

OXYGEN STRESS AND TISSUE DAMAGE



Editors:

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Edited by

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Preface

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Oxidative stress, that is, the free radical reaction plays an important role in both the physiological and pathological conditions of living organisms involving plants, animal and human cells. The intracellular generation of free radicals is an integral part of a number of physiological processes. They are involved in arachidonic acid metabolism, in the microsomal functions or in the mitochondrial electron transport chain during oxidative phosphorylation, furthermore in the oxygen dependent killing mechanism of the phagocytes, in the immune reactions as well as in the reproduction and in the embryonic development.

Pathologic free radical reactions undoubtedly play a role in inflammation processes, ischaemia-reperfusion-induced damage, in hyperlipidaemia, in arteriosclerosis, in diabetes, in the malignant and autoimmune diseases, and in the ageing process.

All reactive oxygen species formed in cells including singlet oxygen can oxidize a number of cellular constituents like lipids, proteins, and DNA. The oxidation of lipids occurs in membranes, especially the intracellular membranes. During lipid peroxidation many different products, e.g., aldehydes, peroxides, and free radicals, are formed. The direct effect of lipid peroxidation is the destruction of the membrane. Secondary effects include the interaction of lipid peroxidation reaction products with other cellular components, e.g., the reaction of aldehydes with DNA, which can result in mutagenicity or carcinogenicity. On the other hand, the oxidation of proteins leads to enzyme inactivation and thereby to toxicity. The direct oxidation of DNA by highly reactive oxygen species gives oxidation products like hydroxylated bases, and the oxidation of the sugar moiety leads to strand breaks of DNA.

This book deals with some of the above-mentioned biological alterations produced by different environmental materials as well as by drugs used in the medical therapy (e.g. amiodarone). The authors discuss the preventive and therapeutic modalities of these phenomena by antioxidative treatment.

We recommend this book to researchers on this topic as well as to specialists of the praxis either in agriculture or in veterinary, and to therapists.

Editors

The Importance of Free Radical Scavengers in the Treatment of Gastrointestinal Disorders

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Abstract

The protection provided by the natural antioxidant system seems to be inappropriate in certain diseases of the gastrointestinal mucus membrane, such as chronic inflammation, immune and malignant diseases of the bowel. Despite the wide range of therapeutic attempts, the treatment of these diseases has not yet been solved, and the anti-inflammatory, immuno-modulatory, and cytostatic drugs currently used, have several side effects. Therefore, the so called natural and synthetic antioxidants have been more widely employed for additional and adjuvant treatment. It also has become clear that the direct free radical scavenging effect and/or the membrane protection play an important role in the action mechanism of several old-established drugs. The recent report summarizes those natural and synthetic antioxidants which have been successfully employed in the clinical treatment of different bowel diseases. Among the natural antioxidants, a protective effect of vitamins A, C and E have been demonstrated in the treatment of gastro-duodenal ulcer and gastric cancer. Among the synthetic antioxidants, 5-amino-salicylic acid was the most effective drug in the treatment of chronic inflammations of the bowel. By getting a more precise knowledge about the details of free radical reactions and by clearing up their role in different pathological processes, it is possible to develop new synthetic antioxidants, and to use them effectively in the treatment of bowel diseases.

Introduction

In human organism, like in any biological system, free radical reactions play an important role in cell-regulation and provide protection against bacteria and other pathogen microorganisms. In certain conditions, however, free radical reactions may be potentially harmful even for the cells. The uncontrolled release and accumulation of free radicals, such as superoxide anion, hydroxyl anion, may lead to

cell-membrane damage and cell-death, due to lipidperoxidation (LPO) and/or direct protein and DNA-damage [1].

Since the release and accumulation of free radicals is metabolism-dependent, the damage caused via this mechanism can be important at places with increased metabolism such as the gastrointestinal mucus membrane. A large amount of free radicals can be released in inflammations of the bowel. A large amount of superoxide anion is released into the extracellular space from phagocytes accumulated at the site of inflammation. Inside the cells, the toxic metabolites produced by the imbalance of cell-metabolism will induce an accumulation of free radicals which may also be responsible for the development of malignant cells.

The natural antioxidant system of the gastrointestinal tract

To protect against the potentially harmful effects of free radicals, all eukariotic organisms have developed a complex and effective system, the so called natural antioxidant system [1]. The intracellular part of this scavenger system is responsible for the protection of the internal millieau, and consists of different enzymes, such as superoxide dismutase (SOD), catalase, glutathion peroxidase and the sulphhydryl groups of other proteins. These enzymes and proteins can also be found in the parietal cells of the gastrointestinal system. The most effective and important protecting molecule in the membrane, is the lipid soluble vitamin E. Vitamin A, having a similar chemical structure to vitamin E, also has its important role.

The protection of the extracellular space, such as the gastrointestinal mucus membrane is extremely weak against free radicals when compared to that of the intracellular millieau. Although the human plasma has its scavengers, such as ceruloplasmin, albumin, albumin-bound bilirubin, vitamin C, transferrin, haptoglobin and hemopexin, the concentrations of these components are very low, almost negligible on the surface of the gastrointestinal mucus membrane. [2].

Being ever so complex the natural antioxidant system, the protection provided by it seems to be insufficient in certain diseases of the gastrointestinal tract, such as chronic inflammations, immune and malignant diseases [1]. Despite the wide range of therapeutic approaches, the treatment of these diseases has not yet been solved, and the immuno-modulatory and cytostatic drugs currently used have several side-effects. Therefore, more and more natural and synthetic scavengers have become widely employed for adjuvant treatment. In addition, very recently became clear that the direct free radical scavenger and/or membrane protective effect plays an important role in the action mechanism of many old-established drugs. Since the too much high concentration of antioxidants can also be potentially harmful for the organism by causing hypoxia, the optimal treatment of choice for normalizing the impaired scavenger concentration would be the selective induction of the intracellular enzyme production at the site of damage inside the cell. This goal, however, could not yet been achieved. Therefore, most of the therapeutic trials have been concentrated on to improve and/or normalize the pathologically impaired extracellular scavenger system. The limitations in the therapeutic use of large-

molecular weight natural antioxidants, such as SOD, catalase, are in their poor absorbency by oral administration and in the rapid clearance from the circulation by intravenous use. Therefore, the low-molecular weight synthetic antioxidants have been more widely employed.

In this study, we concentrate on those natural and synthetic antioxidants which have been successfully employed in the clinical treatment of gastrointestinal diseases.

Free radical scavengers in the treatment of stomach diseases

An elevated LPO has been observed in the serum of patients with duodenal or gastric ulceration, as an indirect proof for the damaging role of free radicals. Almost all of the drugs used in the treatment of these diseases, also exert some scavenger activity. These drugs are the H_2 -receptor antagonist, Cimetidin; Gapona which consists of the mixture of trantelium bromide, benactyzine and the Ca^{++} antagonist, Halidor; cyproheptadine, Peritol; and the beta-blocker, Propranolol [1]. It is well known that vitamin E deficiency may cause ulcer in the gastroduodenal tract, and it has been proved that the treatment with vitamin E has a beneficial effect on the healing rate of patients with ulcer disease [3]. Hungarian authors have demonstrated the protective effect of vitamin A in certain experimental damage of the mucus membrane [4]. Dimethylsulfoxide (DMSO), one of the best known effective scavenger of hydroxyl radicals, has been effectively used in the prevention of the development of stress-ulceration [5]. It has to be noted that in higher doses, DMSO has toxic side effects, therefore, its plasma concentration has to be controlled carefully.

Gastric cancer is still one of the most frequently observed cancer, and the second leading cause of cancer-death in many countries. A recently finished prospective study investigated the effect of nutrition habits on the prevalence of gastric cancer in 1016 patients. The amount of free radical scavengers, vitamins E, C and A in the food had a significant impact on the prevalence of gastric cancer. Gastric cancer was 5 times as frequent in patients on high nitrate, protein and low vitamins E, C and A diet as compared with patients on low nitrate, protein and high vitamins E, C and A diet [6]. In another study, 197 patients with gastric cancer received vitamins C, E and A as antioxidants preoperatively. The postoperative complication rate in these patients decreased to 1.9% as compared to 30.9% of that of the control group [7].

Based on the above results, natural antioxidant vitamins should be more widely used in the effective prevention of the development of gastric cancer.

Free radical scavengers in the treatment of inflammations of the small and large intestine

The first drug of choice in the treatment of chronic inflammatory bowel diseases, such as colitis ulcerosa and Crohn's disease is still sulphasalazine (SASP) originally developed by Svartz 50 years ago [8]. Experiments in the 70's made clear that the biologically active metabolite is not SASP, but one of its metabolite, 5-amino-

salicylic acid (5-ASA) [9]. Studies to clarify the action-mechanism of SASP has cleared that SASP and its metabolites have a significant free radical scavenging activity, and the clinically most effective metabolite, 5-ASA is the strongest in vitro antioxidant [10]. Our own studies using the electron spin resonance method, proved that 5-ASA is the most effective in vitro scavenger of superoxide [11] and hydroxyl radicals [12]. Since the concentration of 5-ASA reaches the millimolar level in the distal lumen of bowel, the drug can exert its direct free radical scavenging activity even in in vivo conditions [13]. There are indirect proofs on that 5-ASA exerts the mucus-membrane protective effect through its free radical scavenging activity; All the other steroidal and non-steroidal anti-inflammatory drugs damage the mucus membrane, and one of their most common side-effect is that they cause erosions, bleedings of the mucus membrane. Although these drugs have a similar chemical structure to 5-ASA, the main difference in their biological effect is that unlike 5-ASA, these drugs have neither an in vitro scavenger activity, nor their concentrations in the plasma and bowel reach the levels necessary to effectively scavenge free radicals in vivo.

Future possibilities of antioxidants in the treatment of ischemic bowel diseases

It is known that the xanthine oxidase-catalyzed superoxide production is responsible for the ischemia-reperfusion damage of the small intestine. The indirect proof for the involvement of free radical-related mechanism in this process, is that the experimentally induced necrosis of the small intestine could be significantly reduced and/or prevented by the previous perfusion of SOD [14]. Based on animal experiments, the administration of SOD and catalase is beneficial in the prevention of ischemia-reperfusion damage of the small intestine, therefore, antioxidants can be recommended even in the human. However, further human experiments are also necessary.

Summary

With the more precise knowledge about the details of free radical reactions and with the understanding of their role in different pathological processes, it is possible the more wider therapeutic use of free radical scavengers in the practice. The beneficial effects of natural antioxidants, such as vitamin A, C and E, in the treatment of gastric and duodenal ulcers and gastric cancers, and the favorable effects of synthetic antioxidant, 5-ASA in the treatment of chronic inflammations of the bowel, give a strong base for further experiments, and for the development of new, effective synthetic antioxidants.

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Effects of Flavonoids on Tissue Regeneration

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Abstract

Higher plants have many free radical scavenger molecules and antioxidant compounds. Among the active compounds of medical plants are the scavenger flavonoids, flavonolignanes, polyphenols and so on. Silibinin is an antioxidant flavonoid from *Silybum marianum* used in therapy of liver diseases of different etiology. *Sempervivum tectorum* (stonecrop) is a well known medical plant for hundreds of years. Although the crude drug *Sempervivum tectorum* has been used for treatment of inflammation, its antioxidant and scavenger properties have not been studied previously. *Sempervivum tectorum* extract contains more than 20 different flavones and flavonol mono- and diglycosides, polyphenols, condensed proanthocyanides, phenolcarboxylic acids as potentially antioxidative compounds etc. The aim of this work was to verify that these flavonoids protect the membrane structures and functions, diminish the lipid peroxidation in different ways, support the natural scavenger capacity of the tissues in different experimental models in which free radical reactions are involved. We examined the in vitro and in vivo effects of these drugs on the changes of lipid peroxidation markers (diene conjugates, thiobarbituric acid reactive products, defense mechanism of tissues by chemiluminometry) and on the changes of membrane bound enzyme activities in microsomes in experimental hyperlipidemia in rat livers and in in vitro irradiation process.

Introduction

Medical plants are part of the treasure-house of nature. Higher plants contain a great variety of free radical scavenger molecules and antioxidant and antiinflammation compounds. Among the active phenolic compounds of medical plants are the free radical scavenger flavanols, flavonones, flavones, flavonols and so on [3].

Silibinin is a well known free radical scavenger and antioxidant flavonoid

originated from *Silybum marianum* [5, 8, 18, 19]. This drug is widely used in the therapy of liver diseases of different etiology [6].

What is *Sempervivum tectorum* extract? The freeze dried extract from *Sempervivum tectorum* (stonecrop) contains flavonol glycosides 0,7% (appr. 20 different flavone and flavonol mono- and diglycosides), polyphenols 4,2% (condensed proanthocyanidines), phenol carboxylic acids, polysaccharides 11,2% etc. (characteristic monosaccharides after strong hydrolysis: rhamnose, arabinose, xylose, mannose, galactose, glucose, uronic acids) [12].

Since flavonoid type compounds exert cytoprotection, we intended to provide further evidence for the drug's antioxidant activity and studied also the free radical scavenger activities and membrane protective properties of silibinin and *Sempervivum tectorum* extract in various free radical producing in vitro and in vivo systems by means of spectrophotometry and chemiluminometry.

Materials and methods

Young male Wistar albino rats weighing 150-200 g were used. Fifteen normolipidemic rats were treated with silibinin 25 mg/kg daily for 2 weeks intraperitoneally for irradiation. Liver microsomes prepared by the method of Fehér et al. [7] were exposed 1.095 KGy/h ^{60}Co -gamma radiation in air saturated aqueous suspension (5-10 mg/ml protein) together control samples from 15 untreated animals. Temperature was 20 °C during irradiation.

Ten normolipidemic animals were fed with LATI chow (Gödöllő, Hungary). Ten hyperlipidemic rats were kept on fat rich diet consisting of 2% cholesterol, 20% sunflower oil and 0.5% cholic acid added to the normal LATI chow for 8 days. Fifteen hyperlipidemic animals were treated with i.p. silibinin injection (25 mg/kg body weight) for 5 days from 3rd day to decapitation [1].

The animals for *Sempervivum tectorum* extract experiments were fed with lipogenic diet (see above) for nine days and treated parallelly with freeze dried extract dissolving in drinking water for 9 days [1].

Protein concentration of liver homogenates and microsomes were assayed by the methods of Lowry et al. [13].

Enzymatically induced lipid peroxidation was measured in a medium of total vol. 0.5 ml with a protein content of 1 mg/ml. The medium contained 20 mM sodium phosphate buffer, pH 7.5, 0.15 mM KCl, 50 μM FeCl_3 , 50 μM sodium pyrophosphate, glucose-6-phosphate dehydrogenase 0.6 IU, 0.5 mM NADPH and 10 mM glucose-6-phosphate, plus various concentration of *Sempervivum tectorum* extract [11]. Malondialdehyde production was monitored by the thiobarbituric acid test of Ottolenghi [15]. A molar absorption coefficient E_{532} 1 cm of 156 $\text{mM}^{-1}\text{cm}^{-1}$ used.

A non-enzymatically induced lipid peroxidation was studied by incubating the protein suspension (1 mg/ml) in a medium of total volume 0.5 ml and containing 50 mM tris-maleate buffer pH 6.8, 1 mM KH_2PO_4 , 5×10^{-5} M FeCl_3 and 5×10^{-4} M ascorbic acid. The incubation temperature was 37 °C.

NADPH cytochrome c reductase and NADH ferricyanide reductase [10], aminopyrin N-demethylase [10, 14] were measured by the standard methods.

Natural scavenger capacity of rat liver homogenates was detected by chemiluminometric method with a CLD-1 Medicor-Medilab Luminometer [1]. Luminol reagent consisted of 0.7 mM luminol, 3.8 μ M hemin and 11.8 mM Na_2CO_3 (pH 11). Temperature was 25 °C in these experiments.

Statistical analysis was done by a two tailed t test with a present probability level of $p < 0.05$. We denoted the confidence limits (h_1 , h_2).

Materials

The bovine serum albumin, glucose-6-phosphate-dehydrogenase were obtained from Calbiochem AG (Lucerne), hemin, luminol from Sigma (St. Louis) and all other reagents from Reanal (Budapest).

Results

Liver microsomes of rats pretreated in vivo with silibinin were subjected to gamma-irradiation. The remaining activities of NADPH cytochrome c reductase, NADH cytochrome b₅ reductase and N-demethylase enzymes of microsomal drug-metabolizing system and amount of thiobarbituric reactive products (malondialdehyde: MDA) were studied. Figure 1 shows the in vivo demonstration of silibinin's antioxidant property. Silibinin did not effect the activity of enzymes

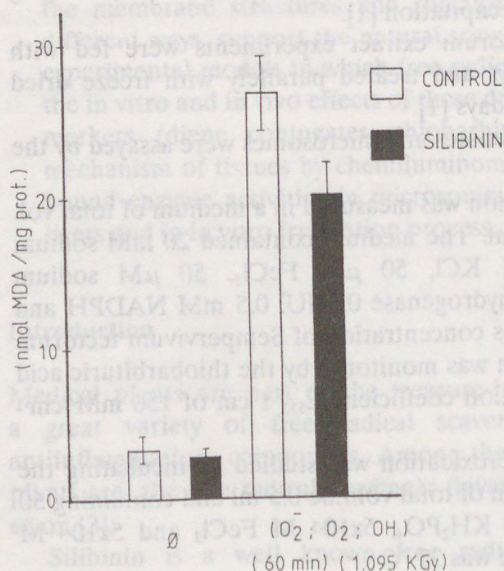
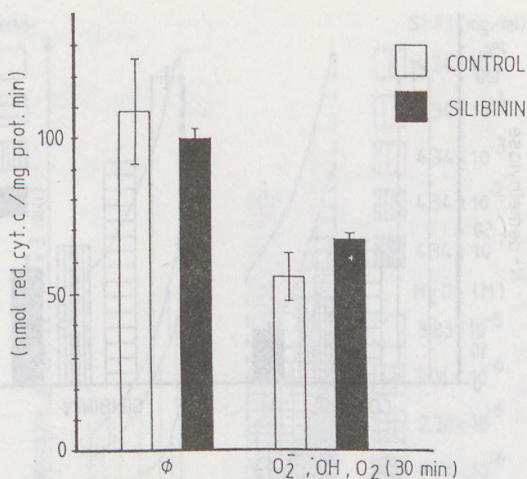


Figure 1. In vivo effect of silibinin treatment on the lipid peroxidation of rat liver microsomes in vitro ^{60}Co gamma irradiated system

Figure 2. Effect of in vivo silibinin treatment on the activity of NADPH cytochrome c reductase in in vitro ^{60}Co gamma irradiated system



NADPH cytochrome c reductase and NADH cytochrome b_5 reductase in vitro and in vivo to a significant extent. Therefore the results indicate that silibinin had a substantial inhibitory effect on the radiation induced degradation of NADPH cytochrome c reductase and NADH cytochrome b_5 reductase (Figs 2, 3). The antioxidant did not increase cytochrome P450 content when the rats were kept on a normal diet (data were published in previous papers [1]), and silibinin treatment also had no significant effect on the activity of N-demethylase in control animals, but increased the membrane stability, protected against radiation-induced

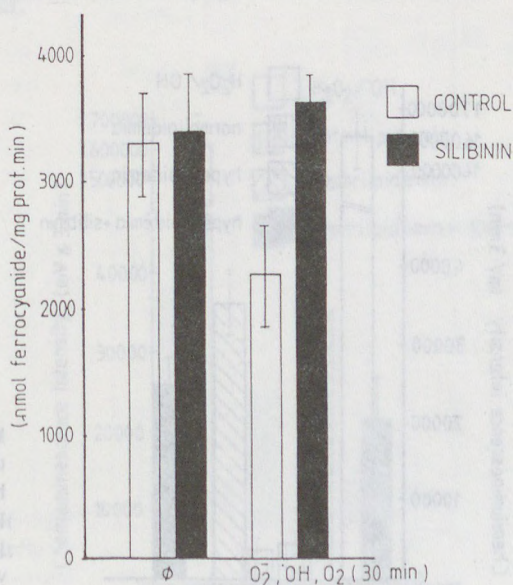


Figure 3. Effect of in vivo silibinin treatment on the activity of NADH ferricyanide reductase in in vitro ^{60}Co gamma irradiated system

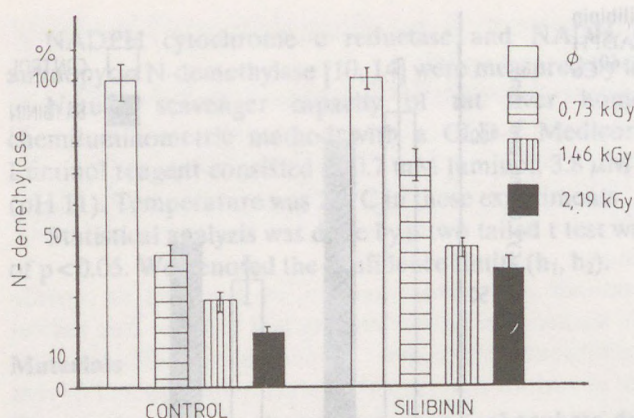


Figure 4. Effect of in vivo silibinin treatment on the activity of N-demethylase in in vitro ^{60}Co gamma irradiated system

degradation of enzymes and membrane lipids (Fig. 4). A non specific scavenger activity could be measured by the chemiluminometric technique. The natural scavenger capacity of liver tissue is changed due to the free radical attack. If the defense mechanisms of the living cell are badly damaged, the total free radical scavenger activity is decreased. Fig. 5 demonstrates, that the protecting activity against $\text{H}_2\text{O}_2/\cdot\text{OH}$ becomes stronger subsequent to antioxidant treatment in rat liver.

To demonstrate the scavenger activity of *Sempervivum tectorum* freeze dried extract, we measured the reduced intensity of the $\text{H}_2\text{O}_2/\cdot\text{OH}$ -luminol system by this drug in vitro. The changes of chemiluminescence intensity of the $\text{H}_2\text{O}_2/\cdot\text{OH}$ -luminol system, and the scavenging effect of *Sempervivum tectorum* extract at increasing concentrations on the $\text{H}_2\text{O}_2/\cdot\text{OH}$ are shown in Fig. 6.

