying degrees in the absence of AM (Figure 2.3). Therefore, to compare the cytotoxicity of AM in different cell fractions, a viability index ((viability of AM treated cells ÷ viability of control cells) x 100%) was calculated. A two-way repeated-measures analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test was used to compare viability index data. A repeated-measures one-way ANOVA followed by Newman-Keuls post-hoc test was used to determine differences in the amount of AM among the different cell types. The cytotoxicity data were presented as mean viability index ± standard deviation. In all cases, $p < 0.05$ was considered statistically significant.

2.3 RESULTS

2.3.1 Enrichment of Cell Fractions

The bronchoalveolar lavage fluid was highly enriched (98%) in alveolar macrophages. The cell digest contained approximately 21% alveolar type II cells and 4% Clara cells (Figures 2.1 and 2.2). Following centrifugal elutriation, E3 contained 55% alveolar type II cells. Percoll density gradient centrifugation of E3 cells further enriched the alveolar type II cells to 75-85% (Figure 2.1). E4 contained 35-50% Clara cells (Figure 2.2). Other major cell types present in each of the elutriation fractions are listed in Table 2.1. Initial viabilities in all of the fractions were greater than 90%, and the viability of all control groups did not decrease below 75% over the course of the experiments (Figure 2.3).

TABLE 2.1

Principle Cell Types Found in Centrifugal Elutriation Fractions

Fraction	Volume Collected	Rotor Speed	Flow Rate	Principal Cell Types
E1	150 ml	2200 RPM	8.75 ml/min	**red blood cells** cell debris
E2	100 ml	2200 RPM	12.5 ml/min	**lymphocytes** alveolar type II cells
E3	100 ml	2200 RPM	24.5 ml/min	**alveolar type II cells (50-65%)** lymphocytes Clara cells
E4	100 ml	1200 RPM	21.5 ml/min	**Clara cells (35-50 %)** ciliated cells clumped red blood cells

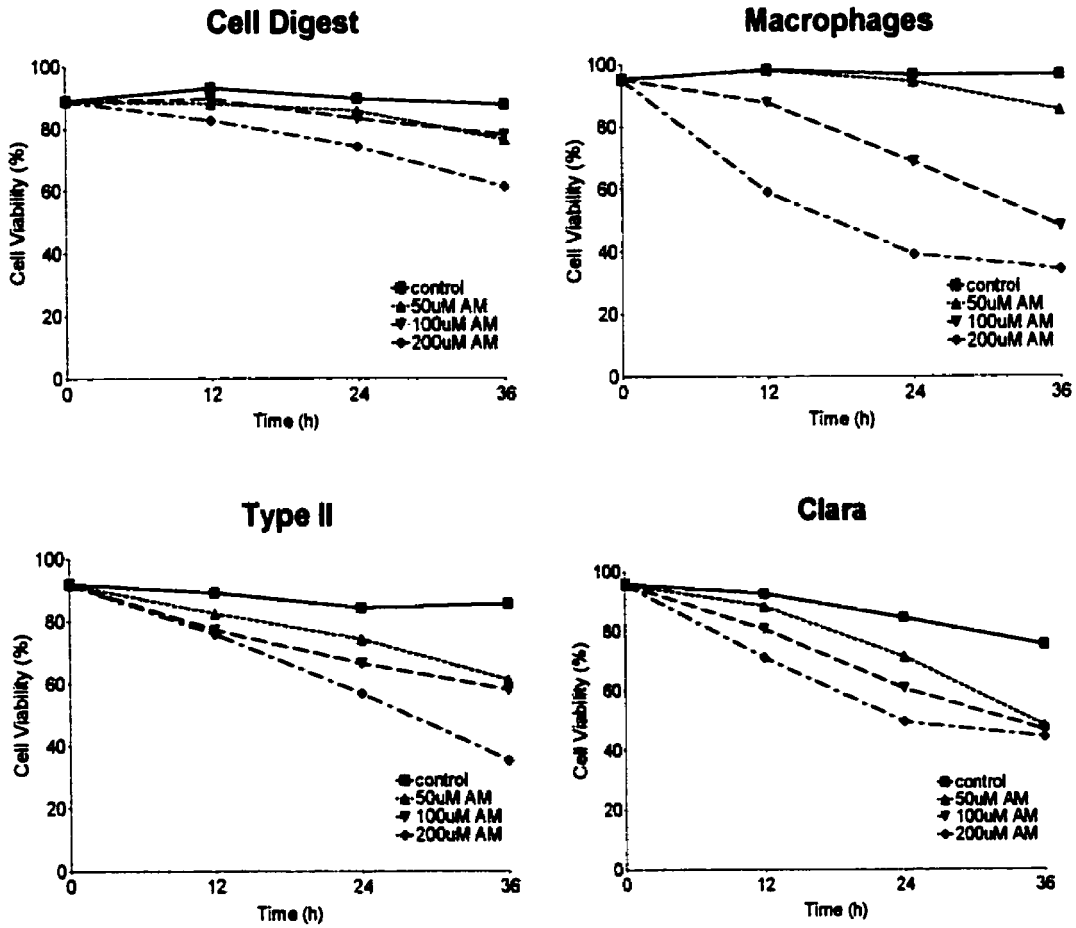


Figure 2.3. Mean viability (n=4) of cell digest, alveolar macrophage, alveolar type II cells, and Clara cells incubated in the presence of AM. n= number of experiments, where for each, cells were isolated from 12 hamsters.

2.3.2 Amiodarone Cytotoxicity

The viability index of the alveolar type II cell and Clara cell fractions decreased at 36 hr relative to t = 0 hr, when incubated with 50 μM AM (Figure 2.4A). In contrast, there was no significant time-dependent decrease in viability for alveolar macrophages or cell digest. The viability index for the Clara cell fraction at 36 hr (63.5 ± 10.7%), was significantly lower than that of the cell digest (86.5 ± 11.6%), macrophage (88.5 ± 17.4%) and alveolar type II cell (79.8 ± 19.2%) fractions at the same time point.

Viability in all fractions decreased during incubation with 100 μM AM (Figure 2.4B). Viability indices were significantly lower after 24 hr for alveolar macrophage (70.7 ± 7.0%), alveolar type II cell (78.9 ± 6.5%) and Clara cell (75.5 ± 9.5%) fractions when compared with their respective indices at 0 hr (98.3 ± 1.4% to 104.3 ± 5.7%). At 36 hr, the viability index was significantly lower in the macrophage fraction (50.0 ± 7.0%) than in all other fractions exposed to 100 μM AM. Viability index values at 36 hr of the alveolar type II and Clara cell fractions were intermediate between those of the cell digest and macrophages.

Two hundred μM AM caused a decrease in cell viability in the alveolar macrophage and the Clara cell fractions at time points equal to and greater than 12 hr. The cell digest and alveolar type II cell fractions showed a decrease at 24 and 36 hr (Figure 2.4C). AM-induced cytotoxicity was greatest in the alveolar macrophages at 12 hr and 24 hr with a viability index of (59.5 ± 6.8%) and (41.0 ± 10.3%), respectively, compared with the other enriched cell fractions.

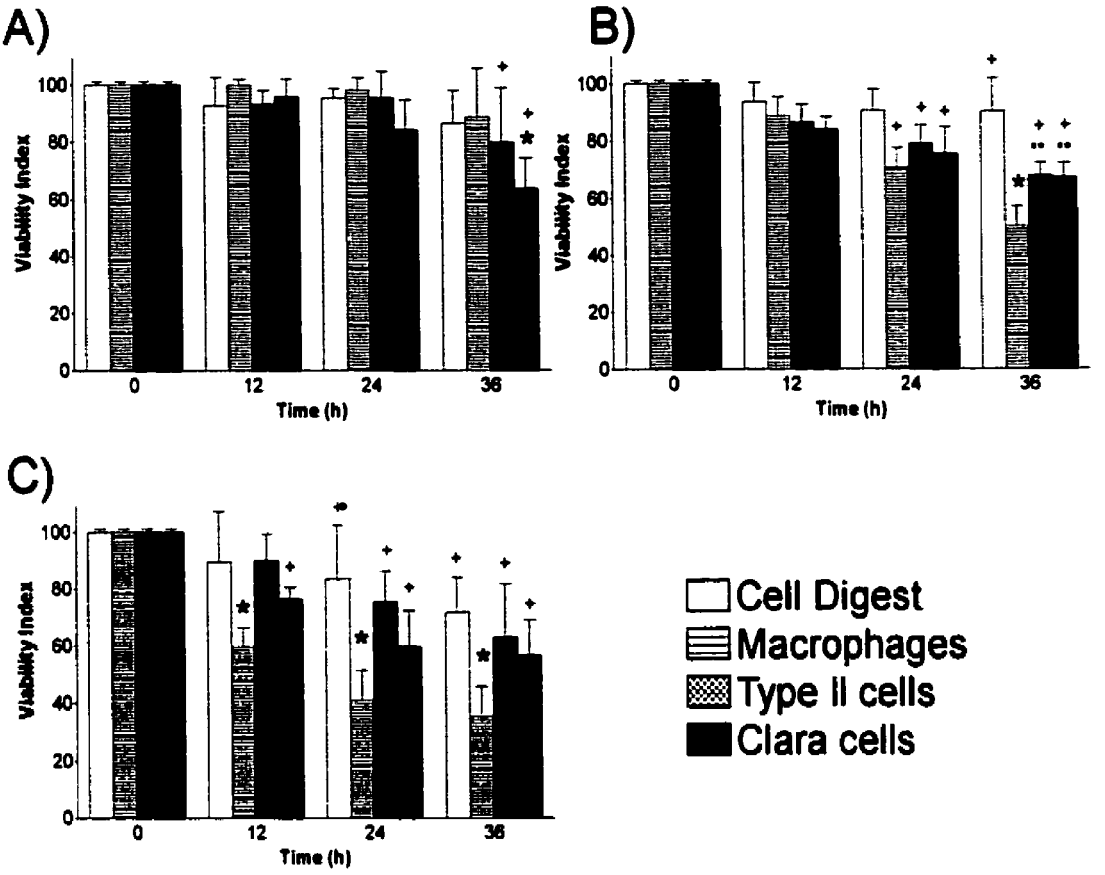


Figure 2.4. Viability indices (mean ± SD, n=4 or 5) of enriched cell fractions incubated with: A) 50 µM; B) 100 µM AM; or C) 200 µM AM. + significant difference ($p < 0.05$, two-way ANOVA with Newman-Keuls test) for that cell fraction compared to t=0 hr. * significant difference from all other cell fractions at that time point. ** significant difference from cell digest at that time point.

2.3.3 Amiodarone Content in Isolated Cells

After incubation of isolated lung cells with 50 μM AM for 24 hr, there was no significant difference in cellular AM content among the different cell fractions (Table 2.2). In contrast, analysis of isolated lung cells incubated with 100 μM AM for 24 hr demonstrated that alveolar macrophages contained a significantly ($p < 0.05$) higher amount of AM (3469 ± 2099 μg / g of cell pellet) than did cells from the other fractions (Table 2.2). HPLC analysis also revealed a very small amount of putative desethylamiodarone (DEA), the primary metabolite of AM, within the Clara cell fraction. However, the amount of DEA present was below the lower limit of quantitative sensitivity (0.18 μg / g of cells) of the procedure.

2.4 DISCUSSION

In the present study, hamster alveolar type II cells and Clara cells were found to be susceptible to AM-induced cytotoxicity *in vitro* when incubated with concentrations of AM greater than or equal to 50 μM. However, alveolar macrophages did not demonstrate AM-induced cytotoxicity unless incubated with 100 μM AM or greater. The susceptibility of both alveolar macrophages and alveolar type II cells to AM-induced cytotoxicity is consistent with previous findings. AM-induced cytotoxicity in alveolar macrophages from Wistar and Fisher-344 rats (Wilson and Lippmann, 1996b) and New Zealand rabbits (Leeder et al., 1996) has been demonstrated, and alveolar macrophages appear "foamy" due to formation of intracytoplasmic lamellar inclusion bodies, in Golden Syrian hamsters and Fisher 344 rats exposed to AM *in vivo* (Kannan et al., 1989a; Rafeiro et al., 1994; Wilson and Lippmann, 1990). Similarly, AM was previously demonstrated to be cytotoxic to rat alveolar type II cells (Wilson and Lippmann, 1996b). However, this is the first report to compare the relative susceptibilities of different lung cell types to AM cytotoxicity. Furthermore, the susceptibility of

TABLE 2.2

Accumulation of Amiodarone in Isolated Lung Cell Fractions Incubated with 50 and 100 μM Amiodarone for 24 hr

Cell Type	50 μM AM μg / g of cell pellet (mean ± SD; n=4)	100 μM AM μg / g of cell pellet (mean ± SD; n=3)
Cell Digest	109 ± 102	665 ± 152
Macrophages	245 ± 115	3469 ± 2099[a]
Type II cells	150 ± 95	971 ± 534
Clara cells	190 ± 163	1156 ± 91

[a] significant difference ($p < 0.05$, repeated measures ANOVA with Newman-Keuls test) from other cell fractions

hamster Clara cells to AM cytotoxicity is a unique finding. Identification of this cellular target for AM toxicity may prove to be valuable in the elucidation of the mechanism(s) of AM-induced pulmonary toxicity.

Susceptibility of isolated hamster lung cell types to AM-induced cytotoxicity was measured *in vitro* by trypan blue exclusion, which required a relatively small number of cells, and allowed for microscopic determination of cell targets for AM-induced cytotoxicity in fractions containing more than one cell type. This proved to be particularly important in determining Clara cell susceptibility (as discussed below). AM-induced pulmonary fibrosis in the hamster, is associated with swelling of the epithelial lining of the lung and proliferation of alveolar type II cells *in vivo* (Cantor et al., 1984), effects consistent with cell loss. Therefore cell death, as indicated by loss of membrane integrity, was used to measure AM-induced cytotoxicity. Other cytotoxicity assays that measure loss of membrane integrity (such as lactate dehydrogenase or ^{51}Cr release) were not used because low cell numbers, particularly in the Clara cell fraction (approximately 1.6 million cells total), precluded the use of spectrophotometric assays. Also, ^{51}Cr release was not used as an indication of cytotoxicity, because a significant loading period is required (e.g., 18h (Kachel et al., 1990)) before its release is measured. This was not practical for the present experiments.

Previous experiments regarding species susceptibility to AM-induced pulmonary toxicity revealed that different cell types do not all react similarly to AM. Rat alveolar macrophages accumulated more drug and phospholipid than did alveolar type II cells (Reasor et al., 1990). Furthermore, cultured bovine pulmonary artery endothelial cells showed greater AM-induced cytotoxicity than did rabbit lung fibroblasts (Wilson and Lippmann, 1990). More recently, it was reported that cultured fibroblasts from Fisher-344 rats were more susceptible to

AM-induced cytotoxicity than were cultured fibroblasts from Wistar rats (Wilson and Lippmann, 1996b). Clearly, the isolation of cells will result in the alteration of cell physiology. However, biotransformation activities of freshly isolated cells more closely approximate *in vivo* activities than do cultured cells (Devereux and Fouts, 1981; Fouts, 1982; Massey, 1989). For this reason, the present study compared the relative susceptibilities of freshly isolated hamster lung cell types to AM-induced cytotoxicity. Because cells were exposed to various concentrations of AM, we were able to show that susceptibility to AM-induced cytotoxicity was dependent upon AM concentration.

Reasons for Clara cell susceptibility to AM are not yet known. Large amounts of biotransformation enzymes (such as cytochrome P450 monooxygenase, NADPH-cytochrome P450 reductase, and epoxide hydrolase) (Plopper et al., 1991) contained within the Clara cells suggest a potential role for AM biotransformation. However, these enzymes are not concentrated in alveolar macrophages, implying the possibility of more than one mechanism of toxicity. In any case, further experimentation will be required to definitively determine the mechanism of AM cytotoxicity. The sensitivity of Clara cells to 50 μM AM apparently was not due to differential accumulation of AM in that cell type, because there was no significant difference in the intracellular concentration of AM among the cell fractions.

Interestingly, HPLC analysis revealed trace amounts of DEA formation within the Clara cell fraction. DEA is recognized as a cytotoxic (Gross et al., 1989; Ogle and Reasor, 1990) and fibrogenic (Daniels et al., 1989) metabolite of AM and may play a significant role in AM-induced toxicity *in vivo*. The amount of DEA produced by the Clara cells in the present study was insignificant in comparison to the amount of AM, such that biotransformation to DEA probably does not contribute to AM-induced cytotoxicity in these experiments.

Nevertheless, it would be of interest to determine if the pattern of susceptibility to DEA is similar to that of AM.

It is important to note that, because Clara cells represented a very small percentage of cells in the cell digest (4 %), enrichment was limited to 35 to 50 % in the Clara cell fraction. Although this purity is within the range of Clara cell enrichment used by other authors with different species (Plopper et al., 1991), it might be argued that non-Clara cells in the Clara cell fraction were actually the cells killed by AM. However, because cell viability was measured by light microscopy, morphological characteristics (size, lack of cilia, lack of surfactant-containing inclusions) confirmed that Clara cells, rather than contaminating cell types, were the principal cells demonstrating AM cytotoxicity in this cell fraction. This means that susceptibility of Clara cells to AM may be greater than the viability results from the Clara cell-enriched fraction would suggest.

Although alveolar macrophages were highly susceptible to 100 and 200 μM AM, 50 μM AM did not produce a cytotoxic effect. Apparently, a threshhold level of AM exposure and / or accumulation within macrophages is necessary for cytotoxicity to occur. HPLC analysis of cell fractions exposed to 100 μM AM revealed that more drug had accumulated in alveolar macrophages than in the other fractions (Table 2.2). Furthermore, the amount of AM within the alveolar macrophages exposed to 100 μM was greater than double the amount of drug in those incubated with 50 μM AM. This effect is consistent with AM-induced inhibition of lysosomal phospholipases which results in an increase in phospholipid within macrophages and consequently enhanced accumulation of AM within this more lipophillic environment. Reasor et al. (1988) demonstrated that sequestration of AM in rat alveolar macrophages was associated with the formation of intracytoplasmic lamellar-inclusion bodies and an increase in total phospholipid. The importance of AM accumulation in cell susceptibility to

cytotoxicity has been recognized. Wilson and Lippmann (1996b) suggested that differences in susceptibility to AM-induced pulmonary toxicity between Wistar and Fisher-344 rats were partially due to differences in the ability of alveolar macrophages to accumulate AM.

Results of these experiments not only demonstrated that alveolar macrophages, alveolar type II cells and Clara cells are all susceptible to AM-induced cytotoxicity, but that the Clara cells were more susceptible than any other cell type incubated with 50 μM AM. Furthermore, Clara cell susceptibility did not correlate with the amount of drug within the cells, indicative that some mechanism other than drug burden (possibly microsomal AM biotransformation, which is very high in the Clara cell) was responsible for Clara cell cytotoxicity. In contrast, alveolar macrophages, which contain relatively low levels of biotransformation enzymes in uninduced animals, were the most susceptible cell type to 100 and 200 μM AM. This suggests that more than one mechanism is responsible for susceptibility to AM-induced toxicity. Understanding the reason(s) for preferential susceptibility of Clara cells to relatively low AM concentrations, and macrophages to higher concentrations, may be important for the elucidation of the mechanism of AM-induced pulmonary toxicity. This is important because it may lead to the production of a new drug therapy which counteracts the effects of AM or the development of new antidysrhythmic agents that are devoid of AM's toxic effects.

CHAPTER 3

DISRUPTION OF MITOCHONDRIAL FUNCTION AND CELLULAR ATP LEVELS BY AMIODARONE AND *N*-DESETHYLAMIODARONE IN THE INITIATION OF AMIODARONE-INDUCED PULMONARY CYTOTOXICITY

3.1 INTRODUCTION

Amiodarone (AM), [2-butyl-3-(3',5'-diiodo-4'-diethylaminoethoxybenzoyl)-benzofuran], is a potent and efficacious class III (Vaughan William's classification) antidysrhythmic agent (Heger et al., 1981; Singh et al., 1992). Because of its high potential for mortality, AIPT is the adverse effect of greatest concern for patients receiving AM therapy. Although the etiology of AIPT has not been fully elucidated, it is generally accepted that, during the development of chemically-induced pulmonary fibrosis, a prolonged cytotoxic insult to the airway epithelium initiates the release of inflammatory mediators, influx of inflammatory cells, fibroblast proliferation, and collagen deposition (Adamson, 1984; Sheppard and Harrison, 1992). Furthermore, in a rat model of AIPT, cytotoxicity occurs shortly after exposure to AM (Taylor et al., 2001), and in the hamster, early alveolar type II cell proliferation consistent with epithelial cell loss has been demonstrated (Cantor et al., 1987).

Previously (Chapter 2), it was demonstrated that hamster alveolar macrophages, alveolar type II cells and non-ciliated bronchiolar epithelial (Clara) cells were all susceptible to cytotoxicity induced by various concentrations of AM (Bolt et al., 1998). Furthermore, these cell types require relatively high levels of energy to maintain normal function (Crystal, 1991; Mason and Williams, 1991;

Plopper et al., 1991). However, the mechanism of AM-induced cytotoxicity that initiates pulmonary fibrosis has not been determined (Massey et al., 1995; Reasor and Kacew, 1996a). In non-pulmonary tissues, AM causes both structural (Guerreiro et al., 1986a; Yasuda et al., 1996) and functional (Fromenty et al., 1990a; Fromenty et al., 1993; Guerreiro et al., 1986b; Ribeiro et al., 1997) perturbations to mitochondria. In addition, AM disrupts oxygen consumption in isolated hamster lung mitochondria (Card et al., 1998). However, the relationship between mitochondrial disruption and lung cell death has not yet been established.

The N-dealkylated AM metabolite, N-desethylamiodarone (DEA), possesses antidysrhythmic properties (Abdollah et al., 1989). However, following AM treatment, DEA accumulates extensively in the lungs of animals and humans (Adams et al., 1985; Daniels et al., 1989; Kannan et al., 1991; Wilson and Lippmann, 1990), often to a greater extent than AM itself (Daniels et al., 1989; Wilson and Lippmann, 1990). Furthermore, DEA is more cytotoxic than AM in rat alveolar macrophages (Ogle and Reasor, 1990) and in non-pulmonary cell types (Beddows et al., 1989; Gross et al., 1989; Ruch et al., 1991), and is a more potent fibrogen in hamsters (Daniels et al., 1989). However, the relative susceptibilities of different lung cell types to DEA-induced cytotoxicity have not been reported. Identification of a particularly susceptible cell type may provide valuable information regarding the etiology of AM and DEA-induced pulmonary toxicity.

The purpose of this study was to determine the effects of AM and DEA on mitochondrial membrane potential and intracellular ATP levels, and the temporal relationship between these effects and loss of viability in different isolated hamster lung cell types. Agents that potentially could attenuate AM-induced mitochondrial disruption or cellular energy loss were also investigated. Furthermore, experiments in isolated whole lung mitochondria compared accumulation of AM and DEA as well as effects on mitochondrial function.

3.2 METHODS AND MATERIALS

3.2.1 Chemicals

Chemicals were obtained from the following suppliers: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) from Molecular Probes (Eugene, OR); trichlorotrifluoroethane (Freon 113) from Ladd Research Chemicals Inc., Burlington, Vermont; amiodarone HCl, protease type I, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), [^{3}H]-2,8-adenosine, RPMI 1640 media with and without phenol red, heparin, tri-n-octylamine, tetrabutylammonium dihydrogen phosphate (TBAP), rotenone (95-98%), adenosine 5'-diphosphate (ADP, free acid), L-glutamate (monosodium salt), L(-) malate (monosodium salt), ethylenediamine tetraacetic acid (EDTA, disodium salt, dihydrate), succinate (disodium salt, hexahydrate), D-mannitol, 3-[N-morpholino]propanesulfonic acid (MOPS), and fatty acid-free bovine serum albumin from Sigma Chemical Co., St. Louis, Mo. ; Percoll® from Pharmacia Biotech AB, Uppsala, Sweden, 4-(2-hydroxyethyl)-1-piperazine-*N*′-2-

ethanesulfonic acid (HEPES) from Boehringer-Mannheim; sodium pentobarbital from M.T.C. Pharmaceuticals (Mississauga, ON, Canada). *N*-desethylamiodarone hydrochloride was generously donated by Wyeth-Ayerst Research (Princeton, NJ). All other reagents were of analytical grade and purchased from standard chemical suppliers.

3.2.2 Animals

Male golden Syrian hamsters (140-160g) were obtained from Charles River Canada Inc., St-Constant, Que. Animals were housed at room temperature on a 12-h light - 12 h dark schedule (lights on at 0700 h) for a minimum of 1 week prior to experimentation. Hamsters were fed Purina rodent chow and water *ad libitum*, and were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

3.2.3 Enriched Lung Cell Preparations

The procedures for isolating alveolar macrophages and obtaining enriched preparations of alveolar type II cells, and non-ciliated bronchiolar epithelial (Clara) cells from unseparated cells (cell digest) are described in detail elsewhere (see Chapter 2 and (Bolt et al., 1998)). Briefly, hamsters were injected with heparin sodium (2000 U in 0.5 ml of saline) 45 min prior to anesthetization with sodium pentobarbital (300 mg/kg i.p.). Alveolar macrophages were obtained by bronchoalveolar lavage. Other lung cells were released by treatment with protease type I, and enriched preparations of alveolar

type II and Clara cells were obtained by centrifugal elutriation. The alveolar type II cell preparation was further enriched by Percoll® density gradient centrifugation (28% (v/v) in RPMI 1640 media). Viabilities and cell numbers were assessed on a haemacytometer by light microscopy using 0.5% trypan blue dye exclusion. Alveolar type II cells and Clara cells were identified using modified Papanicolaou (Kikkawa and Yoneda, 1974) and nitroblue tetrazolium (Devereux and Fouts, 1980) staining, respectively. For each experiment, cells isolated from 12 hamsters were pooled.

3.2.4 Incubations and Viability Assessments

Cells (4×10^5 cells / well) were incubated in flat-bottomed, irradiated, nonadhesive, polystyrene Nunc™ 96 microwell plates (GIBCO Canadian Life Technologies, Burlington, ON) in RPMI 1640 media (with phenol red and buffered with 50 mM $NaHCO_3$) containing vehicle (0.1% ethanol), 100 µM AM, or 100 µM FCCP under 95% air-5% CO_2 at 37^0C, pH 7.4. This concentration of AM was selected because we previously found it to cause appreciable AM-induced cell death at 24 and 36 h (Chapter 2 and (Bolt et al., 1998)) and it is within the range of concentrations of AM present in human lungs following clinical treatment with AM (Brien et al., 1987; Plomp et al., 1984). To minimize clumping, cells were agitated by drawing the cells gently in a 10 ml pipette every 6 to 12 h. In separate experiments, cells were incubated with vehicle or DEA (100, 50, 25 and 10 µM). Because of their relatively high yield and demonstrated susceptibility to AM cytotoxicity (Bolt et al., 1998), alveolar macrophages alone were used in

experiments to assess effects of preincubation with 5.0 mM glucose or 2mM niacin (potential sources for ATP) for 1 h prior to addition of AM, on loss of viability.

3.2.5 Lung Cell Mitochondrial Membrane Potential

Mitochondrial membrane potential in intact lung cells was assessed using the JC-1 probe, a cationic chemical that exists as a green-fluorescing monomer at low membrane potentials (< 120 mV) and as a red-fluorescing dimer (referred to as J-aggregates) at membrane potentials greater than 180 mV (Reers et al., 1995). Following excitation at 488 nm, the ratio of red (595 nm emission) to green (525 nm emission) fluorescence measures the ratio of high to low mitochondrial membrane potential (Reers et al., 1995; Salvioli et al., 1997). Lung cells (5.0×10^5 cells / 0.5 ml of RPMI 1640 media without phenol red) were incubated in irradiated, Nunclon™ Delta 24 multi well plates (GIBCO Canadian Life Technologies) with vehicle (0.1% ethanol), AM, DEA or FCCP, under 95% air- 5% CO_2 at 37^0C for 2 h. JC-1 (5.0 µM) was added to each well, and cells were incubated for 30 min at room temperature in the dark, with gentle agitation. Cells were then washed twice with fresh media and resuspended in 0.5 ml RPMI 1640 media without phenol red. To measure basal fluorescence of each cell preparation, an aliquot was removed prior to addition of JC-1 and analyzed cytofluorometrically. Due to the relatively large number of red blood cells as well as debris present, effects of AM and DEA on mitochondrial membrane potential were not measured in cell digest.

Analysis of cell samples was performed using a Coulter Epics Elite flow cytometer equipped with a Spectra Physics water-cooled, 488 nm argon laser. The settings for the detection system were as follows: channel 1 dichroic, 488nm mirror longpass; channel 1 bandpass, 488 nm bandpass; channel 2 block, 488 nm block; channel 2 dichroic 550nm dichroic mirror longpass; channel 2 bandpass, 525nm bandpass; channel 3 dichroic, 600 nm dichroic mirror longpass; and channel 3 bandpass, 575 nm bandpass. Mean red and green fluorescence intensities of a minimum of 10,000 cells per sample were analyzed with Epics Elite Work Station Software version 4.01. In some cases, flow cytometry gates were adjusted to exclude contaminating red blood cells.

3.2.6 Assessment of Cellular ATP Levels

To label adenine nucleotide pools, cells (1.2×10^6) were loaded with 10.0 µCi of [^{3}H]-2,8-adenosine for 1 h at 37^{0}C, under 95% air-5% CO_2. Cells were centrifuged (500 x *g* for 5.0 min), washed twice with RPMI 1640 media (containing phenol red), and incubated with vehicle or 100 µM AM for 2, 4, or 6 h or 50 µM DEA for 2 h. In some experiments, alveolar macrophages were incubated with 5.0 mM glucose or 2.0 mM niacin for 1 h prior to exposure to 100 µM AM for 6 h to assess the ability of these agents to prevent AM-induced depletion of ATP. ATP measurement was conducted by a previously published method (Tekkanat and Fox, 1988) with modifications. Cells were washed twice with HEPES buffered salt solution (Devereux and Fouts, 1981) and nucleotides extracted by adding 100 µl of 0.5 M ice-cold trichloroacetic acid, followed by

neutralization with 100 µl of 0.25 M tri-n-octylamine in Freon 113. The organic layer was filtered through a 0.45 µm low protein binding Durapore® (PVDF) filter, snap frozen in liquid nitrogen and stored at -80^{0}C for no more than 3 days before analysis.

[^{3}H]ATP was separated on a C_{18} µBondapak column by reverse phase high performance liquid chromatography (U.V. detection, λ=254nm). The mobile phase (flow rate 1.8 ml / min) contained 60 mM KH_2PO_4, 1.32 mM TBAP, and 1.26 M acetonitrile (adjusted to pH 3.4 with phosphoric acid at 21 ^{0}C). Nucleotides were identified by co-elution of tritiated samples with authentic non-radioactive standards. Quantitation was accomplished by liquid scintillation spectroscopy. Preliminary experiments determined that intracellular [^{3}H]adenosine levels were maximal 1 h after loading.

3.2.7 Involvement of Mitochondrial Permeability Transition

To determine if mitochondrial permeability transition occurred during exposure to AM, enriched fractions were loaded with 1.0 µM cyclosporin A 20 min prior to incubation with 100 µM AM (Bernardi, 1996; Walter et al., 1998). Cell viability was measured by 0.5% trypan blue exclusion.

