AMELIORATION OF OXIDATIVE LUNG INJURY BY ANTIARRHYTHMIC AGENTS.

by

Kumuda C. Das

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and state University in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY
in
Veterinary Medical Sciences
(Environmental Toxicology)

APPROVED:

Hara P. Misra

Hara P. Misra, Chairman.

Ryland E. Webb

Ryland E. Webb

S. Ansar Ahmed

Eugene M. Gregory

Eugene M. Gregory

James C. Keith, Jr.

October, 1992
Blacksburg, Virginia.

AMELIORATION OF OXIDATIVE LUNG INJURY BY ANTIARRHYTHMIC AGENTS

BY

Kumuda C. Das

Committee Chairman: Hara P. Misra
Veterinary Medical Sciences

(ABSTRACT)

Class I antiarrhythmic drugs, such as lidocaine, quinidine and procainamide, are known to be effective membrane stabilizers. However, the mechanism of such "membrane stabilization" has not been elucidated. In the present study we found that all three drugs are powerful scavengers of hydroxyl radical. In addition, lidocaine was found to be a quencher of singlet oxygen. These drugs are also found to inhibit NADPH-dependent lipid peroxidation in bovine lung microsomes in a dose dependent manner. Since oxyradicals are implicated in the lipid peroxidation process and antiarrhythmic drugs were found to scavenge/quench reactive oxygen species, we proposed that the membrane stabilizing effects of antiarrhythmic drugs may, in part, be due to their antioxidant properties.

Ischemia-reperfusion injury has been studied in many organs. Despite the evidence of functional, metabolic and structural abnormalities during reperfusion, the precise mechanism of reperfusion lung injury remains obscure. Data from the organ models suggest that toxic oxygen metabolites play an important role in the mechanism of reperfusion tissue injury. Lidocaine has also

been shown to be clinically valuable for the treatment and prevention of ventricular arrhythmia occurring after surgical correction of myocardial infraction. We found that the class I antiarrhythmic drugs are effective in ameliorating post-ischemic lung reperfusion injury in an ex vivo perfused rat lung model exposed to both normoxic and hyperoxic conditions.

Since phagocytes are known to generate reactive oxygen species and play an important role in causing irreversible oxidative tissue injury during reperfusion of organs, we examined the role of antiarrhythmic agents on macrophage function. We found that these drugs inhibit superoxide and hydrogen peroxide production in stimulated macrophages in a dose dependent manner. The diminished production of superoxide was found to be not due to the inactivation of superoxide generating NADPH-oxidase enzyme but by inhibition of the phagocytosis process by these drugs

The results of these studies indicate that the antiarrhythmic drugs, such as, lidocaine, quinidine and procainamide, are effective antioxidants and can protect biomembranes against lipid peroxidation injury and post-ischemic reperfusion injury of the lung. We have investigated the mechanism(s) of action of these drugs in ameliorating oxidative tissue injury and found that these drugs are not only effective in removing reactive oxygen species but also cause inactivation of pulmonary macrophage from inappropriately generating reactive species of oxygen. The fundamental knowledge derived from these studies could lead to enhanced functional improvement of patients following cardiopulmonary bypass, pulmonary arterial embolectomy and acute respiratory distress syndrome, all of which undergo a period of elective/induced ischemia and reperfusion or oxidative stress.

Acknowledgments

I sincerely thank Dr. Hara P. Misra, my advisor and mentor, for his support, guidance and encouragement throughout the course of my graduate program. I would also like to thank Dr. Ryland E. Webb, Dr. Eugene M. Gregory, Dr. S. Ansar Ahmed, and Dr. James C. Keith,Jr., members of my advisory committee for their patience and constructive criticism. Thanks are also due to Dr. Sami I. Said, Professor of Medicine and Physiology, State University of New York Medical Center at Stony Brooke for his patience and agreeing to serve as external examiner.

I gratefully acknowledge the financial support received from the Department of Biomedical Sciences and partial tuition waiver from the Office of the Research and Graduate Studies during my graduate program. Thanks are also due to the technical staff especially to Haywood Pyle for his help in flow cytometry.

I gratefully acknowledge Government of India for granting me leave from Indian Forest Service to undertake my graduate study at VPI & SU.

I will always be thankful to my wife Rita and sons, Chandan and Kundan for their patience and long hours of waiting during my graduate program. I also thank my parents for their patience in my long absence from home.

TABLE OF CONTENTS

CHAPTER II

LIDOCAINE: A hydroxyl radical scavenger and singlet oxygen quencher

CHAPTER III

ANTIARRHYTHMIC AGENTS: Scavengers of hydroxyl radicals and inhibitors of NADPH-dependent lipid peroxidation in bovine lung microsomes

CHAPTER VI

LIST OF TABLES

LIST OF FIGURES

CHAPTER IV

CHAPTER V

List of Abbreviations

$^1O_2$	Singlet oxygen
ATCC	American Type Cell Culture
DCF-DA	Dichloroflurescein Diacetate
DFP	Diisopropyl Fluorophosphate
DMPO	Dimethyl-pyrroline-N-oxide
EPR	Electron Paramagnetic Resonance
GEPS	Gelatin-EDTA-Phosphate-Saline
GX	Glycylxylidide
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
MEGX	Monoethylglycylxylidide
MEM	Minimum Essential Medium
NADPH	Nicotinamide Adenine Dinucleotide (reduced form)
$O_2^{-\cdot}$	Superoxide
.OH	Hydroxyl radical
PMA	Phorbol-12Myrister-13Acetate
PMN	Polymorphonuclear leukocytes
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide Dismutase
TEMP	Tetramethylpiperidine
TEMPO	Tetramethylpiperidine-N-Oxide

Introduction

Class I antiarrhythmic agents (lidocaine, quinidine and procainamide) are widely used drugs with significant stabilizing activity on lipid biomembranes (Stelzner et al., 1987). These drugs have been suggested as a means of reducing ischemic myocardial damage. Prolonged ischemia such as that following myocardial infarction, pulmonary embolism and conditions occurring during long-term coronary bypass procedures cause serious damage to these tissues. The cytoprotective effects of lidocaine, quinidine and procainamide in myocardial ischemia have been attributed to membrane stabilizing properties of these drugs. However, the mechanism of such "membrane stabilization" has not yet been elucidated.

Reoxygenation of ischemic tissue results in extensive tissue damage and this phenomenon has been called reperfusion injury or reperfusion paradox or post-ischemic tissue injury. The biochemical mechanism that underlies the post-ischemic tissue injury involves production of superoxide radical in the reperfused tissue. According to McCord's (McCord, 1985) hypothesis, prolonged ischemia results in the loss of high energy phosphate compounds manifested by a decrease in the cellular ATP levels. As the cell's energy charge drops, it is no longer able to maintain a proper ion gradient across its membrane and this precipitates a redistribution of calcium ions. Concomitantly, the depletion of the cell ATP results in an elevated concentration of AMP. The AMP is catabolized to adenosine, inosine, and then hypoxanthine. The elevated cytosolic calcium concentration, is believed to activate a protease capable of converting xanthine dehydrogenase to xanthine oxidase.

Hypoxanthine as well as xanthine accumulates in the cells during ischemia and are used as substrates for xanthine oxidase. Hence during ischemia two important changes occur in the tissue: a new enzyme activity appears, along with one of its two substrates. Xanthine oxidase uses O_2 as a second substrate to yield both $O_2^{-}\cdot$ and H_2O_2. With reoxygenation, O_2 is introduced and there is a burst of $O_2^{-}\cdot$ production in the reperfused tissue with subsequent H_2O_2 production (Grisham and McCord, 1986). In the presence of iron, $O_2^{-}\cdot$ and H_2O_2 can participate in an iron catalyzed Fenton reaction to produce hydroxyl radical. (Cohen, 1977):

$$O_2^{-}\cdot + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^{-}$$

Hydroxyl radicals are potent oxidants that cause tissue injury by initiating lipid peroxidation in cell membranes. Lipid peroxidation mediated by hydroxyl radicals has been suggested as a possible mechanism of pulmonary injury (Freeman et al., 1985, Jurman et al., 1990, and Martin et al., 1986). The activated species of oxygen ($O_2^{-}\cdot$, H_2O_2, $\cdot OH$) also increase pulmonary arterial pressure (McMurtry et al., 1985, Tate et al., 1982) and increase vascular permeability that results in the formation of a protein rich intra-alveolar edema (Barnard et al., 1989 and Tate et al., 1982). If these toxic species of oxygen are not properly neutralized by endogenous antioxidants, they can interact with cell membranes to generate lipid peroxide, lipid hydroperoxide, lipid endoperoxide and arachidonic acid metabolites (Bertrand, 1985). The generation of short lived free radicals like superoxide anions, hydroxyl radicals and carbon

centered radicals, have been observed during myocardial ischemia and in post-ischemic reperfused myocardium (Bertrand, 1985 and Burghuber, et al., 1984). Lipid peroxidation provokes damage to cellular membranes (Casey et al., 1980). It has been demonstrated that during myocardial ischemia, and especially after reoxygenation of the heart, the concentration of lipid peroxides in the tissues increases (Gauduel and Duvelleray, 1984., Meerson et al., 1982., Rao and Mueller, 1983., Shlafer et al., 1982). Lipid peroxidation mediated by hydroxyl radicals has also been suggested as a possible mechanism of pulmonary injury (Mattin et al., 1986., Kennedy et al., 1989., Freeman et al., 1986).

Since lipid peroxidation can be initiated by reactive species of oxygen which are produced during reperfusion of ischemic tissue, we developed the hypothesis that antiarrhythmic agents might be exerting their beneficial effects, in part, by a free radical scavenging mechanism.

The specific aims of this investigation were: (1) to evaluate the role of antiarrhythmic drugs on oxidative tissue injury and (2) to evaluate their mechanism of action.

It has been shown that free radicals of oxygen are produced in post-ischemic tissues and they are implicated as the mediator of tissue damage during reperfusion of different ischemic organs, such as brain, heart, intestine and kidney. The mechanism of reperfusion injury in lungs has not been clearly understood. We investigated the effects of these drugs (1) on lipid peroxidation of biomembranes isolated from bovine lungs, (2) on reperfusion injury of rat lungs in an ex vivo perfused model and (3) on macrophage function. We find that these drugs (1) effectively scavenge hydroxyl radicals, (2) inhibit lipid

peroxidation of biomembranes, (3) protect lungs against post-ischemic reperfusion injury, and (4) inhibit macrophage function. These findings have obvious clinical implications in managing the lung and other tissues undergoing period of ischemia or hyperoxia.

CHAPTER-I

A review of literature

A. ANTIARRHYTHMICS

Antiarrhythmic drugs are remedies used to suppress dysrhythmic cardiac activity regardless of their mode of action (Hartenstein and Wagner, 1986). According to Vaughan Williams these drugs have been classified into four sub-classes. Class I include the membrane stabilizers, class II includes the B-blockers, drugs in class III lengthen the cardiac action potential and refractory period and class IV includes calcium blockers (Vaughan William's, 1975).

Class I antiarrhythmic drugs are characterized further by the fact that they posses local anesthetic actions and depress myocardial contractile force. These include lidocaine, quinidine and procainamide.

Lidocaine:

Lidocaine was first synthesized by Lofgren for use as a local anesthetic agent in 1943 (Wielding, 1964) and it is still extensively used for that purpose today. It was first used as an antiarrhythmic agent in 1950 by Southworth et al.(Southworth et al., 1950) for the treatment of arrhythmias occurring during cardiac catheterization. This drug is believed to be an effective and safe drug for termination of ventricular arrhythmias.

Chemical structure and properties:

Lidocaine consists of a lipophilic aromatic residue (2, 6 Xylidide) connected through an amide link to diethylglycine, which is the hydrophobic group containing the active tertiary nitrogen (Fig. 1). The non-ester amide link provides stability by preventing hydrolysis by esterases (Hullanger, 1960). Lidocaine is a weak base (pKa = 8.7), and it is marketed in the form of its water soluble salt, lidocaine hydrochloride, which is an acid form of the drug. At pH 7.4, less than 20 percent of lidocaine is believed to be in the form of the free base and it is this nonpolar form that penetrates vascular membrane.

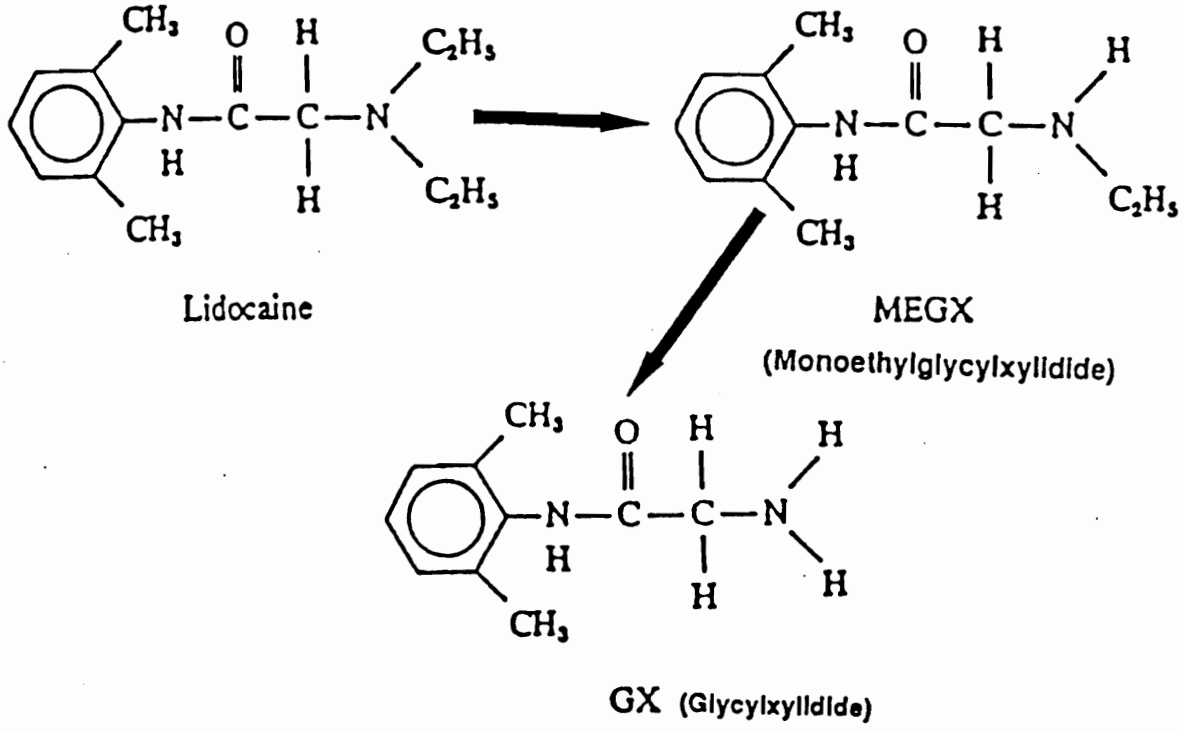


Fig. 1. Lidocaine and its metabolites.

Pharmacokinetics:

More than 70% of orally administered lidocaine is metabolized by the liver before reaching the systemic circulation. Dizziness, nausea, and vomiting may occur in humans after oral lidocaine administration, probably due to the results of high circulating plasma concentrations of the mono-N-deethylated and the di-N-deethylated metabolites of lidocaine, i.e., monoethylglycine xylidide and glycine xylidide, respectively (Fig. 1). Lidocaine is more rapidly absorbed after intramuscular injection into the deltoid muscle (absorption half life 11.7 min.), than into the vastus lateralis and gluteus maximus (absorption half life 25.7 min; Lucchesi and Patterson, 1984).

Metabolism and excretion:

The drug is degraded in the liver by deamination and deethylation, and kidney excretes less than 10 % of the drug unaltered. The first product monoethylglycine xylidide, is formed by N-deethylation of lidocaine and has similar convulsant activity. Monoethylglycine xylididie has a plasma half life of 120 min. and is eliminated from plasma primarily by a second N-deethylation to form glycine xylidide, the second major metabolite of lidocaine. Glycine xylidide possess both antiarrhythmic and convulsant activity, although it is only 10 to 20% as potent as lidocaine. Glycine xylidide is both metabolized and excreted by the kidney. It has a plasma half life of 10 hours.

Doses:

Lidocaine is usually administered intravenously for the treatment of ventricular arrhythmia. In humans after a single intravenous bolus injection, the drug disappears rapidly from the plasma because of redistribution of drug to other tissues. Because of these pharmacokinetic properties, lidocaine is administered as an intravenous bolus followed by a constant intravenous infusion. Injection of 300 to 400 mg/kg gives persistent therapeutic levels lasting for 2 hours (Lucchesi and Patterson, 1984).

Quinidine:

Quinidine has been in active clinical use since 1918 (Frey, 1918). For centuries the quinidine alkaloids from the bark of *Cinchona officinalis* have been used for treatment of malaria. Clinical investigation shows that of the three major alkaloids present in the bark of the Cinchona tree (quinine, quinidine, and cinchona), quinidine is the most effective antiarrhythmic agent (Hartenstein and Wagner, 1986). The efficacy and long history of quinidine use in the treatment of disorders of the cardiac rhythm led to the establishment of quinidine as the prototype antiarrhythmic agent.

Structure and chemical properties:

The chemical structure of quinidine consists of a substituted quinoline ring connected through an alcohol bridge to a quinuclidine ring containing the critically active tertiary nitrogen (Fig. 2). The quinoline ring's strong affinity with proteins, contributes to its slow release from tissue protein sites and it's longer

half life. Quinidine, with its two ionizable ring structures has two dissociation constants (pKa = 4.0 and 8.6). Quinidine may be considered as a weak base, and its net dissociation constant (pKa) is 8.0. This means that in body fluids (pH 7.4) quinidine exists primarily in the ionized form. Quinidine has the same chemical structure as quinine, and these two compounds are optical isomers with opposite stearic configuration at the alcohol bridge (Moss and Patton, 1973).

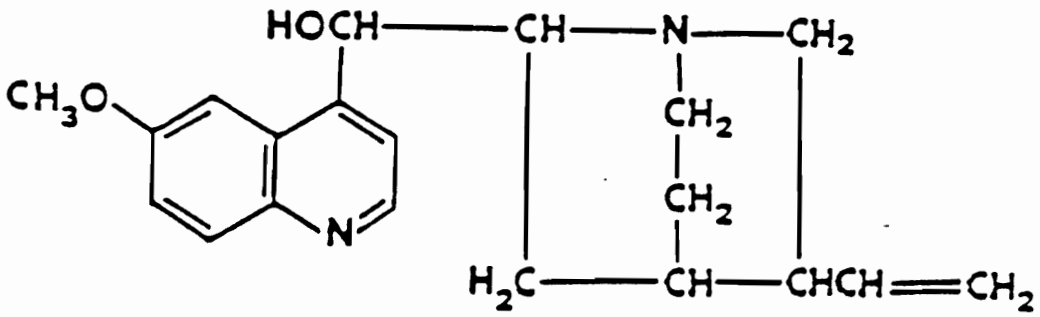


Fig. 2. Chemical structure of Quinidine

Pharmacokinetics:

Quinidine is almost completely absorbed from gastrointestinal tract after oral administration. Peak plasma levels absorbed after intramuscular injection are greater than those absorbed after oral administration.

Metabolism and excretion:

Quinidine is extensively metabolized in the body, primarily by the liver. Major metabolites include 3-hydroxyquinidine and quinidine-N-oxide. Minor metabolites include 2'-oxyquinidine and *o*-desmethylquinidine. The 3' hydroxy metabolites of quinidine possess antiarrhythmic activity. Urinary excretion of conjugated or free metabolites of quinidine accounts for 75 to 95% of administered quinidine. Renal excretion of unaltered quinidine accounts for the remainder.

Doses:

In humans, the initial dose is usually 200 mg/kg, i/v, followed by five doses of 200 mg daily for the treatment of arrhythmia. On this regimen, plasma quinidine concentrations of approximately 4.0, 5.0 and 5.8 μg/ml are attained with doses of 200, 400, and 600 mg/kg, respectively (Lucchesi and Patterson, 1984).

Procainamide:

Procaine hydrochloride was introduced as a local anesthetic in 1905, and in 1936 Mautz, a thoracic surgeon, demonstrated that procaine decreased myocardial excitability when applied directly to the surface of the heart (Mautz, 1936). But the use of procaine for treatment of cardiac arrhythmias had several

drawbacks. The drug was rapidly hydrolyzed in plasma by butyrocholinesterase and thus had very short duration of action, making it difficult to achieve and maintain therapeutic plasma concentrations. A systematic search of compounds structurally similar to procaine, but with antiarrhythmic actions like quinidine, resulted in the isolation of procainamide.

Chemical structure:

Procainamide differs structurally from procaine in that para-aminobenzoic acid and a tertiary nitrogen compound (diethylaminoethanol) are bound through an amide rather than an ester linkage (Fig 3).

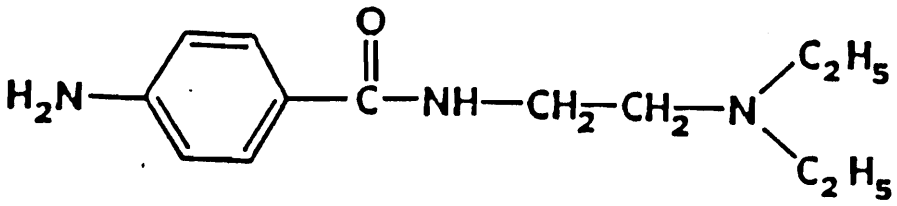


Fig. 3. Chemical structure of procainamide

Pharmacokinetics:

Oral doses of procainamide are well absorbed from the gastrointestinal tract with bioavailability of approximately 75%. Peak plasma concentrations are achieved 60 to 90 minutes after oral administration.

Procainamide is metabolized extensively in the liver by enzyme N-acetyltransferase to N-acetylprocainamide. The rate of metabolism of procainamide varies widely between patients and assumes a bimodal distribution of rapid and slow acetylators. Despite differences observed in the rate of procainamide metabolism by N-acetyltransferase, the plasma clearance and therapeutic responses to procainamide are not markedly different in rapid and slow acetylators. The renal clearance of procainamide is proportional to creatinine clearance (Lucchesi and Patterson, 1984).

Doses:

In humans, maintenance doses for treatment of atrial and ventricular arrhythmias are between 500 and 1000 mg/kg administered every 4 to 6 hours (Lucchesi and Patterson, 1984).

B. FREE RADICALS:

Free radicals are, by definition, species that contain an odd number of electrons. They may be positively charged, negatively charged, or neutral. Radicals vary in their reactivity and are influenced by temperature, pH and by concentration of molecules in their environment. The importance of free radicals in living systems is now clearly established, and impressive evidence has been presented in a number of recent volumes and reviews by Pyror (1973), and Pyror (1977). Most of the radicals that are important in biological systems are oxidants.

Oxygen free radicals:

The thermodynamic properties of oxygen make it an excellent acceptor of electrons. When pairs of electrons are transferred from reduced nutrients such as glucose to oxygen through a series of redox reactions, energy is released due to the large differences in electrochemical potentials between the two molecules. The reactions ultimately result in the reduction of oxygen to water and the simultaneous oxidation of the organic nutrient to carbon dioxide. The energy obtained from these reactions is used to drive the metabolic process of animals (McCord, 1979).

The electrons in the outer shell of an oxygen atom are shared with those of another oxygen atom to form the covalent bond of molecular oxygen (O_2). The orbitals more central to the molecule, such as the 2s orbitals as well as the first three 2p orbitals, are completely filled by electrons. According to the physical rules that govern electron configuration, only two electrons may occupy any atomic orbital, and to do so they must have opposite spins. These electrons are said to be spin-paired. It is actually the opposite spins on the two electrons which counteract their tendency to repel each other and permit them to coexist in an orbital. However, the outer two orbitals (π^*2p) of ground state oxygen are incompletely filled. The molecule attains maximal stability by spreading out the outer two electrons across orbitals in an unpaired arrangement with parallel spins (Hasan and Fridovich, 1979; Fee and Valentine, 1977; Brady and Humiston, 1975). A schematic diagram is presented in Fig. 4.

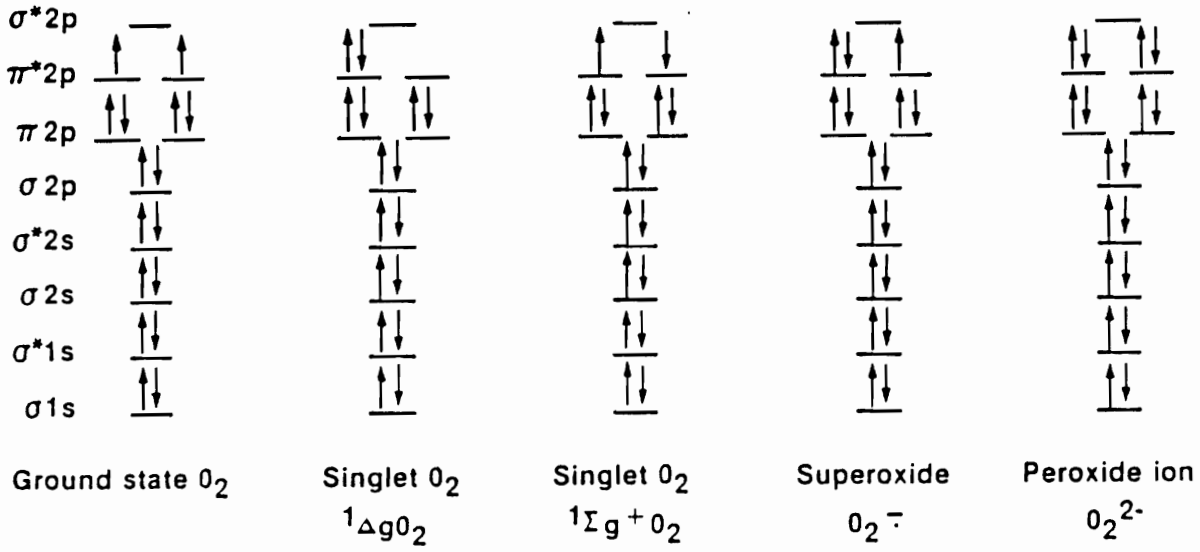


Fig. 4. Molecular orbirals of different species of dioxygen (Redrawn from Haliwell and Gutteridge, 1985)

During metabolic redox reactions, electrons are generally donated to the outer orbital of oxygen in a spin-paired arrangement. These oppositely spinning electrons can not enter the available p orbital of molecular oxygen without one of the outer electrons undergoing a spin inversion. A non-catalyzed spin reversal is slow and unlikely to occur at temperatures that sustain life; thus molecular oxygen is kinetically more stable due to its electronic configuration than it would appear on a thermodynamic basis (McCord, 1979; Fridovich, 1979)

This spin reversal is avoided if oxygen accepts a single electron (a univalent reduction), thereby forming the free radical, superoxide anion (Fridovich, 1979). During non-enzymatic oxidations, organic compounds will more readily transfer an electron pair to two oxygen molecules in two successive univalent reactions than transfer the pair in a single divalent reaction (McCord, 1979). Through a series of non-enzymatic univalent reactions, a molecule of oxygen can ultimately accept four electrons to form two water molecules (Fridovich, 1978). Although oxygen and water are fairly unreactive, the intermediate products (superoxide, hydrogen peroxide and the hydroxyl radical) formed in the intervening reduction and the spin altered species (singlet oxygen) are thought to be responsible for oxygen toxicity observed in both microorganisms, mammalian cells, tissues and whole organisms. (Fridovich, 1976: Fridovich, 1978).

Therapeutic administration of oxygen has found success for nearly 200 years; however, prolonged deliverance of high oxygen concentrations proves deleterious (McCord, 1979; Pfenning, 1979). Administration of 100% oxygen to adult rats up to 72 hours causes diffuse pulmonary capillary endothelial

swelling, degeneration and necrosis, followed by pulmonary edema, hypoxemia and death (Crapo, 1977). The toxic effects of oxygen prove to be more acute under increased pressure. The delivery of 100% oxygen to rats in a chamber pressurized to five atmosphere causes convulsions and death in less than 30 minutes (McCord, 1979; West, 1979). In humans, oxygen toxicity is exacerbated by the high pressure as in deep sea diving. On extremely deep dives, oxygen concentrations must be reduced to under one percent of the inspired gas mixture to avoid deleterious effects (West, 1979).

To prevent pathological changes at normal oxygen concentrations, aerobic organisms have adapted enzymatically to avoid the whole series of univalent reduction reactions and prevent the formation of superoxide, hydrogen peroxide and hydroxyl radical. Oxidative enzymes such as cytochrome oxidase catalyze the tetravalent reduction of oxygen to water without the release of detectable intermediates (Hassan and Fridovich, 1979; Fridovich 1976). Although aerobic cells use cytochrome oxidase pathways for most of their oxygen consumption, many other biological oxidations result in the formation of reactive intermediates.

Hydroxyl radical: Hydroxyl radicals (.OH) have been implicated in a number of biologic phenomenon, generally from the viewpoint of their cytotoxic actions. For example, cellular damage induced by ionizing radiation and the destruction of microorganisms by phagocytes have been attributed, in part, to the action of hydroxyl radicals (Mayers, 1973; Salin and McCord, 1975). There are examples of potentially beneficial actions of hydroxyl radicals, namely, its requirement in prostaglandin biosynthesis (Pangaramala et al., 1976) and ,

indirectly, as protective agents against superoxide mediated lipid peroxidation in liver (Taylor, 1975).

Hydroxyl radicals have not been observed directly in biologic systems. Their presence is generally inferred from the blocking action of hydroxyl radical scavengers. The reaction mechanism leading to formation of .OH in a biological system are a matter of some controversy. One mechanism that has achieved a level of general acceptance is the Fenton reaction (Walling, 1975):

$$H_2O_2 + Fe^{2+} \text{----------} > \cdot OH + OH^- + Fe^{3+}$$

A second reaction, suggested by Haber and Weiss (1934), is the interaction of superoxide radical ($O_2^{-\cdot}$) with hydrogen peroxide.

$$H_2O_2 + O_2^{-\cdot} \text{----------} > \cdot OH + OH^- + O_2$$

This later reaction has sparked considerable debate (Agar and Dainton, 1947; Weiss, 1947, Beauchamp and Fridovich, 1970; Cohen 1977; Fee and Valentine, 1977). It has now generally been believed that the reaction is catalyzed by metal ions such as Fe^{3+} :

$$Fe^{3+} + O_2^{-\cdot} \text{-------} > Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \text{-----} > \cdot OH + OH^- + Fe^{3+}$$

Hydroxyl radical is one of the most reactive radicals known. It reacts very quickly and indiscriminately with all classes of molecules found in biological

systems including sugars, amino acids, phospholipids, nucleotides and organic acids. Hydroxyl radical reacts in three major ways: Hydrogen abstraction, addition, and electron transfer. These radicals react with ethanol by hydrogen abstraction or by removing a hydrogen atom to form water and ethanol radical. The ethanol radical thus contains an unpaired electron on one of the carbons and tends to react with surrounding compounds. Hydroxyl radicals can also react by addition to an aromatic ring. Hydroxyl radical reacts with the aromatic nucleotide bases of DNA and RNA by adding to a double bond. This causes damage to the molecular structure and may include strand breakage. Cells often die after this form of damage or those that survive may undergo mutations. Hydroxyl radical transfers electrons to inorganic compounds such as chloride ion, causing the formation of chloride radicals that are capable of propagating chain reaction (Halliwell and Gutteridge, 1985).

Singlet Oxygen: The potential role of singlet oxygen ($^1O_2$) in biological systems has attracted many investigators over the past 20 years. It was the first review by Foote (1968) that began focusing the attention of biochemists and biologists on this unique electronically excited species of oxygen. The reactions that have been reported to generate $^1O_2$ are represented in the pattern of a clock in Fig. 5.

The singlet oxygen ($^1O_2$), species are much less stable than ground state oxygen. Singlet oxygen is less stable because the two outer electrons are not spread across the two outer orbital, but are spin paired in a single p*2p orbital (Fig. 4). $^1O_2$ is 22.4 kcal above ground state oxygen. The second form of $^1O_2$,

which has an energy of 37.5 kcal above the ground state, has two single electrons in the outer orbital spinning in opposite directions (Fig. 4).

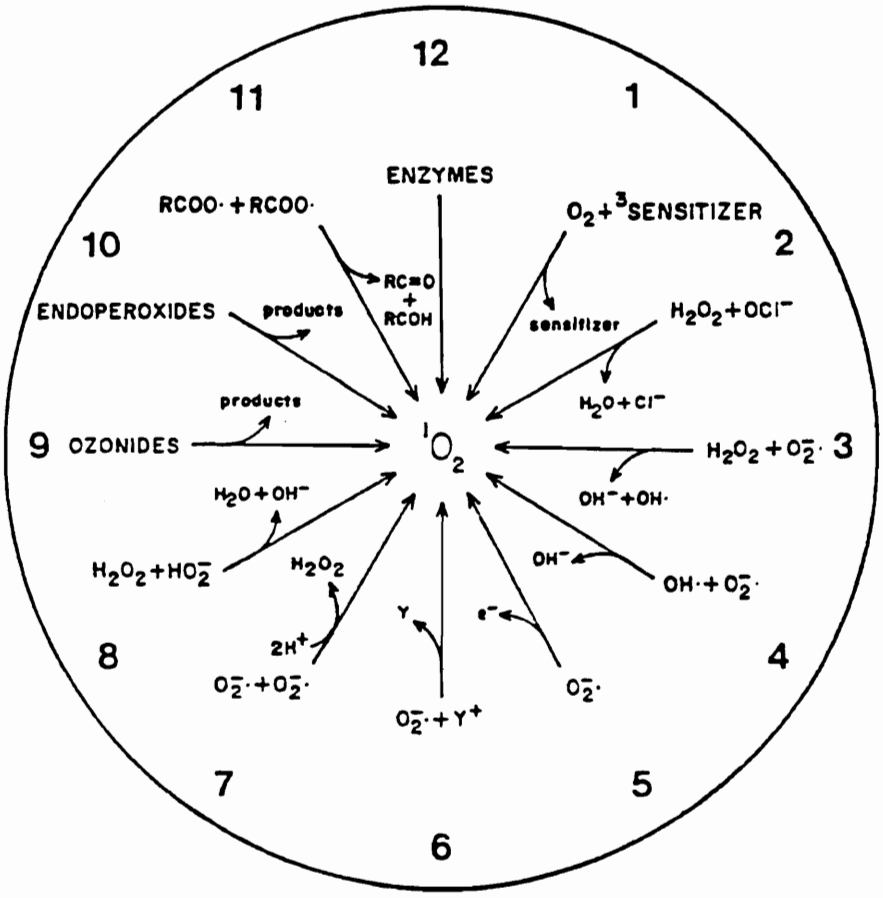


Fig. 5: Photochemical, chemical and biological reactions reported to produce $^1O_2$ (After Khan,1978)

The highly energetic $^1O_2^*$ tends to decay to the $^1O_2$ form that is the only significant species of $^1O_2$ in biological system. These oxygen species do not have the spin restriction found in ground state O_2 and therefore are kinetically much less stable and more ready to accept electron pairs from non radicals.

$^1O_2$ is not itself a free radical, but is a product of some reactions with radicals and tends to form radicals. $^1O_2$ may be formed when hypochlorite ion, a product of phagocyte myeloperoxidase, reacts with hydrogen peroxide. It may

also be generated by photosensitization reactions where a photosensitizer molecule absorbs certain wavelengths of light, raising it to an excited state. This energy is transferred to molecular oxygen to form $^1O_2$. Riboflavin, a flavin mononucleotide, flavin adenine dinucleotide, chlorophylls a and b, bilirubin, retinol and porphyrins may act as photosensitizers. Singlet oxygen may react with surrounding molecules or it may react with and damage the photosensitizer. Singlet oxygen may either combine with molecules in an electron transfer process or transfer its energy to molecules. Singlet oxygens have been shown to peroxidize lipids and damage proteins by these methods (Halliwell and Gutteridge, 1985).

Superoxide radical: The inability of ground state oxygen to accept electron pair is avoided if oxygen accepts electrons one at a time. The addition of an electron to molecular oxygen causes superoxide radical formation. The electron affinity of molecular oxygen has been determined to be between 0.2 - 1.3eV (Khan, 1978). In aqueous solution O_2^- exists in equilibrium with the hydroperoxy radical ·OOH

$$O_2^{-}\cdot + H^+ \text{----> } \cdot OOH$$

A pK of 4.88 for this ionization has been determined by Behar et al. (1970) from studies of ·OOH and $O_2^{-}\cdot$ solutions generated by reactions:

$$O_2 + e_{aq} \text{--------} > O_2^{-}\cdot$$

$$O_2^{-}\cdot + H \text{---------} > \cdot OOH$$

in the pulse radiolysis of oxygenated water. At neutral pH the ratio [·OOH]/[O_2^{-}] is roughly 1:100. A second protonation occurs at lower pH, yielding OOH^{2+} (pK 1.2; Bielski and Allen, 1967). In aqueous solution at acid and neutral pH ranges, the decay of the $O_2^{-}\cdot$ or ·OOH species is due to the disproportionation reaction. This was shown by following decay of $O_2^{-}\cdot$ and ·OOH optical absorption in pulse radiolysis studies of oxygenated water:

$$\cdot OOH + \cdot OOH \text{-------} > O_2 + H_2O_2$$

$$\cdot OOH + .O_2^{-}\cdot \text{ --------} > O_2 + OOH^-$$

The $O_2^{-}\cdot$ is unstable in an aqueous environment , spontaneously dismutating to produce H_2O_2 and O_2 by the reaction:

$$2O_2^{-}\cdot + 2H^+ \text{ ----------} > H_2O_2 + O_2$$

The rate constant for $O_2^{-}\cdot$ dismutation at pH 7.4 is 2 X 10^9 M/s (Fridovich, 1976). Because of this relatively rapid rate of dismutation, production of $O_2^{-}\cdot$ in vivo is always accompanied by production of H_2O_2. The $O_2^{-}\cdot$ is a relatively weak oxidant; it oxidizes only a few compounds, including ascorbate (Nishikimi and Yogi, 1977), sulfhydryl compounds (Quintiliani et al., 1978), sulfite, and certain catecholamines (McCord and Fridovich, 1969). On the other hand Sawyer and Valentine (1981) demonstrated that $O_2^{-}\cdot$ is a potent reductant with a redox potential close to that of sodium hydrosulphite. The $O_2^{-}\cdot$ reduces a variety of compounds, including ferric iron and its complexes (Bielski and Gebicki, 1974), hemeproteins (cytochrome c, methemoglobin, metmyoglobin, and peroxidases (McCord and Fridovich, 1969), and quinones (Patel and Wilson, 1973). Biologically $O_2^{-}\cdot$ may be produced by the autoxidation of epinephrine,

hemoglobin, flavins and quinones or enzymatically by xanthine oxidase (Misra and Fridovich, 1971; Misra and Fridovich, 1972a; Misra and Fridovich, 1972b; Misra and Fridovich, 1972c; Misra, 1974; Fridovich, 1979).

Because of the potentially harmful effects initiated by reactive O_2 metabolites, nature has provided several antioxidant enzymes and scavengers to protect tissue from oxidative insult. They include superoxide dismutase (SOD), catalase, and glutathione peroxidase. Superoxide dismutase exists as an Mn-containing protein in mitochondria and as a CuZn-containing enzyme in the cytoplasm of most mammals.

Spin trapping of free radicals:

A free radical is a species containing unpaired electrons and as such is paramagnetic. When this free radical is placed in a magnetic field, its magnetic moment is directly detected by an electron paramagnetic resonance spectrometer (EPR). Since EPR can only detect free radicals, its use in biological systems is most apparent. Many free radicals of biological interest are so reactive that they never reach the necessary steady state concentration to be detected directly by EPR methodology. Spin trapping can overcome many of these difficulties. This technique consists of using a spin trap, i.e., a compound which forms a stable nitroxide by reacting covalently with an unstable radical. In this way, the unstable radical is trapped as a long lived species which can be observed at room temperature using conventional EPR spectroscopy (Rosen and Rauckman, 1981). The hyperfine splitting constants of the adduct provide information that can aid in the identification and quantification of the original radical. Since the relatively stable nitroxide free

radical accumulates, spin trapping in combination with EPR specroscopy has been an integrative method for measuring free radicals, and is inherently more sensitive than procedures that detect only instantaneous, or steady state, levels of free radicals.

C. PULMONARY OXYGEN TOXICITY:

Most mammalian species die after prolonged exposure to 100% oxygen even at normobaric conditions. The primary damage is to the lung and the clinical signs are those of progressive respiratory distress until death. Ultrastructure studies indicate that major insult is to the alveolar capillary endothelium (Thet, 1986). The mechanism of $O_2^{-\cdot}$ induced pulmonary toxicity has only recently been delineated. Certain autoxidizable compounds such as hydroquinones or reduced flavins reduce O_2 by one electron transfer mechanism to form $O_2^{-\cdot}$ (Misra and Fridovich, 1972) In normal mitochondria, 1% of the electrons that pass down the electron transport chain "leak off" to molecular O_2 by reaction with ubisemiquinone (Coenzyme Q), resulting in a small but significant production of $O_2^{-\cdot}$ in the mitochondria (Fig 6a). The rate of electron leakage or $O_2^{-\cdot}$ production is directly proportional to the partial pressure of O_2 in the mitochondria. Therefore, when an animal is exposed to 100% oxygen the mitochondria of pneumocytes may produce as much as five times the amount of $O_2^{-\cdot}$ produced in normoxic conditions. The $O_2^{-\cdot}$ so generated could undergo a dismutation reaction to produce H_2O_2 and possibly metal-catalyzed reaction to form $\cdot OH$. Most evidence suggests that pulmonary oxygen toxicity is due to increased rates of $O_2^{-\cdot}$ and H_2O_2 production.

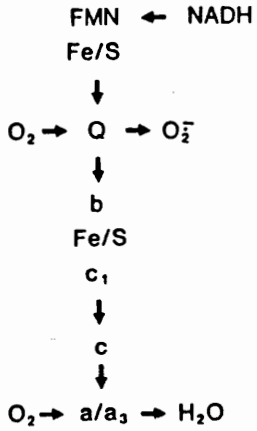


Fig 6a. Mitochondrial electron transport and $O_2^{-\cdot}$ production. (After Turrens et al.)