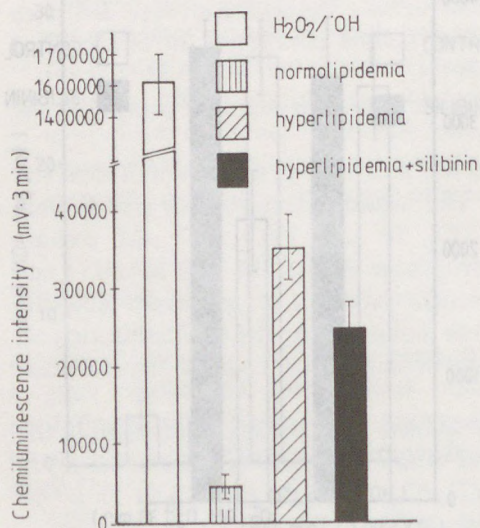
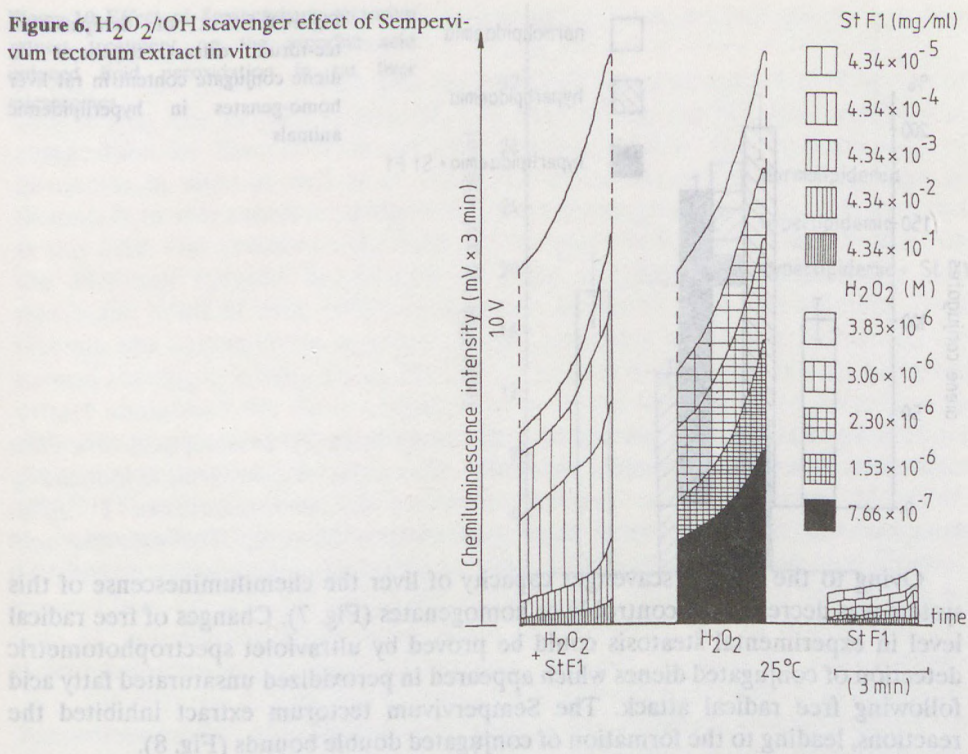


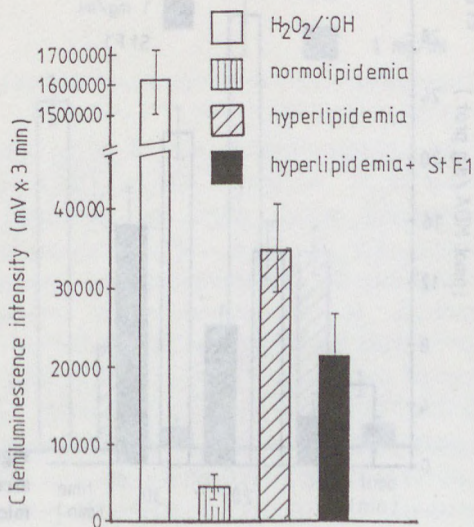
Figure 5. Effect of in vivo silibinin treatment on the natural scavenger capacity of rat liver homogenates
Protein concentration: 1 mg/ml in 5 μl volume;
 H_2O_2 concentration was 2.3×10^{-6} M in the total volume.

Figure 6. $\text{H}_2\text{O}_2/\cdot\text{OH}$ scavenger effect of *Sempervivum tectorum* extract in vitro



The integrated values of light intensity change during a given time interval showed that the *Sempervivum tectorum* treatment protected against $\text{H}_2\text{O}_2/\cdot\text{OH}$. The significant biochemical changes caused by lipid rich diet reflect the changes of values of total scavenger capacity of liver.

Figure 7. Effect of *Sempervivum tectorum* extract treatment on $\text{H}_2\text{O}_2/\cdot\text{OH}$ scavenger function of rat liver homogenates



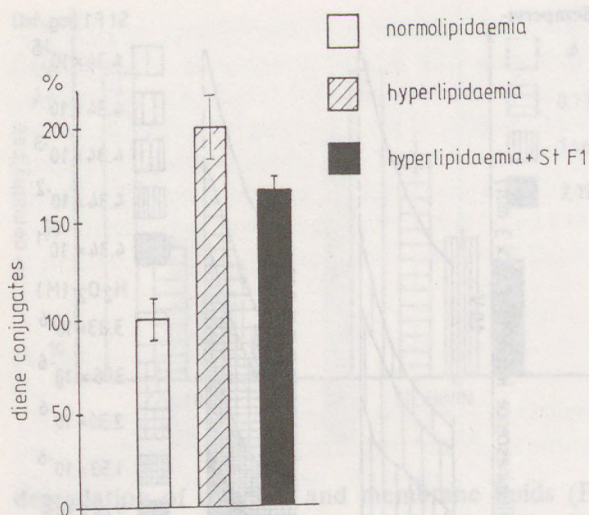


Figure 8. Effect of *Sempervivum tectorum* extract treatment on diene conjugate content in rat liver homo-genates in hyperlipidemic animals

Owing to the natural scavenger capacity of liver the chemiluminescence of this system was decreased by control liver homogenates (Fig. 7). Changes of free radical level in experimental steatosis could be proved by ultraviolet spectrophotometric detection of conjugated dienes which appeared in peroxidized unsaturated fatty acid following free radical attack. The *Sempervivum tectorum* extract inhibited the reactions, leading to the formation of conjugated double bounds (Fig. 8).

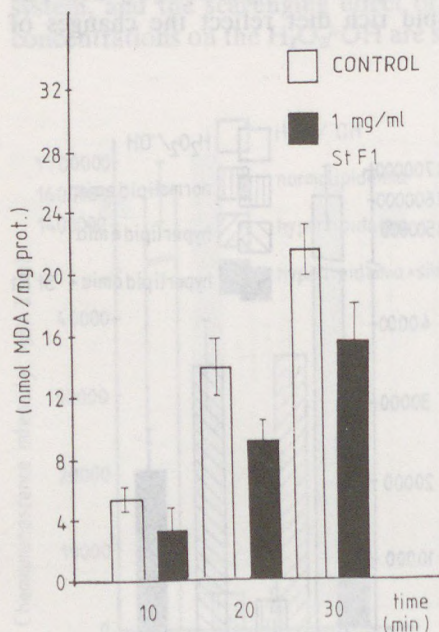


Figure 9. Effect of *Sempervivum tectorum* extract on the ascorbic acid induced lipid peroxidation in rat liver microsomes. Ascorbic acid concentration: 5×10^{-4} M

Figure 10. Effect of *Sempervivum tectorum* extract treatment on the ascorbic acid induced lipid peroxidation in rat liver microsomes

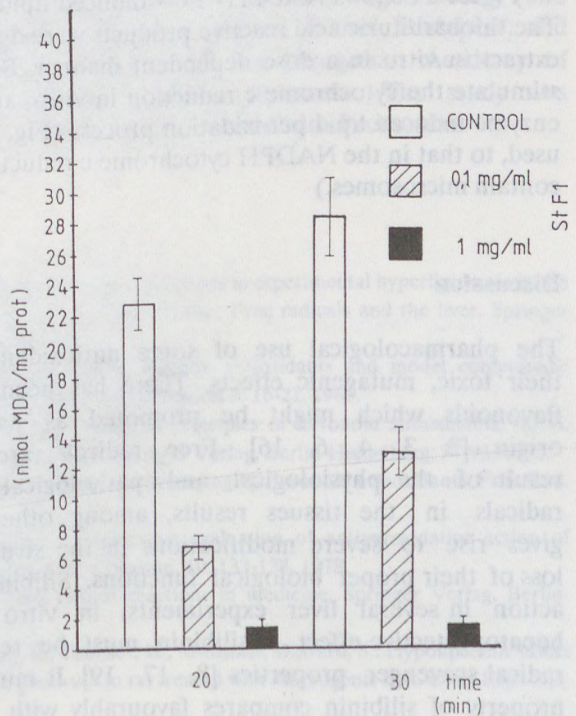
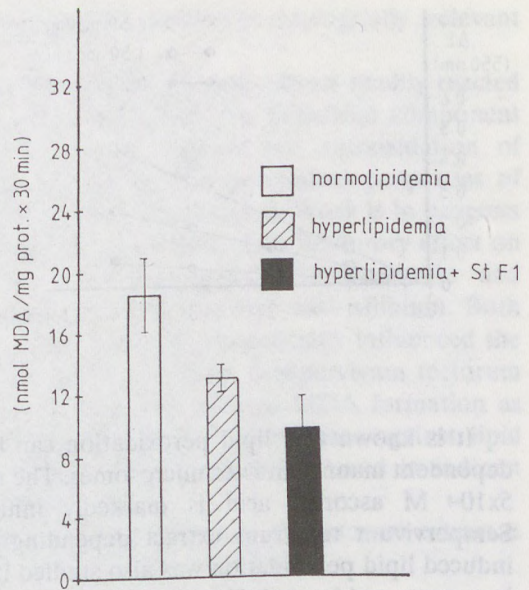


Figure 11. Effect of *Sempervivum tectorum* extract on the NADPH + Fe^{3+} induced lipid peroxidation of rat liver microsomes

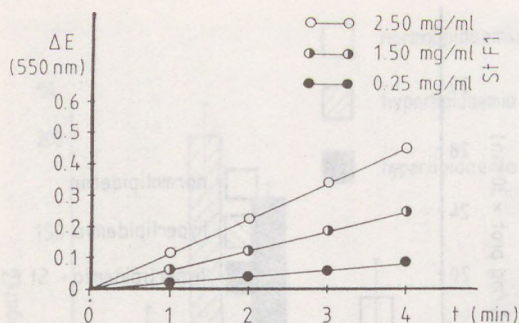


Figure 12. Effect of *Sempervivum tectorum* and NADPH on the reduction of cytochrome c

It is known that lipid peroxidation can be induced by ascorbic acid in a dose dependent manner in liver microsomes. The non enzymatic peroxidation induced by 5×10^{-4} M ascorbic acid is markedly inhibited in the presence of 1 mg/ml *Sempervivum tectorum* extract depending of time (Fig. 9). The ascorbic acid induced lipid peroxidation was also studied in vitro in liver microsome preparation in normo- and hyperlipidemic rats. Considerable differences were detected between the two groups in the production of MDA during lipid peroxidation. In vitro induced lipid peroxidation was also inhibited by *Sempervivum tectorum* extract treatment in the samples derived from animals kept on a fat rich diet (Fig. 10).

Figure 11 shows NADPH+ Fe^{3+} induced lipid peroxidation in liver microsomes. The thiobarbituric acid reactive products were decreased by *Sempervivum tectorum* extract in vitro in a dose dependent manner. *Sempervivum tectorum* extract can stimulate the cytochrome c reduction in vitro, and consequently can influence the enzyme induced lipid peroxidation process (Fig. 12). (Similar reaction mixture was used, to that in the NADPH cytochrome c reductase sample, but the sample did not contain microsomes.)

Discussion

The pharmacological use of some antioxidants has been questioned due to their toxic, mutagenic effects. There has been a growing interest in non-toxic flavonoids which might be promoted as natural antioxidants of vegetable origin [2, 3, 4, 6, 16]. Free radicals are produced in the cell as a result of the physiological and pathological processes. The formation of radicals in the tissues results, among others, in lipid peroxidation, which gives rise to severe modifications in the structures of membranes, resulting in loss of their proper biological functions. Silibinin showed membrane protecting action in several liver experiments, in vitro and in vivo. The wellknown hepatoprotective effect of silibinin must be related to its antioxidant and free radical scavenger properties [8, 17, 19]. It must be mentioned, that scavenger property of silibinin compares favourably with poly-OH substituted flavonoids

which generate toxic oxygen species during autooxidation at biologically relevant pH in vitro [8, 9].

Our earlier results showed, that *Sempervivum tectorum* extract readily reacted with oxygen free radicals in vitro [1, 12]. Presumably, the beneficial component composition of *Sempervivum tectorum* extract inhibits the autooxidation of flavonoids in vitro as well as in vivo, but antioxidant-prooxidant properties of flavonoids in vivo should be addressed in further experiments. Work is in progress in this field. Our present results indicate, that silibinin had an inhibitory effect on the radiation induced degradation of some drug-metabolizing enzymes and membrane lipids of liver microsomes in rats treated in vivo with silibinin. Both silibinin and *Sempervivum tectorum* extract treatments beneficially influenced the natural scavenger activity of rat liver tissue in fatty liver. *Sempervivum tectorum* extract diminished the diene conjugate content and induced MDA formation as well. *Sempervivum tectorum* extract protected the cell membranes against lipid peroxidation in direct (ascorbic acid induced lipid peroxidation) and in indirect (NADPH and iron induced lipid peroxidation) ways.

In conclusion the application of silibinin and *Sempervivum tectorum* extract is justified in several diseases primarily caused by oxygen free radicals.

Acknowledgements

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The Role of Free Radicals in the Pathogenesis of Amiodarone Toxicity

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Abstract

In vitro and in vivo studies were performed to elucidate the pathogenesis of amiodarone toxicity and the potential use of antioxidants in the prevention of the drug's effects. These studies provided direct and indirect evidence that amiodarone in vitro and in vivo produces free radicals that may play a role in the pathogenesis of amiodarone toxicity beside other well-established mechanisms and antioxidants may have a partial protective effect against amiodarone toxicity. An antioxidant silymarin did not diminish the electrophysiological effects of amiodarone in dogs with atrial flutter and in fact have had an additive or potentiating effect on the antiarrhythmic actions of amiodarone. Prospective studies with amiodarone combined with an antioxidant may be justified to reduce amiodarone toxicity without impairing or possibly enhancing its antiarrhythmic effect.

Introduction

Amiodarone is a potent antiarrhythmic drug used in the treatment of refractory arrhythmias. However, therapy is frequently limited by the development of serious and potentially fatal adverse reactions. Side effects reported to result from discontinuation of treatment were mostly pulmonary, liver and cardiac, and occurred in 0.5-22% of patients. In particular, pneumonitis is described to occur in up to 13% of patients in the USA with a mortality rate of 10-20% [1, 2]. Evaluation of potential risk factors including loading, maintenance and cumulative amiodarone dosages as well as serum amiodarone and desethylamiodarone levels has not been helpful in predicting the occurrence of toxicity [1, 2, 3, 4].

Indirect immunologically-mediated [1, 2] and direct cytotoxic mechanisms have been implicated in the pathogenesis of amiodarone toxicity. Direct cellular toxicity may in part be due to the inhibition of lysosomal phospholipase, which results in the accumulation of lysosomal phospholipids leading to cell death above a certain

threshold level [5, 6, 7]. Lamellated lysosomal inclusions, the markers of lysosomal phoepholidosis, are a common finding in patients and animals treated with amiodarone [1, 8, 9, 10, 11]. However, their presence does not signify organ toxicity, but reflects drug therapy with an agent that has amphiphilic (amphiphilic compound is a molecule that equally contains polar and nonpolar moieties) properties.

It is also postulated that direct cytotoxicity may result from amiodarone-induced free radical reactions. Few publications have addressed this topic [14, 15, 16, 17, 18, 19] and without exception were based on *in vitro* studies. Most of these studies investigated the pathomechanism of amiodarone-induced photosensitivity using photolysis. This premise is based in part on the chemical structure of amiodarone. Amiodarone is an iodinated benzofuran derivative capable of redox reactions, and benzofurans are well-known scavengers of singlet oxygen as well [20]. Significantly in patients in whom amiodarone therapy is complicated by skin pigmentation, skin biopsies have demonstrated the presence of lipofuscin, a lipid peroxidation product [21, 22]. Furthermore support for the role of free radical reactions in the pathogenesis of organ toxicity is the similarity of side effect profiles of amiodarone to other free radical generating compounds such as nitrofurantoin, ethanol, and isoniazide [23]. In particular, phenothiazine-induced photosensitivity has been shown to be caused partly by a phototoxic reaction mediated by free radicals [24].

The aim of this study, therefore, was to perform systemic *in vitro* and *in vivo* studies to prove the free radical hypothesis of amiodarone toxicity, and to investigate the potential use of antioxidants in its prevention.

Methods

In vivo studies

1) Rat studies

There were 60 male Wistar rats (150-200 g) comprising six study groups. Each study group contained ten rats. Group 1 was the control study group. Group 2 was the amiodarone study group. Pulverized amiodarone tablets [Cordarone (Novo Mesto, Krka)] were administered in a daily dosage of 500 mg/kg for 30 days. This was admixed with the standard LATI food. Group 3 received amiodarone and the antioxidant 6,6'-methylene-bis 2,2 dimethyl-4-methane sulfonate: sodium 1,2-dihydroquinoline (MTDQ-DA) The antioxidant MTDQ-DA [25] was administered in a daily dosage of 50 mg/kg for 33 days starting 3 days before the amiodarone protocol (outlined above). The MTDQ-DA was added to the drinking water. Group 4 received amiodarone and the antioxidant silibinin. The flavonoid-type silibinin (Madaus GmbH, Cologne) [23] was administered in a daily dosage of 50 mg/kg for 33 days, starting 3 days before the amiodarone protocol (outlined above). The silibinin was added to the drinking water. Group 5 received the antioxidant MTDQ-DA. The MTDQ-DA was administered in a daily dosage of 50 mg/kg for 33 days, and was dissolved in the drinking water.

Food and water consumption were verified daily and the drug dosages were modified accordingly. On completion of the study protocol, the rats were killed by decapitation and their livers and lungs removed for chemiluminometric, biochemical, morphological and biophysical analyses.

Chemiluminometric studies

Chemiluminometric analysis of the liver homogenate was performed to detect and quantitate the presence of free radicals, and to determine the superoxide anion radical (O_2^-) scavenging capacity of the liver. Groups treated with either amiodarone alone (group 2) or amiodarone and silibinin (group 4) were compared with the control group (group 1). Light emission was measured with a CDL-1 Medicor Luminometer (Budapest, Hungary) with an MMT microprocessor (Medilab, Hungary). The intensity of the light emission was expressed either as mV or as its integral mVs. An adrenalin-adrenochrom autooxidative system was used for superoxide radical generation. A total volume of 1,15 ml of a reagent solution composed of 0,7 mM luminol, 3,8 μ mol hemin, and 11,8 mM Na_2CO_3 , adjusted to pH 10-11 and bubbled with N_2 gas [26] was placed the lower chamber of a divided cuvette. Epinephrine 10^{-3} M x 50 μ l was placed in the upper chamber and the intensity of the light emission (E1) was measured after the automatic mixing of the contents of both chambers. E1 is a measure of superoxide radical concentration. A 3g/100 ml concentrate of liver homogenate was prepared and the protein content determined [27]. All homogenates were standardized to the same protein concentration by dilution. The light emission intensity of the homogenate was referred to as unit protein content. Fresh liver homogenate x 20 ml and epinephrine 10^{-3} M x 50 microliter were then placed in the upper cuvette chamber and the intensity of the light emission (E2) was determined after mixing with the reagent in the lower chamber. A measure of the superoxide scavenger capacity of the liver was calculated by subtracting the intensity of light emission E2 from E1. All studies were performed in the presence of air and light.

Biochemical studies

Hepatic microsomes were prepared by ultracentrifugation of liver homogenate. Enzymatic lipid peroxidation was induced using NADPH and Fe^{3+} (28) and quantitated by measuring the malondialdehyde content of samples after the addition of thiobarbituric acid (29). Microsomal malondialdehyde content was normalized to the microsomal protein content determined by the Lowry method (27) using serum albumin as the standard.

Morphological studies

Light microscopy: Liver and lung tissue were stained with haematoxylin-eosin. Histologic evidence of amiodarone induced hepatic and pulmonary toxicity was assessed by a histopathologist blinded to the treatment groups. The number of

alveolar macrophages containing foamy inclusions indicative of phospholipoidosis (so called "foamy cells") was graded on a semiquantitative scale from 1+ to 4+, and the extent of the tissue area involved expressed as a percentage of the whole section area. A "pathological lung" index was determined for each specimen by multiplying the pertinent scale by the involved area. To confirm the reproducibility of this index, all specimens were examined by the same observer on at least two separate occasions and the results were compared.

Electron microscopy: Small pieces of lung and liver tissue were fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer at pH 7.2. After washing, the tissue was postfixated in 1% osmic acid for a 2 hour period and then embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Tesla BS 500 electron microscope. The number of lysosomes and perisomes within a 300 μm^2 area were counted. For each study group, the mean count within a 100 μm^2 area was then determined.