3.2.8 Isolation of Whole Lung Mitochondria

Hamsters were deeply anaesthetized with sodium pentobarbital (300 mg/kg ip), and lungs were perfused *in situ* with ice-cold 0.9% saline solution, removed, blotted dry and weighed. Lung mitochondria (pooled from four to eight hamsters for each experiment) were isolated by differential centrifugation as described previously (Card et al., 1998). Aliquots of mitochondrial suspensions were removed for determination of protein content by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

3.2.9 Polarographic Measurement of Oxygen Consumption

Oxygen consumption of isolated lung mitochondria was measured at 30^{0}C as described previously (Card et al., 1998). Respiration supported by complex I of the respiratory chain was assessed using glutamate (5.0 mM) and malate (5.0 mM), and respiration at complex II was measured using succinate (10 mM, in the presence of 3.0 μM rotenone). Effects of AM or DEA on state 4 respiration at complexes I and II were examined by adding them at least 2 minutes following the total expenditure of 0.2 mM ADP (i.e. following transition from state 3 to state 4 respiration). Respiratory control ratios (RCR) and ADP:O ratios were calculated as indicators of integrity of mitochondrial respiratory function (Estabrook, 1967; Nedergaard and Cannon, 1979).

3.2.10 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 ^{0}C. Isolated mitochondria (1.0-2.0 mg protein) were incubated in 1.5 ml of respiratory buffer 5 min prior to addition of AM or DEA. Following incubation with AM or DEA for 0.5

or 3 min, samples were centrifuged for 1 minute (4000 x *g*) to pellet any precipitated drug. Aliquots (250 µl) of supernatant were used for analysis of drug levels by HPLC (Bolt et al., 1998; Brien et al., 1987), and protein (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 samples were centrifuged (5 min at 13000 x *g*) and the resulting mitochondrial pellets were washed, mixed with 250 µl respiration buffer, and centrifuged again. The supernatants were discarded, and the pellets stored at –20 ^{0}C for up to one month. On the day of analysis, 500 µl of HPLC mobile phase (5% (v/v) acetic acid : acetonitrile, 20:80 (v/v)) were added to the individual pellets, which were mixed vigorously and centrifuged at 13000 x *g* for 5 min. Supernatants were removed and filtered using 0.45 µm syringe filters (Millex®-HV syringe driven filter units, Millipore, Bedford, MA). Fifty µl of these filtrates were analyzed by reverse phase HPLC with UV detection (Brien et al., 1983; Brien et al., 1987). Background drug levels in the buffer were subtracted from the drug concentrations measured in the presence of mitochondrial protein.

3.2.11 Data Analysis

Unless indicated otherwise, all data are expressed as sample group means ± standard deviation (SD). Differences between treatment groups were determined by one-way or two-way repeated measures analysis of variance (ANOVA) followed by Newman-Keuls post hoc test or by paired t-tests, as indicated. Some percentage data underwent arcsine transformation prior to statistical analysis (Sokal and Rohlf, 1973). In all cases, $p<0.05$ was considered statistically significant.

3.3 RESULTS

3.3.1 Lung Cell Isolations

Brochoalveolar lavage fluid contained 98% alveolar macrophages, and cell digest contained approximately 21 % alveolar type II cells and 4 % Clara cells. Centrifugal elutriation of cell digest resulted in preparations containing 50-60% alveolar type II cells, and 35-50% Clara cells. Percoll® density gradient centrifugation of the alveolar type II cell preparation enriched it to 75-85%. As reported previously, the alveolar type II cell preparation also contained lymphocytes (5-10%) and a few Clara cells (2-5%). Ciliated cells (10-15%), clumped red blood cells (10-15%), and a relatively small percentage (2-10%) each of fibroblasts and polymorphonuclear leukocytes were present in the Clara cell preparations (Bolt et al., 1998).

3.3.2 AM Effects in Isolated Lung Cells

Initial viabilities in all fractions were greater than 90%, and control viabilities remained greater than 75 % at 36 h. Total cell numbers in all fractions did not decrease over the course of the incubation period. Incubation with 100 µM AM for 2 h caused a 33-45% decrease in red / green fluorescence ratio with JC-1 (Figure 3.1), indicative of a substantial loss of mitochondrial membrane potential in hamster macrophages, as well as in preparations enriched in type II cells and Clara cells (Figure 3.2). This effect was similar to that seen with the classical mitochondrial uncoupler, FCCP (Figure 3.2). However, neither cellular ATP levels (Figure 3.3) nor viability (Figure 3.4) were significantly affected by AM at this time point. By 4 h of incubation, 100 µM AM caused an approximately 50% decrease in ATP content in cell digest and macrophages (Figure 3.3), although viability was still at control values (Figure 3.4). By 6 h, ATP levels were

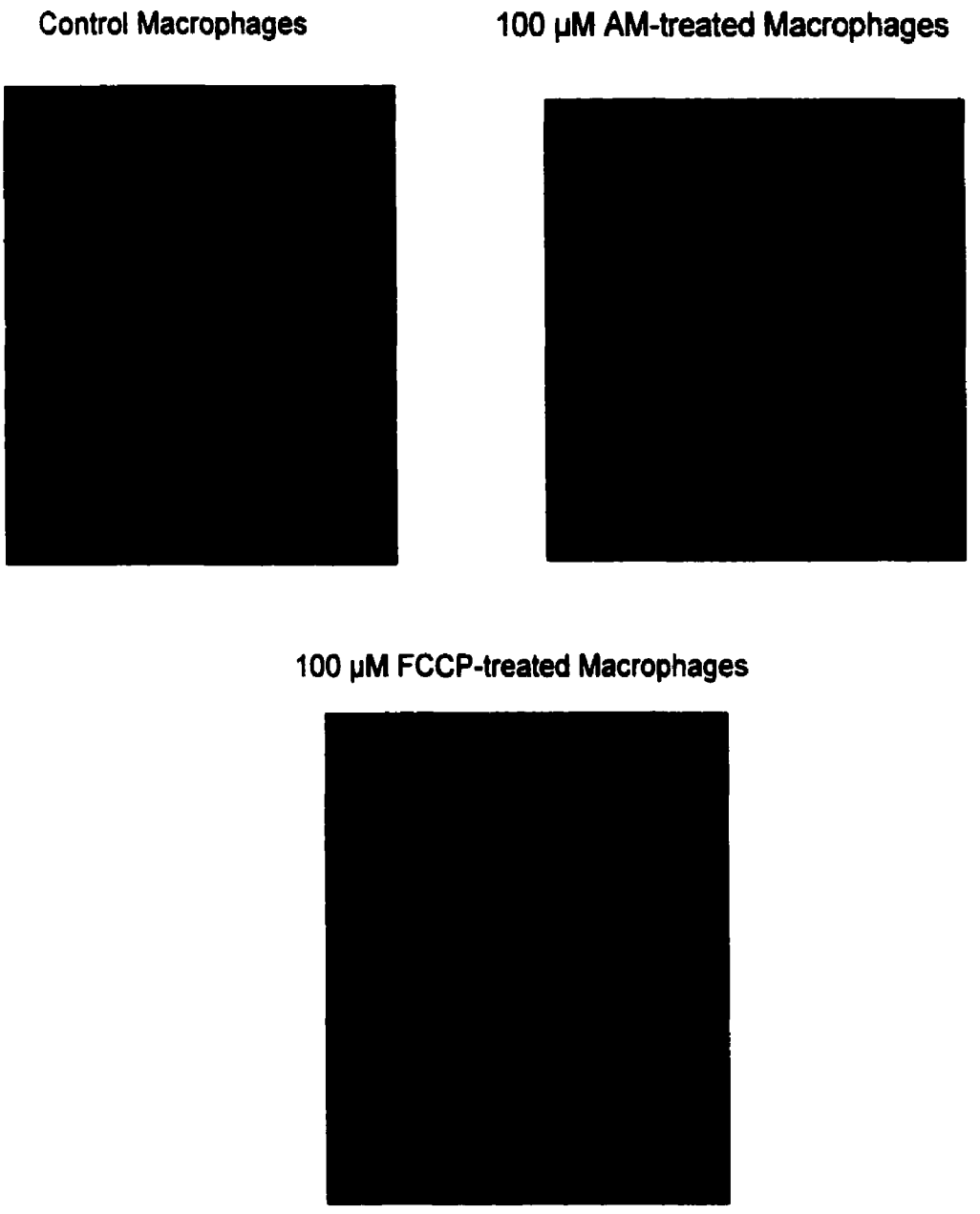


Figure 3.1. Confocal micrographs of alveolar macrophages loaded with JC-1 and incubated with vehicle, 100 μM AM and 100 μM FCCP.

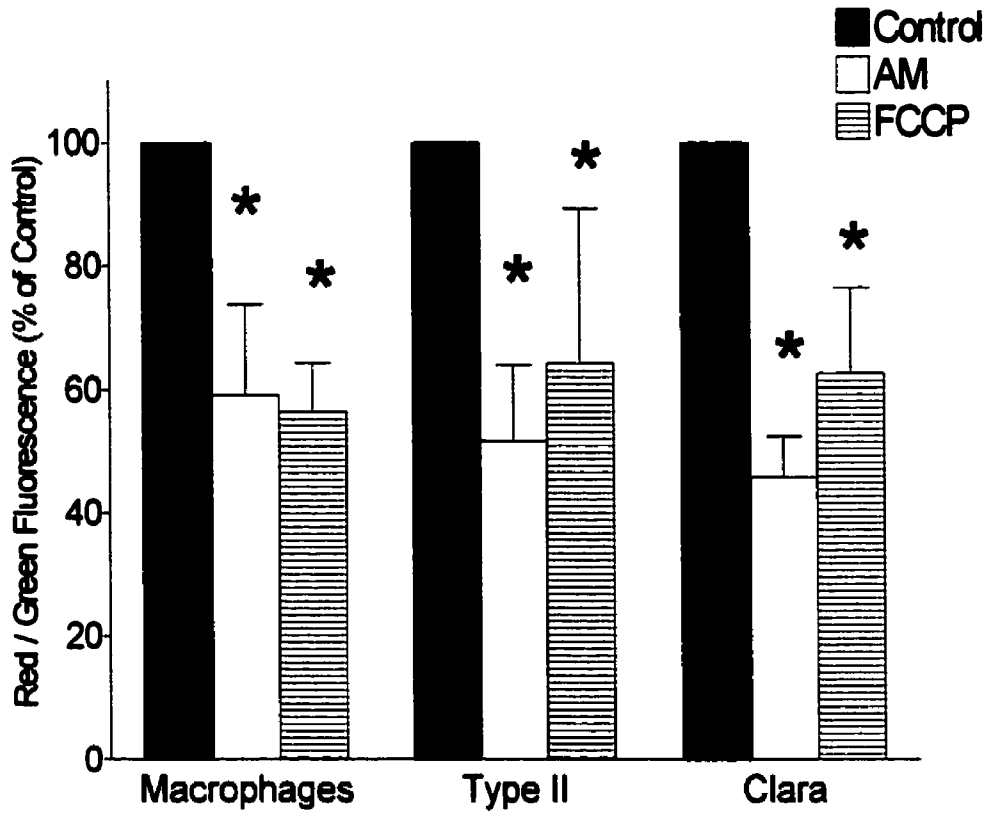


Figure 3.2. Isolated lung cells incubated with vehicle (0.1% ethanol), 100μM AM or 100μM FCCP for 2 h and loaded with JC-1 probe for 30 min. Mean (red / green) fluorescence (± SD, n=4) (measured cytofluorometrically and expressed as percent of control) indicates ratio of high:low mitochondrial membrane potential. *indicates difference ($p<0.05$, repeated measures ANOVA with Newman-Keuls post-hoc) from control. Cells pooled from 12 hamsters were used for each n value.

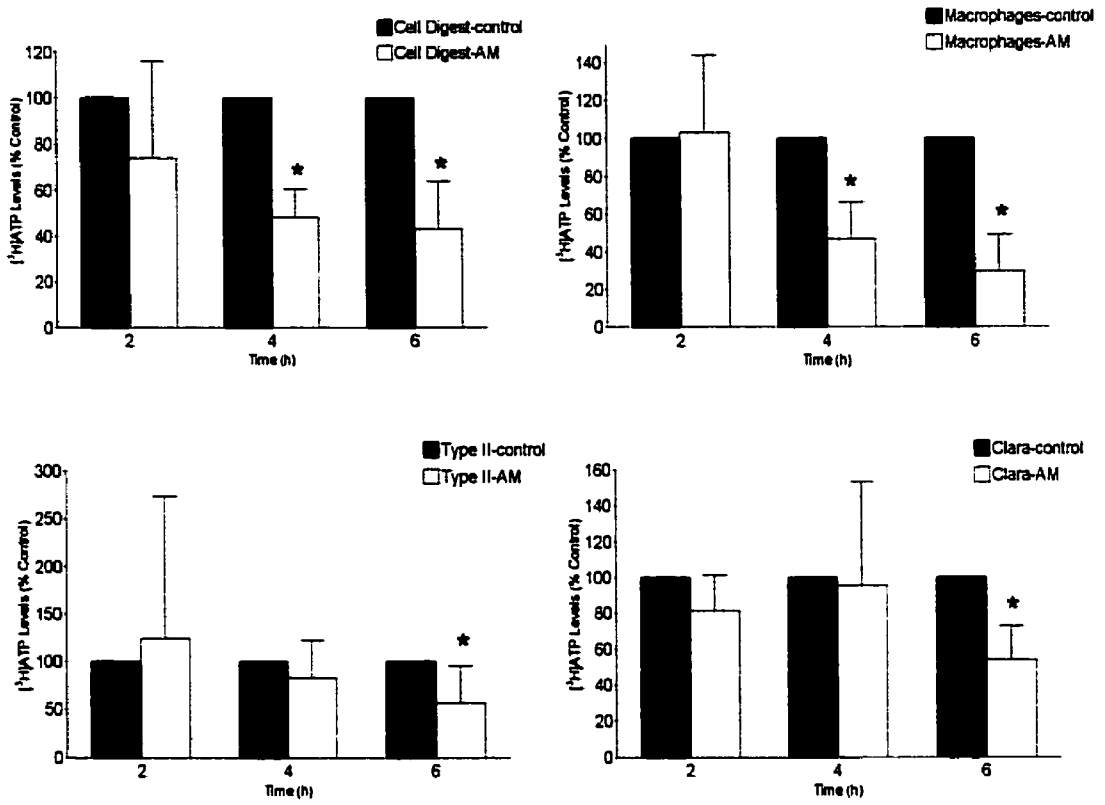


Figure 3.3. [^{3}H]ATP levels in isolated hamster lung cells following incubation with 100 μM AM (mean % of control ± SD). * significant difference from control ($p < 0.05$, paired t-test; n=4). At each time point, cells were pooled from 12 hamsters for each n value.

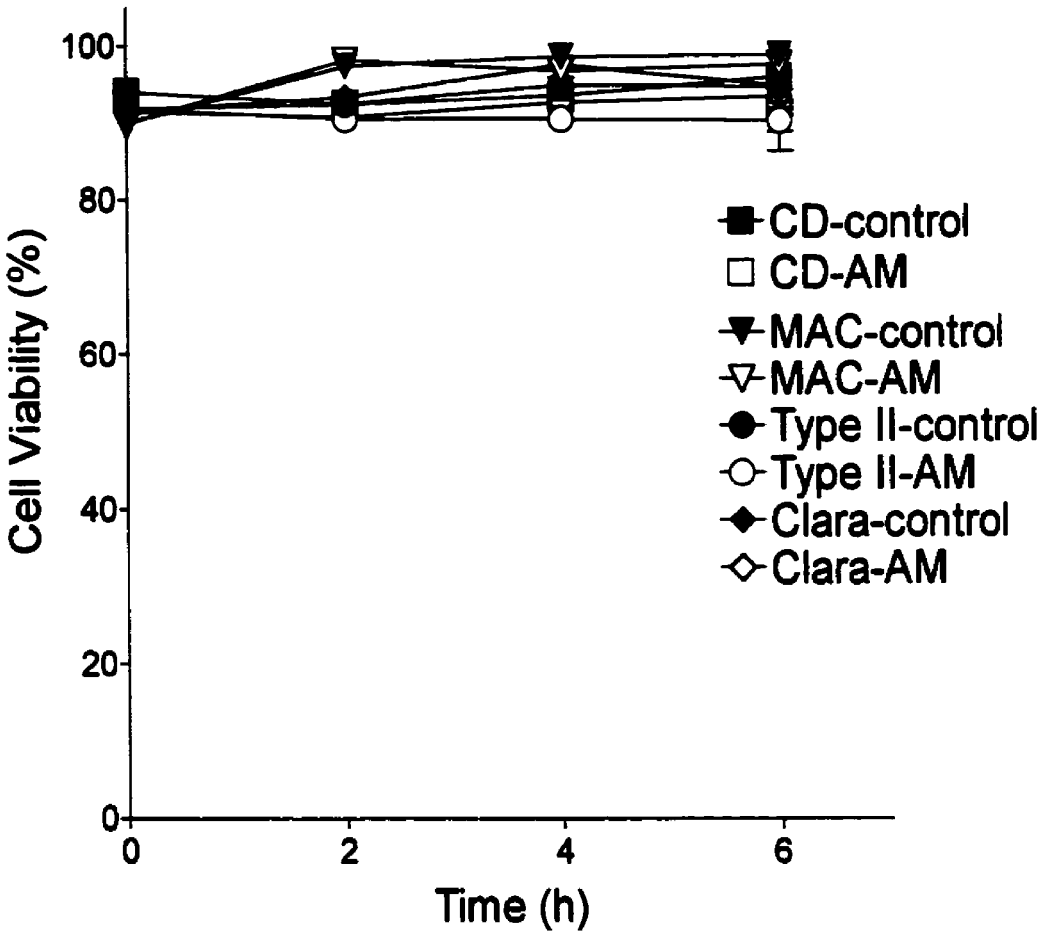


Figure 3.4. Viability of enriched fractions incubated with 100 μM AM for 2, 4, and 6 h.

significantly decreased in all cell fractions exposed to 100 μM AM, with the type II cell fraction showing a lesser degree of depletion than the other cell fractions (Figure 3.3). Despite the pronounced loss of ATP relative to control, viability remained high in all the cell fractions at 6 h (viability in all fractions ranged from 94-98% in control fractions and 90-97% in AM treated fractions, Figure 3.4). Consistent with our previous observation (Chapter 2 and (Bolt et al., 1998)), 100 μM AM caused substantial cytotoxicity in all the cell preparations at 24 and 36 h (Figure 3.6). FCCP (100μM) caused time-dependent loss of viability in all cell preparations, especially during the first 12 h, with cell digest, macrophages and Clara cells showing particular sensitivity (Figure 3.5).

The mitochondrial permeability transition pore inhibitor, cyclosporin A (1.0 μM), did not alter control lung cell viability, and did not affect AM-induced viability loss at 12, 24, and 36 h (Figure 3.6).

In alveolar macrophage preparations, addition of 5.0 mM glucose or 2.0 mM niacin to the incubation media had no significant effect ($p>0.05$, $n= 4$) on AM-induced decreases in cellular ATP levels at 6 h (AM decreased cellular ATP levels to 59.2 ± 19.1% of vehicle control; AM + niacin to 47.3 ± 18.3% of niacin control; and AM + glucose to 59.4 ± 24.1 % of glucose control, Figure 3.7) or loss of viability at 24 and 36 h in alveolar macrophages (AM decreased viability to 70 -75 % of control in the presence and absence of 5.0 mM glucose and to 60-70% of control in the presence and absence of 2.0 mM niacin, Figure 3.8).

3.3.3 DEA Effects in Isolated Lung Cells

Incubation of hamster lung cells with 100 μM DEA for 2 h resulted in 100% cell death in all cell preparations (data not shown). Fifty μM DEA caused a rapid decrease in cell viability relative to 100 μM AM (Figures 3.6 and 3.9) and the viability loss was first observed in the Clara cell enriched preparation, in which a

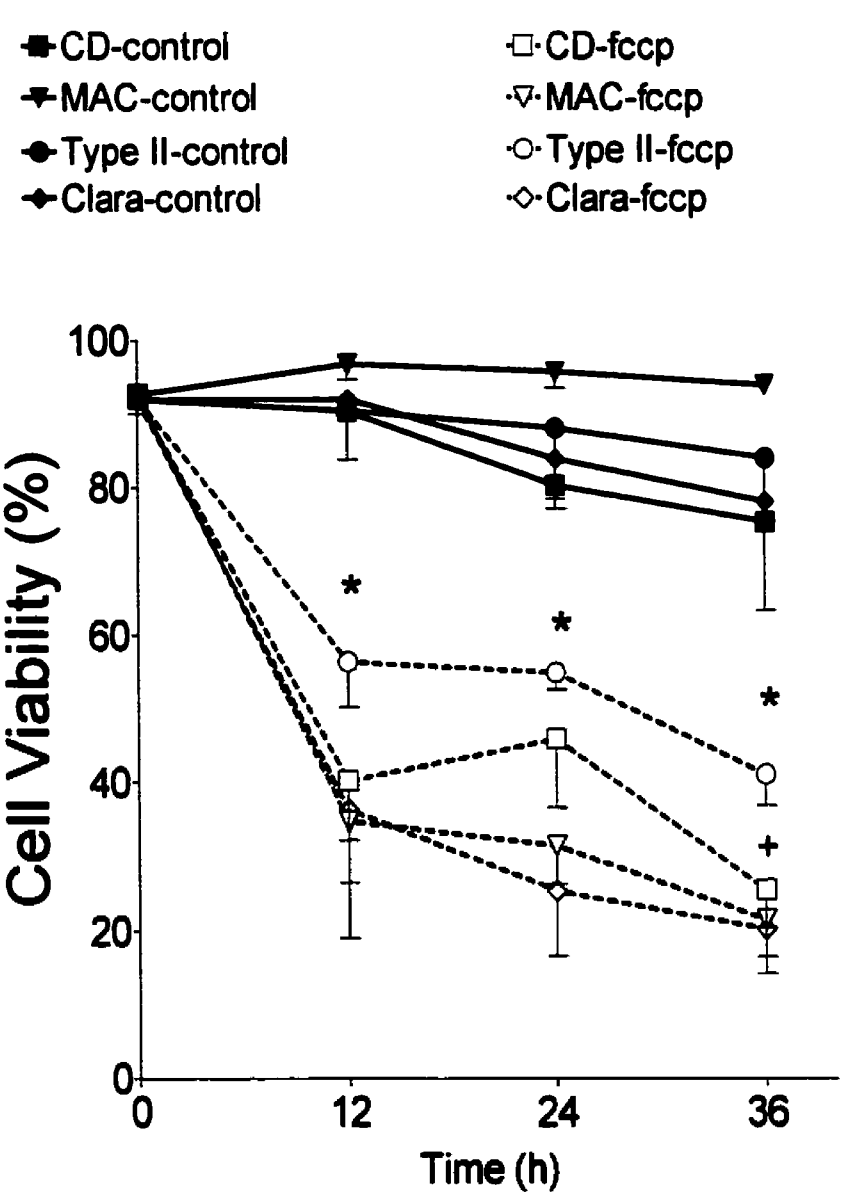


Figure 3.5. Viability (mean ± SD, n=4) of cell digest (CD), macrophages (MAC), alveolar type II cells (Type II) and Clara cells (Clara) incubated with vehicle (0.1% ethanol) or 100μM FCCP. All FCCP-treated fractions were significantly different from respective controls. * difference between FCCP-treated type II preparations and FCCP-treated Clara cell preparations and FCCP-treated type II cell preparations and FCCP-treated macrophage preparations; + significant difference between 36 h FCCP-treated Clara, type II and macrophage fractions from respective 12 h FCCP-treated fractions ($p < 0.05$, repeated measures two-way ANOVA with Newman-Keuls post-hoc test). For each n value, cells from 12 hamsters were pooled and analyzed at 12, 24, and 36 h.

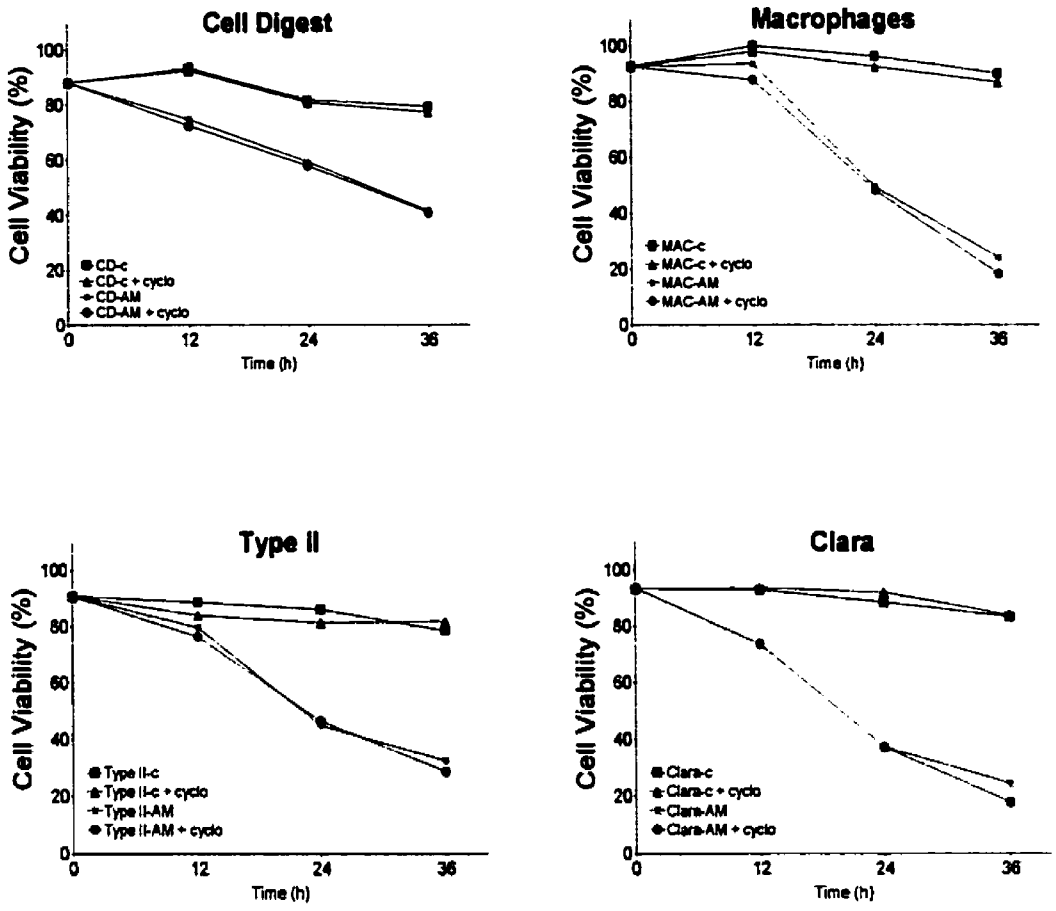


Figure 3.6. Effect of 1.0 μM cyclosporin A (cyclo) on 100 μM AM cytotoxicity in cell digest (CD), macrophages (MAC), alveolar type II cells (Type II) and Clara cells (Clara), (data points represent mean of 2 experiments). Cells from 12 hamsters were pooled for each n value.

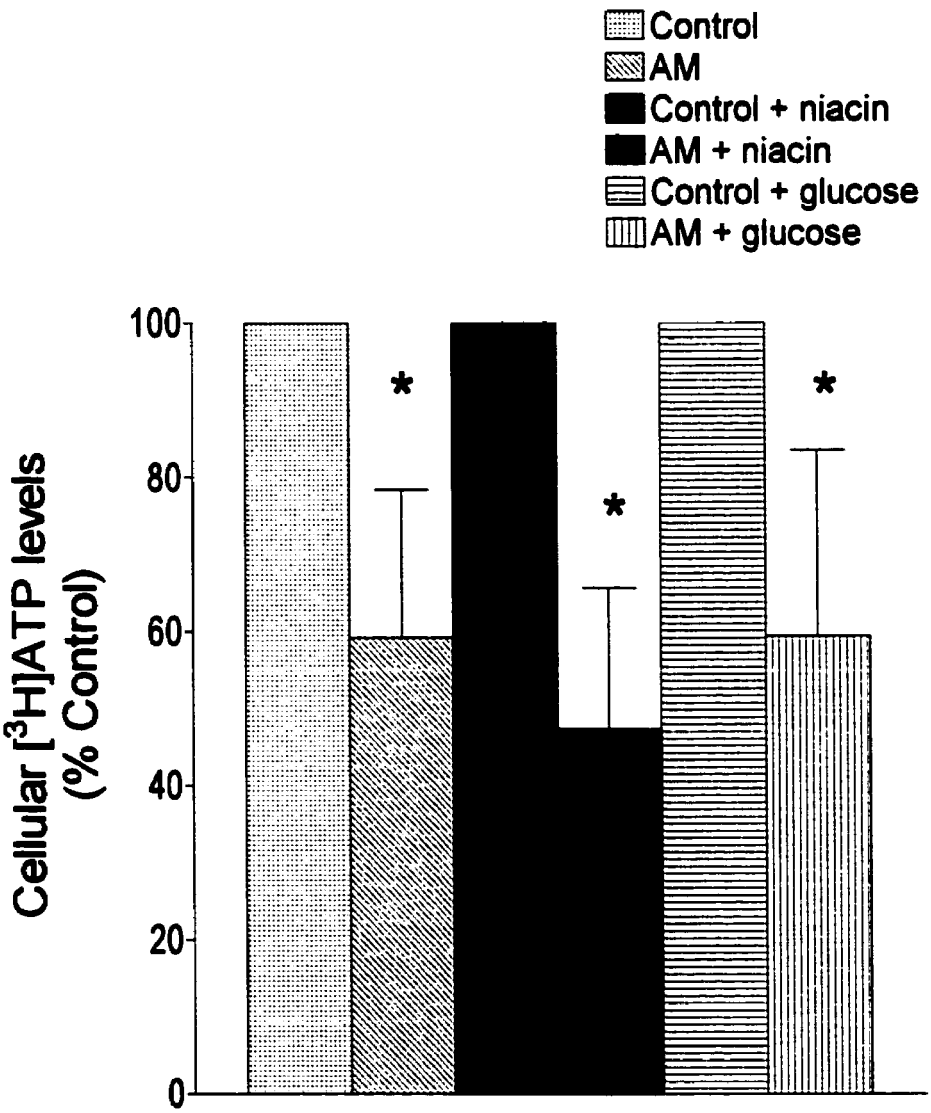


Figure 3.7. Effects of glucose and niacin on AM-induced ATP depletion in alveolar macrophages. * difference between AM-treated fraction and ($p < 0.05$, n=4) respective control.