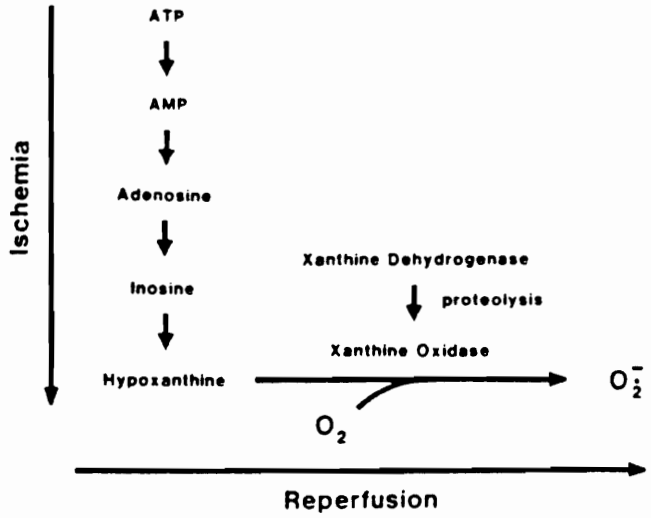


Fig 6b. Proposed mechanism for O2 radical formation in ischemic tissue (After McCord, 1985)

D. POST-ISCHEMIC TISSUE DAMAGE:

Reoxygenation of ischemic tissue results in extensive tissue damage and this phenomenon has been termed as reperfusion injury or reperfusion paradox or post -ischemia tissue injury. The biochemical mechanism that underlies the post-ischemic tissue injury involves production of superoxide radical in the reperfused tissue. Prolonged ischemia results in the loss of high energy phosphate compounds manifested by a decrease in the cellular ATP levels. As cell's energy charge drops, it is no longer able to maintain a proper ion gradient across its membrane and this precipitates a redistribution of calcium ions. Concomitantly, the depletion of the cells ATP results in an elevated concentration of AMP. The AMP is catabolized to adenosine, inosine, and then hypoxanthine (Fig 6b). The elevated cytosolic calcium concentration, is believed to activate a protease capable of converting xanthine dehydrogenase to xanthine oxidase which in turn acts on hypoxanthine and oxygen to generated uric acid and $O_2^{-\cdot}$ (Grisham and McCord, 1986). It is also possible that the mitochondrial "leak off" of electrons is enhanced during reperfusion of ischemic tissue. During hypoxia and ischemia the reducing equivalents in the mitochondria remain at high concentrations. Reperfusion introduces oxygen to the ischemic tissue which, inturn, is reduced by univalent pathways to generate $O_2^{-\cdot}$.

Post-ischemic injury in the lung:

Reperfusion injury mediated by oxygen free radical has been reported in heart, brain, intestine, liver, pancreas, kidney and skin. The reperfusion injury has also been reported after reexpansion of atelectatic lung (Jackson et al.

1986) or reperfusion of mechanically occluded pulmonary artery (Bishop et al. 1986). Investigators have generally felt that the lung is relatively immune from reperfusion injury because oxidative metabolism does not decrease and ATP levels do not fall until alveolar Po_2 drops below 1mm Hg (Fisher and Dodia, 1981). However, other reports indicate that lung is susceptible to reperfusion injury. The source of this injury have been suggested to be reactive oxygen species, such as $O_2^{-\cdot}$ generated during reperfusion. Superoxide dismutase has been shown to protect the lungs from reperfusion injury.

Macrophage and oxidative tissue damage:

The fundamental driving force responsible for oxidative damage by leukocytes is the flow of electrons down the potential gradient from NADPH to a variety of intracellular and extracellular acceptors. The NADPH oxidase, membrane bound enzyme, of phagocytes, is a powerful generator of superoxide radical. The production of superoxide by the leukocyte is normally highly regulated and requires the assembly of a large macromolecular complex of proteins from different cellular compartments after stimulation of the cell. If the leukocyte is appropriately stimulated by invasive microbes or cell debris, the powerful system is used to the advantage of the host to oxidatively kill the microbial cells or degrade the cellular debris. If the phagocyte is inappropriately stimulated then unwanted toxic oxygen species are produced in the host and oxidative tissue damage ensues. Virtually any organ that becomes inflamed, traumatized, or subjected to ischemia or anoxia followed by reperfusion or reoxygenation, will suffer tissue damage from toxic oxygen species originating,

at least in part, as phagocyte-generated superoxide radicals (Jessaitis et al. 1991).

References:

Agar, J.N. and Dainton, F.S. (1947), *Disc Faraday Soc.* 2, 218-219.

Beauchamp, C. and Fridovich, I. (1970), *J. Biol. Chem.* 245, 4641-4646.

Behar, D., Czapski, G., Rabani, J., Dorfman, L.M. and Schwarz, H. (1970), *J. Phys. Chem.* 74: 3209-3213

Bielski, B.H.J. and Allen, A.O. (1967), *Proc. Tihany. Symp. Radiat. Chem.* 2nd, 81

Bielski, B. H. J. and Gebicki, J. M. (1974), Study of peroxidase mechanisms by pulse radiolysis. *Biochim. Biophys. Acta* 364: 233-235.

Bishop, M.J., Boatman, E.S., Ivey, T.D., Jordan, J.P. and Cheney, F. W. (1986), Reperfusion of ischemic dog lung results in fever, leukopenia, and lung edema. *Am. Rev. Respir. Dis.* 134:752-756.

Brady, J.E. Humiston, G.E. (1975), General Chemistry: Principles and structure. New York, John Wiley & Sons, Inc.

Cohen, G. (1977), In Defense of Haber-Weiss. In: *Superoxide and superoxide dismutases.* Michaelson, A.M., McCord, J.M., Fridovich, I. (eds.). New York, Academic Press: 323-334.

Crapo, J.D. (1977), The role of the superoxide and superoxide dismutases in pulmonary oxygen toxicity. In: *Superoxide and Superoxide Dismutases.* Michaelson, A.M., McCord, J.M., Fridovich, I. (eds.). New York, Academic Press: 231-239.

Fee, J.A. and Valentine, J.S. (1977), Chemical and physical properties of superoxide.In: *Superoxide and Superoxide Dismutases.* Michaelson,

A.M., McCord, J.M., Fridovich, I. (eds.). New York, Academic Press: 19-60.

Fisher, A.B., and Dodia, C. (1981), The lung as a model for evaluation of critical intracellular pO2 and pCO_2. *Am. J. Physiol.* 242:E47-50.

Foote, C.S. (1968), Mechanism of photosensitized oxidation. *Science,* 162:963

Frey W. (1918),*Weitere Erfahrungen mit chinidin bei absoluter herzun-regel-massigkeit. Wein klin wchnschr, 55:849-853.*

Fridovich, I. (1976), Oxygen radicals, hydrogen peroxide and oxygen toxicity. In: *Free Radicals in Biology*, edited by Pyror, W.A. New York: Academic Press, Vol. 1, p. 239-277.

Fridovich, I. (1978), The biology of oxygen radicals, *Science*, 201:875-880.

Fridovich, I. (1979) Superoxide and superoxide dismutases. In: *Advances in Inorganic Biochemistry.* Eichhorn, G.L, Marzelli, L.G. (eds.). New York, Elsevier North Holland: 67-90.

Grisham, M.B. and McCord,J.M. (1986), Chemistry and cytotoxicity of reactive oxygen metabolites. In: *Physiology of Oxygen Radicals,* American Physiological Society Bethesda, Maryland,1-18.

Haber, F. and Weiss, J. (1934), Proc. R. Soc. A 147, 332-351

Halliwell, B., and Gutteridge, J.M.C. (1985), Oxygen is poisonous-an introduction to oxygen toxicity and free radicals. In: *Free Radicals in Biology and Medicine.* Oxford, Clarendon Press: 1-19.

Hartenstein, J. and Wagner, B. (1986), Antiarrhythmic agents. In: *Cardiovascular Drugs* (Bistol, J.A. Ed.) pp 309-415, John Wiley & Sons, New York.

Hassan, H. M. and Fridovich, I. (1979), Superoxide dismutase and its role for survival in the presence of oxygen. In: *Strategies of Microbial Life in Extreme Environments.* Shilo M (ed.). New York, Verlag Chemie, 1979: 179-193.

Hullanger, G., (1960), On the metabolism of lidocaine. I. The properties of the enzyme system responsible for the oxidative metabolism of lidocaine. *Acta. Pharmacol. Toxicol.,* 17:356-365.

Jackson, R. M., Veil, C.F., Alexander, C.B., Brqnnen, A.L. and Fulmer, J.D. (1988), Reexpansion pulmonary edema. A potential role for free radicals in its pathogenesis. *Am. Rev. Respir. Dis.* 137:1165-1171.

Jessaitis, A. J., Quinn, M.T., Mukherjee, G., Ward, P.A., and Dratz, E. A. (1991), The molecular basis of oxidative damage by leukocytes. In: *Molecular basis of Oxidative Damage By Leukocytes.* pp1-8 (Jessaitis, A. J.and Dratz, E. A., eds.), CRC Press, Boca Raton

Khan, A.U. (1978), Activated oxygen : Singlet molecualr oxygen and superoxide anion, *Photochem. Photobiol.* 28:615-627.

Lucchesi, B. R. and Patterson, E. S. (1984), Antiarrhythmic drugs. In : *Cardiovascular Pharmacology,* (Antonaccio, M. Ed.), pp 329-414, Raven press, New York.

Mautz, F. R.(1936), The reduction of cardiac irritability by epicardial and systemic administration of drugs as a protection in cardiac surgery. *J. Thorac. Surg.* 5:612-628.

McCord, J.M. (1979), Superoxide, superoxide dismutase and oxygen toxicity. In: *Reviews in Biochemical Toxicology.* Hodgson, Bend Philpot (eds.). Elsevier North Holland, 1979: 109-123.

McCord, J.M. (1979), Superoxide, superoxide dismutase and oxygen toxicity. In: *Reviews in Biochemical Toxicology.* Hodgson, Bend, Philpot (eds.). Elsevier North Holland, 1979:109-123.

McCord, J.M. and Fridovich, I. (1969), Superoxide dismutase. An enzymatic function of erythrocuprein (hemocupreine). *J. Biol. Chem.* 244: 6049-6055.

Misra, H. P. (1974), The generation of superoxide radical during autoxidation of thiols. *J. Biol. Chem.* 249: 2151-2155.

Misra, H. P. and Fridovich, I. (1971), The generation of superoxide radical during autoxidation of ferredoxins. *J. Biol. Chem.* 146: 6886-6890.

Misra, H. P. and Fridovich, I. (1972a), The univalent reduction of oxygen by reduced flavins and quinones. *J. Biol. Chem.* 147: 188-192.

Misra, H. P. and Fridovich, I. (1972b), The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247: 3170-3175.

Misra, H. P. and Fridovich, I. (1972c), The generation of superoixde radical during the autoxidation of hemoglobin. *J. Biol. Chem.* 247: 6960-6962.

Moss, J. A. and Patton, R. D. (1973), Antiarrhythmic agents, pp 159, Charles C. Thomash, Publishers, Springfield, Illinois

Myers, L.S., Jr. (1973), Fed. Proc. 32:1882-1894.

Nishikimi, M. and Yogi, K. (1977), Oxidations of ascorbic acid and α–tocopherol by superoxide. In: *Biochemical and Medical Aspects of Active Oxygen,* edited by Hayaishi, O and Asada, K. Tokyo, Univ. of Tokyo Press, p. 79-87.

Panganamala, R.V., Sharma, H.V., Heikkila, R.E., Geer, J.C. and Cornwell, D.G. (1976), *Prostaglandins.* 11, 599-607

Patel, K. B. and Wilson, P. L. (1973), Semiquinone free radicals and oxygen. *J. Chem. Soc. Faraday Trans.* 1: 814-825.

Pfenning, N. (1979), Formation of oxygen and microbial processes establishing and maintaining anaerobic environments. In: *Strategies of Microbial LIfe in Extreme Environments.* Shilo, M (eds). New York, Verlag Chemie: 137-148.

Pyror, W. A. (1973), Free radical reactions and their importance in biological systems. Fed. Proc. 32:1862-1868.

Pryor, W. A. (1977), The involvement of free radical reactions in aging and carcinogenesis. In: Medicinal Chemistry V, Mathieu, J. Ed., Elsevier, Amsterdam, pp. 331-359.

Quintiliani, M., Badiello, R., Yamba, M., Esfondi, A and Garin, G.(1977), Radiolysis of glutathione in oxygen-containing solution of pH 7. *Int. J. Radiat. Biol.* 32: 195-202.

Rosen, G. M. and Rauckman, E. (1981) The spin trapping of biologically generated free radicals. In: *Oxygen and Oxy-radicals in Chemistry and Biology,* Academic Press, Inc. New York.

Salin, M.L., and McCord, J. M. (1975), Free Radicals and Inflammation. *J. Clin. Invest.* 56: 1319-1323.

Sawyer, D. T. and Valentine, J. S. (1981), How super is superoxide? *Acc. Chem. Res.* 14: 393-400.

Southworth, J. L., McKusick, V. A., Pierce, E. C., (1950), Ventricullar fibrillation precipitated by cardiac catheterization. Complete recovery of a patient after forty-five minutes, *JAMA,* 143:217-230.

Thet, L.A. (1986), Repair of oxygen induced lung Injury, In: *Physiology of Oxygen Radicals,* American Physiological Society Bethesda, Maryland,87-108.

Tyler, D.D. (1975) *FEBS Lett.* 51: 180-183

Vaughan W. E. M., (1975), *Pharmacol. Theraup.* B., 1, 115.

Walling, C., Partch, R. E. and Weil, T. (1975), *Proc. Natl. Acad. Sci.* U.S.A. 72, 140-142.

Weiss, J. (1947), *Disc. Faraday Soc.* 2, 217-218..

West, J.B. (1979), *Respiratory Physiology,* Baltimore, Williams and Wilkins.

Wielding, S. (1964),. *Xylocaine: The pharmacologic basis of its clinical use,* 2nd Ed. Stockholm, Almquist & Wilksell, 1964.

CHAPTER-II

LIDOCAINE: A HYDROXYL RADICAL SCAVENGER AND SINGLET OXYGEN QUENCHER

(Accepted for publication in Molecular and Cellular Biochemistry)

ABSTRACT

Lidocaine, a local anaesthetic, has been shown to reduce ventricular arrhythmias associated with myocardial infarction and ischemic myocardial injury and its protective effects have been attributed to its membrane stabilizing properties. Since oxygen radicals are known to be produced during ischemia induced tissue damage, we have investigated the possible antioxidant properties of lidocaine and found that lidocaine does not scavenge $O_2^{-\cdot}$ radicals at 1 to 20mM concentrations. However, lidocaine was found to be a potent scavenger of hydroxyl radicals and singlet oxygen. Hydroxyl radicals were produced in a Fenton type reaction and detected as DMPO-OH adducts by electron paramagnetic resonance spectroscopic techniques. Lidocaine inhibited DMPO-OH adduct formation in a dose dependent manner. The amount of lidocaine needed to cause 50% inhibition of that rate was found to be approximately 80μM and at 300μM concentration it virtually eliminated the DMPO-OH adduct formation. The production of OH·-dependent TBA reactive products of deoxyribose was also inhibited by lidocaine in a dose dependent manner. Lidocaine was also found to inhibit the $^1O_2$-dependent 2,2,6,6-

tetramethylpiperidine N-oxyl (TEMPO) formation in a dose dependent manner. $^1O_2$ was produced in a photosensitizing system using Rose Bengal or Methylene Blue as photosensitizers and was detected as TEMP-$^1O_2$ adduct by EPR spectroscopy. The amount of lidocaine required to cause 50% inhibition of TEMP-$^1O_2$ adduct formation was found to be 500μM. These results suggest that the protective effect of lidocaine on myocardial injury may, in part, be due to its reactive oxygen scavenging properties. These results may also explain the "membrane stabilizing actions" of lidocaine by scavenging OH. and $^1O_2$ that are implicated in membrane lipid peroxdation.

INTRODUCTION

A major concern during cardiopulmonary bypass procedures is minimizing ischemic damage to the myocardium, thereby avoiding depressed myocardial performance in the post operative-period. Membrane stabilizing agents have been suggested as a means of reducing ischemic myocardial damage. Lidocaine (*d*-diethyl-amino-2, 6-acetoxylidide), a local anaesthetic, has been shown to be clinically valuable for the treatment and prevention of ventricular arrhythmias occurring after myocardial infarction and cardiac surgery (1-4). More recently lidocaine has been shown to reduce myocardial infract size (5) and ischemic myocardial injury (6). It has been reported that lidocaine is rapidly and extensively metabolized (over 90%) into monoethylglycylxylidide and glycylxylidide which may also have antiarrythmic activity (7).Prolonged ischemia such as that following myocardial infarction or occurring during long-term coronary bypass procedures causes serious damage to the myocardium. It has been suggested that free radicals are involved in the patho-physiology of ischemia-induced tissue damage (8-11). During ischemia, increased reducing equivalents are produced(8) due to the decreased concentrations of oxygen, and this may favor the production of superoxide anion and other free radical species upon reoxygenation (9). The generation of short-lived free radicals, like superoxide anion, hydroxyl radicals and carbon-centered radicals, have been observed during myocardial ischemia and in post-ischemic reperfused myocardium(13-14). Since lidocaine has a protect ve role in ischemic myocardial damage (15-17, 19), and has been used in cardioplegic solutions (26), we investigated the possible antioxidant

properties of lidocaine. We report here evidence of singlet oxygen quenching and the hydroxyl radical scavenging properties of lidocaine as detected by colorimetric and spin trapping EPR spectroscopic techniques.

MATERIALS AND METHODS

5,5-dimethyl-1-pyrroline N- oxide (DMPO) was obtained from Sigma Chemical Co. The DMPO was further purified by stirring aqueous solutions of DMPO (900mM) with activated charcoal at 10 mg/ml and filtered through 0.22μ Millipore filter catridges and centrifuged at 1000 X g for 2 minutes. The purified DMPO did not give any EPR signal when scanned at a concentration of 1.12mM.

Rose bengal, methylene blue, bovine erythrocyte superoxide dismutase, cytochrome c (Type III), histidine, mannitol, thiourea, xanthine, xanthine oxidase, lidocaine, deoxyribose, thiobarbituric acid (TBA), trichloroacetic acid (TCA), ferrous sulfate, ß-carotene, boric acid, sodium borate and hydrogen peroxide were obtained from Sigma Chemical Company. 2,2,6,6-Tetramethylpiperidine (TEMP) was obtained from Aldrich. Lidocaine was dissolved in 0.1N HCl and adjusted to pH 7.8 by boric acid-borax buffer.

Hydroxyl radicals were generated in a Fenton type reaction and were detected as DMPO-OH adduct. The reaction mixture contained the following reagents at the final concentration: 31μM H_2O_2, 33.2μM ferrous sulfate, 0.83mM EDTA, 1.12mM purified DMPO in 0.2M boric acid-borax buffer pH 7.8 . The reaction was initiated by adding ferrous sulfate. Various levels of lidocaine, or other scavengers of hydroxyl radicals were used in the above system. Lidocaine did not react with H_2O_2 at indicated concentrations. This is in agreement with previoulsy reported studies (20). Lidocaine had also no effect on the stability of DMPO-OH adduct. The adduct remained stable for at least 30

minutes. Addition of 300 μM lidocaine to the reaction mixture at the end of 10 minutes incubation had trivial effect on the above adduct. The studies were repeated for at least three times. The generation of hydroxyl radicals was observed as DMPO-OH adduct on a Bruker D-200 X-Band EPR spectrometer. Scan conditions, unless indicated otherwise, were as follows: microwave frequency, 9.78 KHz; power, 10mW; modulation amplitude 1.0; modulation frequency, 100 KHz; time constant, 0.64s, scan time 200s; receiver gain 4.0 $X10^5$; field setting 3483G.

Hydroxyl radicals are known to damage deoxyribose which in turn produces a TBA reactive chromogen that can be monitored at 535nm (21). The reaction system contained 1.6mM deoxyribose in phosphate buffered saline, pH 7.4. The reaction was initiated by adding freshly prepared ferrous ammonium sulfate (42 μM) and the mixture was incubated at 37^0C for 15 minutes. One ml of 1%(w/v) TBA was added along with 1 ml of 2.8% (w/v)TCA. The mixture was heated at 100^0C for 10 minutes, cooled and the chromogen concentration was determined by a Shimadzu 160UV spectrophotometer at 535nm. Lidocaine had no effect on the chromogen when added after the incubation and before the addition of TBA and TCA.

Photolysis studies were performed at room temperature, in the presence of dissolved air, in quartz capillary tubes. Samples were irradiated for 5 minutes at a distance of 30 cm from the lens of a Viewlex VR-25 remote controlled slide projector. EPR measurements were made on a Bruker D-200 X-Band spectrometer using a magnetic field modulation frequency of 100KHz. The microwave power was maintained at 10 milliwatts and scans were traced

with a modulation amplitude of 1.0G, time constant of 0.64s, scan rate of 200s, and the receiver gain of $4X10^5$.

RESULTS

Effects of lidocaine on Superoxide Anion:

Superoxide anion (O_2^-) is known to be produced when xanthine oxidase acts on xanthine in the presence of molecular oxygen. The O_2^- radicals so generated can reduce ferricytochrome c. Superoxide dismutase inhibits this reaction by effectively competing with ferricytochrome c for the flux of O_2^-. This reaction has been used as a convenient assay for superoxide dismutase (22). When 10^{-9}M xanthine oxidase was added to 10^{-5}M ferricytochrome c, 5 X 10^{-5} M xanthine in 0.05M potassium phosphate, pH 7.8 plus 10^{-4}M EDTA and was monitored at 550nm, a linear rate of reduction of ferricytochrome c was observed (ΔAbs at 0.025/min) for at least 5 minutes and superoxide dismutase at 0.1µg/ml inhibited this rate by 50%, indicating that the reduction of ferricytochrome c was dependent on O_2^-. We tested the effects of lidocaine in this system as possible superoxide dismutase mimics. Lidocaine at 1 to 20mM concentrations did not inhibit the rate of ferricytochrome c reduction. We further tested the O_2^- scavenging activities of lidocaine in an epinephrine autoxidation assay. Thus, when 2.8mM epinephrine was added to a 0.05M bicarbonate buffer at pH 10.2 and monitored at 480nm (23) a linear rate of accumulation of adrenochrome, after a short lag, was observed. Lidocaine did not inhibit the rate of adrenochrome formation at 1 and 20mM. These results indicate that lidocaine is not an effective superoxide scavenger.

Effects of Lidocaine on Hydroxyl Radical:

The hydroxyl radicals generated in a Fenton type system ($Fe^{2+} + H_2O_2$ --> $OH\cdot + OH^- + Fe^{3+}$) yield a spin adduct with DMPO(21). Thus, as presented in the Figure 1 (line 1), a well characterized 1:2:2:1 pattern of DMPO-OH with $A_N = A^{HB}$ =14.92 G was obtained when 33.2μM ferrous sulfate was added to 31μM H_2O_2 in presence of 1.12mM DMPO and 0.83mM EDTA in 0.2M borate buffer, pH 7.8. The EPR signal of DMPO-OH adduct was stable for several minutes. Addition of OH· scavengers inhibited the signal in a dose dependent manner, and 1mM thiourea and 3mM mannitol inhibited the signal almost completely The effects of lidocaine was tested in this system. As shown in Figure 1, lidocaine inhibited the DMPO-OH adduct formation in a dose dependent manner. Thus, lidocaine at 300μM concentration virtually eliminated the DMPO-OH adduct formation. The molar concentration of lidocaine required to cause 50% inhibition in this system was found to be 80μM. Compared to the molar concentration of inhibitors required to cause 50% inhibition of the rate of DMPO-OH adduct formation, lidocaine was found to be 18 times more sensitive than mannitol and 1.6 times more sensitive than thiourea. That lidocaine is a potent OH· scavenger was further confirmed in another assay in which OH· reacts with deoxyribose to generate a TBA reactive substance. As shown in Figure 2, OH· generated in a Fenton type reaction oxidized deoxyribose to produce a TBA-reactive product that can be monitored at 535nm and lidocaine inhibited this reaction in a dose dependent manner. The molar concentration of lidocaine required to cause 50% inhibition in this system was found to be 3mM.

Effects of lidocaine on Singlet Oxygen:

The generation of singlet oxygen by photochemical reactions of rose bengal or methylene blue was studied by EPR spectroscopy using TEMP as a singlet oxygen trap. We have demonstrated the formation of TEMPO as a nitroxyl radical by the attack of singlet molecular oxygen, generated during photoactivation of Rose Bengal, on TEMP (24-25). The characteristic EPR spectral pattern of three equal intensity lines of TEMPO nitroxide radical was observed when an air saturated aqueous solution of rose bengal was irradiated in the presence of TEMP at room temperature (Fig 3). The hyperfine splitting constants and g-value of the radical were found to be A^N=17.2 G and g= 2.0056, respectively, consistent with our previously reported values (24-25).

The formation of TEMPO during photoirradiation of rose bengal and TEMP was further charcterized. The known singlet oxygen scavengers such as ß-carotene, sodium azide and histidine had profound inhibitory effects on the TEMPO formation, consistant to our previous report (25). As shown in figure 3, lidocaine inhibited the TEMPO formation in a dose dependent manner. Singlet oxygen dependent TEMPO signal was virtually eliminated at 1mM lidocaine. Lidocaine inhibited this reaction more effectively than the above singlet scavengers. Thus, compared to histidine, azide and ß-carotene, lidocaine was 20, 12 and 1.2 times more sensitive in this system respectively. When Rose Bengal was replaced with Methylene Blue (600µM) as a photosensitizer and TEMPO accumulation was monitored for a period of 10 minutes, lidocaine at 4.18mM concentration inhibited this reaction by 50%. This difference probably

depends on the rate of $^1O_2$ generation by the two photosensitizers, which have been discussed in another context (24).

DISCUSSION:

In the present study we have shown that lidocaine is not an effective superoxide radical scavenger. This was confirmed by using two different assays. In cytochrome c assay O_2^- acts as a reductant where as in epinephrine assay this radical acts as an oxidant. In both these assays lidocaine up to 20mM concentration had little effect. Our results are in accord with Mark and Weglicki (17), who reported that lidocaine did not inhibit O_2^- -dependent lipid peroxidation.

Lidocaine was found to be a potent scavenger of hydroxyl radicals. This was shown in two different assays. Thus, when OH. radicals were generated in a Fenton-type reaction and detected as DMPO-OH adduct by EPR spectroscopic techniques or as OH.-dependent TBA reactive products of deoxyribose, lidocaine inhibited these reactions in a dose dependent manner. It is noteworthy to indicate that the rate of reaction of DMPO with OH. is extremely fast, 3.4 $X10^9M^{-1}$ sec^{-1} (18), and 80μM lidocaine was able to compete with 1.12mM DMPO for the available OH. to cause a 50% reduction on the rate of DMPO-OH adduct formation. Compared to some of the known hydroxyl radical scavengers, lidocaine was found to be 1.6 times more sensitive than thiourea and 18 times more sensitive than mannitol. The amount of lidocaine required to cause 50% inhibition was different in two different systems because the sensitiveness of the spin-trapping study and deoxyribose assay were different as determined by known OH. scavenger intervention. In spin-trapping study 3mM mannitol virtually eliminated the DMPO-OH signal, where as in deoxyribose assay 10mM mannitol caused 80% inhibition.

Lidocaine was also found to be a potent quencher of singlet oxygen. Using two different sensitizers, rose bengal and methylene blue, we have shown that lidocaine inhibits $^1O_2$-dependent TEMPO formation in a dose dependent manner. TEMP reacts with $^1O_2$ to form TEMPO at $5.3X10^5$ M^{-1} sec^{-1} (24). In this reaction 500μM lidocaine was able to compete with 65mM TEMP for the available $^1O_2$ to cause a 50% reduction in the rate of formation of TEMPO. When compared with other known scavengers/quenchers of $^1O_2$, the singlet oxygen scavenging ability of lidocaine was found to be 20 times better than azide and was 1.2 times more effective than ß-carotene in these reaction systems.

Although She et al (16) have presented evidence that the alveolar epithelial fluid containing lidocaine(used as an anesthetic) quenches hypochlorous acid, the antioxidant properties of lidocaine, however, were not investigated in detail. We now present evidence to confirm the antioxidant properties of this drug which is found to be a potent inhibitor of both singlet oxygen and hydroxyl radical.

The cytoprotective effects of lidocaine in myocardial ischemia have been attributed to its membrane stabilizing properties(19, 21 22). However, the mechanism of such "membrane stabilization" has not been elucidated. Recently, Woodward and Zakaria (30) and Manning et al. (31) have proposed the hypothesis that oxygen-derived free radicals generated during the early moments of reperfusion may initiate membrane injury, leading to the development of severe ventricular arrhythmias. Singlet oxygen and hydroxyl radical are proposed to be the direct initiator of lipid peroxidation by concerted

addition-abstraction reactions with the diene bonds of unsaturated lipids giving rise to lipid hydroperoxides(32-36). It was suggested that singlet oxygen and OH· are involved in the propagation of lipid peroxidation(32) which in turn can produce membrane damage and cell dysfunctions (33). During myocardial ischemia, especially after reoxygenation of the heart, there is an apparent accumulation of lipid peroxides in the tissue (10,11,29,34). Canine sarcolemmal membranes were also found to be readily peroxidized by reactive species of oxygen and the phospholipid-rich sarcolemma of ventricular myocytes were proposed to be a major site of free radical attack (32). The results of the present study show that lidocaine is a powerful antioxidant which can scavenge hydroxyl radical and quench singlet oxygen. Therefore, the "membrane stabilization" ability of lidocaine may, in part, be attributed to the reactive oxygen scavenging properties of this compound.

Recent studies have indicated the involvement of reactive oxygen species in myocardial ischemia. Thus, Bernier et al. (28) found that superoxide dismutase, catalase, mannitol, methionine, glutathione and desferroxamine reduced the incidence of reperfusion-induced ventricular fibrillation from 80 to 0, 7, 7, 7, 20, and 7%, respectively. They also demonstrated a clear dose dependency for each intervention. In another study (29) it was found that superoxide dismutase plus catalase enhanced the efficacy of hypothermic cardioplegia in protecting the globally ischemic, reperfused heart. The production of oxygen-centered free radicals during ischemia and reperfusion of myocardium have been demonstrated and a burst of oxygen radical generation was shown to occur within moments of reperfusion (36). Electron spin resonance studies of free radical production in ischemic/reperfused rat hearts

have also been shown to produce oxygen free radicals (35). These studies suggest that excessive free radicals are produced in the extracellular compartment and these radicals in turn are responsible for the sarcolemmal membrane damage. Protective effects of lidocaine has also been demonstrated in acute haemorrhagic shock (38). Circulatory shock induced by trauma and endotoxin have been shown to be prevented by phenyl t-butyl nitrone (PBN), a spin trapping agent (38). Since spin traps behave as free radical scavengers, the protective effects of PBN provided an indirect evidence for the involvement of free radicals in circulatory shock.

In view of our present findings, we propose that lidocaine exerts its protective effects by scavenging highly cytotoxic reactive species of oxygen generated during ischemia and reperfusion. Furthermore, the membrane stabilizing properties of lidocaine can be attributed to its removal of $OH\cdot$ and $^1O_2$ which in turn cause membrane lipid peroxidation.

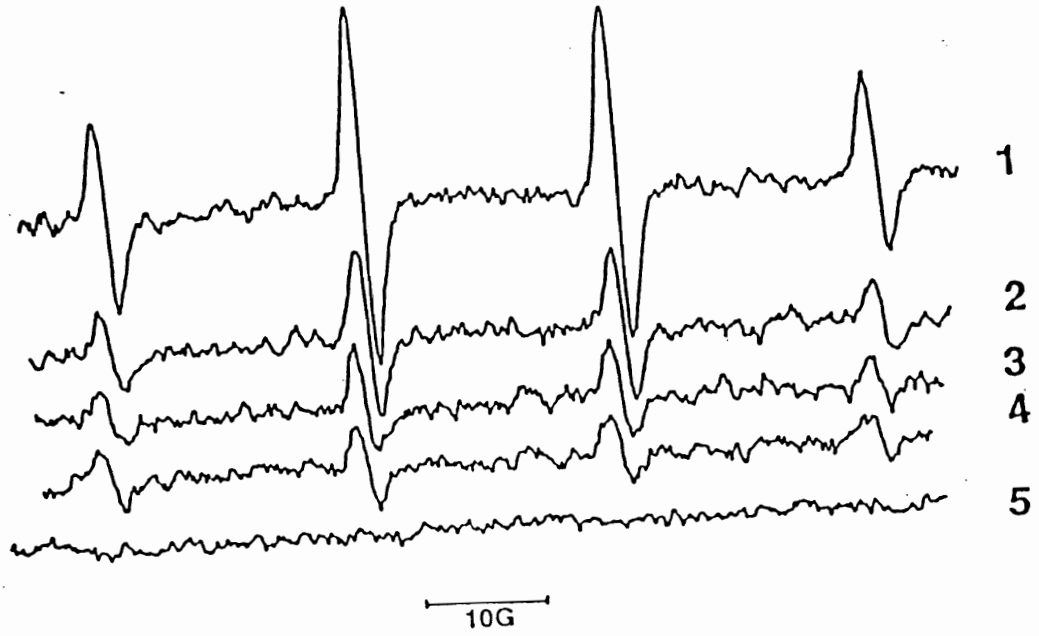


Fig 1. Effect of Lidocaine on the formation of DMPO-OH adduct. The experimental conditions are as described under "Materials and Methods". Lidocaine at indicated concentrations were added to 31μM H_2O_2, 1.12mM DMPO, in boric acid-borax buffer, pH 7.8 in the presence of 33.2μM ferrous sulfate and 0.83mM EDTA. The DMPO-OH adduct formation was recorded immediately. Receiver gain was 4.0X 10^5, and the scan rate of 200 seconds. Other EPR parameters were same as described in "Materials and Methods". Line 1: control without lidocaine; Line 2: 100μM lidocaine; Line 3: 200μM lidocaine; Line 4: 300μM lidocaine; Line 5: 1.2mM DMPO only.

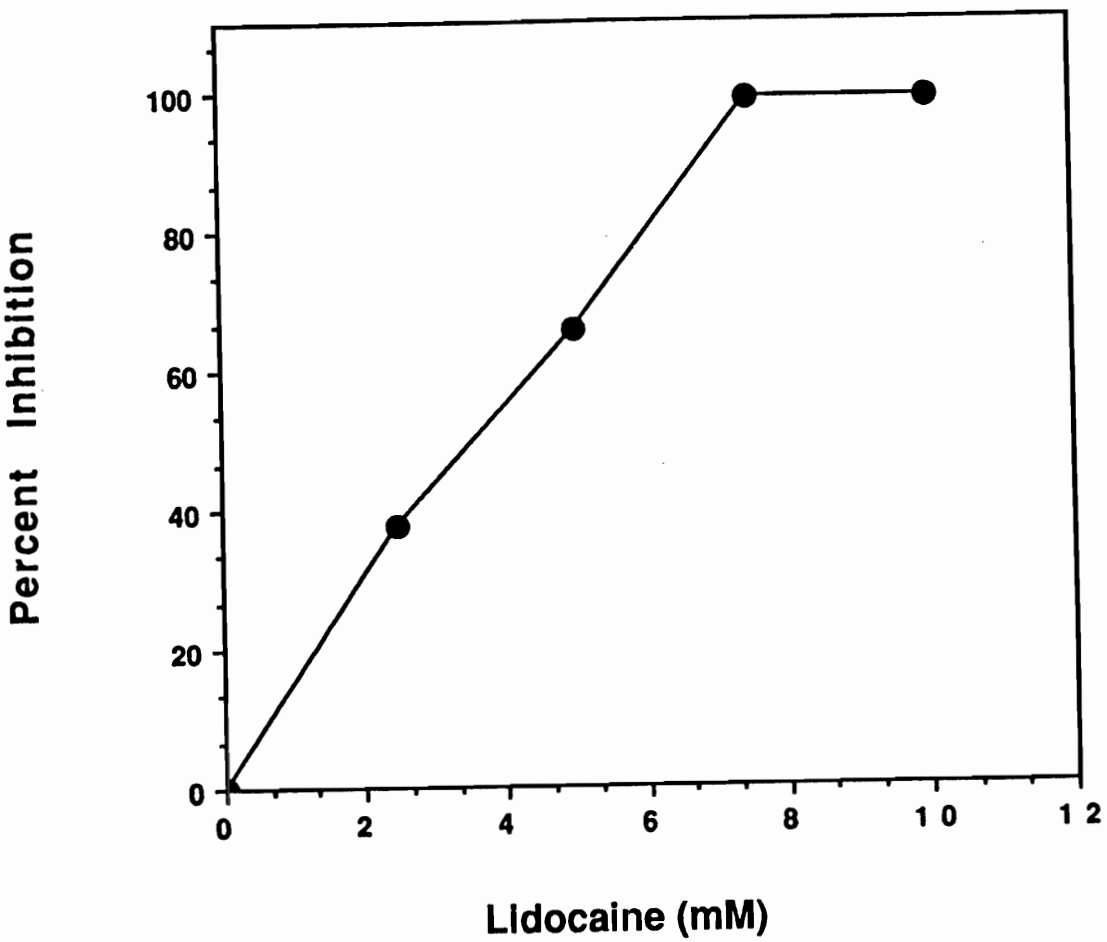


Fig 2. Effects of lidocaine on the formation of TBA reactive substance from deoxyribose. Conditions are as described under "Materials and Methods". Lidocaine at indicated concentrations was added to 1.6mM deoxyribose, 42μM ferrous ammmonium sulfate in phosphate buffered saline (pH 7.4) and the mixture was incubated at 37^0C for 15 minutes. TBA (1ml of 1% w/v) was added followed by 1ml of 2.8% (w/v)TCA. The mixture was heated at 100^0C for 10 minutes, cooled, and the chromogen was measured at 535nm.

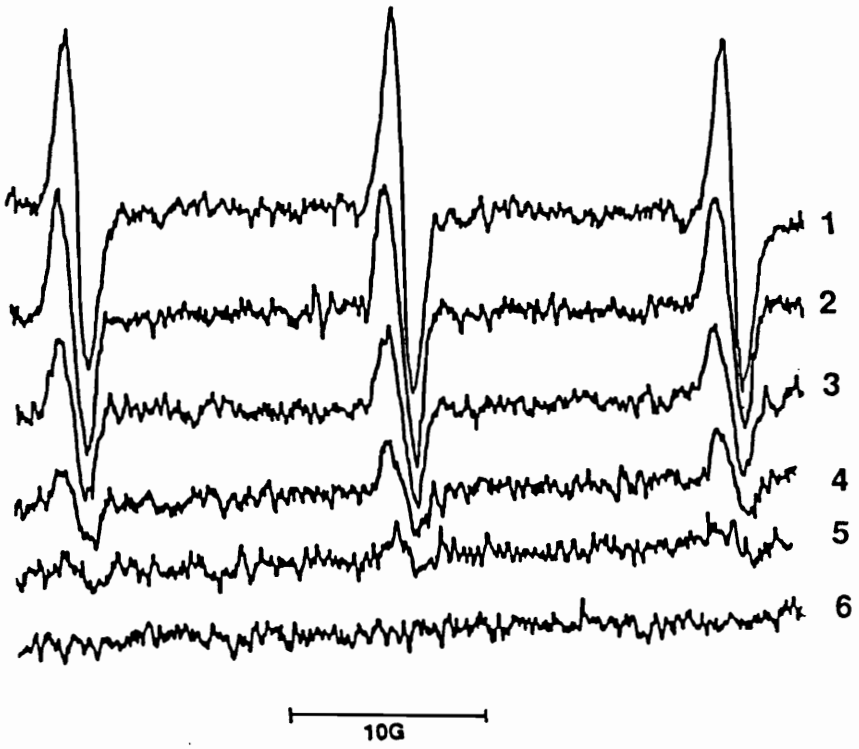


Fig 3. Effects of lidocaine on the formation of TEMP-$^1O_2$ adduct. The experimental conditions are as described under "Materials and Methods". Lidocaine at indicated concentrations were added to 40μM Rose Bengal, 65mM TEMP dissolved in ethanol (final concentraton of ethanol was 10%), in 0.05M potassium phosphate buffer with 10^{-4} M EDTA. TEMP-$^1O_2$ adduct formation was recorded after irradiating the reaction mixture for 5 minutes. Line 1: without lidocaine; Line 2: 200μM lidocaine; Line 3: 500μM lidocaine; Line 4: 700μM lidocaine; Line 5: 1mM lidocaine; Line 6: No photosensitizer or light.

REFERENCES

1. Harrison DC, Sprouse JH and Morrow AG: Antiarrhythmic properties of lidocaine and procainamide: Clinical and physiologic studies of their cardiovascular effects in man. *Circulation* 28: 486-491, 1963.
2. Gianelly R, von der Groeben JO, Spivack AP and Harrison DC: Effects of lidocaine on ventricular arrhythmias in patients with coronary heart disease. *New Engl. J. Med.* 277: 1215-1221, 1967.
3. Jewitt DE, Kishon Y, and Thomas M: Lignocaine in the management of arrhythmias after acute myocardial infraction. *Lancet* 2: 266-268, 1968.
4. Pfeiffer CJ, Keith JC, Cho CH, DeRolf S, Pfeiffer DC and Misra HP: Gastric and cardiac protection by lidocaine. *Acta Phys. Hung.* 73:129-136, 1989.
5. Nasser FN, Walls JT, Edwards WD and Harison CE, Jr: Lidocaine-induced reduction in size of experimental myocardial infraction. *Am. J. Cardiol.* 46: 967-975, 1980.
6. Boudolulars H, Karayannacos PE, Lewis RP, Kakos GS, Kilman JW, and Vasko JS: Potential effect of lidocaine on ischemic myocardial injury: Experimental and clinical observations. *J. Surg. Res.* 24: 469-476, 1978.
7. Narang KP, Crouthamel WG, Carliner HN, Fisher ML: Lidocaine and its active metabolites. *Clin. Pharmacol. Ther,* 654-622, 1978.
8. Guarnieri C, Flamigni F, Caldarera CM: Role of oxygen in the cellular damage induced by reoxygenation of hypoxic heart. *J. Mol. Cell. Cardiol.* 12: 797-808, 1980.

9. Hess ML, Manson NH, and Okabe E: Involvement of free radicals in the pathophysiology of ischemic heart disease. *Can. J. Physiol. Pharmacol.* 60: 1382-1389, 1982.

10. McCord JM, Roy RS: The pathophysiology of superoxide: Roles in inflammation and ischemia. *Can. J. Physiol. Pharmacol.* 60: 1346-1352, 1982.

11. Rao PS and Mueller HS: Lipid peroxidation and myocardial ischemia. In: *Myocardial injury* (Eds. Spitzer, J. J.),pp341-367. Plenum Publishing Co, New York, 1983.

12. Fridovich I: Hypoxia and oxygen toxicity. *Adv. Neurol.* 26: 255-259,1979.

13. Misra HP, Weglicki WB, Abdula R and Mc Cay, PB: Identification of a carbon-centered free radical during reperfusion injury in ischemic heart. *Circulation*, 70: II-260 , 1984.

14. Carmen MA, Kramer JH, Benjamin FD, and Weglicki WB: Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO. *FEBS Lett.*. 221: 101-104,1987.

15. Baron DW, Sunamori M, and Harrison CE: Preservation of oxidative phosphorylation by lidocaine in ischemic and reperfused myocardium. *Adv. Myocardiol.* 4: 567-573, 1983.

16. She, ZW, Liming, JD, Fagan, JB, Pacht, ER, and Davis, WB: Inhibition of hypochlorous acid by lidocaine and native components of alveolar epithelial lining fluid, *Am. Rev. Respir. Dis.* 144:227-230, 1991.

17. Mak,IT and Weglicki, WB: Protection by B-Blocking agents against free radical-mediated sarcolemmal lipid peroxidation., *Circ. Res.* 63:262-266, 1988.

18. Kalyanaraman, B: Detection of toxic free radicals in biology and medicine. In: *Reviews in Biochemical Toxicology* (Eds. Hodgson, E., Bend, JR. and Philpot, RM.) pp 73-139, Elsevier Biomedical, New York.

19 Schaub RG, Stewart G, Strong M, Ruofolo R, Lemole G: Reduction of ischemic myocardial damage in the dog by lidocaine infusion. *Am. J. Pathol*, 87: 388-414, 1977.

20. Stelzner, TJ., Welsh, CH., Berger, E., McCullough, RG., Morris, K., Repine, JE., and Weil, JV. Antiarrhythmic agents diminish thiourea induced pulmonary vascular protein leak in rats. *J. Appl. Physiol.* 63: 1877-1883, 1987

21. Halliwell B and Gutteridge JMC: Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts. *FEBS Lett..*, 128: 347-351, 1981.