Biophysical studies

Cobalt 60-gamma (^{60}Co -gamma) irradiation of hepatic microsomes: Lipid peroxidation of hepatic microsomes was induced with stationary radiolysis and quantitated at 40-minute intervals using the thiobarbituric acid test. The 1,600 TBq nominal activity source at this institute was used for the irradiation of the hepatic microsomes. The dose rate was usually 1,100 Gy/hours as measured by Fricke dosimetry and corrected for electron density. Three groups of hepatic microsomes were irradiated: microsome group I: This group was composed of liver microsomes from rats treated with amiodarone alone (group 2), amiodarone and MTDQ-DA (group 3), MTDQ-DA alone (group 5), and control rats (group 1); microsome group II: This group was composed of hepatic microsomes from rats treated with amiodarone alone (group 2), to which either MTDQ-DA or the antioxidant sodium 2,2-dimethyl 1-2, dihydroquinoline-4-yl-methane sulfonate (CH 402 (USA Patent No. 4363910) were added in vitro; and microsome group III: This group was composed of liver microsomes derived from control rats (group 1), to which either MTDQ-DA or CH 402 was added in vitro.

2) Dog studies

Forty one mongrel dogs of either sex (14-26 kg) were divided into 3 groups. Group 1 consisted of 12 dogs treated with oral amiodarone (Cordarone, Wyeth Laboratories) (600 mg/d for 8 weeks). Group 2 had 12 dogs that received oral amiodarone (600 mg/d for 8 weeks) and silymarin (Legalon caps., Madaus GmbH, Cologne, Germany) (70 mg bid for 8 weeks) and Group 3 was made up of 17 untreated control dogs. All control animal were used for the electrophysiological part of the protocol while 4 were also used for the biochemical-biophysical studies.

Surgical procedures

Dogs were anesthetized with pentobarbital (30 mg/kg IV; additional drug was injected as needed to maintain anesthesia). Dogs were then intubated and ventilated with room air using a constant volume-cycled respirator (model 607, Harvard Apparatus, South Natick, Massachusetts). To monitor arterial pressure, a fluid-filled cannula was placed in the right femoral artery and connected to a transducer (Statham P23ID, Gould, Cleveland, Ohio). A femoral venous cannula was used to infuse 100-200 ml/hr normal saline to replace spontaneous fluid losses. The chest was opened in the 4th right intercostal space and the heart exposed and suspended in a pericardial cradle. The epicardial temperature was monitored with a thermistor (model 400, Yellow Springs Instrument, Yellow Springs Ohio) and was maintained between 36 °C and 38 °C by use of an operating room table lamp and a heating pad. To minimize drying of cardiac surfaces, the thoracotomy was covered by a plastic sheet. Then the right atrium was incised on a line extending from the superior to inferior venae cavae and sewn together. A second incision ran from the inferior margin of the first incision to the right atrial appendage parallel to the atrioventricular (AV) groove (Fig. 1) [30, 31]. During this procedure the sinus node was crushed. The right common carotid artery was isolated, and a USCI 6F quadripolar catheter inserted and advanced to the noncoronary cusp of the aortic valve to record His bundle electrograms. Bipolar plunge electrodes placed 5 mm apart in the right atrial appendage and in the right ventricle or tripolar contact electrodes were used to record bipolar atrial and ventricular electrograms. Plunge

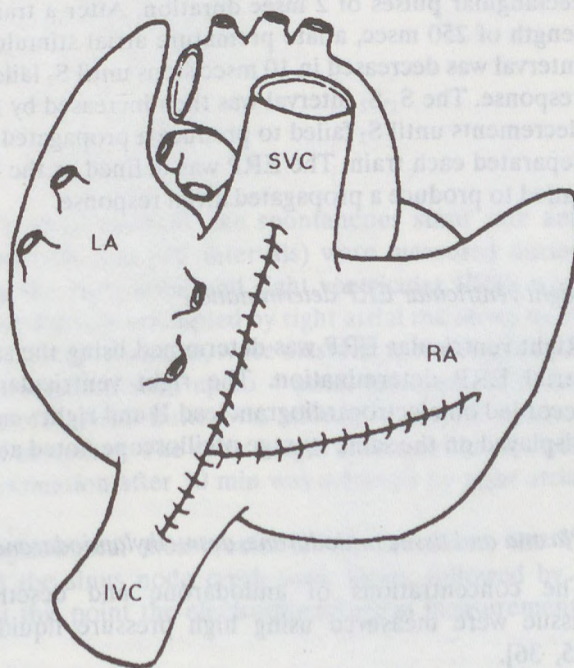


Figure 1. The Y-shaped atrial incision used to create acute canine atrial flutter

LA=left atrium;

RA=right atrium;

SVC=superior vena cava;

IVC=inferior vena cava

electrodes for unipolar cathodal stimulation were placed in the right atrium and right ventricle, and a 33 mm diameter anodal disc electrode was placed subcutaneously over the abdominal muscles. Electrocardiogram lead II, the His bundle electrogram, the bipolar atrial and ventricular electrograms and the arterial blood pressure trace were displayed simultaneously on a Bard electrophysiology monitor and stored on analog tape for subsequent analysis. Atrial and ventricular electrograms were amplified and filtered at 30-500 Hz and His electrogram was amplified and filtered at 30-1,000 Hz.

When the electrophysiological part of the study was completed, further preparations were made for the biochemical-biophysical protocol in the same animal [32, 33]. Briefly a snare was placed around the mid left anterior descending coronary artery. A catheter was advanced into the anterior interventricular vein and the tip was positioned at least 0.5 cm distal to the snare, so as to minimize any contamination of blood samples with venous effluent from nonischemic vascular beds. The left anterior descending coronary artery was occluded for 15 min and reperused for 30 min.

Electrophysiological measurements

Right atrial effective refractory period (ERP) determination

The right atrium was paced at a constant cycle length using twice diastolic threshold rectangular pulses of 2 msec duration. After a train of eight stimuli (S_1) at a cycle length of 250 msec, a late premature atrial stimulus (S_2) was introduced. The S_1 - S_2 interval was decreased in 10 msec steps until S_2 failed to produce a propagated atrial response. The S_1 - S_2 interval was then increased by 10 msec and decreased by 1 msec decrements until S_2 failed to produce a propagated atrial response. A pause of 2 sec separated each train. The ERP was defined as the longest S_1 - S_2 interval at which S_2 failed to produce a propagated atrial response.

Right ventricular ERP determination

Right ventricular ERP was determined using the same protocol described for right atrial ERP determination. The right ventricular responses to S_1 and S_2 were recorded on electrocardiogram lead II and right ventricular bipolar electrogram and displayed on the same storage oscilloscope noted above.

Plasma and tissue amiodarone, desmethyamiodarone measurement

The concentrations of amiodarone and desethylamiodarone in plasma and tissue were measured using high pressure liquid chromatography (HPLC) [34, 35, 36].

EPR spectroscopy analysis

We infused the spin trapping agent N-tert-butyl-alpha-phenyl-nitrone (PBN) into the femoral vein. After collection, all blood samples were immediately centrifuged to separate the plasma. The plasma specimens were frozen at -70°C liquid nitrogen for subsequent analysis by EPR spectroscopy. Total lipids were extracted from the entire volume (approx. 1-3 mls) of each plasma sample by adding 15 vol of chloroform/methanol (2:1 vol/vol). To this single phase system, one-fifth volume of 0.5% NaCl was added to bring about phase partitioning in a separatory funnel. The chloroform layer was then recovered and evaporated to dryness at room temperature, purging on top of the solution with nitrogen gas in hood. The lipid residue (which would include any radical adducts of PBN) was taken up in 500 microliter of degased chloroform and transferred to the flat cell for EPR analysis. EPR spectra were recorded in flat cells at room temperature with a Bruker-IBM ER 300 (Bruker Analytical Instruments, Karlsruhe, Germany) spectrometer operating at X band using 100 kHz modulation frequency and a TM 110 cavity. The microwave frequency and magnetic field were precisely measured using an EIP 575 Microwave Frequency Counter and Bruker ER 035M NMR Gaussmeter (Bruker Analytical Instruments, Karlsruhe, Germany). Spectral simulations were performed using simulation which assume isotropic g and A tensors. Quantitation of the free radical signals was performed by comparing double integral of the observed signal to that of a known concentration of the tempo free radical in aqueous solution measured under identical circumstances [37].

Protocol

Electrophysiological study

Prior to the placement of the Y atrial incision, the spontaneous sinus rate and atrioventricular conduction times (AH and HV intervals) were measured during spontaneous sinus rhythm. Then the right atrial and right ventricular ERPs were determined and atrial flutter induction was attempted by right atrial overdrive burst pacing using rectangular pulses of 2 msec duration, twice diastolic to 4 mA strength, or by programmed atrial extrastimulation with up to 4 atrial extrastimuli. If the atrial flutter induction was successful, atrial flutter was allowed to last 30 minutes and monitored by videotape and computer. The cycle length and duration of atrial flutter was then determined. Termination after 30 min was achieved by right atrial overdrive burst pacing.

After the baseline electrophysiological measurements were obtained, the Y-shaped right atrial incision and the sinus node crush were made, followed by a waiting period of 15 minutes. At this point the electrophysiological measurements were repeated.

The aim of these studies was to confirm the *in vivo* antioxidant effect of silymarin and to investigate the free radical generating action of amiodarone using electron paramagnetic resonance (EPR) spectroscopy analysis. Two of the 4 control dogs used for the biochemical-biophysical studies were infused the spin trapping agent N-tert-butyl-alpha-phenyl-nitrone (PBN) without subsequent ischemia-reperfusion; the other 2 control dogs received PBN infusion with subsequent ischemia-reperfusion. Into these 4 control dogs PBN was infused and plasma and tissue samples were collected in the same way and at the same time as in treated dogs. Electron paramagnetic resonance (EPR) spectroscopy analysis was accomplished from the plasma samples of 6 animals of each treated group (group 1 and group 2). During the 1st phase of the study, baseline samples for EPR spectroscopy analysis were collected (Fig. 2). In the 2nd phase of the study, ischemia-reperfusion was applied to all treated dogs and 2 control dogs, because it was uncertain that baseline samples could produce appropriate signal intensity in the presence of physiological

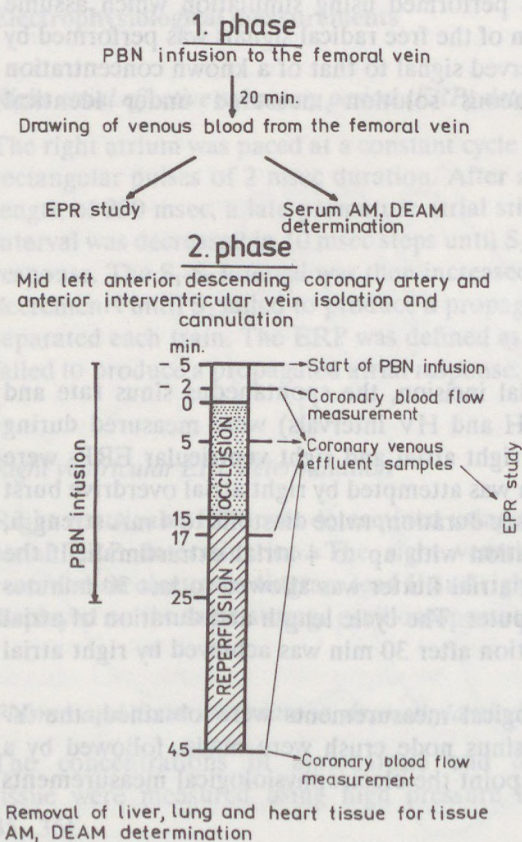


Figure 2. Flow chart of the biochemical-biophysical study protocol

EPR=electron paramagnetic resonance spectroscopy, AM=amiodarone; DEAM=desethyl amiodarone, PBN=N-tert-butyl-alpha-phenyl-nitrone

antioxidants and/or silymarin and we assumed that by intensifying free radical production this way detectable signals could be obtained. In the first phase of the biochemical-biophysical studies, PBN was infused into the femoral vein for 20 min in a total dose of 50 mg/kg. Then 6 ml of venous blood from the opposite femoral vein was drawn into a syringe containing 100 U heparin for EPR analysis. Also 6 ml blood was collected from the femoral vein for plasma amiodarone and desethylamiodarone determination and was immediately frozen.

During the 2nd phase, the mid left anterior descending coronary artery was occluded for 15 min and reperfused for 30 min. PBN was infused starting 5 min before coronary occlusion and continued until 10 min after reperfusion (11 mg/ml concentration, total dose: 30 mg/kg). Blood samples (4-6 ml) were drawn over 60 sec into a syringe containing 100 U heparin from the coronary vein alongside the left anterior descending coronary artery 2 min before occlusion, and at various time points during ischemia-reperfusion. The latter samples were not used for final EPR analysis since we obtained excellent baseline free radical signals. All blood samples were immediately centrifuged to separate the plasma for subsequent EPR analysis. The plasma specimens for EPR analysis were frozen at -70 °C in liquid nitrogen. The concentration of free radicals in the sample ($\mu\text{mol/ml}$) was multiplied by the coronary blood flow (ml/min) measured at the same time and the value obtained is an estimation of myocardial release of spin adducts ($\mu\text{mol/min}$) from the area drained by the anterior interventricular vein. At the end of the experiment 5 gm tissue samples were removed from the lung, liver and heart (right ventricle) for tissue amiodarone and desethylamiodarone determination. The tissues samples were frozen immediately after removal. Finally before taking the 5 gm sample from the heart the dog was overdosed with phenobarbital.

Data analysis

Using an Apple Macintosh LC computer, all analog signals displayed on the monitor were digitized at 333 SPS and stored on a hard disk. Interactive analysis of the data was performed using a Labview 2 application (National Instruments Corporation, 1990).

In vitro studies

Both in vitro chemiluminometric and biophysical studies were performed.

Chemiluminometric studies

These studies were performed to determine whether free radicals were able to be quantitatively detected in the presence of amiodarone alone or combined with antioxidants. The intensity of light emission of varying concentrations of 0.2 ml aliquots of amiodarone alone or in combination with the dihydroquinoline antioxidants MTDQ-DA and CH 402 was determined after mixing with the reagent previously described. Three separate amiodarone preparations were analyzed and

included amiodarone tablets, amiodarone HCl substance (Sanofi Pharma, Paris, France), and amiodarone injection [Cordarone (Novo Mesto, Krka)]. The amiodarone tablets and amiodarone.HCl substance were suspended in water. Measurement conditions were: mixing velocity 3, sensitivity 1, temperature 25 °C, and total volume 1.15 ml.

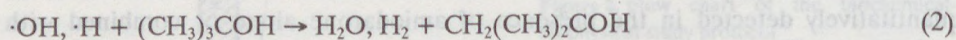
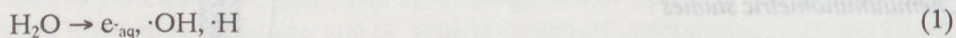
Biophysical studies

Pulse radiolysis and stationary ^{60}Co -gamma radiolysis were performed to differentiate the free radicals generated by amiodarone.HCl.

Electron pulse radiolysis: The computer-controlled pulse radiolysis facility and the fast optical detection system of the Institute of Isotopes have recently been described [38]. Briefly, a Tesla-made LINAC LPR-4 linear accelerator produced 80-2,600 nsec pulses of electrons with a mean energy of 4 MeV. The dose/pulse was varied between 10 and 60 Gy, as measured by conventional thiocyanate dosimetry. The formation and decay of transient species were followed by a kinetic spectrophotometer (Applied Photophysics, Ltd., London, United Kingdom) using a xenon arc analyzing light source. Analog-to-digital conversion handling and storage of data as well as the operation of the accelerator was done by an Iwatsu TS 8123 storage oscilloscope and a Cromemco CS-2H computer. For pulse radiolysis study, amiodarone.HCl substance was used, which was a generous gift from Sanofi Pharma, Paris.

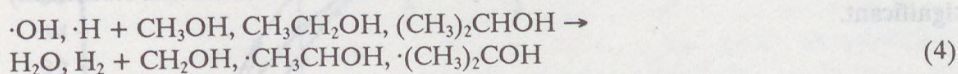
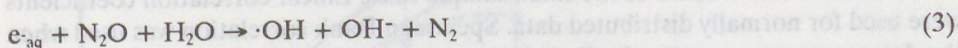
^{60}Co -gamma radiolysis: Stationary radiolysis was carried out using the 1,600 TBq nominal activity source of the Institute. The dose rate was usually 1,100 Gy/hour as measured by Fricke dosimetry and corrected for electron density.

Sample preparation and analysis: The chemicals used throughout the study were of analytical grade. Reducing free radicals were generated using diluted aqueous solutions (30% v/v) of different alcohols [tert-butanol = $(\text{CH}_3)_3\text{COH}$, methanol = CH_3OH , ethanol = $\text{CH}_3\text{CH}_2\text{OH}$, isopropanol = $(\text{CH}_3)_2\text{CHOH}$] prepared from doubly distilled water containing 1 mM amiodarone.HCl saturated with either N_2 or N_2O by gentle bubbling for 30 minutes before the experiment. The different alcohols were used in order to study the behavior of amiodarone in various reducing environments. On subjecting the aqueous solution of amiodarone containing tert-butanol to pulses or radiation, the major free radical products of the radiolysis of water $\cdot\text{OH}$ and $\cdot\text{H}$ were transformed into relatively unreactive tert-butanol radicals (Equations 1 and 2), the hydrated electron (e_{aq}^-) is a strong reducing agent and reacts very rapidly with the electron acceptor amiodarone.



When using alcohols other than tert-butanol in water as solvents and saturating the solution with N_2O to scavenge hydrated electrons, alpha-hydroxy radicals will be the major reactive transients known to exhibit weaker reducing properties

(Equations 3 and 4).



The silica optical cells were of 1 cm optical path length and of flow through type, whenever an effect of the dose could be noticed.

Difference spectra of irradiated samples were taken using a Varian DMS-80 spectrophotometer (Varian Assoc. Medical and Industrial Products Group, Palo Alto, CA, USA).

The concentration of iodide ion was determined by potentiometry using an iodide ion selective electrode and a calomel reference electrode.

Products of radiolysis were separated by thin layer chromatography using silica gel plates (Merck Kieselgel 60 F₂₅₄, 0.2 mm) and a benzene : methanol (3 : 1) solvent. The irradiated samples were subjected to high performance liquid chromatography (HPLC) applying a PIC method, which we developed specifically for separation of highly polar amino compounds. Analyses were performed on a BST Nevisorb 10 micrometer C 18 (250 X 4 mm) column and a methanol : water (7 : 3) solvent containing 5 mmol dibutyl-amine and phosphoric acid to attain a pH of 2.6. The spectrophotometric detector was operated at a wavelength of 200 to 400 nm.

As standard samples of amiodarone metabolites were not available, we prepared partially and fully deiodinated compounds by hydrogenating amiodarone dissolved in methanol using a 10% (Pd) alumina catalyst.

Statistical analysis

1. Rat studies and in vitro studies

All data were expressed as the mean $\pm$ the standard error of the mean (SEM). The following statistical analyses were applied: analysis of variance, and paired and unpaired Student's t-test. The modified Student's t-test was used for between group comparative studies when the analysis of variance resulted in a significant difference.

2. Dog studies

The data in this study are expressed as mean $\pm$ SD. Comparisons among groups for baseline data use analysis of variance (ANOVA) with the Tukey's honestly significant difference procedure for multiple comparisons. Changes during the study were analyzed with two way repeated measures ANOVA. When the data were not normally distributed, the Kruskal-Wallis nonparametric ANOVA test was applied.

Comparison of atrial flutter inducibility in the treated groups was done by using Fisher's Exact test because of the small sample sizes. Linear correlation coefficients were used for normally distributed data. Spearman Rank correlation was used when the data were not normally distributed. A p value <0.05 was considered statistically significant.

Results

In vivo studies

1. Rat studies

Chemiluminometric studies

Liver homogenate (Fig. 3): The superoxide scavenging capacity of rats treated with amiodarone (group 2) or amiodarone and silibinin (group 4) was diminished compared to the control group (group 1). However importantly, in the rats treated

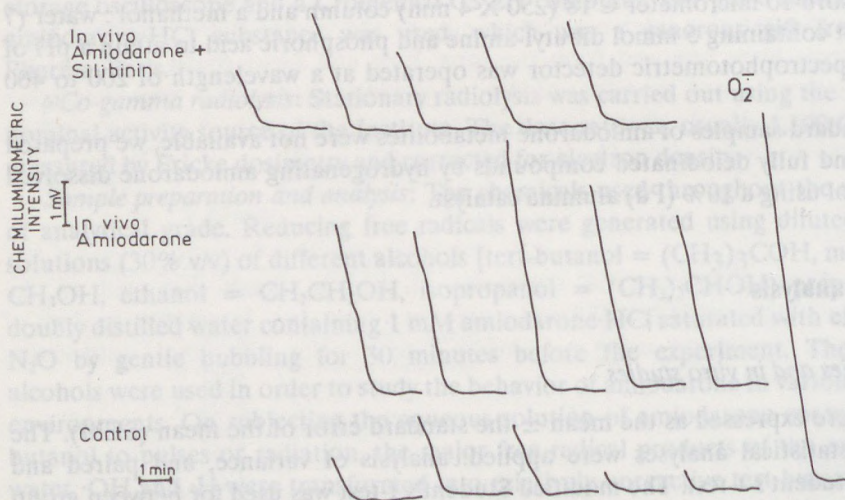


Figure 3. The effect of in vivo silibinin and amiodarone treatment on rat liver homogenate superoxide radical ($O_2^{\bullet-}$) scavenger capacity. Time in minutes is plotted on the abscissa, and intensity of light emission in mV on the ordinate. The right side of the figure shows the chemiluminescence signal (E1) induced by the autooxidation of adrenaline to adrenochrom, associated with $O_2^{\bullet-}$ production indicated by $O_2^{\bullet-}$ on the upper right. E1 is a measure of $O_2^{\bullet-}$ concentration. The addition of the liver homogenate of treatment groups and control to the adrenaline-adrenochrom autooxidative system resulted in chemiluminescence signals of lower intensity (E2) than E1, because of the $O_2^{\bullet-}$ scavenger capacity of liver homogenates, calculated by subtracting E2 from E1. The $O_2^{\bullet-}$ scavenger capacity of rats treated with amiodarone (group 2) or amiodarone and silibinin (group 4) was substantially diminished compared to control group (group 1). However, in rats treated with both amiodarone and silibinin (group 4), the effect was significantly reduced compared to rats treated with amiodarone alone.