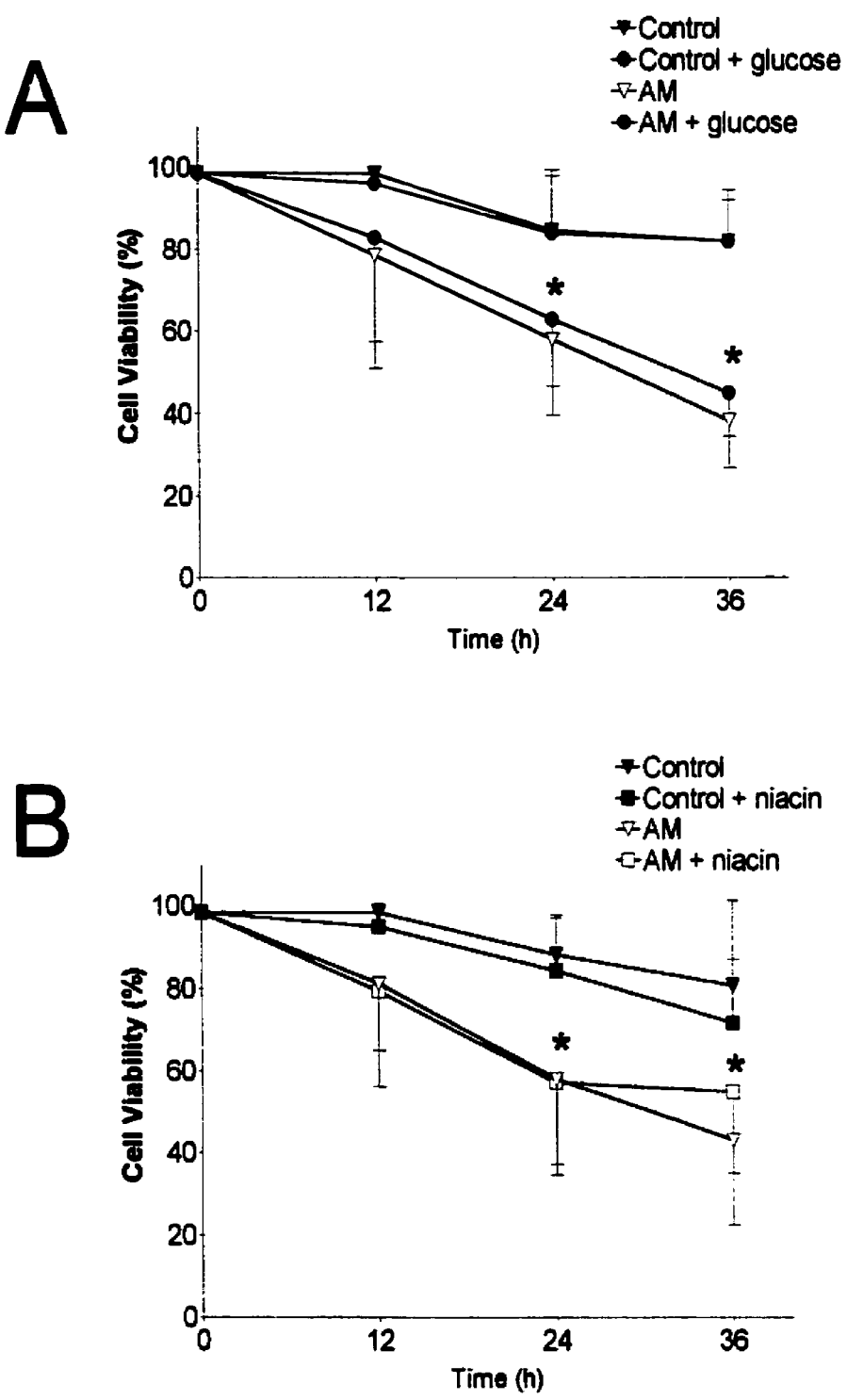


Figure 3.8. Effects of glucose (A) and niacin (B) on AM-induced cytotoxicity in alveolar macrophages. * difference of both AM-treated fractions from ($p < 0.05$, n=4) respective controls.

significant loss (approximately 20%) was apparent by 2 h (Figure 3.9). Following incubation with 50 μM DEA for 6 h, substantial and similar degrees of viability loss had occurred in all the cell fractions, and the decreases in viability progressed to 24 h (Figure 3.9). With 25 μM DEA, time-dependent decreases in viability were evident in the cell digest beginning at 6 h and in alveolar type II cells and Clara cells beginning at 12 h. Loss of viability in alveolar macrophages did not occur during exposure to 25 μM DEA. At 10 μM, DEA did not cause significant cytotoxicity in any enriched preparation at any time point (Figure 3.9).

As indicated by JC-1 fluorescence ratios, 50 μM DEA caused substantial disruption of mitochondrial membrane potential in all cell preparations examined at 2 h (Figure 3.10). The alterations in fluorescence were similar to those induced by 100 μM FCCP. At the same timepoint, and in contrast to 100 μM AM, 50 μM DEA significantly decreased ATP levels in all preparations, and the effects were most pronounced in the Clara cell enriched preparation, in which virtually complete depletion of ATP occurred (Table 3.1).

3.3.4 Effects of AM and DEA on Isolated Whole Lung Mitochondria

In mitochondria isolated from whole hamster lungs, calculated respiratory control ratios (RCRs) were 2.90 ± 0.43 and 1.52 ± 0.10 for complex I and II, respectively. The ADP:O ratio values were 4.27 ± 0.42 and 2.20 ± 0.43 for complex I and II, respectively. As indicated by the higher RCR values (i.e. >2.50), tight coupling was observed at complex I but not at complex II, similar to our previous report (Card et al., 1998).

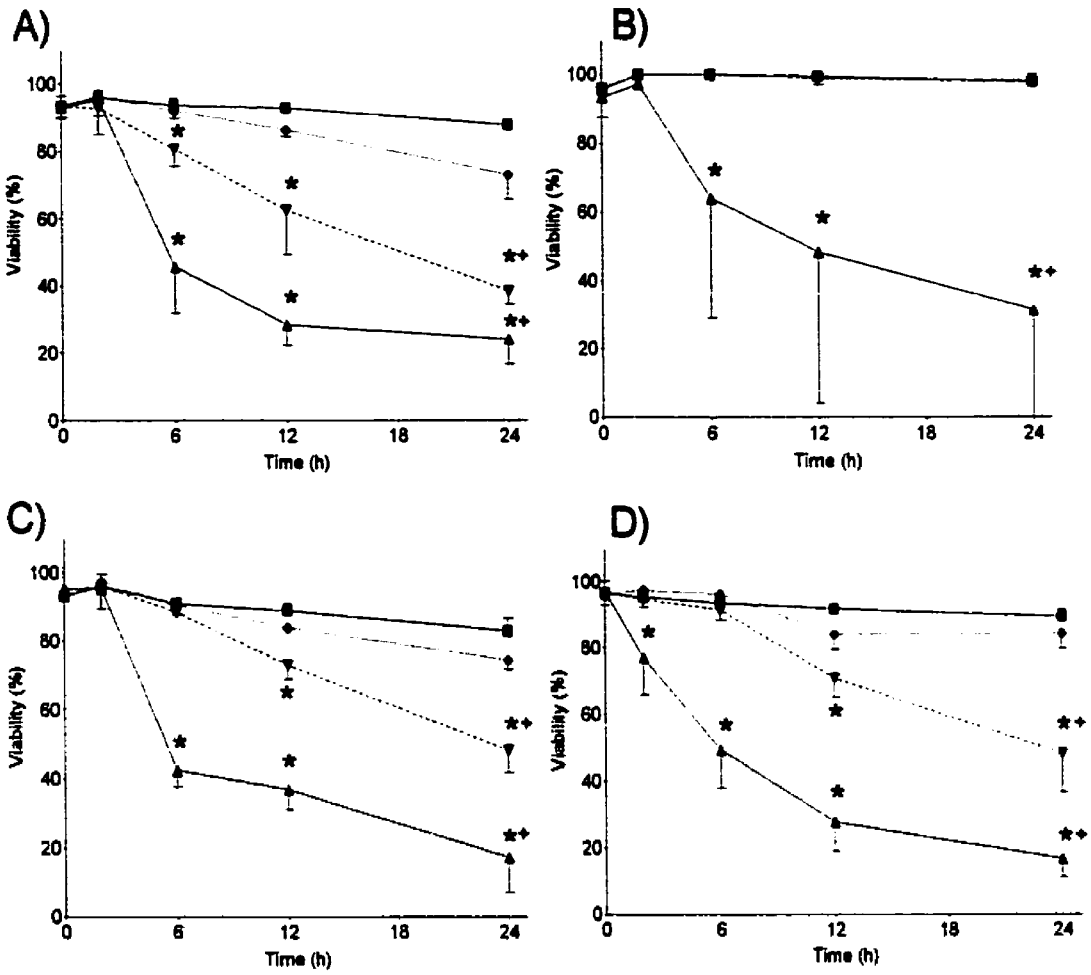


Figure 3.9. Viability (mean ± SD, n=4) of: A) Cell Digest, B) macrophages, C) type II cells and D) Clara cells incubated with vehicle (0.1% ethanol, (■)) or 10 (◆), 25 (▼) or 50 µM (▲) DEA. * significant difference from control; + significant difference between 36 h DEA-treated fraction from respective 6 h DEA-treated fraction ($p<0.05$, repeated measures two-way ANOVA with Newman Keuls post hoc test). For each n value, cells from 12 hamsters were pooled and analyzed at 2, 6, 12, and 24 h. Separate cell preparations were used for the time course of each concentration of DEA.

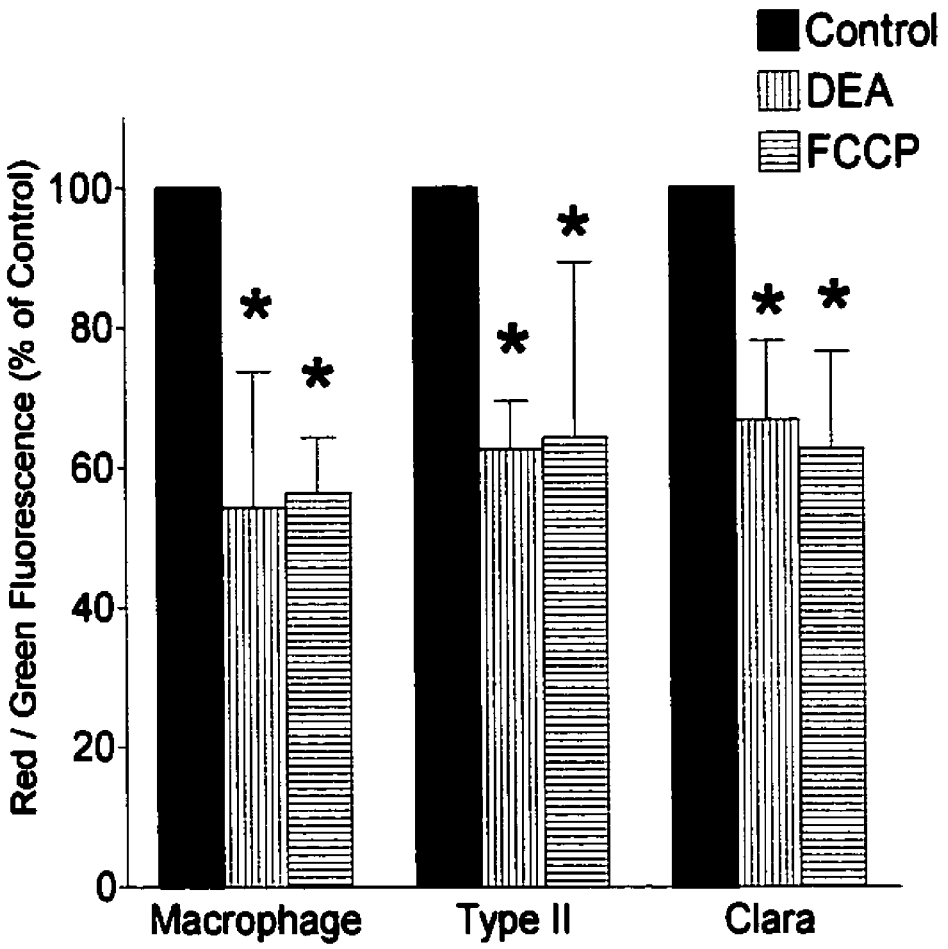


Figure 3.10. Cytofluorometrically measured red / green fluorescence (mean % of control ± SD, n=4) representative of mitochondrial membrane potential in isolated cells incubated with vehicle (0.1% ethanol), 50 μM DEA or 100 μM FCCP for 2 h and loaded with JC-1 for 30 min. * indicates difference ($p < 0.05$, repeated measures ANOVA with Newman-Keuls post hoc test) from control. Cells from 12 hamsters were pooled for each n value.

Table 3.1: [^{3}H]ATP levels in isolated hamster lung cells following incubation with 50μM DEA for 2 h.

Cell Preparation	ATP Levels in Cells Treated with DEA (Mean % control ± SD)
Cell Digest	[a] 35.4 ± 50 %
Macrophages	[a] 57.6 ± 25 %
Type II Cells	[a] 42.5 ± 29 %
Clara Cells	[a] 3.7 ± 4 %

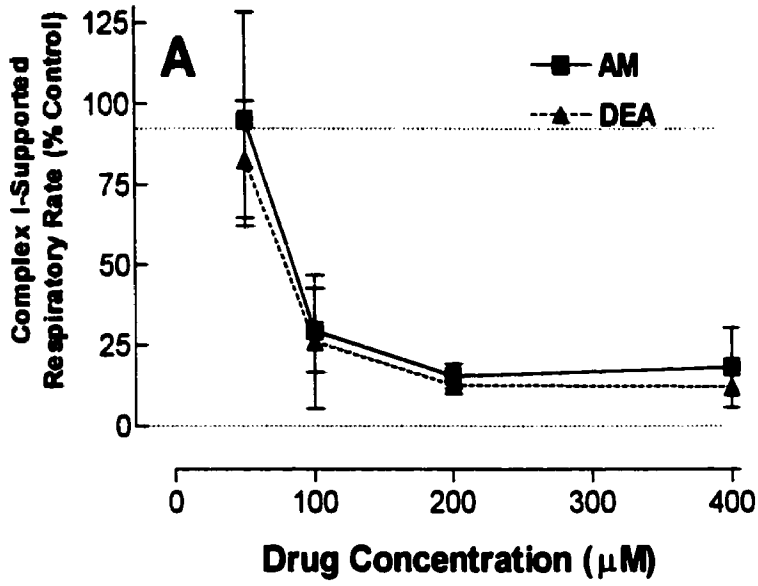
[a] significant difference from control ($p < 0.05$, paired t-tests, $n=4$)

Baseline state 4 respiratory rates (i.e. before drug addition) were 31.8 ± 9.8 nmol O / min / mg protein for complex I, and 79.6 ± 22.3 nmol O / min / mg protein for complex II. Complex I-supported respiration was inhibited to similar extents by equimolar concentrations of AM or DEA (Figure 3.11A). For complex II-supported respiration, concentrations of AM or DEA greater than 100 μM inhibited respiration (Figure 3.11B). However, inhibition by DEA was more pronounced than that of AM at complex II, resulting in virtually complete inhibition at concentrations above 100 μM.

A diagrammatic representation of the respiratory effects of various concentrations of AM or DEA is shown in Figure 3.12. Inhibition of both complex I and complex II-supported respiration by AM was gradual, occurring after a lag period ranging from 2 to 5 min after drug addition, and often after an initial stimulation of respiration. In contrast, inhibitory effects of DEA were rapid, occurring immediately after drug addition, and no initial stimulatory phase was observed. Effect of AM or DEA on state 3 respiration (i.e. ADP-stimulated) could not be determined, as preincubation of lung mitochondria with either drug resulted in complete inhibition of oxygen consumption in response to the addition of respiratory substrates. At both 0.5 min and 3 min, DEA accumulated in mitochondria to a greater extent than did AM (Figure 3.13).

3.4 DISCUSSION

Although the etiology of AIPT still has not been fully elucidated, the results of the present investigation suggest a sequence of events involving disruption of



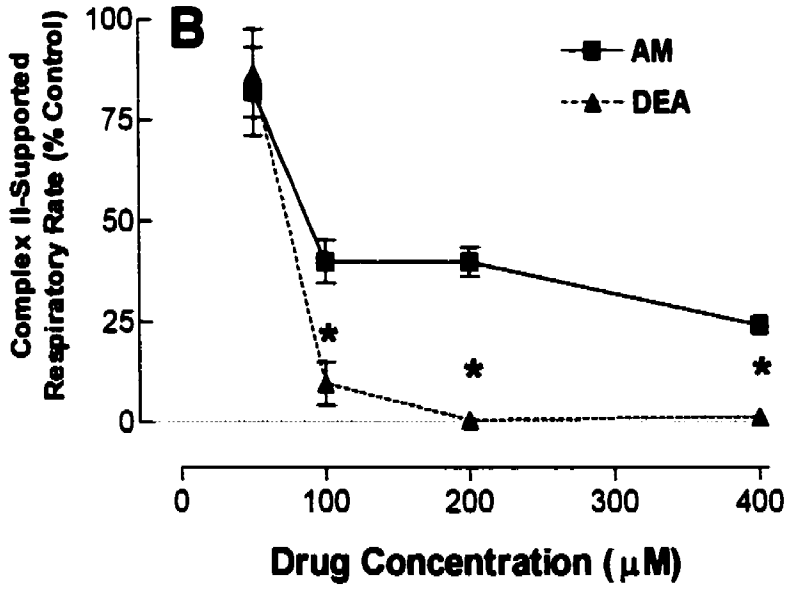


Figure 3.11. Effect of AM and DEA on A) complex 1-supported and B) complex II-supported state 4 (resting) oxygen consumption in isolated hamster lung mitochondria. * significant difference ($p<0.05$; mean ± SD, n=4, paired t-tests) from equimolar concentrations of AM. Mitochondria were pooled from 8 hamsters for each n value.

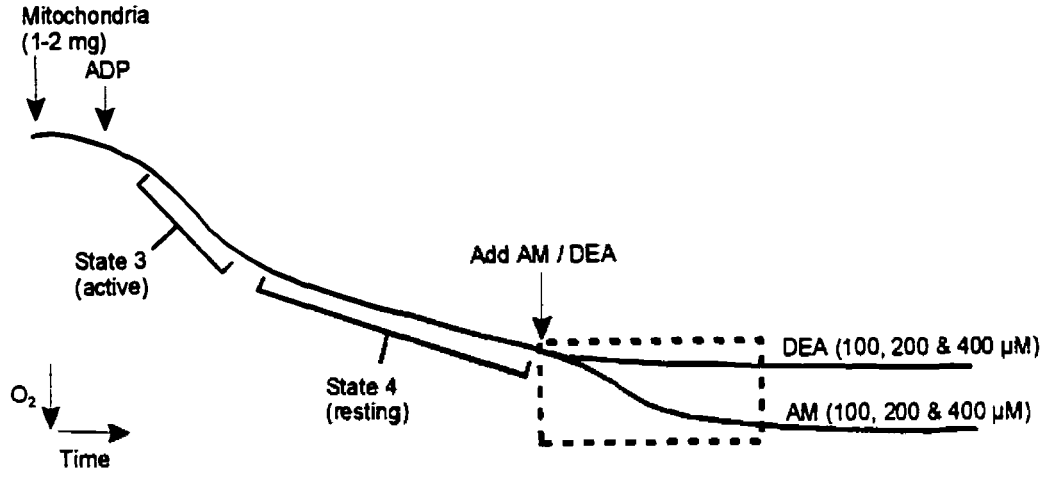


Figure 3.12. Representative lung mitochondrial oxygen consumption tracing depicting the effect of AM or DEA on state 4 respiration supported by complex I. Note the immediate inhibition of respiration as a result of DEA addition, compared to the initial stimulation followed by secondary inhibition as a result of AM. Similar effects were observed for respiration supported by complex II in the presence of AM or DEA.

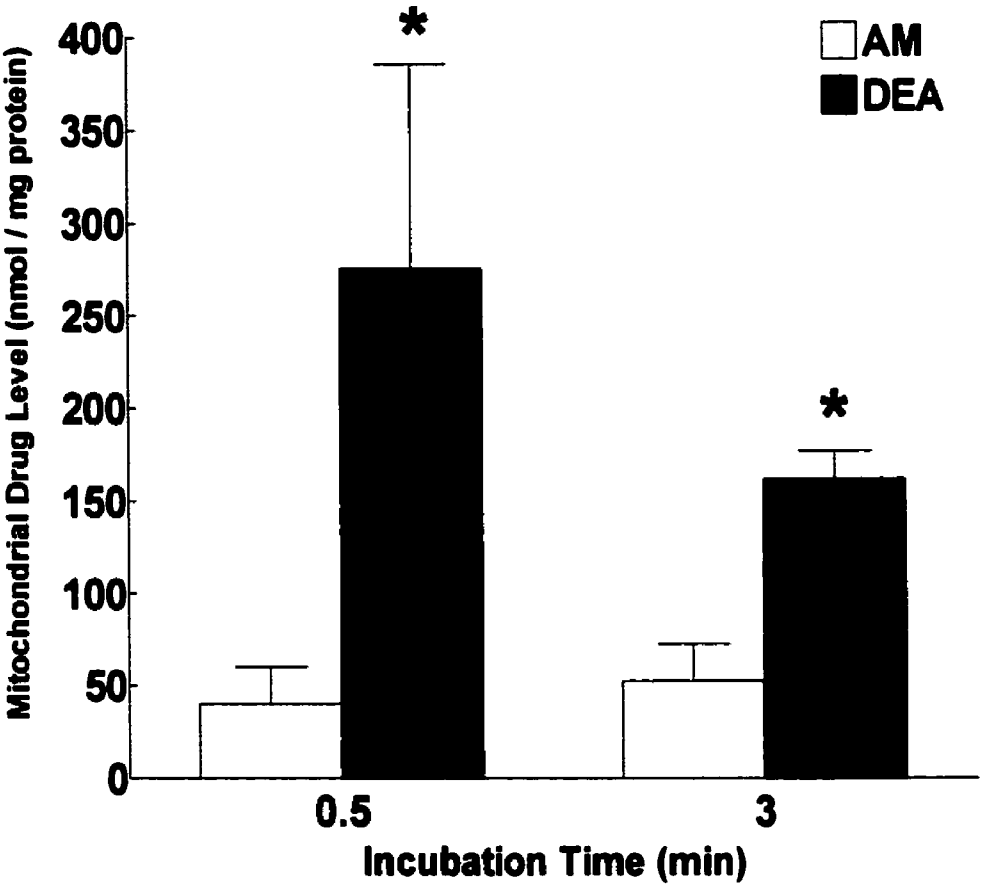


Figure 3.13. Mitochondrial AM and DEA levels (mean ± SD) in isolated whole lung mitochondria following incubation with 400 μM AM or DEA. * significant difference from AM at the same time point ($p<0.05$, n=3, repeated measured two-way ANOVA). Mitochondria were pooled from 8 hamsters for each n value.

mitochondrial function and loss of cellular ATP as pivotal events in initiation of AM cytotoxicity in the lung. Incubation with AM for 2 h resulted in decreased intracellular mitochondrial membrane potential in all cell preparations. However, no effects on ATP levels or viability were observed at this time. At 4 h in macrophages and 6 h in type II cell and Clara cell preparations, ATP levels decreased. Overt AM-induced cell death in alveolar macrophages, type II cells and Clara cells did not occur until 12 h and was more pronounced at 24 and 36 h (Bolt et al., 1998), (Figure 3.6). Therefore, (as summarized in Figure 3.14) during AM-induced cytotoxicity, mitochondrial function is disrupted prior to ATP depletion and ultimately cell death in freshly isolated target lung cell types. The early occurrence of AM-induced perturbation of mitochondrial function is suggestive of an initiating event in cytotoxicity.

AM-induced perturbations of mitochondrial structure (Guerreiro et al., 1986a; Yasuda et al., 1996), and function (Card et al., 1998; Fromenty et al., 1990a; Guerreiro et al., 1986b; Matola et al., 2000) have been reported previously. However, those experiments were performed in isolated mitochondria or non-pulmonary cells. Our experiments confirmed disruptive effects of AM on mitochondrial function and examined, for the first time, these effects in freshly isolated, intact, target lung cell types. This was important because it allowed for the assessment of AM-induced effects in the presence of factors such as biotransformation enzymes, reducing agents, antioxidants, etc. which are greatly altered in isolated mitochondria and cultured cells. Although there are advantages and disadvantages to isolated organelles and cultured

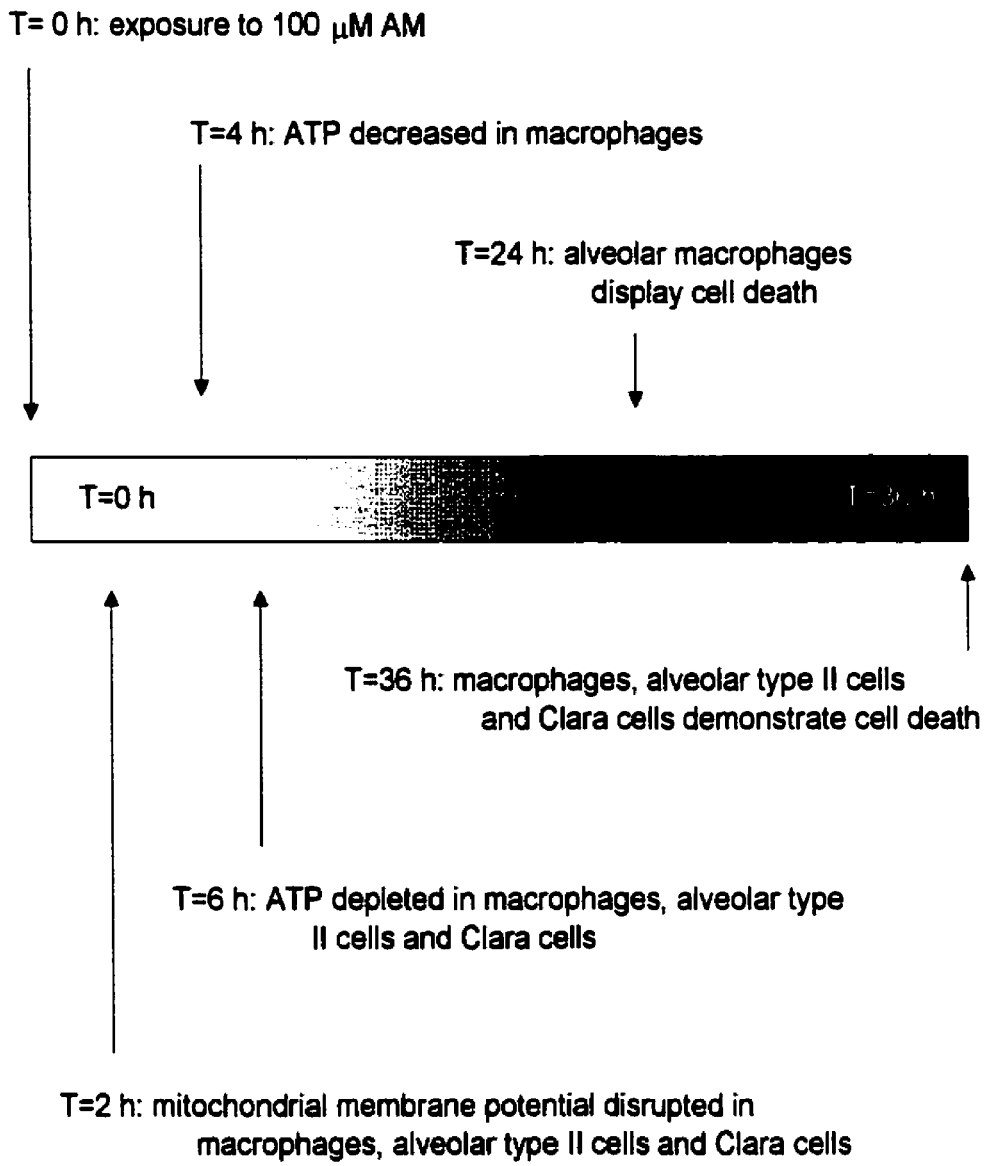


Figure 3.14. Time course of events in AM-induced cytotoxicity.

cells, freshly isolated cells more accurately reflect cellular properties as they occur *in situ* (Massey, 1989).

JC-1 has been used to measure mitochondrial membrane potential in other isolated cell types (Di Lisa et al., 1995; Salvioli et al., 1998; White and Reynolds, 1996). However, to our knowledge, this is the first report to use JC-1 in freshly isolated lung cells. JC-1 has the advantage of being more specific for mitochondrial (as opposed to plasma) membrane potential and more consistent in its response to depolarization, than other dyes such as rhodamine 123, and 3,3'-dihexyloxacarbocyanin iodide ($DiOC_6$) (Salvioli et al., 1997). Furthermore, flow cytometry allows measurement of fluorescence of each cell, a property particularly useful in the Clara cell preparations, where flow cytometer gates can be adjusted to measure fluorescence from only the larger cells (thereby excluding contributions of contaminating blood cells).

Previous studies have indicated the ability of AM to uncouple mitochondrial electron transport (Fromenty et al., 1990a; Ribeiro et al., 1997). Our observation that the time-course of AM-induced disruption of lung mitochondrial membrane potential relative to cytotoxicity resembled that of FCCP, an established uncoupler of electron transport, suggests that the mechanism of mitochondrial disruption resulting in cytotoxicity for AM and FCCP may be the same.

Mitochondrial permeability transition (MPT), a phenomenon recognized to be important in both necrosis and apoptosis for some agents, is a sudden increase in permeability of the inner mitochondrial membrane, resulting in

membrane depolarization, uncoupling of oxidative phosphorylation, mitochondrial swelling and release of intramitochondrial ions (Lemasters et al., 1998). MPT is regulated by a voltage dependent channel that is inhibited by micromolar concentrations of cyclosporin A, and cyclosporin A protects against cell death involving MPT-activating substances or events (Seaton et al., 1998). The failure of cyclosporin A to reduce AM-induced cytotoxicity suggests that MPT is not involved in AM-induced disruption of mitochondrial function. Also, exposure of isolated hamster lung cells to cyclosporin A concentrations above 1.0 μM caused loss of viability (data not shown).

Despite occurrence of mitochondrial disruption in AM-treated cells at 2 h, [^{3}H]ATP levels did not differ between control and AM-treated cells until approximately 4 to 6 h. Interestingly, we report, for the first time, that alveolar macrophages, the cell type most susceptible to 100 μM AM-induced cytotoxicity, demonstrated AM-induced ATP depletion earlier than other cell types. The reason for this phenomenon is not known but could be related to particularly high energy requirements of macrophages. Although it is not possible to completely rule out potential effects of AM on ATP utilization, observed decreases in ATP levels are consistent with disrupted mitochondrial function and hence, ATP synthesis.

Since AM decreased ATP levels prior to cell death, we hypothesized that AM-induced cytotoxicity would be reduced by preventing ATP depletion. Theoretically, ATP depletion might be diminished by adding glucose or niacin to the incubation medium. Glucose, via either glycolysis or the pentose phosphate

pathway, can generate ATP. Although glucose previously has been shown to decrease AM-induced lymphocyte cytotoxicity (Fromenty et al., 1993), its lack of effectiveness against lung cell cytotoxicity correlated with its inability to prevent AM-induced depletion of ATP.

Niacin has been found to prevent chemically-induced cytotoxicity by maintaining cellular NAD levels (Weitberg, 1989). However, similar to the situation for glucose, we found niacin to be ineffective at preventing AM-induced ATP depletion and cytotoxicity in hamster macrophages. Although the metabolic pathway for the synthesis of NAD may differ between cell types, this lack of effectiveness is consistent with that seen in cultured rat pulmonary macrophages (Nadeau and Lane, 1988). Thus, the partial attenuation of AM-induced pulmonary fibrosis previously reported for niacin (Wang et al., 1992) is likely attributable to some mechanism other than maintenance of ATP levels, such as decreased procollagen gene expression (Gurujeyalakshmi et al., 1996).