22. McCord JM and Fridovich I: The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* 243: 5753-5760, 1968.

23. Misra HP and Fridovich.I: The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247: 3170-3175, 1972.

24. Zang L, Zhang Z and Misra HP: EPR studies of trapped singlet oxygen ($^1O_2$) generated during photoirradiation of Hypocrellin A. *Photochem.Photobiol.* **52:** 677- 684,1990.

25 Misra BR and Misra HP: Vasoactive intestinal peptide, a singlet oxygen quencher. *J. Biol. Chem.* 265: 15371-15374,1990.

26. Hearse JD, O' Brian OK, Braimbridge M: Protection of the myocardium during ischemic arrest. *J. Thorac. Cardiovasc. Surg.* 81: 873-879,1981.

27. Schaub RG, Lemole MG, Pinder GC, Black P: Effects of lidocaine and epinephrine on myocardial preservation following cardiopulmonary bypass in the dog. *J. Thorac. Cardiovasc. Surg.* 74: 571-576, 1977.

28. Bernier M, Hearse JD, Manning AS: Reperfusion-induced arrhythmias and oxygen derived free radicals. Studies with anti-free radical interventions and a free radical generating system in the isolated perfused heart. *Circ. Res.* 58: 331-340, 1986.

29. Shlafer M, Kane PF and Kirsh MM: Superoxide dismutase plus catalase enhances the efficacy of the hypothermic cardioplegia to protect globally ischemic, reperfused heart. *J. Thorac. Cardiovasc. Surg.* 83: 830-839, 1982.

30. Woodward B, Zakaria M: Effects of some free radical scavengers on reperfusion induced arrhythmias in the isolated rat heart. *J. Mol. Cell. Cardiol.* 16: 497-517, 1984.

31. Manning AS, Coltart JD and Hearse DJ: Ischemia and reperfusion-induced arrhythmias in the rat. *Circ. Res.* 55: 545-550, 1984.

32.Svingen AS, O' Neal FO and Aust SD: The role of superoxide and singlet oxygen in lipid peroxidation. *Photochem. Photobiol.* 28: 803-8091978.

33 Niki E, Yamamoto Y and Kamiya Y: Inhibition of Peroxidations of liposomal and biomembranes by water soluble antioxidants. In: *Lipid Peroxidation in Biological Systems* (Ed. Sevanian, A.), pp 32-50, American Oil Chemists Society, Champaign, Illinois.1988.

34. Gauduel Y and Duvelleray MA: Oxygen radicals in cardiac injury due to reoxygenation. *J. Mol. Cell. Cardiol.* 16: 458-470, 1984.

35. Cohen G: The generation of hydroxyl radicals in biologic systems: Toxicological aspects. *Photochem. Photobiol.* 28: 669-675, 1978.

36. Zweier JL , Flaherty JT, and Weisfeldt ML: Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc. Natl. Acad. Sci.* 84: 1404-1407, 1987.

37. Keith JC, Jr: Effect of lidocaine pretreatment on acute haemorragic shock in the anaesthetized rat. *Circ. Shock* 19: 283-292, 1986.

38. Novelli GP: Free radicals and circulatory shock. In: *The role of Oxygen Radicals in Cardiovascular Diseases* (Eds. L' Abbate, A. , and Ursini, F.), pp13-22, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1986.

CHAPTER-III

ANTIARRHYTHMIC AGENTS:
Scavengers of Hydroxyl Radicals and Inhibitors of NADPH-Dependent Lipid Peroxidation in Bovine Lung Microsomes

(Published in the Journal of Biological Chemistry Vol.267, No 27, pp19172-19178, 1992)

Abstract

Antiarrhythmic drugs, e.g. lidocaine, quinidine and procainamide have been suggested as a means of reducing myocardial damage. The mode of action of these drugs have been attributed to their "membrane stabilizing" properties. However, as tissue ischemia-reperfusion is reported to generate toxic species of oxygen, we investigated the oxygen radical scavenging properties of these drugs and their effect on NADPH-dependent lipid peroxidation. These antiarrhythmic drugs are found to be ineffective as superoxide radical scavengers but are potent scavengers of hydroxyl radical with rate constants of 1.8×10^{10} $M^{-1}s^{-1}$, 1.61×10^{10} $M^{-1}s^{-1}$ and 1.45×10^{10} $M^{-1}s^{-1}$ for quinidine, lidocaine and procainamide, respectively, as determined by deoxyribose assay. In EPR study, using DMPO as a spin trap, lidocaine, quinidine and procainamide caused a dose dependent inhibition of DMPO-OH adduct formation. These drugs also caused a dose dependent inhibition of NADPH-

dependent lipid peroxidation when lung microsomes were incubated with NADPH in presence of Fe^{3+}-ADP. We propose that the antiarrhythmic agents exert their beneficial effects, in part, by their ability to scavenge toxic species of oxygen and by reducing membrane lipid peroxidation.

Introduction

Class I antiarrhythmic agents (lidocaine, quinidine and procainamide) are widely used drugs with significant stabilizing activity on lipid biomembranes (1). These drugs have been suggested as a means of reducing ischemic myocardial damage. Prolonged ischemia such as that following myocardial infarction, pulmonary embolism and conditions occurring during long-term coronary bypass procedures cause serious damage to these tissues. During ischemia, increased reducing equivalents are produced (2) due to the decreased concentration of oxygen, and this may favor the production of superoxide anion and other free radical species upon reoxygenation (3). The generation of short lived free radicals like superoxide anions, hydroxyl radicals and carbon centered radicals, have been observed during myocardial ischemia and during post-ischemic reperfused myocardium (4, 5). Lipid peroxidation provokes damage to cellular membranes (6). It has been demonstrated that during myocardial ischemia, and especially after reoxygenation of the heart, the concentration of lipid peroxides in the tissues increases (7-10). Lipid peroxidation mediated by hydroxyl radicals has also been suggested as a possible mechanism of pulmonary injury (11-13). Since lipid peroxidation can be initiated by reactive species of oxygen which are produced during reperfusion of ischemic tissue, we developed the hypothesis that antiarrhythmic agents might be exerting their beneficial effects, in part, by a free radical scavenging mechanism thereby inhibiting lipid peroxidation. In this study we

present evidence that antiarrhythmic drugs such as lidocaine, quinidine and procainamide are potent inhibitors of hydroxyl radicals and are capable of inhibiting NADPH-dependent lipid peroxidation.

Materials and Methods

5,5-dimethyl-1-pyrroline N-oxide (DMPO) was obtained from Sigma Chemical Co.. The DMPO was purified by stirring aqueous solutions of DMPO (900 mM) with activated charcoal at 10 mg/ml and filtered through 0.22 μ Millipore filter cartridges, and then centrifuged at 2000 X g for 2 minutes. The purified DMPO did not give any EPR signal when scanned at 45 and 90 mM concentrations.

Rose bengal, methylene blue, bovine superoxide dismutase, catalase, cytochrome c (Type III), mannitol, thiourea, sodium benzoate, ethanol, lidocaine, quinidine, procainamide, thiobarbituric acid (TBA), Trichloro acetic acid (TCA), adenosine diphosphate (ADP), ferric chloride and deoxyribose were obtained from Sigma Chemical Co. 2,2,6,6-Tetramethyl piperidine (TEMP) was obtained from Aldrich. All other materials were purchased at the highest available purity.

Preparation of bovine microsomes:

One bovine lung lobe was collected on ice after sacrifice and was minced with scissors. The minced lung was homogenized for 3 min in a Waring blender with 5 volumes of ice cold 0.15 M potassium phosphate buffer pH 7.6 and filtered through a triple layered cheese cloth. The filtrate was centrifuged at 24,000 X g for 10 minutes to remove mitochondria, nuclei and cell debris. The supernatant was then centrifuged at 100,000 X g for 90 minutes and the microsomal pellet was collected. The microsomes were washed by resuspending in 0.15 M phosphate buffer, pH 7.6, to the original volume and by sedimenting at 100,000 X g for 90 minutes. The washed microsomal pellet was then resuspended in Tris-HCl buffer, pH 7.6, to yield a final concentration of 10 mg microsomal protein per ml. The washing procedure removes most contaminants of hemoglobin, superoxide dismutase and catalase from microsomal protein. Protein concentration was determined by Bradford protein assay using bovine serum albumin as the standard (17).

Assay of lipid peroxidation:

Lipid peroxidation was determined by the thiobarbituric acid method (18-19). The reaction mixture, containing 3 mg of microsomal protein in Tris-HCl buffer pH 7.6, 200 μM NADPH, 1 mM ADP, 50 μM Ferric chloride was incubated at 37°C for 15 min. Lipid peroxidation was initiated by addition of NADPH and was terminated by addition of 2 ml of 0.5% (w/v) TBA and 2% TCA. This mixture was heated at 95°C for 10 minutes. Three ml of chloroform was added after

cooling and the mixture was vortexed for 30 s. Samples were centrifuged and malondialdehyde (MDA) concentration was determined by reading at 535 nm against a blank that contained all reagents except NADPH and by using an extinction coefficient of 1.56 X 10^5 M cm^{-1} (19). The effect of various concentrations of lidocaine, quinidine and procainamide was tested in this system.

Assay of NADPH-Cytochrome P-450 reductase:

NADPH-Cytochrome P-450 reductase was measured by its NADPH-cytochrome c reductase activity (20). One unit of NADPH-Cytochrome c reductase is defined as one μmole ferricytchrome c reduced per minute. The reaction mixture contained the following reagents at final concentrations: potassium cyanide 1 mM, NADPH 4.2 X 10^{-5} M, nicotinamide 188 mM, ferricytochrome c 6 X 10^{-5} M. The effects of lidocaine, quinidine and procainamide at various concentrations on the activity of cytochrome P-450 reductase was determined in this system.

EPR studies:

Hydroxyl radicals were generated in a Fenton type reaction and were detected as DMPO-OH adduct. The reaction mixture contained the following reagents at the final concentration: 31 μM H_2O_2, 33.2 μM ferrous sulfate, 0.83 mM EDTA, 1.12 mM purified DMPO in 0.2 M boric acid-borax buffer pH 7.8. The reaction was initiated by the addition of ferrous sulfate. Various levels of lidocaine, quinidine and procainamide or other scavengers of hydroxyl radicals

were used in the above system. The generation of hydroxyl radicals was observed as DMPO-OH adduct on a Bruker D-200 X-Band EPR spectrometer. Scan conditions, unless otherwise indicated were as follows: microwave frequency, 100 KHz; power, 10 mW; modulation amplitude 1.0 G; modulation frequency,100 KHz; time constant, 0.64 s, scan time 200 s; receiver gain 4.0 X 10^5; center field setting 3483 G.

Photolysis studies were performed at room temperature, in the presence of dissolved air, in quartz capillary tubes. Samples were irradiated for various time periods at a distance of 30 cm from the lens of a Viewlex VR-25 remote controlled slide projector. EPR measurements were made as described above.

Determination of rate constant

The degradation of deoxyribose by hydroxyl radicals and the production of a pink chromogen monitored spectrophotometrically has been used to determine the rate constants of various compounds (14-16) and are in agreement with the rate constants determined by the pulse radiolysis method. Hence, we used the deoxyribose method (14) to determine the rate constant for reaction of ·OH with of the antiarrhythmic agents (lidocaine, quinidine and procainamide).

Reaction mixtures contained, in a final volume of 1.0 ml, the following reagents at the final concentrations stated: Deoxyribose (2.8 mM), KH_2PO_4-KOH buffer, pH 7.4 (20 mM), $FeCl_3$ (25 μM), EDTA (30 μM), H_2O_2 (1 mM), and ascorbate (100 μM). Solutions of $FeCl_3$ and ascorbate were made up immediately before use in deaerated water. Reaction mixtures were incubated at 37°C for 1 h, and one ml of 0.5% TBA (w/v) and 1ml of 1.4% TCA(w/v) was

added and the mixtures were heated at 80°C for 30 minutes. The rate of deoxyribose degradation was constant over the 1hr incubation period (14).

Results

Effect of antiarrhythmic agents on the production of MDA:

Lipid peroxidation of lung microsomes were induced by incubating the lung microsomes with 200 μM NADPH and 1 mM ADP chelated ferric chloride and the production of TBA reactive MDA was considered as an index of lipid peroxidation. Malondialdehyde production by lung microsomes was linear with respect to the concentration of microsomal protein, over the range of 1 to 3 mg of protein per ml (Fig. 1A), and was dependent on the presence of NADPH (Fig. 1B). As shown in Figure 1B, increasing the concentration of NADPH beyond 600 μM did not increase malondialdehyde production. A linear rate of MDA production was observed with incubation time and appeared to have reached a maximum after 10 min (Fig. 1C). Preincubation with lidocaine, quinidine and procainamide inhibited the NADPH-induced lipid peroxidation in a dose dependent manner (Fig. 2). Lidocaine was found to be the most potent inhibitor of NADPH-dependent lipid peroxidation followed by quinidine and procainamide. Quinine, a stereoisomer of quinidine but a less effective antiarrythmic agent (21), was only 60-66% as effective as quinidine at 200, 300 and 400 μM concentraions in this reaction. The dose-dependent effects of lidocaine on the extent of lipid peroxidation was further examined. As shown in Figure 2, lipid peroxidation was strongly inhibited by lidocaine and the maximum inhibition actually achieved was 72%. When these data were presented on reciprocal coordinates (Fig. 2 inset), inhibitions in all cases appeared to be kinetically simple and the extrapolation of the data indicate that

there was a maximum of 75% inhibition on lipid peroxidation by lidocaine indicating the existance of a competing alternate pathway for lipid peroxidation.

Effect of scavengers of reactive oxygen species on MDA Production:

It was previously shown that liver microsomes generate H_2O_2 in the presence of NADPH (22-23). Since $O_2^{-}\cdot$ can dismutate to form H_2O_2 and in a metal catalyzed reaction, $H_2O_2 + O_2^{-}\cdot$ can form ·OH, it has been suggested that the reactive species of oxygen may be involved in lipid peroxidation initiation reaction. In order to compare the action of antiarrhythmic drugs with other known scavengers, data were obtained on NADPH-dependent MDA production by lung microsomes in presence or absence of several free radical scavengers. Thus, as shown in Table I, the hydroxyl radical scavengers thiourea and ethanol inhibited MDA formation 100 and 61%, respectively, at 10 mM concentrations. DMPO, a spin trap which removes $O_2^{-}\cdot$ and OH. by forming DMPO-OOH and DMPO-OH adduct, respectively, also inhibited the formation of MDA by 100%. Catalase at 5000 units/ml inhibited the formation of MDA by 81%. Superoxide dismutase 1 to 10 μg/ml did not inhibit the formation of MDA, rather a slight increase was noted. This is in agreement with our previous report (24).

Mannitol and benzoate increased the formation of MDA by 20 and 30%, respectively, at 10 mM concentrations. This is in agreement with Cohen and Cederbaum (25) who observed similar effects of mannitol in a lipid peroxidation-dependent chemiluminescence reaction. It is likely that mannitol and benzoate, being hydrophilic, can not partition to the membrane bilayer for the effective scavenging of ·OH at the site of its production. In order to test this

hypothesis, 1 mM mannitol was added to a n-octanol / water (50 : 50) mixture, vigorously shaken for 5min and allowed the phases to separate. Mannitol was measured in the two phases colorimetrically by the chromotropic acid-formaldehyde reaction using periodic acid to oxidize the sugar alcohol (26). This assay was found to be sensitive at 20 μM mannitol and a linear standard curve was obtained with mannitol concentrations from 20 μM to 1 mM. Almost 100% of the mannitol was found to be partitioned to the aquous phase and no trace of mannitol was detected in the n-octanol phase. Since n-octanol has dielectric constant similar to most biomembranes, it is likely that the lack of protection of microsomes against lipid peroxidation by mannitol is due to its inability to reach the membrane lipid bilayer.

Effect of antiarrhythmic agents on the activity of NADPH-Cyt P_{450} reductase:

In NADPH-dependent lipid peroxidation, NADPH-Cyt P_{450} reductase is the key enzyme causing one electron reduction of molecular oxygen or Fe^{3+} (27). This enzyme serves as electron carrier from NADPH to oxygen via cytochrome P450. Although the antiarrhythmic drugs inhibited the NADPH-dependent microsomal lipid peroxidation in a dose dependent manner, there is still reason to believe that these drugs could inhibit the NADPH-cyt P_{450} reductase and thus lower the flux of univalent oxygen reduction to generate fewer oxyradicals. To exclude the possibility of this subtle artifact we have examined the effect of antiarrhythmic agents on the activity of NADPH-Cyt P_{450} reductase. As shown in Table II, the activity of the enzymes remain unchanged when various concentrations of lidocaine, quinidine and procainamide were

incubated with microsomes in presence of NADPH. These studies established that the inhibition of microsomal lipid peroxidation by antiarrhythmic drugs was not due to decreased production of active oxygen species and is more likely due to scavenging of these species

Effects of antiarrhythmic agents on active oxygen species:

In an attempt to identify the radical species directly involved in initiating lipid peroxidation, and which can be scavenged by antiarrhythmic agents, we investigated the role of these agents in various known activated oxygen generating systems.

Superoxide anions are known to be produced when xanthine oxidase acts on xanthine in the presence of molecular oxygen. The $O_2^{-}\cdot$ so generated can reduce ferricytochrome c and this has been used as a convenient assay for superoxide dismutase (25). We tested the effects of antiarrhythmic agents lidocaine, quinidine and procainamide at 0.5 and 1.0 mM concentrations and found that these agents are not effective in scavenging $O_2^{-}\cdot$ (data not shown).

The hydroxyl radicals generated in a Fenton type system ($Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+}$) yield spin adducts with DMPO (21). Thus, as presented in Fig. 3 (inset) a well characterized 1:2:2:1 patteren of DMPO-OH with $A_N = AH^B =$ 14.92 G was obtained when 33.2 μM ferrous sulfate was added to 31 μM H_2O_2 in the presence of 1.12 mM DMPO and 0.83 mM EDTA in 0.2 M borate buffer, pH 7.8. The EPR signal of DMPO-OH adduct was stable for several minutes. Addition of ·OH scavengers inhibited the signal intensity in a dose dependent manner. Thus, 1mM thiourea and 3 mM mannitol inhibited the signal almost completely (data not shown). The effect of the antiarrhythmic agents lidocaine,

quinidine and procainamide were tested in this system. None of the antiarrhythmic agents reacted with H_2O_2 which is in accord with the previous findings (1). As shown in Figure 3A --> C, lidocaine, quinidine and procainamide inhibited the DMPO-OH adduct formation in a dose dependent manner. The percent inhihition was calculated from the signal heights and are presented in Figure 3D. The molar conconcentration of drugs required to cause 50% inhibition was found to be 108, 108 and 300 μM for lidocaine, quinidine and procainamide, respectively.

If inhibition by antiarrhythmic agents of ·OH, as shown in Figure 3, are truly a reflection of interaction of these agents with the ·OH, then identical results should be obtained with a different assay. That this was the case is illustrated in Figure 4 where a different assay, a deoxyribose colorimetric assay (14), was adopted. In this assay a mixture of $FeCl_3$-EDTA, H_2O_2 and ascorbic acid at pH 7.4, produce ·OH radicals which can be detected by their ability to degrade the sugar deoxyribose into fragments and which generate a pink chromogen upon heating with TBA at low pH :

Fe^{3+}-EDTA + ascorbate ------> Fe^{2+}-EDTA + oxidized ascorbate [1]

Fe^{2+}-EDTA + H_2O_2 ------> Fe^{3+}-EDTA + ·OH + OH [2]

·OH + Deoxyribose ------> Degraded Sugar $\xrightarrow[\text{TBA}]{}$ Color product [3]

The ·OH so generated (reaction 2) is equally accessible to deoxyribose (the detector molecule) and to any other scavenger of ·OH added. Thus, the ability of a substance to inhibit competitively with deoxyribose under these conditions is a measure of its ability to scavenge ·OH and can be used to calculate the rate

constant for reaction of ·OH with scavengers (14). Lidocaine, quinidine and procainamide were able to compete with deoxyribose effectively in preventing the TBA reactive color product formation. The second order rate cnstants for the reaction of these agents with ·OH were calculated (Ref 14) and were found to be 1.8 X 10^{10} $M^{-1}s^{-1}$, 1.61 X 10^{10} $M^{-1}s^{-1}$ and 1.45 X 10^{10} $M^{-1}s^{-1}$ for quinidine, lidocaine and procainamide, respectively (Fig. 4). Control experiments showed that none of the antiarrhythmic drugs interfere with the measurement of deoxyribose degradation or itself react with ·OH to give TBA reactive color products. Thus, when 500 μM each of the drug was added to the reaction mixture at the end of the incubation time, before the addition of TBA, little protection of deoxyribose degradation was observed. Moreover, when these drugs were allowed to react with the ·OH generating system in the absence of deoxyribose, no TBA reactive products were observed at 535 nm.

Since singlet oxygen is thought to be involved in the lipid peroxidation process, we have investigated the role of antiarrhythmic agents in known singlet oxygen generating systems. The generation of singlet oxygen by photochemical reactions of rose bengal or methylene blue was studied by EPR spectroscopy using TEMP as a singlet oxygen trap. We have demonstrated the formation of TEMPO as a nitroxyl radical by the attack of singlet molecular oxygen, generated during photoactivation of various sensitizers, on TEMP (29,30). The characteristic EPR spectral pattern of three lines of equal intensity for the TEMPO nitroxide radical was observed when air saturated aqueous solution of rose bengal were irradiated in the presence of TEMP at room temperature (Fig.5 inset). The hyperfine splitting constants and g-value of the radical were found to be A_N=17.2 G and g= 2.0056, respectively, consistent with

our previously reported values (29,30). Lidocaine, quinidine and procainamide inhibited the TEMPO formation in a dose dependent manner (Fig.5). However, as presented in Table III, when rose bengal was replaced with methylene blue (600 μM) as a photosensitizer and TEMPO accumulation was monitored for a period of 10 minutes, quinidine (1 to 10 mM) and procainamide (1 to 10 mM) did not inhibit the formation of TEMPO. This difference is suggestive of sensitizer quenching rather than singlet quenching. Lidocaine on the other hand was found to inhibit TEMPO formation at higher concentrations in a dose-dependent manner. Thus, at a concentration of 4.18 mM, lidocaine inhibited 50% of the TEMPO signal.

Discussion

In the present study we have demonstrated that antiarrhythmic agents (lidocaine, quinidine and procainamide) inhibit NADPH-dependent lipid peroxidation at micromolar concentrations. Among the three antiarrhythmic agents tested, the order of ability to inhibit lipid peroxidation was: lidocaine > quinidine > procainamide (Fig. 2), which is consistent with the therapeutic potential of the drugs (31-33).

Lidocaine, quinidine and procainamide are found to be scavengers of hydroxyl radical. This was demonstrated both in the EPR spin-trapping study as well as in the deoxyribose degradation assay (Fig. 3 and 4). Thus, when ·OH radicals were generated in a Fenton type reaction and detected as DMPO-OH adduct by EPR spectroscopic techniques, lidocaine, quinidine and procainamide inhibited these reactions in a dose-dependent mannner. Compared to some of the known hydroxyl radical scavengers, lidocaine, quinidine and procainamide were more sensitive than ethanol or thiourea.

Lidocaine, quinidine and procainamide inhibited $^1O_2$-dependent TEMPO formation (Fig.5) when rose bengal was used as a sensitizer. However, when rose bengal was replaced by methylene blue there was no inhibition of TEMPO formation by both quinidine (1 to 10 mM) and procainamide (1 to 10 mM). Lidocaine inhibited this reaction at high concentrations which is consistent with our previous findings (34). The inhibition of TEMPO formation may be due to ionic interaction between the cationic drugs quinidine and procainamide with rose bengal (2^-) since these drugs had minimal effects when cationic sensitizer

methylene blue ($_1^+$) was used to generate $^1O_2$. Therefore it appears likely that quinidine and procainamide are quenching the sensitizer rather than the singlet oxygen. Lidocaine, however, was able to inhibit TEMPO formation in both rose bengal and methylene blue systems in a dose dependent manner indicating that it may posses singlet oxygen scavenging/quenching activity. Although mM concentrations of the drug are required to inhibit singlet oxygen-dependent TEMPO formation, these concentrations are comparable to the concentrations of other known singlet quenchers such as histidine, dimethylfuran and diphenylfuran (24) in inhibiting lipid peroxidation. At pharmacologically relevant concentrations, however, it may be that none of the three antiarrhymic drugs are efficient in scavenging singlet oxygen.

The cytoprotective effects of lidocaine, quinidine and procainamide in myocardial ischemia have been attributed to membrane stabilizing properties (31-33). However, the mechanism of such "membrane stabilization" has not been elucidated. Recently, Woodward and Zakaria (35) and Manning et al. (36) have proposed the hypothesis that oxygen-derived free radicals generated during the early moments of reperfusion may initiate membrane injury, leading to the development of severe ventricular arrhythmias. During myocardial ischemia, especially after reoxygenation of the heart, there is an apparent accumulation of lipid peroxides in the tissue (2,7,9,10). Canine sarcolemmal membranes were also found to be readily peroxidized by reactive species of oxygen and the phospholipid-rich sarcolemma of ventricular myocytes were proposed to be a major site of free radical attack (37). Hydroxyl radicals are proposed to be the direct initiator of lipid peroxidation by concerted addition-abstraction reactions with the diene bonds of unsaturated lipids (38-41). It was

suggested that singlet oxygen and ·OH are involved in the propagation of lipid peroxidation (38) which in turn can produce membrane damage and cell dysfunctions (39). The results of the present study show that lidocaine, quinidine and procainamide are powerful antioxidants, which can scavenge hydroxyl radical and prevent membrane lipid peroxidation. Therefore, the "membrane stabilization" ability of lidocaine as reported earlier may, in part, be attributed to the reactive oxygen scavenging properties of this compound.

Recent studies have indicated the involvement of reactive oxygen species in myocardial ischemia. Thus, Bernier et al. (42) found that superoxide dismutase, catalase, mannitol, methionine, glutathione and desferroxamine reduced the incidence of reperfusion-induced ventricular fibrillation from 80% to 0, 7, 7, 7, 20, and 7%, respectively. They also demonstrated a clear dose dependency for each intervention. In another study (10) it was found that superoxide dismutase plus catalase enhanced the efficacy of hypothermic cardioplegia in protecting the globally ischemic, reperfused heart. The production of oxygen-centered free radicals during ischemia and reperfusion of myocardium have been demonstrated and a burst of oxygen radical generation was shown to occur within moments of reperfusion (41). Electron spin resonance studies have conformed the production of oxygen free radicalsin ischemic/reperfused rat hearts (4,5,41). These studies suggest that excessive free radicals are produced in the extracellular compartment and these radicals in turn are responsible for the sarcolemmal membrane damage.

In our present study, lidocaine, quinidine and procainamide inhibited NADPH-dependent lipid peroxidation. The scavengers of hydroxyl radicals,

such as ethanol, thiourea and DMPO (43), were found to inhibit MDA formation. However, mannitol and benzoate were found to increase the MDA formation when used in higher concentration (Table I). Mannitol is known to increase the production of MDA when citrate-Fe^{2+} is used as a source of chelated iron but the mechanism of this increase is not known (44). Our data indicate that mannitol can not partition to n-octanol phase. Since n-octanol has a dielectric constant similar to most biomembranes, it is possible that the inability of mannitol to protect microsomal membrane against lipid peroxidation may, in part, be due to its inability to reach the site of free radical production.

Isolated rat liver microsomes in the presence of NADPH and iron can catalyze the production of ·OH-like species by a reaction sensitive to catalase but not to superoxide dismutase (24). The involvement of hydroxyl radical in the NADPH-dependent lipid peroxidation has been a subject of controversy. Our observed inhibition of lipid peroxidation by the scavengers of hydroxyl radical are in accord with the findings of some investigators (25, 45-47), but not with those of others (48, 49). The controversy regarding the involvment of hydroxyl radicals in lipid peroxidation may, in part, be due to the different lipid composition of different membranes and the multiplicity of mechanisms of oxygen activation which cause lipid peroxidation. The composition of the reaction medium (presence and absence of exogenous iron) may also play an important role in generating these reactive species. The mechanism proposed below seems to be operating in our system in generating hydroxyl radicals:

$$2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (1)$$

$$O_2^{-\cdot} + \text{ADP-}Fe^{3+} \longrightarrow O_2 + \text{ADP-}Fe^{2+} \quad (2)$$

$$\text{ADP-}Fe^{2+} + H_2O_2 \longrightarrow \text{ADP-}Fe^{3+} + \cdot OH + OH^- \quad (3)$$

$H_2O_2 + O_2^{-}\cdot$ --------> $O_2 + OH^- + \cdot OH$ (4)

$\cdot OH$ + lipid --------> lipid peroxides

The NADPH-microsomal system generates $O_2^{-}\cdot$ which can dismutate as in reaction 1 or it can react with H_2O_2 as in reaction 4. The later reaction generates the powerful oxidant ·OH which can continue the chain reaction to peroxidize the membrane lipids. Reactions 2 and 3 presented here are similar to Fenton's reaction (50-51). Since superoxide dismutase slightly enhanced the lipid peroxidation and catalase inhibited the reaction, we propose that reduced cytochrome P450 would form a complex, superoxoferricheme (Fe^{2+}-O_2 ----> Fe^{3+}--$O_2^{-}\cdot$) by reacting wih molecular oxygen and that a small part of this complex will dissociate to form Fe^{3+} and $O_2^{-}\cdot$ (52). $O_2^{-}\cdot$ so generated can form H_2O_2 catalyzed by superoxide dismutase (Reaction 1). Here superoxide dismutase should enhance lipid peroxidation by forming H_2O_2 and catalase would inhibit the reaction by preventing the formation of ·OH (Reaction 3). Peroxidation of membrane lipids by ·OH then would be initiated by hydrogen abstraction from a methylene carbon cation. That the hydroxyl radical scavengers such as thiourea, ethanol and DMPO inhibited lipid peroxidation is strong evidence that the reactive species, regardless of how it is generated, must be ·OH.

The fact that lidocaine, quinidine and procainamide inhibited the lipid peroxidation as well as scavenged the ·OH, generated in a chemical reaction, suggests that these drugs protect the membrane lipids by scavenging ·OH. However, it does not conclusively establish a cause-effect between these two phenomena. Two points need to be considered: (1) the concentration dependence for inhibition of lipid peroxidation by these drugs is not paralleled

by a similar concentration dependence for scavanging hydroxyl radical and, (2) it is possible that the inhibition of lipid peroxidation could be due to the ability of these drugs to interact directly with lipids thus indirectly protecting the membrane from ·OH attack. The first phenomenon may be explained by the fact that lidocaine is known to be further metabolized in the body rapidly and extensively into compounds (monoethylglycylxylidide and glycylxylidide) which have antiarrythmic activities (53); it is possible that in our microsomal-NADPH system these metabolic products are produced and are better antioxidants than lidocaine itself. Further investigation is needed to resolve this issue. To lessen the possibility of the second issue, a rather subtle artifact, we have added stearic acid to the lipid peroxidation reaction mixture to examine if addition of a saturated lipid diminishes the effectiveness of these drugs. In a similar reaction system the activity of these drugs(500 μM) remained unchanged in the presence of 500 μM stearic acid (data not presented). These results strongly suggest that the "membrane stabilization" effects of these drugs may, in part, be due to their ability to remove the reactive oxygen species

Table I. Effect of oxygen radical scavengers on MDA formation in bovine lung microsomes

Scavengers	nmoles/mg protein/min (% Control)
None	100
Ethanol, 10 mM	39
Thiourea, 10 mM	0
Mannitol, 10 mM	120
Benzoate, 10 mM	130
DMPO, 10 mM	0
Catalase, 5000 Units/ml	19
Superoxide dismutase, 10 µg/ml	110

The reaction mixture contained 3 mg of microsomal protein in Tris-HCl buffer pH 7.6, 200 µM NADPH, 1 mM ADP and 50 µM $FeCl_3$. Various scavengers at indicated concentrations were added to this reaction mixture and incubated for 10 minutes at 37^0C. Lipid peroxidation was initiated by the addition of NADPH and measured as described in "Materials and Methods".

Table II **Effect of antiarrhythmic drugs on cytochrome P-450 reductase activity.**

	Cytochrome P-450 reductase, units/mg protein/min		
Drug, (μM)	Lidocaine	Quinidine	Procainamide
25	14.83	16.58	15.41
50	14.80	15.36	15.03
100	14.84	16.33	15.26
200	13.41	12.94	13.59
500	15.41	16.20	15.98

NADPH Cytochrome P-450 reductase activity was measured by its cytochrome c reductase activity. One unit of NADPH-Cytochrome c reductase is defined as one μmole ferricytochrome c reduced per minute per mg protein. The reaction conditions were as described in the methods section.

Table III. Effects of antiarrhythmic drugs on methylene blue sensitized singlet oxygen generating system.

Concentration of Drugs	Percent Inhibition		
	Lidocaine	Quinidine	Procainamide
2 mM		35	0	0
5 mM		52	0	0
7 mM		70	0	0
10 mM		83	0	0

Experimental conditions are as described under "Materials and Methods". Lidocaine, quinidine and procainamide at indicated concentrations were added to 600μM methylene blue, 65 mM TEMP and 0.05 M potassium phosphate buffer, pH 7.8 with 10^{-4} M EDTA. The TEMPO formation was recorded after irradiating the mixture for 5 min. The percent inhibition was calculated from the intensity of the first signal.

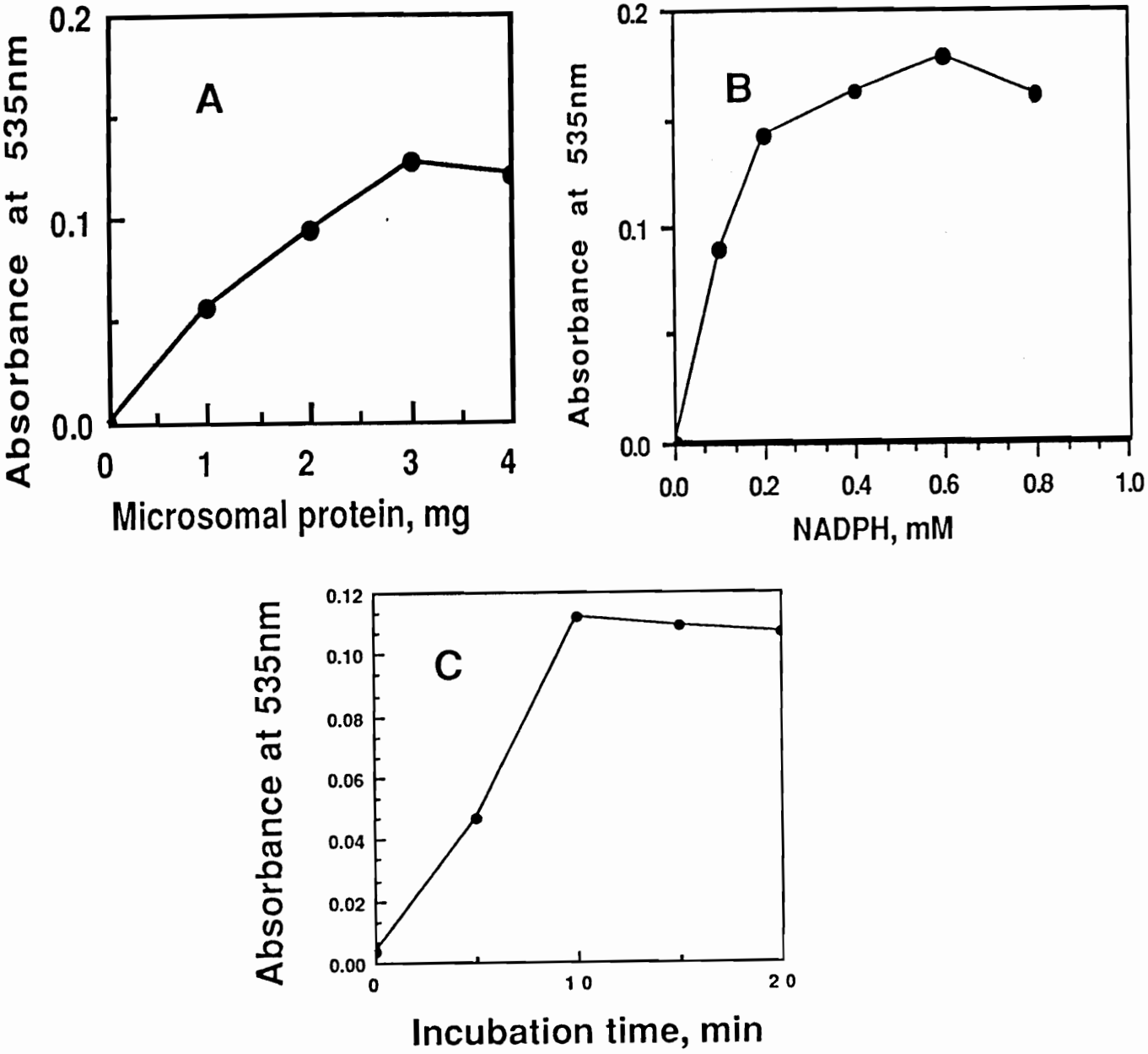


Fig 1. Optimization of NADPH-dependent lipid peroxidation assay. The experimental conditions are as described in "Materials and Methods". The reaction mixture contained 1 mM ADP, 50 μM $FeCl_3$, in Tris-HCl buffer, pH 7.6, in the presence of **(A):** indicated concentrations of microsomal protein, **(B):** varing concentrations of NADPH and **(C):** incubated for varying time periods at 37^0C. Lipid peroxidation was initiated by addition of NADPH and was terminated by the addition of 2 ml of 0.5% (w/v) TBA and 2% TCA. This mixture was heated at 95^0C for 10 minutes.

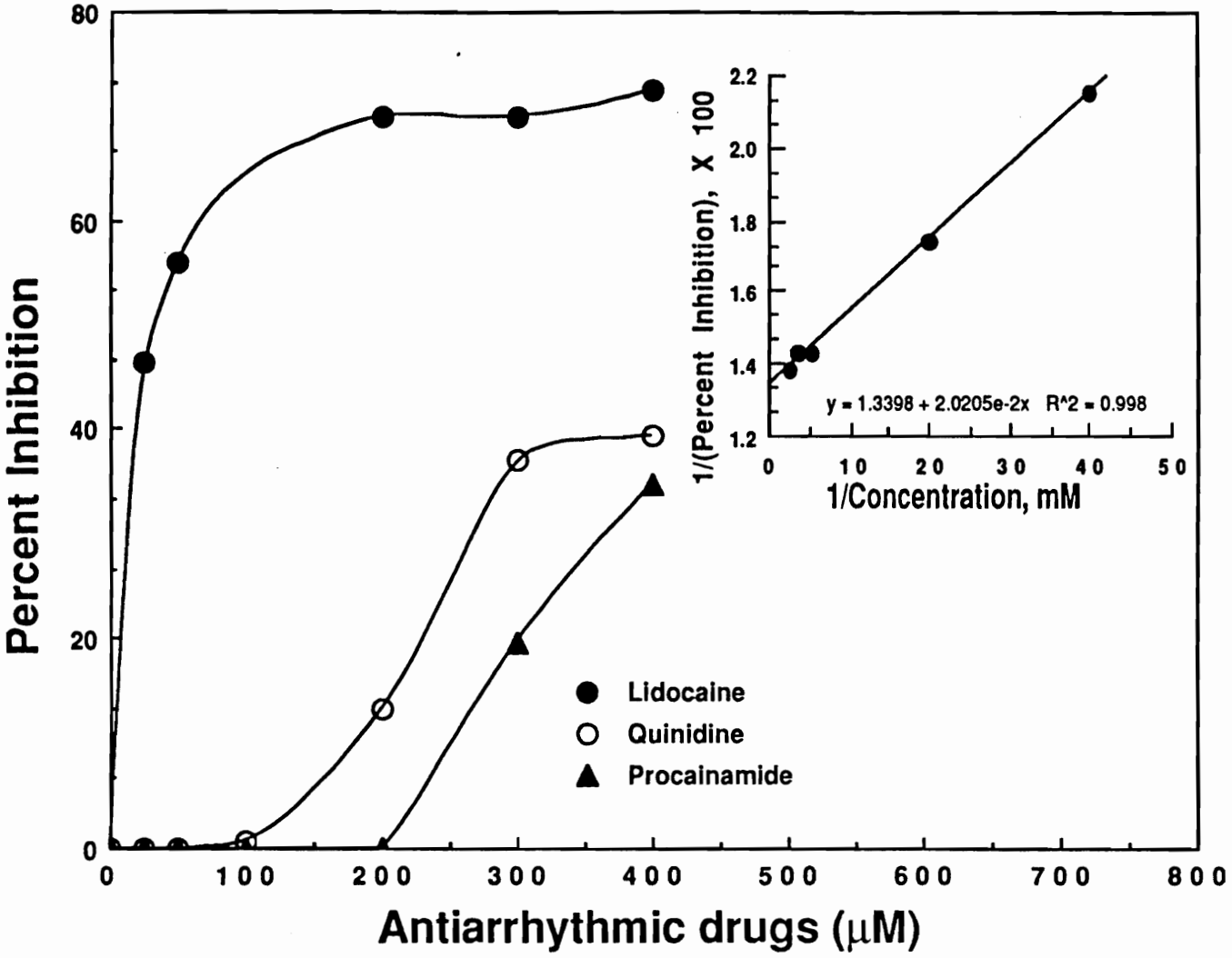


Fig 2 Effect of antiarrhythmic drugs (lidocaine, quinidine and procainamide) on NADPH dependent lipid peroxidation. The experimental conditions are as described in "Materials and Methods". The reaction mixture contained 3mg/ml of microsomal proteins, 200 μM NADPH, 1 mM ADP, 50 μM $FeCl_3$, and the indicated concentrations of antiarrhythmnic drugs in Tris-HCl buffer, pH 7.6 and was incubated at 37^0C for 10 min. Lipid peroxidation was initiated by addition of NADPH and was terminated by the addition of 2 ml of 0.5% (w/v) TBA and 2% TCA. This mixture was heated at 95^0C for 10 minutes.