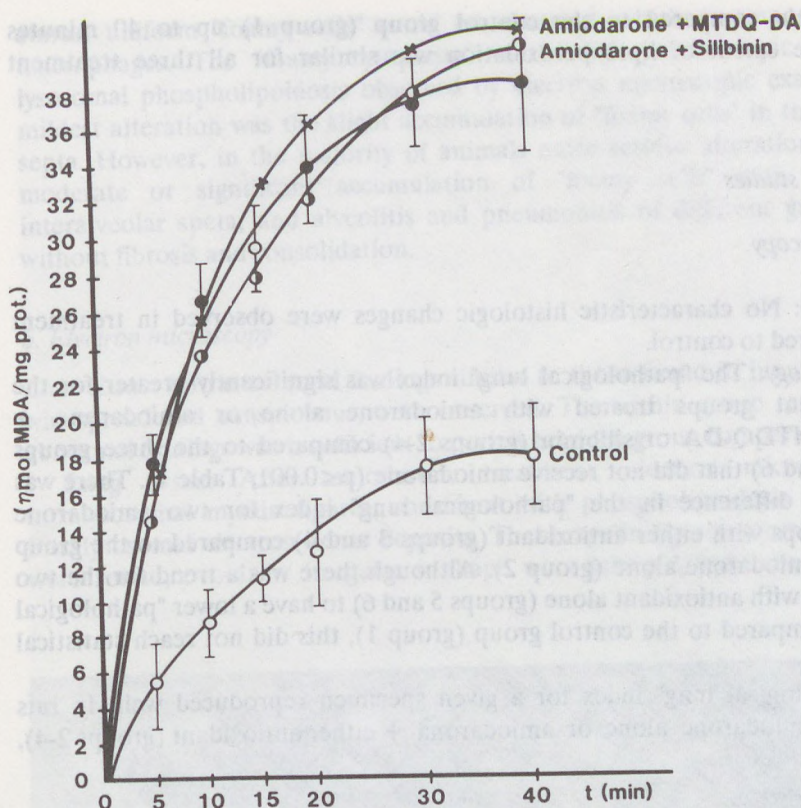


Figure 4. NADPH and Fe^{3+} induced microsomal lipid peroxidation in rat hepatic microsomes. Quantitation of induced enzymatic lipid peroxidation of liver microsomes was performed by measuring the microsomal malondialdehyde content using the thiobarbituric acid test. Microsomal malondialdehyde content expressed as micromol/mg of microsomal protein is plotted on the y axis against time in minutes on the x axis (mean $\pm$ SEM). Data for the control, amiodarone alone, amiodarone + MTDQ-DA, and amiodarone + silibinin treatment groups are shown. All three treatment groups show a significant increase in lipid peroxidation compared to the control group at each sampling interval ($p < 0.01$). There is no significant difference in lipid peroxidation between the three treatment groups. For clarity, the SEM has not been plotted for the amiodarone + MTDQ-DA or amiodarone + silibinin treatment groups

with both amiodarone and silibinin (group 4), this effect was substantially reduced compared to rats treated with amiodarone alone (group 2). The results of this preliminary study prove indirectly the free radical generating effect of amiodarone, which was partially prevented by the simultaneous silibinin treatment.

Biochemical studies

NADPH and Fe^{3+} induced liver microsomal lipid peroxidation (Fig. 4): The induced enzymatic lipid peroxidation was significantly greater in the three treatment groups (groups 2-4) treated with amiodarone alone or amiodarone + antioxidants MTDQ-

DA or silibinin compared to the control group (group 1) up to 40 minutes ($p < 0.01$). The extent of lipid peroxidation was similar for all three treatment groups.

Morphological studies

a. Light microscopy

Liver histology: No characteristic histologic changes were observed in treatment groups compared to control.

Lung histology: The "pathological lung" index was significantly greater for the three treatment groups treated with amiodarone alone or amiodarone + antioxidants MTDQ-DA or silibinin (groups 2-4) compared to the three groups (groups 1, 5 and 6) that did not receive amiodarone ($p < 0.001$; Table 1). There was no significant difference in the "pathological lung" index for two amiodarone treatment groups with either antioxidant (groups 3 and 4) compared to the group treated with amiodarone alone (group 2). Although there was a trend for the two groups treated with antioxidant alone (groups 5 and 6) to have a lower "pathological lung" index compared to the control group (group 1), this did not reach statistical significance.

The "pathological lung" index for a given specimen reproduced well. In rats treated with amiodarone alone or amiodarone + either antioxidant (groups 2-4),

Table 1. "Pathological lung" index values determined by light microscopy from rat lung tissue in the different treated groups

Group	"Pathological lung" index = the number of (+)-s denoting foamy cells x pathological area (%)
	"Pathological lung" index: $\bar{x} \pm \text{SEM}$
Control (n = 10)	87.5 $\pm$ 36.14
MTDQ-DA (n = 9)	9.45 $\pm$ 4.50
Amiodarone (n = 10)	127.0 $\pm$ 48.47
Amiodarone + MTDQ-DA (n = 10)	142.0 $\pm$ 34.77
Silibinin (n = 8)	7.51 $\pm$ 4.12
Amiodarone + silibinin (n = 10)	152.0 $\pm$ 42.91

The line (amiodarone) effect is highly significant ($p < 0.001$) according to two way analysis of variance, which means that the values of amiodarone treated groups are significantly different from those of groups not treated with amiodarone. Although the "pathological lung" index values of MTDQ-DA and silibinin treated groups seem to be essentially lower than those of control, this difference didn't reach statistical significance. The simultaneous antioxidant treatment with amiodarone didn't influence the "pathological lung" index compared to the group treated with amiodarone alone.

almost uniform "foamy cells" could be observed, which correspond to alveolar macrophages. The "foamy cytoplasm" is the light microscopic equivalent of lysosomal phospholipoidosis observed by electron microscopic examination. The mildest alteration was the slight accumulation of "foamy cells" in the interalveolar septa. However, in the majority of animals more serious alterations were found: moderate or significant accumulation of "foamy cells" with thickening of interalveolar septa, and alveolitis and pneumonitis of different grades with and without fibrosis and consolidation.

b. Electron microscopy

Liver tissue: Ultrastructural findings similar to those found in lung tissue, but less widespread and conspicuous, were observed. The within group reproducibility of abnormal findings was insufficient to permit between group comparative studies.

Lung tissue: After amiodarone treatment, electron microscopy showed intralysosomal myelin figures, consistent with phospholipoidosis associated with intralysosomal electron dense deposits. These myelin figures containing lysosomes were round, ovoid, or irregular in shape, were enlarged, and their diameter varied



Figure 5. Electron micrograph of an alveolar epithelial cell in the control animal. The cell contains dense, small lysosomes (arrows) of 0.3-1.0 micrometer in diameter with an internal structure consisting of thin parallel or concentric lamellae distributed throughout the cytoplasm. Scale bar = 1 micrometer (x 42,000)

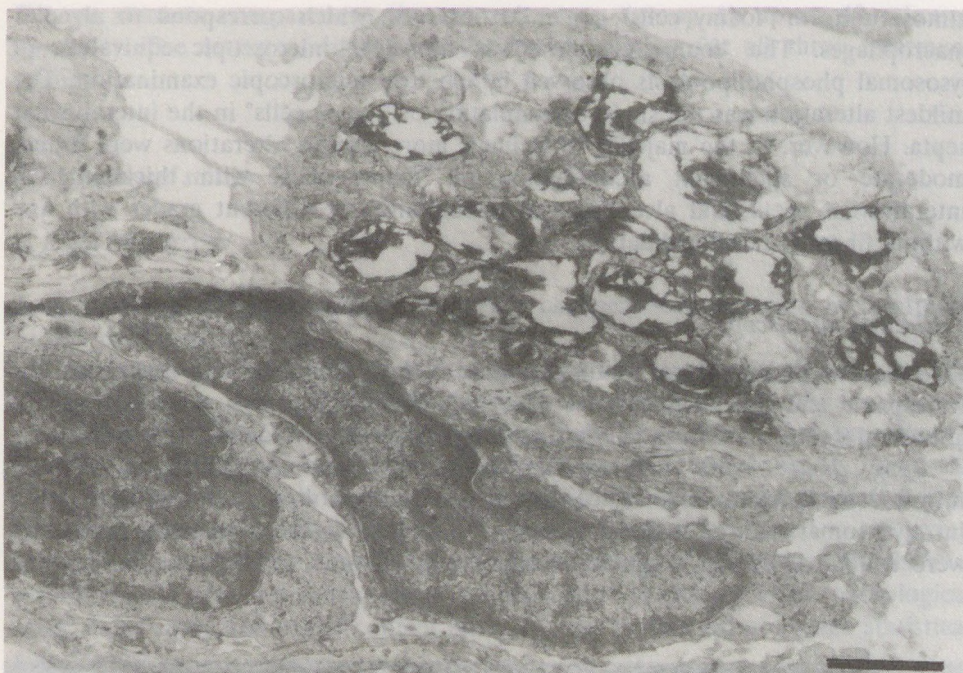


Figure 6. Electron micrograph of an alveolar epithelial cell in an amiodarone-treated animal. After amiodarone treatment membranous structures arranged in whorled arrays (myelin figures) and electron dense deposits could be observed in the lysosomes of an alveolar epithelial cell. The number and size of lysosomes increased compared to control. Scale bar = 1 micrometer (x 18,600)

from 0.5 to 2.0 μm . Their number was increased compared to control group ($p < 0.001$) (Figs. 5 and 6, Table 2). Lysosomes with the myelin figures were noted in most alveolar epithelial cells, alveolar phagocytes, and in some endothelial cells. In all cases intralysosomal deposits of either granular or homogenous electron dense material were also observed. Similar deposits were observed in large multivesicular

Table 2. Number of lysosomes referred to 100 μm^2 tissue area

Group	$\bar{x} \pm \text{SEM}$		
Control (n = 8)	7.31 ± 1.4	p < 0.001	p < 0.05
Amiodarone (n = 8)	26.2 ± 2.8		
Amiodarone + silibinin (n = 8)	13.7 ± 2.5	p < 0.01	
Amiodarone + MTDQ-DA (n = 8)	23.4 ± 3.8	ns	

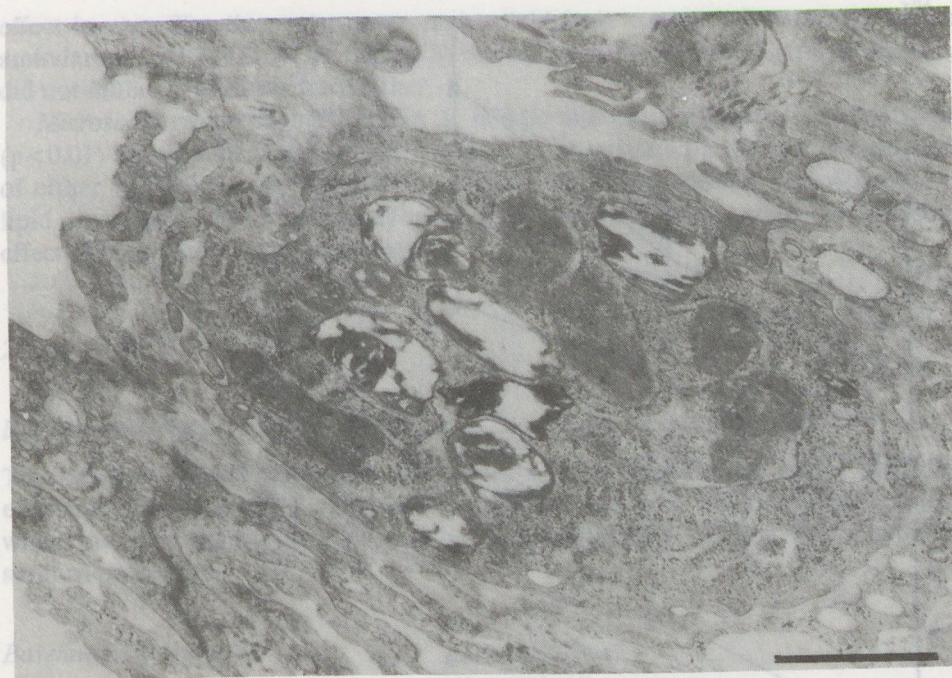


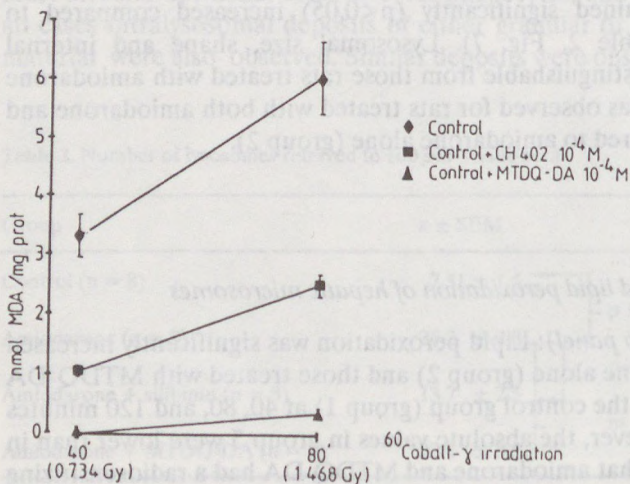
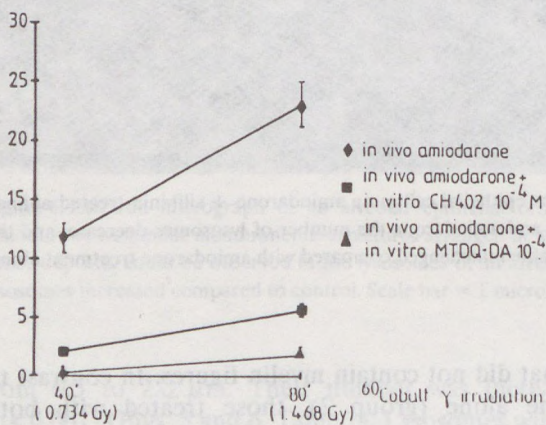
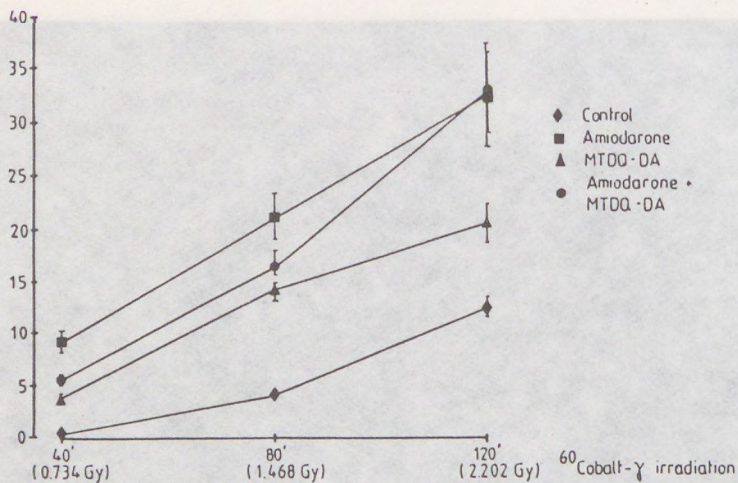
Figure 7. Electron micrograph of an alveolar epithelial cell in an amiodarone + silibinin-treated animal. After simultaneous treatment with silibinin and amiodarone the number of lysosomes decrease, and the size, shape, and internal characteristics remained unchanged compared with amiodarone treatment alone. Scale bar = 1 micrometer (x 24,000)

bodies (diameter 1.0 to 4.0 μm) that did not contain myelin figures. In contrast to the rats treated with amiodarone alone (group 2), those treated with both amiodarone and silibinin (group 4) had significantly fewer lysosomes ($p < 0.01$) although this number remained significantly ($p < 0.05$) increased compared to control rats (group 1) (Table 2, Fig. 7). Lysosomal size, shape and internal characteristics remained indistinguishable from those rats treated with amiodarone alone. No significant effect was observed for rats treated with both amiodarone and MTDQ-DA (group 3) compared to amiodarone alone (group 2).

Biophysical studies

^{60}Co -gamma radiolysis-induced lipid peroxidation of hepatic microsomes

Microsome group I (Fig. 8, top panel): Lipid peroxidation was significantly increased in rats treated with amiodarone alone (group 2) and those treated with MTDQ-DA alone (group 5) compared to the control group (group 1) at 40, 80, and 120 minutes of irradiation ($p < 0.01$); however, the absolute values in group 5 were lower than in group 2. These results show that amiodarone and MTDQ-DA had a radiosensitizing



effect in vivo. The liver microsome malondialdehyde content of rats treated with amiodarone and MTDQ-DA (group 3) fell between the values of groups 2 and 5, did not differ significantly from either.

Microsome groups II and III (Fig. 8, middle and bottom panels): A significant ($p < 0.01$) antioxidant radioprotective effect was observed with the in vitro addition of either MTDQ-DA or CH 402 to control amiodarone-treated microsomes, i.e. lipid peroxidation was significantly reduced at 40 and 80 minutes of irradiation. The effect of both antioxidants were similar.

2. Dog studies

Electrophysiological (EP) measurements

The baseline (measured before the creation of Y-shaped right atrial incision) electrophysiological (EP) data demonstrate the effect of drug treatment alone, while the postsurgical data reflect the combined effect of drug treatment and surgical procedure.

Baseline EP parameters

The AH interval and right ventricular ERP (RV-ERP) values were significantly longer in the amiodarone + silymarin treated group than in the control group (Fig. 9). Although these values tended to be greater in the amiodarone group than in the control, the difference did not reach statistical significance. The atrial flutter mean cycle length (AF MEAN CL) and right atrial ERP (RA-ERP) were significantly longer in both treated groups compared to control. Both amiodarone and amiodarone + silymarin treatment tended to lengthen the spontaneous sinus

Figure 8. In vivo biophysical studies. The malondialdehyde content in nmol malondialdehyde/ mg microsomal protein was plotted on the ordinate against irradiation time in minutes on the abscissa. (Top) Lipid peroxidation induced by ^{60}Co -gamma irradiation in liver microsomal fraction of in vivo treated rats. According to two-way analysis of variance, the in vivo amiodarone treatment increased significantly ($p < 0.01$) the lipid peroxidation induced by ^{60}Co -gamma irradiation, consistent with the radiosensitizing effect of amiodarone. The simultaneous MTDQ-DA treatment together with amiodarone did not exert a protective effect. The radiosensitizing effect of MTDQ-DA treatment alone was also significant ($p < 0.01$) compared to control. (Middle) The in vitro effect of CH 402 and MTDQ-DA antioxidants on lipid peroxidation measured in liver microsomal fraction of rats treated with ^{60}Co -gamma irradiation and in vivo amiodarone. Amiodarone treatment increased significantly ($p < 0.01$) the lipid peroxidation induced by ^{60}Co -gamma irradiation according to two-way analysis of variance. The protective effect of in vitro added antioxidants proved to be significant ($p < 0.01$) as well. (Bottom) The in vitro effect of CH 402 and MTDQ-DA antioxidants on lipid peroxidation measured in the microsomal fraction of rats treated with ^{60}Co -gamma irradiation. The protective effect of antioxidants proved to be significant ($p < 0.01$) as well. The microsomal fraction malondialdehyde content data show triplicate measurements done with two parallels. The microsomal fraction was prepared from the liver homogenate of 5-5 animals

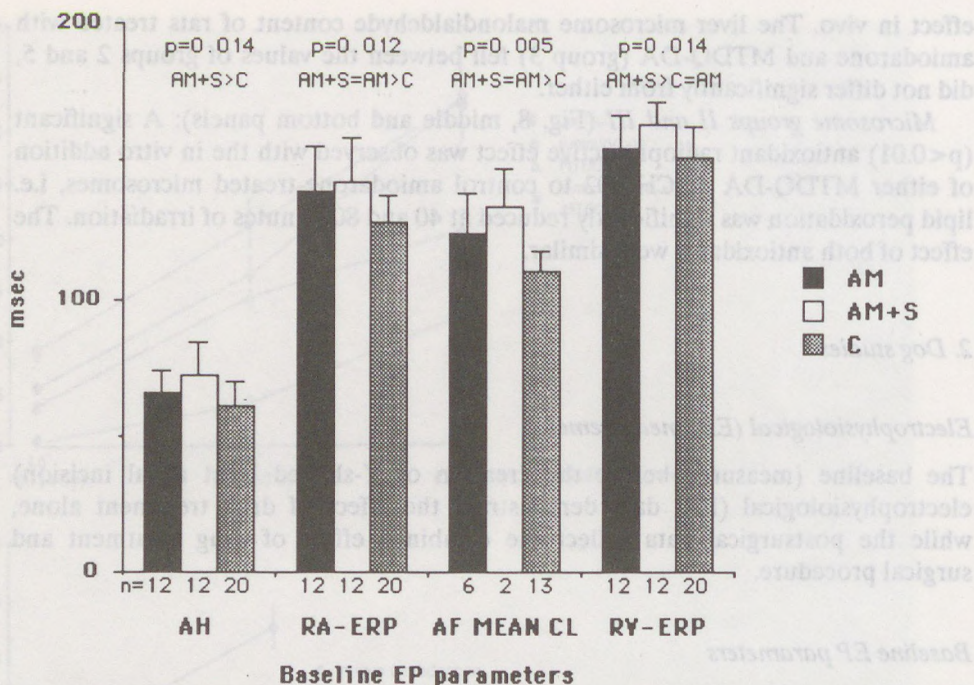


Figure 9. Baseline electrophysiological parameters showing significant differences between groups

There was significant difference in all illustrated electrophysiological parameters between amiodarone + silymarin treated and control dogs. The AF MEAN CL and RA-ERP was also significantly longer in amiodarone treated dogs than in control dogs. The RV-ERP was significantly ($p = 0.014$) longer in amiodarone + silymarin treated dogs than in amiodarone treated dogs. There was no significant between-groups difference in other measured baseline parameters (not shown here).