Although AM, DEA, and FCCP were cytotoxic in all of the cell preparations examined, intercellular differences were observed. Notably, 50 μM DEA caused significant loss of ATP and viability in Clara cell enriched preparations before effects were observed in macrophages and type II cells (Figure 3.9 and Table 1). Furthermore, Clara cells and type II cells were more susceptible to lower concentrations of DEA (25 μM) than macrophages. Similarly, in our earlier study, we found Clara cells to be more susceptible to a lower concentration of AM (50 μM) than were other hamster lung cell types (Bolt et al., 1998). Since both Clara cells and type II cells contain appreciable levels of cytochromes P450 (Massaro et al., 1993; Plopper et al., 1991), one might suggest a role for P450-catalyzed

biotransformation of AM and DEA in cytotoxicity. However, if P450-catalyzed bioactivation were central to the cytotoxicity of these agents, then macrophages, which have very low expression of P450 unless treated with a P450 inducer (Crystal, 1991; Raunio et al., 1900), should not be sensitive to AM or DEA toxicity. While this prediction holds true for macrophages exposed to 25 μM DEA (Figure 3.9) and 50 μM AM (Bolt et al., 1998), loss of macrophage viability due to 50 μM DEA (Figure 3.9) and 100 μM AM (Figure 3.6, and (Bolt et al., 1998) was substantial. Furthermore, the macrophage and Clara cell enriched preparations showed greater susceptibility to FCCP cytotoxicity than did the type II cell enriched preparation (Figure 3.5). Since FCCP is a direct-acting mitochondrial uncoupler, not requiring P450-catalyzed bioactivation, then the sensitivity of macrophages and Clara cells to FCCP may reflect a relative inability to overcome or survive disruption of cellular energy metabolism. This could also explain the early loss of Clara cell viability seen with DEA and a low concentration of AM (Bolt et al., 1998). It is also supported by the observation that cell preparations demonstrating the greatest depletion of ATP tended to show the greatest loss of viability later during incubations (Figure 3.3 and Table 1).

In all hamster cell types examined, DEA was much more cytotoxic than AM, causing cell death at a lower concentration (as summarized in Figure 3.15). This suggests a potentially important contribution of DEA in clinical AIPT, particularly considering the fact that substantial accumulation of DEA occurs in lung tissues of patients undergoing chronic AM therapy (Brien et al., 1983; Brien et al., 1987). The explanation for the relatively high inter-experiment variability in susceptibility of macrophages to 50 μM DEA-induced cytotoxicity (Figure 3.9b), is not readily apparent.

The link between disruption of mitochondrial function and loss of cell viability was also supported by the inhibition by AM and DEA of state 4

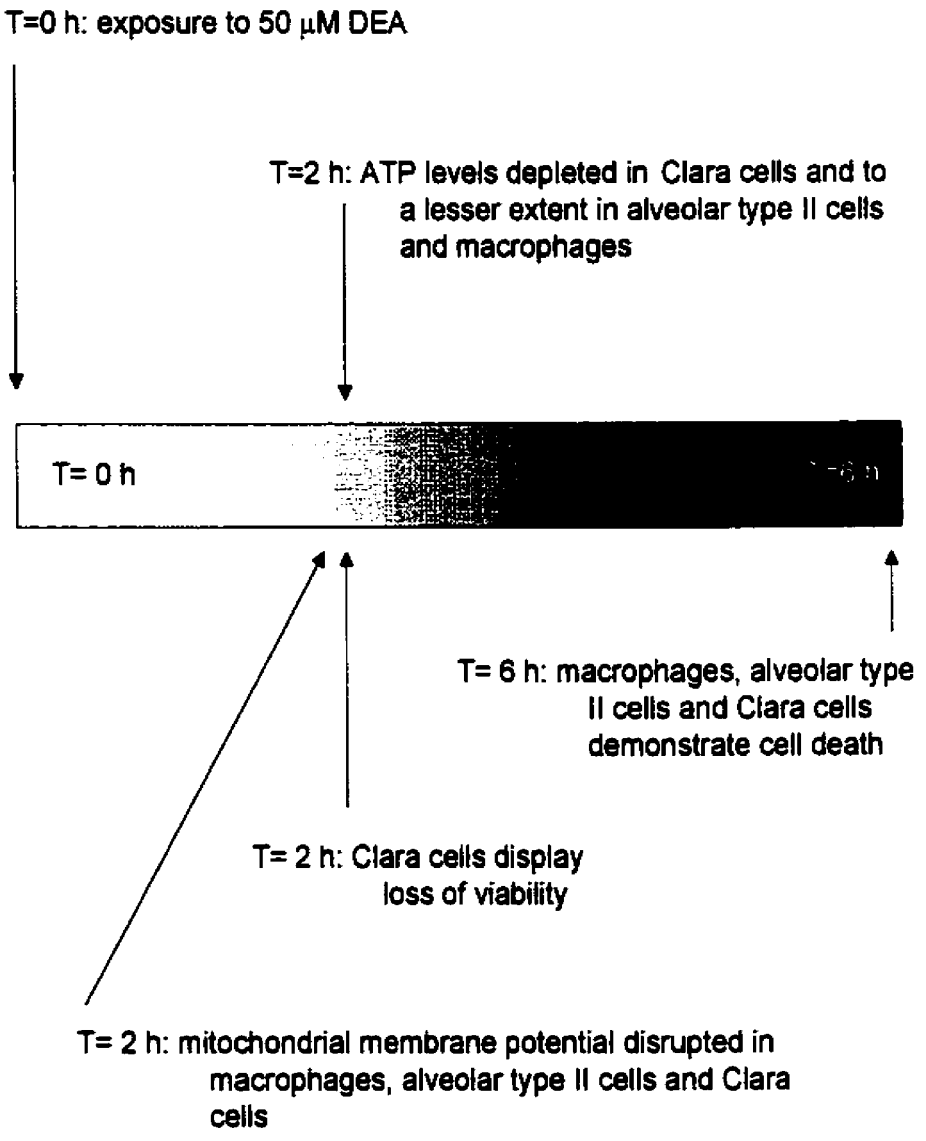


Figure 3.15. Time course of events in DEA-induced cytotoxicity.

respiration in whole lung mitochondria. The biphasic effects of AM may be associated with rapid accumulation of protonated AM within the mitochondrial matrix, resulting in release of protons, and hence initial stimulation of respiration due to an uncoupling effect; the subsequent inhibition is presumed to be due to further accumulation of AM (Card et al., 1998; Fromenty et al., 1990a). The monophasic inhibition caused by DEA has been observed in other tissues (Fromenty et al., 1990a). Of particular note were the more rapid inhibition of respiration by DEA, which was associated with greater accumulation into the mitochondria relative to AM, and greater inhibition of complex II by DEA. Although the relative accumulation of AM and DEA into mitochondria may differ in intact cells, the greater effects of DEA in isolated mitochondria coincided with the greater cytotoxicity of the N-dealkylated AM metabolite compared to AM itself. Also, the rapidity of onset of effects in isolated mitochondria relative to intact cells is consistent with a lack of barriers for diffusion in the former.

In conclusion, AM and DEA disrupt mitochondrial function prior to cellular ATP depletion and subsequent cell death in freshly isolated hamster lung cells, suggesting that perturbation of lung cell mitochondrial function initiates AM-induced cytotoxicity (Figures 3.14 and 3.15). Since cell death occurs early relative to the onset of fibrosis in animal models of AIPT (Taylor et al., 2001), then preventing collapse of mitochondrial membrane potential and / or subsequent ATP depletion might potentially prevent AM-induced cytotoxicity and ultimately pulmonary fibrosis. Furthermore, DEA is more cytotoxic and causes cytotoxicity faster than AM suggesting an important contribution of DEA to the development of AIPT.

CHAPTER 4

EFFECTS OF VITAMIN E ON CYTOTOXICITY OF AMIODARONE AND *N*-DESETHYLAMIODARONE IN ISOLATED HAMSTER LUNG CELLS

4.1 INTRODUCTION

Many adverse effects are associated with the clinical use of AM, including pulmonary fibrosis. AM-induced pulmonary toxicity (AIPT), occurring in 1-13% of patients receiving relatively high doses of AM (Martin and Rosenow, 1988) and in 1.6% of patients receiving less than or equal to 400 mg / day (Sunderji et al., 2000), is the adverse effect of greatest concern due to its high potential for mortality.

Formation of an AM radical has been proposed to be involved in AM cytotoxicity (Li and Chignell, 1987; Vereckei et al., 1993). However, reactive oxygen species do not appear to be involved (Leeder et al., 1996; Matola et al., 2000). Therefore, preventing the formation of, or the damage caused by, an AM radical may be effective for preventing AM-induced lung cell death, and ultimately ameliorating AIPT.

Vitamin E is a lipid soluble radical-scavenging agent. It has been demonstrated by others that dietary supplementation with vitamin E for 6 weeks prior to a single intratracheal dose of AM decreases AM-induced histological and biochemical indices of pulmonary fibrosis (Card et al., 1999a). However, the mechanism of vitamin E protection against AM-induced pulmonary fibrosis *in vivo* is not known.

Previously (Chapter 2 and 3), it was shown that hamster alveolar macrophages, alveolar type II cells and non-ciliated bronchiolar epithelial (Clara) cells are susceptible to cytotoxicity induced by AM and its N-dealkylated metabolite, *N*-desethylamiodarone (DEA) (Bolt et al., 1998; Bolt et al., 2001). Although vitamin E has been shown to decrease AM-induced cytotoxicity in cultured pulmonary endothelial cells (Futamura, 1996b) and in non-pulmonary cell types (Honegger et al., 1995; Kachel et al., 1990; Ruch et al., 1991), the effects of this antioxidant on different AM-susceptible lung cell types have not been investigated.

The present study investigated the effects of vitamin E on AM- and DEA-induced cytotoxicity, cellular drug levels, and lipid peroxidation in freshly isolated hamster lung cells. Identification of (a) particularly sensitive lung cell type(s) for vitamin E protection against AM-induced cytotoxicity may provide information about the mechanism of cell death responsible for initiating AIPT and potentially the mechanism by which vitamin E decreases AM-induced pulmonary fibrosis.

4.2 METHODS AND MATERIALS

4.2.1 Chemicals

Chemicals were obtained from the following suppliers: amiodarone HCl, DNAse type I, protease type I, trypsin inhibitor, heparin sulphate, RPMI 1640 media, antibiotic/antimycotic (containing penicillin, streptomycin, and amphotericin B), ethylene glycol-bis(-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), and (±)α-tocopherol (vitamin E) acetate from Sigma Chemical Co., St.

Louis, Mo.; Percoll® from Pharmacia Biotech AB, Uppsala, Sweden; 4-(2-hydroxyethyl)-1-piperazine-*N*'-2-ethanesulfonic acid (HEPES) from Boehringer-Mannheim, Germany; and [^{3}H]Prostaglandin $F_{2\alpha}$, from Cayman Chemical Company, Ann Arbour, MI. *N*-desethylamiodarone was generously provided by Wyeth-Ayerst Research, Princeton, N.J. All other reagents were of analytical grade or better and purchased from standard chemical suppliers.

4.2.2 Animals

Male golden Syrian hamsters (140-160g) were obtained from Charles River Canada Inc., St-Constant, Que. Animals were housed at room temperature on a 12-h light - 12 h dark schedule (lights on at 0700 h) for a minimum of 1 week prior to experimentation. Hamsters were fed Purina rodent chow and water *ad libitum*, and were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

4.2.3 Enriched Lung Cell Preparations

The procedures followed for isolating alveolar macrophages and obtaining enriched preparations of alveolar type II cells, and non-ciliated bronchiolar epithelial (Clara) cells from unseparated cells (cell digest) are described in detail elsewhere (Chapter 2 and (Bolt et al., 1998). Briefly, hamsters were injected with heparin sodium (2000 IU in 0.5 ml of saline) 45 min prior to anesthetization with sodium pentobarbital (300 mg/kg i.p.). Alveolar macrophages were obtained by bronchoalveolar lavage. Other lung cells were released by treatment with

protease type I, and enriched preparations of alveolar type II and Clara cells were obtained by centrifugal elutriation. The alveolar type II cell preparation was further enriched by Percoll® density gradient centrifugation (28% (v/v) in RPMI 1640 media). Viabilities and cell numbers were assessed on a haemacytometer by light microscopy using 0.5% trypan blue dye exclusion. Alveolar type II cells and Clara cells were identified using modified Papanicolaou (Kikkawa and Yoneda, 1974) and nitroblue tetrazolium (Devereux and Fouts, 1980) staining, respectively. For each experiment, cells isolated from 12 hamsters were pooled.

4.2.4 Cell Incubations and Viability Assessment

Following the enrichment procedure, cell digest, and fractions enriched with alveolar macrophages, alveolar type II cells and Clara cells (8.0×10^5 / well) were incubated in flat-bottomed, irradiated, nonadhesive, polystyrene Nunc™ 96 microwell plates (GIBCO Canadian Life Technologies, Burlington, ON) with vehicle (0.1% ethanol) ± 300 µM vitamin E, 50 µM DEA ± 300 µM vitamin E, or 100 µM AM ± 300 µM vitamin E under 95% air-5% CO_2 at 37^0C. These concentrations of AM and DEA were selected because they were representative of postmortem concentrations in the lungs of patients who had been treated with AM (Brien et al., 1987). Furthermore, we previously found these concentrations of DEA and AM to cause reproducible cytotoxicity in isolated hamster lung cells (Bolt et al., 2001). In other studies investigating protective effects of vitamin E against AM-induced cytotoxicity, reported concentrations of vitamin E ranged from one-half to 10 times the concentration of AM (Futamura, 1996b; Kachel et

al., 1990; Ruch et al., 1991). However, concentrations of vitamin E above 300 μM were insoluble in the incubation media we employed, so 300 μM vitamin E (i.e. three times the AM concentration) was used. Cell viability was measured by 0.5% trypan blue dye exclusion because it represents irreversible loss of plasma membrane integrity, a late event in processes leading to cell death (Tyson and Green, 1987).

4.2.5 Quantitation of AM and DEA Content in lung cells

Intracellular levels of AM or DEA were measured by reverse-phase HPLC with UV-visible spectrophotometric detection at 254nm (Bolt et al., 1998; Brien et al., 1987). AM or DEA content was determined by interpolation of the chromatographic signal area on an aqueous AM or DEA standard curve that was prepared for each day of analysis.

4.2.6 Measurement of Lipid Peroxidation

Cells (1×10^6 / well) were incubated with 50 μM DEA ± 300 μM vitamin E, 100 μM AM ± 300 μM vitamin E or 500 μM carbon tetrachloride (CCl_4) for 4 h. Samples were removed from the wells, centrifuged (1000 x *g* for 3 min) and the supernatants removed. The inside of the tube containing the pellet was wiped with a cotton swab to remove any excess media. Pellets were weighed and both pellet and supernatant fractions snap frozen in liquid nitrogen and stored at -80^{0}C for later analysis.

F2-isoprostanes, indicative of lipid peroxidation, were measured by enzyme immunoassay (Caymen Chemicals, Ann Arbour, MI, USA). On the day of analysis, total (i.e. free plus esterified) 8-isoprostanes were extracted from the pellets according to the procedure provided with the assay kit. Briefly, 2.0 ml of ethanol was added to each pellet which was then mixed vigorously, allowed to stand at 4^0C for 5 min, and centrifuged at 1500 x *g* for 10 min to remove precipitated proteins. 5000 cpm of [^{3}H]$PGF_{2\alpha}$ (which acted as an internal standard) and 2.0 ml of 15% KOH was added to the supernatant. The mixture was incubated at 40^0C for 1 h and diluted to 10.0 ml with water (pH adjusted to below 4 with HCl). The samples were purified using Sep-Pak C_{18} columns, dried under nitrogen and resuspended in 1.0 ml of enzyme immunoassay buffer. Tritium in a portion of the sample was quantitated radiometrically to calculate the percent recovery of the internal standard. Isoprostanes extracted and purified from sample pellets were determined using the enzyme immunoassay and measured spectrophotometrically (λ=405 nm).

4.2.7 Data Analysis

All data are expressed as sample group means ± standard deviation (SD). Differences between treatment groups were determined by repeated measures analysis of variance (ANOVA) followed by Newman-Keuls post hoc test or by paired t-tests, as indicated (Sokal and Rohlf, 1973). In all cases, $p<0.05$ was considered statistically significant.

4.3 RESULTS

Bronchoalveolar lavage fluid contained 98% alveolar macrophages, and cell digest contained approximately 21% alveolar type II cells and 4% Clara cells. Following centrifugal elutriation, the third elutriation fraction (E3) contained 50-60% alveolar type II cells and E4 contained 35-50% Clara cells. Percoll® density gradient centrifugation of E3 further enriched the alveolar type II cell fraction to 75-85%. As reported previously, the alveolar type II cell preparation also contained lymphocytes (5-10%) and a few Clara cells (2-5%). Ciliated cells (10-15%) clumped red blood cells (10-15%), and a relatively small percentage (2-10%) each of fibroblasts, polymorphonuclear leukocytes and type II cells were present in the Clara cell preparation, (Bolt et al., 1998).

The cell isolation procedures resulted in considerable enrichment of both type II cells and Clara cells. Since Clara cells represented a very small percentage of cells in the cell digest (4 %), enrichment was limited to 35 to 50 % in the Clara cell fraction. This purity is within the range of Clara cell enrichment previously obtained by us (Bolt et al., 1998) and by other authors studying different species (Plopper et al., 1991). Initial viabilities in all fractions were greater than 90%, and control viabilities did not decrease below 75% over the course of experiments.

Following incubation for 24 and 36 h, 100 μM AM decreased ($p < 0.05$) viability in cell digest and in fractions enriched with alveolar macrophages, alveolar type II cells and Clara cells, by 20-40% relative to control (Figure 4.1).

Addition of 300 μM vitamin E had no significant effect on control viability in any fraction or on AM-induced cell death in cell digest or in alveolar type II cell and Clara cell fractions. However, vitamin E decreased ($p<0.05$) AM-induced cytotoxicity in macrophages by approximately 12% at 24 h and 21% at 36h. (Figure 4.1).

Following incubation for 6 h, 50 μM DEA decreased ($p< 0.05$) viability in all cell preparations by 35 - 50 %, and loss of cell viability progressed to 24 h (Figure 4.2). Vitamin E had no effect on DEA-induced cytotoxicity (Figure 4.2). Alveolar macrophages demonstrated variable responses to DEA, as demonstrated by the standard deviations calculated for each group mean. Sometimes, DEA caused a substantial amount of cell death, while on other occasions DEA caused minimal cytotoxicity.

Following incubation for 24 h, vitamin E did not alter cellular accumulation of AM or DEA in cell digest, macrophage, type II cell or Clara cell fractions (Table 4.1).

CCl_4 increased ($p<0.05$) F_2-isoprostanes levels in cell digest, alveolar type cell and Clara cell fractions (Figures 4.3 and 4.4) by approximately 19 fold, 4 fold and 5 fold respectively. Relative to control, 100 μM AM and 50 μM DEA had no effect on isoprostane content in macrophages, or in enriched type II cell or Clara cell preparations, but did increase ($p<0.05$) cell digest isoprostane levels 18 and 12 fold respectively (Figures 4.3 and 4.4). No differences in isoprostane levels were observed between AM-treated and AM + vitamin E-treated or between DEA- and DEA + vitamin E-treated cells for alveolar macrophage,

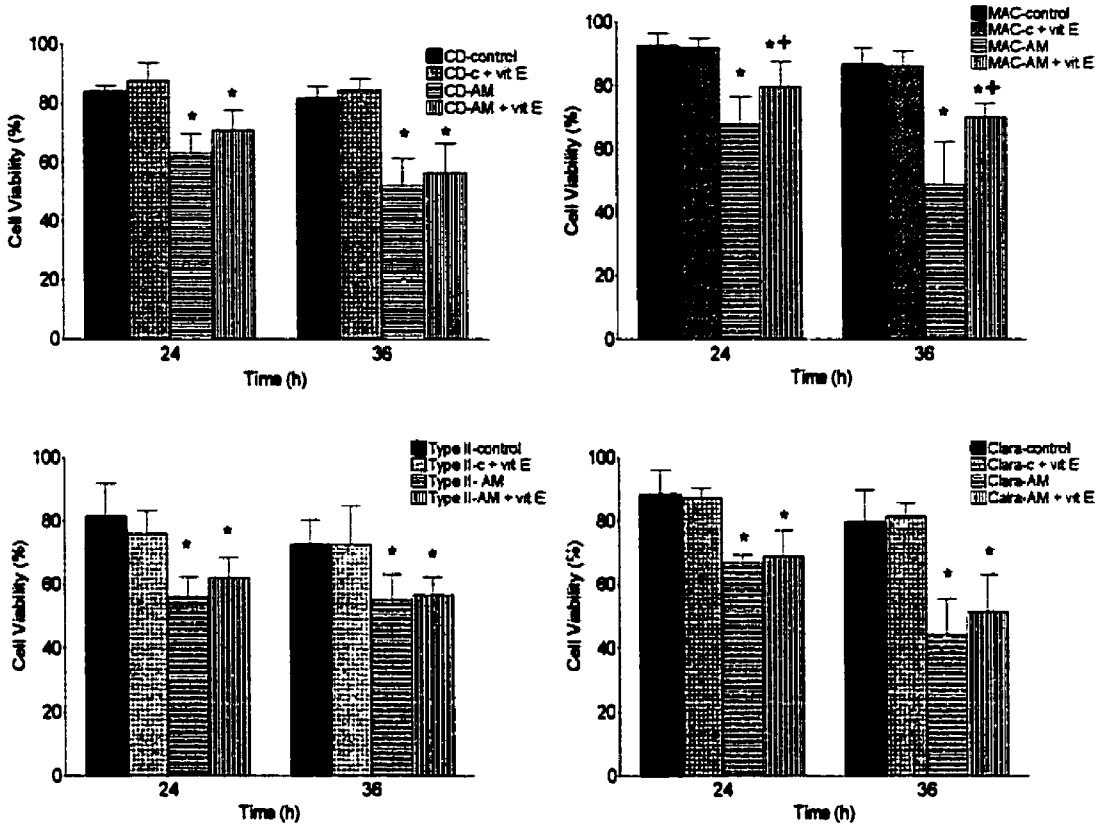


Figure 4.1. Effects of 300 μM vitamin E on 100 μM AM-induced loss of cell viability in cell digest (CD) and in fractions enriched with macrophages (MAC), type II cells (Type II) and Clara cells (Clara). * significant difference from respective control ($p < 0.05$, repeated-measures ANOVA with Newman-Keuls post hoc test, n=4). + significant difference ($p<0.05$) from all other macrophage experimental conditions.

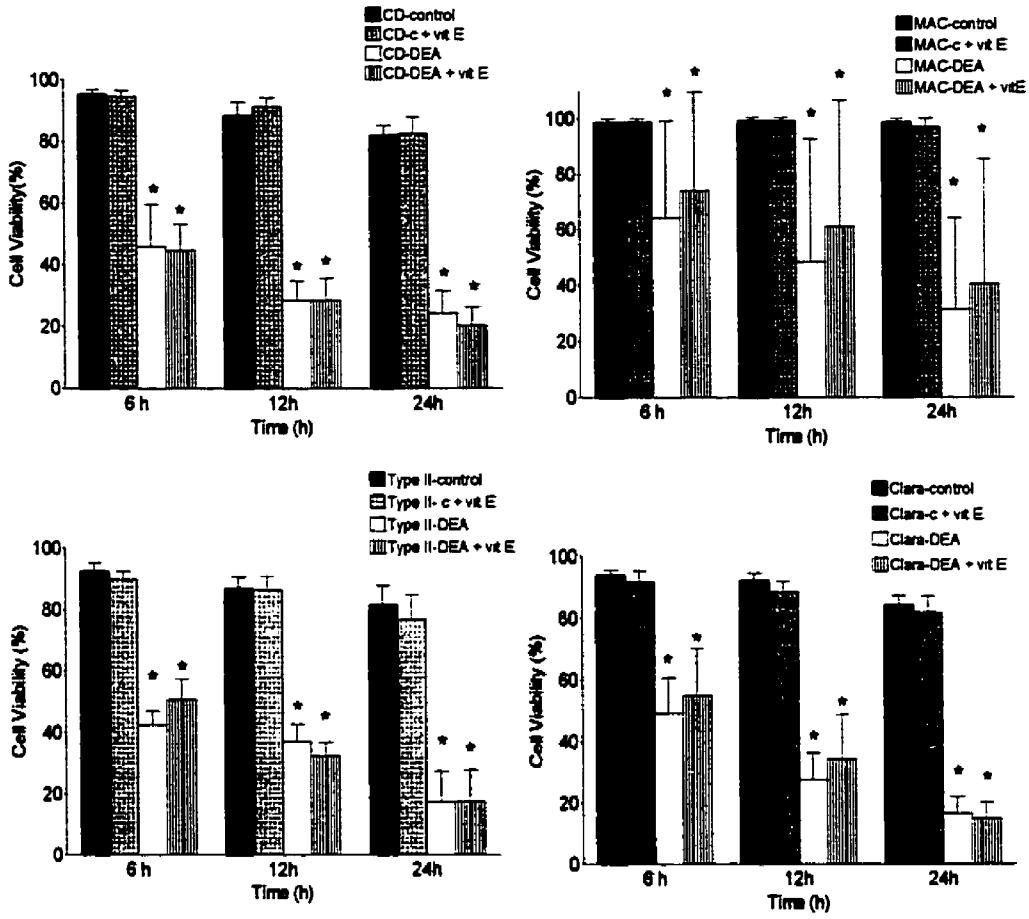


Figure 4.2. Effects of 300 μM vitamin E on 50 μM DEA-induced loss of cell viability in cell digest (CD) and in fractions enriched with macrophages (MAC), type II cells (Type II) and Clara cells (Clara). * significant difference from respective control ($p < 0.05$, repeated-measures ANOVA with Newman-Keuls post hoc test, n=4).

Table 4.1. Cellular levels of AM or DEA after incubation with 100 μM AM or 50 μM DEA with or without vitamin E for 24 h ($p > 0.05$, paired t-tests, n=4).

Cell Fraction	AM content (μg / g cell pellet) (Mean ± SD, n=4)		DEA content (μg / g cell pellet) (Mean ± SD, n=4)	
	AM	AM + Vitamin E	DEA	DEA + Vitamin E
Cell Digest	398 ± 271	394 ± 329	493 ± 389	428 ± 157
Macrophages	539 ± 231	412 ± 150	427 ± 119	479 ± 176
Type II Cells	581 ± 413	440 ± 242	743 ± 565	442 ± 169
Clara Cells	472 ± 293	387 ± 208	537 ± 288	402 ± 222

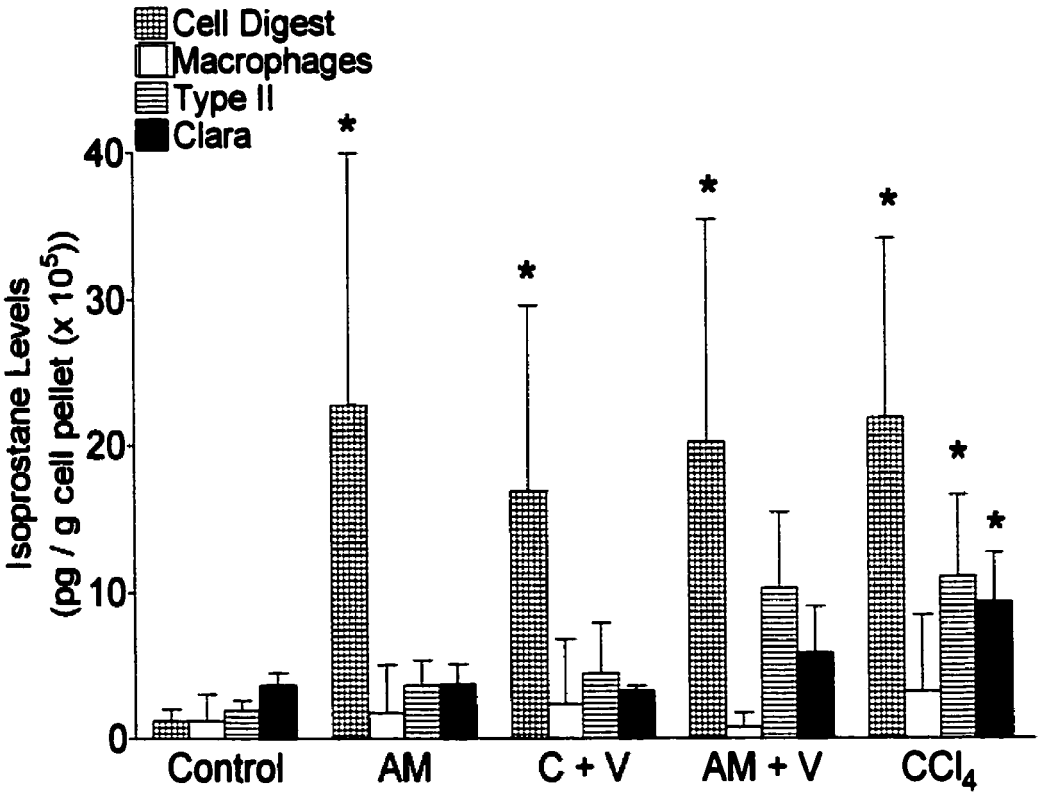


Figure 4.3. Isoprostane levels extracted from cells following incubation with vehicle (0.1% ethanol) control (C) ± 300 μM vitamin E (V), 100 μM AM ± 300 μM vitamin E or 500 μM CCl_4 for 4 h. * significant difference from control ($p<0.05$, repeated-measures ANOVA with Newman-Keuls post hoc test, n=5).

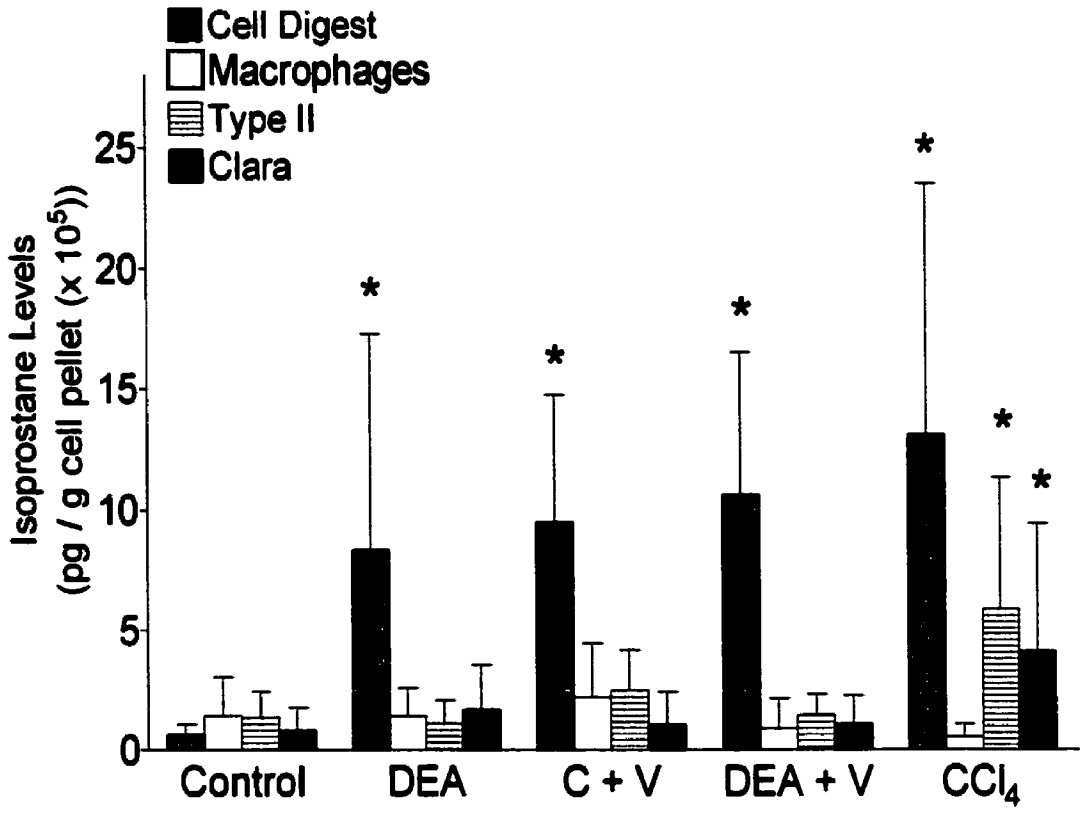


Figure 4.4. Isoprostane levels extracted from cells following incubation with vehicle (0.1% ethanol) control (C) ± 300 μM vitamin E (V), 50 μM DEA ± 300 μM vitamin E or 500 μM CCl_4 for 4 h. * significant difference from control ($p<0.05$, repeated-measures ANOVA with Newman-Keuls post hoc test; n=5).

alveolar type II cell or Clara cell fractions. Paradoxically, vitamin E alone increased control isoprostane levels in all cell digest fractions but not in any of the enriched fractions (Figures 4.3 and 4.4).