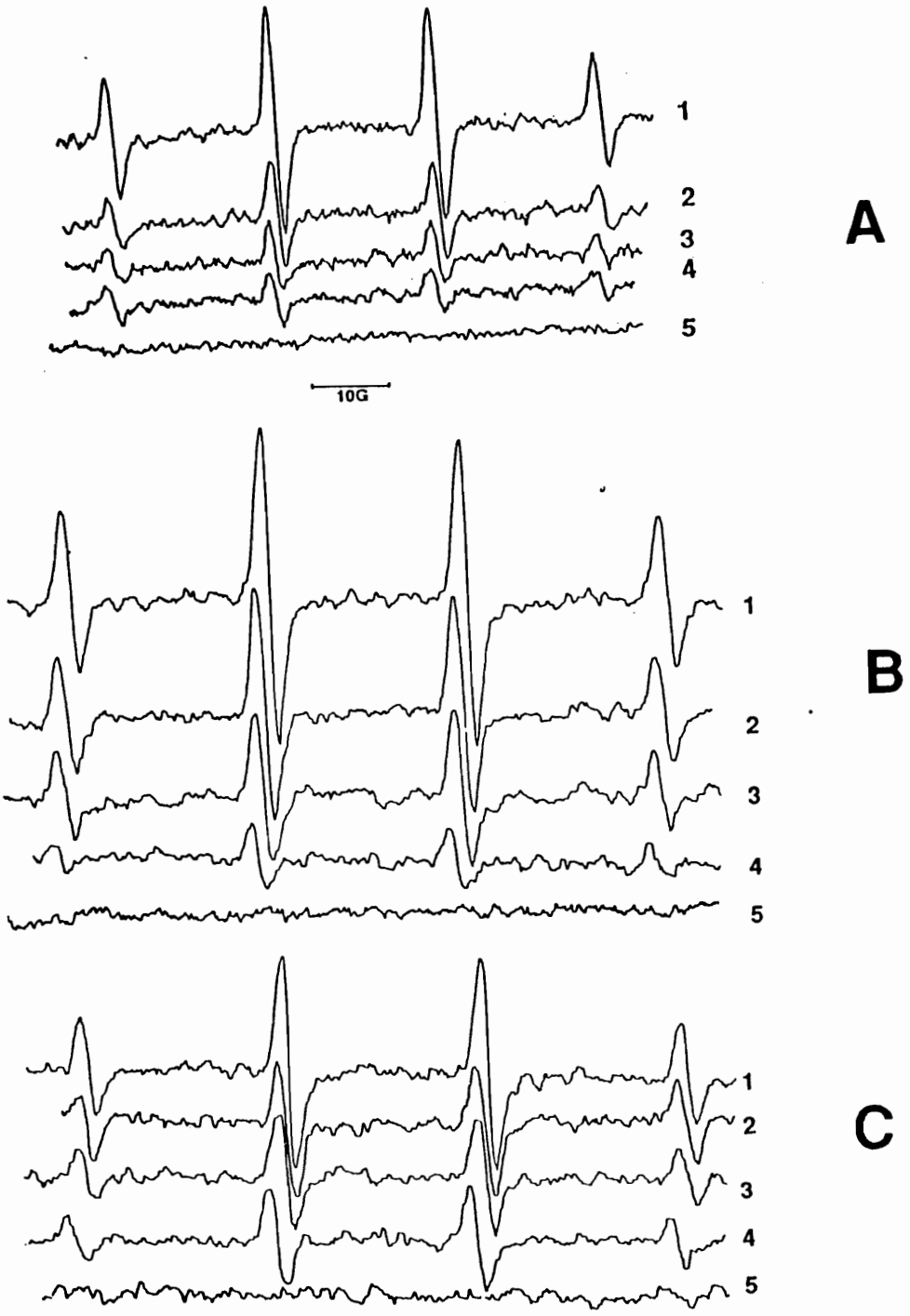


Fig 3. Effect of antiarrhythmic agents on the formation of DMPO-OH adduct. The experimental conditions are as described under "Materials and Methods". The reaction mixture contained: 31 μM H_2O_2, 1.12 mM DMPO, in boric acid-borax buffer, pH 7.8, in the presence of 33.2 μM ferrous sulfate and 0.83 mM EDTA. The DMPO-OH adduct formation was recorded immediately. Receiver gain was 4.0X 10^5, and the scan rate of 200 seconds. Other EPR parameters were same as described in "Materials and Methods". **A:** Lidocaine, Line 1: control , Line 2: 100 μM lidocaine; Line 3: 200 μM lidocaine; Line 4: 300 μM lidocaine; Line 5: 1.2 mM DMPO only; **B:** Quinidine, Line 1 control; Line 2: 50 μM quinidine; Line 3: 100 μM quinidine; Line 4: 200 μM quinidine; Line 5: 1.2 mM DMPO only **C:** Procainamide, Line 1: control; Line 2: 100 μM procainamide; Line 3: 200 μM procainamide; Line 4: 400 μM procainamide; Line 5: 1.2 mM DMPO only.

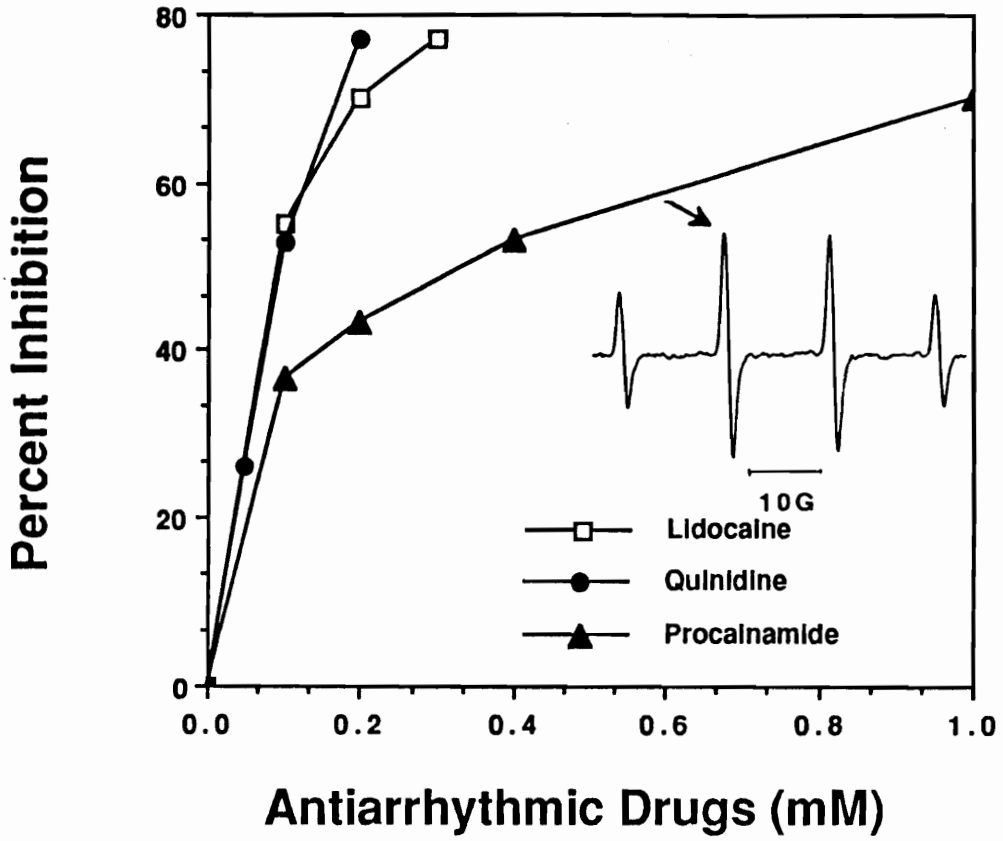


Fig 3. D: Percent inhibition of DMPO-OH adduct signal intensity byindicatedconcentration of antiarrhythmic drugs.

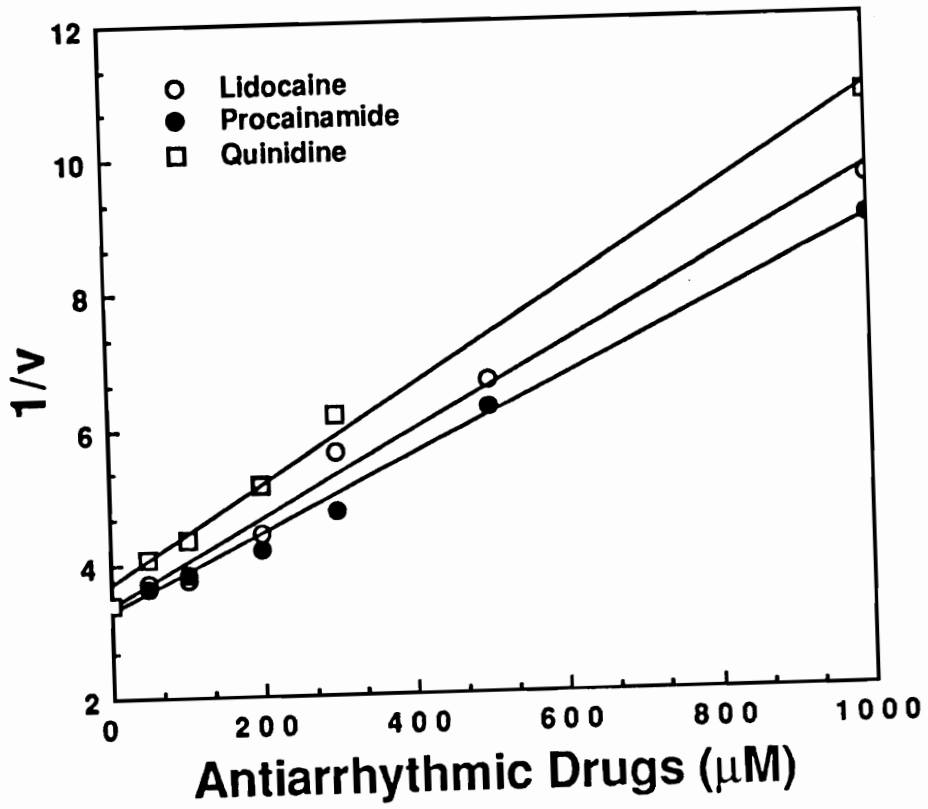


Fig 4. Hydroxyl radical scavenging by antiarrhythmic drugs: determination of rate constants. Deoxyribose degradation in the presence of various concentrations of antiarrhythmic drugs was followed as described under "Materials and Methods" using a final deoxyribose concentration of 2.8 mM in the reaction mixture. The rate constant was determined form the slope of the line (k = slope X k_{DR} X [DR] X A) as described in the text giving the value of lidocaine, 1.6 X 10^{10} $M^{-1}s^{-1}$; quinidine, 1.8 X 10^{10} $M^{-1}s^{-1}$; procainamide 1.45 X 10^{10} M^{-1} s^{-1} in this experiment

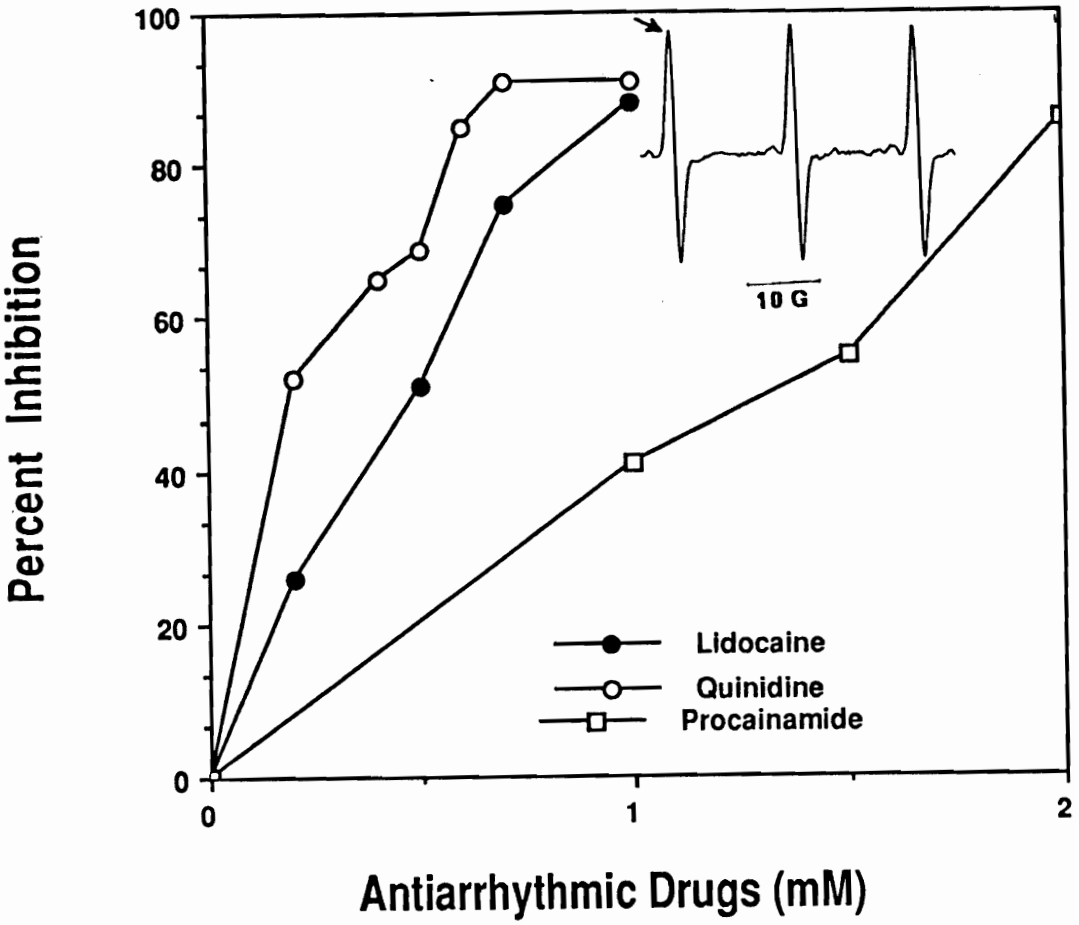


Fig 5 Effect of antiarrhythmic drugs on the formation of TEMP-$^1O_2$ adduct. The experimantal conditons are as described under "Materials and Methods". Lidocaine, quinidine and procainamide at indicated concentrations were added to 40 μM rose bengal, 65 mM TEMP dissolved in ethanol (final concentration of ethanol was 10%), in 0.05M potassium phosphate buffer, pH 7.8, with 10^{-4} EDTA. The TEMP-$^1O_2$ adduct formation was recorded after irradiating the reaction mixture for 3 minutes. The percent inhibition was calculated from the signal intensity of EPR signal indicated by an arrow.

References

1. Stelzner, T. J., Welsh, C.H., Berger, E., McCullough, R.G., Morris, K., Repine, J.E., and Weil, J.V. (1987) *J. Appl. Physiol.* **63** ,1877-1883
2. Guarnieri, C., Flamigni F,. Caldarera, C.M. (1980) *J. Mol. Cell. Cardiol.* **12,** 797-808
3. McCord, J.M. (1985) *N. Eng. J. Med.*, **312,** 159-163
4. Misra, H.P, Weglicki, W.B., Abdula, R. and Mc Cay, P.B. (1984) *Circulation*, **70**, II-260
5. Carmen, M.A., Kramer, J.H., Benjamin, F.D. and Weglicki, W.B. (1987) *FEBS Lett* **221,** 101-104
6. Kako, K.J.(1987) *J. Mol. Cell. Cardiol.* **19,** 209-211
7. Gauduel, Y. and Duvelleray, M.A. (1984) *J. Mol. Cell. Cardiol.* **16,** 458-470
8. Meerson, F. Z., Kagan, V.E., Kuzlov, Yu. P., Belkina, L.M and Arkhipenko, Yu.V (1982) *Basic Res. Cardiol.* **77,** 465-485
9. Rao, P.S. and Mueller, H.S. (1983) in *Myocardial injury* (Spitzer,J.J. Ed), pp341-367, Plenum Publishing Co, New York, 1983.
10. . Shlafer, M., Kane, P.F. and Kirsh, M.M. (1982) *J. Thorac. Cardiovasc. Surg* **83,** 830-839
11. Martin, D., Allison, R.C. and Taylor, A.E.(1986) in *Physiology of oxygen radicals* (Taylor, A.E., Matalon, S and Wand,P.A.,Eds) pp. 131-143, American Physiological Society, Bethesda, Maryland.
12. Kennedy, T.P., Rao, N.V., Hopkins, C., Pennington, L., Tolley, E., and Hoidal, J.R (1989) *J. Clin. Invest.* **83,** 1326-1335

13. Freeman, B.A., Panus, P., Beckman, J.S., Matalan, S. (1986) in *The role of oxygen radicals in cardiovascular diseases* (L'Abbatt, A. and Ursini, F. Eds.)pp 201-215, Kluwer Academic Press, London.

14. Halliwell, B., Gutteridge, J. M. C. and Aruoma, O. I. (1978) *Anal. Biochem.* **165**, 215-219

15. Moorehouse, C.P., Grootveld, M., Halliwell, B., Quinlan, G. J. and Gutteridge J.M.C. (1987) *FEBS Lett.* **213,** 23-28

16. Akanmu, D., Cecchini, R., Aruoma, O.I. and Halliwell, B. (1991) *Arch. Biochem Biophysic.* **288,** 10-16

17. Bradford, M. (1976) *Anal. Biochem.* **72,** 248-254.

18. Bernheim, F., Bernheim, M.L.C. and Wilbur, K.M. (1948) *J. Biol. Chem.* **174**, 257-264

19. Wills, E.D. (1969) *Biochem. J.* **313,** 315-324

20. Philips, A.H. and Langdon, R.G.(1962) *J. Biol. Chem.* **237,** 2652-2660

21. Hartenstein, J. and Wagner, B. (1986) in *Cardiovascular drugs* (Bristol, J. A., Ed pp. 331-333, John Wiley & Sons, New York.

22. Thurman, R.G., Ley, H.G., and Scholz, R. (1972) *Eur. J. Biochem.* **25,** 420-430

23. Boveris, A., Oshino,N., and Chance, B. (1972) *Biochem.J.* **128,** 617-630

24. Misra, H.P. and Gorsky, L.D.(1981) *J. Biol. Chem.* **256,** 9994-9998

25.Cohen, G., and Cederbaum, A.I.(1980) *Arch. Biochem. Biophys.* **199,** 438-447

26. Burton, R.M. (1955) *Methods in Enzymol.* **3,** 246-248

27. Kukiela, E. and Cederbaum, A.I. (1986) *Arch. Biochem. Biophys..* **275,** 540-550

28. McCord, J.M. and Fridovich, I. (1968) *J. Biol. Chem.* **243,** 5753-5760

29. Zang, L, Zhang, Z and Misra H.P. (1990) *Photochem.Photobiol.* **52,** 677- 684

30. Misra, B.R. and Misra, H.P. (1990)*J. Biol. Chem.* **265,** 15371-15347

31. Interian, A., Jr., Zaman, L., Velez-Robinson, E., Kozlovskis, P.,Castellanos, A., Myerburg, R.J. (1991) *J. Am. Coll. Cardiol.* **17,** 1581-1586

32. Undrovinas, A.I., Burnashev, N., Eroshenko,D., Fleidervish,I., Starmer,C.F., Makielski,J.C. and Rosenshtraukh, L.V.(1990) *Am. J. Physiol.* **259,** H1609-12

33.Schaub, R.G., Lemole, M.G., Pinder, G.C., Black, P. (1977) *J. Thorac. Cardiovasc. Surg.* **74,** 571-576

34. Das, K.C. and Misra, H.P. (1992) *Mol. Cell. Biochem*, in press

35. Woodward, B., Zakaria, M.(1984)*J. Mol. Cell. Cardiol.* **16,** 497-517

36. Manning, A.S., Coltart, J.D. and Hearse, D.J. (1984) *Circ. Res.* **55,** 545-550

37. Mak, T.I., and Wegliki, W.B. (1988) *Circ Res.* **63,** 262-266

38.Yamamoto, Y. and Kamiya, Y. (1988) in *Lipid Peroxidation in Biological Systems* (Sevanian, A. Ed) pp 32-50, American Oil Chemists Society, Champaign Illinois.1988.

39.Svingen, A.S., O' Neal, F.O. and Aust, S.D. (1978) *Photochem. Photobiol.* **28**, 803-809

40. Cohen, G. (1978) *Photochem. Photobiol.* **28,** 669-675

41. Zweier, J.L., Flaherty, J.T., and Weisfeldt, M.L. (1987)*Proc. Natl. Acad. Sci.* **84**, 1404-1407

42. Bernier, M., Hearse, J.D., Manning, A.S., (1986) *Circ. Res.* **58,** 331-340.

43.Neta, P. and Dorfman, L. M. (1968) *Adv. Chem. Ser.* **81,** 222-230

44.Puntarulo, S. and Cederbaum, A.I. (1988) *Arch. Biochem. Biophys.* **264,**482-491

45.King, M. M., Lai, E.K., and McCay, P.B.(1975) *J. Biol. Chem.* **250,** 6496-6502

46.Lai, C.S. and Piette, L.H. (1978) *Arch. Biochem. Biophys.* **190,** 27-38

47.Lai, C.S. and Piette, L.H. (1977) *Biochem. Biophys. Res. Commun.* **78,** 51-59

48.Kronbrust, D.J and Maris, R.D., (1980), *Molec. Pharmac.* **17,** 400-407

49.Svingen, B.A. and Aust, S.D. (1980) in *Molecular basis of environmental toxicity* (Bhatnagar, R.S., Ed) pp. 69-110, Ann Arbor Science Publishing, Ann Arbor, M

50. Fenton, H. J (1895) Proc. Chem. Soc. (London) **9,** 113

51. Weiss, J. J. (1964) Nature **203,** 183

52. Misra, H. P. and Fridovich, I. (1972) *J. Biol. Chem.* **247,** 6960-6962.

53. Burney, R.G., Difazio, C.A., Peach, J.J., Petrie, K.A. and Silvester, M.J. (1974) *Am. Heart J.* **88,** 765-769

CHAPTER-IV

AMELIORATION OF POST-ISCHEMIC REPERFUSION INJURY BY ANTIARRHYTHMIC DRUGS IN ISOLATED PERFUSED RAT LUNG.

(Submitted for publication in the American Journal of Physiology)

Abstract

Antiarrhythmic drugs, such as lidocaine, quinidine and procainamide, have been shown to be effective against post-ischemic reperfusion injury in isolated rat lungs. Rat lungs were perfused at constant flow with Krebs-Henseilet buffer supplemented with 4% bovine serum albumin and ventilated with air containing 5% CO_2. The lungs were subjected to ischemia by stopping perfusion and ventilation for 60 minutes followed by a 30 minutes of reperfusion. Lung injury was determined by measuring increased wet-to-dry lung weight ratio, pulmonary arterial pressure and peak airway pressure. Lidocaine, quinidine and procainamide at doses of 5, 10 and 20 mg/kg b.w. were found to attenuate the post-ischemic lung injury significantly ($p< 0.0001$). The formation of cyclooxygenase products which were elevated during reperfusion were also significantly ($p< 0.0001$) inhibited by these drugs. Since these antiarrhythmic agents are found to be powerful scavengers of hydroxyl radical and can prevent membrane lipid peroxidation (Das and Misra, 1992), the above findings suggest that the antioxidant properties of these drugs may, in part, be responsible for protecting the lungs against reperfusion injury.

Introduction

Lung transplant is rapidly becoming a clinical alternative for patients with end-stage lung disease. Preservation of the cadaver lung during transplant has been a major impediment to the wide clinical use of transplant procedures. Reperfusion of transplanted lung previously subjected to brief period of ischemia causes irreversible tissue injury (11). Ischemia-reperfusion injury has been extensively studied in many organs, including brain, heart, intestine and kidney (7, 20, 21, 23, 25, 26). Little work has been done to elucidate the mechanism of reperfusion injury in the lung. Reactive oxygen species has been implicated in the etiology of ischemia-reperfusion injury (14, 18, 20, 29). Antiarrhythmic drugs have been used as membrane stabilizers and were found to prevent microvascular permeability resulting from acute lung injury (27). The mechanism by which these antiarrhythmic agents diminish lung edema, however, is unclear and their ability to reduce post-ischemic reperfusion injury of the lung has not been tested. Recently we have reported that antiarrhythmic agents such as lidocaine, quinidine and procainamide, are potent hydroxyl radical scavengers and were found to inhibit lipid peroxidation (6). Since free radicals of oxygen play an important role in initiating lipid peroxidation (3, 9), and lipid peroxides are known to be produced during post-ischemic reperfusion of lung (8), we developed the hypothesis that antiarrhythmic drugs may protect lungs from reperfusion injury. Here we present evidence that the antiarrhythmic drugs, such as lidocaine, quinidine and procainamide attenuate post-ischemic reperfusion injury in isolated perfused rat lung and these drugs were effective in preventing the accumulation of cyclooxygenase products during reperfusion of ischemic lung.

Materials and Methods

Ex vivo lung preparation

Male Sprague-Dawley rats (Harlan's Sprague-Dawley) weighing 300-500 gms, were anesthetized with 64.8 mg/kg ip sodium pentobarbital (Anthony products Co., Arcadia,CA). A tracheostomy was performed that permitted ventilation with a Harvard rodent ventilator (Model 683) at 62 strokes per min, a tidal volume of 2.3 to 3ml, and positive end expiratory pressure of 2.5 cm H_2O. The inspired gas mixture was air mixed with 5% CO_2 (Analyzed, Industrial gas Co.). Subsequently a median sternotomy was performed, heparin (200 IU) was injected into the right ventricle, and cannulas were placed in the pulmonary artery and left ventricle. The heart, lungs and mediastinal structures were removed *en bloc* and suspended from a Fort-250 (World Precision Instruments, New Haven, CT) rigid linear force transducer to monitor any weight change and placed in a humidified chamber. The lungs were perfused by a masterflex (Cole Parmer Instruments) pump with Krebs-Hanseilet buffer at a constant flow of 0.05ml/min/gm of b.w.. The Krebs-Hanseilet buffer contained (in mM) 118 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.18 KH_2PO_4, 1.90 $CaCl_2$, 11.1 glucose and 4% bovine serum albumin (66000 MW, Sigma Chemical Co, St Louis, MO). The pH of the perfusate was maintained between 7.35 - 7.45 by periodic addition of sodium bicarbonate and was constantly monitored.

The first 50 ml of lung effluents were discarded to eliminate circulating blood elements from the vascular space of the lung. Subsequently a recirculating mode was established with 50 ml of perfusate. Pulmonary artery

pressure was constantly monitored with a transducer blood pressure BPLR-0111 (World Precision Instruments., WPI). Peak air way pressure was constantly monitored by a PNEU-01 (WPI) pressure transducer. The pressure transducers were calibrated with a blood pressure measurement instrument (The Lumiscope, Co. Japan).

Mean PA pressures, change in lung weight and mean peak air way pressure were constantly monitored through the pressure and linear force transducers connected to an amplifier bridge (WPI). The bridge was connected to a MacLab-4 (WPI) which in turn was connected to a Macintosh SE computer. The data were recorded by Scope v 3.1 software for the MacLab system (WPI).

Induction of Lung ischemia

Isolated lungs were perfused for 10 minutes to ensure a stable preparation and were then subjected to ischemic injury for 60 min by stopping ventilation and perfusion. The lungs were inflated by instillating of 2 ml of gas mixture into tracheal cannula before occlusion at the start of the ischemic period. Lung inflation was done to facilitate reperfusion after ischemia. Throughout the 60 minute ischemia the lungs and perfusate were kept at 37^0C.

Lung reperfusion

Reperfusion after ischemic interval was started slowly and the flow rate was increased such that a mean pulmonary arterial pressure (Pa) of 14mm Hg was never exceeded. Within 5 minutes of the onset of the reperfusion the perfusate flow was increased to the original flow rate present before the ischemic period

(0.05ml/min/g b.w.). During reperfusion the perfusate resorvoir and the lungs were maintained at 37-38^{0}C. Lungs were reperfused for 30 min while they are ventilated with the same gas mixture.

Experimental groups

Five experimental groups were studied. The first group of 6 lungs underwent 60 minutes of ischemia followed by 30 minutes of reperfusion. Same protocol was maintained for the 2nd, 3rd, and 4th groups (n=6) with the exception that lidocaine, quinidine and procainamide at 5, 10 and 20 mg/Kg b.w., respectively were added to the perfusate. This dose was calculated using at 8% of the body weight as blood volume. The 5th group served as control and underwent no ischemia. The drugs were added to the lung perfusate at the onset of lung perfusion prior to ischemia.

Measurement of Lung injury

Wet-to-dry lung weight ratio

At the end of each experiment the left main stem bronchus was transected and the left lung was isolated for the determination of the wet-to-dry lung weight ratio. Lungs were weighed and placed in a convection oven (Model 605, Precision Scientific Inc.) at 120^{0}C and weighed daily for 3 days. Seventy two hours lung weight was reported as dry weight because no further weight loss occurred after that time.

Pulmonary artery pressure

Mean pulmonary artery pressure (Pa) was measured for 10 minutes of pre-ischemic period and entire post-ischemic period after the full flow was resumed.

Percentage change was calculated taking the difference of the mean pre- and post-ischemic pulmonary artery pressures.

Peak airway pressure

Mean peak airway pressure (Paw) was monitored for 10 minutes of pre-ischemic period and thirty minutes of post-ischemic period. Percentage change was calculated taking the difference of pre- and post-ischemic pressures.

Measurement of cyclooxygenase metabolites

TxB_2 and 6-Keto-$PGF_{1\alpha}$, the stable metabolites of TxA_2 and prostacyclin, respectively, were measured as indicators of cyclooxygenase metabolite production. Samples (1.8 ml) of pulmonary venous effluent were collected immediately before the onset of ischemia, and at 5,10, and 20 minutes of reperfusion. Time matched samples were also obtained in the un-injured controls. Measurements of TxB_2 and 6-Keto-$PGF_{1\alpha}$ were made in thromboxane B_2 [^{3}H] and 6-Keto-$PGF_{1\alpha}$[^{3}H] scintillation proximity assay (SPA) systems (Amersham) on methyl formate extracted samples in duplicate (2).

Statistical Analysis:

Values were expressed as mean±SEM. Groups were compared using one way analysis of variance and the Tukey's multiple comparison test using SAS statistical package. A p value less than 0.05 was accepted as significant.

Results

Effect of antiarrhythmic drugs on wet-to-dry lung weight ratio

Lung weight remained stable in un-injured control lungs during the 100 minutes of perfusion. Lungs subjected to ischemia-reperfusion had increased lung weight at the end of 30 minutes of reperfusion as recorded by MacLab-4 connected to a linear force transducer (data not shown). Lung wet weight to dry weight ratio, a measure of edema formation, was significantly higher ($p < 0.0001$) in ischemia-reperfused lungs compared to un-injured controls (Fig. 1). Antiarrhythmic drugs, such as lidocaine, quinidine and procainamide at single doses of 5, 10 and 20 mg/kg b.w., respectively, significantly ($p < 0.0001$) reduced the wet weight to dry weight ratios compared to ischemia-reperfused lungs (Fig. 1).

Effect on pulmonary pressure

Pulmonary artery pressure (Pa) remained stable over the 100 minutes of perfusion in the control lungs not subjected to ischemia. Lungs subjected to 60 min ischemia were allowed to reach a stable Pa within 10 min after onset of reperfusion. The Pa was found to be significantly higher ($p < 0.008$) ten min after the onset of reperfusion, compared to the pressures present in the same lung during pre-ischemia or compared to the Pa measured in the time-matched control lungs (Fig. 2). The Pa remained elevated compared to pre-ischemic values in the same lung at 10 and 30 min after the onset of reperfusion. Addition

of lidocaine, quinidine, and procainamide at 5, 10 and 20 mg/kg b.w, respectively, significantly reduced the Pa pressure of ischemia-reperfused lungs compared to untreated reperfused lungs (Fig. 2).

Effect on Peak air way pressure

Peak airway pressure (Paw) remained stable over 100 minutes of perfusion in the un-injured control lungs. Lungs subjected to ischemia and reperfusion had significantly higher ($p< 0.0001$) Paw after 30 minutes of reperfusion. Lungs subjected to ischemia-reperfusion but treated with lidocaine (5 mg/kg b.w), quinidine (10 mg/kg b.w.) and procainamide (20 mg/kg b.w) significantly ($p < 0.0001$) reduced Paw compared to the untreated ischemia reperfused lungs. These data are presented in Figure 3.

Effect of antiarrhythmic drugs on cyclooxygenase product formation

Measurements of 6-Keto-$PGF_{1\alpha}$ and TxB_2 in lung effluents collected prior to ischemia and in the time matched lung effluents from the un-injured control lungs were not different (p=not significant) (Table 1). Five minutes after reperfusion a seven fold and two fold increase in the production of cyclooxygenase metabolites, 6-Keto-$PGF_{1\alpha}$ and TxB_2 (Table 1), respectively, was observed. The time course of accumulation of these metabolites in lung effluents were studied at 5, 10, and 20 min post-ischemia. Both the 6-Keto-$PGF_{1\alpha}$ and TxB_2 levels were found to remain elevated ($p<0.0001$) in the lung effluent collected up to 20 min after reperfusion compared to effluent collected from the same lung prior to ischemia or when compared to time matched lung effluent samples from control lungs not subjected to ischemia (Fig. 4). Tweny

minutes after reperfusion, 6-Keto-PGF1α remain elavated compared to the controls (Fig. 4). TxB_2 levels were also increased ($p<0.0001$) in lung effluents collected at 5, 10 and 20 minutes after the onset of reperfusion compared to effluent collected from the same lungs prior to ischemia or from time matched control lungs (Fig. 5). Lungs subjected to ischemia-reperfusion and treated with lidocaine, quinidine and procainamide at 5, 10 and 20 mg/kg b.w, respectively, had significantly less 6-keto-PGF1α and TxB_2 ($p<0.0001$) compared to non treated lungs subjected to similar ischemia-reperfusion conditions (Table 1, Fig. 4 and Fig. 5))

Discussion

A major source of damage in post-ischemic reperfusion injury is believed to be the generation of oxygen-free radicals and other toxic oxygen metabolites (10, 19, 29). These radicals and metabolites have been implicated in post-ischemic reperfusion injury in the heart, kidney, intestine, brain and other organs (9, 19, 22, 30). Studies in the post-ischemic-reperfusion injury in the lungs also implicate toxic oxygen metabolite as a source of damage (10, 12-14). In our present study the antiarrhythmic drugs, lidocaine, quinidine and procainamide, significantly reduced pulmonary edema, pulmonary arterial pressure and peak air way pressure. These drugs also inhibited the formation of cyclooxygenase metabolites that are known to be produced during reperfusion of ischemic lungs (16). Although these agents have numerous systemic and local effects on various biological tissues both, *in vivo* and *in vitro* (1, 24), their ability to ameliorate post-ischemic reperfusion injury in the lungs had not been previously recognized.

The mechanism, by which the antiarrhythmic agents are effective against reperfusion injury may be explained, in part, by their antioxidant properties. In a recent study Stelzner et al. (27) found that these drugs protect lungs against thiourea induced injury in rats. As oxygen radicals are likely to be produced during metabolism of thiourea, Stelzner et al. suggested an antioxidant action of antiarrhythmic drugs. However, they found that these drugs are not scavengers of $O_2^{-\cdot}$ or H_2O_2. Recently we have demonstrated that these drugs are powerful hydroxyl radical scavengers and inhibitors of lipid peroxidation

(6). Therefore it is likely that these drugs protect pulmonary tissue against post-ischemic reperfusion injury by scavenging toxic ·OH radicals.

Since toxic oxygen metabolite can directly cause cell injury or lead to the production of other mediators, such as arachidonate metabolites, we measured the effect of antiarrhythmic agents on cyclooxygenase metabolites, thromboxane and prostacyclin, during reperfusion of ischemic lung. Post-ischemic reperfusion caused significant elevation of both the thromboxane and prostacyclin levels as measured by their stable metabolites TxB_2 and 6-Keto-$PGF_{1\alpha}$. Similar increases of these metabolites have been observed by Ljungman et al. (16). It is believed that cyclooxygenase metabolites, produced secondary to the production of oxygen radicals, may in turn, cause tissue damage (16). These authors have, however, concluded that cyclooxygenase metabolites may not be the sole source of injury because protection by inhibitors of cyclooxygenase product formation was not complete. Burghuber et al. (4) had also reported similar findings and concluded that cyclooxygenase products are not primarily responsible for lung damage. It has been shown that lidocaine is not an inhibitor of prostglandin biosynthesis in vitro (15), rather it increases the production of prostacyclin (5). Our data demonstrate that all these antiarrhythmic drugs inhibit cyclooxygenase product formation. It is possible that these drugs may not inhibit prostaglandin formation *per se* but might inhibit the liberation of arachidonic acid from phospholipids by scavenging hydroxyl radicals. However, this hypothesis needs to be tested. Nevertheless, the lung damage imposed by ischemia-reperfusion could be a concommitant actions of both the toxic oxygen products (12) and cyclooxygenase metabolites (16). We conclude that the inhibition of

cyclooxygenase product formation and attenuation of post-ischemic lung injury by these drugs may, in part, be due to the removal of toxic oxygen metabolites generated during the reperfusion of the lung.

Table 1.

Effect of antiarrhythmic drugs on cyclooxygenase product formation in isolated rat lung subjected to ischemia-reperfusion

	6-Keto-$PGF_{1\alpha}$ (pg/ml)		TxB_2 (pg/ml)	
	Pre-ischemia	Post-ischemia	Pre-ischemia	Post-ischemia
Control	220 ± 10	200 ± 20	58 ± 1.95	64 ± 1.47
I/R	163 ± 15	1428 ± 315*	68. ± 1.28	132 ± 9.68*
I/R+Lidocaine	205± 11	312 ± 63**	62 ± 1.68	85± 2.62**
I/R+Quinidine	250 ± 36	209 ± 73**	55 ± 1.75	63 ± 1.31**
I/R+ Procainamide	251 ± 15	282 ± 74**	55 ± 2.80	65± 1.48**

Control: lungs not subjected to ischemia but perfused for 100 min. I/R : 60 min ischemia followed by 30 min reperfusion. Lidocaine, quinidine and procainamide at doses of 5, 10 and 20 mg/kg b.w., respectively, were added to the perfusate at the begining of perfusion. Pre-ischemia: first 10 minutes of perfusion; Post-ischemia: samples taken 5 min after reperfusion.

* $p < .0001$, compared to control; ** $p < 0.001$, compared to I/R.

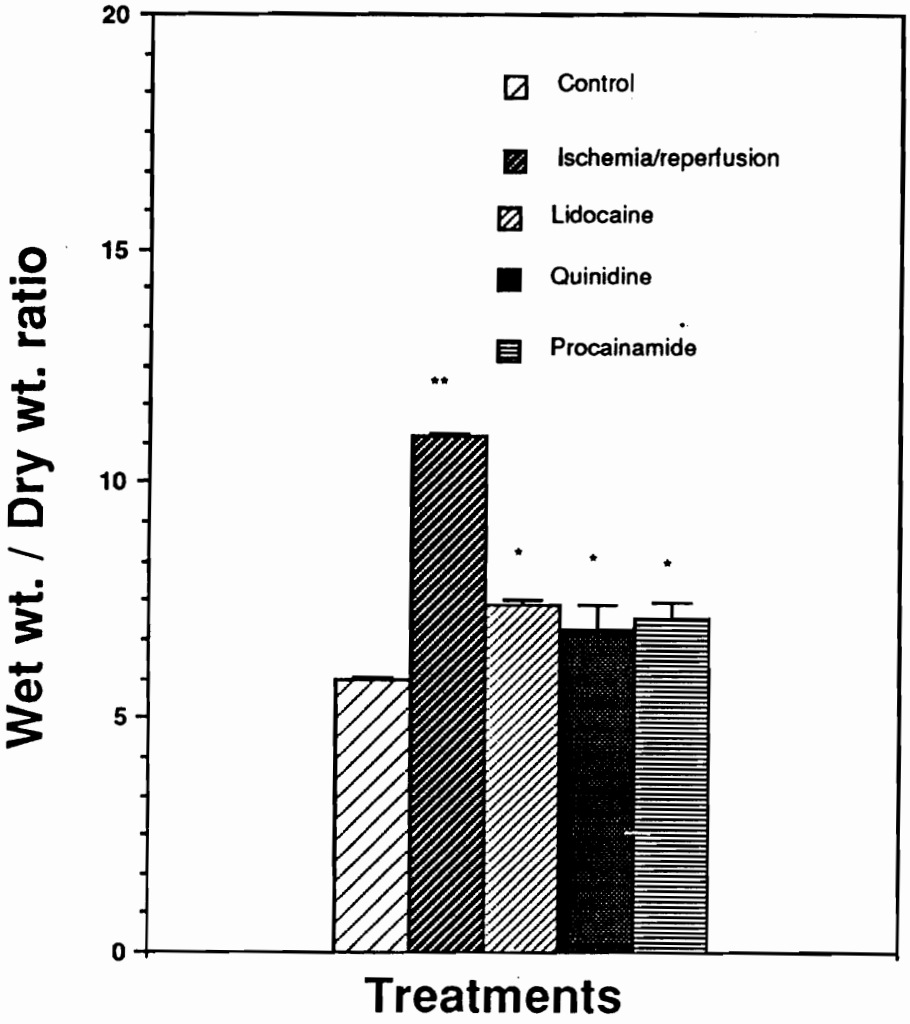


Fig. 1. Effects of antiarrhythmic drugs on wet-to-dry lung weight ratio of ischemic-reperfused rat lung. Wet-to-dry lung weight ratios (n=6) after 100 minutes of lung perfusion in experiments exposed to ischemia-reperfusion (I/R) and I/R with lidocaine, quinidine and procainamide at doses of 5, 10 and 20 mg/Kg b.w., respectively. Values are mean±SE.. *$p < .0001$ compared with ischemia-reperfusion lungs. ** $p < .001$ compared to the control.

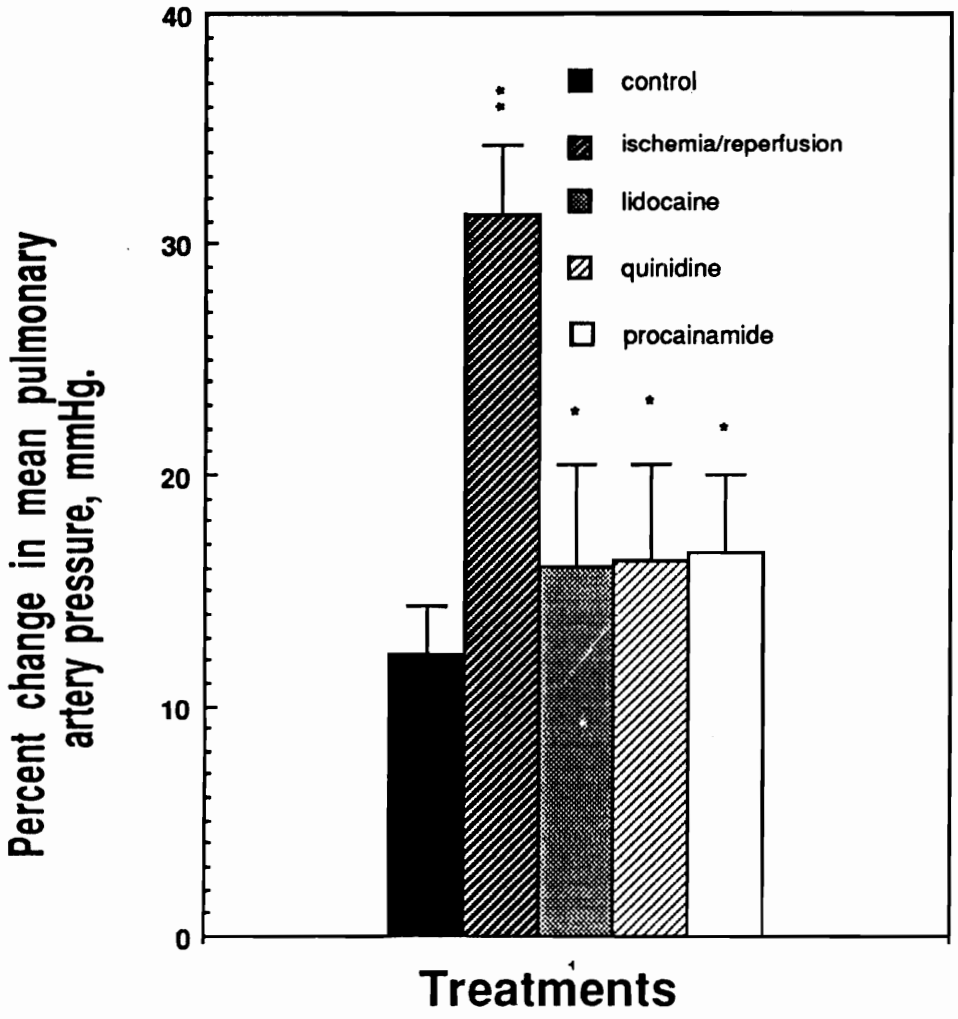


Fig. 2. Effects of antiarrhythmic drugs on pulmonary arterial pressure of Ischemic-reperfused rat lung. Un-injured control lungs were perfused for a total period of 100 min. Lungs subjected to ischemia-reperfusion underwent, 10 minutes of pre-ischemia followed by 60 minutes of ischemia and 30 minutes of reperfusion. Lidocaine, quinidine and procainamide at 5, 10 and 20 mg/kg b.w doses, respectively, were added to the perfusion buffer at the beginning of perfusion. Results were expressed as percent change in mean pulmonary arterial pressure between pre-ischemia and post-ischemic reperfusion. *$p < 0.0001$and ** $p < 0.001$ compared to the control.