AM = amiodarone; AM + S = amiodarone + silymarin; C = control; RA-ERP = right atrial effective refractory period; RV-ERP = right ventricular effective refractory period; AF MEAN CL = atrial flutter mean cycle length; EP = electrophysiological

rhythm cycle length (SRCL), compared with control, but the difference was not statistically significant.

Evaluation of presurgical (baseline) and postsurgical EP data together

The following significant changes in EP parameters occurred in the amiodarone + silymarin treated group compared to control group: increase of baseline AH (Fig. 9), increase of pre- and postsurgical RV-ERP, RA-ERP, AF MEAN CL (Fig. 10), decrease of postsurgical atrial flutter maximal duration (AF MAX DUR) (Table 3). ($p = 0.014$, $p = 0.005$, $p = 0.027$, $p = 0.01$, $p = 0.006$ respectively). The pre- and postsurgical RA-ERP, AF MEAN CL and postsurgical right atrial diastolic threshold (RA-TH) were significantly ($p = 0.027$, $p = 0.01$, $p = 0.033$ respectively) increased in the amiodarone group compared with control. There was no significant

difference between the EP parameters of amiodarone + silymarin and amiodarone groups except for the baseline RV-ERP (163 ± 8 vs 154 ± 12 ; $p = 0.014$) in the amiodarone + silymarin group, and the postsurgical RA-TH (0.51 ± 0.36 vs 0.34 ± 0.20 ; $p = 0.033$) in the amiodarone group (Fig. 10).

Atrial flutter

Sustained (>30 min) atrial flutter was produced in 12/17 (71%) control dogs, 8/12 (66%) amiodarone treated dogs and 1/12 (8%, amiodarone + silymarin treated dog. Amiodarone treatment alone did not prevent sustained atrial flutter (NS from

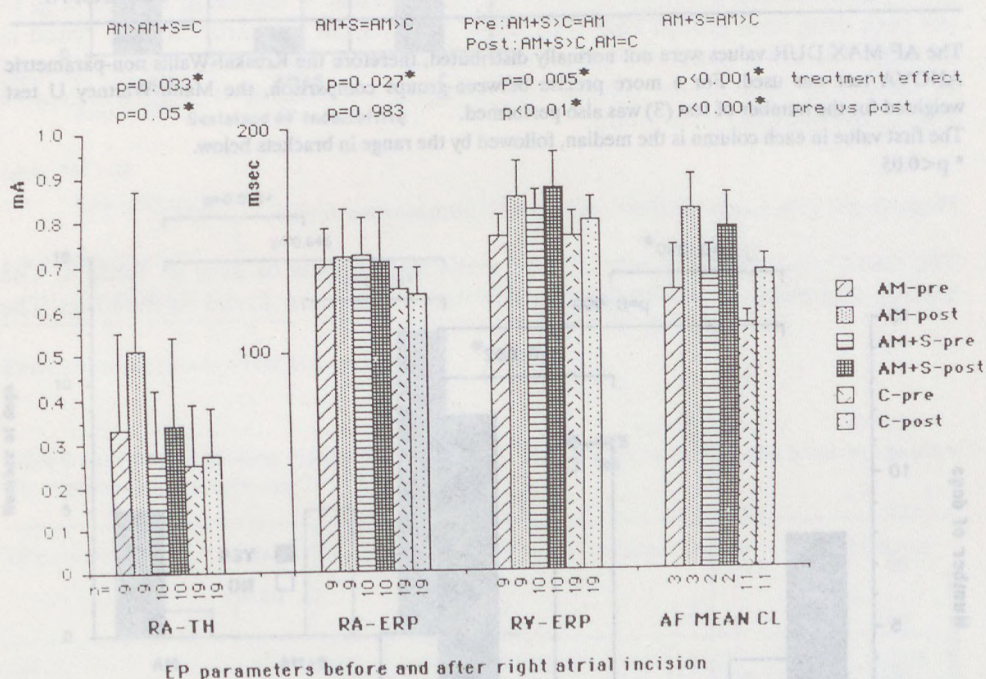


Figure 10. Pre- and postsurgical electrophysiological parameters showing significant between-groups treatment effect with or without surgical intervention effect. In the amiodarone + silymarin treated group significant prolongation of pre- and postsurgical RV-ERP, RA-ERP, AF MEAN CL ($p = 0.005$, $p = 0.027$, $p = 0.01$ respectively) occurred. The pre- and postsurgical RA-ERP, AF MEAN CL and postsurgical RA-TH were significantly ($p = 0.027$, $p = 0.01$, $p = 0.033$ respectively) increased in the amiodarone treated group. There was no significant difference between the electrophysiological parameters of amiodarone + silymarin and amiodarone groups, except for two parameters: the baseline RV-ERP was significantly ($p = 0.014$) longer in the amiodarone + silymarin group and the postsurgical RA-TH was significantly ($p = 0.033$) higher in the amiodarone group.

AM = amiodarone; AM + S = amiodarone + silymarin; C = control; pre = presurgical; post = postsurgical; RA-TH = right atrial diastolic threshold; RA-ERP = right atrial effective refractory period; RV-ERP = right ventricular effective refractory period; AF MEAN CL = atrial flutter mean cycle length; EP = electrophysiological

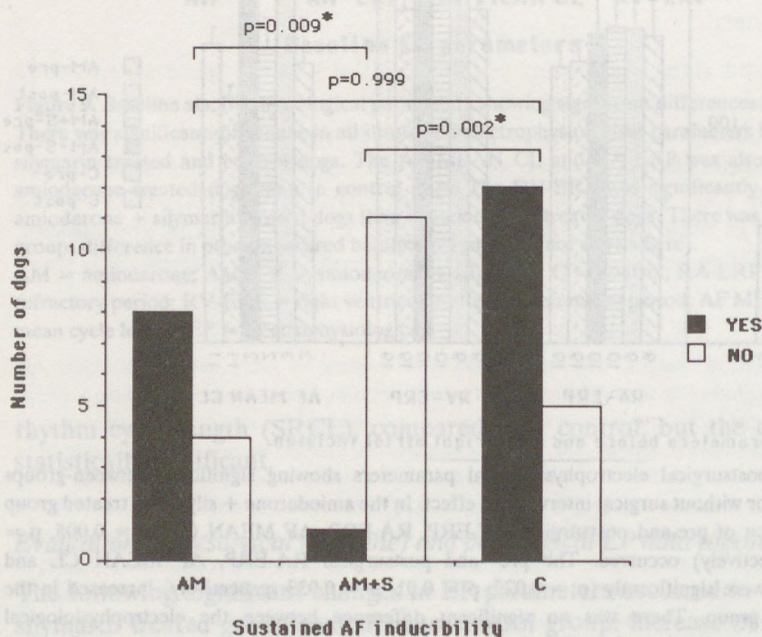
Table 3. Maximal duration of atrial flutter (AF MAX DUR) before and after right atrial incision

	AM	AM+S	C	p	Comment
pre	158 (4->1800) (12)	15 (2->1800) (10)	125 (16->1800) (18)	= 0.162	
AF MAX DUR (sec)					
post	>1800 (1->1800) (11)	185 (2-1262) (9)	>1800 (16->1800) (19)	= 0.006*	AM+S<C AM=C AM=AM+S

The AF MAX DUR values were not normally distributed, therefore the Kruskal-Wallis non-parametric ANOVA test was used. For a more precise between-groups comparison, the Mann-Whitney U test weighted for the number of test (3) was also performed.

The first value in each column is the median, followed by the range in brackets below.

* $p < 0.05$

**Figure 11.** Induction of sustained atrial flutter in all treated dog.

Amiodarone + silymarin treatment reduced significantly ($p = 0.02$) the induction of sustained (> 30 min) atrial flutter compared with the control group; amiodarone treatment alone failed to prevent ($p = 0.999$) induction of sustained atrial flutter. Sustained atrial flutter could be induced in fewer dogs ($p = 0.009$) in the amiodarone + silymarin treated group than in the amiodarone alone treated group.

AM = amiodarone; AM + S = amiodarone + silymarin; C = control.

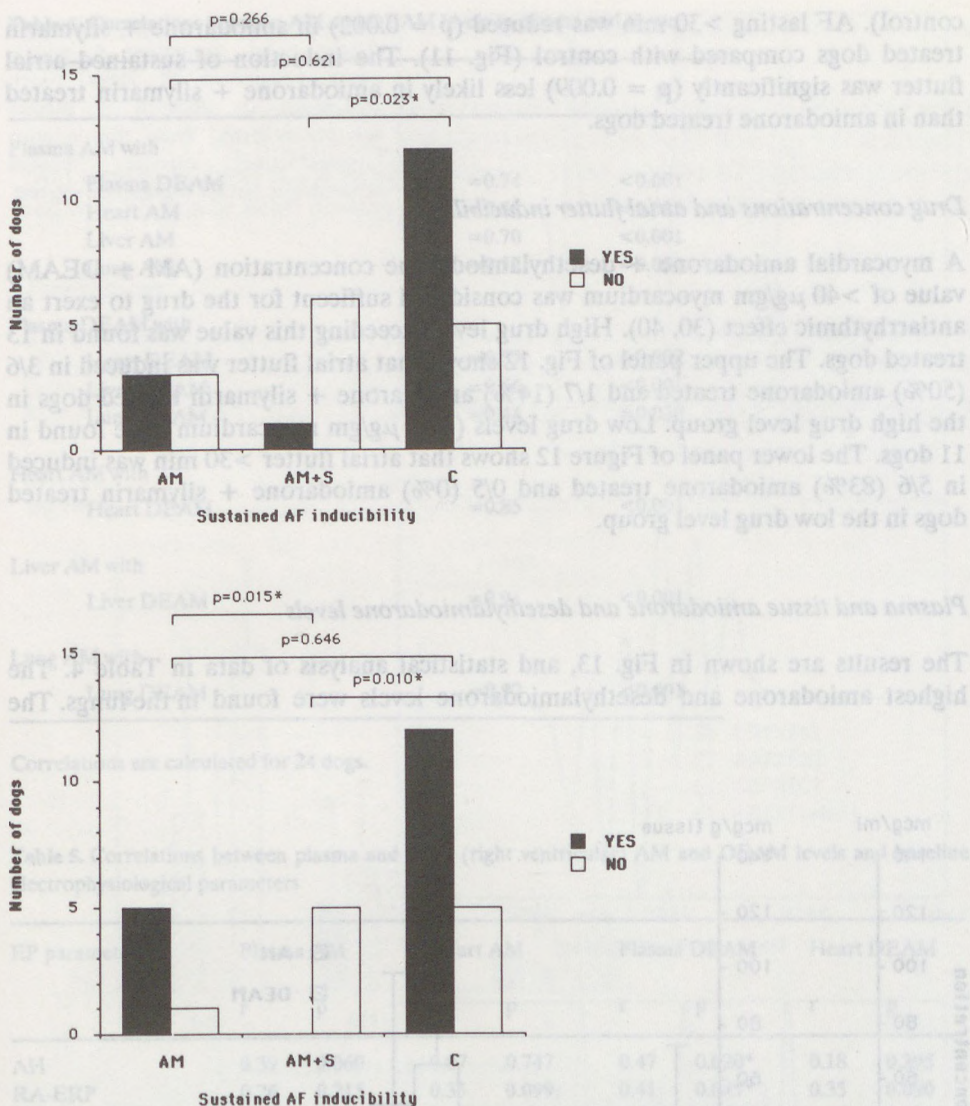


Figure 12. Induction of sustained atrial flutter in high and low drug level group

The upper panel shows the prevalence of sustained (> 30 min) atrial flutter results for the high drug level ([AM + DEAM] ≥ 40 microgram/mg myocardium) group. Amiodarone + silymarin treatment significantly reduced ($p = 0.023$) the number of dogs with sustained atrial flutter compared with the control group, while amiodarone treatment alone did not ($p = 0.621$). No significant difference resulted between amiodarone + silymarin and amiodarone groups probably because of the small sample size.

The lower panel shows the prevalence of sustained atrial flutter for low drug level ([AM + DEAM] < 40 $\mu\text{gm/gm}$ myocardium) dogs. Amiodarone + silymarin treatment significantly reduced the prevalence of sustained atrial flutter ($p = 0.01$) even in the low drug level group, while amiodarone treatment alone did not ($p = 0.646$). Sustained atrial flutter could be induced in significantly ($p = 0.05$) less amiodarone + silymarin treated dogs than in amiodarone treated dogs.

AM = amiodarone; AM + S = amiodarone + silymarin; C = control

control). AF lasting >30 min was reduced ($p = 0.002$) in amiodarone + silymarin treated dogs compared with control (Fig. 11). The induction of sustained atrial flutter was significantly ($p = 0.009$) less likely in amiodarone + silymarin treated than in amiodarone treated dogs.

Drug concentrations and atrial flutter inducibility

A myocardial amiodarone + desethylamiodarone concentration (AM + DEAM) value of $>40 \mu\text{g/gm}$ myocardium was considered sufficient for the drug to exert an antiarrhythmic effect (30, 40). High drug level exceeding this value was found in 13 treated dogs. The upper panel of Fig. 12 shows that atrial flutter was induced in 3/6 (50%) amiodarone treated and 1/7 (14%) amiodarone + silymarin treated dogs in the high drug level group. Low drug levels ($<40 \mu\text{g/gm}$ myocardium) were found in 11 dogs. The lower panel of Figure 12 shows that atrial flutter >30 min was induced in 5/6 (83%) amiodarone treated and 0/5 (0%) amiodarone + silymarin treated dogs in the low drug level group.

Plasma and tissue amiodarone and desethylamiodarone levels

The results are shown in Fig. 13, and statistical analysis of data in Table 4. The highest amiodarone and desethylamiodarone levels were found in the lungs. The

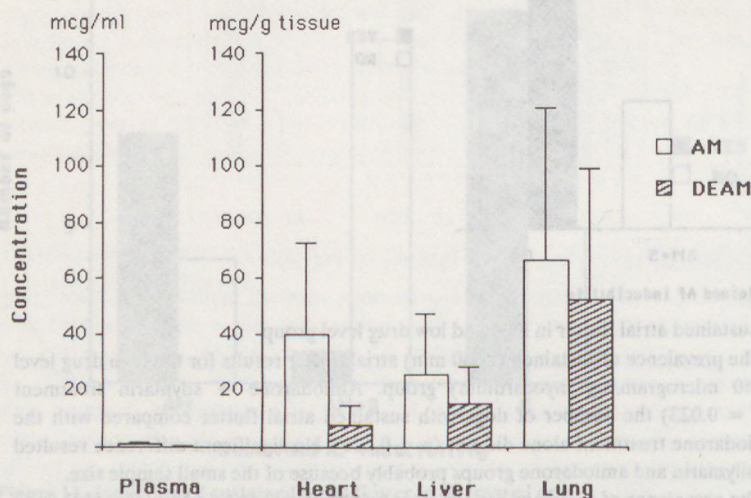


Figure 13. Plasma and tissue amiodarone and desethylamiodarone concentrations

The highest amiodarone and desethylamiodarone concentrations were found in the lungs, the lowest values in the plasma. The heart and liver amiodarone and desethylamiodarone concentration values fell in between those of the plasma and the lung

AM = amiodarone; DEAM = desethylamiodarone

Table 4. Correlations between AM and DEAM levels in plasma and tissue

	r	p
Plasma AM with		
Plasma DEAM	=0.74	<0.001
Heart AM	=0.58	=0.003
Liver AM	=0.70	<0.001
Lung AM	=0.57	=0.004
Plasma DEAM with		
Heart DEAM	=0.59	=0.002
Liver DEAM	=0.66	<0.001
Lung DEAM	=0.44	=0.030
Heart AM with		
Heart DEAM	=0.85	<0.001
Liver AM with		
Liver DEAM	=0.91	<0.001
Lung AM with		
Lung DEAM	=0.92	<0.001

Correlations are calculated for 24 dogs.

Table 5. Correlations between plasma and heart (right ventricular) AM and DEAM levels and baseline electrophysiological parameters

EP parameter	Plasma AM		Heart AM		Plasma DEAM		Heart DEAM	
	r	p	r	p	r	p	r	p
AH	0.39	0.060	0.07	0.747	0.47	0.020*	0.18	0.395
RA-ERP	0.26	0.215	0.35	0.099	0.41	0.045*	0.35	0.090
AF-MEAN CL (n=8)	0.23	0.518	-0.16	0.706	0.79	0.020*	0.56	0.151
RV-ERP	0.36	0.088	0.23	0.282	0.38	0.068	0.46	0.023*

N=24 unless marked otherwise.

There was no correlation between other measured baseline EP parameters (SRCL, HV, RA-TH, AF MAX DUR) and plasma and heart AM and DEAM levels (not shown here).

heart and liver tissue amiodarone and desmethyamiodarone levels fell in between the plasma and lung levels. Plasma concentrations correlated closely with tissue concentrations for amiodarone and desethylamiodarone ($p < 0.02$).

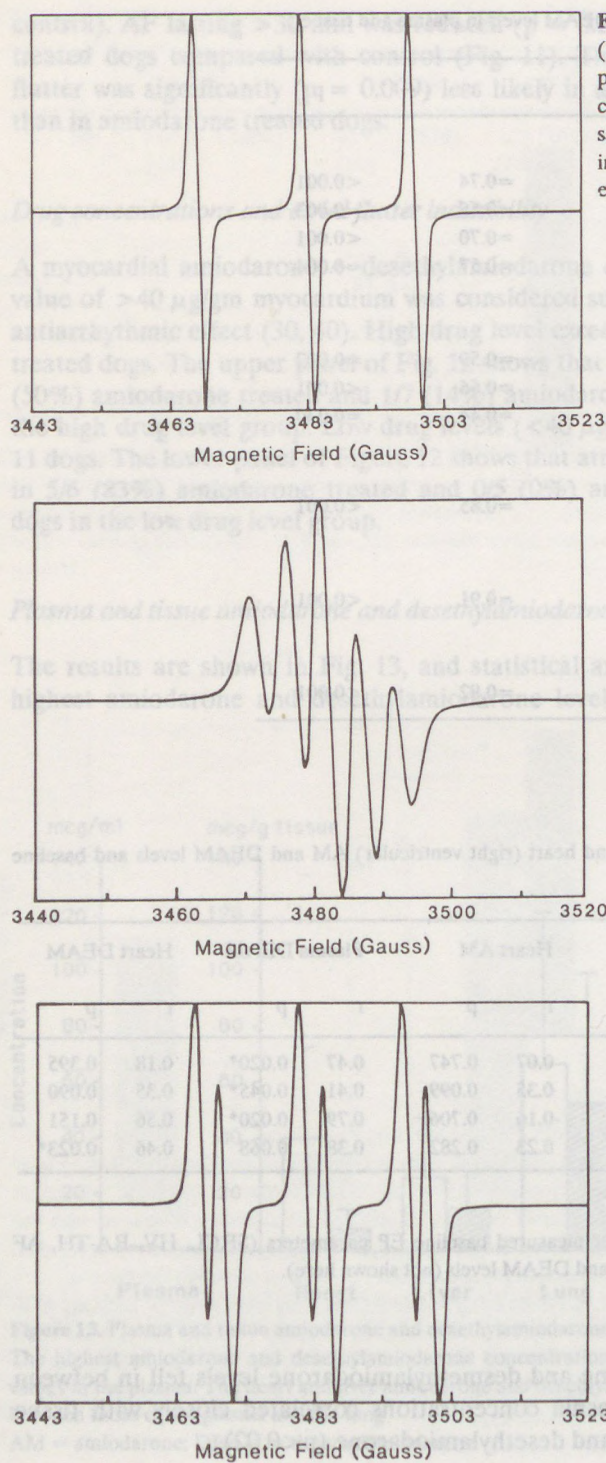


Figure 14. Simulated free radical signals (Upper panel: component A, middle panel: component B, lower panel: component C) detected in plasma samples collected from the anterior interventricular coronary venous effluent. For further explanation see text

Relationship between plasma- and tissue amiodarone, desethylamiodarone levels and baseline EP parameters

As Table 5 shows, the baseline EP parameters showing correlation with drug levels (AH, RA-ERP, AF MEAN CL, RV-ERP) were the same as those in which a significant treatment effect could be demonstrated. These baseline EP parameters correlated with plasma or heart desethylamiodarone levels but not with amiodarone levels.

Free radicals

Three different radical signals were detectable and were defined as components A, B and C (Fig. 14). Component C is a signal similar to that reported by Bolli et al. [32, 33]. The hyperfine couplings are $a_N = 15.1$ G $a_H = 3.15$ G which is most likely

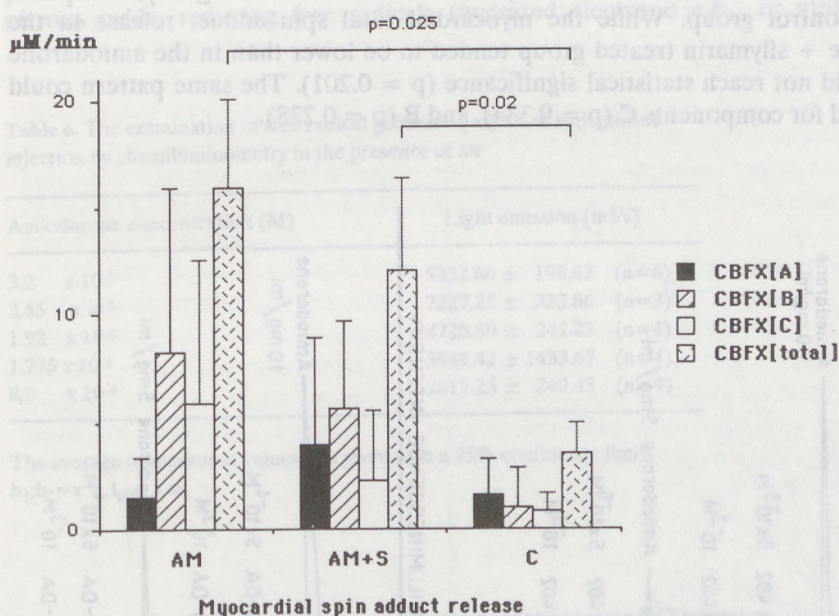


Figure 15. Myocardial spin adduct release from the myocardial bed drained by the anterior interventricular coronary vein

The myocardial release ($\mu\text{mol}/\text{min}$) for the total spin adduct and each component was calculated as follows: the spin adduct concentration ($\mu\text{mol}/\text{ml}$) was multiplied by the coronary blood flow (ml/min). The myocardial total spin adduct release is a sum of the myocardial release of the three components (A, B and C). The total spin adduct release increased significantly in the treated groups ($p = 0.025$ for amiodarone vs control and $p = 0.02$ for amiodarone + silymarin vs control). The total spin adduct and component C, B release tended to be consistently lower in the amiodarone + silymarin group compared with the amiodarone group, however it did not reach statistical significance because of the small sample size and non-normal distribution of data.