CCl_4 caused a small decrease in viability in macrophage (10%) and Clara cell (14%) enriched fractions relative to respective controls at 4h, the timepoint at which cellular isoprostane levels was determined (Figure 4.5). AM had no effect in any enriched fraction (Figure 4.5A) but DEA caused approximately 25% loss of viability in the Clara cell fraction at 4 h (Figure 4.5B).

4.4 DISCUSSION

In the present study, we report that vitamin E decreases AM-induced cytotoxicity in hamster alveolar macrophages, but not in cell digest or enriched type II cell or Clara cell fractions (Figure 4.1 and 4.2). Previous investigations of vitamin E protection against AM cytotoxicity in different cultured cell types have produced variable results (Futamura, 1996b; Honegger et al., 1995; Kachel et al., 1990; Ruch et al., 1991). Results from Chapter 4 demonstrated that partial protection against 100 μM AM cytotoxicity by vitamin E is cell-type dependent in the lungs of the hamster, a species that manifests AM-induced pulmonary fibrosis resembling human AIPT (Daniels et al., 1989; Kennedy, 1990). The variable response of macrophages to 50 μM DEA suggests that this xenobiotic concentration may be near a threshhold for DEA-induced cytotoxicity in macrophages. The fact that vitamin E did not provide any protection against DEA cytotoxicity suggests that,

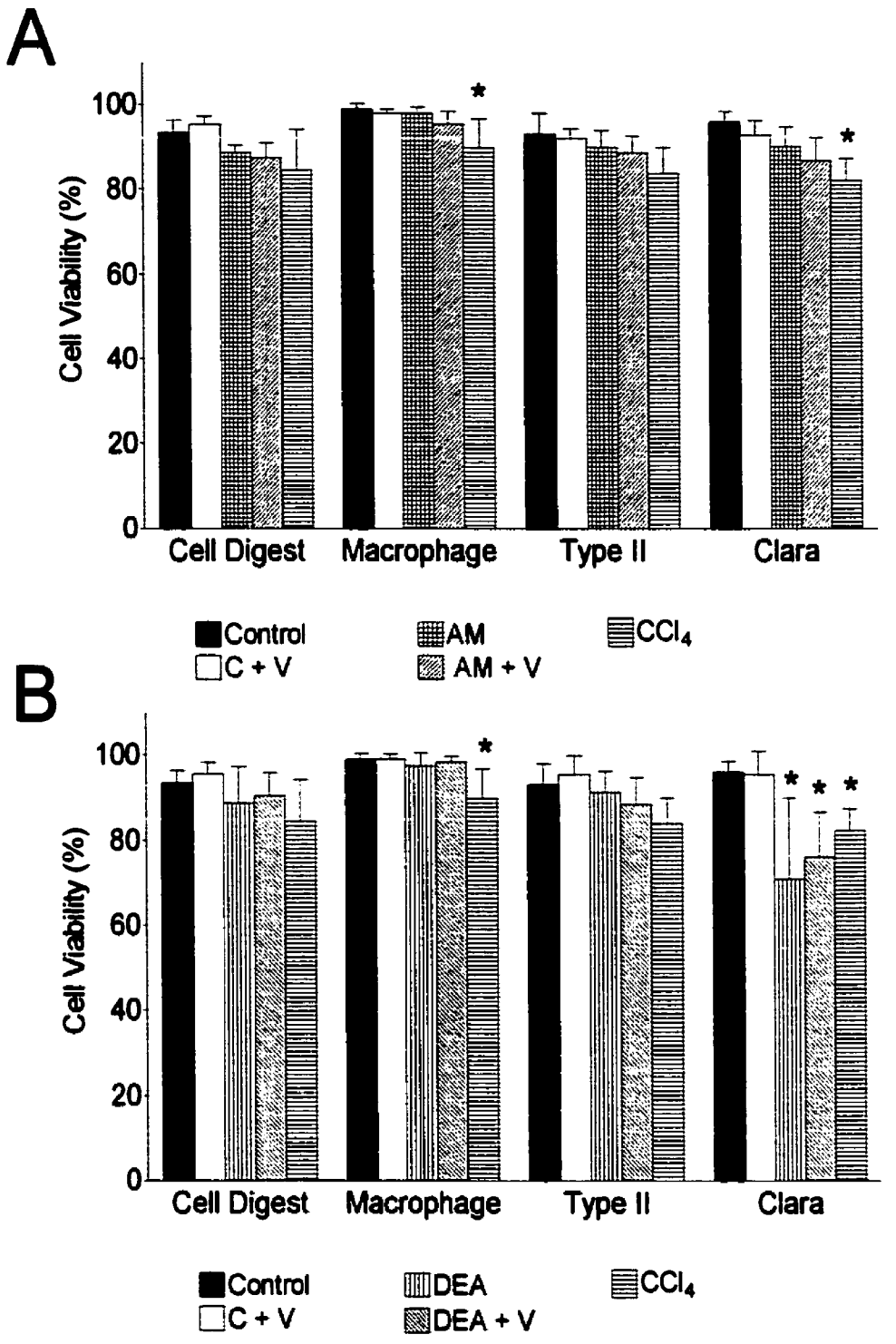


Figure 4.5. Viability of cells incubated with vehicle (0.1% ethanol) ± 300 μM vitamin E, 500 μM CCl_4, and A) 100 μM AM ± 300 μM vitamin E or B) 50 μM DEA ± 300 μM vitamin E for 4 h. * significant difference from control ($p<0.05$, repeated-measures ANOVA with Newman-Keuls post hoc test, n=4).

due to its greater toxic potency, the cytotoxic insult of DEA was too great to be overcome by vitamin E, or that differences exist between the mechanisms of AM- and DEA-induced cytotoxicity.

Several potential explanations exist for the macrophage-selective protective effects of vitamin E against AM-induced cytotoxicity. Since vitamin E can stabilize membranes and alter membrane fluidity (Urano et al., 1988) it might decrease drug accumulation in target cells. However, no effect of vitamin E on cellular levels of either AM or DEA was observed in any cell fraction, including macrophages.

Another possibility is that vitamin E, a radical scavenging agent, prevents cellular damage due to an AM radical (Li and Chignell, 1987; Massey et al., 1995; Vereckei et al., 1993) or it acts by arresting lipid peroxidation initiated by AM. Although we cannot rule out a direct scavenging action of vitamin E on an AM radical, inhibition of lipid peroxidation is not a likely explanation for vitamin E's effects in the present study. Although the ability of AM to initiate lipid peroxidation in various biological systems has been equivocal (Blake and Reasor, 1995b; Card et al., 1998; Mansani et al., 1999; Vereckei et al., 1993), an important observation of the present study was that none of the cell fractions demonstrated evidence of AM- or DEA-initiated lipid peroxidation at 4 h, a time point prior to cell death in most fractions. In these experiments, we used the isoprostane assay because of its reliability, low susceptibility to artifactual positive results, and lack of interference by vitamin E relative to other assays (Halliwell and Chirico, 1993; Moore and Roberts II, 1998; Morrow and Roberts,

1997). The positive lipid peroxidation stimulated by CCl_4 in the type II cell and Clara cell fractions is consistent with the requirement for CCl_4 bioactivation by cytochrome P450, which is localized particularly in Clara cells and, to a lesser extent, in type II cells (Devereux, 1984; Nichols et al., 1992; Plopper et al., 1991), and hence validates the use of the isoprostane assay in this system. Since CCl_4 had no effect on isoprostane levels in macrophages, which contain much lower levels of cytochrome P450 enzymes than type II and Clara cells, the observed CCl_4-induced increases in isoprostane levels in alveolar type II cells and Clara cells were not likely due to nonspecific solvent effects. Although post-cell death cellular degradation must be carefully considered as a contributor to positive lipid peroxidation data, the relatively small degrees of loss of cell viability due to CCl_4 in macrophage and Clara cell enriched fractions at 4 h (figure 4.5) are unlikely to account for the magnitude of increased isoprostane formation produced by CCl_4 in the type II cell and Clara cell enriched fractions.

The unexpected stimulation of isoprostane production by AM, DEA and vitamin E in cell digest may be explained by the fact that this fraction contains large amounts of plasma and cellular debris, which are sources of free ferric ion (Fe^{3+}). Vitamin E can reduce Fe^{3+} to Fe^{2+}, and Fe^{2+}, via Fenton chemical reactions, can lead to production of oxyl radicals and, ultimately, lipid peroxidation (Halliwell and Gutteridge, 1999e). Similarly, AM has been shown to increase Fe^{3+} -induced lipid peroxidation (Vereckei et al., 1991).

In cultured human skin fibroblasts, vitamin E also decreases AM- and DEA-induced phospholipidosis (Honegger et al., 1995), a phenomenon observed

in alveolar macrophages exposed to AM or DEA both *in vitro* (Ogle and Reasor, 1990) and *in vivo* (Kannan et al., 1989b). However, a clear relationship between the appearance of phospholipidosis and AM-induced cell death has not been established.

In contrast to earlier results (Chapter 2), cellular levels of AM in macrophages were not greater than AM levels present in other cell types. Therefore, higher susceptibility of macrophages to AM-induced cytotoxicity is not likely due to greater AM accumulation (as proposed is Chapter 2).

In conclusion, vitamin E decreases AM-induced cytotoxicity in alveolar macrophages, but has no effect in other cell types or against DEA-induced cytotoxicity. Therefore, the protective effects of vitamin E *in vivo* may involve decreased AM-induced cytotoxicity in macrophages. AM and DEA do not cause hamster lung cell death by stimulating lipid peroxidation, and the protective mechanism of vitamin E does not appear to involve altering cellular AM or DEA accumulation or lipid peroxidation.

CHAPTER 5

TOXICITY OF AMIODARONE AND *N*-DESETHYLAMIODAREONE IN FRESHLY ISOLATED HUMAN LUNG CELLS

5.1 INTRODUCTION

The etiology of AM- and DEA-induced pulmonary toxicity is not known and may be multi-factorial (as reviewed in (Massey et al., 1995; Reasor and Kacew, 1996a)). Many different *in vitro* models have been used to investigate AM- and DEA-induced effects. Although cultured human and animal lung cells have been used (Bargout et al., 2000; Beddows et al., 1989; Matola et al., 2000; Wilson and Lippmann, 1990), cellular changes that occur due to the culturing process hamper these investigations. Rodent models demonstrate AM cytotoxicity *in vitro* (Bolt et al., 1998; Card et al., 1998; Ogle and Reasor, 1990; Padma and Setty, 1997; Rafeiro et al., 1990) and AM-induced pulmonary fibrosis *in vivo* (Daniels et al., 1989; Reinhart and Gairola, 1997; Taylor et al., 2000), similar to that produced in humans. However, an *in vitro* model that accurately assesses events occurring in human lung cells *in situ* does not exist.

Previously, investigations from our laboratory and others have demonstrated *in vitro* that alveolar macrophages and alveolar type II cells are susceptible to AM and DEA-induced cytotoxicity in both the hamster (Bolt et al., 1998) and the rat (Reasor et al., 1990; Wilson and Lippmann, 1996b). Furthermore, vitamin E, a lipid soluble radical scavenging agent, has been shown to decrease AM-induced toxicity both *in vivo* (Card et al., 1999b) and *in vitro* (Bolt et al., 1999; Bolt et al., 2001; Honegger et al., 1995; Kachel et al., 1990). In the

present study, we investigated effects of AM and DEA on viability of preparations enriched with freshly isolated, human alveolar macrophages, and alveolar type II cells as well as in a fraction of isolated, unseparated cells (cell digest). Also, the effect of vitamin E on AM-induced cell death and drug accumulation was investigated. Assessing effects of AM in human lung cells provides new relevant information regarding the actions of AM in human lung and allows the validation of animal-based models for studying mechanisms of AM-induced pulmonary toxicity.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

Chemicals were obtained from the following suppliers: amiodarone HCl, protease type XIV, trypsin inhibitor type II-S, deoxyribonuclease (DNAse) type I, α-tocopherol (vitamin E) acetate, RPMI 1640 media, antibiotic/antimycotic (containing penicillin, streptomycin, and amphotericin B), glucose-6-phosphate, ethylenediaminetetraacetic acid (EDTA), β-nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate dehydrogenase from Sigma Chemical Co., St. Louis, Mo.; Percoll® from Pharmacia Biotech AB, Dorval, PQ., 4-(2-hydroxyethyl)-1-piperazine-*N'*-2-ethanesulfonic acid (HEPES) was purchased from Boehringer-Mannheim; sodium pentobarbital from M.T.C. Pharmaceuticals (Mississauga, ON, Canada). *N*-desethylamiodarone hydrochloride was generously donated by Wyeth-Ayerst Research (Princeton, NJ). All other

reagents were of analytical grade and purchased from standard chemical suppliers.

5.2.2 Tissue Procurement

Human lung tissue devoid of macroscopically visible tumours, was obtained from Kingston General Hospital, in accordance with procedures approved by the Queen's University Ethics Review Committee for Research on Human Subjects. Following informed consent, peripheral lung sections (10-100 g) were removed during clinically indicated lobectomy. Immediately following its removal, tissue is placed in 0.9% NaCl solution and kept on ice. Elapsed time between surgical resection and initiation of cell isolation was approximately 15 min. Prior to lung cell isolation, a 1.5 cm^3 section was removed from the tissue and placed in buffered formalin. The fixed tissue was dehydrated, embedded in paraffin and 5 mm sections were stained with haematoxylin and eosin (Culling, 1976). They were then examined by light microscopy to confirm the absence of microscopic tumours. Patients were characterized with respect to age, gender, surgical diagnosis, possible occupational exposure to carcinogens, drug treatment one month prior to surgery, and smoking history. Patients were classed as former smokers if smoking cessation was greater than two months before surgery. This time interval was chosen to eliminate the inductive effects of cigarette smoke on biotransformation enzymes (McLemore et al., 1990).

5.2.3 Lung Cell Isolation

Human lung cells were isolated as described previously (Donnelly et al., 1996; Smith et al., 1999). Briefly, following protease digestion, a 2.0 ml aliquot of unseparated cells, referred to as cell digest, was saved, and the remaining 10.0 ml underwent centrifugal elutriation using a Beckman J2-21M/E centrifuge with a JE-6B elutriation rotor. Three elutriation fractions, each having a different profile of cell types, were collected. The first fraction (150 ml, 2200 rpm, 13.0 ml/min) contained primarily red blood cells and cellular debris and was not used in experiments. For cells from poorly perfused tissue specimens, which contained large amounts of red blood cells, up to 250 ml were collected in the first fraction. The second fraction (100 ml, 2200 rpm, 21.0 ml / min) comprised of 20 - 60 % alveolar type II cells and was saved for further enrichment. The third fraction (100 ml, 1200 rpm, 18.0 ml/min) consisting of 60 - 80 % macrophages, was saved for use in experiments.

Alveolar type II cells were further enriched from the second elutriation fraction by Percoll® density gradient centrifugation. Cells were layered on a 35 % (v/v) solution of Percoll® in RPMI-1640 medium and centrifuged at 1000 x *g* for 15 min. The layer of cells remaining on top of the Percoll® contained 65 - 85 % type II cells and was saved for use in experiments.

Cell viability and yield were estimated by 0.5% trypan blue dye exclusion on a haemacytometer (Seglen, 1972). Using the modified Papanicolaou stain (Kikkawa and Yoneda, 1974), alveolar type II cells were identified by the presence of stained lamellar bodies in the cytoplasm, while macrophages were

identified by the absence of lamellar bodies, large size, round shape and nucleus, and the absence of stained cytoplasmic granules.

5.2.4 Incubations and Viability Assessments

Cells (4 x 10^5 cells / well) were incubated in RPMI 1640 media in flat-bottomed, irradiated, nonadhesive, polystyrene Nunc 96 microwell plates (GIBCO Canadian Life Technologies, Burlington, ON) with vehicle (0.1% ethanol) ± 300 μM vitamin E, 100 μM AM ± 300 μM vitamin E, or 50 μM DEA ± 300 μM vitamin E under 95% air-5% CO_2 at 37^0C. These concentrations of AM and DEA were selected because we previously found them to cause appreciable AM and DEA-induced loss of viability in hamster lung cells (Bolt et al., 1998; Bolt et al., 2001) and they are within the range of concentrations of AM and DEA present in human lungs following clinical treatment with AM (Brien et al., 1987; Plomp et al., 1984). A concentration of 300 μM vitamin E was used because it provided significant protection against AM-induced cytotoxicity in hamster alveolar macrophages (Bolt et al., 1999), and because concentrations of vitamin E above 300 μM were insoluble in the incubation media. Following incubation for 12, 24 and 36 h, viability was measured by 0.5% trypan blue dye exclusion.

5.2.5 Quantification of AM in Lung Cells

Intracellular levels of AM and DEA were measured by reverse phase HPLC with spectrophotometric detection at 254nm (Bolt et al., 1998; Brien et al., 1983; Brien et al., 1987). Amounts of AM or DEA were determined by

interpolation of the chromatographic signal peak area on an aqueous AM or DEA standard curve that was prepared for each day of analysis.

5.2.6 Lung Microsomes

Peripheral human lung microsomes were prepared from frozen lung tissues using standard differential centrifugation and subcellular fractionation techniques (Daniels et al., 1990b; Donnelly et al., 1996). Lung tissue was initially cut into 1.5cm^3 sections, wrapped in aluminum foil and snap frozen in liquid N_2 and stored at –80 ^{0}C until microsomal preparation. Protein concentration was determined by the method of Lowry *et al.* (1951). Microsomes (1mg) were incubated in 1 ml of 0.2 M phosphate buffer containing 200 mM glucose-6-phosphate, 100 mM $MgCl_2$, 20 mM ethylenediaminetetraacetic acid (EDTA), 25 mM β-nicotinamide adenine dinucleotide phosphate (NADP), and 20 U /ml glucose-6-phosphate dehydrogenase. Reactions were initiated by adding AM (500 μM) dissolved in 10 μl of absolute ethanol. Samples were incubated for 1 h in a shaking water bath at 37^{0}C. Reactions were stopped by adding 2.0 ml of HPLC mobile phase (acetonitrile : 5% (v/v) aqueous acetic acid, 80:20 (v/v) adjusted to pH 5.9 with ammonium hydroxide) to the incubation mixture. The contents were mixed and allowed to stand for 15 min before being centrifuged at 1350 x *g* for 5 min. Aliquots were analyzed by HPLC as described above in section 5.2.5. Because the amount of human lung microsomes was extremely limited, the DEA standard curve was generated using various concentrations of DEA dissolved in microsomal incubation mixture containing rat lung microsomes.

5.2.7 Statistical Analysis

Data are expressed as mean ± standard deviation and as individual patient's results. In all cases, $p < 0.05$ was considered statistically significant. Differences in viability of single determinanats were obtained by repeated measures one way analysis of variance (ANOVA) followed by Newman Keuls post hoc. Effects of vitamin E on AM and DEA accumulation were examined by paired t-tests.

5.3 RESULTS

5.3.1 Patient Demographics

Cells isolated from sections of peripheral lung obtained from a total of five human subjects (four males and one female) from Eastern Ontario, aged 58.2 ± 7.2 years, were used to assess AM- and DEA-induced cytotoxicity ± vitamin E (Table 5.I). Based on reported smoking histories, four individuals were current smokers, and one was a former smoker.

Microsomes isolated from sections of peripheral lung obtained from a total of five human subjects (four males and one female) from Eastern Ontario, aged 61.6 ± 10.2 years, were used to measure AM biotransformation (Table 5.2). Based on reported smoking histories, four individuals were current smokers, and one was a former smoker.

Table 5.1. Patient Demographics for Isolated Lung Cell Experiments

Patient [a]	Age (yrs)	Sex	Smoking History	Diagnosis leading to surgery	Possible occupatinal exposure to carcinogens	Drug Treatment [b] 1 month prior
137	45	M	Current (49.5 pckyrs)	Adenocarcinoma / pulmonary embolism	None	Diazepam, Fluvoxamine Maleate (luvox), Warfarin Sodium (Coumadin)
139	59	M	Current (26.9 pckyrs)	Adenocarcinoma (poorly differentiated)	None	Metoprolol, ASA
146	58	M	Current (69 pckyrs)	Adenocarcinoma (poorly differentiated)	None	Chlordiazapoxide (Librium), Carbamazapine (tegretol), ASA (Novasen), floctafenine (Idarac), Lamictal (Lamictal)
151	66	F	Former (20 yrs) 15 pckyrs)	past history of breast cancer/ clusters of large abnormal cells, suspicious for adenocarcinoma	None	Nifedipine, (adalat), levothyroxine (synthroid)
152	63	M	Current (45 pckyrs)	non-small cell carcinoma/ large cell carcinoma	None	none

M, male; F, female; pckyrs, pack years (no. of packs smoked per day x no. of years; 1 pack year = 1 pack per day for 1 year); ASA, acetylsalicylic acid.

[a] For confidentiality, patients are coded and assigned numbers.

[b] Generic drug name (proprietary name)

Table 5.2. Patient Demographics for Whole Lung Microsome Experiments

Patient [a]	Age (yrs)	Sex	Smoking History	Diagnosis leading to surgery	Possible Occupational Exposure to carcinogens	Drug Treatment [b] 1 month prior
9DM	60	F	Current (lifelong) (pckyrs N/A)	Hemoptysis/ carcinoid tumour	None	None
1EM	77	M	Former for 20 yrs (40 pckyrs)	Bronchogenic adenocarcinoma	None	Salbutamol (Ventolin)
3GM	67	M	Current (heavy) (pckyrs N/A)	Squamous cell carcinoma	None	None
3JM	46	M	Current (50 pckyrs)	Adenocarcinoma	None	Diazepam (Valium), fluvoxamine maleate (Luvox), warfarin sodium (Coumadin)
9JM	58	M	Current (69 pckyrs)	Adenocarcinoma (poorly differentiated)	None	Chlodiazepoxide HCl (Librium), Carbamazapine, Lamitrogine (Lamictal), ASA (Novasen)

M, male; F, female; pckyrs, pack years (no. of packs smoked per day x no. of years; 1 pack year = 1 pack per day for 1 year).

[a] For confidentiality, patients are coded and assigned numbers.

[b] Generic drug name (proprietary name)

5.3.2 Lung Cell Isolations

The isolated unseparated cell preparations (cell digest) contained 20 - 30% alveolar type II cells, 10-30% macrophages, 20-50% red blood cells, and less than 10% polymorphonuclear leukocytes. Preparations enriched in type II cells (65 - 85%) also contained macrophages and polymorphonuclear leukocytes, each comprising less than 10% of the total number of cells. Preparations enriched in macrophages (60-80%) contained approximately 10 - 15% type II cells and few polymorphonuclear leukocytes. Initial viability in all three fractions was at least 90%.

5.3.3 Viability and Drug Accumulation Studies

Following exposure for 36 h, AM decreased cell digest viability by approximately 20% in cells from 3 patients, but in the other cells from 2 patients, AM decreased cell digest viability approximately 60-80% (Figure 5.1). Vitamin E diminished AM-induced cytotoxicity (by approximately 10%) in cell digest of patient 139 and to a lesser extent in patient 151 (Figure 5.1). In alveolar macrophages, AM caused a 20-30% decrease in viability in cells from 3 patients (Figure 5.2). Patient 151 demonstrated a progressive loss of viability (up to 80% cell death) over 36 h while patient 152 demonstrated a 60% decrease in viability, but only after a 24 h lag period (Figure 5.2). In cells from patient 139, vitamin E decreased AM-induced cytotoxicity at 24 h by 10% in macrophages (Figure 5.2). Alveolar type II cell preparations demonstrated AM-induced cytotoxicity earlier and to a greater extent than the other cell fractions in patients 136, 151, and 152,

Cell Digest

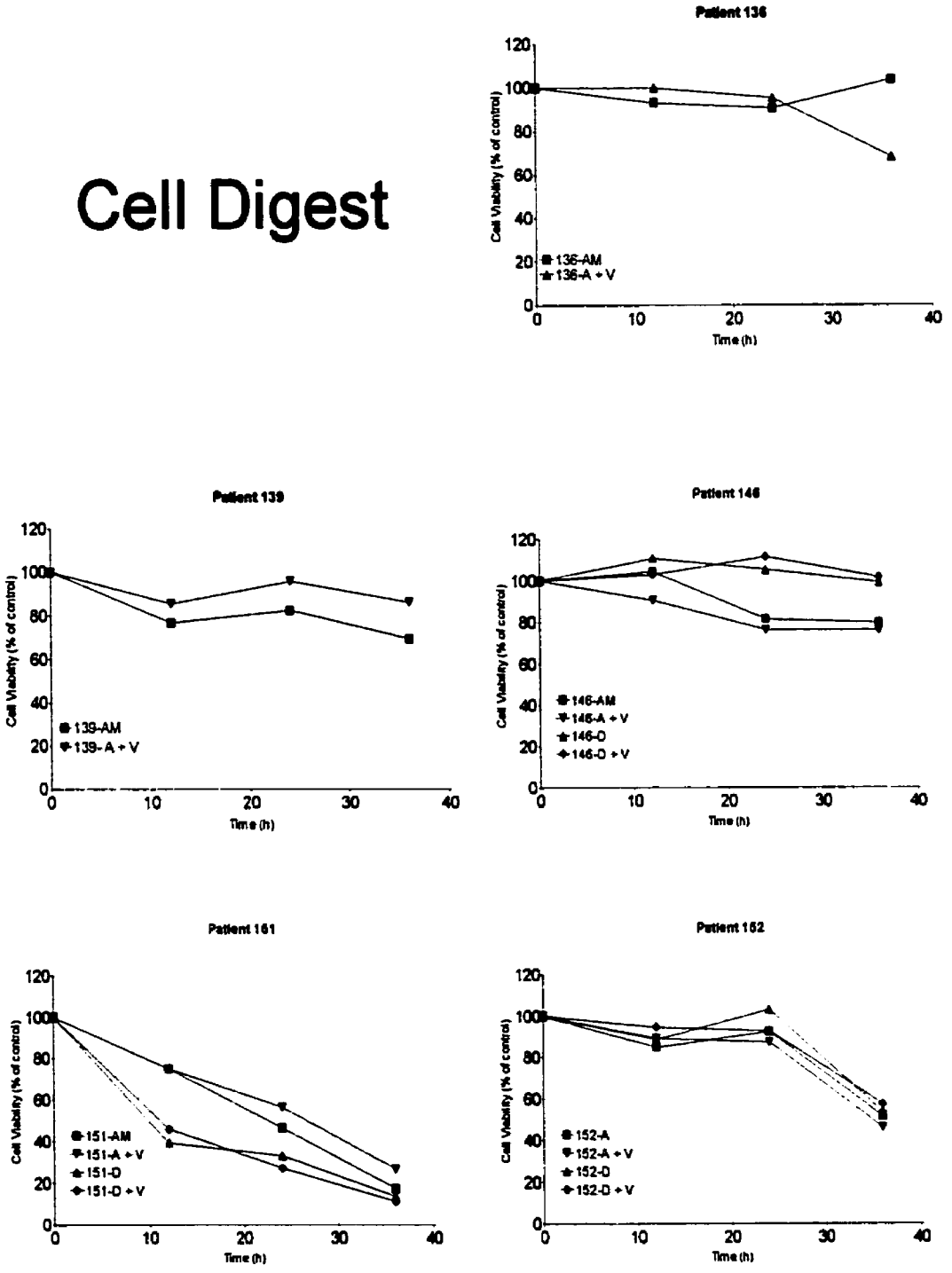


Figure 5.1. Cell digest viability (% of control) of individual patients incubated with 100 μM AM ± 300 μM vitamin E (v) or 50 μM DEA ± 300 μM vitamin E.

Macrophages

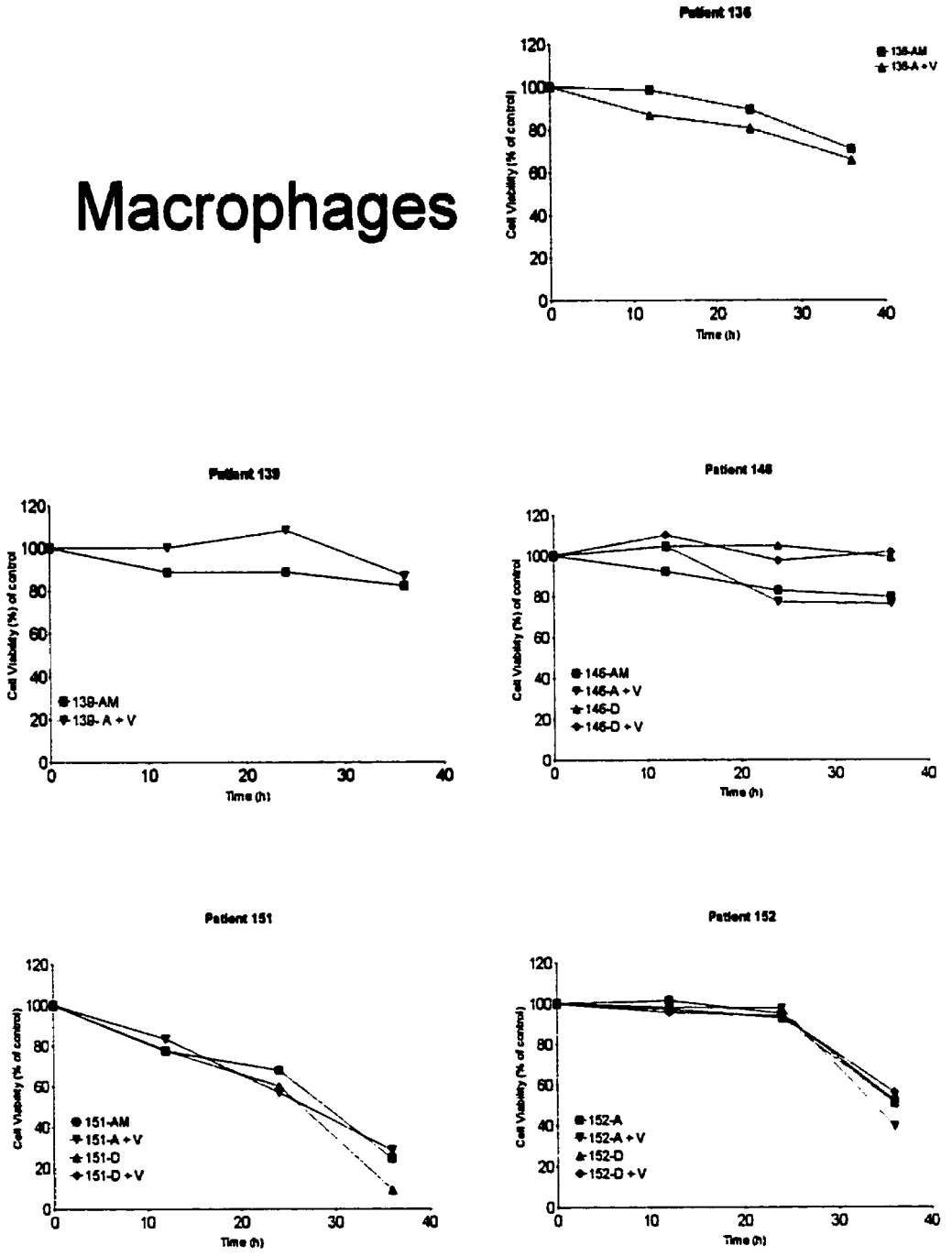


Figure 5.2. Macrophage viability (% of control) of individual patients incubated with 100 μM AM ± 300 μM vitamin E (v) or 50 μM DEA ± 300 μM vitamin E.