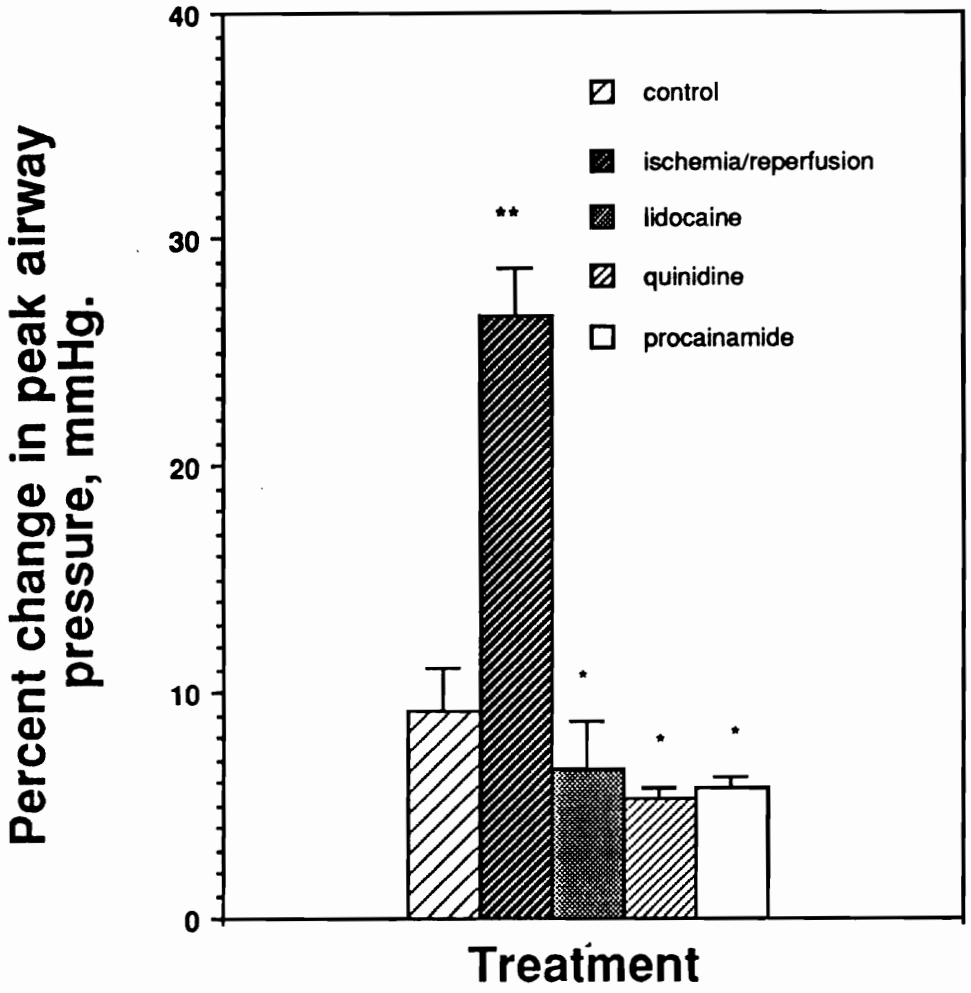


Fig. 3. Effects of antiarrhythmic drugs on peak airway pressure of ischemic-reperfused rat lung. Un-injured control lungs were perfused for a total period of 100 min. Lungs subjected to ischemia-reperfusion underwent, 10 minutes of pre-ischemia followed by 60 minutes of ischemia and 30 minutes of reperfusion. Lidocaine, quinidine and procainamide at 5, 10 and 20 mg/kg b.w. doses were added to the perfusion buffer at the beginning of perfusion. Results were expressed as percent change in mean peak airway pressure between pre-ischemia and post-ischemic reperfusion. *$p < 0.0001$ compared to control. ** $p < 0.001$ compared to ischemia reperfused lung.

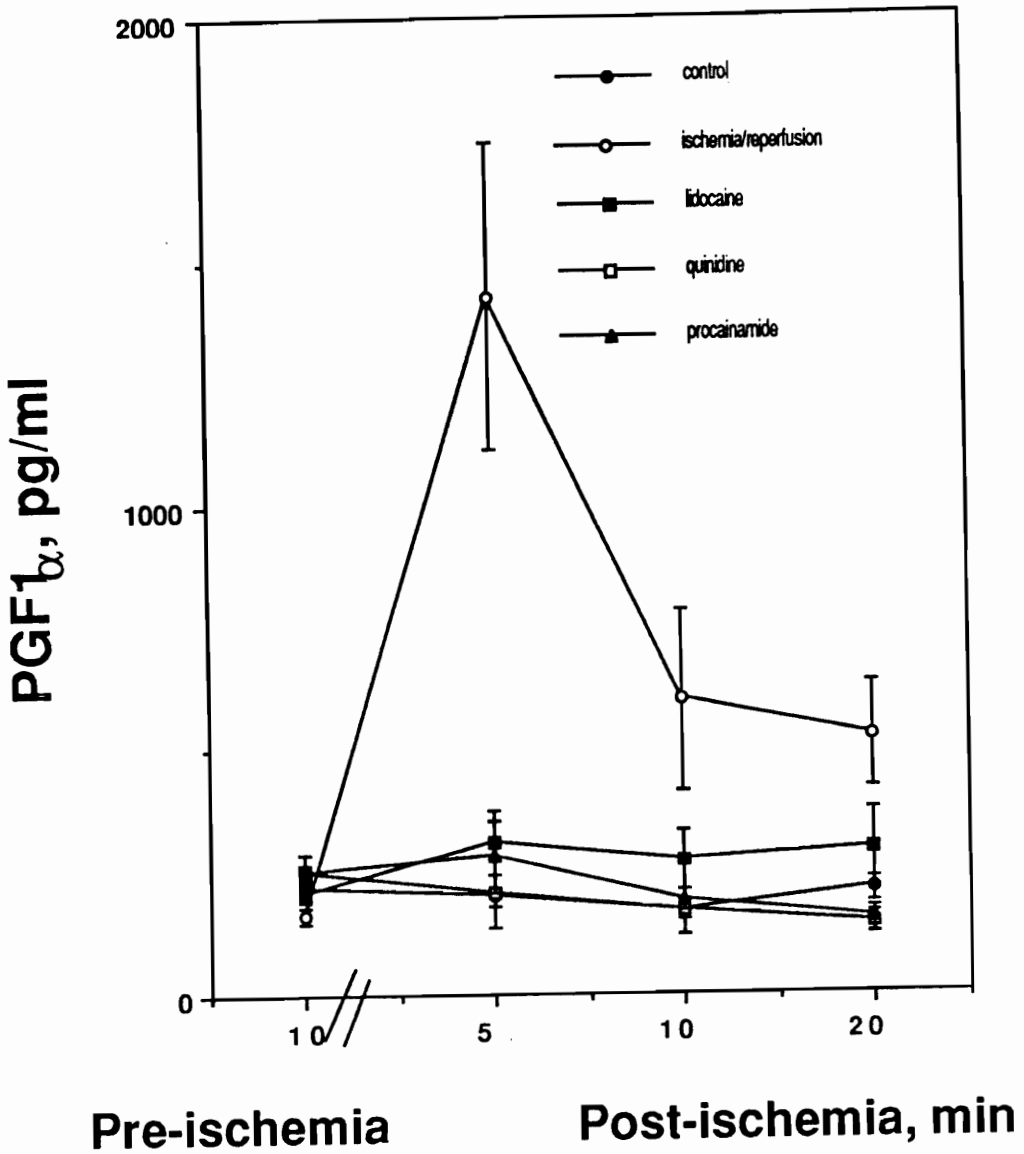


Fig. 4. Effects of antiarrhythmic drugs on 6-Keto-$PGF_{1\alpha}$ on ischemic-reperfused rat lung. Samples for 6-Keto-$PGF_{1\alpha}$ measurements were obtained prior to ischemia as well as 5, 10, 20 minutes after reperfusion or at time matched points in control lungs not subjected to ischemia. Lidocaine, quinidine and procainamide at 5, 10 and 20 mg/kg b.w. were added to the perfusate at the beginning of the perfusion period. 6-Keto-$PGF_{1\alpha}$ was measured by the scintillation proximity assay method on extracted samples in duplicate.

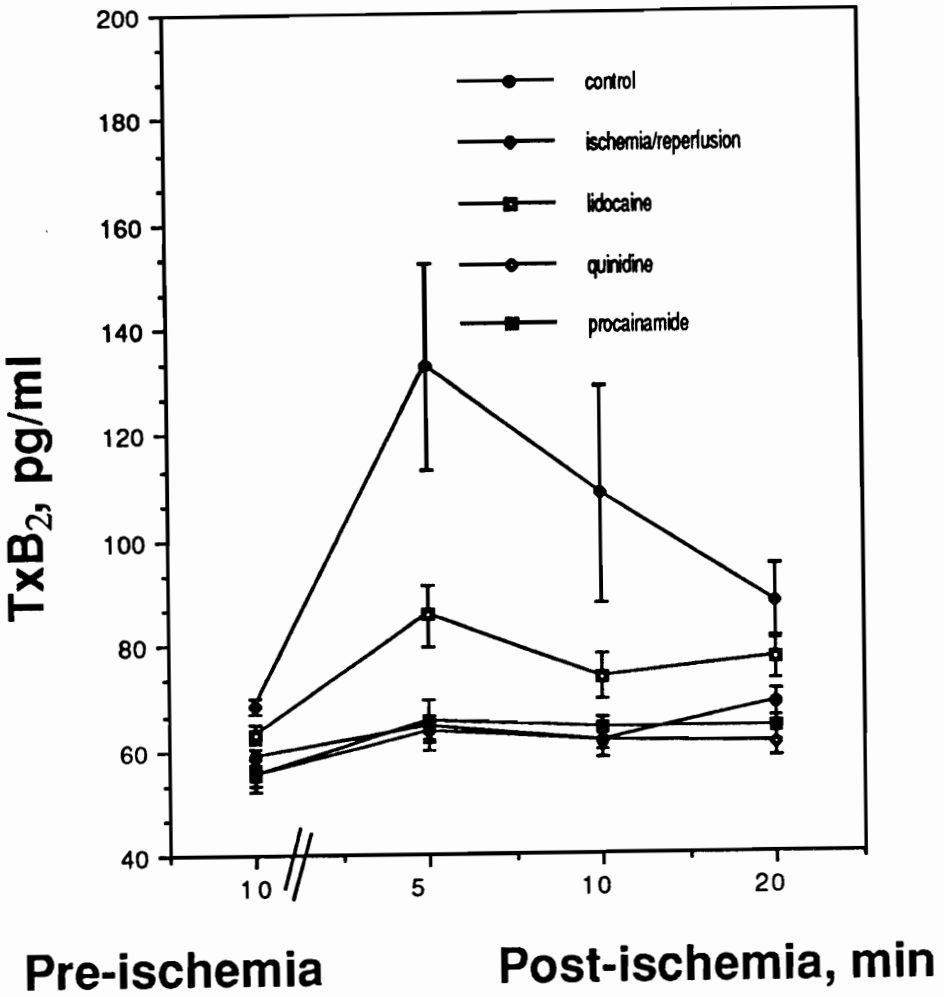


Fig. 5. Effects of antiarrhythmic drugs on TxB_2 on ischemic-reperfused rat lung. Samples for TxB_2 measurements were obtained prior to ischemia as well as 5, 10, 20 minutes after reperfusion or at time matched points in control lungs not subjected to ischemia. Lidocaine, quinidine and procainamide at 5, 10 and 20 mg/kg b.w. were added to the perfusate at the beginning of the perfusion period. TxB_2

References

1. Arnsdorf, M. F. Electrophysiologic properties of antidysrhythmic drugs as a rational basis for therapy. *Med. Clin. N. Am.* 60:213-232, 1976.
2. Bakerbond application, PH-06, Extraction of prostaglandins from serum. J.T. Baker Co.
3. Bertrand, Y. Oxygen-free radicals and lipid peroxidation in adult respiratory distress syndrome. *Intensive Care Med.* 11:56-60, 1985.
4. Burghuber, O., M. E. Mathias, I. F. McMurtry, J. T. Reeves, and N. F. Voelkel. Lung edema due to hydrogen peroxide is independent of cyclooxygenase products. *J. Appl. Physiol.* 56(4):900-905, 1984.
5. Casey, L. C., M. C. Armstrong, J. R. Fletcher, and P. W. Ramwell. Lidocaine increases prostacyclin in the rat. *Prostaglandins,* 19:976-984.
6. Das, K. C., and H. P. Misra. Antiarrhythmic agents: scavengers of hydroxyl radicals and inhibitors of NADPH-dependent lipid peroxidation in bovine lung microsomes. *J. Biol. Chem.*, in press.
7. Dempsey, R. J., M. W. Roy, K. Meyer, D.E. Cowen, and H.H. Tai. Development of cyclooxygenase and lipooxygenase metabolites of arachidonic acid after transient cerebral ischemia. *J. Neurosurg.* 64:118-124, 1986.
8. Fisher, A. B., C. Dodia, Z. Tan, I. Ayene, and R.G. Eckenhoff, Oxygen-dependent lipid peroxidation during lung ischemia. *J. Clin. Invest.* 88:674-679, 1991.
9. Granger, D. N., G. Rutili, and J. M. McCord. Role of superoxide radicals in intestinal ischemia. *Gastroenterology,* 81:22-29, 1981.

10. Granger, D. N., M. E. Hollwarth, and D. A. Parks. Ischemia-Reperfusion injury: role of oxygen derived free radicals. *Acta. Physiol. Scand. Suppl.* , 1986.

11. Haverich, A., W.C.Scott, and S.W. Jamieson. Twenty years of lung preservation: a review. *J. Heart Transplant.* 4:234-240, 1985

12. Jurmann, M.J., L. Dammenhayn, H. J. Schaefers, and A. Haverich. Pulmonary reperfusion injury: evidence for oxygen-derived free radical mediated damage and effects of different free radical scavengers. *Eur. J. cardio-thorac. Surg.* 4:665-670, 1990.

13. Kennedy, T. P., N.V. Rao, C. Hopkins, L. Pennington, E. Tolley, and J. R. Hoidal. Role of reactive oxygen species in reperfusion injury of the rabbit lung. *J. Clin. Invest.* 83:1326-1335, 1989.

14. Kennedy,T., N. Rao, C. Hopkins, E. Tolhey, and J. Hoidal. Reperfusion injury occurs in the lung by free-radical mechanism. *Chest,* 93:1495-, 1988

15. Kunze, H. E., E. Bohn, and W. Vogt. Effects of local anaesthetics on prostaglandin biosynthesis in vitro. *Biochim. Biophys. Acta.* 360:260-269, 1974.

16. Ljungman, G. A., C. M. Grum, G. M. Deeb, S. F. Bolling, and M. L. Morganroth. Inhibition of cyclooxygenase metabolite production attenuates ischemia-reperfusion lung injury. *Am. Rev. Respir. Dis.* 143:610-617, 1991.

17. Martin, D., R. C. Allison, and A. E. Taylor, (1986) in *Physiology of Oxygen Radicals* (Taylor, A.E., Matalon, S and Wand,P.A.,Eds) pp. 131-143, American Physiological Society, Bethesda, Maryland.

18. Martin, D., R. J. Korthuis, M. Perry, M. I. Townsley, and A. E. Taylor. Oxygen radical mediated lung damage associated with α– napthylthiourea. *Acta Physiol. Scand. Suppl.* 548:119-125, 1986.

19. Marubayashi, S., K. Dohi, H. Ezaki, K. Yamada, and T. Kawasaki. Preservation of ischemic liver cells prevention of damage by coenzyme Q10. *Transplant Proc.* 15:1297-1299, 1983.

20. McCord, J. M.. Oxygen-derived free radicals in post-ischemic tissue injury. *N. Engl. J. Med.* 312:159-163, 1985.

21. Otamiri, T. (1989), Oxygen radicals, lipid peroxidation, and neutrophil infiltration after small-intestinal ischemia and reperfusion. *Surgery* 105:593-597

22. Pallar, M. S. , J. R. Hoidal, and T. F. Ferris. Oxygen free radicals in ischemic acute renal failure in rats. *J. Clin. Invest.* 74:1156-1164, 1984.

23. Parks, D. A., G. B. Bulkley, D.N. Granger, S.R. Hamilton, and J. M. McCord. Ischemic injury in the small intestine: Role of superoxide radicals. *Gastroenterology* 82:9-15, 1982.

24. Scherphof, G. L., A. Scarpa, and A. Van Toorenbergen. The effects of local anesthetics on the hydrolysis of free and membrane bound phospholipids catalysed by various phospholipases. *Biochim. Biophys. Acta.* 270: 226-240, 1972.

25. Shlafer, M, P. K. Kane, M. M. Krish. Superoxide dismutase plus catalase enhances the efficacy of hypothermic cardioplegia to protect the globally ischemic reperfused heart. *J. Thoracic. Cardiovasc. Surg.* 83:830-839, 1982.

26. Simpson, P. J., R. F. Todd III, J. C. Fantone, J. K. Mickelson, J. D. Griffin and B. R. Lucchesi. Reduction of experimental canine myocardial reperfusion injury by monoclonal antibody (anti-mol, anti-CD116) that inhibits leukocyte adheson. *J. Clin. Invest.* 81:624-629, 1988.

27. Stelzner, T. J., C. H. Welsh, E. Berger, R. G. McCullough, K. Morris, J. E. Repine, and J. V. Weil. Antiarrhythmic agents diminish thiourea-induced pulmonary vascular protein leak in rats. *J. Appl. Physiol.* 63:1877-1883, 1987.

28. Tate, R. M., K. M. vanbenthuysen, D. M. Shasby, I. F. McMurtry, and J. E. Repine. Oxygen radical mediated permeability edema and vasoconstriction in isolated perfused rabbit lungs. *Am. Rev. Respir. Dis.* 126:802-806, 1982.

29. Weisfeldt, M. L. Reperfusion and reperfusion injury. *Clin. Res.* 2:311-319, 1987.

30. Zweier, J. R. Measurement of superoxide-derived free-radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. *J. Blol. Chem.* 263:1353-1357, 1988

CHAPTER-V

PREVENTION OF REPERFUSION LUNG INJURY BY LIDOCAINE IN A HYPEROXIC VENTILATED RAT LUNG MODEL

(Submitted for publication in the American Review of Respiratory Disease)

Abstract

Lidocaine, an antiarrhythmic drug has been shown to be effective against post-ischemic reperfusion injury in heart. However, its effect on pulmonary reperfusion injury has not been investigated. We investigated the effects of lidocaine on a post-ischemic reperfused rat lung model. Lungs were isolated and perfused at constant flow with Krebs-Henseilet buffer containing 4% bovine serum albumin and ventilated with 95% oxygen mixed with 5% CO_2. Lungs were subjected to ischemia by stopping perfusion for 60 minutes and then reperfused for 10 minutes. Post-ischemic reperfusion caused significantly ($p < 0.001$) higher wet-to-dry lung weight ratio, pulmonary arterial pressure and peak airway pressure than the control lungs. Lidocaine, at a dose of 5 mg/kg b.w. was found to significantly ($p<.001$) attenuate the rises in the wet-to-dry lung weight ratio, pulmonary arterial pressure and peak airway pressure observed in post-ischemic lungs. We conclude that lidocaine is effective in preventing post-ischemic reperfusion injury in isolated perfused rat lung.

Introduction

High levels of O_2 ventilation have been associated with acute lung injury (1-3). Lungs exposed to higher O_2 tension in the absence of circulation was found to produce large amounts of free radicals (4). Koyama et al. (5) have demonstrated the involvement of oxygen derived radicals in acute lung injury when canine lung lobes were ventilated with 95% O_2. In another recent study, Fisher et al.(6) have shown increased production of lipid peroxides when 95% O_2 was administered during ischemic ventilation in isolated rat lung.

Lipid peroxidation mediated by hydroxyl radicals has been suggested as a possible mechanism of pulmonary injury (7-9). These activated species of oxygen also increase pulmonary arterial pressure (10-11) and increase vascular permeability that results in the formation of a protein rich intra-alveolar edema (11-12). If these toxic species of oxygen are not properly neutralized by endogenous antioxidants, they can interact with cell membranes to generate lipid peroxides, lipid hydroperoxides, lipid endoperoxides and arachidonic acid metabolites (11). Llidocaine, is a widely used drug with significant stabilizing activity on lipid biomembranes (12). In a recent study lidocaine was found to reduce thiourea induced pulmonary vascular protein leak in rats (12). Lidocaine was also reported to reduce canine infarct size and was shown to decrease the release of conjugated diene, a marker of lipid peroxidation (13). We have recently found that lidocaine is a potent hydroxyl radical scavenger and quencher of singlet oxygen (14). Lidocaine was also found to inhibit NADPH-dependent lipid peroxidation in bovine lungs microsomes (15). Since

lipid peroxidation can be initiated by reactive species of oxygen (7-9), and lipid peroxide products accumulate in O_2 ventilated ischemic lungs (6), we developed the hypothesis that lidocaine can prevent post-ischemic reperfusion injury in O_2 ventilated lungs. We present evidence in this report that lidocaine prevents ischemia-reperfusion lung injury in isolated O_2 ventilated perfused rat lung.

Materials and Methods

Ex vivo lung preparation:

Male Sprague-Dawley rats (Harlan's Sprague-Dawley) weighing 300-500 gms, were anesthetized with 64.8 mg/kg ip sodium pentobarbital (Anthony products Co., Arcadia,CA). A tracheostomy was performed that permitted ventilation with a Harvard rodent ventilator (model 683) at 62 strokes per min, a tidal volume of 2.3 to 3ml, and positive end expiratory pressure of 2.5 cm H_2O. The inspired gas mixture was 95% oxygen with 5% CO_2 (Analyzed, Industrial gas Co,). Subsequently a median sternotomy was performed, heparin (200 IU) was injected into the right ventricle, and cannulas were placed in the pulmonary artery and left ventricle. The heart, lungs and mediastinal structures were removed *en bloc* and suspended from a Fort-250 (World Precision Instruments, New Haven, Connecticut) rigid linear force transducer to monitor any weight change and placed in the humidified chamber. The lungs were perfused by a masterflex (Cole Parmer Instruments) pump with Krebs-Henseilet buffer at a constant flow of 0.05ml/min/gm of b.w. . The Krebs-Hanseilet buffer contained (in mM) 118 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.18 KH_2PO_4, 1.90 $CaCl_2$, 11.1 glucose and 4% bovine serum albumin (66000 MW, Sigma Chemical Co, St Louis, MO). The pH of the perfusate was maintained between 7.35 - 7.45 by periodic addition of sodium bicarbonate and constantly monitored.

The first 50 ml of lung effluents were discarded to eliminate circulating rat blood elements from the vascular space of the lung. Subsequently a

recirculating mode was established with 50 ml of perfusate. Pulmonary artery pressure (Pa) was constantly monitored with a blood pressure transducer BPLR-0111 (WPI). Peak air way pressure was constantly monitored by a PNEU-01 (WPI) pressure transducer. The pressure transducers were calibrated with a blood pressure measurement instrument (The Lumiscope, Co. Japan). Mean Pa, change in lung weight and mean peak air way pressure were constantly monitored through the pressure transducer and linear force transducers, respectively, connected to an amplifier bridge (WPI). The bridge was connected to a MacLab-4 (WPI) which was in turn connected to a Macintosh SE computer. The data were recorded by Scope V3.1 software for the MacLab system (WPI).

Induction of Lung ischemia

After initiation of the closed recirculating system, isolated lungs were observed for 10 minutes to ensure a stable preparation. After a total 10 minutes of perfusion, the lungs were subjected to ischemic injury for 60 min by stopping perfusion. Ventilation was continued with 95% oxygen throughout the ischemic period. During the 60 minute ischemia the lungs and perfusate were kept at 37^0C.

Lung reperfusion

Reperfusion after ischemic interval was started slowly and the flow rate was increased such that a Pa of 14 mm Hg was not exceeded. Within 5 minutes of the onset of the reperfusion the perfusate flow was increased to the same flow rate present before the ischemic period (0.05ml/min/gm b.w.). During

reperfusion, the perfusate reservoir and the lungs were maintained at 37^{0}C. Lungs were reperfused for 10min.

Experimental groups

Three experimental groups were studied. The first group of 6 lungs underwent 60 minutes of ischemia followed by 10 minutes of reperfusion. The same protocol was maintained for the 2nd group (n=6) with the exception that lidocaine at 5mg, /kg b.w. was added to the perfusate. This dose was calculated taking the blood volume at 8% of the body weight. The 3rd group served as control and underwent no ischemia. The drug was added to the lung perfusate at the onset of lung perfusion prior to ischemia.

Measurement of Lung injury

Wet-to-dry lung weight ratio

At the end of each experiment the left main stem bronchus was transected and the left lung was isolated for the determination of the wet-to-dry lung weight ratio. Lungs were weighed and placed in a convection oven (Model 605, Precision Scientific Inc.) at 120^{0}C and weighed daily for 3 days. Seventy two hours lung weight was reported as dry weight because after this time no further weight loss occurred.

Pulmonary artery pressure

Mean Pa was measured for 10 minutes of pre-ischemic period and entire post ischemic period after the full flow was resumed. Percentage change was calculated taking the difference of the mean pre- and post-ischemic pulmonary artery pressure.

Peak airway pressure

Mean peak airway pressure (Paw) was monitored for 10 minutes of pre-ischemic period and entire post-ischemic period. Percent change was calculated taking the pre- and post-ischemic pressure differences.

Statistical Analysis

Values were expressed as mean±SEM. Groups were compared using one way analysis of variance and the Tukey's multiple comparison test using SAS statistical package. A p value less than 0.05 was accepted as significant.

Results

Effect of lidocaine on wet-to-dry lung weight ratio

Lung weight remained stable in control lungs during the 70 minutes of perfusion. Lungs subjected to ischemia and reperfusion increased in weight by the end of 10 minutes of reperfusion as recorded in the MacLab-4 (data not shown). Lung wet-to-dry weight ratio was significantly higher ($p< 0.001$) in ischemic reperfused lungs compared to controls (Fig. 1). Lungs subjected to ischemia-reperfusion but treated with lidocaine at 5mg/kg b.w. had significantly lower wet-to-dry weight ratios compared to untreated ischemia-reperfused lungs (Fig. 1).

Effect on pulmonary artery pressure

The Pa remained stable over the 70 minutes of perfusion in the control lungs not subjected to ischemia. Lungs subjected to 60 min ischemia reached a stable Pa within 10 minutes after the onset of reperfusion. The Pa was found to be significantly higher ($p < 0.001$) ten minutes after the onset of reperfusion, compared to the pressures observed in the same lung pre-ischemia or compared to the Pa measured in the time-matched control lungs (Fig. 2). The Pa remained elevated compared to pre-ischemic values in the same lung at 10 minutes after the onset of reperfusion. Lidocaine treatment (5mg/kg b.w.) significantly attenuated increase in the Pa observed in post -ischemic O_2 ventilated reperfused lungs (Fig. 2).

Effect on Peak air way pressure

Paw remained stable over 70 minutes of perfusion in the control group. In lungs subjected to 60 min ischemia, Paw was significantly ($p < 0.001$) higher (Fig. 3) at 10 minutes of reperfusion. There was no difference in Paw of control lungs and lungs subjected to ischemia-reperfusion but treated with 5mg/kg b.w. lidocaine.

Discussion

A major source of tissue damage in post-ischemic reperfusion is believed to be via the generation of oxygen-free radicals and other toxic oxygen metabolites (16-18). These radicals and metabolites have been implicated in post-ischemic reperfusion injury in the heart, kidney, intestine, brain and other organs (19-22). Studies in the post-ischemic-reperfusion injury in the lungs also implicate toxic oxygen metabolite as a source of damage (2,3,8,16). In our present study, lidocaine at 5mg/kg b.w. significantly reduced pulmonary edema, pulmonary arterial pressure and peak air way pressure. Although lidocaine has numerous systemic and local effects on various biological tissues, both in vivo and in vitro (23-24), their ability to ameliorate post-ischemic reperfusion injury in the lungs have not been previously recognized. We have demonstrated earlier that lidocaine is a hydroxyl radical scavenger and it inhibits NADPH-dependent lipid peroxidation in bovine lung microsomes(15). Fox et al. (25) and Martin et al. (26) have demonstrated decreased lung injury by pre-treating the tissue with several scavengers of reactive oxygen metabolites including DMSO, DMTU, SOD, catalase, ethanol suggesting a prominent role for oxygen metabolites in lung injury. Kennedy et al. (27) have shown that ischemia-reperfusion injury can be prevented by SOD, catalase and an iron chelator, desferoxamine, suggesting that an iron-mediated Fenton reaction is necessary for the injury to occur. In O_2 ventilated lung there is an increase in production of lipid peroxides (6). This increase is dependent on percent of O_2 in the inspired gas during ventilation in the ischemic period (6). In rat intestine, using salicylate as a

radical trap, increased production of hydroxyl radicals have also been demonstrated during ischemia (28). Therefore, it is likely that tissue damage, such as membrane lipid peroxidation, observed during the reperfusion of ischemic lungs was due to the production of these reactive metabolites.

The mechanism(s) by which lidocaine afforded protection against reperfusion injury, may be explained, in part, by its antioxidant properties. In a recent study we found that lidocaine is a powerful hydroxyl radical scavenger and it inhibits lipid peroxidation in bovine lung microsomes (15). Since lidocaine was found to protect lungs against thiourea induced lung injury in rats (12), and was found to decrease the release of conjugated diene, a lipid peroxidation product, in a canine myocardial ischemia-reperfusion model (13), it is likely that it protects pulmonary tissue against O_2 ventilated reperfusion injury by scavenging toxic ·OH radicals.

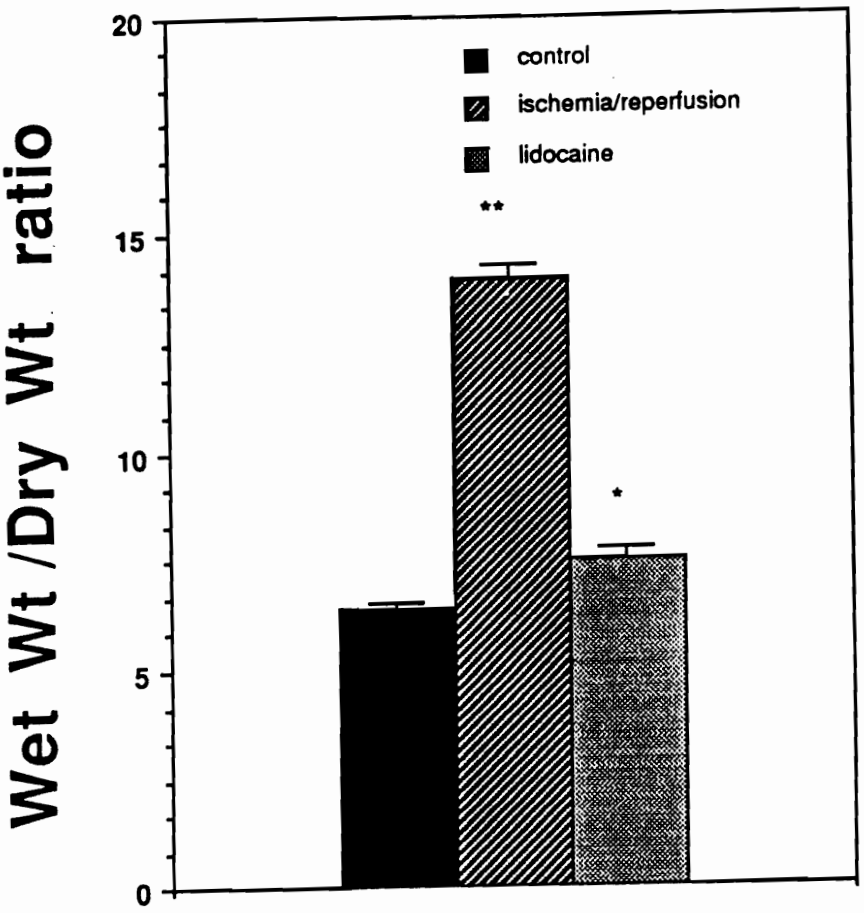


Fig.1. Effect of lidocaine on wet-to-dry lung weight ratios. Wet-to-dry lung weight ratio (n=6) after 70 minutes of lung perfusion in experiments exposed to ischemia-reperfusion (I/R) and I/R with lidocaine at a dose of 5 mg/Kg b.w. Values are mean±SE. *p <0.001 compared to ischemia-reperfusion lungs; ** p < 0.001 when compared to the control group.

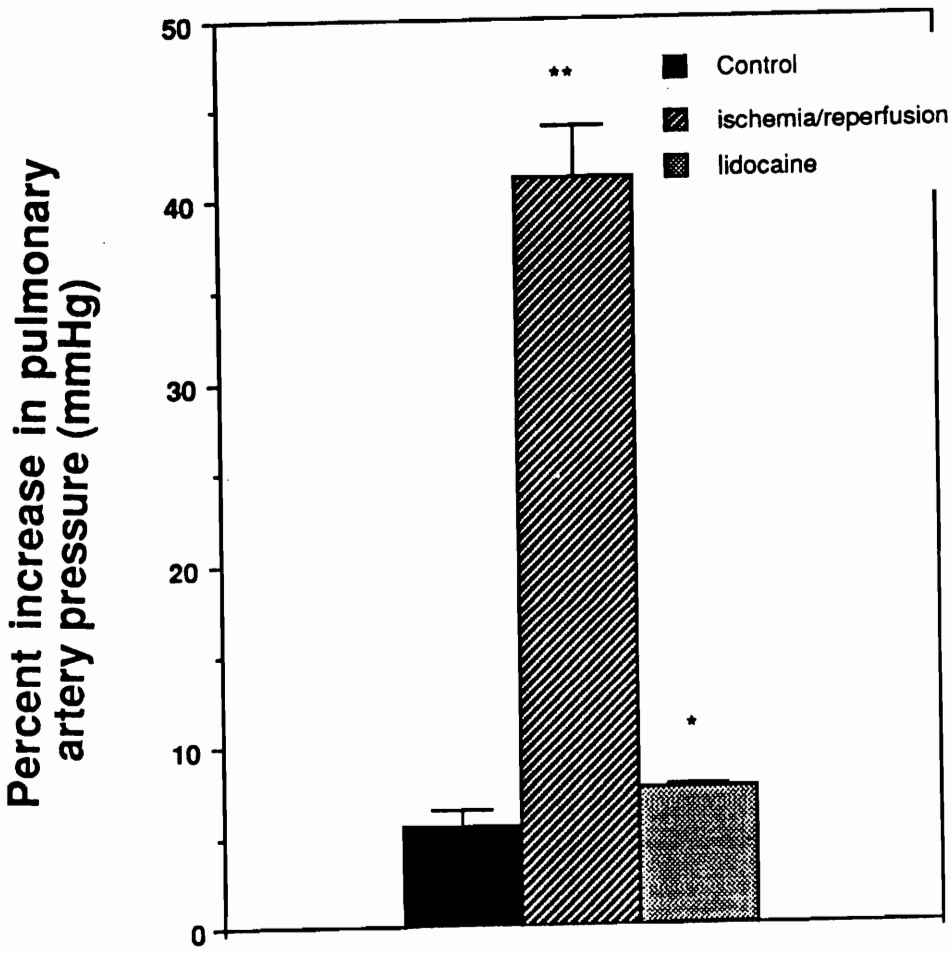


Fig. 2. Effect of lidocaine on mean pulmonary artery pressure. Percent change in pulmonary arterial pressure between pre-ischemia and post-ischemic reperfusion (n=6). Lungs in the control group were perfused for a total period of 70 min. Lungs in the ischemia group were perfused for 10 minutes which constiute the pre-ischemic period and followed by a 60 minutes of ischemia and 10 minutes of reperfusion. * $p < 0.001$ compared to ischemia-reperfusion lungs; ** $p < 0.001$ compared to control group..

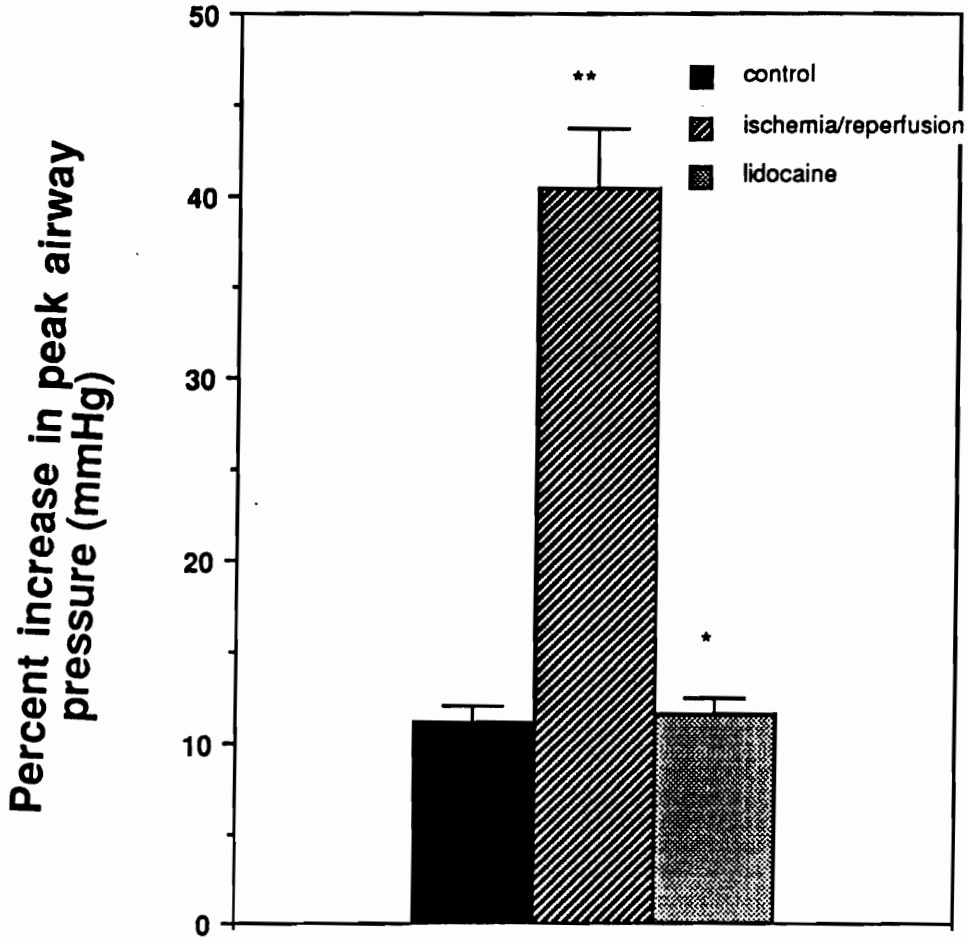


Fig. 3. Effect of lidocaine on the mean peak airway pressure. Percent change in peak airway pressure between pre-ischemia and post-ischemic reperfusion (n=6). Control lungs were perfused for a total period of 70 min. Lungs undergoing ischemia-reperfusion were perfused for 10 minutes which constitute the pre-ischemia period and then followed by a 60 minutes of ischemia and 10 minutes of reperfusion. * $p < 0.001$ compared to ischemia-reperfusion lungs; ** $p < 0.001$ compared to control group.

References

1. Cross CE. Oxygen radicals and human disease. Ann Intern Med 1987; 107:525.
2. Kennedy TP, Rao NV, Hopkins C, Tolhey E, Hoidal J. Reperfusion injury occurs in the lung by free-radical mechanism. Chest 1988; 93:1495-,
3. Ljungman GA, Grum CM, Deeb GM, Bolling SF, Morganroth ML. Inhibition of cyclooxygenase metabolite production attenuates ischemia-reperfusion lung injury. Am Rev Respir Dis 1991; 143:610-17.
4. McMurtry IF, Davidson AB, Reeves JT, Grover RT. Inhibition of hypoxic vasoconstriction by calcium antagonist in isolated rat lungs. Circ Res 1976; 38:99-104.
5. Koyama I, Toung JKT, Rogers MC, Gurtner GH, Traystman RJ. $O_2^{-\cdot}$ radical mediate reperfusion lung injury in ischemic O_2 ventilated canine pulmonary lobe. J Appl Physiol 1987; 63:111-15.
6. Fisher AB, Dodia C, Tan Z, Ayene I, Eckenhoff RG. Oxygen-dependent lipid peroxidation during lung ischemia. J Clin Invest 1991; 88:674-79.
7. Freeman BA, Panus P, Beckman JS, Matalan, S. Pulmonary vascular injury from reactive oxygen species. In: L'Abbatt A, Ursini F, eds. The role of oxygen radicals in cardiovascular diseases. London: Kluwer Academic Press, 1988; 201-215,.
8. Jurmann MJ, Dammenhayn L, Schaefers HJ, Haverich A. Pulmonary reperfusion injury: evidence for oxygen-derived free radical mediated

damage and effects of different free radical scavengers. Eur. J. cardio-thorac Surg 1990; 4:665-70.

9. Lynch MJ, Grum CM, Gallagher KP, Bolling SF, Deeb GM, Morganroth ML. Xanthine oxidase inhibition attenuates iscahemic-reperfusion lung injury. J Surg Res 1988; 44:538-44.
10. Tate RM, Vanbenthuysen KM, Shasby DM, McMurtry IF, Repine JE. Oxygen radical mediated permeability edema and vasoconstriction in isolated perfused rabbit lungs. Am Rev Respir Dis 1982; 126:802-6.
11. Bertrand Y. Oxygen-free radicals and lipid peroxidation in adult respiratory distress syndrome. Intensive Care Med.1985; 11:56-60.
12. Stelzner TJ., Welsh CH, Berger E, McCullough RG, Morris K, Repine JE, Weil JV. Antiarrhythmic agents diminish thiourea-induced pulmonary vascular protein leak in rats. J Appl Physiol 1987; 63:1877-83.
13. Lesnefsky EJ, VanBenthuysen KM, McMurtry IF, Shikes RH, Johnston RB, Jr., Horwitz LD. Lidocaine reduces canine infarct size and decreases release of a lipid peroxidation product. J Cardio Pharmacol 1989; 13:895-901.
14. Das KC, Misra HP. Lidocaine: A hydroxyl radical scavenger and singlet oxygen quencher. Mol Cell Biochem (In Press).
15. Das KC, Misra HP. Antiarrhythmic agents: scavengers of hydroxyl radicals and inhibitors of NADPH-dependent lipid peroxidation in bovine lung microsomes. J Biol Chem (In Press).
16. Granger DN, Hollwarth ME, Parks DA. Ischemia-Reperfusion injury: role of oxygen derived free radicals. Acta Physiol Scand Suppl 1986;

17. McCord JM. Oxygen-derived free radicals in post-ischemic tissue injury. N. Engl J. Med 1985; 312:159-163.

18. Weisfeldt ML. Reperfusion and reperfusion injury. Clin Res 1987; 2:311-319.

19. Granger DN, Rutili G, McCord JM, Role of superoxide radicals in intestinal ischemia. Gastroenterology 1981; 81:22-29.