AM = amiodarone; AM + S = amiodarone + silymarin; C = control; CBF = coronary blood flow; [A] = component A concentration; [B] = component B concentration; [C] = component C concentration; [total] = total spin adduct concentration

consistent with either an alkyl ($R\cdot$) or an alkoxy ($RO\cdot$) radical, which may be generated in vivo. Component B appears to be a dimer of PBN since the observed hyperfine coupling arise from 2 identical nitrogens, $a_N = 5.04$ G. The origin of this dimer is uncertain and it could have been formed in vivo or possibly on sample extraction and processing where high PBN concentrations are achieved. Component A is a simple nitroxide triplet $a_N = 15.9$ G. This may arise from a different PBN radical adduct where the H coupling is very small or absent and may be formed either in vivo or on sample extraction and processing.

There was no between-groups difference in the coronary blood flow values (35.2 ± 14.9 ml/min for amiodarone, 26.5 ± 12.8 ml/min for amiodarone + silymarin, and 27.1 ± 3.7 ml/min for the control group). Figure 15 shows that the myocardial total spin adduct release, which is the sum of the myocardial release of the three components, was significantly ($p = 0.025$ for amiodarone vs control, and $p = 0.02$ for amiodarone + silymarin vs control) increased in the treated groups compared with the control group. While the myocardial total spin adduct release in the amiodarone + silymarin treated group tended to be lower than in the amiodarone group, it did not reach statistical significance ($p = 0.201$). The same pattern could be observed for components C ($p = 0.384$), and B ($p = 0.228$).

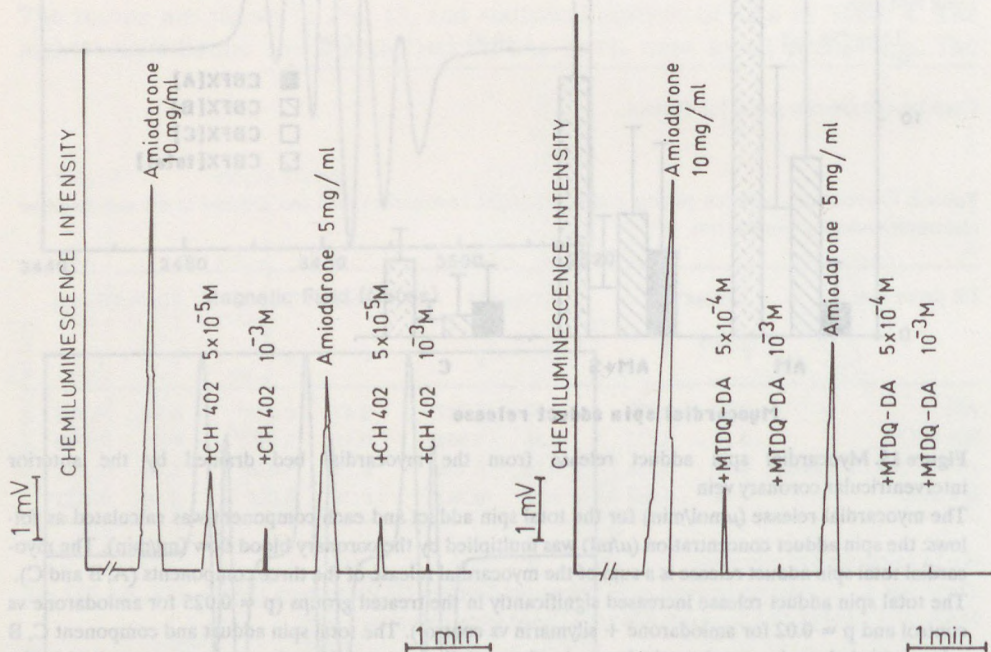


Figure 16. In vitro chemiluminescence induced by amiodarone tablet. The dose-dependent chemiluminescence signal induced by the water suspension of amiodarone tablet and its dose-dependent inhibition by dihydroquinoline-type antioxidants CH 402 and MTDQ-DA. The light emission induced by amiodarone injection solution is not shown here, but it produced a similar chemiluminescence signal and the effect of antioxidants was essentially the same. See text for further explanation

In vitro studies

Chemiluminometric studies

Dose-dependent chemiluminescent signals were observed for all three amiodarone preparations. The addition of either MTDQ-DA or CH 402 to these preparations resulted in dose-dependent inhibition of these signals (Fig. 16, Tables 6-8).

Biophysical studies

The aim of these studies was to identify the putative free radical products formed from amiodarone in vitro in a reducing environment. The activity of amiodarone toward reducing agents was examined using the stationary ^{60}Co -gamma and electron pulse radiolysis. On irradiating a diluted aqueous solution of an alcohol-containing nitrous oxide, reducing free radicals (hydrated electron = e_{aq} or alpha-hydroxy

Table 6. The examination of free radical generating effect of amiodarone injection by chemiluminometry in the presence of air

Amiodarone concentration (M)	Light emission (mVs)
3.2×10^{-3}	9332.66 ± 198.82 (n=6)
2.55×10^{-3}	7227.25 ± 333.86 (n=3)
1.92×10^{-3}	4720.50 ± 245.23 (n=4)
1.775×10^{-3}	3949.42 ± 1433.67 (n=4)
8.0×10^{-4}	2617.25 ± 240.43 (n=4)

The average of measured values was given with a 95% confidence limit:
 $h_1; h_2 = x \pm t_{p5\%} \times s_x$

Table 7. The study of free radical generating property of amiodarone.HCl substance by chemiluminometry in the presence of air

The concentration of amiodarone.HCl substance (M)	Light emission (mVs)
3.04×10^{-4}	10126.25 ± 1966.64 (n=4)
2.28×10^{-4}	7569.50 ± 2561.02 (n=3)
1.52×10^{-4}	2940.16 ± 2317.22 (n=3)
7.51×10^{-5}	1447.50 ± 1093.86 (n=3)

The average of measured values was given with a 95% confidence limit:
 $h_1; h_2 = x \pm t_{p5\%} \times s_x$

Table 8. The study of free radical generating property of amiodarone.HCl substance by chemiluminometry in the presence of MTDQ-DA and air

Concentration		Light emission (mVs)
3.04×10^{-4}	amiodarone.HCl substance	10126 ± 1966.64 (n=4)
8.7×10^{-4}	MTDQ-DA	339.93 ± 45.28 (n=3)
8.7×10^{-4}	"	1647.50 ± 273.26 (n=3)
8.7×10^{-4}	"	5065.50 ± 2675.45 (n=3)
4.35×10^{-4}	"	8307.50 ± 2326.63 (n=3)
2.6×10^{-4}	"	9144.15 ± 1612.31 (n=3)
8.7×10^{-4}	"	11980.46 ± 2105.25 (n=3)

The average of measured values was given with a 95% confidence limit:

$$h_1; h_2 = x \pm t_{p5\%} \times s_x$$

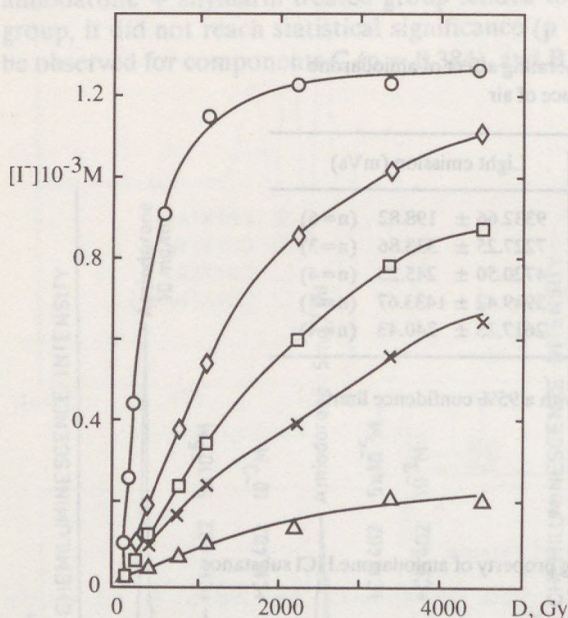
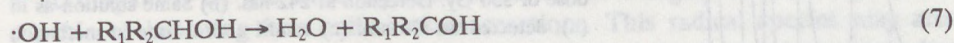
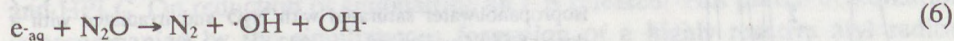
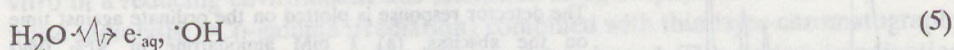


Figure 17. Potentiometric determination of I⁻ concentration in aqueous alcohol (30% v/v) solution containing 1 mM amiodarone, gamma irradiated with various doses. The concentration of I⁻ in mM is plotted on the ordinate against the irradiation dose (D) in Gy plotted on the abscissa.

O = isopropanol, bubbled with N₂O; ◊ = ethanol, N₂O; ◻ = methanol, N₂O; X = tert-butanol, N₂; Δ = tert - butanol, N₂O.

The yield of I⁻ was the greatest in isopropanol solution, because isopropanol is the strongest reducing agent, having the greatest negative redox potential, and its alpha-C-H bond is the weakest among the alcohols used, therefore the H atom is available for absorption. The concentration of I⁻ in isopropanol solution irradiated with doses greater than 1 kGy in the presence of 1 mM amiodarone was well in excess of 1 mM, indicating that at the higher conversion of amiodarone full deiodination takes place by releasing both iodine atoms in succession

radicals = $\cdot\text{OH}$) can be produced as shown by Equations 5 through 7:



In the presence of amiodarone ($1 \times 10^{-3} \text{ M}$) at neutral pH, formation of iodide ion was observed by potentiometry using an iodide ion selective electrode (Fig. 17). Using various alcoholic solutions, the yield of iodide ion increased in the order:

tert-butanol < methanol < ethanol < isopropanol

The concentration of iodide ion (I^-) in isopropanol/water irradiated with doses greater than 1 kGy in the presence of 1 mM amiodarone was well in excess of 1 mM, indicating that at higher conversion of amiodarone full deiodination takes place, by releasing both iodine atoms in succession. Analyses at early stages of the reaction at low doses of stationary or electron pulse irradiation confirmed that partially deiodinated amiodarone was the major product in each case. On reduction of amiodarone, I^- is released, accompanied by a simultaneously formed aryl radical (Fig. 18). The aryl radical is highly reactive, readily abstracts hydrogen atoms from the neighboring organic molecules, and thereby initiates lipid peroxidation or other free radical reactions.

In various alcoholic solutions of amiodarone, a stable product absorbing at 370 nm is formed as a consequence of stationary ^{60}Co -gamma or electron pulse irradiation under reducing conditions. The identification of this stable product was tried using thin layer chromatography and HPLC. Since standard samples of amiodarone metabolites necessary for the evaluation of chromatograms were not available, partially and fully deiodinated compounds by hydrogenating amiodarone dissolved in methanol using a 10% Pd/alumina catalyst were prepared.

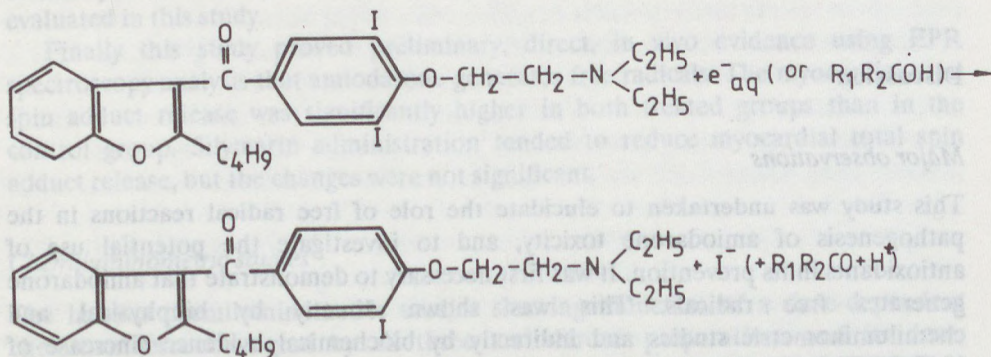


Figure 18. The formation of aryl radical from amiodarone in a reducing environment. e_{aq}^- = hydrated electron; $\text{R}_1\text{R}_2\text{C}\cdot\text{OH}$ = alpha hydroxy radical situated on an organic molecule; $\text{R}_1\text{R}_2\text{CO}$ = carbonyl group situated on an organic molecule; H^+ = hydrogen ion. See text for further explanation

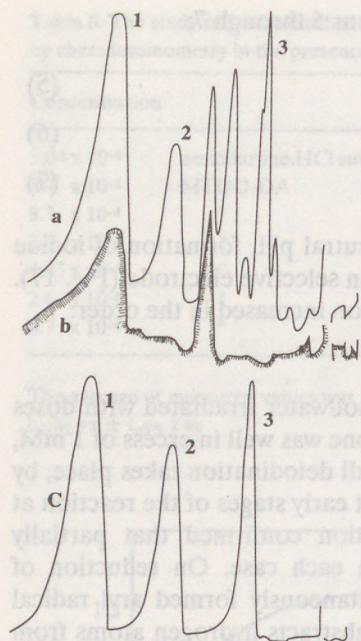


Figure 19. High performance liquid chromatogram of alcoholic amiodarone solution exposed to various reducing conditions. The detector response is plotted on the ordinate against time on the abscissa. (a) 1 mM amiodarone in 30% (v/v) isopropanol/water saturated with N_2O and irradiated with a dose of 550 Gy. Detection at 242 nm. (b) Same solution as in (a), detected at 370 nm. (c) 1 mM amiodarone in methanol, subjected to catalytic hydrogenation, detected at 242 nm. Peak 1 is assigned to amiodarone, peak 2 to partially and peak 3 to fully deiodinated amiodarone. The three chromatograms are aligned in time. The second peak on chromatogram b, which is different from peaks 1, 2 and 3 assigned to amiodarone, partially and fully deiodinated amiodarone, respectively, shows the stable product formed from amiodarone beside the main product (partially deiodinated amiodarone).

Chromatogram b of fig. 8 shows the stable product absorbing at 370 nm, which is also generated beside the main product (the partially deiodinated amiodarone). The identification of the stable product did not succeed. It is conspicuous on Fig. 19 that the stable product is different from the deiodinated products prepared by catalytic hydrogenation of amiodarone. An unpleasant property of the stable product, that it decomposes within a couple days, largely impedes its identification. The stable product might be formed in recombination processes of free radicals generated in the system.

Discussion

Major observations

This study was undertaken to elucidate the role of free radical reactions in the pathogenesis of amiodarone toxicity, and to investigate the potential use of antioxidants in its prevention. It was first necessary to demonstrate that amiodarone generates free radicals. This was shown directly by biophysical and chemiluminometric studies and indirectly by biochemical evidence (increase of NADPH and Fe^{3+} induced microsomal lipid peroxidation). The radiosensitizing effect of amiodarone was also verified using ^{60}Co -gamma irradiated liver microsomal preparations; this is also consistent with the free radical producing

activity of the drug. The main free radical product emerging from amiodarone in vitro in a reducing environment was identified using biophysical methods (electron pulse radiolysis, ^{60}Co -gamma irradiation) combined with thin layer chromatography and HPLC. On reduction of amiodarone an I $^{\cdot-}$ is released. This partial deiodination is accompanied by the simultaneous formation of a highly reactive aryl radical capable of initiating free radical chain reactions. This radical species may arise during in vivo metabolism of amiodarone in the hepatic microsomal drug metabolizing system or elsewhere in the organism, and may play an important role in the pathogenesis of amiodarone toxicity in addition to those of other well-established mechanisms such as the inhibition of lysosomal phospholipase activity. The ultrastructural studies led one step further, showing that the simultaneous treatment with the antioxidant silibinin and amiodarone decreased the lysosomal phospholipoidosis induced by amiodarone treatment. Since lysosomal phospholipoidosis is most likely not only a marker of amiodarone and amphiphilic drug treatment, but also may result in cell death above a certain level, these results established a potential pathogenetic link between free radical production, lysosomal phospholipoidosis and amiodarone toxicity.

The rat studies and in vitro studies suggested that simultaneous antioxidant therapy with amiodarone might be used to reduce amiodarone toxicity if it did not impair amiodarone's antiarrhythmic action [41, 42], therefore in the dog studies, we tested whether simultaneous administration of a flavonoid antioxidant, silymarin altered the electrophysiologic actions of amiodarone in open chest dogs with induced atrial flutter. Amiodarone + silymarin reduced the prevalence of dogs with sustained atrial flutter, while amiodarone alone did not. Further, the changes in AH, RV-ERP, and postsurgical AF MAX DUR produced by amiodarone + silymarin treatment exceeded those produced by amiodarone alone when compared with control, but only the baseline RV-ERP was longer in the amiodarone + silymarin treated group when the comparison was made between the two treated groups. These data suggest that silymarin has an additive or potentiating effect on the antiarrhythmic and electrophysiologic actions of amiodarone and might have an antiarrhythmic activity alone as well, however this latter possibility was not evaluated in this study.

Finally this study proved preliminary, direct, in vivo evidence using EPR spectroscopy analysis that amiodarone generates free radicals. The myocardial total spin adduct release was significantly higher in both treated groups than in the control group. Silymarin administration tended to reduce myocardial total spin adduct release, but the changes were not significant.

Chemiluminometric studies

The in vivo chemiluminometric studies showing induction of a dose-dependent chemiluminescence signal by all three amiodarone preparations and its dose-dependent inhibition by dihydroquinoline-type antioxidants (CH 402, MTDQ-DA) furnished direct evidence that amiodarone produces free radicals. The free radical scavenger activity of these antioxidants was confirmed in previous experiments [25,

43]. By also using amiodarone.HCl substance in the chemiluminescence studies, the primary role of injection solvent or tablet vehicle in the induction of the chemiluminescence signal was excluded.

The chemiluminescence in this system is caused by excited luminol derivatives. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) is oxidized to an excited aminophthalate anion by reactive oxygen intermediates (e.g. superoxide anion radical: O_2^- , hydrogen peroxide: H_2O_2 , hydroxyl radical: $\cdot OH$, singlet oxygen: 1O_2), which, as it returns to its ground state, emits light [44].

The mechanism of chemiluminescence in this system is not clear. Amiodarone may react with O_2 in the presence of air and light, and may generate reactive oxygen intermediates or 1O_2 directly as well. However, this is only a suggestion, and further studies are needed to clarify this question.

The chemiluminometric study of liver homogenates from treated rats indirectly supports the free radical producing effect of amiodarone, which was partially prevented by simultaneous silibinin treatment.

In summary, the chemiluminometric studies proved that amiodarone generates free radicals in the presence of air and light. Since all secondary reactive oxygen intermediates are able to produce chemiluminescence in the presence of luminol, these studies did not elucidate the mechanism of free radical generation, or the nature of the free radicals formed.

Biochemical studies

The purpose of the biochemical studies was to provide independent indirect in vivo evidence for free radical generation by amiodarone. NADPH and $FeCl_3$ induced microsomal lipid peroxidation was measured in the presence of amiodarone with or without antioxidants. The microsomal lipid peroxidation was determined using the thiobarbituric acid test. In vivo amiodarone treatment significantly increased the NADPH and $FeCl_3$ induced lipid peroxidation in the liver microsomal fraction. Antioxidants (MTDQ-DA, silibinin) were not protective against the amiodarone-induced increase in induced enzymatic lipid peroxidation. A merely speculative explanation for the ineffectiveness of antioxidants might be that, during preparation of the microsomal fractions, most of the antioxidants that could get access to this site during in vivo treatment might have been lost. Consequently, the concentration of antioxidants might be orders of magnitude smaller in liver microsomal fractions than in fresh liver homogenate; however this suggestion needs to be verified. The induced lipid peroxidation study is also related to the chemiluminometric data, since chemiluminescence intensity and the accumulation of malondialdehyde (an end-product of lipid peroxidation) are proportional to each other, because they are indicators of the same fundamental process, although of its different stages. According Sugioka and Nakano [45], the light intensity in the iron-induced lipid peroxidation of mitochondrial and microsomal membranes was proportional to the square of accumulated lipid hydroperoxide concentration.

Kennedy et al. [18] reported similar chemiluminometric and biochemical results. In their in vitro perfused and ventilated rabbit lung model, the application of

antioxidants prevented pulmonary edema caused by amiodarone. Amiodarone treatment significantly increased the chemiluminescence of lung tissue in the presence of luminol and its glutathione disulfide content. They considered oxidant injury to have a primary role in the pulmonary toxicity of amiodarone.

Morphological studies

a) Light microscopy

The aim of this study was to check that the dosage and length of amiodarone treatment were sufficient to produce pathological findings compatible with amiodarone toxicity, serving as an endpoint, because further *in vivo* studies to investigate the pathogenesis of amiodarone toxicity were performed with the premise that amiodarone toxicity was induced by the applied regimen. The light microscopic results confirmed the data published by many other authors [3, 4, 8, 9, 46-51]. The "pathological lung" index was significantly ($p < 0.001$) higher in amiodarone-treated groups than in groups not receiving amiodarone. The simultaneous treatment with antioxidants (MTDQ-DA, silibinin) and amiodarone did not influence the "pathological lung" index compared to the group treated with amiodarone alone.