(40-60 % cell death by 36 h) (Figure 5.3). However, type II cells of patients 139 and 146 demonstrated relatively little to no AM-induced cytotoxicity (10-15 % cytotoxicity) (Figure 5.3). Vitamin E decreased AM-induced cytotoxicity (5-20%) in type II cells from patients 136 and 151 (Figure 5.3).

For most patients' cells, 50 μM DEA-induced cytotoxicity was very similar to 100 μM AM-induced cytotoxicity, except in the case of patient 151, where DEA appeared to cause greater cell death in cell digest and alveolar type II cell fractions. Vitamin E had no effect on 50 μM DEA-induced cytotoxicity in any cell preparations (Figures 5.1, 5.2, and 5.3).

When data from all patients were pooled, cell digest and fractions enriched with alveolar macrophages and alveolar type II cells demonstrated statistically significant ($p < 0.05$) susceptibility to 100 μM AM- and 50 μM DEA-induced cytotoxicity (Figure 5.4). However, alveolar type II cells demonstrated loss of viability earlier ($p < 0.05$) than macrophages or cell digest. All fractions contained similar intracellular levels of AM and DEA, and vitamin E did not alter these levels (Figure 5.5). However, type II cell preparations from patients #146, and #152 contained an analyte that co-eluted with authentic DEA.

5.3.4 Whole Lung Microsomes

In whole lung microsomes incubated with AM, a peak which co-eluted with authentic DEA was observed. Putative DEA amounts formed in whole lung microsomes are listed in Table 5.3. Mass spectrometry and HPLC with UV spectra detection were used, unsuccessfully, to identify the putative DEA peak.

Type II Cells

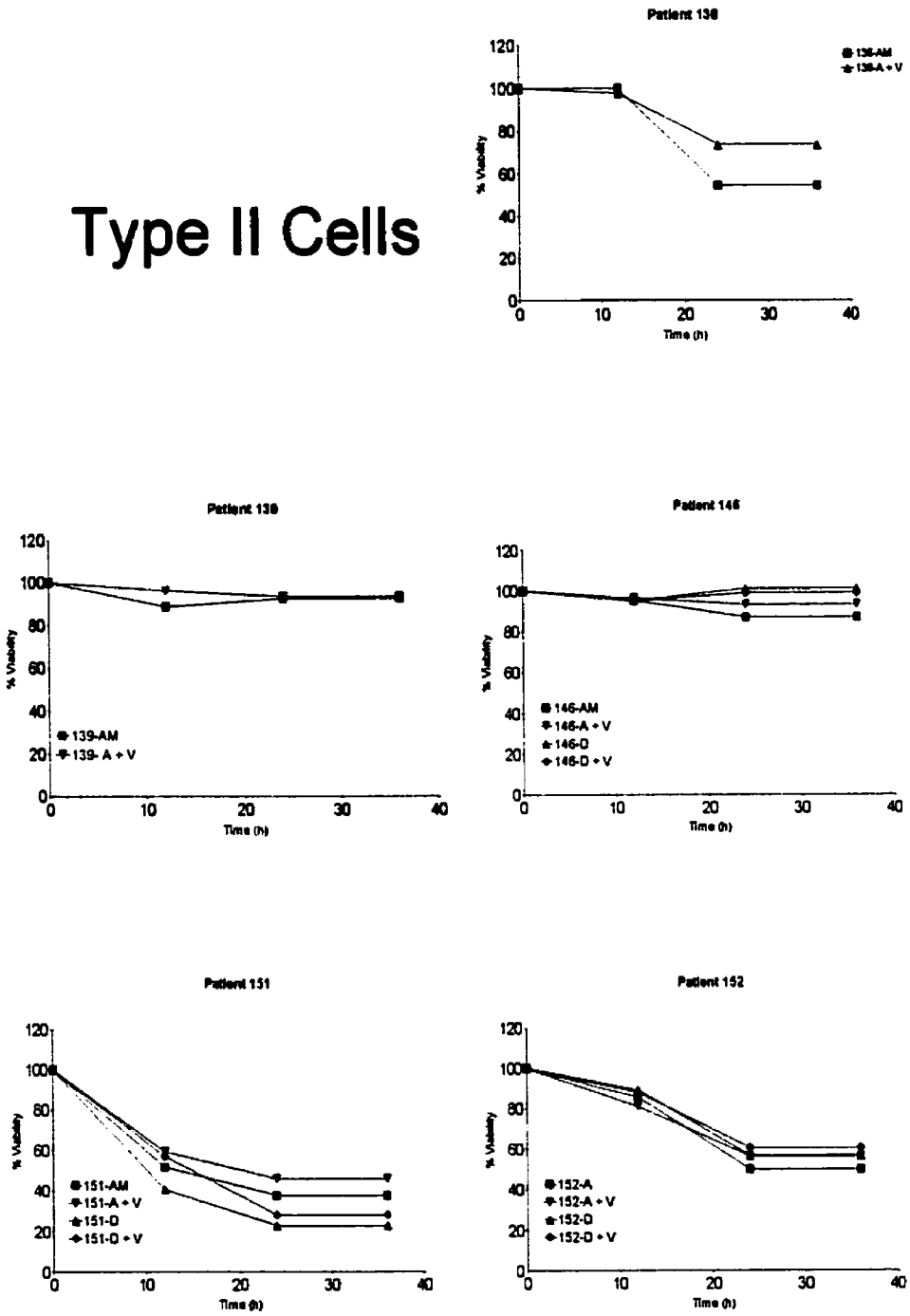


Figure 5.3. Alveolar type II cell viability (% of control) of individual patients incubated with 100 μM AM ± 300 μM vitamin E (v) or 50 μM DEA ± 300 μM vitamin E.

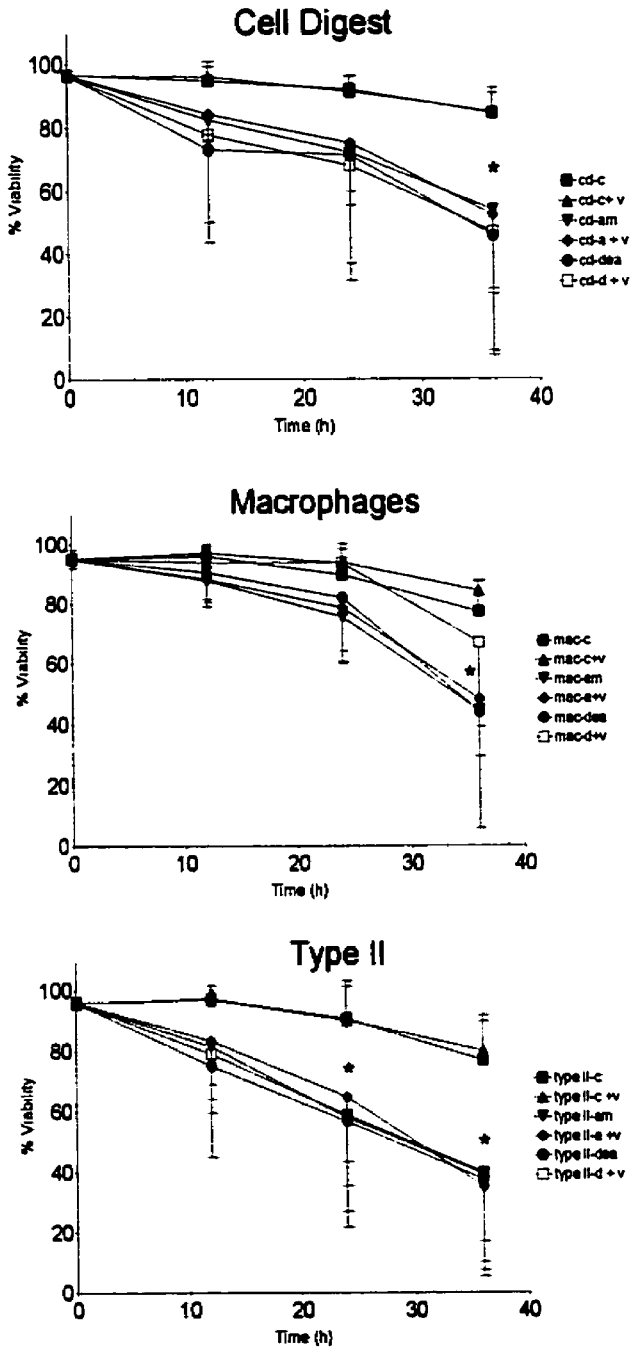


Figure 5.4. Viability (mean ± SD, n=5) of cell digest (cd), alveolar macrophages (mac) and alveolar type II cells (type II) incubated with vehicle (0.1% ethanol, c), 100 μM AM ± 300 μM vitamin E (v) or 50 μM DEA ± 300 μM v for 12, 24 and 36 h. *indicates difference from control.

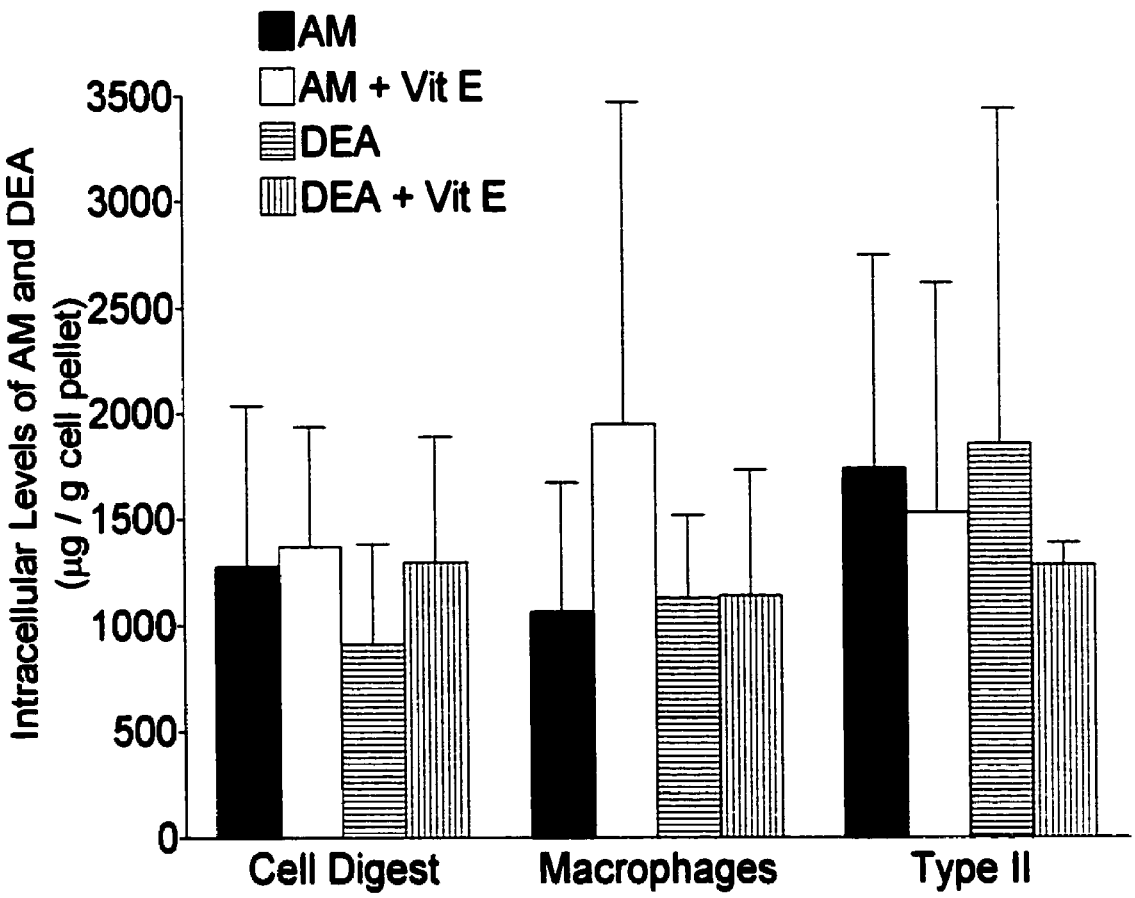


Figure 5.5. Cellular drug content in enriched preparations following incubation with 100 µM AM ± 300 µM vitamin E or 50 µM DEA ± 300 µM vitamin E for 24 h.

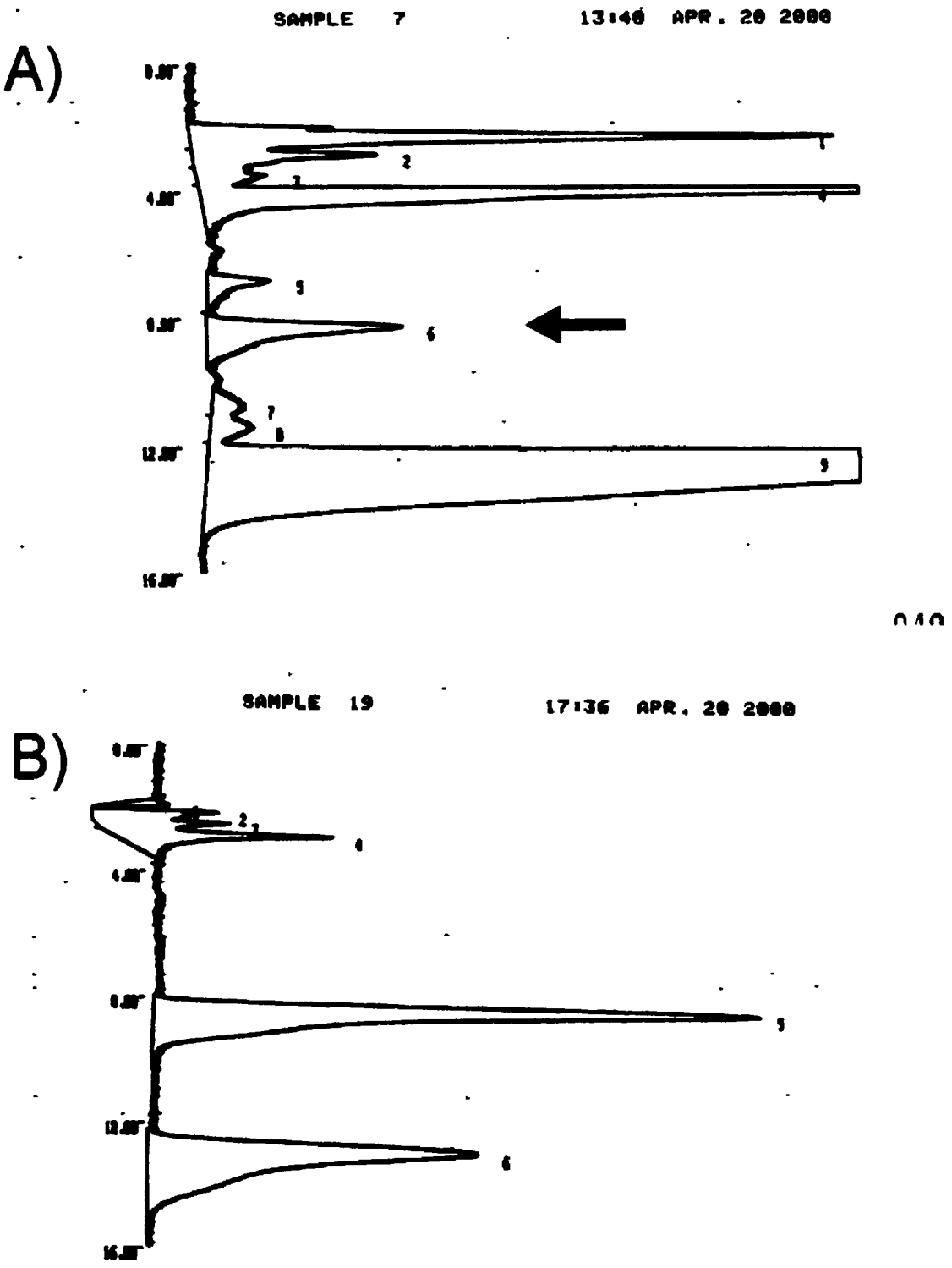


Figure 5.6. A) Chromatograph of putative DEA peak (peak #6). B) Chromatograph of DEA (peak #5)and AM (peak #6) standards.

Table 5.3. Putative DEA Formed in Human Lung Microsomes

Patient	Putative [DEA] formed (μg / mg of human lung microsomal protein) from 0.5μmoles of AM
3JM	1.736
9DM	1.564
9JM	1.484
3GM	Detected but unable to quantitate
1EM	Detected but unable to quantitate

5.4 DISCUSSION

The present study investigated, for the first time, *in vitro* effects of AM and DEA in freshly isolated human lung cells. Freshly isolated cells advantageously possess factors such as biotransformation enzymes, reducing agents, antioxidants, etc. which are not present in isolated organelles (such as isolated mitochondria or microsomes) and are greatly altered in cultured cells (Massey, 1989). Thus, freshly isolated human lung cells more accurately reflect cellular responses to AM as they occur *in situ* in patients receiving AM therapy. Results from these experiments demonstrated both AM- and DEA-induced cell death in cell digest and in fractions enriched with alveolar macrophages and alveolar type II cells. These same cell types isolated from both Syrian hamster (Bolt et al., 1998; Bolt et al., 2001) and Fisher and Wistar rat (Reasor et al., 1990; Wilson and Lippmann, 1996b) have previously demonstrated susceptibility to AM and DEA-induced cytotoxicity. Consistent with the animal models (Bolt et al., 2001; Reasor et al., 1990; Wilson and Lippmann, 1996b), the human cells revealed greater cytotoxic potency of DEA relative to AM. Furthermore, concentrations of AM and DEA that caused cytotoxicity in our model (100 and 50 μM respectively) also caused comparable quantitative loss of viability in the animal models. Therefore, similarities between the present results and those observed in animal cells support the validity of animal studies for investigation of cytotoxic mechanisms of AM and DEA in the lungs.

Despite similarities between human and animal models, results from this chapter also demonstrated some unique features of the human cells. For

example, in the hamster, alveolar macrophages demonstrated the greatest susceptibility to 100 μM AM-induced cytotoxicity. However, human alveolar type II cells demonstrated earlier AM-induced cytotoxicity than did human lung macrophages. This may be related to the preliminary evidence which suggests that human alveolar type II cells have the ability to N-dealkylate AM to form DEA. Thus type II cells in the human lung (and not just the liver as presently thought (Ohyama et al., 2000a; Rafeiro et al., 1990; Trivier et al., 1993)) may be a source of DEA production *in vivo*. The greater cytotoxic potency of DEA relative to AM (Beddows et al., 1989; Bolt et al., 2001; Gross et al., 1989) (Figures 5.1, 5.2 and 5.3), may contribute to the increased susceptibility of human type II cells to AM-induced cytotoxicity.

Our results also demonstrated inter-patient differences in response to AM and DEA, which typically are not observed when using animal models. This suggests that some patients may ultimately have increased susceptibility to pulmonary fibrosis. Similarly, vitamin E protected against AM-induced cytotoxicity in some patients, but not in others. However, before conclusions regarding the protective effects of vitamin E can be made, differences in patient diet and demographics would have be considered.

In lung microsomes from some patients, incubation with AM resulted in the production of an analyte which co-eluted with DEA. This provided further evidence that human lung may biotransform AM to DEA. HPLC eluate corresponding with suspected DEA from human alveolar type II cells of some patients was collected, and analysis by mass spectrometry was attempted.

However, the small amounts of DEA produced precluded successful mass spectrometry analysis.

In conclusion, freshly isolated human lung cells can be used for studying AM-induced toxicity *in vitro*. Furthermore, human alveolar type II cells may possess the ability to biotransform AM to DEA and are particularly susceptible for DEA- and AM-induced cytotoxicity relative to other cell types. Also, interpatient differences in cytotoxicity and responsiveness to vitamin E protection against AM-induced cytotoxicity were evident, suggesting that some patients may be at higher risk than others for developing AM-induced pulmonary toxicity.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 GENERAL DISCUSSION

Results from initial experiments demonstrated that alveolar macrophages, alveolar type II cells and Clara cells are all susceptible to AM-induced cytotoxicity. Furthermore, compared to other enriched fractions, alveolar macrophages demonstrated the greatest loss of viability when incubated with 100 and 200 μM AM. In contrast, 50 μM AM did not significantly alter alveolar macrophage viability, but caused cytotoxicity in Clara cells (Chapter 2). From these experiments, the reason(s) for differences in susceptibility to AM-induced cytotoxicity were unclear, but may have been attributed to higher AM levels in macrophages. In addition, greater Clara cell susceptibility to 50 μM AM suggests that metabolism or bioactivation of AM may have a role in AM-induced cytotoxicity.

AM perturbed mitochondrial function and decreased ATP long before the onset of AM-induced loss of viability based on trypan blue exclusion (Chapter 3). This suggests that AM-induced disruption of cellular energy homeostasis initiates cell death and may initiate AIPT *in vivo*. Also, alveolar macrophages (the most susceptible cell type to 100 μM AM-induced cytotoxicity) demonstrated AM-induced loss of ATP before other cell types. Therefore, susceptibility to AM-induced cytotoxicity may be related to susceptibility to ATP depletion.

Cyclosporin A, an inhibitor of MPT did not affect AM-induced cytotoxicity (Chapter 3). Therefore MPT does not appear to be involved in AM-induced loss

of viability. Neither glucose nor niacin protected against AM-induced ATP depletion or cell death in macrophages (Chapter 3). This suggests that protection against AM-induced toxicity observed by others with these compounds (Fromenty et al., 1993; Wang et al., 1992) may have been due to some mechanism other than preventing ATP depletion.

Alveolar macrophages, alveolar type II cells and Clara cells are all susceptible to 50 μM DEA-induced cytotoxicity (Chapter 3). These experiments demonstrated that DEA is more cytotoxic and causes cytotoxicity earlier than AM. Although 10 μM DEA did not produce cell death in any enriched cell fraction, 25 μM DEA was cytotoxic to alveolar type II cells and Clara cells. Similar to 50 μM AM, 25 μM DEA had no effect on alveolar macrophage viability, suggesting that a thresh hold level must be surpassed before AM- or DEA-induced cytotoxicity in alveolar macrophages can occur. Differences in susceptibility to 50 μM DEA were not observed between enriched cell fractions suggesting that the mechanism of DEA-induced cell death may be the same in all cell types.

DEA disrupted mitochondrial function and depleted ATP prior to DEA-induced cell death (Chapter 3), suggesting that perturbation of energy homeostasis also initiates DEA-induced cytotoxicity. DEA-induced ATP depletion in the Clara cell fraction was greater than in any other enriched preparation. In addition, alveolar macrophages and Clara cells demonstrated greater FCCP (a mitochondrial uncoupler)-induced cell death than alveolar type II cells. Combining of all these data, results in the following suggestion for

increased susceptibility of macrophages and Clara cells to AM or DEA-induced cytotoxicity. After AM or DEA reaches a threshhold level for initiating cell death, mitochondrial function is disrupted, and ATP levels decrease. Consequently, less free energy is formed by the conversion of ATP to ADP, thereby preventing both the synthesis of macromolecules from precursors, and active transport of molecules and ions, and causing cell death. Because alveolar macrophages and Clara cells are more susceptible than alveolar type II cells to disruptions of mitochondrial function, ATP depletion and ultimately cell death occurs earlier or to a greater extent than in other enriched lung cell fractions. Also consistent with the observation is the following: because DEA accumulates in isolated mitochondria to a greater extent than does AM, and DEA disrupts isolated mitochondrial function to a greater extent than does AM, DEA is more cytotoxic than AM.

Experiments indirectly investigating the role of free radicals in initiating AM-induced cell death demonstrated that vitamin E decreases AM-induced cytotoxicity in alveolar macrophages but not in other enriched lung cell fractions (Chapter 4). Furthermore, vitamin E has no effect on DEA-induced cytotoxicity in any enriched preparation. Therefore, protective effects of vitamin E against AM-induced pulmonary fibrosis *in vivo* (Card et al., 1999a) may possibly be attributed to decreasing alveolar macrophage cell death. Paradoxically, differences in cellular AM levels were not observed between cell types (Chapter 4) and therefore, greater AM accumulation can no longer explain higher susceptibility of alveolar macrophages (Chapter 2). The reason for this discrepancy is not

known. Perhaps differences in the immune status caused by: exposure to airborne microbes; change in season; or changes in housing of hamsters used in Chapter 2 experiments resulted in macrophage activation prior to the isolation procedure and ultimately, greater AM phagocytosis and higher cellular AM levels than in experiments in Chapter 4.

Based on isoprostane formation, AM and DEA do not cause lipid peroxidation prior to cell death and vitamin E does not decrease isoprostane formation in any fraction. In addition, vitamin E does not alter intracellular levels of AM or DEA (Chapter 4). Moreover, in our laboratory, Jeffrey Card demonstrated that vitamin E does not prevent AM-induced decreases in oxygen consumption in isolated hamster lung mitochondria (unpublished observation). Therefore, protective effects of vitamin E in macrophages against AM-induced cytotoxicity are apparently due to some other mechanism.

Freshly isolated, human alveolar macrophages and alveolar type II cells are susceptible to AM-induced cytotoxicity. Furthermore, cytotoxicity similar in magnitude to that observed in rodents, occurs in human lung cells incubated with 100 μM AM or 50 μM DEA, and therefore, supports the use of animal models for studying AM-induced cytotoxicity *in vitro*. In a few cases, vitamin E protected human lung cells from AM-induced cytotoxicity. However, human alveolar type II cells were more susceptible than alveolar macrophages to AM-induced cell death and the extent of AM-induced cytotoxicity differed between patients. Interestingly, analysis of human alveolar type II cells and human whole lung microsomes incubated with AM revealed a HPLC analyte that eluted at the same

time as DEA, suggesting that human lung may be capable of metabolizing AM to DEA. Therefore, *in situ* DEA formation may contribute to AM-induced cytotoxicity in human lung.

In conclusion, these experiments were the first to show that Clara cells are susceptible to AM- and DEA-induced cytotoxicity and that, depending on the concentration of AM or DEA, alveolar macrophages or Clara cells are most susceptible to AM-induced cell death. This research also demonstrated for the first time that AM- and DEA-induced perturbations of mitochondrial membrane potential and depletion of cellular ATP levels occur early relative to cell death. Therefore, AM- and DEA-induced disruptions of cellular energy homeostasis are likely to initiate AM-induced cell death and possibly AIPT. Experiments in this thesis also demonstrated that vitamin E decreases AM-induced cytotoxicity in hamster macrophages and that AM and DEA do not cause lipid peroxidation prior to cell death. Finally, this thesis is the first to report an *in vitro* system for assessing AM-induced events occurring in freshly isolated human lung cells. Also, research from this thesis may show that metabolism of AM to DEA in human lung does occur and may influence AM-induced cytotoxicity. However, further investigations are required to unequivocally identify the formation of DEA.

6.2 FUTURE DIRECTIONS

6.2.1 Identification of DEA Formation in Isolated Human Lung Cells

Although HPLC data from isolated human lung cells and from human lung microsomes suggest that the peak which co-elutes with DEA is actually DEA, it

would be of value to determine the identity of this analyte. Therefore, more human lung cells per incubate should be incubated with AM, possibly producing a larger putative DEA peak which could be collected and analyzed by mass spectrometry. However, this approach is complicated by the fact that the putative DEA peak is not produced by all patients and obtaining freshly isolated human lung cells is limited by tissue availability. Nevertheless it would be beneficial to investigate whether there is a relationship between the ability to produce DEA and susceptibility to AM-induced cytotoxicity.

6.2.2 Susceptibility of Lung Cell Types to Mitochondrial Dysfunction

Higher susceptibility of alveolar macrophages and Clara cells to FCCP-induced cytotoxicity suggests that, for reasons which remain to be elucidated, these cell types are more susceptible to perturbations of mitochondrial function than are alveolar type II cells. Determination of metabolic requirements for different lung cell types (i.e. % of functional mitochondria and / or ATP synthesis necessary for cell survival) could provide useful information regarding potential pulmonary targets for agents that disrupt mitochondrial function.

6.2.3 Mechanism of Vitamin E-induced Protection Against AM Cytotoxicity

The mechanism by which vitamin E decreases AM-induced cytotoxicity in alveolar macrophages remains to be elucidated. Therefore, effects of vitamin E on AM-induced mitochondrial membrane potential or on processes which occur after AM-induced mitochondrial disruption or ATP depletion (such as activation of

caspases), or effects of vitamin E on other possible mechanisms of AM-induced cytotoxicity, such as phospholipidosis or disruption of Ca^{2+} homeostasis, should be investigated.

6.2.4 Effects of Pirfenidone on AM-Induced Cytotoxicity

Pirfenidone is an antifibrotic agent shown to ameliorate chemically-induced pulmonary fibrosis in hamsters (Iyer et al., 1995) and mice (Kehrer and Margolin, 1997) and is regarded as encouraging for treating idiopathic pulmonary fibrosis in humans (Raghu et al., 1999). Currently, experiments within our laboratory are investigating the ability of pirfenidone to reduce AM-induced pulmonary fibrosis in hamsters. If pirfenidone ameliorates AM-induced fibrosis *in vivo*, then effects of pirfenidone on: 1) AM-induced cytotoxicity and 2) AM-induced perturbations of mitochondrial function and ATP depletion *in vitro* should be investigated. Identification of the mechanism of protection by pirfenidone may provide additional information about the etiology of AIPT and chemically-induced pulmonary fibrosis.

6.2.5 Development of a Mouse Model of AIPT

Recent development of new molecular tools, such as DNA microarrays, and transgenic and knockout animal models, allows for a more in depth investigation into which genes and / or proteins are involved in the development of pulmonary fibrosis. However, the majority of these techniques are available mostly for either mouse or human samples. In order to take advantage of these

tools, an *in vivo* mouse model of pulmonary fibrosis would be advantageous. DNA microarray technology could identify which genes are upregulated or downregulated prior to onset of pulmonary fibrosis. Knockout mouse models could investigate whether preventing AM-induced alterations in gene expression would prevent pulmonary fibrosis.

Reference List

Abdollah H., Brien J.F., and Brennan F.J. (1989). Antiarrhythmic effects of desethylamiodarone in dogs with subacute myocardial infarction and inducible ventricular arrhythmias. J. Cardiovasc. Pharmacol. 13:37-44.