20. Marubayashi S, Dohi K, Ezaki H, Yamada K, Kawasaki T, Preservation of ischemic liver cells - prevention of damage by coenzyme Q10. Transplant Proc 1983; 15:1297-1299.

21. Pallar MS, Hoidal JR, Ferris TE, Oxygen free radicals in ischemic acute renal failure in the rat. J Clin Invest 1984; 74:1156-1164.

22. Zweier JR. Measurement of superoxide-derived free-radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. J Biol Chem 1988; 263:1353-1357.

23. Arnsdorf MF, Electrophysiologic properties of antidysrhythmic drugs as a rational basis for therapy. Med Clin N Am 1976; 60:213-232.

24. Scherphof GL, Scarpa A, Van Toorenenbergen A. The effects of local anesthetics on the hydrolysis of free and membrane bound phospholipids catalyzed by various phospholipases. Biochim Biophys Acta 1972; 270: 226-240.

25. Fox RB, Harada RN, Tate RM, Repine JE. Prevention of thiourea-induced pulmonary edema by hydroxyl-radical scavengers . J Appl Physiol 1983; 55:1456-1459.

26. Martin D, Allison RC, Taylor AE. Hydroxyl radical lung damage produced by α-naphthylthiourea. In: Taylor AE, Matalan S,Ward PA, eds. Physiology

of oxygen radicals. Bethesda: American Physiological Society 1986; 131-143.

27. Kennedy TP, Rao NV, Hopkins C, Pennington L, Tolley E, Hoidal JR. Role of reactive oxygen species in reperfusion injury of the rabbit lung. J Clin Invest 1989; 83:1326-1335.

CHAPTER VI

IMPAIRMENT OF RAW 264.7 MACROPHAGE FUNCTION BY ANTIARRHYTHMIC DRUGS

Abstract

The effects of antiarrhythmic drugs (lidocaine, quinidine and procainamide) on O_2^{-}. and H_2O_2 production was investigated in a RAW 264.7 mouse monocytic macrophage cell line. Cells stimulated by either zymosan or phorbol ester were found to generate both superoxide ($O_2^{-}\cdot$) and H_2O_2. $O_2^{-}\cdot$ production was detected as superoxide dismutase inhibitable ferricytochrome c reduction. H_2O_2 production was monitored in both a chemical and flowcytometric fluorescent assay. Quinidine at 250 and 300 μM caused a 50% inhibition in $O_2^{-}\cdot$ production, and at 50 and 100 μM inhibited 50% H_2O_2 production, in zymosan and phorbol ester stimulated cells, respectivelly. All three antiarrhythmic drugs inhibited phagocytosis of fluorescent latex beads in a dose-dependent manner as studied by folw cytometry. The effect of these drugs on respiratory burst oxidase was investigated and procainamide was found to have significant effect ($p < 0.001$) in inhibiting oxidase activity. Lidocaine and quinidine had no effect on the activation of the respiratory burst oxidase. The result of these studies does not conclusively establish a mechanism of action of these drugs on the inhibition of $O_2^{-}\cdot$ and H_2O_2 generation by stimulated macrophages. Nevertheless, it is interesting to note that all three drugs

inhibited the phagocytic activity in a dose dependent manner and their statutary role in acute inflammatory diseases needs further investigation.

Introduction

Antiarrhythmic drugs lidocaine, quinidine and procainamide are widely used for treatment and prevention of arrhythmias in patients with cardiovascular diseases(1). Lidocaine is a local anesthetic and is routinely employed to anesthetise the air ways during broncoscopic subsegmental lavage (2). In addition, lidocaine is known to impair certain metabolic functions of leukocytes in vitro. Phagocytic cells respond to certain membrane stimulants by the production of $O_2^{-}\cdot$, H_2O_2, and .OH. This metabolic event is known as "oxidative" or "respiratory burst". There exists some controversy in the literature regarding the effects of antiarrhythmic drugs on phagocytic $O_2^{-}\cdot$ and H_2O_2 production. Thus, it was shown that human neutrophil $O_2^{-}\cdot$ production was inhibited by quinidine and procainamide but not by lidocaine whereas H_2O_2 production was inhibited by all three antiarrhythmnic drugs (4). However, in another study lidocaine was shown to inhibit human neutrophil $O_2^{-}\cdot$ production (5). Hoidal et al. (2) have shown that lidocaine at 16 mM inhibited superoxide production by alveolar macrophages. It was suggested that these drugs might have some inhibitory effect on the $O_2^{-}\cdot$ generating enzyme, NADPH oxidase (5). Recently we have reported that these drugs are effective in scavenging hydroxyl radical and in inhibiting NADPH-dependent lipid peroxidation on lung microsomes (3). Since monocytic macrophages have striking differences in oxidative metabolism compared with alveolar macrophages and (polymorphonuclear leukocytes) PMN in the same species (6-8) , it was of interest to determine the effect of these drugs on monocytic macrophage function and to delineate the

mechanism of action of these drugs. We present evidence that these drugs impair macrophage function not by inhibiting NADPH oxidase activation but possibly by inhibiting macrophage phagocytosis.

Materials and Methods

Cell Culture of RAW 264.7 monocytic macrophage line:

RAW 264.7 (ATCC) cells were grown in standard 75 cm^2 tissue culture flasks in Dulbeco's MEM supplemented with 10% fetal calf serum and 10 units of penicillin G with 10 μg of streptomycin per ml (Sigma Chemical Co). Cells were harvested to desired numbers and washed in HBSS (with Ca^{2+} and Mg^{2+} without phenol red). The cells were counted using a Coulter counter (Coulter Electronics).

Superoxide production:

Superoxide production was determined by measuring superoxide dismutase (SOD) inhibitable reduction of horse heart ferricytochrome c spectrophotometrically (9). Reaction mixtures contained 10^6 cells in 1 ml Hank's balanced solution (with Ca^{2+} and Mg^{2+} without phenol red). The cells were stimulated by either PMA (phorbol-12-myristate 13-acetate, 100 ng/ml) or opsonized zymosan (500-600 particles/phagocyte) to which antiarrhythmic agents or vehicle control were added. Superoxide dismutase (40 μg/ml) was added to some of the solutions to determine $O_2^{\cdot-}$-dependent cytochrome c reduction. The reactions were carried out in triplicate. Reaction mixtures were incubated at 37^0C for 20 min. Control cells were kept on ice and used as blanks. At the end of incubation time reaction mixtures were centrifuged at 4 ^{0}C at 800 X g for 10 min. The absorbance of the supernatants at 550nm was then monitored in a Shimadzu UV-160 double beam spectrophotometer using SOD added cuvettes as references. The results were expressed as nanomoles of

superoxide dismutase inhibitable cytochrome c reduced using an extinction coefficient of E_{550}19.6 $mM^{-1}cm^{-1}$ (10).

Macrophage H_2O_2 production:

Macrophage H_2O_2 production was determined by the modification of method of Weiss et al.(11). Reaction mixtures contained 10^6 cells in Hank's balanced solution to which 1 mM sodium azide, and antiarrhythmic agents were added in a total volume of 1ml. Samples were incubated at 37^0C for 30 minutes before addition of 0.1 ml of trichloroacetic acid (50%, w/v). Samples were than chilled and centrifuged at 500 X g for 5 min in a refrigerated centrifuge. Ferrous ammonium sulfate (10 mM, 0.2 ml) was then added to the supernatant and incubated for 5 min followed by the addition of 0.1 ml of potassium thiocyanate (2.5 mM) and incubated for additional 5 min. The absorption of the ferrithiocyanate complex was measured at 480 nm in a Shimadzu-UV160 spectrophotometer and compared with a standard H_2O_2 curve.

Flow cytometry:

Flow cytometric analysis was performed on a Coulter EPICS V Flow Cytometer interfaced with an MDADS data analysis computer (Coulter Electronics, Hialeah, FL.) with a hard copy display provided by a Tektronix 4612 Video Hard Copy Unit (Tektronix, Wilsonville, OR). Standardization of the flow cytometer was performed using fluorescent calibration beads (Coulter Electronics, Hialeah, FL.) using 488nm laser (Coherent, Palo Alto, CA) excitation at 300mW. The cell population of interest was displayed on a two

parameter histogram of forward angle light scatter (FALS) and 90^0 angle light scatter (90^0LS). FALS was measured linear using an ND2 interference filter and 90^0LS was measured log integral using a 488 dichroic filter. A 452-515 laser blocker was employed to block laser light from other fluorescent measurements. The fluorescent distribution was displayed as a 256 channel, single parameter histogram. Green fluroscence was measured through 550nm longpass dichroic and 525 band pass filter. A gated amplifier was used to electronically ignore small particles allowing the number of cells to be determined by the total count of particles that had scattered light.

Determination of H_2O_2 by DCF-DA in flow cytometer:

Dichlorofluorescin diacetate (DCF-DA) is a non-fluorescent probe becomes fluorescent in the presence of hydrogen peroxide. DCF-DA diffuses in to the cells, is hydrolyzed by intracellular esterases to a nonfluorescent analog 2', 7'-Dichlorofluorescene (DCFH) and is trapped within the cell. With H_2O_2 production during oxidative respiratory burst, DCFH is oxidized to highly fluroscent 2' 7'-dichlorofluorescein (DCF). H_2O_2 was measured using DCF-DA method of Bass et al. (12) with following modifications. Macrophages at 1×10^6 cells/ml was suspended in Hank's balanced salt solution with 1 µl of DCF-DA (5 mM) and desired concentration of antiarrhythmic drugs. The mixture was pre-incubated at 37^0C for 15 min. The cells were stimulated with the addition of PMA (100ng/ml or 100 µl of opsonized zymosan , 1mg/ml, about 600 particles per phagocyte and incubated for a period of 30 minutes. The cells were than read in the flow cytometer as described above.

Determination of phagocytosis by fluorescent microspheres in flow cytometer:

Phagocytosis was assayed as per the method of Dunn and Tyrer (13). The cell suspension (1 X 10^6cells/ml) in KRH-Gelatin buffer (4.8 mM KCl, 0.7% NaCl, 2.34 mM $MgSO_4$, 15.60 mM HEPES-Triethanolamine (pH 7.4) and 0.1% gelatin, from sheep skin, Sigma Chemical Co.) was divided into 0.5 ml aliquots in 50 ml plastic round bottom centrifuged tubes. Homologous plasma (mouse serum, Sigma Chemical Co, 10%) was added followed by 10 µl of (1:10 diluted) 1.16 µ diameter Fluoresbrite microspheres (Polyscience, Inc., Warrington, Pa). The dilution of beads gave a final ratio of 50 beads/cell. After incubation in a 37 ^{0}C waterbath for 60 min, 5 ml of PEG buffer (PBS with 0.1% EDTA disodium salt and 0.1% unflavoured gelatin, from sheep skin, Sigma Chemical Co) was added to each tube. The cells were washed twice with PEG buffer (500 X g) to eliminate most extracellular beads. The final cell pellet was suspended in 0.5 ml of 1% glutaraldehyde (from 25% aqueous grade I glutaraldehyde, Fisher Chemical Co.).

Since the flow cytometer was standardized for fluroscence using a solution of free beads, the peak on the fluroscence histogram due to cells containing single beads could be identified. For each sample analysis 10000 cells were counted and the number of cells phagocytosing beads was expressed as a percentage of 10000.

Isolation of components of NADPH Oxidase:

Macrophages (4 X 10^8 cells/ml) were treated with diisopropyl-fluorophosphate (DFP) (Sigma Chemical Co) before homogenization, to

prevent proteolysis by the modification of method of Crowley et al. (14). Briefly, cells were suspended in 10 mM glycylglycine buffer at pH 8.0 containing 2.7 mM KCl and 138mM NaCl, DFP (5mM) in ethylene glycol was added at a ratio of 50µl of DFP solution per ml of cell suspension. The cells were then allowed to stand on ice for 10 min, then sedimented at room temperature in a table top centrifuge at lowest speed that would pellet the cells completely in 2.5 minutes. DFP treated cells were washed two times with PBS, then disrupted by French Cell Press at a 350 psi of cavity pressure. The homogenate was than fractionated on a discontinuous percoll gradient by the method of Borregard et al. (15). The tonicity of percoll (Sigma Chemical Co.) was adjusted by adding one-tenth the final volume of a 10 times concentrated Borregard's relaxation buffer (1 M KCl, 30 mM NaCl, 35 mM $MgCl_2$, 10 mM ATP[Na]$_2$, 12.5 mM EGTA, 100 mM PIPES, pH 6.8. Fourteen ml of percoll, (density 1.120 g/ml) was layered under 14 ml of percoll (density 1.050 g/ml) through a long needle. Ten ml of cell homogenate was applied to the top and centrifugation was carried out at 4^0C for 15 min at 20,000 rpm in an SS34 rotor in Sovrall RC 5B refrigerated ultra centrifuge. The density of the gradient was estimated from the bands of calibration beads of known density (Sigma Chemical Co.) in gradient run in parallel. The uppermost band was collected by a Pasteur pipette and centrifuged at 230,000 X g for 2 hours at 4^0C to obtain the membrane fraction of the oxidase. The pooled membranes were suspended at a concentration of 3 X 10^8 cell eq/ml in half strength Borregaards relaxation buffer (50 mM KCl, 1.5 mM NaCl, 5 mM PIPES, pH 7.3, 1.75 mM $MgCl_2$, 0.5 mM ATP, 0.62 mM EGTA (15) containing 0.34 M sucrose. The suspension was divided into aliquats and stored at -70 ^{0}C for future use. The supernatant from the 230,000 X g fraction

(designated as "cytosol") was divided into aliquats and stored at -70^{0}C. This material served as the source of the cytosolic factor.

Preparation of solubilized enzyme:

Membranes from macrophages were solubilized by the modification of method of Curnutte et al. (16). Membrane fractions were thawed at room temperature, mixed with an equal volume of extraction buffer (1mM NaN_3, 1.7 μM, $CaCl_2$, 20 mM sodium glycinate, pH 8.0, 2.33% sodium deoxycholate (w/v), and 50% (v/v) glycerol , vortexed briefly, incubated on ice for 30 min with occasional agitation, and finally centrifuged at 435,000 X g for 1 hour in SW60 rotor in Beckman L8-80M ultracentrifuge using polycarbonate tubes. Dormant oxidase was found in the supernatant.

Activation of NADPH oxidase in cell-free system:

Superoxide production in cell free system was assayed by the method of Curnutte et al.(16) as modified by Umeki and Soejima (17). Assay mixtures contained 0.1 mM cytochrome c, 89 mM KCl, 2.7 mM NaCl, 0.5 mM, PIPES, pH 7.3, 0.9 mM ATP, 3.6 mM $MgCl_2$, 1.2 mM EGTA, 0.04 mM sodium dodecyl sulfate (SDS), and 0.16 mM NADPH plus 6 X 10^6 equivalent of cytosol and 1.5 X 10^6 cell equivalent of membranes solubilized in sodium deoxycholate in a total volume of 1 ml. The reference cuvette contained 50 μg of SOD. After mixing of the reaction constituents (NADPH omitted), absorbance at 550nm was followed for 2.15 min at 25 ^{0}C, superoxide generation was initiated by adding NADPH 25 μl (4μM) to each cuvette, and the change in absorbance at 550 was followed for 2.15 min on a Shimadzu-UV double beam

spectrophotometer. Superoxide production was calculated using an extinction coefficient, E^{mM}_{550nm} 19.6 $mM^{-1}cm^{-1}$(10). Protein was determined by the method of Bradford (18).

Since quinidine with the deoxycholate solubilized membrane extract formed a white precipitate, it was not possible to determine the effect of quinidine on the activation of NADPH oxidase in cell free system with deoxycholate solubilized membrane. NADPH oxidase components were therefore isolated using the method of Sha'ag (19).

Briefly, RAW 264.7 mouse monocyte macrophages were disupted with digitonin (0.20 mM) in phosphate buffer- Na phosphate (10 mM), pH 7.2; NaCl (130 mM); KCl (10mM); azide (2 mM); at 1 X 10^8 cells/ml by mixing gently for 2-2.5 min at room temperature. The lysate was centrifuged at 400 X g for 6 min. The supernatant was removed carefully and immediately centrifuged again at 265,000 X g for 1 hr in a SW 60 rotor in Beckman L8-80 ultracentrifuge using polycarbonate tubes. The 265,000 X g supernatant was used as cytosol. Solubilized membranes were prepared as described previously (20): Cellular debris from digitonized cells (400 X g pellet) was suspended in solubilizing buffer (sodium phosphate, 120 mM, pH 7.3; glycerol 20% v/v, azide 1.6 mM, EGTA 0.8 mM, $MgCl_2$ 0.8 mM) to 10^8 cell equivalents/ml. The cell suspension was sonicated with a probe (Heat Systems) at 10 W for 3 X 15s in 2 ml aliquots, the sonicate was pooled and centrifuged at 2000 X g for 10 min. The 2000 X g supernatant was centrifuged at 48,000 X g for 1 hr. The pellet thus obtained was suspended mechanically to 4 X 10^8 cell equivalent/ml in solubilization buffer supplemented with solid octyl-glucoside and KCl to a final concentration of 40 mM and 200 mM, respectively. Solubilization was by gentle

rotation by hand for 30 min, after which the solubilized preparation was centrifuged at 48,000 X g for 1 hour. The clear supernatant was decanted and diluted 8 fold in solubilization buffer. This was the solubilized membrane preparation used throughout the experiment.

Assay of superoxide generation in the cell free system was done as per the method of Sha'ag and Pick (21) The assay mixture contained: sodium/potassium phosphate (65 mM, pH 6.8), EGTA (1 mM), cytochrome c (0.10 mM), NaN_3 (2 mM), 20 µl of pellet extract and 60 µl of cytosol. Materials were added in the order listed above. The mixture was incubated in the room temperature for 1 min, and reaction was then started by the addition of 20µl of NADPH (10 mM). Assay was performed in Shimadzu-UV160 double beam spectrophotometer where the reference cuvettee contained reaction mixture with 40 µg of SOD. Superoxide generation was calculated using an extinctinction coefficient of E_{550} 19.6$mM^{-1}cm^{-1}$ (10).

Results

Effect of antiarrhythmic drugs on generation of superoxide by macrophages:

The ability of macrophages to release superoxide anion was measured using PMA (100ng/ml) or opsonized zymosan (1mg/ml) as stimuli in the presence and absence of antiarrhythmic drugs. As shown in Figure 1, $O_2^{-\cdot}$ release in quinidine treated cells was reduced in a dose dependent manner when expressed as percent inhibition of ferricytochrome c reduction. The trend was similar in both, PMA and zymosan stimulated cells. Thus, quinidine at 250 μM and 300 μM caused a 50% inhibition of superoxide production in zymosan and PMA stimulated cells, respectively (Fig. 1 & Fig. 2). Lidocaine at 1mM inhibited only 18.86% and 10.52% in zymosan and PMA stimulated cells, respectively. Similarly, procainamide had only 12.63% inhibition on superoxide release at 1mM in PMA stimulated cells where as it had 28.30% inhibition in zymosan stimulated cells. Thus, all three drugs inhibited superoxide release in a dose dependent manner, and quinidine had the maximal effect.

Effect of antiarrhythmic drugs on H_2O_2 production by macrophages.

The production of H_2O_2 in PMA and opsonized zymosan stimulated cells was inhibited in a dose dependent manner when cells were treated with quinidine and procainamide. Thus, quinidine caused 50% inhibition at 50 μM

and 100 μM (Fig. 3 & Fig. 4) in zymosan and PMA stimulated cells, respectively. Procainamide at 75 and 500 μM inhibited 50% production of H_2O_2 (Fig. 3 & Fig. 4) in opsonized zymosan and PMA treated cells. On the other hand lidocaine had no effect on H_2O_2 production in PMA stimulated cells even at 1 mM concentration (Fig. 4). However, lidocaine inhibited the H_2O_2 production in zymosan stimulated cells and at 1mM concentration it caused 26% inhibition (Fig. 3).

When H_2O_2 was assayed in a flow cytometer using DCF-DA as fluorescent probe, and mean channel number as a measure of intensity of fluorescence, zymosan stimulated cells showed more inhibitory response to quinidine and procainamide (Fig. 5) than in PMA stimulated cells (Fig. 6). Thus, in opsonized zymosan stimulated cells quinidine at 500 μM concentration inhibited a maximum of 40% of fluorescence and procainamide was only 11.4% inhibitory at 500 μM . On the other hand lidocaine inhibited only 5.2% at 1 mM concentration in opsonized zymosan stimulated cells (Fig. 5). In PMA stimulated cells (Fig. 6) quinidine, procainamide and lidocaine at 1mM inhibited 18.21, 10.14 and 6.0% respectively. The trends of these results are similar to the trends observed in the chemical assays.

Effect of antiarrhythmic drugs on Phagocytosis:

When macrophages were incubated with fluorescent microspheres they ingested varying number of particles. The fluorescence distribution (Fig. 7b) exhibits the multiple populations characterized by distint peaks representing the number of beads in the cell. As shown in this figure, there was minimal autofluorescence of macrophages. The population resolved as follows: the

peak nearest to the origin is the peak for the population of cells containing single beads. Using homogenous population of beads as a standard (Fig. 7a) the first peak was identified as cells containing single beads. This peak appears at channel 39. The peak with twice the fluorescence intensity of single beads, was regarded as population of cells ingested two beads, cells with three beads appeared as third peak and so on (Fig 7b). Cells that have ingested five or more fluorescent beads are not easily discernible in the fluorescence distribution although some structure is evident in the three dimensional histogram (Fig 7c). A negative control (cells kept at 0^0C with fluroscent microspheres) and a positive control (cells incubated at 37^0C for 60 min with fluorescent microspheres) are clearly discernible in the two dimensional as well as in a three dimensional histograms (Fig 7b, 7c, and Fig. 8a, 8b). In this system the total intensity of fluorescence was taken as average phagocytosis and we determined the effects of antiarrhythmic drugs on this average phagocytotic process. As shown in Figure 9, cells in positive control group phagocytose 60% of the beads during 60 min incubation. The negative controls ingested to a maximum of 11% of the beads when they were incubated at 0^0C. When 50, 100 and 500 μM of lidocaine, quinidine and procainamide were added in the medium, phagocytosis was inhibited in a dose dependent manner (Fig. 9). When cells were separately counted for their fluorescence depending on the number of beads they ingested, i.e. 1,2 and 3 or more beads per cell, all the three antiarrhythmic drugs were found to inhibit the ingestions in a dose dependent manner (Fig. 10).

Effect of antiarrhythmic drugs on NADPH oxidase in a cell free system:

When the membrane and cytosolic components of NADPH oxidase, the respiratory burst enzymes, were isolated and assayed by the method of Curnutte et al. (16), quinidine reacted with the deoxycholate solubilized membrane component producing a white precipitate. However, lidocaine and procainamide did not form such precipitates with the deoxycholate extracts. As shown in Figure 11, lidocaine had no significant effect on NADPH oxidase activation at 1 mM concentration. However, procainamide had significant inhibitory effect on the activation of NADPH oxidase in cell free system. When components of NADPH oxidase was isolated by the method of Sha'ag et al (19) and membrane fraction were solubilized in n-octylglucoside, quinidine remained homogenous with the solubilized membrane preparation. Lidocaine and quinidine had no significant effect on the inhibition of activation of NADPH oxidase in this cell free system. However, as in case of deoxycholate solubilized membrane extract, procainamide had a significant inhibitory effect ($p < 0.001$) on the activation of NADPH oxidase (Fig. 12).

Discussion

Antiarrhythmic drugs are widely used drugs with significant stabilizing activity on lipid biomembranes (4). In addition, lidocaine and procainamide are used as local anesthetics. All three drugs were found to scavenge .OH and inhibit membrane lipid peroxidation in bovine lung microsomes (3). Recently we found that these agents can protect lungs against oxidative injury induced during post-ischemic reperfusion period in isolated perfused rat lungs (34). The results of the present study suggest that all these drugs are effective in reducing the metabolic activity of monocytic macrophages in vitro.

Superoxide radicals were generated when macrophages were stimulated by either PMA or zymosan particles. All three antiarrhythmic drugs inhibited $O_2^{-}\cdot$ production. It was apparent that quinidine (Fig. 1 and Fig. 2) was most potent in inhibiting $O_2^{-}\cdot$ production in vitro. Our results are in accord with the findings of Stelzner et al. (4) who demonstrated that $O_2^{-}\cdot$ production by stimulated human neutrophils are inhibited by quinidine. These authors also reported that lidocaine has no effect on on human neutrophil $O_2^{-}\cdot$ production. However, in our study on monocytic macrophage, both lidocaine and procainamide were found to inhibit $O_2^{-}\cdot$ generation in both the zymosan stimulated as well as PMA stimulated cells. Although the inhibitory effect of lidocaine and procainamide were not as intensive as quinidine, their effects were consistantly observed. We concur with the findings of Hoidal et al (2). and Peck et al. (5) that lidocaine is effective, although less sensitive compared to quinidine, in preventing $O_2^{-}\cdot$ production by stimulated phagocytes.

Hydrogen peroxide is produced as a result of oxidative burst by leukocytes. However, the effect of antiarrhythmic drugs on H_2O_2 has not been studied in detail. In a recent study (4) the three antiarrhythmic drugs were shown to have inhibitory effects on H_2O_2 production when human neutrophils were stimulated by PMA. However, in our study on monocytic macrophage, lidocaine at 1mM concentration had minimal effect on opsonized zymosan stimulated cells (Fig 5) and had little effect on PMA treated cells. Quinidine and procainamide on the other hand had significant inhibitory effect on H_2O_2 production. Flow cytometric analysis in combination with DCF-DA as the fluorescent probe, revealed that the production of H_2O_2 in presence of quinidine had a maximum inhibitory effect which is in accord with the previous study on human neutrophils (4). Lidocaine, although had least effects, was distinctly inhibitory at about 1 mM concentration.

All three drugs inhibited phagocytosis in a dose dependent manner (Fig. 9). Previous studies using human leukocytes have documented inhibition of phagocytosis of latex particles by lidocaine (22). However, we not only concurred the above findings but also found that the other antiarrhythmic agents such as quinidine and procainamide also have inhibitory effects on monocytic macrophage phagocytosis.

Phagocytes upon activation by stimuli are known to induce release of granule enzymes. Since $O_2^{-\cdot}$ is known to induce a change in membrane polarization and which allows plasma membrane for influx of sodium and calcium ions, these events are believed to be involved during oxidative metabolism of phagocytes (23). Since NADPH oxidase is a membrane bound enzyme and antiarrhythmic drugs impose their action on membrane

organization, there is reason to believe that these drugs might inhibit the activity of this enzyme. However as shown in Fig. 11 and Fig. 12, only procainamide had trivial inhibitory action on NADPH oxidase at 500 μM concentration. Lidoaine and quinidine had little effect on the activity of this enzyme.

In conclusion, it appers likely that the mechanism of action of antiarrhythmic drugs on macrophages function is not consistant with the therapeutic potential of the drugs as antiarrhythmic agents. However, all these drugs were found to suppress $O_2^{-\cdot}$ and H_2O_2 production by macrophages stimulated by both soluble agent (PMA) and phagocytizable particles such as zymosan. Further studies are warranted to establish the mode of action of antiarrhythmic drugs on respiratory burst .

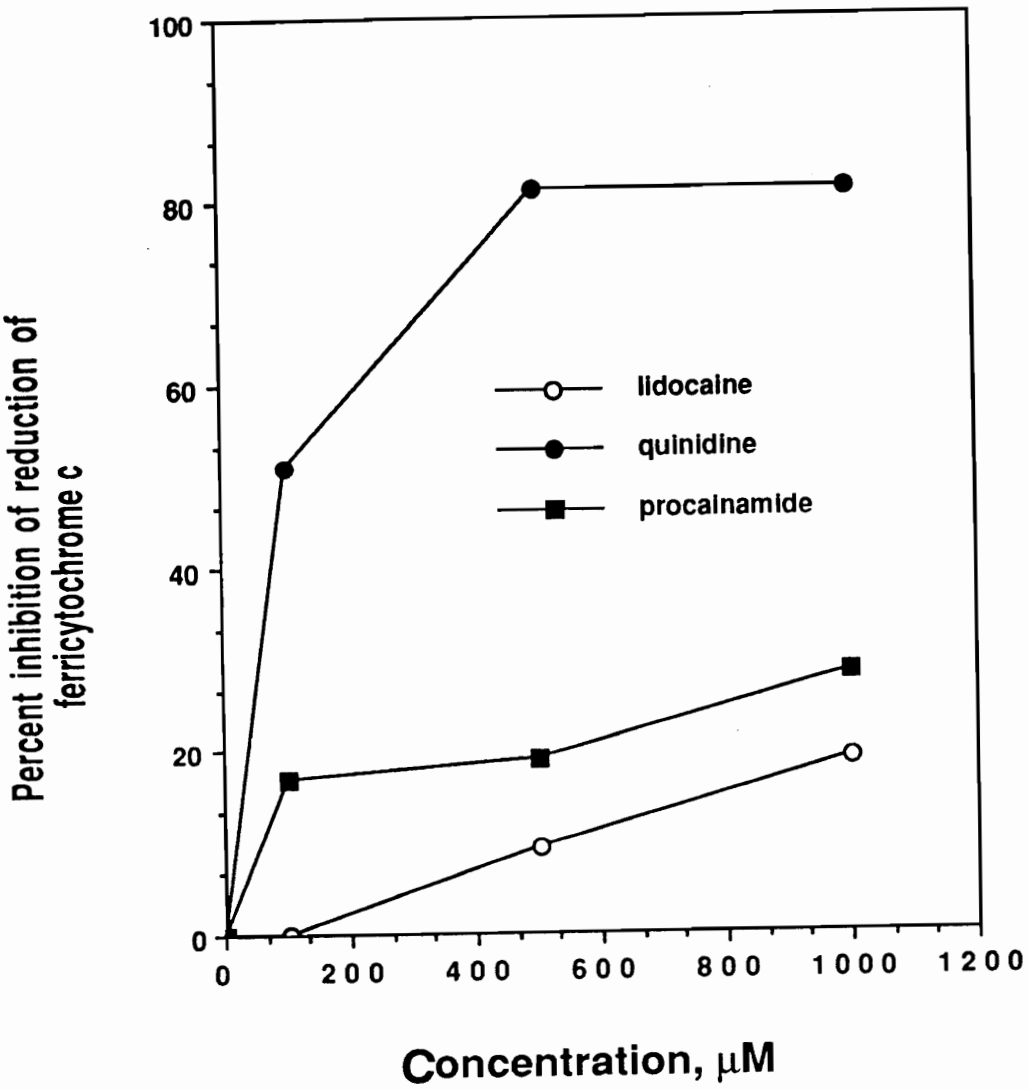


Fig 1. Effect of antiarrhythmic drugs on $O_2^{-\cdot}$ generation by macrophages in opsonized zymosan stimulated cells. The experimental conditions are as described under "Materials and Methods". Lidocaine, quinidine and procainamide at indicated concentrations were added to 1 ml of Hank's balanced salt solution containing and 10^{-3} M ferricytochrome c and 1 X 10^6 cells. Opsonized zymosan at 1mg/ml (600 particles per phagocyte) was added to the reaction mixture and the mixture was incubated for 20 min at 37^0C. The percent inhibition was calculated from positive control where no drug was added to the reaction mixture. Spectra of supernatant were measured at 550 nm.

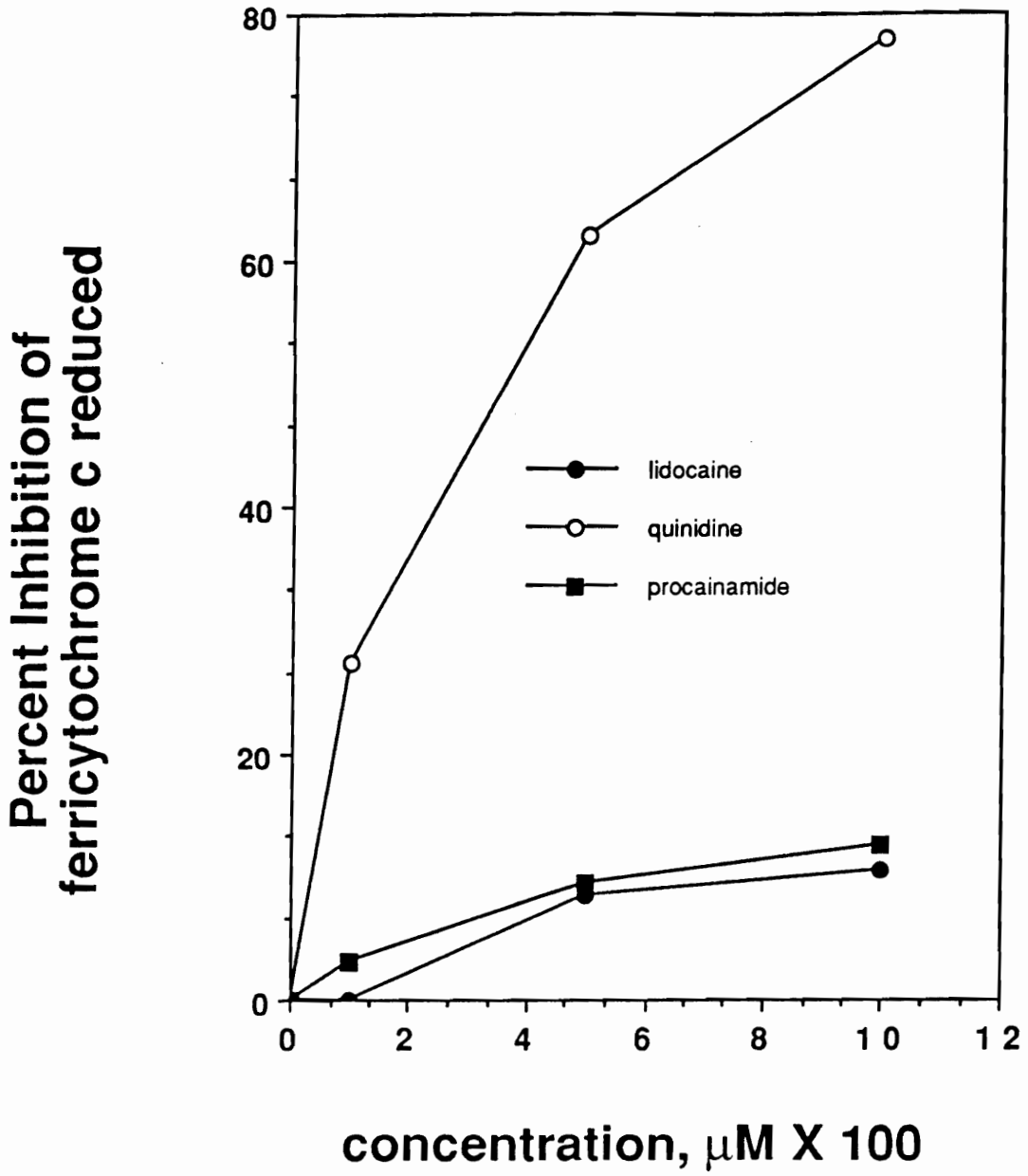


Fig 2. Effect of antiarrhythmic drugs on $O_2^{-\cdot}$ generation by macrophages in PMA stimulated cells. The experimental conditions are as described under "Materials and Methods". Lidocaine, quinidine and procainamide at indicated concentrations were added to 1 ml of Hank's balanced salt solution containing and 10^{-3} M ferricytochrome c and $1 X 10^6$ cells. PMA (100ng/ml) was added to the reaction mixture and the mixture was incubated for 20 min at 37^0C. The percent inhibition was calculated from positive control where no drug was added to the reaction mixture. Spectra of supernatant was measured at 550 nm.

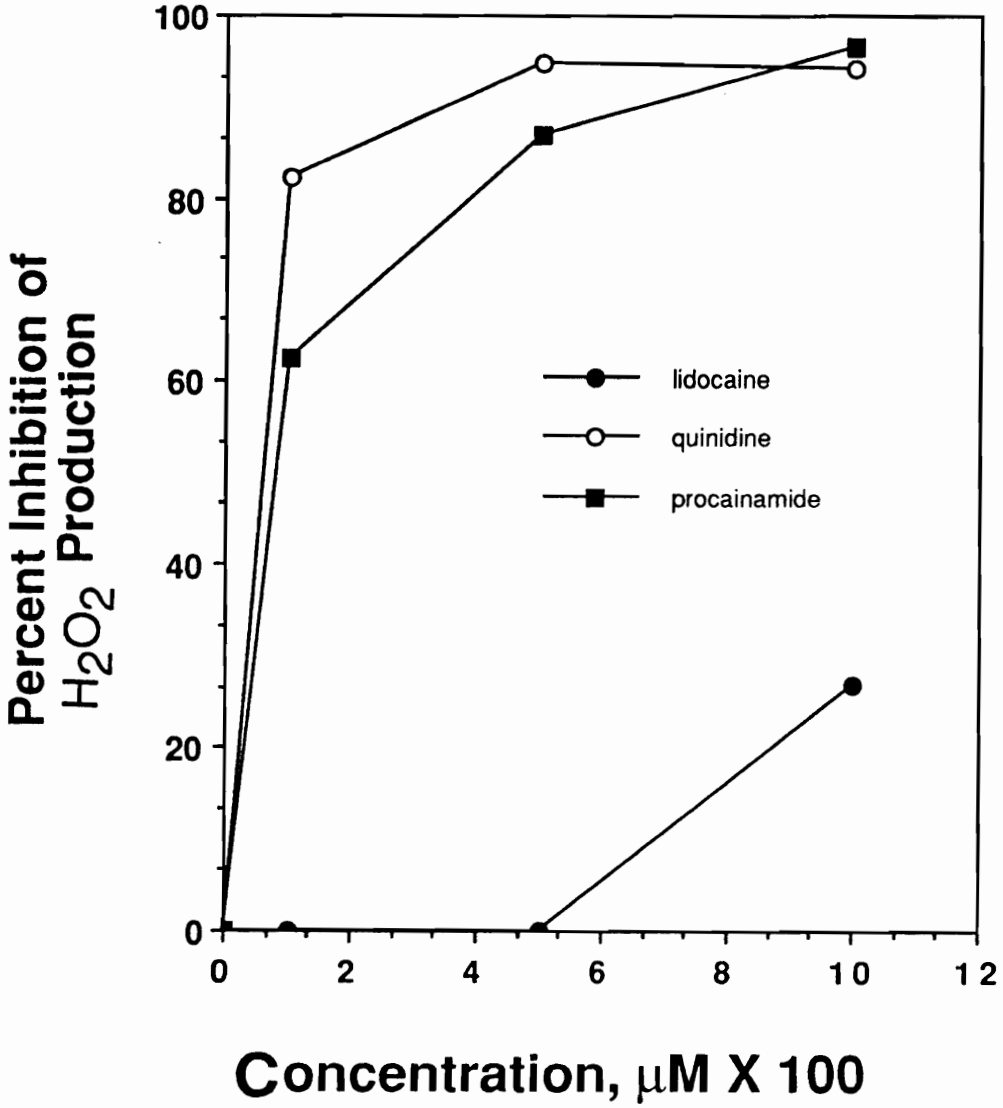


Fig 3. Effect of antiarrhythmic drugs on H_2O_2 production by macrophages stimulated with opsonized zymosan. The experimental conditions were as described under "Materials and Methods". Lidocaine, quinidine and procainamide at indicated concentrations were added to 1 X 10^6 cells in Hank's balanced solution with opsonized zymosan (1mg/ml) and incubated for 30 min at 37^0C. Reaction was terminated by the addition of 0.1 ml of TCA. and centrifuged. Then ferrous ammonium sulphate 200 μl (10 mM) and potassium ferrithiocyanate 100 μl (10 mM) were added to the supernatant and the absorbance were recorded at 480 nm. Percent inhibition was calculated from control reactions containing no drugs.

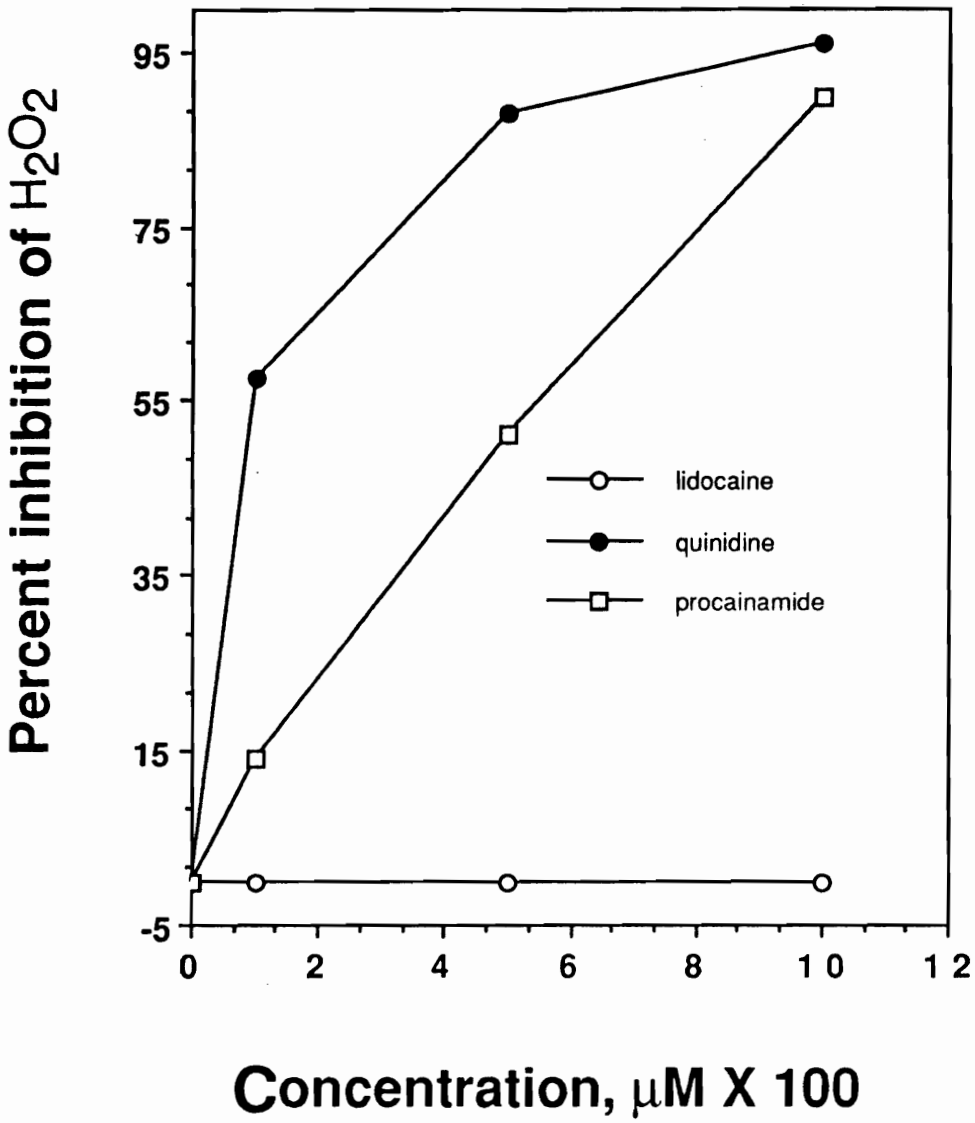


Fig 4. Effect of antiarrhythmic drugs on H_2O_2 production by macrophages stimulated with PMA The experimental conditions were as described under "Materials and Methods". Lidocaine, quinidine and procainamide at indicated concentrations were added to 1 X 10^6 cells in Hank's balanced solution with PMA (100ng/ml) and incubated for 30 min at 37^0C. Reaction was terminated by the addition of 0.1 ml of TCA. and centrifuged. Then ferrous ammonium sulphate 200 μl (10 mM) and potassium ferrithiocyanate 100 μl (10 mM) were added to the supernatant and the absorbance were recorded at 480 nm. Percent inhibition was calculated from control reactions containing no drugs.