This latter fact does not necessarily mean that antioxidants are not protective against amiodarone lung toxicity. The "pathological lung" index is not unequivocally characteristic to the extent of lung toxicity. One component of the "pathological lung" index formula is the number of foamy cells, which was also increased in patients taking amiodarone without any clinical or laboratory signs of lung toxicity. When the other component of the formula, the pathological area, was calculated, severe pneumonitis and fibrosis and minor-to-moderate alterations (e.g. the thickening of interalveolar septa with or without mild to moderate alveolitis) were included. Thus the "pathological lung" index can reach high values in patients taking amiodarone without any clinical sign of lung toxicity. On the other hand, it is difficult and probably impossible to define a border line based solely on morphological grounds, which differentiates a lung reaction to amiodarone treatment from amiodarone lung toxicity [3, 48, 52]. For example, Adams et al. [47] reported the presence of alveolitis in two patients and prominent foamy macrophages in the lungs of six of eight patients treated with amiodarone without clinical toxicity. Serious pneumonitis with fibrosis is probably more compatible with toxicity, but the diagnosis of amiodarone lung toxicity can be made only on clinical grounds with the consideration of all laboratory and imaging data. The myelin figures consistent with phospholipoidosis and electron dense deposits seen on electron micrographs taken from amiodarone-treated animals are not indicators of amiodarone organ toxicity, only markers of amiodarone treatment. Therefore, it is conceivable that a partial protective effect of an antioxidant seen with electron microscopy is not detectable by calculating the "pathological lung" index from light microscopic sections.

b) Electron microscopy

Many authors have described lysosomal phospholipoidosis in the organs of amiodarone treated animals and patients [1, 10, 11, 53, 54]. The purpose of the study was not merely to confirm these previous findings, but also to study the influence of simultaneous treatment with antioxidants and amiodarone on lysosomal phospholipoidosis.

What are the possible relationships between lysosomal phospholipoidosis and free radical reactions? The lysosomal damaging effect of free radical reactions is well-established [55, 56]. In the skin, lysosomes of amiodarone-treated patients accumulated lipofuscin, which was visible by light microscopy. Electron microscopic examination in the same patients demonstrated myelin figures suggesting lysosomal phospholipoidosis [21, 22]. Lipofuscin is a lipid (phospholipid) protein complex derived from lysosomal degradation of the cellular organelles' membranes. Consequently, lipofuscin is a component of lysosomal phospholipids accumulated in amiodarone-treated patients. Since lipofuscin is the endproduct of *in vivo* lipid peroxidation, it is probable that free radical reactions play some role in the accumulation of lysosomal phospholipids. Lipofuscin has many cross-links that render the compound resistant to phospholipase and proteolytic enzyme action. For this reason the accumulation of lipofuscin in lysosomes may result in lysosomal phospholipoidosis, which is in fact an acquired lipid storage disease. The increased extralysosomal lipid peroxidation may also lead to lysosomal lipofuscin accumulation, resulting in increased organelle damage and increased lysosomal autophagocytosis of damaged organelles [56].

There are some indirect lines of evidence that free radical reactions are able to induce morphological alterations similar to lysosomal phospholipoidosis. In cerebral lipofuscinosis where free radical reactions are also primary pathogenic factors, the accumulation of the lipopigment ceroid in the brain and retina is primarily responsible for the symptoms. It is proved that ceroid is a product of *in vivo* lipid peroxidation. The electron microscopic morphology in cerebral lipofuscinosis is to a great extent similar to that of organ damage caused by amphiphilic compounds [57].

The results of the present study also confirm that a relationship may exist between free radical reactions and lysosomal phospholipoidosis. Simultaneous antioxidant (silibinin) treatment was able to decrease significantly the amiodarone-induced increase of lysosome number, while the size, shape, and internal characteristics of lysosomes remained the same, although it could not entirely prevent the formation of myelin figures and electron dense deposits. Since myelin figures in lysosomes correspond to lysosomal phospholipoidosis, these results mean that the total quantity of lysosomal phospholipids also decreased.

In summary, the following conclusions can be drawn: 1. free radical reactions are able to induce morphological alterations similar to lysosomal phospholipoidosis; 2. the lipofuscin, which is a lipid peroxidation product, probably participates at least in part to the composition of lysosomal phospholipids accumulating as a consequence of amiodarone treatment; and 3. the simultaneous

administration of an antioxidant (silibinin) together with amiodarone may significantly decrease the extent of lysosomal phospholipidosis but cannot prevent the formation of lysosomal phospholipids.

The reason for the much more conspicuous manifestation of amiodarone toxicity in lung tissue than in liver tissue in our morphological studies is unclear, since the hepatic microsomal drug metabolizing system may be one of the most probable suggested sites of aryl radical production from amiodarone. An explanation might be that during amiodarone treatment, the amiodarone and desethylamiodarone concentration in rat liver is lower than in the lung [58]. On the other hand, the endogenous antioxidant defense capacity of the liver is better than that of the lung, and may provide a partial protective effect against amiodarone toxicity. If amiodarone toxicity is mediated partly by free radical mechanisms the high O_2 concentration of lung tissue also predisposes to free radical damage. Another potential cause is that weaker reducing agents occurring *in vivo* react quite slowly with amiodarone. Therefore the reactive aryl radical is produced at a relatively slow rate. Thus it is conceivable that the 1-month treatment period was enough to induce toxicity in the more sensitive lung tissue, but was too short to induce liver toxicity. Kannan et al. [59] reported similar results. The percentage of vacuoles containing inclusions was not significantly different in liver macrophages in rats treated with amiodarone compared to control. On the other hand, the percentage of vacuolar area in the alveolar macrophages in rats treated with amiodarone was significantly higher than that in the controls.

Biophysical studies

The pulse radiolysis and stationary ^{60}Co -gamma radiolysis studies clarified the structure and mechanism of formation of main free radical species produced from amiodarone. In a reducing environment I- is released; this partial deiodination is accompanied by the simultaneous formation of a highly reactive aryl radical, which is the main free radical product, able to initiate free radical chain reactions like lipid peroxidation. With respect to the main products, similar results were reported by several authors using photolysis [15, 16]. The results of Kannan et al. [59] also support the aryl radical hypothesis. In serum samples taken from rats treated with amiodarone, they found monodeiodinated desethylamiodarone in a significant amount beside amiodarone and desethylamiodarone using HPLC. This may be *in vivo* proof of the aryl radical hypothesis, because the monodeiodinated desethylamiodarone might be a stable product formed from the aryl radical after H atom abstraction from the neighboring molecules (the initiation step of free radical chain reactions) and after desethylation. So far the effect of reducing environment produced stationary ^{60}Co -gamma radiolysis or pulse radiolysis on amiodarone has not yet been investigated. This is important because during the *in vivo* metabolism of amiodarone in the liver microsomal electron transport chain, amiodarone may meet this reductive environment, and in the organism, under physiological and pathological conditions as well, free radicals with reducing properties may occur,

which when reacting with amiodarone are able to induce the reactions that took place in our experimental system. Exploring these *in vitro* data to *in vivo* circumstances, it is supposed that during *in vivo* metabolism of amiodarone in the hepatic drug metabolizing system and elsewhere, the reactive aryl free radical may be produced, which may play an important role in the pathogenesis of amiodarone toxicity. Amiodarone reacts very rapidly with e_{aq}^- (a strong reducing agent) in a diffusion controlled reaction. However, its reaction with other weaker reducing agents such as α -hydroxy radicals is about three orders of magnitude slower (György, unpublished observation). *In vivo*, these weaker reducing agents predominate. Therefore the aryl radical might be produced at a low rate *in vivo*. This might explain the chemical experience that amiodarone toxicity usually is manifested after a longer treatment period. The reactive aryl radical reacts at once at its site of production with neighboring biomolecules; antioxidants are probably unable to prevent this reaction. They may be capable of partially repairing the oxidative damage sites, or scavenging secondary reactive oxygen intermediates if they are produced.

The pulse radiolysis and ^{60}Co -gamma radiolysis studies did not elucidate whether or not secondary reactive oxygen intermediates are formed from amiodarone in reducing environments. This question awaits further investigation. Bennett et al. [17] suggested the formation of a carbonyl radical on the amiodarone molecule. We cannot exclude that possibility, but found nothing to support this concept. This is the first report on the radiosensitizing effect of amiodarone, which is not unexpected given its free radical generating property.

There was a discrepancy between the *in vitro* radioprotective and *in vivo* radiosensitizing effects of MTDQ-DA. Investigating the mechanism of its radiosensitizing effect in tumor tissues, it was shown that the compound selectively accumulates in tumor tissues and oxygen is released *in situ* as a result of its interaction with peroxides in the neoplastic tissue, which may play a major role in its radiosensitizing action [60]. The compound might have an *in vivo* radiosensitizing effect only in special circumstances and acts as an antioxidant *in vitro*; however, this suggestion needs further experimental verification.

EP measurements

Amiodarone treatment failed to reduce sustained atrial flutter inducibility, while amiodarone + silymarin treatment did, even when the drug concentration (AM+DEAM) was $<40 \mu\text{g/gm}$ myocardium. An arbitrary myocardial (AM+DEAM) $>40 \mu\text{g/gm}$ myocardium was considered a high drug concentration sufficient for the drug to exert an antiarrhythmic effect based on references [39, 40] and our experience. It is not completely clear why amiodarone was ineffective while amiodarone + silymarin treatment was effective in this model. The dose (600 mg/d) and duration (8 weeks) of amiodarone therapy seems to be sufficient, since other authors observed significant antiarrhythmic and electrophysiologic effects using similar amiodarone dose for similar time [61, 62, 63, 64]. The plasma amiodarone

concentration ($0.938 \pm 0.736 \mu\text{g/ml}$) was at the lower end of the range typically found in patients receiving long-term amiodarone therapy, but the mean plasma desethylamiodarone level ($0.208 \pm 0.153 \mu\text{g/ml}$) was lower [39, 64, 65, 66, 67]. The myocardial amiodarone and desethylamiodarone concentrations (39.771 ± 32.727 and $8.071 \pm 6.472 \mu\text{g/gm}$ myocardium respectively) were comparable to the data reported in the literature [39, 40] in studies where amiodarone effectively prevented ventricular arrhythmias. There was no significant difference in the plasma and myocardial amiodarone and desethylamiodarone concentrations between the amiodarone and amiodarone + silymarin treated groups. Some investigators [61, 68, 69, 70, 71] showed that there is a significant relationship between plasma amiodarone, desethylamiodarone concentrations and the EP-antiarrhythmic effects of these compounds. Mitchell et al. [61] found that early effects tend to correlate with serum amiodarone concentrations and late effects with serum desethylamiodarone concentrations. Our results are consistent with this observation, since EP parameters reflecting atrial and ventricular refractoriness - a change of which is only apparent later during chronic amiodarone treatment -, showed correlation with either plasma or myocardial desethylamiodarone levels. However some authors [63, 72, 73, 74, 75] demonstrated that the maximal antiarrhythmic activity which becomes apparent only during chronic administration of amiodarone or desethylamiodarone had no systematic relationship to plasma and tissue concentrations of these compounds.

We supposed that the distribution of amiodarone in the different organs is relatively homogenous, that's why we correlated for example the right ventricular concentration of amiodarone and desethylamiodarone not only with right ventricular but also with right atrial refractoriness.

Since the most relevant EP parameter for atrial flutter induction the RA-ERP was equally prolonged in both treated groups, there is no clear explanation for the difference in the effectivity of amiodarone and amiodarone + silymarin treatments to prevent sustained atrial flutter induction. An explanation may be, that in the presence of the created right atrial barrier, which once induced helps to maintain atrial flutter [31] amiodarone could not be as effective as in patients.

The EP markers for the maximal antiarrhythmic activity of amiodarone were more completely present in the amiodarone + silymarin treated group (prolongation baseline AH, pre- and postsurgical RV-ERP, RA-ERP, AF MEAN CL; decrease of AF MAX DUR) than in the amiodarone treated group (prolongation of pre- and postsurgical RA-ERP, AF MEAN CL) compared with the control group, except a significant bradycardia (the SRCL tended to be longer in both treated groups but it wasn't significant), and the baseline RV-ERP was significantly longer in the amiodarone + silymarin treated than in the only amiodarone treated group. These and atrial flutter inducibility data show the additive or potentiating effect of silymarin on the EP action of amiodarone and silymarin might have an antiarrhythmic effect alone in this model. The antiarrhythmic activity of several antioxidant compounds has been established for ventricular tachyarrhythmia during coronary artery reperfusion [76, 77, 78, 79, 80].

Free radicals

The EPR analysis of plasma samples taken from the anterior interventricular coronary venous effluent demonstrated that in both treated groups the myocardial total spin adduct release of the area drained by the anterior interventricular coronary vein was significantly higher compared with the control group. The myocardial total spin adduct-, component C-, and component B release tended to be consistently lower in the amiodarone + silymarin group compared with amiodarone group, indicating the possible *in vivo* antioxidant effect of silymarin in this system; and component C and B concentrations tended to be higher in both treated groups compared with the control group. However the small sample size and the large variability leading to non-normal distribution of data made it impossible to find statistical significance.

Component C is a signal similar to that reported by Bolli et al. [32, 33] and most likely corresponds to an alkyl ($R\cdot$) or an alkoxy ($RO\cdot$) radical generated *in vivo*. These are secondary free radicals. Conceivably both of them may be produced by lipid peroxidation induced by the generated reactive and consequently short half life primary free radical. Bolli et al. [32] showed that superoxide dismutase and catalase made the production of the PBN radical adduct similar to the component C undetectable during ischemia and markedly inhibited following reperfusion. This finding might suggest that the component C might also be derived from the reactions initiated by superoxide radical ($O_2^{\cdot-}$) and/or H_2O_2 .

Limitations

Rat and in vitro studies

Amiodarone was administered in a rather high dose for a relatively short time in order to produce side effects during this period. These experiments were started in 1987; in the meantime a reliable experimental rat model of amiodarone lung toxicity has been developed [51], which uses the more sensitive Fisher 344 rats. These authors used 175 mg/kg per day of amiodarone and the histologic sections were abnormal after 3 weeks of drug treatment.

Measurement of antioxidant, amiodarone, and desethylamiodarone levels unfortunately was not possible at this time. Another limitation was that *in vivo* studies, with the exception of morphological studies, were only conducted in the liver. These studies should also be performed in the lung to obtain more complete evidence for the free radical hypothesis.

Evidence was furnished that amiodarone generates free radicals but further studies are needed to confirm that these free radicals are important mediators of amiodarone toxicity. Therefore, the electron microscopic studies should be repeated in the future using the more precise volume density analysis method [81] and applying more antioxidants, including the main lipid phase antioxidant vitamin E.

In the in vitro systems, antioxidants were unequivocally effective, but in vivo their protective effect was ambiguous. The fact that an antioxidant is effective in one system and ineffective in another one, or in the same system one antioxidant is effective and another antioxidant is not, in itself is not surprising and does not rule out the role of free radical reactions in the investigated process (for further explanation the following reviews are recommended [23, 82, 83]).

Dog studies

Our ERP spectroscopy results should be considered preliminary because of the small sample size. Since free radical signals could be detected in the baseline samples, collection of plasma samples from the venous effluent of any central vein will be appropriate in the future which renders the preparations for the study more simple.

Another limitation is the animal model used. Future studies in different arrhythmia (e.g. ventricular tachycardia, ventricular fibrillation) models are necessary to confirm the additive or potentiating antiarrhythmic and electrophysiological actions of simultaneous silymarin treatment with amiodarone without impairing amiodarone's antiarrhythmic action. Biggest limitation is not testing the electrophysiological and antiarrhythmic effects of silymarin treatment alone in this atrial flutter model. The trial of other antioxidant compounds (particularly vitamin E) is also desirable.

Conclusions

The rat and in vitro studies provided direct and indirect evidence that amiodarone in vitro, and probably in vivo, produces free radicals. The electron microscopic results show that simultaneous antioxidant (silibinin) treatment with amiodarone could probably diminish the extent of lysosomal phospholipoidosis induced by amiodarone treatment, although it could not entirely prevent its formation. Since the lysosomal phospholipoidosis is not only a specific marker of treatment with amiodarone and other amphiphilic compounds, but above a certain threshold may be involved in the toxicity of these compounds, the capability of an antioxidant to decrease lysosomal phospholipoidosis may mean that the free radicals generated by amiodarone may play a role in the pathogenesis of amiodarone toxicity.

Although the simultaneous administration of antioxidants with amiodarone seems to be justified (provided they do not interfere with the drug's main antiarrhythmic effect) to diminish amiodarone toxicity the synthesis of an amiodarone derivative with the same antiarrhythmic effect but with less free radical generating properties, may be a better approach to this problem, because the protective effect of antioxidants could be only partial even in optimal conditions. However, the development of an amiodarone analog with the desired properties may take a long time and meanwhile the simultaneous administration of antioxidants with amiodarone might prove to be useful to achieve partial protection against amiodarone toxicity.

The dog study provided direct in vivo evidence of free radical generation by amiodarone treatment. An antioxidant silymarin did not diminish the electrophysiological effects of amiodarone in dogs with atrial flutter and in fact have had an additive or potentiating effect on the antiarrhythmic actions of amiodarone. Prospective studies with amiodarone combined with an antioxidant may be justified to reduce amiodarone toxicity without impairing or possibly enhancing its antiarrhythmic efficacy.

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Effect of Free Radicals on Renal Ischemia/Reperfusion in Dogs.

Free Radicals and Renal Perfusion

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Abstract

Recent observations indicate that the reperfusion of an ischemic organ can increase tissue damage. The aim of the study was to investigate the pathological role of oxygen derived free radicals during renal ischemia and reperfusion. Ischemia was evoked in dogs in one kidney, by clamping the left renal artery for 45 min. This was followed by a 90 min period of reperfusion when diuresis, GFR, PAH clearance and sodium and potassium excretion were studied. Besides a control group (n=6), the following treatment groups were investigated. Allopurinol (n=7): 50 mg/d for two days p.o. and 50 mg/kg in physiological saline infusion during the experiment; a small dose of SOD (n=6): 0.5 mg/kg in infusion started 1 min before reperfusion and given continuously for 10 min; a high dose of SOD (n=7): 5 mg/kg as above and silibinin (n=6) 4 mg/kg in physiological saline infusion started 10 min before reperfusion and administered continuously for 20 min. In the first 15 min following reperfusion, the renal functions significantly worsened in all groups. Later on, the renal functions gradually improved and in the last period after reperfusion, GFR in the ischemic kidney was 64%, cPAH was 59%, diuresis was 60%, and sodium and potassium excretion were 65% and 76%, respectively of basal values in the control group.

Treatment with free radical scavengers did not cause any considerable changes in the renal functions. In some respects, the worst results were observed with low-level SOD treatment (cPAH, diuresis, as well as sodium and potassium excretion). At the end of reperfusion, there was a significant drop in sodium excretion by the right (intact circulation) kidney of the treated animals.

Our experiment indicate that in early phase of renal reperfusion, the pathological role of free radicals is not of great importance (the xanthine oxidase enzyme is not a critical mediator of the reperfusion injury). It seems reasonable to study the role of activated neutrophils which generate free radicals during several hours or days after restart of circulation

Abbreviations: FR = free radical; SOD = superoxide dismutase; XO = xanthine oxidase

Introduction

Acute ischemic renal failure is of great clinical importance of its frequent occurrence and the high mortality it causes [1-3]. Any interventions to improve circulation, carried out soon enough can help to preserve organ function. Recent observations indicate however, that reperfusion has its own dangers [4]. It seems likely, that the degree of tissue damage after reperfusion depends on both the preceeding ischemia, and the injury caused by restarting of the circulation [5]. It has been proposed that the reperfusion injury is caused by the oxygen derived FRs which are generated in great quantity from several sources during reoxygenation [6, 7]. Since FRs, these extremely reactive intermediates which are produced during physiological circumstances too, virtually all aerobic beings processes systems against these toxic agents [8]. It is thought, that especially in the early phase of recirculation there is a "burst" of generation of FRs which overwhelm the capacity of the defense system. It has been proposed that exogenously administered antioxidant agents may protect against the injury caused by oxygen derived toxic substances [9-11], and may therefore ameliorate tissue damage. By these reason, the protection afforded by different antioxidant agents have been widely investigated especially under experimental conditions. These studies gave contradictory results [12-16], so the problem need further investigations. Trials of this kind with dogs have been presented relatively rarely in the literature so we chose this species for our experiments.