Adams G.D., Kehoe R., Lesch M., and Glassroth J. (1988). Amiodarone-induced pneumonitis: assessment of risk factors and possible risk reduction. Chest 93:254-263.

Adams P.C., Gibson G.J., Morley A.R., Wright A.J., Corris P.A., Reid D.S., and Campbell R.W.F. (1986). Amiodarone pulmonary toxicity: clinical and subclinical features. Quart. J. Med. 59:449-471.

Adams P.C., Holt D.W., Storey G.C., Morley A.R., Callaghan J., and Campbell R.W. (1985). Amiodarone and its desethyl metabolite: tissue distribution and morphologic changes during long-term therapy. Circulation 72:1064-1075.

Adamson I.Y.R. (1984). Drug-induced pulmonary fibrosis. Environ. Health Perspect. 55:25-36.

Akoun G.M., Gauthier-Rahman S., Milleron B., Perrot J., and Mayaud C. (1984). Amiodarone-induced hypersensitivity pneumonitis. Chest 85:133-135.

Akoun G.M., Cadranel J.L., Blanchette G., Milleron B.J., and Mayaud C.M. (1991). Bronchoalveolar lavage cell data in amiodarone-associated pneumonitis: Evaluation in 22 patients. Chest 99:1177-1182.

Amiodarone Trials Meta- Analysis Investigators (1997). Effect of prophylactic amiodarone on mortality after acute myocardial infarction and in congestive heart failure: meta-analysis of individual data from 6500 patients in randomised trials. Lancet 350:1417-1424.

Anastasiou-Nana M., Levis G.M., and Moulopoulos S. (1982). Pharmacokinetics of amiodarone after intravenous and oral administration. Int. J. Clin. Pharmacol. Ther. Toxicol. 20:524-529.

Anderson K.P., Walker R., Dustman T., Lux R.L., Ershler P.R., Kates R.E., and Urie P.M. (1989). Rate-related electrophysiologic effects of long-term

administration of amiodarone on canine ventricular myocardium in vivo. Circulation 79:948-958.

Andreasen F., Agerbaek H., Bjerregaard P., and Gotzsche H. (1981). Pharmacokinetics of amiodarone after intravenous and oral administration. Eur. J. Clin. Pharmacol. 19:293-299.

Antonini J.M., McCloud C.M., and Reasor M.J. (1994). Acute silica toxicity: Attenuation by amiodarone-induced pulmonary phospholipidosis. Environ. Health Perspect. 102:372-378.

Antunes-Madeira M.C., Videira R.A., Kluppel M.L.W., and Madeira V.V.M.C. (1995). Amiodarone effects on membrane organization evaluated by fluoresence polarization. International Journal of Cardiology 48:211-218.

Arinc E (1993). Extrahepatic microsomal forms. Lung microsomal cytochrome P450 isozymes. In: Cytochrome P450, Schenkman,J.B. and Greim H., Eds., pp 373-386, Springer-Verlag, Berlin.

Aust S.D., Chignell C.F., Bray T.M., Kalyanaraman B., and Mason R.P. (1993). Free Radicals in Toxicology. Toxicol. Appl. Pharmacol. 120:168-178.

Bargout R., Jankov A., Dincer E., Wang R., Komodromos T., Ibarra-Sunga O., Filippatos G., and Uhal B. (2000). Amiodarone induces apoptosis of human and rat alveolar epithelial cells in vitro. Am. J. Physiol. (Lung Cell. Mol. Physiol.) 278:L1039-L1044.

Baudin B., Beneteau-Burnat B., and Giboudeau J. (1996). Cytotoxicity of amiodarone in cultured human endothelial cells. Cardiovascular Drugs and Therapy 10:557-560.

Beddows S.A., Page S.R., Taylor A.H., McNerney R., Whitley G.S., Johnstone A.P., and Nussey S.S. (1989). Cytotoxic effects of amiodarone and desethylamiodarone on human thyrocytes. Biochem. Pharmacol. 38:4397-4403.

Bernardi P. (1996). The permeability transition pore. Control points of a cyclosporin A-sensitive mitochondrial channel involved in cell death. Biochim. Biophys. Acta 1275:5-9.

Blake T.L. and Reasor M.J. (1995a). Metabolism of amiodarone following intratracheal instillation in hamsters. Toxicol. Lett. 81:223-228.

Blake T.L. and Reasor M.J. (1995b). Acute pulmonary inflammation in hamsters following intratracheal administration of amiodarone. Inflammation 19:55-65.

Blake T.L. and Reasor M.J. (1995c). Pulmonary responses to amiodarone in hamsters: Comparison of intratracheal and oral administrations. Toxicol. Appl. Pharmacol. 131:325-331.

Bolt M.W., Racz W.J., Brien J.F., Bray T.M., and Massey T.E. (1998). Differential susceptibilities of isolated hamster lung cell types to amiodarone toxicity. Can. J. Physiol. Pharmacol. 76:721-727.

Bolt,M.W., Racz,W.J., Brien,J.F., Bray,T.M., and Massey,T.E. (1999). Effects of vitamin E on amiodarone-induced cytotoxicity in isoalted hamster lung cells. Toxicologist 48.

Bolt,M.W., Racz,W.J., Brien,J.F., Bennett,B.M., and Massey,T.E. (2001). Effects of vitamin E on desethylamiodarone cytotoxicity in isolated hamster lung cells. Toxicologist.

Bonati M., Gaspari F., D'Aranno V., Benfenati E., Neyroz P., Galletti F., and Tognoni G. (1984). Physicochemical and analytical characteristics of amiodarone. J. Pharm. Sci. 73:829-831.

Brien J.F., Jimmo S., and Armstrong P.W. (1983). Rapid high-performance liquid chromatographic analysis of amiodarone and N-desethylamiodarone in serum. Can. J. Physiol. Pharmacol. 61:245-248.

Brien J.F., Jimmo S., Brennan F.J., Armstrong P.W., and Abdollah H. (1990). Disposition of amiodarone and its proximate metabolite, desethylamiodarone, in the dog for oral administration of single-dose and short-term drug regimens. Drug Metab. Dispos. 18:846-851.

Brien J.F., Jimmo S., Brennan F.J., Ford S.E., and Armstrong P.W. (1987). Distribution of amiodarone and its metabolite, desethylamiodarone in human tissues. Can. J. Physiol. Pharmacol. 65:360-364.

Brigelius-Flohe R. and Traber M.G. (1999). Vitamin E: function and metabolism. FASEB J 13:1145-1155.

Broekhuysen J., Laruel R., and Sion R. (1969). Etude comparee du transit et du metabolisme de l'amiodarone chez diverses especes animales et chez l'homme. Arch. Int. Pharmacodyn. 177:340-359.

Burlakova E.B., Krashakov S.A., and Khrapova N.G. (1998). The role of tocopherols in biomembrane lipid peroxidation. Membr. Cell Biol. 12:173-211.

Cairns J.A., Connolly S.J., Roberts R., and Gent M. (1997). Randomised trial of outcome after myocardial infarction in patients with frequent or repetitive ventricular premature depolarisations: CAMIAT. The Lancet 349:675-682.

Campbell T.J. (1997). The place of amiodarone: an overview of the four recent large controlled trials. Aust. N. Z. J Med. 27:582-590.

Camus P. and Mehendale H.M. (1986). Pulmonary sequestration of amiodarone and desethylamiodarone. J. Pharmacol. Exp. Ther. 237:867-873.

Cantor J.O., Keller S., Mandl I., and Turino G.M. (1987). Increased synthesis of elastin in amiodarone-induced pulmonary fibrosis. J. Lab. Clin. Med. 109:480-485.

Cantor J.O., Osman M., Cerreta J.M., Suarez R., Mandl I., and Turino G.M. (1984). Amiodarone-induced pulmonary fibrosis in hamsters. Exp. Lung Res. 6:1-10.

Card J.W., Lalonde B.R., Rafeiro E., Tam A.S., Racz W.J., Brien J.F., Bray T.M., and Massey T.E. (1998). Amiodarone-induced disruption of hamster lung and liver mitochondrial function: lack of association with thiobarbituric acid-reactive substance production. Toxicol. Lett. 98:41-50.

Card J.W., Leeder R.G., Racz W.J., Brien J.F., Bray T.M., and Massey T.E. (1999a). Effects of dietary vitamin E supplementation on pulmonary morphology and collagen deposition in amiodarone- and vehicle-treated hamsters. Toxicology. 133:75-84.

Card,J.W., Leeder,R.G., Racz,W.J., Brien,J.F., Bray,T.M., and Massey,T.E. (1999). Lung morphology and collagen deposition in amiodarone- and vehicle-treated hamsters following vitamin E supplementation. Toxicologist 48:128-128.

Card,J.W., Racz,W.J., Brien,J.F., Bennett,B.M., and Massey,T.E. (2001). Down regulation of procollagen gene expression in hamster lung by dietary vitamin E supplementation: Relationship to amiodarone-induced pulmonary toxicity. Toxicologist 60:427.

Cazzadori A., Braggio P., Barbieri E., and Ganassini A. (1986). Amiodarone-induced pulmonary toxicity. Respiration 49:157-160.

Charlier R., Delaunois G., Bauthier J., and Deltour G. (1969). Recherches dans la serie des benzofuranes. XL. Proprietes antiarythmiques de l'amiodarone. Cardiology 54:83-90.

Chatelain P. and Laruel R. (1985). Amiodarone partitioning with phospholipid bilayers and erythrocyte membranes. J. Pharm. Sci. 74:783-784.

Chatelain P., Laruel R., and Gillard M. (1985). Effect of amiodarone on membrane fluidity and Na^+/K^+ ATPase activity in rat-brain synaptic membranes. Biochem. Biophys. Res. Commun. 129:148-154.

Chung,W.H., Bennett,B.M., Racz,W.J., Brien,J.F., and Massey,T.E. (2001). An investigation of *C-JUN* and TGF-β_1 gene expression in Fisher 344 rats during amiodarone-induced pulmonary fibrosis. Toxicologist 60:427.

Cohen J.J. (1993). Apoptosis. Immunol. Today 14:126-130.

Colgan T., Simon G.T., Kay J.M., Pugsley S.O., and Eydt J. (1984). Amiodarone pulmonary toxicity. Ultrastruct. Pathol. 6:199-207.

Connolly S.J. (1999). Evidence-based analysis of amiodarone efficacy and safety. Circulation 100:2025-2034.

Cooper J.A.D., White D.A., and Matthay R.A. (1986). Drug-induced pulmonary disease. Part 2: Noncytotoxic drugs. Am. Rev. Respir. Dis. 133:488-505.

Costa-Jussa F.R., Corrin B., and Jacobs J.M. (1984). Amiodarone lung toxicity: a human and experimental study. J. Pathol. 143:73-79.

Coudert B., Bailly F., Lombard J.N., Andre F., and Camus P. (1992). Amiodarone pneumonitis. Chest 102-4:1005-1012.

Crystal RG (1991). Alveolar macrophages. In: The lung., Crystal,R.G. and West J.B., Eds., pp 527-538, Raven Press, Ltd., New York.

Crystal RG, Ferrans VJ, and Basset F (1991). Biological basis of pulmonary fibrosis. In: The Lung: Scientific Foundations, Crystal,R.G. and West J.B., Eds., pp 2031-2046, Raven Press, Ltd., New York.

Culling CFA (1976). Histology. In: Lynch's Medical Laboratory Technology, Raphael,S.S., Ed., pp 876-1062, W.B. Saunders Company, Philadelphia.

D'Amico D.J., Kenyon K.R., and Ruskin J.N. (1981). Amiodarone keratopathy: drug-induced lipid storage disease. Arch Ophthalmol. 99:257-261.

Dake M., Madison J., Montgomery C., Shellito J., Hinchcliffe W., Winkler M., and Bainton D. (1985). Electron microscopic demonstration of lysosomal inclusion bodies in lung, liver. lymph nodes, and blood leukocytes of patients with amiodarone pulmonary toxicity. Am. J. Med. 78:506-512.

Daniels J.M., Brien J.F., and Massey T.E. (1989). Pulmonary fibrosis induced in the hamster by amiodarone and desethylamiodarone. Toxicol. Appl. Pharmacol. 100:350-359.

Daniels J.M., Leeder R.G., Brien J.F., and Massey T.E. (1990a). Repeated amiodarone exposure in the rat: toxicity and effects on hepatic and extrahepatic monooxygenase activities. Can. J. Physiol. Pharmacol. 68:1261-1268.

Daniels J.M., Liu L., Stewart R.K., and Massey T.E. (1990b). Biotransformation of aflatoxin B_1 in rabbit lung and liver microsomes. Carcinogenesis 11:823-827.

Darmanata J., van Zandwijk N., Duren D., van Royen E., Mooi W., Plomp T.A., Jansen H., and Durrer D. (1984). Amiodarone pneumonitis: three further cases with a review of published reports. Thorax 39:57-64.

Davies K.J.A. (1995). Oxidative stress: the paradox of aerobic life. Biochem. Soc. Symp. 61:1-31.

Dean P.J., Groshart K.D., Poerterfield J.G., Iansmith D.H., and Golden E.B. (1987). Amiodarone-associated pulmonary toxicity; a clinical and pathological study of eleven cases. Am. J. Clin. Pathol. 87:7-13.

Deltour G., Broekhuysen J., and Charlier R. (1970). Etude du mecanisme biochimique d'action d'un agen antiangoreux: l'amiodarone. J. Pharmacol. (Paris) 1:39-51.

Desai A.D., Chun S., and Sung R.J. (1997). The role of intravenous amiodarone in the management of cardiac arrhythmias. Ann. Intern. Med. 127:294-303.

Devereux T.R. (1984). Alveolar type II and Clara cells: Isolation and xenobiotic metabolism. Environ. Health Perspect. 56:95-101.

Devereux T.R. and Fouts J.R. (1980). Isolation and identification of Clara cells from rabbit lung. In Vitro 16:958-968.

Devereux T.R. and Fouts J.R. (1981). Isolation of pulmonary cells and use in studies of xenobiotic metabolism. Methods Enzymol. 77:147-154.

Di Lisa F., Blank P.S., Colonna R., Gambassi G., Silverman H.S., Stern M.D., and Hansford R.G. (1995). Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition. J. Physiol. 486:1-13.

Domin B.A., Devereux T.R., and Philpot R.M. (1986). The cytochrome P-450 monooxygenase system of rabbit lung: enzyme components, activities, and induction in the nonciliated bronchiolar epithelial (Clara) cell, alveolar type II cell, and alveolar macrophage. Mol. Pharmacol. 30:296-303.

Donnelly P.J., Stewart R.K., Ali S.L., Conlan A.A., Reid K.R., Petsikas D., and Massey T.E. (1996). Biotransformation of aflatoxin B_1 in human lung. Carcinogenesis 17:2487-2494.

Dunn M. and Glassroth J. (1989). Pulmonary complications of amiodarone toxicity. Prog. Cardiovasc. Dis. 31:447-453.

Dusman R.E., Stanton M.S., Miles W.M., Klein L.S., Zipes D.P., Fineberg N.S., and Heger J.J. (1990). Clinical features of amiodarone-induced pulmonary toxicity. Circulation 82:51-59.

Dzimiri N. and Almotrefi A.A. (1993). Actions of amiodarone on mitochondrial ATPase and lactate dehydrogenase activities in guinea pig heart preparations. Eur. J. Pharmacol. 242:113-118.

Ellis R.E., Yuan J.Y., and Horvitz H.R. (1991). Mechanisms and functions of cell death. Annu. Rev. Cell Biol. 7:663-698.

Endoh Y., Hanai R., Uto K., Uno M., Nagashima H., Narimatsu A., Takizawa T., Onishi S., and Kasanuki H. (2000). KL-6 as a potential new marker for amiodarone-induced pulmonary toxicity. Am. J. Cardiol. 86:229-231.

Erich J. (2000). Amiodarone: the AHA's new drug of choice for victims of ventricular arrhythmias. Emerg. Med. Serv. 29:22.

Estabrook R.W. (1967). Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Methods Enzymol. 10:41-47.

Fan K., Bell R., Eudy S., and Fullenwider J. (1987). Amiodarone-associated pulmonary fibrosis. Evidence of an immunologically mediated mechanism. Chest 92:625-630.

Flanagan R.J., Storey G.C.A., Holt D.W., and Farmer P.B. (1982). Identification and measurement of desethylamiodarone in blood plasma specimens from amiodarone-treated patients. J. Pharm. Pharmacol. 34:638-643.

Forman HJ and Thomas MJ (1992). Oxygen Metabolism in the Lungs. In: Comparative Biology of the Normal Lung, Parent,R.A., Ed., pp 485-510, CRC Press, Ann Arbour, Michigan.

Fouts J.R. (1982). The metabolism of xenobiotics by isolated pulmonary and skin cells. Trends Pharmacol. Sci. 3:164-165.

Fraire A.E., Guntupalli K.K., Greenberg S.D., Cartwright Jr.J., and Chasen M.H. (1993). Amiodarone pulmonary toxicity:a multidisciplinary review of current status. Southern Med. J. 86-1:67-77.

Freedman M.D. and Somberg J.C. (1991). Pharmacology and pharmacokinetics of amiodarone. J. Clin. Pharmacol. 31:1061-1069.

Freeman B.A. and Crapo J.D. (1982). Biology of disease: free radicals and tissue injury. Lab. Invest. 47:412-426.

Fromenty B., Fisch C., Berson A., Letteron P., Larrey D., and Pessayre D. (1990a). Dual effect of amiodarone on mitochondrial respiration. Initial protonophoric uncoupling effect followed by inhibition of the respiratory chain at the levels of complex I and complex II. J. Pharmacol. Exp. Ther. 255:1377-1384.

Fromenty B., Fisch C., Labbe G., Degott C., Deschamps D., Berson A., Letteron P., and Pessayre D. (1990b). Amiodarone inhibits the mitochondrial β-oxidation of fatty acids and produces microvesicular steatosis of the liver in mice. J. Pharmacol. Exp. Ther. 255:1371-1376.

Fromenty B., Letteron P., Fisch C., Berson A., Deschamps D., and Pessayre D. (1993). Evaluation of human blood lymphocytes as a model to study the effects of drugs on human mitochondria. Biochem. Pharmacol. 46:421-432.

Fujii Y., Johnson M.E., and Gores G.J. (1994). Mitochondrial dysfunction during anoxia/reoxygenation injury in liver sinusoidal endothelial cells. Hepatology 20:177-185.

Futamura Y. (1995). Effect of amiodarone on release of cytokines from mouse alveolar macrophages pretreated with eicosapentaenoic acid. Jpn. J. Pharmacol. 69:335-341.

Futamura Y. (1996a). Effect of amiodarone on cytokine release and on enzyme activities of mouse alveolar macrophages, bone marrow macrophages, and blood monocytes. J. Toxicol. Sci. 21:125-134.

Futamura Y. (1996b). Toxicity of amiodarone on mouse pulmonary endothelial cells cultured with or without alveolar macrophages. J. Toxicol. Sci. 21:253-267.

Futamura Y. (1997). Amiodarone induces two different types of disorders in mouse alveolar macrophages. Jpn. J. Pharmacol. 74:21-28.

Gefter W.B., Epstein D.M., Pietra G.G., and Miller W.T. (1983). Lung disease caused by amiodarone, a new antiarrhythmic agent. Radiology 147:339-344.

Gibb P. and Melendez L.J. (1986). Segmental pulmonary consolidation due to amiodarone. Can. Med. Assoc. J. 134:611-613.

Gill J., Heel R.C., and Fitton A. (1992). Amiodarone: An overview of its pharmacological properties, and review of its therapeutic use in cardiac arrhythmias. Drugs 43:69-110.

Gillis A. and Kates R.E. (1984). Pharmacokinetics of the newer antiarrhythmics. Clin. Pharmacokinetics 9:375-403.

Gross S.A., Bandyopadhyay S., Klaunig J.E., and Somani P. (1989). Amiodarone and desethylamiodarone toxicity in isolated hepatocytes in culture. Proc. Soc. Exp. Biol. Med. 190:163-169.

Guerreiro M.H.C.P.M., Lopes L.C.V., Campello A.P., and Kluppel M.L.W. (1986a). Effect of amiodarone on mitochondrial energy-linked reactions and on mitochondrial morphology. Arq. Biol. Tecnol. 29:621-631.

Guerreiro M.H.C.P.M., Campello A.P., Lopes L.C.V., and Kluppel M.L.W. (1986b). Effect of amiodarone on mitochondrial membranes. Arq. Biol. Tecnol. 29:675-684.

Gurujeyalakshmi G., Iyer S.N., Hollinger M.A., and Giri S.N. (1996). Procollagen gene expression is down-regulated by taurine and niacin at the transcriptional level in the bleomycin hamster model of lung fibrosis. J. Pharmacol. Exp. Ther. 277:1152-1157.

Ha H.R., Bigler L., Binder M., Kozlik P., Stieger B., Hesse M., Altorfer H.R., and Follath F. (2001). Metabolism of amiodarone (part I): identification of a new hydroxylated metabolite of amiodarone. Drug Metab Dispos. 29:152-158.

Ha H.R., Wyss P.A., Stieger B., Meier P.J., and Meyer U.A. (1993). Amiodarone metabolism in human liver microsomes. FASEB J. 6:A1845-A1845.

Haffajee C.I., Love J.C., Canada A.T., Lesko L.J., Asdourian G.A., and Alpert J.S. (1983). Clinical pharmacokinetics and efficacy of amiodarone for refractory tachyarrhythmias. Circulation 67:1347-1355.

Hageluken A., Nurnberg B., Harhammer R., Grunbaum L., Schunack W., and Seifert R. (1995). The class III antiarrhythmic drug amiodarone directly activates pertussis toxin-sensitive G proteins. Mol. Pharmacol. 47:234-240.

Halliwell B. and Chirico S. (1993). Lipd peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. 57:715S-725S.

Halliwell B and Gutteridge JMC (1999a). Oxygen is a toxic gas-an introduction to oxygen toxicity and reactive oxygen species. In: Free radicals in biology and medicine, Halliwell,B. and Gutteridge J.M.C., Eds., pp 1-35, Oxford Universtiy Press, New York.

Halliwell B and Gutteridge JMC (1999b). Teh chemistry of free radicals and related 'reactive species'. In: Free radicals in biology and medicine, Halliwell,B. and Gutteridge J.M.C., Eds., pp 36-104, Oxford University Press, New York.

Halliwell B and Gutteridge JMC (1999c). Oxidative stress: adaptation, damage, repair and death. In: Free radicals in biology and medicine, Halliwell,B. and Gutteridge J.M.C., Eds., pp 246-350, Oxford University Press, New York.

Halliwell B and Gutteridge JMC (1999d). Antioxidant defenses. In: Free radicals in biology and medicine, Halliwell,B. and Gutteridge J.M.C., Eds., pp 105-245, Oxford University Press, New York.

Halliwell B. and Gutteridge J.M.C. (1999e). Free Radicals in Biology and Medicine. Oxford University Press, New York, New York, USA.

Heath M., Costa-Jussa F., Jacobs W., and Jacobson W. (1985). The induction of pulmonary phospholipidosis and the inhibition of lysosomal phospholipases by amiodarone. Br. J. Exp. Path. 66:391-397.

Heger J.J., Prystowsky E.N., Jackman W.M., Naccarelli G.V., Warfel K.A., Rinkenberger R.L., and Zipes D.P. (1981). Amiodarone:Clinical efficacy and electrophysiology during long term therapy for recurrent ventricular tachycardia or ventricular fibrillation. New Eng. J. Med. 305:539-549.

Heger J.J., Prystowsky E.N., Miles W.M., and Zipes D.P. (1984). Clinical use and pharmacology of amiodarone. Med. Clin. North Am. 68:1339-1366.

Heger J.J., Prystowsky E.N., and Zipes D.P. (1983). Relationships between amiodarone dosage, drug concentrations, and adverse side effects. Am. Heart J. 106:931-935.

Himmel H.M., Dobrev D., Grossmann M., and Ravens U. (2000). N-desethylamiodarone modulates intracellular calcium concentration in endothelial cells. Naunyn Schmiedebergs Arch Pharmacol. 362:489-496.

Holt D.W., Tucker G.T., Jackson P.R., and Storey G.C.A. (1983). Amiodarone pharmacokinetics. Am. Heart J. 106:840-846.

Honegger U.E., Scuntaro I., and Wiesmann U.N. (1995). Vitamin E reduces accumulation of amiodarone and desethylamiodarone and inhibits phospholipidosis in cultured human cells. Biochem. Pharmacol. 49:1741-1745.

Honegger U.E., Zuehlke R.D., Scuntaro I., Schaefer M.H.A., Toplak H., and Wiesmann U.N. (1993). Cellular accumulation of amiodarone and desethylamiodarone in cultured human cells. Biochem. Pharmacol. 45:349-356.

Hostetler K.Y., Giordano J.R., and Jellison E.J. (1988). In vitro inhibition of lysosomal phospholipase A1 of rat lung by amiodarone and desethylamiodarone. Biochim. Biophys. Acta 959:316-321.

Hostetler K.Y., Reasor M.J., Walker E.R., Yazaki P.J., and Frazee B.W. (1986). Role of phospholipase A inhibition in amiodarone pulmonary toxicity in rats. Biochim. Biophys. Acta 875:400-405.

Imberti R., Nieminen A.L., Duncan P.R., Herman B., and Lemasters J.J. (1990). Mitochondrial inhibition and uncoupling preceeding lethal injury to rat hepatocytes by *t*-butly hydroperoxide: protection by fructose, oligomycin, cyclosporin A and trifluoperazine. Hepatology 12:933.

Imberti R., Nieminen A.L., Herman B., and Lemasters J.J. (1992). Synergism of cyclosporin A and phopholipase inhibitors in protection against lethal injury to rat hepatocytes oxidant chemicals. Res. Commun. Chem. Pathol. Pharmacol. 78:27-38.

Israel-Biet D., Venet I., Caubarrere I., Bonan G., Dennewald G., Pechaud D., Danel C., and Chretien J. (1985). Amiodarone pneumonitis mechanism: evaluation by bronchoalveolar lavage (BAL). Am. J. Respir. Dis. 131:A74.

Isreal-Biet D., Venet A., Caubarrere I., Bonan G., Danel C., Chretien J., and Hance A. (1987). Bronchoalveolar lavage in amiodarone pneumonitis: cellular abnormalities and their relevance to pathogenesis. Chest 91:214-221.

Iyer S.N., Wild J.S., Schiedt M.J., Hyde D.M., Margolin S.B., and Giri S.N. (1995). Dietary intake of pirfenidone ameliorates bleomycin-induced lung fibrosis in hamsters. J Lab Clin Med 125:779-785.

Johnston D.E. and Kroening C. (1998). Mechansim of early carbon tertachloride toxicity in cultured rat hepatocytes. Pharmacol. Toxicol. 83:231-239.

Julian D.G., Camm A.J., Frangin G., Janse M.J., Schwartz P.J., and Simon P. (1997). Randomised trial of effect of amiodarone on mortality in patients with left-ventricular dysfunction after recent myocardial infarction: EMIAT. The Lancet 349:667-674.

Kachel D.L., Moyer T.P., and Martin W.J. (1990). Amiodarone-induced injury of human pulmonary artery endothelial cells: Protection by α-tocopherol. J. Pharmacol. Exp. Ther. 254:1107-1112.

Kannan R., Sarma J.S., Guha M., and Venkataraman K. (1989a). Tissue drug accumulation and ultrastructural changes during amiodarone administration in rats. Fundam. Appl. Toxicol. 13:793-803.

Kannan,R., Sarma,J.S.M., Guha,M., Swaminathan,N., and Venkataraman,K. (1990). Role of vitamin-E in amiodarone-induced phospholipidosis in rats. FASEB Journal 4:1127A.

Kannan R., Sarma J.S.M., Guha M., and Venkataraman K. (1989b). Tissue drug accumulation and ultrastructrual changes during amiodarone administration in rats. Fund. Appl. Toxicol. 13:793-803.

Kannan R., Sarma J.S.M., Guha M., and Venkataraman K. (1990a). Desethylamiodarone-induced phospholipidosis in lung, liver, and pulmonary macrophages and its relationship to tissue drug accumulation in rats. Eur. J. Pharmacol. 183:2452-2453.

Kannan R., Sarma J.S.M., Guha M., and Venkataraman K. (1991). Amiodarone toxicity. II. Desethylamiodarone-induced phospholipidosis and ultrastructural changes during repeated administration in rats. Fundam. Appl. Toxicol. 16:103-109.

Kehrer J.P. and Margolin S.B. (1997). Pirfenidone diminshes cylophosphamide-induced lung fibrosis in mice. Toxicol. Lett. 90:125-132.

Kennedy J.I. (1990). Clinical aspects of amiodarone pulmonary toxicity. Clin. Chest Med. 11:119-129.

Kennedy T.P., Gordon G.B., Paky A., McShane A., Adkinson N.F., Peters S.P., Friday K., Jackman W., Sciuto A.M., and Gurtner G.H. (1988). Amiodarone causes acute oxidant lung injury in ventilated and perfused rabbit lungs. J. Cardiovasc. Pharmacol. 12:23-36.

Kerin N.Z., Blevins R.D., Frumin H., Faitel K., and Rubenfire M. (1985). Intravenous and oral loading versus oral loading alone with amiodarone for chronic refractory ventricular arrhythmias. Am. J. Cardiol. 55:89-91.

Kikkawa Y. and Yoneda K. (1974). The type II epithelial cell of the lung. I. Method of Isolation. Lab. Invest. 30:76-84.

Kikuta Y., Kusunose E., Matsubara S., Funae Y., Imaoka S., Kubota I., and Kusunose M. (1989). Purification and characterization of hepatic microsomal prostaglandin omega-hydroxylase cytochrome P-450 from pregnant rabbits. J. Biochem. (Tokyo.) 106:468-473.

Kodama I., Kamiya K., and Toyama J. (1999). Amiodarone: ionic and cellular mechanisms of action of the most promising class III agent. Am. J. Cardiol. 84:20R-28R.

Kodavanti P.R., Pentyala S.N., Yallapragada P.R., and Desaiah D. (1992). Amiodarone and desethylamiodarone increase intrasynaptosomal free calcium through receptor mediated channel. Naunyn Schmiedebergs Arch Pharmacol. 345:213-221.

Kodavanti U.P. and Mehendale H.M. (1991). Amiodarone- and desethylamiodarone-induced pulmonary phospholipidosis, inhibition of

phospholipases in vivo, and alteration of [14C]amiodarone uptake by perfused lung. Am. J. Respir. Cell Mol. Biol. 4:369-378.

Kudenchuk P., Pierson D., Greene H., Graham E., Sears G., and Trobaugh G. (1984). Prospective evaluation of amiodarone pulmonary toxicity. Chest 86:541-548.

Kudenchuk P.J., Cobb L.A., Copass M.K., Cummins R.O., Doherty A.M., Fahrenbruch C.E., Hallstrom A.P., Murray W.A., Olsufka M., and Walsh T. (1999). Amiodarone for resuscitation after out-of-hospital cardiac arrest due to ventricular fibrillation New Eng. J. Med. 341:871-878.

Lag M., Becher R., Samuelsen J.T., Wiger R., Refsnes M., Huitfeldt H.S., and Schwarze P.E. (1996). Expression of CYP2B1 in freshly isolated and proliferating cultures of epithelial rat lung cells. Exp. Lung Res. 22:627-649.

Larrey D., Tinel M., Letteron P., Geneve J., Descatoire V., and Pessayre D. (1986). Formation of an inactive cytochrome P-450Fe(II)-metabolite complex after administration of amiodarone in rats, mice and hamsters. Biochem. Pharmacol. 35:2213-2220.