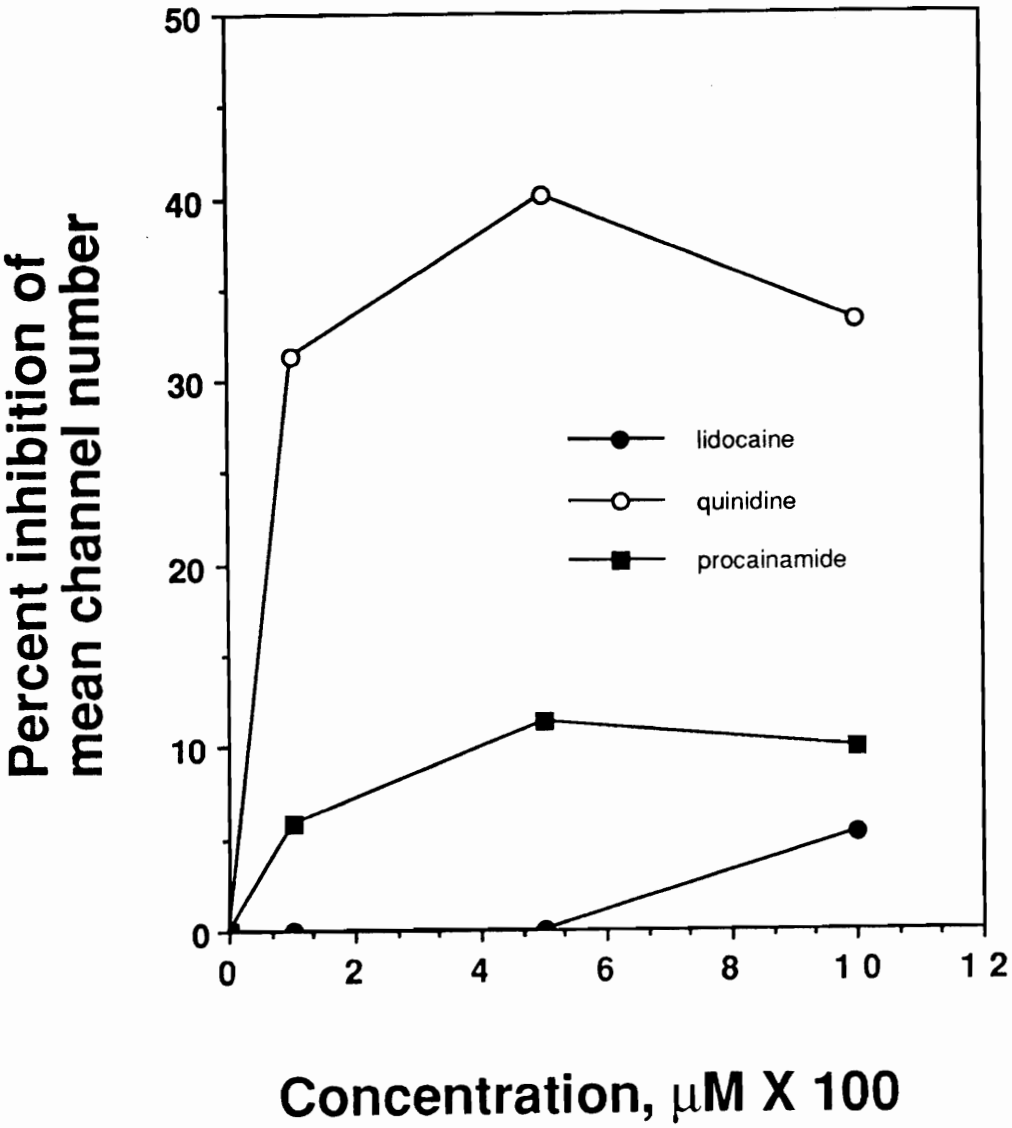


Fig 5. Effect of antiarrhythmic drugs on flow cytometric determination of H_2O_2 production using DCF-DA. The experimental conditions are as described under " Materials and Methods". Mean channel number indicates the amount of fluorescence. Cells at 1 X 10^6 concentrations were incubated with DCF-DA containing indicated concentrations of antiarrhythmic drugs. Cells were than stimulated with opsonized zymosan (0.5mg/ml) and incubated for a further period of 30 min. Then the cells were read in the flow cytometer for the amount of fluorescence. Percent inhibition was calculated from the mean channel number of control reactions.

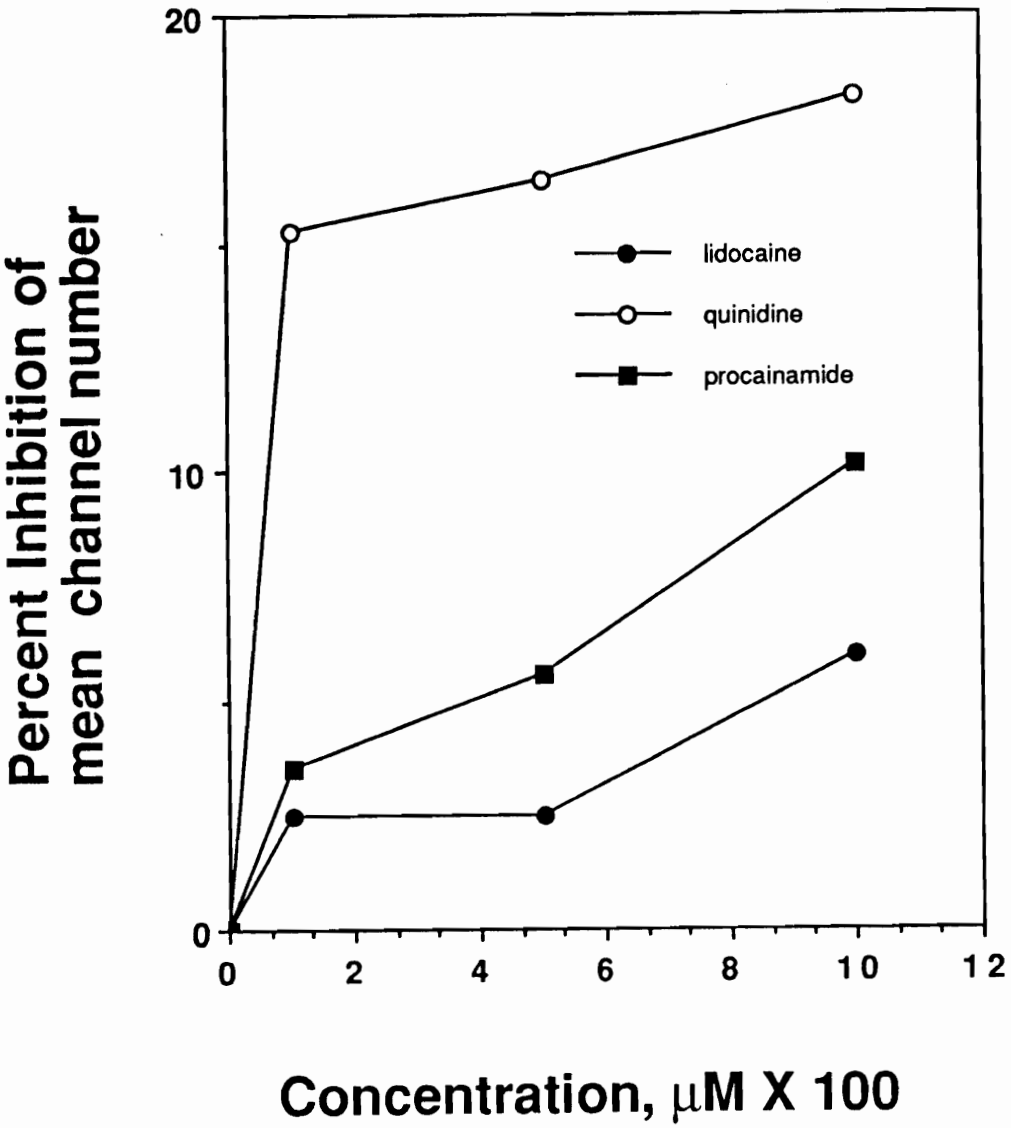


Fig 6. Effect of antiarrhythmic drugs on flow cytometric determination of H_2O_2 production using DCF-DA. The experimental conditions are as described under " Materials and Methods". Mean channel number indicates the amount of fluorescence. Cells at 1 X 10^6 concentrations were incubated with DCF-DA containing indicated concentrations of antiarrhythmic drugs. Cells were than stimulated with PMA (100ng/ml) and incubated for a further period of 30 min. Then the cells were read in the flow cytometer for the amount of fluorescence. Percent inhibition was calculated from the mean channel number of control reactions.

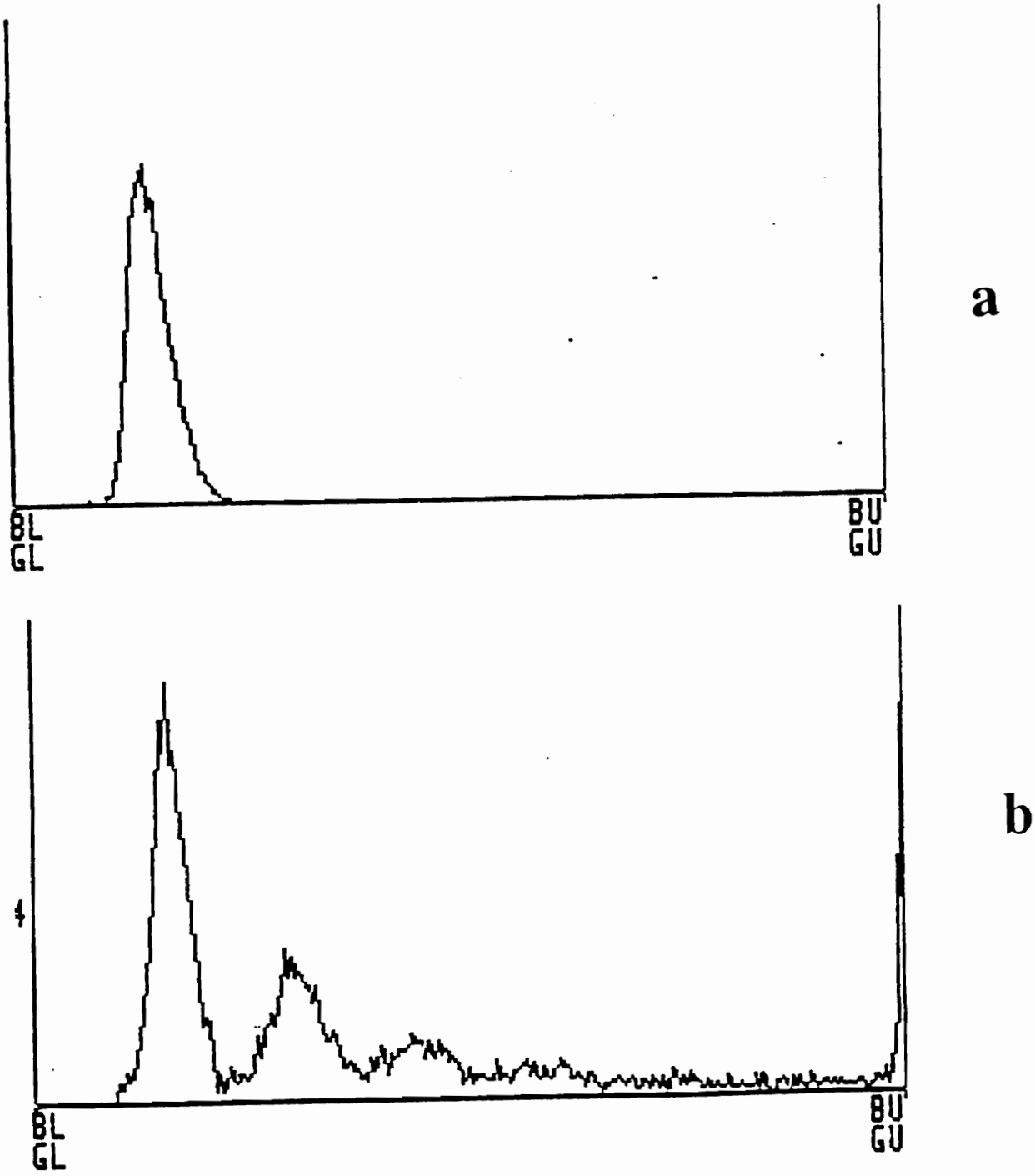
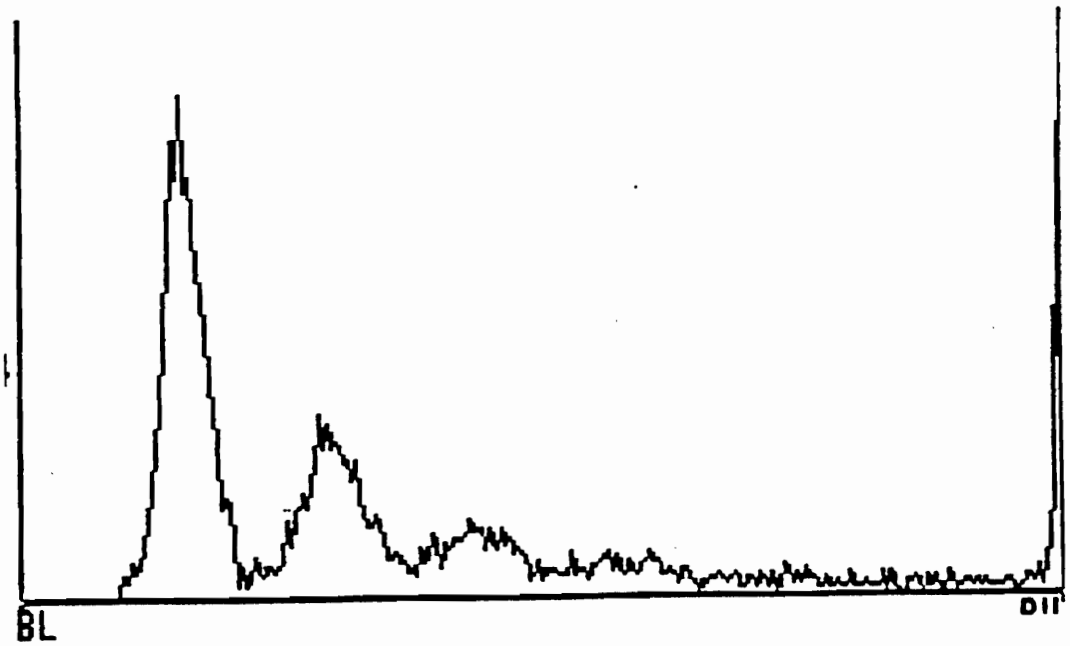


Fig 7. A two parameter histogram of frequency (y-axis) and fluorescence intensity (x-axis) of latex beads. (a): Histogram of single beads with peak at channel 39. **(b):** Histogram of a positive control of phagocytosis where cells were incubated with the latex beads for 60 min at 370C. The 1st peak corresponds to the peaks of single bead ingested cells as determined from the standard, the 2nd peak corresponds to the cells phagocytosing two beads, the third peak corresponds to the cells phagocytosing 3 beads and so on.



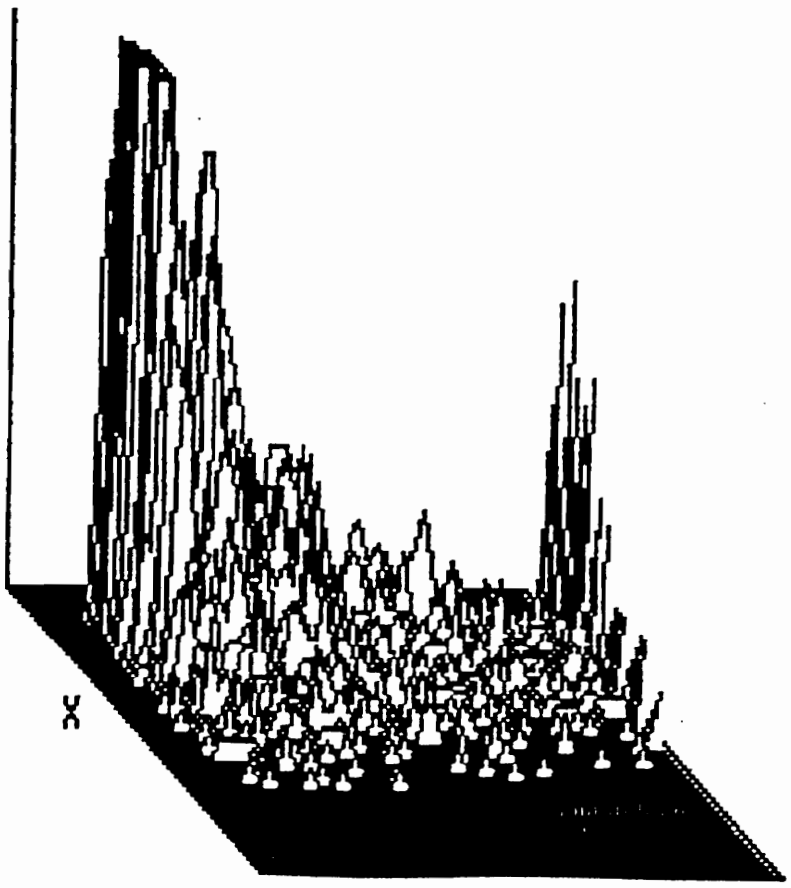


Fig 7c. A two parameter histogram of a positive control reaction and same histogram in a three dimensional setting. Experimental conditions were as described for Fig. 7.b.

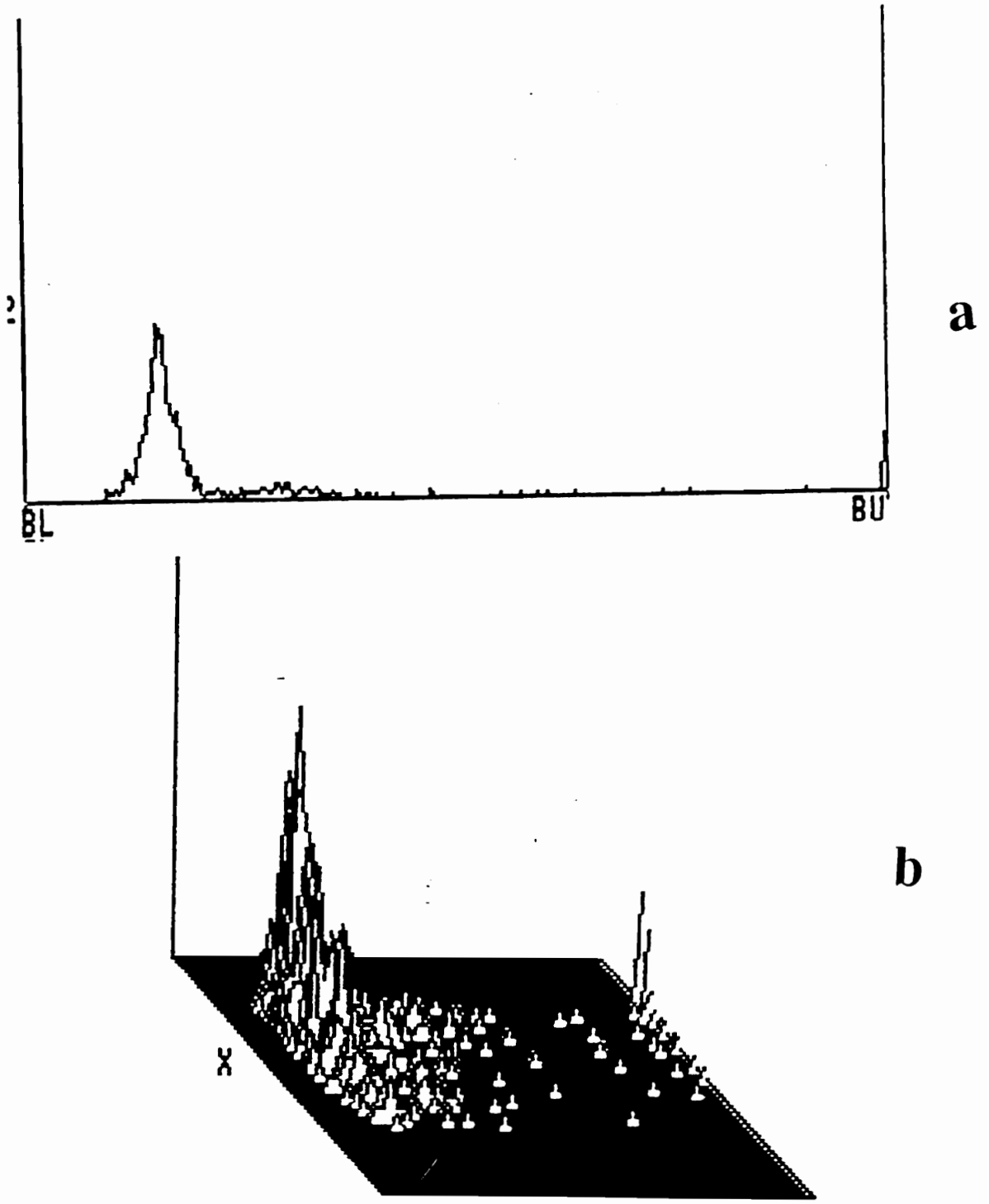


Fig 8. A two parameter histogram of negative control (a): two dimensional histogram (b): a three dimensional histogram. Experimental conditions were similar to the legends on Fig. 7.b.

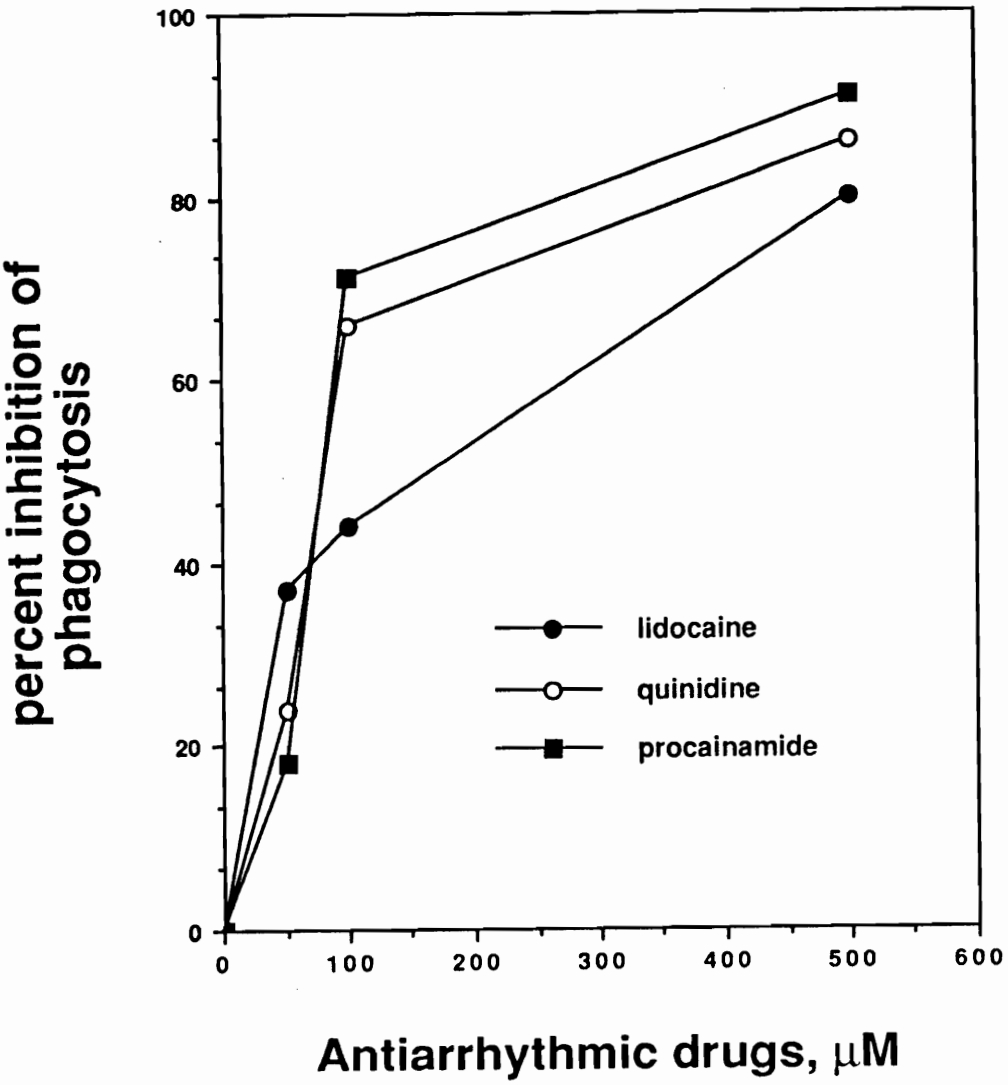


Fig 9. Effect of antiarrhythmic drugs on percent inhibition of phagocytosis as determined by flow cytometry using fluorescent latex beads. The experimental conditions were as in "Materials and Methods". Antiarrhythmic drugs at indicated concentrations were added to incubation mixture containing 1 X 10^6 cells/ml with 10 μl of fluorescent latex beads (50 beads/cell). The mixture was incubated for 60 min at 37^0C. Percent inhibition of phagocytosis was calculated comparing to control reactions without any drugs.

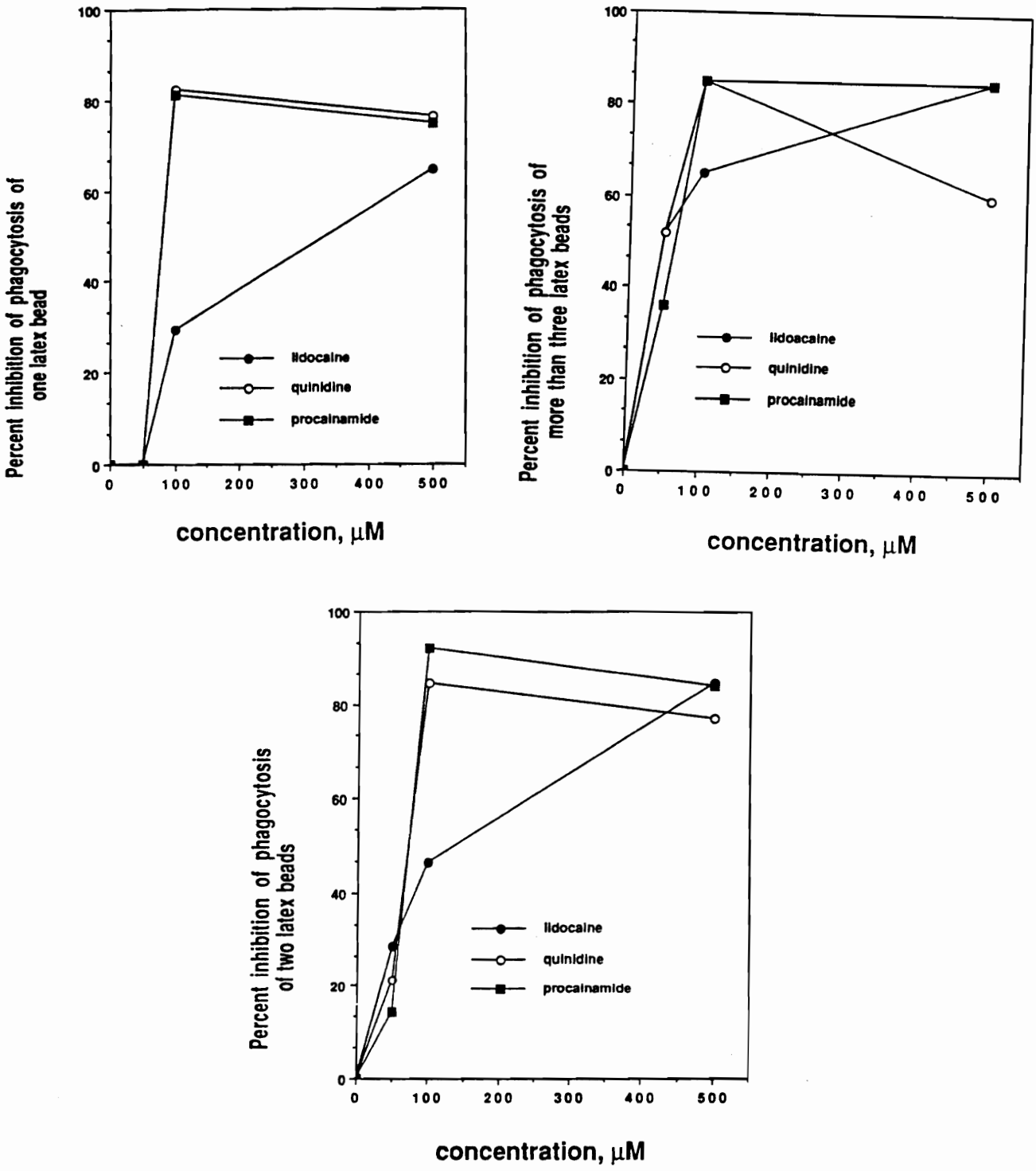


Fig 10. Percent inhibition of phagocytosis by antiarrhythmic drugs on cells ingesting specific number of fluorescent latex beads Experimental conditions were as described under "materials and methods". Cells at 0.5 X 10^6 were incubated at 37^0C for 60 min with fluorescent latex beads,10% homologous serum in KRH buffer and lidocaine, quinidine and procainamide were added to the reaction mixture at indicated concentrations. At the end of the incubation cells were washed with PEG buffer and fixed in 1% glutaraldehyde for flow cytometric analysis.

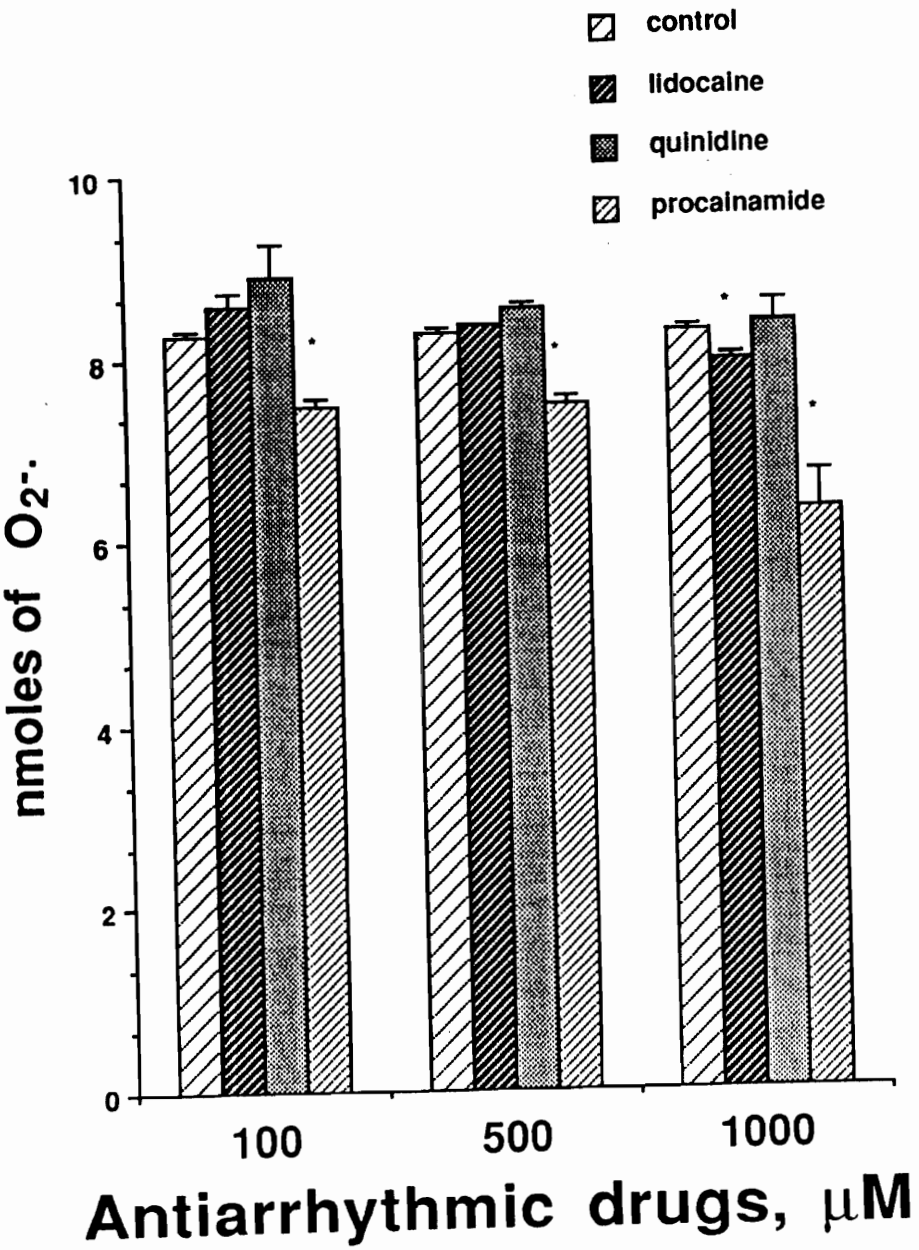


Fig 11. Effect of antiarrhythmic drugs on NADPH oxidase activation in a cell free system (Curnutte et al.) Experimental conditions are described as under "Materials and Methods". Antiarrhythmic drugs at indicated concentrations were used the superoxide production by 1.5 X 10^6 cell equivalent of membrane fraction and 6 X 10^6 cell equivalent of cytosol fraction were monitored.

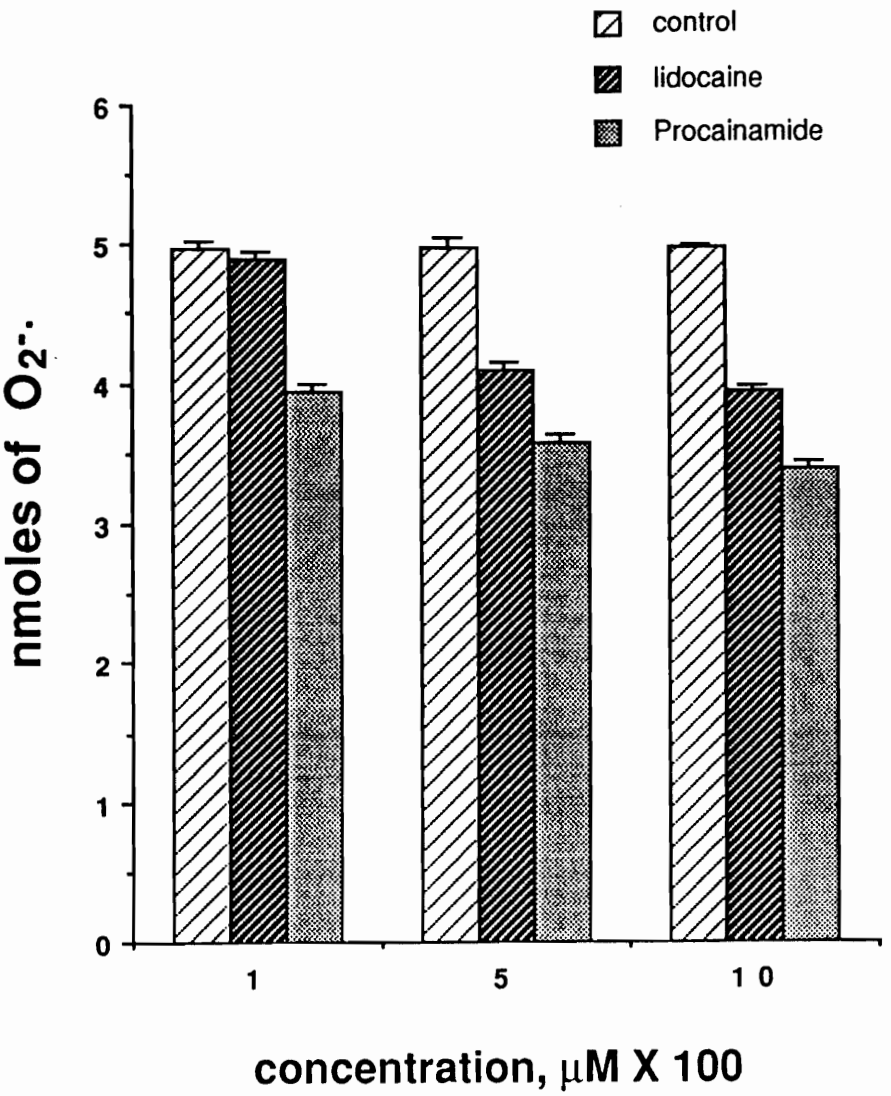


Fig 12. Effect of antiarrhythmic drugs on NADPH oxidase activation in a cell free system (Sha'ag et al.) Experimental conditions are described as under "Materials and Methods". Antiarrhythmic drugs at indicated concentrations were used. The superoxide production by 1 X 10^6 cell equivalent of membrane fraction and 2 X 10^6 cell equivalent of cytosol fraction were monitored.

References

1. Arnsdorf, M. F. Electrophysiologic properties of antidysrhythmic drugs as a rational basis for therapy. Med. Clin. N. Am. 60:213-232, 1976
2. Hoidal, J.R., White, J.G. and Repine, J.E. Influence of cataionic local anestehetics on the metabolism and ultrastructure of human alveolar macrophages. J. Lab. Clin. Med 93:857-866, 1979.
3. Das, K.C. and Misra, H.P. Antiarrhythmic agents: hydroxyl radical scavengers and inhibitors of NADPH-dependent lipid peroxidation. J. Biol. Chem.(In press).
4. Stelzner, T.J., Welsn, C.H., Berger, E, McCllough, R., Morris, K., Repine, J.E. and Weil, J.V. Antiarrhythmic agents diminish thio-urea induced pulmonary vascular protein leak in rats. J. Appl. Physiol. 63:1877-1883, 1987.
5. Peck, S.L, Johnston, R.B.,Jr., Horwitz, L.D. Reduced neutrophil superoxide anion release after prolonged infusions of lidocaine. J. Pharmacol. Exp. Ther. 235:418-422, 1985.
6. Drath, D.B., and Karnovsky, L. Superoxide production by phagocytic leukocytes. J. Exp. Med. 142:257-262. 1975.
7. Drath, D.B., Karnovsky, M. L. and Huber, G. L. Hydroxyl radical formation in phagocytic cells of the rat. J. Appl. Physiol. 46:136-140., 1979

8. Oren, R., Farnham, A.E., Saito, K., Milofsky, E. and Karnovsky, M.L. Metabolic patterns in three types of phagocytosing cells. J. Cell. Biol. 17:487, 1963.

9. Johnston, R.B.,Jr., Keele, B.B.,Jr., Misra, H.P., Lehmeyer, J.E., Webb, LS., Baehner, R.L. and Rajgopalan, K.V. The role of superoxide anion genaration in phagocytic bactericidal activity: studies with normal andchronic granulomatous disease leukocytes. J. Clin. Invest. 55:1357-1372, 1975.

10. Yonetani, T. Studies on cytochrome c peroxidase.II stoichiometry between enzyme, H_2O_2, and ferricytochrome c and enzymatic determination of extinction coefficients of cytochrome c. J. Biol. Chem. 240:4509-4514, 1965.

11. Weiss, S.J., Young, J.,Lobuglio, A.F., Slivka, A. and Nimeh, N.F. Role of hydrogen peroxide in neutrophil mediated destruction of cultured endothelial cells. J. Clin. Invest. 68:714-721, 1981.

12. Bass, D. A., Parce, J. W., Dechatelet, L.R., Szejda, P., Seeds, M.C. and Thomas, M. Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. J. Immunol. 130:1910-1917, 1980.

13. Dunn, P.A. and Tyrer, H.W. Quantitation of neutrophil phagocytosis using fluorescent latex beads. J. Lab. Clin. Med. 98:374-381, 1981.

14. Crowley, C.A., Curnuttee, J.T., Rosin R. E., Andre-schwartz, J., Gallin, J.I., Klempner, M., Snyderman, R., Southwick, F.S., Stossel, T.P. and Babior, B.M. An inherited abnormality of neutrophil adheson. N. Engl. J. Med. 302:1163-1168, 1980.

15. Borregaard, N., Heiple, J.M., Simons, E.R., and Clark, R.A. Subcellular localization of the b-Cytochrome component of the human neutrophil microbicidal oxidase: Translocation during activation. J. Cell. Biol. 97:52-61, 1983.

16. Curnutte, J.T., Kuver, R. and Babior B.M. Activation of the respiratory burst oxidase in a fully soluble system from human neutrophils. J. Blol. Chem. 262:6450-6452, 1987.

17. Umeki, S. and Soejima, R. Hydrocortisone inhibits the respiratory burst oxidase from human neutrophils in whole cell and cell free systems. Biochim. Biophys. acta. 1052:211-215, 1990.

18. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254,

19. Sha'ag, D. Sodium dodecyl sulphate dependent NADPH oxidation: an alternative method for assaying NADPH Oxidase in a cell free system. J.Biochem. Biophys. Meth. 19:121-128, 1089.

20. Pick, E., Bromberg, Y., Shpungin, S. and Gabda, R. Activation of the superoxide forming oxidase in a cell free system by sodium dodecyl sulphate, characterization of the membrane associated component. J. Biol. Chem. 262:16476-16483

21. Sha'ag, D. and Pick, E. Macrophage-derived superoxide-generating NADPH Oxidase in an amphile-activated, cell free system; partial purification of the cytosolic component and evidnce that ir may contain the NADPH binding site. Biochim. Biophys. Acta, 952:213-219, 1988.

22. Cullen, B. F. and Haschke, R. H. Local anesthetic inhibition of phagocytes and metabolism of human leukocytes. Anesthesiology, 40:142-148, 1974.

23. Weissman, G., Smolen, J.E. and Korchak, H.K. Release of inflammatory mediators from stimulated neutrophils. N. Engl. J. Med. 303:27-34, 1980.

24. Rossi, F., Romeo, D., and Patriarca, P. Mechanism of phagocytosis associated oxidative metabolism in polymorphonuclear leucocytes and macrophages. J. Reticuloendothelial. Soc. 12: 127-149, 1972.

25 Repine, J.E., White, J.G., Clawson, C.C. and Holmes, B.M. The influence of phorbol myristate acetate on oxygen consumption by polymorphonuclear leucocytes. J. Clin. Med. 83:911-920, 1974.

26. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature, 308:693-698, 1984.

27. Myers, M.A., McPhail, L. C. and Snydrman, R. Redistribution of protein kinase C activity in human monocytes: correlation with activities of the respiratory burst. J. Immunol. 135:3411-3416, 1985.

28. Krebs, E. G, and Beavo, J.A. Phosphorylation-dephosphorylation of enzyme. Annu. Rev. Biochem, 48:923-925, 1979.

29. Stephens, C.G., and Snyderman, R. Cyclic nucleotides regulate the morphologic alterations required for chemotaxis in monocytes. J. Immunol. 128:1192- ,1982.