Latini R., Tognoni G., and Kates R. (1984). Clinical pharmacokinetics of amiodarone. Clin. Pharmacokinetics 9:136-156.

Leech J.A., Gallastegui J., and Swiryn S. (1984). Pulmonary toxicity of amiodarone. Chest 85:444-445.

Leeder R.G., Brien J.F., and Massey T.E. (1994). Investigation of the role of oxidative stress in amiodarone-induced pulmonary toxicity in the hamster. Can. J. Physiol. Pharmacol. 72:613-621.

Leeder R.G., Rafeiro E., Brien J.F., Mandin C.C., and Massey T.E. (1996). Evaluation of reactive oxygen species involvement in amiodarone pulmonary toxicity in vivo and in vitro. J. Biochem. Toxicol. 11:147-159.

Lemasters J.J., Nieminen A.L., Qian T., Trost L.C., Elmore S.P., Nishimura Y., Crowe R.A., Cascio W.E., Bradham C.A., Brenner D.A., and Herman B. (1998). The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta 1366:177-196.

Li A.S.W. and Chignell C.F. (1987). Spectroscopic studies of cutaneous photosensitizing agents -IX. A spin trapping study of the photolysis of amiodarone and desethylamiodarone. Photochem. Photobiol. 45:191-197.

Lowry O.H., Rosebrough N.J., Farr A.L., and Randall R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Lullmann H., Lullmann-Rauch R., and Wasserman O. (1978). Lipidosis induced by amphiphilic cationic drugs. Biochem. Pharmacol. 27:1103-1108.

Magro S.A., Lawrence E.C., Wheeler S.H., Krafchek J., Lin H.T., and Wyndham C.R. (1988). Amiodarone pulmonary toxicity: prospective evaluation of serial pulmonary function tests. J. Am. Coll. Cardiol. 12:781-788.

Mahmarian J.J., Smart F.W., Moye L.A., Young J.B., Francis M.J., Kingry C.L., Verani M.S., and Pratt C.M. (1994). Exploring the minimal dose of amiodarone with antiarrhythmic and hemodynamic activity. Am. J. Cardiol. 74:681-686.

Mansani F.P., Dinis T.C., Carnieri E.G., and Madeira V.M. (1999). Neutrality of amiodarone on the initiation and propagation of membrane lipid peroxidation. Cell Biochem. Funct. 17:131-142.

Marchlinski F.E. (1987). Amiodarone: therapeutic but toxic. N. Y. State J. Med. 87:322-324.

Marchlinski F.E., Gansler T.S., Waxman H.L., and Josephson M.E. (1982). Amiodarone pulmonary toxicity. Annals Intern. Med. 97:839-845.

Martin J., Dinsdale D., and White I.N. (1993). Characterization of Clara and type II cells isolated from rat lung by fluorescence-activated flow cytometry. Biochem. J 295 (Pt 1):73-80.

Martin W.J. (1990a). Mechanisms of amiodarone pulmonary toxicity. Clin. Chest Med. 11:131-138.

Martin W.J. (1990b). Pulmonary toxicity induced by chemical agents. Eur Respir. J 3:375-376.

Martin W.J. and Howard D.M. (1985). Amiodarone-induced lung toxicity; *in vitro* evidence for the direct toxicity of the drug. Am. J. Pathol. 120:344-350.

Martin W.J. and Rosenow E.C. (1988). Amiodarone pulmonary toxicity: recognition and pathogenesis (Part I). Chest 93:1067-1075.

Martin W.J. and Standing J.E. (1988). Amiodarone pulmonary toxicity: biochemical evidence for a cellular phospholipidosis in the bronchoalveolar lavage of human subjects. J. Pharmacol. Exp. Ther. 244:774-779.

Mason J.W. (1987). Amiodarone. New Eng. J. Med. 316:455-466.

Mason J.W., Hondeghem L.M., and Katzung B.G. (1983). Amiodarone blocks inactivated cardiac sodium channels. Pflugers Arch. 396:79-81.

Mason J.W., Hondeghem L.M., and Katzung B.G. (1984). Block of inactivated sodium channels and of depolarization-induced automaticity in guinea pig papillary muscle by amiodarone. Circ. Res. 55:278-285.

Mason RJ and Williams MC (1991). Alveolar Type II cells. In: The Lung: Scientific Foundations, Crystal,R.G. and West J.B., Eds., pp 235-246, Raven Press, Ltd., New York.

Massaro G.D., Singh G., Mason R., Plopper C.G., Malkinson A.M., and Gail D.B. (1993). Biology of the clara cell. Conference ReportL101-L106.

Massey TE (1989). Isolation and use of lung cells in toxicology. In: *In Vitro* Toxicology: Model Systems and Methods, McQueen,C.A., Ed., pp 35-66, Telford Press, Caldwell.

Massey T.E., Leeder R.G., Rafeiro E., and Brien J.F. (1995). The 1994 Veylien Henderson Award of the Society of Toxicology of Canada. Mechanisms in the pathogenesis of amiodarone-induced pulmonary toxicity. Can. J. Physiol. Pharmacol. 73:1675-1685.

Matola T.D., D'Ascoli F., Fenzi G., Rossi G., Martino E., Bogazzi F., and Vitale M. (2000). Amiodarone induces cytochrome *c* release and apoptosis through an iodine-independent mechanism. J. Clin. Endocrinol. Metab. 85:4323-4330.

Mazue G., Vic P., Gouy D., Remandet B., Lacheretz F., Berthe J., Barchewitz G., and Gagnol J. (1984). Recovery from amiodarone-induced lipidosis in laboratory animals: a toxicological study. Fund. Appl. Toxicol. 4:992-999.

McKenna W.J., Rowland E., and Krikler D.M. (1983). Amiodarone: the experience of the past decade. Br. Med. J. 287:1654-1656.

McLemore T.L., Adelberg S., Liu M.C., McMahon N.A., Yu S.J., and Hubbard W.C. (1990). Expression of CYP1A1 gene in patients with lung cancer: Evidence for cigarette smoke-induced gene expression in normal lung tissue and for altered gene regulation in primary pulmonary carcinomas. J. Natl. Cancer Inst. 82:1333-1339.

McNeil K.D., Oliver W., Firouz-Abadi A., and Zimmerman P.V. (1992). Amiodarone pulmonary toxicity - three unusual manifestations. Aust. N. Z. J. Med. 22:14-18.

Miaskiewicz S.L., Amico J.A., Follansbee W.P., and Levey G.S. (1987). Amiodarone-associated thyrotoxicosis masquerading as painful thyroiditis. Annals Intern. Med. 107:118-119.

Moore K. and Roberts II L.J. (1998). Measurement of lipid peroxidation. Free Rad. Res. 28:659-671.

Morrow J.D. and Roberts L.J. (1997). The isoprostanes: unique bioactive products of lipid peroxidation. Prog. Lipid Res. 36:1-21.

Mostow N.D., Vrobel T.R., Noon D., and Rakita L. (1986). Rapid suppression of complex ventricular arrhythmias with high-dose oral amiodarone. Circulation 73:1231-1238.

Myers J.L., Kennedy J.I., and Plumb V.J. (1987). Amiodarone lung: pathologic findings in clinically toxic patients. Hum. Pathol. 18:349-354.

Myles J.E., Geddes B.A., and Massey T.E. (1989). Biotransformation activities in Clara and alveolar type II cells isolated from hamster lungs. Res. Commun. Chem. Pathol. Pharmacol. 66:297-309.

Myung S., Chang Y., Min H., Kim D., Kim M., Kang T., Yoo E., Sohn Y.T., and Yin H. (2000). Characterization of amiodarone metabolites and impurities using liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Rapid Commun. Mass Spectrom. 14:2046-2054.

Nadeau D. and Lane D.A. (1988). The cytotoxicity of chrysotile asbestos fibers to pulmonary alveolar macrophages. I. Effects of inhibitors of ADP-ribosyl transferase. Cell Biol. Toxicol. 4:13-30.

Nalos P.C., Kass R.M., Gang E.S., Fishbein M.C., Mandel W.J., and Peter T. (1987). Life-threatening postoperative pulmonary complications in patients with previous amiodarone pulmonary toxicity undergoing cardiothoracic operations. J. Thorac. Cardiovasc. Surg. 93:904-912.

Nattel S. (1986). Pharmacodynamic studies of amiodarone and its active N-desethyl metabolite. J. Cardiovasc. Pharmacol. 8:771-777.

Nattel S. and Talajic M. (1988). Recent advances in understanding the pharmacology of amiodarone. Drugs 36:121-131.

Nattel S., Talajic M., Quantz M., and De Roode M. (1987). Frequency-dependent effects of amiodarone on atrioventricular nodal function and slow-channel action potentials:evidence for channel-blocking activity. Circulation 76:442-449.

Nazareth W., Nasser Y., and Crompton M. (1991). Inhibition of anoxia-induced injury in heart myocytes by cyclosporin A. J. Mol. Cell. Cardiol. 23:1351-1354.

Nedergaard J. and Cannon B. (1979). Overview- Preparation and properties of mitochondria from different sources. Methods Enzymol. 55:3-12.

Nichols W.K., Covington M.O., Seiders C.D., Safiullah S., and Yost G.S. (1992). Bioactivation of halogenated hydrocarbons by rabbit pulmonary cells. Pharmacol. Toxicol. 71:335-339.

Nicolet-Chatelain G., Prevost M.C., Escamilla R., and Migueres J. (1991). Amiodarone-induced pulmonary toxicity; Immunoallergologic tests and bronchoalveolar lavage phospholipid content. Chest 99:363-369.

Nicotera P., Bellomo G., and Orrenius S. (1992). Calcium-mediated mechanisms in chemically induced cell death. Annu. Rev. Pharmacol. Toxicol. 32:449-470.

O'Neill C.A. and Giri S.N. (1994). Biochemical mechanisms for the attenuation of bleomycin-induced lung fibrosis by treatment with niacin in hamsters: the role of NAD and ATP. Exp. Lung Res. 20:41-56.

Ogle C.L. and Reasor M.J. (1990). Response of alveolar macrophages to amiodarone and desethylamiodarone, in vitro. Toxicology. 62:227-238.

Ohar J.A., Jackson F., Dettenmeier P.A., Bedrossian C.W., Tricomi S.M., and Evans R.G. (1992). Bronchoalveolar lavage cell count and differential are not reliable indicators of amiodarone-induced pneumonitis. Chest 102:999-1004.

Ohyama K., Nakajima M., Nakamura S., Shimada N., Yamazaki H., and Yokoi T. (2000a). A significant role of human cytochrome P450 2C8 in amiodarone N-deethylation: An approach to predict the contribution with relative activity factor Drug Metab. Dispos. 28:1303-1310.

Ohyama K., Nakajima M., Suzuki M., Shimada N., Yamazaki H., and Yokoi T. (2000b). Inhibitory effects of amiodarone and its N-deethylated metabolite on human cytochrome P450 activities: prediction of in vivo drug interactions. Br. J. Clin. Pharmacol. 49:244-253.

Osowole O.A., Tonukari J.N., and Owaifo A.O. (1992). Isolation and partial characterization of pigeon pea protease inhibitor: Its effect on the genotoxic action of aflatoxin B_1. Biochem. Pharmacol. 43:1880-1882.

Padma P. and Setty O.H. (1997). Protective effect of vitamin E against ethionine toxicity. Biochem. Mol. Biol. Int. 41:785-795.

Padmavathy B., Devaraj H., and Devaraj N. (1993). Amiodarone-induced changes in surfactant phospholipids of rat lung. Naunyn-Schmiedeberg's Arch. Pharmacol. 347:421-424.

Pichler W.J., Schindler L., Staubli M., Stadler B.M., and de Weck A.L. (1988). Anti-amiodarone antibodies: detection and relationship to the development of side effects. Am. J. Med. 85:197-202.

Pirovino M., Muller O., Zysset T., and Honegger U. (1988). Amiodarone-induced hepatic phospholipidosis: Correlation of morphological and biochemical findings in an animal model. Hepatology 8:591-598.

Pitcher W.D. (1992). Southwestern international medicine conference:amiodarone pulmonary toxicity. Am. J. Med. Sci. 303:206-212.

Plomp T.A., van Rossum J.M., Robles de Medina E.O., van Lier T., and Maes R.A.A. (1984). Pharmacokinetics and body distribution of amiodarone in man. Arzneimittelforschung 34:513-520.

Plomp T.A., Wiersinga W.M., and Maes R.A.A. (1985). Tissue distribution of amiodarone and desethylamiodarone in rats after repeated oral administration of various amiodarone dosages. Arzneimittelforschung 35:1805-1810.

Plopper CG and Hyde DM (1992). Epithelial Cells of Bronchioles. In: Comparative Biology of the Normal Human Lung, Parent,R.A., Ed., pp 85-92, CRC Press, Ann Arbour, Michigan.

Plopper CG, Hyde DM, and Buckpitt AR (1991). Clara cells. In: The lung., Crystal,R.G. and West J.B., Eds., pp 215-228, Raven Press Ltd., New York.

Podrid P.J. (1995). Amiodarone: reevaluation of an old drug. Ann. Intern. Med. 122:689-700.

Pollak P.T. (1999). Clinical organ toxicity of antiarrhythmic compounds: ocular and pulmonary manifestations. Am. J. Cardiol. 84:37R-45R.

Pollak P.T., Bouillon T., and Shafer S.L. (2000). Population pharmacokinetics of long-term oral amiodarone therapy. Clin. Pharmacol. ther. 67:642-652.

Pollak P.T. and Sami M. (1984). Acute necrotizing pneumonitis and hyperglycemia after amiodarone therapy. Am. J. Med. 76:935-939.

Poucell S., Ireton J., Downer E., Larratt L., Patterson J., Blendis L., and Phillips M.J. (1984). Amiodarone-associated phospholipidosis and fibrosis of the liver. Gastroenterology 86:926-936.

Powis G., Olsen R., Standing J.E., Kachel D., and Martin W.J. (1990). Amiodarone-mediated increase in intracellular free Ca2+ associated with cellular injury to human pulmonary artery endothelial cells. Toxicol. Appl. Pharmacol. 103:156-164.

Pratico D. (1999). F_2-isoprostanes: sensitive and specific non-invasive indices of lipid peroxidation in vivo. Atherosclerosis 147:1-10.

Pre J., Perret G., Nicolas P., and Vassy R. (1991). Effect of a short term oral treatment by phenobarbital propranolol and amiodarone in rat on serum levels of fluorescent lipid-peroxidation products. Fundam. Clin. Pharmacol. 5:139-141.

Rafeiro,E. An investigation of mechanisms in the pathogenesis of amiodarone-induced pulmonary toxicity. (1997). PhD. Thesis. Kingston, Ontario, Queen's University.

Rafeiro E., Leeder R.G., Brien J.F., Kabalka G.W., Chatla N., and Massey T.E. (1994). Comparison of the in vivo pulmonary toxicity of amiodarone and des-oxo-amiodarone in the hamster. Toxicol. Appl. Pharmacol. 127:275-281.

Rafeiro E., Leeder R.G., Daniels J.M., Brien J.F., and Massey T.E. (1990). In vitro hepatic, renal, and pulmonary N-dealkylation of amiodarone. Biochem. Pharmacol. 39:1627-1629.

Raghu G., Johnson W.C., Lockhart D., and Mageto Y. (1999). Treatment of idiopathic pulmonary fibrosis with a new antifibrotic agent, pirfenidone: results of a prospective, open-label Phase II study. Am. J. Respir. Crit. Care Med. 159:t-9.

Rakita L., Sobol S., Mostow N., and Vrobel T. (1983). Amiodarone pulmonary toxicity. Am. Heart J. 106:906-913.

Rao R.H., McCready V.R., and Spathis G.S. (1986). Iodine kinetic studies during amiodarone treatment. J. Clin. Endocrinol. Metab. 62:563-568.

Raunio H., Hakkola J., Hukkanen J., Lassila A., Paivarinta K., Pelkonen O., Anttila S., Piipari R., Boobis A., and Edwards R.J. (1900). Expression of xenobiotic-metabolizing CYPs in human pulmonary tissue. [Review] [49 refs]. Exp. Toxicol. Pathol. 51:412-417.

Reasor M.J. (1989). A review of the biology and toxicologic implications of the induction of lysosomal lamellar bodies by drugs. Toxicol. Appl. Pharmacol. 97:47-56.

Reasor M.J. and Kacew S. (1996a). An evaluation of possible mechanisms underlying amiodarone-induced pulmonary toxicity. Proc. Soc. Exp. Biol. Med. 212:297-304.

Reasor M.J., McCloud C.M., Beard T.L., Ebert D.C., Kacew S., Gardner M.F., Aldern K.A., and Hostetler K.Y. (1996b). Comparative evaluation of amiodarone-induced phospholipidosis and drug accumulation in Fischer-344 and Sprague-Dawley rats. Toxicology. 106:139-147.

Reasor M.J., McCloud C.M., DiMatteo M., Schafer R., Ima A., and Lemaire I. (1996a). Effects of amiodarone-induced phospholipidosis on pulmonary host defense functions in rats. Proc. Soc. Exp. Biol. Med. 211:346-352.

Reasor M.J., Ogle C.L., and Miles P.R. (1990). Response of rat lungs to amiodarone: Preferential accumulation of amiodarone and desethylamiodarone in alveolar macrophages. Exp. Lung Res. 16:577-591.

Reasor M.J., Ogle C.L., Walker E.R., and Kacew S. (1988). Amiodarone-induced phospholipidosis in rat alveolar macrophages. Am. Rev. Respir. Dis. 137:510-518.

Reers M., Smiley S.T., Mottola-Hartshorn C., Chen A., Lin M., and Chen L.B. (1995). Mitochondrial membrane potential monitored by JC-1 dye. Methods Enzymol. 260:406-417.

Reinhart P.G. and Gairola C.G. (1997). Amiodarone-induced pulmonary toxicity in Fisher rats: release of tumor necrosis factor alpha and transforming growth factor beta by pulmonary alveolar macrophages. J. Toxicol. Environ. Health 52:353-365.

Reiser K.M. and Last J.A. (1986). Early cellular events in pulmonary fibrosis. Exp. Lung Res. 10:331-355.

Rekka E., Mannhold R.M., Bast A., and Timmerman H. (1990). Molecular pharmacological aspects of antiarrhythmic activity. I Class I and class III compounds and lipid peroxidation. Biochem. Pharmacol. 39:95-100.

Ribeiro S.M.R., Campello A.P., Nascimento A.J., and Kluppel M.L.W. (1997). Effect of amiodarone (AMD) on the antioxidant enzymes, lipid peroxidation, and mitochondrial metabolism. Cell Biochem. Funct. 15:145-152.

Rimbach G., Hohler D., Fischer A., Roy S., Virgili F., Pallauf J., and Packer L. (1999). Methods to assess free radicals and oxidative stress in biological systems. Arch Tierernahr. 52:203-222.

Riva E., Gerna M., Neyroz P., Urso R., Bartosek I., and Guaitani A. (1982). Pharmacokinetics of amiodarone in rats. J. Cardiovasc. Pharmacol. 4:270-275.

Riva E., Marchi S., Pesenti A., Bizzi A., Cini M., Veneroni E., Tavbani E., Boeri R., Bertani T., and Latini R. (1987). Amiodarone induced phospholipidosis. Biochemical, morphological and functional changes in the lungs of rats chronically treated with amiodarone. Biochem. Pharmacol. 36:3209-3214.

Roden D.M. (1993). Pharmacokinetics of amiodarone:implications for drug therapy. Am. J. Cardiol. 72:45f-50f.

Roden D.M. (1999). Mechanisms underlying variability in response to drug therapy: implications for amiodarone use. Am. J. Cardiol. 84:29R-36R.

Rosen G.M. and Rauckman E.J. (1977). Formation and reduction of a nitroxide radical by liver microsomes. Biochem. Pharmacol. 26:675-678.

Rothenberg F., Franklin J.O., and DeMaio S.J. (1994). Use, value, and toxicity of amiodarone. Heart Disease & Stroke 3:19-23.

Rotmensch H.H., Liron M., Tupilski M., and Laniado S. (1980). Possible association of pneumonitis with amiodarone therapy. Am. Heart J. 100:412-413.

Rotmensh H.H., Belhassen B., Swanson B.N., Shoshani D., Spielman S.R., Greenspon A.J., Greenspan A.M., Vlasses P.H., and Horowitz L.N. (1984). Steady-state serum amiodarone concentrations: relationships with antiarrhythmic efficacy and toxicity. Annals Intern. Med. 101:462-469.

Roussel J.P., Grazzini E., Guipponi M., and Astier H. (1995). Dihydropyridine-like effects of amiodarone and desethylamiodarone on thyrotropin secretion and

intracellular calcium concentration in rat pituitary. Eur. J. Endocrinol. 133:489-498.

Ruch R.J., Bandyopadhyay S., Somani P., and Klaunig J.E. (1991). Evaluation of amiodarone free radical toxicity in rat hepatocytes. Toxicol. Lett. 56:117-126.

Salvioli S., Ardizzoni A., Franceschi C., and Cossarizza A. (1997). JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett. 411:77-82.

Salvioli S., Maseroli R., Pazienza T.L., Bobyleva V., and Cossarizza A. (1998). Use of flow cytometry as a tool to study mitochondrial membrane potential in isolated, living hepatocytes. Biochemistry. Biokhimiia. 63:235-238.

Sandron D., Israel-Biet D., Venet A., and Chretien J. (1986). Immunoglobulin abnormalities in bronchoalveolar lavage specimens from amiodarone-treated subjects. Chest 89:617-618.

Saraste M. (1999). Oxidative phosphorylation at the fin de siecle. Science 283:1488-1493.

Sautereau A., Tournaire C., Suares M., Tocanne J., and Paillous N. (1992). Interactions of amiodarone with model membranes and amiodarone-photoinduced peroxidation of lipids. Biochem. Pharmacol. 43:2559-2566.

Scuntaro I., Kientsch U., Wiesmann U.N., and Honegger U.E. (1996). Inhibition by vitamin E of drug accumulation and of phospholipidosis induced by desipramine and other cationic amphiphilic drugs in human cultured cells. Br. J. Pharmacol. 119:829-834.

Seaton T.A., Cooper J.M., and Schapira A.H.V. (1998). Cyclosporin inhibition of apoptosis induced by mitochondial complex I toxins. Brain Res. 809:12-17.

Seglen P.O. (1972). Preparation of rat liver cells. I. Effects of Ca^{2+} on enzymatic dispersion of isolated, perfused liver. Exp. Cell Res. 74:450-454.

Sheppard M.N. and Harrison N.K. (1992). Lung injury, inflammatory mediators, and fibroblast activation in fibrosing alveolitis. Thorax 47:1064-1074.

Singh B.N. (1983). Amiodarone: historical development and pharmacologic profile. Am. Heart J. 106:788-797.

Singh B.N. (1996). Antiarrhythmic actions of amiodarone: a profile of a paradoxical agent. Am. J. Cardiol. 78:41-53.

Singh B.N. and Vaughan Williams E.M. (1970). The effect of amiodarone, a new anti-anginal drug, on cardiac muscle. Br. J. Pharmacol. 39:657-667.

Singh S., Fletcher R.D., Fisher S., Deedwania P., Lewis D., Massie B., Singh B., and Colling C.L. (1992). Congestive heart failure: survival trial of antiarrhythmic therapy. Control. Clin. Trials 13:339-350.

Smith G.B.J., Castonguay A., Donnelly P.J., Reid K.R., Petsikas D., and Massey T.E. (1999). Biotransformation of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in freshly isolated human lung cells. Carcinogenesis 20:1809-1818.

Snyder J.W., Pastorino J.G., Attie A.M., and Farber J.L. (1992). Portection by cyclosporin A of cultured hepatocytes from the toxic consequences of the loss of mitochondrial energization produced by 1-methyl-4-phenylpyridinium. Biochem. Pharmacol. 44:833-835.

Sokal R.R. and Rohlf F.J. (1973). Introduction to biostatistics. W.H.Freeman and Company, San Francisco.

Sorokin SP (1970). The cells of the lung. In: Morphology of Experimental Respiratory Carcinogenesis, Nettesheim,P., Hanna M.G., and Deatherage J.W., Eds., pp 23-44, US Atomic Energy Commission Symposium Series, Oak Ridge,TN.

Staubli M., Troendle A., Schmidt B., Palmer P., Kohler B., Studer H., and Bircher J. (1985). Pharmacokinetics of amiodarone, desethylamiodarone, and other iodine-containing amiodarone metabolites. Eur. J. Clin. Pharmacol. 29:417-423.

Suarez L.D., Poderoso J.J., Elsner B., Bunster A.M., Esteva H., and Bellotti M. (1983). Subacute pneumopathy during amiodarone therapy. Chest 83:566-658.

Sunderji R., Kanji Z., and Gin K. (2000). Pulmonary effects of low dose amiodarone: A review of the risks and recommendations for surveillance. Can. J. Cardiol. 16:1435-1440.

Taylor,M.D., Antonini,J.M., Roberts,J.R., Van Dyke,K., Bowman,L.L., Castranova,V., Hubbs,A.F., Shi,X., Leonard,S.S., and Reasor,M.J. (2001). Intratracheal amiodarone adminstration to Fisher 344 rats causes acute damage to airways and parenchymal cells; the possible role of free radical generation. Toxicologist 60:425-425.

Taylor M.D., Van Dyke K., Bowman L.L., Miles P.R., Mason R.J., Shannon K., and Reasor M.J. (2000). A characterization of amiodarone-induced pulmonary toxicity in F344 rats and identification of surfactant protein-D as a potential biomarker for the development of the toxicity. Toxicol. Appl. Pharmacol. 167:182-190.

Tekkanat K.K. and Fox I.H. (1988). Isocratic separation of ATP and its degradation products from biological fluids by automated liquid chromatography. Clin. Chem. 34:925-932.

Trivier J.M., Libersa C., Belloc C., and Lhermitte M. (1993). Amiodarone N-deethylation in human liver microsomes: Involvement of cytochrome P450 3A enzymes (first report). Life Sci. 52:PL-91-PL-96.

Turrens J.F. (1997). Superoxide production by the mitochondrial respiratory chain. Biosciences Reports 17:3-7.

Tyson CA and Green CE (1987). Cytotoxicity measures: choices and methods. In: The Isolated Hepatocyte: Use in Toxicology and Xenobiotic Biotransformations, Rauckman,E.J. and Padilla G.M., Eds., pp 119-158, Academic Press, Orlando.

Urano S., Yano K., and Matsuo M. (1988). Membrane-stabilizing effects of vitamin E: effect of α-tocopherol and its model compounds on fluidity of lecithin liposomes. Biochem. Biophys. Res. Commun. 150:469-475.

Van Schepdael J. and Solvay H. (1970). [Clinical study of amiodarone in cardiac rhythmic disorders]. Presse Med. 78:1849-1850.

van Zandwijk N., Darmanata J.I., Duren D.R., Alberts C., Durrer D., and Wagenvoort C. (1983). Amiodarone pneumonitis. Eur. J. Respir. Dis. 64:313-317.

Venet A., Bonan G., and Caubarrere I. (1984). Five cases of immune-mediated amiodarone pneumonitis. Lancet 1:962-963.

Vereckei A., Blazovics A., Gyorgy I., Feher E., Toth M., Szenasi G., Zsinka A., Foldiak G., and Feher J. (1993). The role of free radicals in the pathogenesis of amiodarone toxicity. J. Cardiovasc. Electrophysiol. 4:161-177.

Vereckei A., Blazovics A., Szenasi G., Konya L., Lang I., Zsinka A., and Feher J. (1991). The role of oxidative stress, caused by amiodarone, in the side effects of the drug. Orv. Hetil. 132:483-484.

Vig P.J.S., Yallapragada P.R., Kodavanti P.R.S., and Desaiah D. (1991). Modulation of calmodulin properties by amiodarone and its major metabolite desethylamiodarone. Pharmacol. Toxicol. 68:26-33.

Vrobel T.R., Miller P.E., Mostow N.D., and Rakita L. (1989). A general overview of amiodarone toxicity: its prevention, detection, and management. Prog. Cardiovasc. Dis. 31:393-426.

Wallace K.B., Eells J.T., Madeira V.M.C., Cortopassi G., and Jones D.P. (1997). Symposium overview: Mitochondria-mediated cell injury. Fundam. Appl. Toxicol. 38:23-37.

Walter D.H., Haendeler J., Galle J., Zeiher A.M., and Dimmeler S. (1998). Cyclosporin A inhibits apoptosis of human endothelial cells by preventing release of cytochrome C from the mitochondria. Circulation 98:1153-1157.

Wang Q., Hollinger M.A., and Giri S.N. (1992). Attenuation of amiodarone-induced lung fibrosis and phospholipidosis in hamsters by taurine and/or niacin treatment. J. Pharmacol. Exp. Ther. 262:127-132.

Weitberg A.B. (1989). Effect of nicotinic acid supplementation in vivo on oxygen radical-induced genetic damage in human lymphocytes. Mutat. Res. 216:197-201.

White R.J. and Reynolds I.J. (1996). Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. J. Neurosci. 16:5688-5697.

Willey J.C., Coy E., Brolly C., Utell M.J., Frampton M.W., Hammersley J., Thilly W.G., Olson D., and Cairns K. (1996). Xenobiotic metabolism enzyme gene expression in human bronchial epithelial and alveolar macrophage cells. Am. J Respir. Cell Mol. Biol. 14:262-271.

Wilson AGE (1982). Toxicokinetics of uptake, accumulation, and metabolism of chemicals by the lung. In: Mechanisms in Respiratory Toxicology, Vol. 1, Witschi,H.P. and Nettesheim P., Eds., pp 161-185, CRC Press, Boca Raton, Florida.

Wilson B.D., Clarkson C.E., and Lippmann M.L. (1991). Amiodarone-induced pulmonary inflammation. Correlation with drug dose and lung levels of drug, metabolite, and phospholipid. Am. Rev. Respir. Dis. 143:1110-1114.

Wilson B.D. and Lippmann M.L. (1990). Pulmonary accumulation of amiodarone and N-desethylamiodarone: Relationship to the development of pulmonary toxicity. Am. Rev. Respir. Dis. 141:1553-1558.

Wilson B.D. and Lippmann M.L. (1996b). Susceptibility to amiodarone-induced pulmonary toxicity: relationship to the uptake of amiodarone by isolated lung cells. Lung 174:31-41.

Wilson J.S. and Podrid P.J. (1991). Side effects from amiodarone. Am. Heart J. 121(1 PT 1:158-171.

Witting P.K., Upston J.M., and Stocker R. (1998). The molecular action of alpha-tocopherol in lipoprotein lipid peroxidation. Pro- and antioxidant activity of vitamin E in complex heterogeneous lipid emulsions. Subcell. Biochem. 30:345-390.

Yasuda S.U., Sausville E.A., Hutchins J.B., Kennedy T., and Woosley R.L. (1996). Amiodarone-induced lymphocyte toxicity and mitochondrial function. J. Cardiovasc. Pharmacol. 28:94-100.

Young R.A. and Mehendale H.M. (1987). Effect of cytochrome P-450 and flavin-containing monooxygenase modifying factors on the in vitro metabolism of amiodarone by rat and rabbit. Drug Metab. Dispos. 15:511-517.

Zitnik R.J., Cooper J.A.D., Rankin J.A., and Sussman J. (1992). Effects of in vitro amiodarone exposure on alveolar macrophage inflammatory mediator production. Am. J. Med. Sci. 304:352-356.