30. Lehmeyer, J.E., and Johnston,Jr, R.B. Effect of anti-inflammatory drugs and agents that elevate intracellular cAMP on the release of toxic oxygen

metabolites by phagocytosis: studies in a model of tissue-bound Ig. Clin. Immunol. Immunopathol. 9:482-, 1978.

31. Gallin, J.I., Sandler, J.A., Chyman, R.I., Mangniello, V.C. and Vaughan, M. Agents that increase cAMP inhibit accumulation of cGMP and depress human monocyte locomotion. J. Immunol. 128:492-,1982.

32. Smolen, J.E., Korchak, H.M. and Weissman, G. The role of extracellular and intracellular calcium in lysosomal enzyme release and superoxide anion generation by human polymorphonuclear leukocytes. Biochim. Biophys. Acta. 677:512-520.

33. Tomoda, M., Ueda, W., Tsuchiya, M., and Hirakawa, M. Effects of lidocaine on stimulation coupled responses of neutrophils and protein kinase c activities. Masui, 41:369-375.

Summary

Class I antiarrhythmic drugs (lidocaine, quinidine and procainamide) are widely used in clinical practice for management of ventricular arrhythmias. These drugs are also effective in preventing post-ischemic reperfusion injury of heart. Since toxic oxygen metabolites are implicated as causative agents in reperfusion tissue injury, we hypothesized that these drugs could protect the pulmonary tissue against reperfusion injury by scavenging the reactive metabolites. We used an isolated rat lung model to evaluated the effect of these drugs on reperfusion injury. We also studied the effect of these drugs on monocytic macrophage function to evaluate the effect of these drugs on oxidative tissue injury contributed by stimulated phagocytes.

The results of our studies indicate that reperfusion injury to the isolated rat lung could be prevented by antiarrhythmic drugs. Lidocaine was found to protect reperfusion injury in both normoxic reperfusion and hyperoxic (O_2 ventilated) perfused rat lungs. Since lipid peroxides are associated with reperfusion tissue injury, we studied the effects of these drugs on membrane lipid peroxidation. All three drugs were found to be effective in protecting biomembranes against free radical induced lipid peroxidation. Thus, all three antiarrhythmic drugs inhibited NADPH-dependent lipid peroxidation in bovine lung microsomes in a dose dependent manner. Their order of ability to inhibit lipid peroxidation was lidocaine > quinidine > procainamide which is consistent with the therapeutic potential of these drugs. The antioxidant properties of these drugs were investigated and found that these drugs are not effective in

scavenging superoxide radicals but are found to be potent scavengers of hydroxyl radicals with a rate constant of 1.8 X 10^{10}, 1.61 X 10^{10} and 1.45 X 10^{10} $M^{-1}Sec^{-1}$ for quinidine, lidocaine and procainamide, respectively. Lidocaine was also found to be a quencher of singlet oxygen.

To evaluate the effect of these drugs on oxidative tissue injury caused by activated macrophages, we studied the effect of these drugs on oxidative burst of macrophages stimulated either by phorbol ester or opsonized zymosan. All three drugs were found to inhibit the production of $O_2^{-}\cdot$ and H_2O_2 by stimulated cells. However, none of these drugs had any significant effect on superoxide generating NADPH oxidase system. These drugs, however, were found to be potent inhibitor of phagocytosis. We propose that these drugs could ameliorate oxidative tissue damage, in part, mediated by inappropriately stimulated phagocytes.

We conclude that the antiarrhythmic drugs such as lidocaine, quinidine and procainamide protect ischemia-reperfusion induced pulmonary tissue injury in a perfused ex vivo rat lung model. The mechanism(s) of protection may, in part, be due to their ability to scavenge hydroxyl radical, prevent lipid peroxidation and inhibit phagocytes in inappropriately generating toxic oxygen metabolites.

Literature cited

Agar, J.N., and Dainton, F.S. (1947), *Disc Faraday Soc.* 2:218-219.

Akanmu, D., Cecchini, R., Aruoma, O.I. and Halliwell, B. (1991), The antioxidant action of ergothionine. *Arch. Biochem. Biophysic.* 288:10-16

Arnsdorf, M. F. (1970), Electrophysiologic properties of antidysrhythmic drugs as a rational basis for therapy. *Med. Clin. N. Am.* 60:213-232.

Aust, S. D., and White, B. C. (1985), Iron chelation prevents tissue injury following ischemia. *Adv. Free Rad. Biol. Med.* 1:1-17.

Bakerbond application, PH-046, Extraction of prostaglandins from serum. J. T. Baker Inc. 222 Red School Lane, Phillipsburg, NJ 08865.

Barnard, J. W., Patterson, C. E., Hull, M. T., Wagner, W. W., Jr. and Rhoades, R. A. (1989), Role of microvascular pressure in reactive oxygen-induced lung edema. *J. Appl. Physol.* 66:1486-1493.

Baron, D. W., Sunamori, M., and Harrison, C. E. (1983), Preservation of oxidative phosphorylation by lidocaine in ischemic and reperfused myocardium. *Adv. Myocardiol.* 4: 567-573.

Bass, D. A., Parce, J. W., Dechatelet, L.R., Szejda, P., Seeds, M.C. and Thomas, M. (1980), Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J. Immunol.* 130:1910-1917.

Beauchamp, C. and Fridovich, I. (1970), A mechanism for the production of ethylene from methional. *J. Biol. Chem.* 245:4641-4646.

Behar, D., Czapski, G., Rabani, J., Dorfman, L.M. and Schwarz, H. (1970), The acid dissociation constant and decay kinetics of the perhydroxyl radical *J. Phys. Chem.* 74: 3209-3213

Bernheim, F., Bernheim, M.L.C. and Wilbur, K.M. (1948) The reaction between thiobarbituric acid and the oxidation products of certain lipids. *J. Biol. Chem.* 174:257-264

Bernier, M., Hearse, J. D., Manning, A.S. (1986), Reperfusion-induced arrhythmias and oxygen derived free radicals. Studies with anti-free radical interventions and a free radical generating system in the isolated perfused heart. *Circ. Res.* 58: 331-340.

Bertrand, Y. (1985), Oxygen-free radicals and lipid peroxidation in adult respiratory distress syndrome. *Intensive Care Med.* 11:56-60.

Bielski, B.H.J. and Allen, A.O. (1967), *Proc. Tihany. Symp. Radiat. Chem.* 2nd,

81

Bielski, B. H. J. and Gebicki, J. M. (1974), Study of peroxidase mechanisms by pulse radiolysis. *Biochim. Biophys. Acta* 364: 233-235.

Bishop, M.J., Boatman, E.S., Ivey, T.D., Jordan, J.P. and Cheney, F. W. (1986), Reperfusion of ischemic dog lung results in fever, leukopenia, and lung edema. *Am. Rev. Respir. Dis.* 134:752-756.

Borregaard, N., Heiple, J.M., Simons, E.R., and Clark, R.A. (1983), Subcellular localization of the b-Cytochrome component of the human neutrophil microbicidal oxidase: Translocation during activation. *J. Cell. Biol.* 97:52-61.

Boudolulars, H., Karayannacos, P. E., Lewis, R. P., Kakos, G. S., Kilman J. W., and Vasko, J. S. (1978), Potential effect of lidocaine on ischemic myocardial injury: Experimental and clinical observations. *J. Surg. Res.* 24: 469-476.

Boveris, A., Oshino,N., and Chance, B. (1972) The cellular production of hydrogen peroxide *Biochem.J.* 128:617-630

Bradford, M.M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Brady, J.E. Humiston, G.E. (1975) General Chemistry: Principles and structure. New York, John Wiley & Sons, Inc.

Burghuber, O., Mathias, M. E., McMurtry, I. F.,Reeves, J. T. , and Voelkel, N. F. (1984), Lung edema due to hydrogen peroxide is independent of cyclooxygenase products. *J. Appl. Physiol.* 56:900-905.

Burney, R.G., Difazio, C.A., Peach, J.J., Petrie, K.A. and Silvester, M.J. (1974), Anti-arrhythmic effects of lidocaine metabolites. *Am. Heart J.* 88:765-769

Burton, R.M. (1955), The determination of glycerol and dihydroxyacetone. *Methods in Enzymol.* 3:246-248

Carmen, M. A., Kramer, J. H., Benjamin, F. D., and Weglicki, W. B. (1987), Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO. *FEBS Lett.*. 221: 101-104.

Casey, L. C., Armstrong, M. C., Fletcher, J. R., and Ramwell, P. W. (1980), Lidocaine increases prostacyclin in the rat. Prostaglandins, 19:976-984.

Cohen, G. (1977), In Defense of Haber-Weiss. In: Superoxide and Superoxide Dismutases. Michaelson, A.M., McCord, J.M., Fridovich, I. (eds.). New York, Academic Press pp. 323-334.

Cohen, G. The Fenton reaction. In: CRC handbook of Methods for oxygen radical research research. R. A Greenwald, editor. CRC Press, Inc., Boca Raton, FL pp. 55-64.

Cohen, G. (1978), The generation of hydroxyl radicals in biologic systems: Toxicological aspects. *Photochem. Photobiol.* 28: 669-675.

Cohen, G. and Cederbaum, A.I.(1980), Microsomal metabolism of hydroxyl radical scavenging agents relationship to microsomal oxidation of alcohol. *Arch. Biochem. Biophys.* 199, 438-447

Crapo, J.D. (1977) The Role of the Superoxide and Superoxide Dismutases in Pulmonary Oxygen Toxicity. In: Superoxide and Superoxide Dismutases. Michaelson, A.M., McCord, J.M., Fridovich, I. (eds.). New York, Academic Press: 231-239.

Cross, C. E. (1987), Oxygen radicals and human disease. *Ann. Intern. Med.* 107:525.

Crowley, C.A., Curnuttee, J.T., Rosin R. E., Andre-schwartz, J., Gallin, J.I., Klempner, M., Snyderman, R., Southwick, F.S., Stossel, T.P. and Babior, B.M. (1980), An inherited abnormality of neutrophil adhesion. *N. Engl. J. Med.* 302:1163-1168.

Cullen, B. F. and Haschke, R. H. (1974), Local anesthetic inhibition of phagocytes and metabolism of human leukocytes. *Anesthesiology,* 40:142-148.

Curnutte, J.T., Kuver, R. and Babior B.M. (1987), Activation of the respiratory burst oxidase in a fully soluble system from human neutrophils. *J. Blol. Chem.* 262:6450-6452.

Das, K. C. and Misra, H. P. (1992), Antiarrhythmic agents: scavengers of hydroxyl radicals and inhibitors of NADPH-dependent lipid

peroxidation in bovine lung microsomes. *J. Biol. Chem.*, 267:19172-19178.

Das, K. C. and Misra, H. P. (1992), Lidocaine: Hydroxyl radical scavenger and singlet oxygen quencher. *Mol. Cell. Biochem.*, in press.

Dempsey, R. J., Roy, M. W., Meyer, K., Cowen, D. E. and Tai, H. H. (1986), Development of cyclooxygenase and lipooxygenase metabolites of arachidonic acid after transient cerebral ischemia. *J. Neurosurg.* 64:118-124.

Drath, D.B., and Karnovsky, L. (1975), Superoxide production by phagocytic leukocytes. *J. Exp. Med.* 142:257-262.

Drath, D.B., Karnovsky, M. L. and Huber, G. L.(1979), Hydroxyl radical formation in phagocytic cells of the rat. *J. Appl. Physiol.* 46:136-140.

Dunn, P.A. and Tyrer, H.W.(1981), Quantitation of neutrophil phagocytosis using fluorescent latex beads. *J. Lab. Clin. Med.* 98:374-381.

Fee, J.A. and Valentine, J.S. (1977) Chemical and physical properties of superoxide. In: *Superoxide and Superoxide Dismutases.* Michaelson, A.M., McCord, J.M., Fridovich, I. (eds.). New York, Academic Press: 19-60.

Fenton, H. J (1895) Proc. Chem. Soc. (London) 9, 113

Fisher, A. B., Dodia, C., Tan, Z., Ayene, I., and Eckenhoff, R. G. (1991), Oxygen-dependent lipid peroxidation during lung ischemia. *J. Clin. Invest.* 88:674-679.

Fisher, A.B. and Dodia, C. (1981) The lung as a model for evaluation of critical intracellular pO_2 and pCO_2. *Am. J. Physiol.* 242:E47-50.

Fox, R. B., Harada, R. N., Tate, R. M. and Repine, J. E. (1983), Prevention of thiourea-induced pulmonary edema by hydroxyl-radical scavengers . *J. Appl. Physiol.* 55:1456-1459.

Freeman, B.A., Panus, P., Beckman, J.S., Matalan, S. (1986), in *The role of oxygen radicals in cardiovascular diseases* (L'Abbatt, A. and Ursini, F. Eds.)pp. 201-215, Kluwer Academic Press, London.

Frey, W. (1918), *Weitere Erfahrungen mit chinidin bei absoluter herzun-regel-massigkeit. Wein klin wchnschr, 55:849-853.*

Fridovich, I. (1979), Hypoxia and oxygen toxicity. *Adv. Neurol.* 26: 255-259.

Fridovich, I. (1976), Oxygen radicals, hydrogen peroxide and oxygen toxicity. In: *Free Radicals in Biology.* Vol 1, Pryor W.A. (ed), New York, Academic Press: 239-271.

Fridovich, I. (1978), The biology of oxygen radicals, *Science*, 201:875-880.

Fridovich, I. (1979) Superoxide and superoxide dismutases. In: *Advances in Inorganic biochemistry.* Eichhorn, G.L, Marzelli, L.G. (eds.). New York, Elsevier North Holland pp. 67-90.

Gallin, J.I., Sandler, J.A., Chyman, R.I., Mangniello, V.C. and Vaughan, M. (1982), Agents that increase cAMP inhibit accumulation of cGMP and depress human monocyte locomotion. *J. Immunol.* 128:492-495.

Gauduel, Y. and Duvelleray, M. A. (1984), Oxygen radicals in cardiac injury due to reoxygenation. *J. Mol. Cell. Cardiol.* 16: 458-470.

Gianelly, R., von der Groeben, J. O., Spivack, A. P. and Harrison, D.C. (1967), Effects of lidocaine on ventricular arrhythmias in patients with coronary heart disease. *New Engl. J. Med.* 277: 1215-1221.

Granger, D. N., Rutili, G., and McCord., J. M. (1981), Role of superoxide radicals in intestinal ischemia. *Gastroenterology,* 81:22-29.

Granger, D. N., Hollwarth, M. E. and Parks, D. A. (1986), Ischemia-Reperfusion injury: role of oxygen derived free radicals. *Acta. Physiol. Scand. Suppl.* 548:47-63.

Grisham, M.B. and McCord,J.M. (1986) Chemistry and cytotoxicity of reactive oxygen metabolites. *Physiology of Oxygen Radicals*, American Physiological Society Bethesda, Maryland,1-18.

Guarnieri, C., Flamigni, F., Caldarera, C. M. (1980), Role of oxygen in the cellular damage induced by reoxygenation of hypoxic heart. *J. Mol. Cell. Cardiol.* 12: 797-808.

Haber, F. and Weiss, J. (1934) Proc. R. Soc. A 147, 332-351

Halliwell, B. and Gutteridge, J. M. C. (1981), Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts. *FEBS Lett..*, 128: 347-351.

Halliwell, B., and Gutteridge, J.M.C. (1985) Oxygen is poisonous-an Introduction to oxygen toxicity and free radicals. In: *Free Radicals in Biology and Medicine.* Oxford, Clarendon Press: 1-19.

Halliwell, B., Gutteridge, J. M. C. and Aruoma, O. I. (1978), The deoxyribose method: A simple "Test-Tube" assay for determination of rate constants for reaction of hydroxyl radicals. *Anal. Biochem.* 165, 215-219

Harrison, D. C., Sprouse, J. H. and Morrow, A. G. (1963), Antiarrhythmic properties of lidocaine and procainamide: Clinical and physiologic studies of their cardiovascular effects in man. *Circulation* 28: 486-491, 1963.

Hartenstein, J. and Wagner, B. (1986), Antiarrhythmic agents: In cardiovascular drugs (Bistol, J.A. Ed.) pp 309-415, John Wiley & Sons, New York.

Hassan, H. M and Fridovich, I. (1979), Superoxide dismutase and its role for survival in the presence of oxygen. In: *Strategies of Microbial Life in extreme environments.* Shilo M (ed.). New York, Verlag Chemie, 1979: 179-193.

Haverich, A., Scott, W.C. and Jamieson, S.W.(1985), Twenty years of lung preservation: a review. *J. Heart Transplant.* 4:234-240.

Hearse, J. D., O' Brian, O. K., Braimbridge, M. (1981), Protection of the myocardium during ischemic arrest. *J. Thorac. Cardiovasc. Surg.* 81: 873-879.

Hess, M. L., Manson, N. H., and Okabe, E. (1982), Involvement of free radicals in the pathophysiology of ischemic heart disease. *Can. J. Physiol. Pharmacol.* 60: 1382-1389.

Hoidal, J.R., White, J.G. and Repine, J.E. (1979), Influence of cataionic local anesthetics on the metabolism and ultrastructure of human alveolar macrophages. *J. Lab. Clin. Med* 93:857-866.

Hullanger, G., (1960), On the metabolism of lidocaine. I. The properties of the enzyme system responsible for the oxidative metabolism of lidocaine. *Acta. Pharmacol. Toxicol.,* 17:356-365.

Interian, A., Jr., Zaman, L., Velez-Robinson, E., Kozlovskis, P.,Castellanos, A., Myerburg, R.J. (1991), Paired comparisons of efficacy of intravenous and oral procainamide in patients with inducible sustained ventricular tachyarrhythmias. *J. Am. Coll. Cardiol.* 17, 1581-1586

Jackson, R. M., Veil, C.F., Alexander, C.B., Brqnnen, A.L. and Fulmer, J.D. (1988) Reexpansion pulmonary edema. A potential role for free radicals in its pathogenesis. *Am. Rev. Respir. Dis.* 137:1165-1171.

Jessaitis, A. J., Quinn, M.T., Mukherjee, G., Ward, P.A., and Dratz, E. A. (1991), The molecular basis of oxidative damage by leukocytes. In: *Molecular*

Basis of Oxidative Damage By Leukocytes. pp1-8 (Jessaitis, A. J.and Dratz, E. A., eds.), CRC Press, Boca Raton

Jewitt, D. E., Kishon, Y. and Thomas, M. (1968), Lignocaine in the management of arrhythmias after acute myocardial infraction. *Lancet* 2: 266-268.

Johnston, R.B.,Jr., Keele, B.B.,Jr., Misra, H.P., Lehmeyer, J.E., Webb, LS., Baehner, R.L. and Rajgopalan, K.V.(1975), The role of superoxide anion genaration in phagocytic bactericidal activity: studies with normal and chronic granulomatous disease leukocytes. *J. Clin. Invest.* 55:1357-1372.

Jurmann, M.J., Dammenhayn, L., Schaefers,H. J, and Haverich, A. (1990), Pulmonary reperfusion injury: evidence for oxygen-derived free radical mediated damage and effects of different free radical scavengers. *Eur. J. cardio-thorac. Surg.* 4:665-670.

Kako, K.J.(1987), Free radical effects on membrane protein in myocardial ischemia/reperfusion injury. *J. Mol. Cell. Cardiol.* 19, 209-211

Kalyanaraman, B: Detection of toxic free radicals in biology and medicine. In: *Reviews in Biochemical Toxicology* (Eds. Hodgson, E., Bend, JR. and Philpot, RM.) pp 73-139, Elsevier Biomedical, New York.

Keith, J. C, Jr., (1986), Effect of lidocaine pretreatment on acute haemorragic shock in the anesthetized rat. *Circ. Shock,* 19:283-292.

Kennedy, T., Rao., N.V., Hopkins, C., Tolhey., E. and Hoidal, J.(1988), Reperfusion injury occurrs in the lung by free-radical mechanism. *Chest,* 93:149S.

Kennedy, T. P., Rao, N.V., Hopkins, C., Pennington, L. Tolley, E. and Hoidal, J. R. (1989), Role of reactive oxygen species in reperfusion injury of the rabbit lung. *J. Clin. Invest.* 83:1326-1335.

Khan, A.U. (1978), Activated oxygen : Singlet molecualr oxygen and superoxide anion, *Photochem. Photobiol.* 28:615-627.

King, M. M., Lai, E.K., and McCay, P.B.(1975) Singlet oxygen production associated with enzyme catalyzed lipid peroxidation in liver microsomes. *J. Biol. Chem.* 250, 6496-6502

Koyama, I., Toung, J. K. T., Rogers, M. C., Gurtner, G. H., and Traystman, R. J. (1987), O_2 radical mediate reperfusion lung injury in ischemic O_2 ventilated canine pulmonary lobe. *J. Appl. Physiol.* 63:111-115.

Krebs, E. G, and Beavo, J.A. (1979), Phosphorylation-dephosphorylation of enzyme. *Annu. Rev. Biochem*, 48:923-925.

Kronbrust, D.J and Maris, R.D., (1980), Microsomal lipid peroxidation I. Characterization of the role of iron and NADPH. *Molec. Pharmac.* 17:400-407

Kukiela, E. and Cederbaum, A.I. (1989) NADH-dependent microsomal interaction with ferric complexes and production of reactive oxygen intermediates. *Arch. Biochem. Biophys.*. 275, 540-550.

Kunze, H. E., Bohn, E. and Vogt, W. (1974), Effects of local anesthetics on prostaglandin biosynthesis in vitro. *Biochim. Biophys. Acta.* 360:260-269.

Lai, C.S. and Piette, L.H. (1977), Hydroxyl radical production involved in lipid peroxidation of rat liver microsomes. *Biochem. Biophys. Res. Commun.* 78:51-59

Lai, C.S. and Piette, L.H. (1978), Spin-trapping studies of hydroxyl radical production involved in lipid peroxidation. *Arch. Biochem. Biophys.* 190: 27-38

Lehmeyer, J.E., and Johnston,Jr, R.B.(1978), Effect of anti-inflammatory drugs and agents that elevate intracellular cAMP on the release of toxic oxygen metabolites by phagocytosis: studies in a model of tissue-bound Ig. *Clin. Immunol. Immunopathol.* 9:482-490.

Lesnefsky, E. J., VanBenthuysen, K.M., McMurtry, I.F., Shikes, R.H., Johnston, R.B, Jr., and Horwitz, L. D. (1989), Lidocaine reduces canine infarct size and decreases release of a lipid peroxidation product. *J. Cardio. Pharmacol.* 13:895-901.

Ljungman, G. A., Grum, C. M., Deeb, G. M, Bolling, S. F. and Morganroth, M. L. (1991), Inhibition of cyclooxygenase metabolite production attenuates ischemia-reperfusion lung injury. *Am. Rev. Respir. Dis.* 143:610-617.

Lucchesi, B. R. and Patterson, E. S. (1984), Antiarrhythmic drugs: In *Cardiovascular Pharmacology,* (Antonaccio, M. Ed.), pp 329-414, Raven press, New York.

Lynch, M. J., Grum, C. M., Gallagher, K. P., Bolling, S. F., Deeb, G. M., Morganroth, M. L.(1988), Xanthine oxidase inhibition attenuates ischemic-reperfusion lung injury. *J. Surg. Res.* 44:538-544.

Mak, I. T. and Weglicki, W. B. (1988), Protection by B-Blocking agents against free radical-mediated sarcolemmal lipid peroxidation., *Circ. Res.* 63:262-266.

Manning, A. S., Coltart, J. D. and Hearse, D. J. (1984), Ischemia and reperfusion-induced arrhythmias in the rat. *Circ. Res.* 55: 545-550.

Martin, D., Allison, R.C. and Taylor, A.E.(1986) in *Physiology of oxygen radicals* (Taylor, A.E., Matalon, S and Wand,P.A.,Eds) pp. 131-143, American Physiological Society, Bethesda, Maryland.

Martin, D., Korthuis, R. J.,Perry, M., Townsley, M. I. and Taylor, A. E.(1986), Oxygen radical mediated lung damage associated with α-napthylthiourea. *Acta Physiol. Scand. Suppl.* 548:119-125.

Marubayashi, S., Dohi, K., Ezaki, H., Yamada, K. and Kawasaki, T.(1983), Preservation of ischemic liver cells prevention of damage by coenzyme Q10. *Transplant Proc.* 15:1297-1299.

Mautz, F. R.(1936), The reduction of cardiac irritability by epicardial and systemic administration of drugs as a protection in cardiac surgery. *J. Thorac. Surg.* 5:612-628.

McCord, J. M. and Fridovich, I. (1968), The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* 243: 5753-5760.

McCord, J. M, Roy R.S. (1982), The pathophysiology of superoxide: Roles in inflammation and ischemia. *Can. J. Physiol. Pharmacol.* 60: 1346-1352.

McCord, J. M..(1985), Oxygen-derived free radicals in post-ischemic tissue injury. *N. Engl. J. Med.* 312:159-163.

McCord, J.M. (1979) ,Superoxide, superoxide dismutase and oxygen toxicity. In: *Reviews in Biochemical Toxicology.* Hodgson, Bend Philpot (eds.). Elsevier North Holland, pp. 109-123.

McCord, J.M. and Fridovich, I. (1969), Superoxide dismutase. An enzymatic function of erythrocuprein (hemocupreine). *J. Biol. Chem.* 244: 6049-6055.

McMurtry, I. F., Davidson, A. B., Reeves, J. T. and Grover, R. T. (1976), Inhibition of hypoxic vasoconstriction by calcium antagonist in isolated rat lungs. *Circ. Res.* 38:99-104.

Meerson, F. Z., Kagan, V.E., Kuzlov, Yu. P., Belkina, L.M and Arkhipenko, Yu.V. (1982), The role of lipid peroxidation in pathogenesis of ischemic

damage and the antioxidant protection of the heart. *Basic Res. Cardiol.* 77: 465-485

Misra, B.R. and Misra, H.P. (1990), Vasoactive intestinal peptide, a singlet oxygen quencher. *J. Biol. Chem.* 265: 15371-15374.

Misra, H. P., Weglicki, W. B., Abdula, R. and McCay, P. B. (1984), Identification of a carbon-centered free radical during reperfusion injury in ischemic heart. *Circulation*, 70: II-260 .

Misra, H. P. (1974), The generation of superoxide radical during autoxidation of thiols. *J. Biol. Chem.* 249: 2151-2155.

Misra, H. P. and Fridovich, I. (1971), The generation of superoxide radical during autoxidation of ferredoxins. *J. Biol. Chem.* 146: 6886-6890.

Misra, H. P. and Fridovich, I. (1972a), The univalent reduction of oxygen by reduced flavins and quinones. *J. Biol. Chem.* 147: 188-192.

Misra, H. P. and Fridovich, I. (1972b), The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247: 3170-3175.

Misra, H. P. and Fridovich, I. (1972c), The generation of superoixde radical during the autoxidation of hemoglobin. *J. Biol. Chem.* 247: 6960-6962.

Misra, H.P. and Gorsky, L.D.(1981) Paraquat and NADPH-dependent lipid peroxidation in lung microsomes. *J. Biol. Chem.* 256, 9994-9998

Moorehouse, C.P., Grootveld, M., Halliwell, B., Quinlan, G. J. and Gutteridge, J.M.C. (1987) *FEBS Lett.* 213:23-28.

Moss, J. A. and Patton, R. D. (1973). Antiarrhythmic agents, pp 159, Charles C. Thomash, Publishers, Springfield, Illinois

Myers, L.S., Jr. (1973), Free radical damage of nucleic acids and their components by ionizing radiation. *Fed. Proc.* 32:1882-1894.

Myers, M.A., McPhail, L. C. and Snydrman, R. (1985)Redistribution of protein kinase C activity in human monocytes: correlation with activities of the respiratory burst. *J. Immunol.* 135:3411-3416.

Narang, K. P., Crouthamel, W. G., Carliner, H. N., Fisher , M. L. (1978), Lidocaine and its active metabolites. *Clin. Pharmacol. Ther*, 12: 654-662.

Nasser, F. N, Walls, J. T, Edwards, W. D. and Harison, C. E, Jr. (1980), Lidocaine-induced reduction in size of experimental myocardial infraction. *Am. J. Cardiol.* 46: 967-975.

Neta, P. and Dorfman, L. M. (1968) *Adv. Chem. Ser.* 81, 222-230

Niki, E, Yamamoto, Y. and Kamiya, Y. (1988), Inhibition of Peroxidations of liposomal and biomembranes by water soluble antioxidants. In: *Lipid Peroxidation in Biological Systems* (Ed. Sevanian, A.), pp 32-50, American Oil Chemists Society, Champaign, Illinois.

Nishikimi, M. and Yogi, K. (1977), Oxidations of ascorbic acid and α–tocopherol by superoxide. In: *Biochemical and Medical Aspects of Active Oxygen,* edited by Hayaishi, O and Asada, K. Tokyo, Univ. of Tokyo Press, p. 79-87.

Nishizuka, Y. (1984), The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature,* 308:693-698.

Oren, R., Farnham, A.E., Saito, K., Milofsky, E. and Karnovsky, M.L. (1963), Metabolic patterns in three types of phagocytosing cells. *J. Cell. Biol.* 17:487-490.

Otamiri, T. (1989), Oxygen radicals, lipid peroxidation, and neutrophil infiltration after small-intestinal ischemia and reperfusion. *Surgery* 105:593-597

Pallar, M. S. , J. R. Hoidal, and T. F. Ferris.(1984), Oxygen free radicals in ischemic acute renal failure in rats. *J. Clin. Invest.* 74:1156-1164.

Pangaramala, R.V., Sharma, H.V., Heikkila, R.E., Geer, J.C. and Cornwell, D.G. (1976) Prostaglandins. 11:599-607

Parks, D. A. and Granger, D. N. (1986), Xanthine oxidase: biochemistry, distribution and physiology. *Acta. Physiol. scand. (Suppl.)* 548:87-99.

Parks, D. A., Bulkley, G. B., Granger, D.N., Hamilton, S.R. and McCord., J. M.. (1982), Ischemic injury in the small intestine: Role of superoxide radicals. *Gastroenterology* 82:9-15.

Patel, K. B. and Wilson, P. L. (1973), Semiquinone free radicals and oxygen. *J. Chem. Soc. Faraday Trans.* 1: 814-825.

Peck, S.L, Johnston, R.B.,Jr., Horwitz, L.D.(1985), Reduced neutrophil superoxide anion release after prolonged infusions of lidocaine. *J. Pharmacol. Exp. Ther.* 235:418-422.

Pfeiffer, C. J, Keith, J. C, Jr., Cho, C. H, DeRolf, S., Pfeiffer, D. C. and Misra H. P. (1989), Gastric and cardiac protection by lidocaine. *Acta Phys. Hung.* 73:129-136.

Pfenning, N. (1979) Formation of oxygen and microbial processes establishing and maintaining anaerobic environments. In: *Strategies of Microbial LIfe in Extreme Environments.* Shilo, M (eds). New York, Verlag Chemie:137-148.

Philips, A.H. and Langdon, R.G.(1962), Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. *J. Biol. Chem.* 237: 2652-2660.

Pick, E., Bromberg, Y., Shpungin, S. and Gabda, R. (1987), Activation of the superoxide forming oxidase in a cell free system by sodium dodecyl sulphate, characterization of the membrane associated component. *J. Biol. Chem.* 262:16476-16483

Puntarulo, S. and Cederbaum, A.I. (1988), Comparison of the ability of ferric complex to catalyze microsomal chemiluminescence, lipid peroxidation and hydroxyl radical generation. *Arch. Biochem. Biophys.* 264,482-491.

Pyror, W. A. (1973), Free radical reactions and their importance in biological systems. Fed. Proc. 32:1862-1868.

Pryor, W. A. (1977), The involvement of free radical reactions in aging and carcinogenesis. In: Medicinal Chemistry V, Mathieu, J. Ed., Elsevier, Amsterdam, pp. 331-359.

Quintiliani, M., Badiello, R., Yamba, M., Esfondi, A and Garin, G.(1977), Radiolysis of glutathione in oxygen-containing solution of pH 7. *Int. J. Radiat. Biol.* 32: 195-202.

Rao, P. S. and Mueller , H. S. (1983), Lipid peroxidation and myocardial ischemia. In: *Myocardial injury* (Eds. Spitzer, J. J.),pp341-367. Plenum Publishing Co, New York.

Ratych, R. E., Chuknyiska, R. S., and Bulkley, G. B. (1987), The primariy localization of free radical generation after anoxia-reoxygenation in isolated endothelial cells. *Surgery,* 102:122-131.

Repine, J.E., White, J.G., Clawson, C.C. and Holmes, B.M.(1974), The influence of phorbol myristate acetate on oxygen consumption by polymorphonuclear leucocytes. *J. Clin. Med.* 83:911-920.

Rosen, G. M. and Rauckman, E. (1981), The spin trapping of biologically generated free radicals. In: Oxygen and oxy-radicals in chemistry and biology, Academic Press, Inc. New York.

Rossi, F., Romeo, D., and Patriarca, P. (1972), Mechanism of phagocytosis associated oxidative metabolism in polymorphonuclear leucocytes and macrophages. *J. Reticuloendothelial. Soc.* 12: 127-149.

Salin, M.L. and McCord, J. M. (1975) Free Radicals and Inflammation. *J. Clin. Invest.* 56: 1319-1323.

Sawyer, D. T. and Valentine, J. S. (1981), How super is superoxide? *Acc. Chem. Res.* 14: 393-400.

Schaub, R. G., Lemole, M. G., Pinder, G.C., Black, P. (1977), Effects of lidocaine and epinephrine on myocardial preservation following cardiopulmonary bypass in the dog. *J. Thorac. Cardiovasc. Surg.* 74: 571-576.

Schaub, R. G., Stewart, G., Strong, M., Ruofolo, R., Lemole, G. (1977), Reduction of ischemic myocardial damage in the dog by lidocaine infusion. *Am. J. Pathol*, 87: 388-414.

Scherphof, G. L., Scarpa, A., and Van Toorenbergen, A. (1972), The effects of local anesthetics on the hydrolysis of free and membrane bound phospholipids catalyzed by various phospholipases. *Biochim. Biophys. Acta.* 270: 226-240.

Seeman, P. (1972), The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* 24:583-646.

Sha'ag, D. and Pick, E. (1988), Macrophage-derived superoxide-generating NADPH Oxidase in an amphile-activated, cell free system; partial purification of the cytosolic component and evidence that It may contain the NADPH binding site. *Biochim. Biophys. Acta,* 952:213-219, 1988.

Sha'ag, D.(1989), Sodium dodecyl sulphate dependent NADPH oxidation: an alternative method for assaying NADPH Oxidase in a cell free system. *J.Biochem. Biophys. Meth.* 19:121-128.

She, Z. W, Liming, J. D, Fagan, J. B, Pacht, E. R, and Davis, W. B. (1991), Inhibition of hypochlorous acid by lidocaine and native components of alveolar epithelial lining fluid, *Am. Rev. Respir. Dis.* 144:227-230.

Shlafer, M., Kane, P.F and Kirsh, M. M. (1982), Superoxide dismutase plus catalase enhances the efficacy of the hypothermic cardioplegia to

protect globally ischemic, reperfused heart. *J. Thorac. Cardiovasc. Surg.* 83: 830-839.

Simpson, P. J., Todd, R. F. III., Fantone, J. C., Mickelson, J. K., Griffin, J. D. and Lucchesi, B. R.(1988), Reduction of experimental canine myocardial reperfusion injury by monoclonal antibody (anti-mol, anti-CD116) that inhibits leukocyte adheson. *J. Clin. Invest.* 81:624-629.

Smolen, J.E., Korchak, H.M. and Weissman, G. The role of extracellular and intracellular calcium in lysosomal enzyme release and superoxide anion generation by human polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* 677:512-520.

Southworth, J. L., McKusick, V. A., Pierce, E. C., (1950), Ventricullar fibrillation precipitated by cardiac catheterization. Complete recovery of a patient after forty-five minutes, JAMA, 143:217-230.

Steinberg, H., Greenwald, R.A., Sciubba, J. and Das, D.K.(1982), The effect of oxygen-derived free radicals on pulmonary endothelial cell function in the isolated perfused rat lung. *Exp. Lung Res.* 3:163-173.

Stelzner, T. J., Welsh, C. H., Berger, E., McCullough, R. G., Morris, K. Repine, J. E. and Weil,. J. V. (1987), Antiarrhythmic agents diminish thiourea-induced pulmonary vascular protein leak in rats. *J. Appl. Physiol.* 63:1877-1883, 1987.

Stephens, C.G., and Snyderman, R.(1982) Cyclic nucleotides regulate the morphologic alterations required for chemotaxis in monocytes. *J. Immunol.* 128:1192-1197.

Suttorp, H., and Simon, L.M. (1982), Lung cell oxidant injury: enhancement of polymorphnuclear leukocyte mediated cytotoxicity in lung cells exposed to sustained in vitro hyperoxia. *J. Clin. Invest.* 70;342-350.

Svingen, A. S, O' Neal, F. O. and Aust, S. D. (1978), The role of superoxide and singlet oxygen in lipid peroxidation. *Photochem. Photobiol.* 28: 803-809.

Svingen, B.A. and Aust, S.D. (1980) in *Molecular basis of environmental toxicity* (Bhatnagar, R.S., Ed) pp. 69-110, Ann Arbor Science Publishing, Ann Arbor, MI

Tate, R. M., Vanbenthuysen, K. M., Shasby, D. M., McMurtry, I. F. and Repine, J. E. (1982), Oxygen radical mediated permeability edema and vasoconstriction in isolated perfused rabbit lungs. *Am. Rev. Respir. Dis.* 126:802-806.

Thet, L.A. (1986), Repair of Oxygen Induced Lung Injury, In: *Physiology of Oxygen Radicals,* American Physiological Society Bethesda, Maryland,87-108.

Thurman, R.G., Ley, H.G., and Scholz, R. (1972), Hepatic microsomal ethanol oxidation hydrogen peroxide formation and the role of catalase. *Eur. J. Biochem.* 25: 420-430

Tomoda, M., Ueda, W., Tsuchiya, M., and Hirakawa, M.(1992), Effects of lidocaine on stimulation coupled responses of neutrophils and protein kinase c activities. *Masui,* 41:369-375.

Tyler, D.D. (1975), Role of superoxide radicals in the lipid peroxidation of intracellular membranes. *FEBS Lett.* 51, 180-183

Umeki, S. and Soejima, R.(1990), Hydrocortisone inhibits the respiratory burst oxidase from human neutrophils in whole cell and cell free systems. *Biochim. Biophys. acta.* 1052:211-215.

Undrovinas, A.I., Burnashev, N., Eroshenko,D., Fleidervish,I., Starmer,C.F., Makielski,J.C. and Rosenshtraukh, L.V.(1990) *Am. J. Physiol.* 259: H1609-12

Vaughan W. E. M., (1975), *Pharmacol. Theraup. B.*, 1, 115.

Walling, C., Partch, R. E. and Weil, T. (1975), Kinetics of the decomposition of hydrogen peroxide catalyzed by ferric ethylenediaminetetraacetate complex. *Proc. Natl. Acad. Sci.* U.S.A. 72, 140-142.

Weisfeldt, M. L.(1987), Reperfusion and reperfusion injury. *Clin. Res.* 2:311-319.

Weiss, J. (1947), *Disc. Faraday Soc.* 2, 217-218..

Weiss, J. J. (1964), Nature of the iron-oxygen bond in oxyhaemoglobin. *Nature* 203: 183

Weiss, S.J., Young, J.,Lobuglio, A.F., Slivka, A. and Nimeh, N.F. (1981), Role of hydrogen peroxide in neutrophil mediated destruction of cultured endothelial cells. *J. Clin. Invest.* 68:714-721.

Weissman, G., Smolen, J.E. and Korchak, H.K. (1980), Release of inflammatory mediators from stimulated neutrophils. *N. Engl. J. Med.* 303:27-34.

West, J.B. (1979), *Respiratory Physiology,* Baltimore, Williams and Wilkins, pp 169.

Wielding, S. (1964),. Xylocaine: The pharmacologic basis of its clinical use, 2nd Ed. Stockholm, Almquist & Wilksell.

Wills, E.D. (1969) Lipid peroxide formation in microsomes. *Biochem. J.* 113, 315-324

Woodward B, Zakaria M. (1984), Effects of some free radical scavengers on reperfusion induced arrhythmias in the isolated rat heart. *J. Mol. Cell. Cardiol.* 16: 497-517.

Yamamoto, Y. and Kamiya, Y. (1988) in *Lipid Peroxidation in Biological Systems* (Sevanian, A. Ed) pp 32-50, American Oil Chemists Society, Champaign, Illinois.

Yonetani, T. (1965), Studies on cytochrome c peroxidase.II stoichiometry between enzyme, H_2O_2, and ferricytochrome c and enzymatic determination of extinction coefficients of cytochrome c. *J. Biol. Chem.* 240:4509-4514, 1965.

Zang, L., Zhang, Z., and Misra, H. P. (1990), EPR studies of trapped singlet oxygen ($^1O_2$) generated during photoirradiation of Hypocrellin A. *Photochem.Photobiol.* 52: 677- 684.

Zweier, J. L., Flaherty, J.T., and Weisfeldt, M.L. (1987), Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc. Natl. Acad. Sci.* 84:1404-1407.

Zweier, J. R.(1988), Measurement of superoxide-derived free-radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. *J. Biol. Chem.* 263:1353-1357

VITA

The author was born on September 9, 1954 in Orissa, India. He went to high school at various places in Orissa. In 1975 he obtained his B.Sc (Honors) with distinction in Zoology from Utkal University, Bhubaneswar, Orissa. He obtained his M.Sc in Zoology specializing in Cell biology in 1978 from Gujarat University, Ahmedabad, India. In 1979 he was selected in an all India competitive basis to join Indian Forest Service. In 1981, while in Forest Service, he earned a Master's in Forestry from the Indian Forest Research Institute, Dehradun, India. In 1986 he obtained a post graduate diploma in Wild Life management from Wildlife Institute of India, Dehradun, India. In 1989 spring, he joined graduate program in Department of wildlife at Virginia Tech on a study leave from Indian Forest Service. Later, in 1990 summer he joined in the Graduate program of Veterinary Medical Sciences. He is married to Rita and they have two sons living with him at Blacksburg, Virginia

PUBLICATIONS:

1.Das, K. C. and H.P. Misra 1992. The role of oxyradicals during post-ischemic reperfusion injury in isolated perfused rat lung and amelioration by antiarrhythmic drugs, Submitted to American Journal of Physiology.

2. Das, K. C. and H.P. Misra, 1992. Antiarrhythmic agents: Hydroxyl radical scavengers and inhibitors of NADPH-dependent lipid peroxidation in bovine lung microsomes, Journal of Biological Chemistry , 267:19172-19178.

3. Das, K. C. and H.P. Misra.,1992 Lidocaine, a hydroxyl radical scavenger and singlet oxygen quencher. Molecular and Cellular Biochemistry, in press

4. Das, K. C. and H.P. Misra, 1992. Prevention of ischemia-reperfusion injury in a hyperoxic ventilated perfused rat lung model . Submitted to American Review of Respiratory Diseases.

5. Das, K. C. and H.P. Misra, 1992. Effect of antiarrhythmic drugs on oxidative burst of macrophage cell line RAW 264.7, in preparation.

Abstracts

1. Das, K. C. and Misra, H.P. 1991.Lidocaine, a hydroxyl radical scavenger and singlet oxygen quencher. *Presented at the Third International Symposium on lipid metabolism in the normoxic and ischemic heart, Erasmus University, Rotterdam, The Netherlands September 9-10, 1991*

Kumuda C. Das.
October 29, 1992.