Methods

The experiments were conducted in mongrel dogs of either sex with a body-weight of 10 to 34 kg. The animals were narcotized with Nembutal (sodium pentobarbital, 30 mg/kg body-weight i.v.), and steady narcosis was provided at 3 mg/kg body-weight i.v. maintenance doses. A cuffed endotracheal tube was inserted, continuous artificial respiration was maintained (respirator RO-5, Moscow, USSR) and regular blood-gas analysis was carried out during the trial. Surgery was performed on a heatable operating table, with the rectal temperature maintained between 37 and 38 °C. Under the aims of giving infusion, taking blood and measuring blood pressure, a jugular vein and both femoral arteries and veins were catheterized. Urine was obtained through catheters from the ureters by median laparotomy. Venous blood from the kidney was obtained through the v. spermatica or ovarica, as appropriate, by the aid of a catheter led into the left v. renalis. The left renal artery was exposed, with the renal nerves left intact. On finishing the operation, saline infusion was administered (20 ml/kg body-weight) for 30 minutes, which was given followed by a dose of 10 ml/kg body-weight/h until the end of the trial. For the purpose of measuring GFR and the effective renal plasma flow (ERPF), inulin and paraaminohippuric acid (PAH) was infused in physiological saline into the v. jugularis to reach a plasma concentration of 30-50 mg/100 ml and 1-3 mg/100 ml, respectively, depending on the animal's body-weight (0.21-0.58 ml/min). After the

Table 1. The effect of the antioxidant therapy on renal function and arterial blood pressure in narcotized dogs after 45 min unilateral warm renal ischemia

	Before ischemia					90 min after ischemia				
	A	B	C	D	E	A	B	C	D	E
Diuresis (ml/min)	I: 1.8 ± 0.4	1.9 ± 0.5	1.8 ± 0.3	3.1 ± 0.4	1.7 ± 0.4	2.3 ± 0.6	0.6 ± 0.1	1.7 ± 0.4	2.8 ± 0.5	1.4 ± 0.2
	II: 1.8 ± 0.4	2.1 ± 0.6	1.9 ± 0.3	3.4 ± 0.7	1.8 ± 0.5	1.4 ± 0.3	1.4 ± 0.9 ^b	1.3 ± 0.3	1.6 ± 0.5 ^b	2.3 ± 0.2
GFR (ml/min)	I: 65.0 ± 5.0	77.0 ± 6.0	80.0 ± 5.0	72.0 ± 4.0	62.0 ± 5.0	66.0 ± 4.0	82.0 ± 6.0	82.0 ± 7.0	82.0 ± 6.0	67.0 ± 7.0
	II: 63.0 ± 5.0	75.0 ± 6.0	76.0 ± 4.0	72.0 ± 4.0	58.0 ± 3.0	39.0 ± 4.0 ^c	40.0 ± 5.0 ^c	32.0 ± 5.0 ^c	37.0 ± 7.0 ^c	46.0 ± 8.0
cPAH (ml/min)	I: 144.0 ± 10.0	219.0 ± 13.0	237.0 ± 22.0	209.0 ± 15.0	170.0 ± 16.0	160.0 ± 12.0	189.0 ± 13.0	213.0 ± 12.0	196.0 ± 19.0	141.0 ± 14.0
	II: 155.0 ± 6.0	204.0 ± 14.0	223.0 ± 17.0	212.0 ± 15.0	166.0 ± 19.0	91.0 ± 10.0 ^a	106.0 ± 14.0 ^c	86.0 ± 11.0 ^c	93.0 ± 19.0 ^c	95.0 ± 14.0
Sodium excretion (μmol/min)	I: 276.0 ± 57.0	365.0 ± 90.0	302.0 ± 60.0	455.0 ± 99.0	229.0 ± 85.0	253.0 ± 61.0	211.0 ± 40.0 ^b	205.0 ± 34.0 ^b	308.0 ± 65.0 ^b	198.0 ± 50.0
	II: 319.0 ± 93.0	383.0 ± 95.0	308.0 ± 54.0	491.0 ± 11.0	267.0 ± 63.0	165.0 ± 46.0	78.0 ± 10.0 ^c	163.0 ± 34.0	167.0 ± 70.0 ^a	174.0 ± 58.0
Potassium excretion (μmol/min)	I: 67.0 ± 8.0	79.0 ± 12.0	50.0 ± 6.0	99.0 ± 18.0	76.0 ± 11.0	76.0 ± 8.0	84.0 ± 17.0	52.0 ± 10.0	98.0 ± 12.0	68.0 ± 8.0
	II: 70.0 ± 9.0	77.0 ± 11.0	49.0 ± 6.0	99.0 ± 18.0	70.0 ± 9.0	58.0 ± 10.0	42.0 ± 6.0 ^b	44.0 ± 7.0	55.0 ± 13.0 ^b	61.0 ± 6.0
Blood pressure (mmHg)	I: 130.0 ± 7.0	133.0 ± 4.0	123.0 ± 5.0	129.0 ± 4.0	119.0 ± 7.0	139.0 ± 4.0	138.0 ± 6.0	131.0 ± 4.0	139.0 ± 4.0	124.0 ± 6.0

Abbreviations: I = right kidney (intact circulation)

II = left (ischemic) kidney

A = control group (n=6)

B = small dose of SOD group (n=6) (0.5 mg/kg body-weight)

C = large dose of SOD (n=7) (5 mg/kg body-weight)

D = allopurinol group (n=7)

E = silibinin group (n=6)

a = p<0.05, b = p<0.01, c = p<0.001 vs. basal level in the same group

The data (mean ± SEM) are expressed for 100 g wet kidney weight

operation, a 60-minute equilibration period was maintained, then urine was collected at 15-minute intervals. After two control periods, the left renal artery was occluded for 45 minutes and urine was collected from the right kidney alone. During the occlusion, the quantity of infusion was halved. Following the release of the occlusion, urine was collected from both kidney for further six periods of 15 minutes, each. Arterial blood samples were taken at the midpoint of the periods.

Blood pressure, measured with a Statham P 23 db pressure transducer, was continuously recorded (Rikadenki, B 381, Tokyo, Japan).

Morphologic studies

Kidney tissue from the animals were examined by light and electron microscopy. One part of the specimens were fixed in 4% formalin, were embedded in paraffin and stained with hematoxylin eosin, additional specimens for ultrastructure analysis were fixed osmium-glutaraldehyde and were ambedded in Durcupan ACM, than stained with uranylacetate citrate or toulidine-blue. Jeol Sem 100 S electron microscope was used for assessment of cell ultrastructure.

The following groups were examined:

1. *Control group* ($n=6$): Animals given salina alone.
2. *Small-dose SOD* (Peroxinorm, Grünenthal, Germany) *group* ($n=6$): 0.5 mg/kg body-weight SOD infusion started 1 minute before reperfusion and given continuously for 10 minutes.
3. *Large-dose SOD group* ($n=7$): 5 mg/kg body-weight SOD administered as above.
4. *Allopurinol* (Milurit, EGIS, Hungary) ($n=7$): Animals pretreated with allopurinol at dose of 50 mg/d for two days. During the experiment, allopurinol was given i.v. at a dose of 50 mg/kg in physiological saline solution.
5. *Silibinin* (Legalon, Madaus, Germany) ($n=6$): 4 mg/kg/h in physiological saline infusion started 10 min. before reperfusion and was given continuously for 20 min.

Inulin and PAH concentration in the plasma and the appropriately diluted urine samples were measured by anthrone and diphenylamine method. Sodium and potassium concentration were measured with a Flapho 4 type flame photometer (Carl Zeiss, Jena, Germany). Table 1 contains mean values of kidney parameters expressed for 100 g kidney $\pm$ SEM. The data were analyzed by Student's t test.

Results

In the course of statistical data processing, the parameters of the ischemic kidney were compared to the basal ones. It was observed that 15 minutes after the release of the renal artery occlusion, all renal parameters significantly worsened, especially

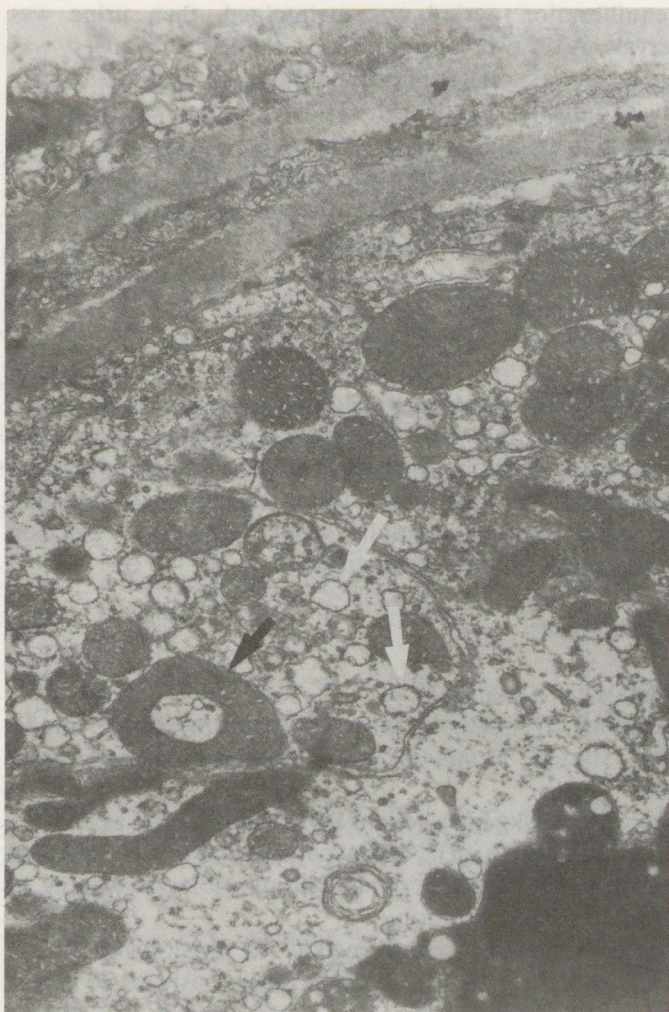
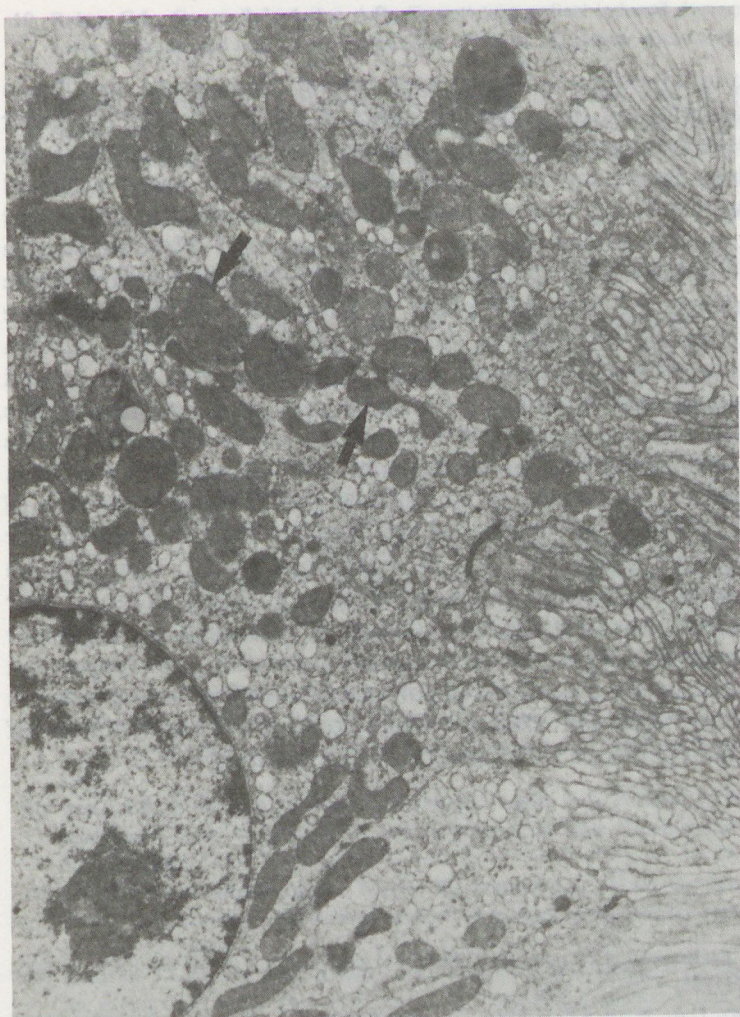


Figure 1. Kidney tubular cells after reperfusion. A: Fragmented swollen mitochondria (↑), dilated endoplasmic reticulum (↗). Treatment with 5 mg/kg b.m SOD. (EM 8,300 x).

GFR, which was 44% in the silibinin ($p < 0.05$), 21% in the control, 13% in the allopurinol, 10% in the small-dose, and 8% in the large-dose SOD group, respectively, compared to the basal level ($p < 0.001$). From that time on, the renal functions gradually improved and 90 minutes after recirculation, the GFR of the ischemic kidney was 64%, cPAH 59%, diuresis 60%, whereas sodium and potassium excretion 65% and 76%, respectively, of the basal level in the control group. Table 1 shows the data of two relevant periods in the experiment. Treatment with FR scavengers did not cause any improvement in the functions of the ischemic kidney, and some functions (sodium and potassium excretion, diuresis) were even less



(↑) control animal (EM 5,000 x)

B: Different shaped mitochondria

favourable in the animals treated with a low dose of SOD and allopurinol. At the end of reperfusion, we observed a significant decrease of sodium excretion by the right (intact circulation) kidney. There was no difference in blood pressure between the different groups.

On light microscopy examination no significant injury of the cells of the ischemic tissue was found. However, analysis of cellular ultrastructure demonstrated swollen, fragmented mitochondria in the tubular cells, accompanied by dilated endoplasmic reticulum and autophagic cytolysosomes, and an increased nuclear density was observed in the distorted cells (Fig. 1).

Discussion

An indirect, but important evidence of the oxygen derived FR mediated reperfusion injury if one can demonstrate the protective effect of the free radical scavenger therapy. There are several sources of the FR generation during reperfusion [8], but it seems, that the most important are the role of the enzyme XO and of activated neutrophil granulocytes [17, 18]. Therefore we investigated the effect of allopurinol which inhibits XO, and the efficacy of SOD a natural scavenger of the superoxide radical and silibinin which agent was protective in the FR mediated liver disease [19]. In our experiment no protective effect of antioxidant therapy could be demonstrated: there was no difference in the tissue damage observed by electron microscopy, and the function of the ischemic kidney doesn't improved in the treated animals. These observations indicate that in the early phase of the reperfusion the role of the FR mediated reactions and the XO enzyme no critical in the canine. The reason of this observation may be, that the XO content of the tissue of the dog is lower as in other species, for example in the rat liver [20]. If the major source of superoxide radical during reperfusion in the activated polymorphonuclear neutrophils as have been stated by some in vestigators [21-23], there will be continuous FR generation, because the number of neutrophils increase after restarting of circulation in the ischemic tissue [24]. So it is possible, that in a later period of reperfusion toxic oxygen metabolites generated by the granulocytes can increase the tissue damage. Because of the importance of the question the problem needs further investigation, for example with prolonged administration of antioxidant agents and reperfusion with blood, free from granulocytes.

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The Evaluation of an Important Cell Defense Mechanism into an Activator of Viral Replication and Tissue Destruction

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Introduction

A primitive defense mechanism in all species of animals is the generation of oxygen radicals. The source of these radicals has evolved in the species from lower vertebrates to man. In the lower species, xanthine oxidase is the main source of oxygen radical generation in macrophages. In addition, xanthine oxidase is located and active in the gastrointestinal tract and liver as well as other organs. Where it is not initially located in the membrane, it is incorporated into the area either by the influx of macrophages, in rodents, or directly through the blood. The generation of oxygen radicals including superoxide, hydrogen peroxide and hydroxy radicals are important factors for destroying foreign substances, be they viruses, bacteria, tumors, or endogenous tissue not recognized by the host.

In man, the generation of oxygen radicals in granulocytes is via NADPH oxidase and myeloperoxidase. However, xanthine oxidase still plays a relatively important initial role in attracting neutrophils and macrophages to the source of defense. This is obvious in the gastrointestinal tract, where defense is primarily needed. Xanthine oxidase is active at the membrane where the first exposure of foreign substrates arrives via the oral route. Following this initial source of xanthine oxidase, the liver appears to be the next relevant organ showing an elevation of xanthine oxidase to various stimuli. Here additional defense is required to protect against foreign substance. When the substances are no longer there, then the generation of oxygen radicals subsides. Normally, a natural protection exists in the cells and blood to protect the host from its own destructive elements, here oxygen radicals. This protection is complex but relatively efficient if the generation is limited. The defense consists of superoxide dismutase, glutathione peroxidase, catalase and vitamins E, C and riboflavin as well as numerous phenols that appear to help in reduction and prevention of peroxidation damage to the cell.

The oxygen radical defense system however, can be not only destructive to the host, when it is not kept within its boundaries, but appears to be utilized by viruses for their replication.

Viruses

The local, and systemic elevation of xanthine oxidase is an indicator of viral infection and a source of viral toxicity. The possibility exists that viruses are utilizing an endogenous defense system of their host, namely the generation of oxygen radicals to enhance their replication. The oxygen radicals activate a major part of the intracellular enzymes important in replication, including ornithine decarboxylase, cyclic guanosine monophosphate, and the protein kinases, and inactivate protease inhibitors which protect the cell and prevent replication. These enzymes are also active in tumor promotion and may explain how a chronic viral infection can lead to cancer.

It has been only recently discovered that sources of oxygen radicals, such as U-V, phorbol esters etc. could activate the replication of HIV, and antioxidants could inhibit the replication/activation of HIV in vitro. In HIV patients, the lymphocytes have been reported to show an increased intensity of free radical oxidation together with the activation of the xanthine oxidase D-form to the O-form. Normally where xanthine oxidase exists, it exists in the D-form which does not generate oxygen radicals, but is activated by superoxide or proteases to the radical generating O-form. When the substrates xanthine and hypoxanthine are elevated, the generation of radicals commences. Since HIV replication is enhanced by oxygen radicals, the use of xanthine oxidase as a source of these radicals would be a very practical approach to be used by the virus for its incorporation into the cell. Glycyrrhizin, a xanthine oxidase inhibitor and preventer of peroxidation, inhibits HIV replication in vitro. It is not yet known if in vivo, a xanthine oxidase inhibitor, alone or in combination with other antioxidants will be effective in inhibiting replication, but may be worthy of investigation.

HIV is not the only virus to increase xanthine oxidase. In the brain of man and in mice infected with cytomegalovirus, uric acid, the product of xanthine oxidase, has been shown to be elevated. In mice, CMV in the lung elevates xanthine oxidase activity, presumably from the influx of macrophages into the area. Similar results were seen with Influenza B virus in the lung, and protection was offered by xanthine oxidase inhibition, as well as oxygen radical reducing compounds.

In the liver of mice, the elevation of xanthine oxidase was seen with Coxsackievirus B1, Vaccinia virus, Herpes virus hominis, Hart park virus, Nyamanini virus and Dengue virus.

Further data from patients with HBV and EBV indicate a serum elevation of xanthine oxidase. In HBV patients, this elevation was 200 times above the upper normal limits, presumably derived from its source in the infected liver. In Japan, glycyrrhizin has been used for many years as a treatment of viral hepatitis.

Bacteria

Bacteria generate the release of purines which are substrates for xanthine oxidase. As a primary local defense against bacteria xanthine oxidase generation of oxygen radicals helps to protect the host from invasion. In addition, the chemotactic activity

of superoxide radicals attract in the granulocytes for additional defense through generation of oxygen radicals. When the bacteria replicate too rapidly, and/or protect themselves with their own antioxidant system, then the toxicity to the host continues. This may be the situation in stomach ulcers, with helicobacter pylori. Carbenoxolone, a glycyrrhizin derivative protects in stomach ulcers and enhances healing. Glycyrrhizin as previously mentioned, inhibits xanthine oxidase.

Endotoxins from *E. coli* increase the levels of xanthine oxidase in the arterioles in the lung and in the kidney. This appears to be one of the main sources of toxicity in endotoxic shock. Horses pretreated with allopurinol, an inhibitor of xanthine oxidase, and given endotoxin showed no morbidity.

Interferon

Interferon and interferon inducers increase the levels of mRNA for xanthine dehydrogenase in the liver. This results in an elevation of xanthine oxidase and enhancement of liver toxicity in mice and rats.

It is interesting to note that the oxygen radicals appear to enhance the efficacy of interferon while at the same time increasing the destruction of host cells. It may be that the host defense system wishes to destroy the cells that are infected in order to enhance the replication of healthy cells to replace those destroyed. Further evidence in this direction is the serum elevation of xanthine oxidase and the distant elevation of xanthine oxidase far from its initial source of stimulation. Is interferon sending out xanthine oxidase from the liver to bind on to cells that are infected? It is known that oxygen radicals elevate the intracellular pH, and extracellularly a decreased pH increases the binding of xanthine oxidase to the sulphated glycosaminoglycans on the endothelial cell surface. The xanthine oxidase is somehow signaled to be released into the blood and circulates until it finds a cell with an altered membrane surface such as one finds in acidic inflammatory situations and in cellular toxicity where it can bind and destroy the cell provided a substrate is available. When viruses invade other neighboring cells, with the combined effects of interferon and xanthine oxidase, the result is the destruction of the majority of cells and the demise of the infected cells or when the process is excessive, the organ itself is destroyed.

Gastrointestinal disorders

The role of xanthine oxidase in gastrointestinal disease is not well known. Recent evidence indicates a protective effect in ulcerative colitis with the combination of allopurinol and 5-ASA. A longer remission seen in the treated patients.

Hypoxia and reperfusion injury

With hypoxia and reperfusion there is a breakdown of ATP which is metabolized to substrates for xanthine oxidase, xanthine and hypoxanthine. This is thought to be the main pathway for tissue destruction in reperfusion. Allopurinol has been shown to be effective. In addition, in adult respiratory distress syndrome, allopurinol has been reported to be effective in preventing lung toxicity.

Similar data indicating protection with allopurinol and reperfusion was seen in pancreatic hypoxic toxicity, with greater protection offered by allopurinol combined with protease inhibitors.

In other reperfusion studies, the combination of verapamil, a calcium channel blocker with allopurinol appeared more effective.

Summary

Xanthine oxidase is an enzyme that appears to be an early, primitive and yet effective defense system. In humans it has evolved to include activation from interferon as a defense against viruses. The viruses however, can utilize the generation of oxygen radicals to enhance their replication and their release from the cell as a result of oxygen radical cell destruction.

The mechanism of its toxicity is complex. Once a substrate is present, xanthine oxidase generates oxygen radicals. When histamine is released, as in asthma, or other allergies, the K_m for xanthine oxidase is lowered and more substrate can be metabolized with the generation of greater amounts of oxygen radicals. The oxygen radicals normally reduced to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide is then reduced to water by catalase. The peroxides generated are reduced to inactive products via glutathione peroxidase. The problem arises when too many oxygen radicals are generated, and when the reducing enzymes are not sufficient to reduce fast enough or efficiently enough. In addition, when free iron is available, the reaction goes in the direction of hydroxyl radicals, which are extremely toxic. Some antioxidants including cysteine and vitamin C can actually enhance toxicity by their reduction of iron. This may be one of the reasons that N-acetylcysteine at low doses in vitro actually enhances HIV replication.

Combinations of inhibitors appear to be more effective than the individual compounds alone including xanthine oxidase inhibitors. For example, the release of calcium by the oxygen radicals activates a protease which also enhances toxicity. There is often a cascade effect in toxicity where one inhibitor alone is unable to stop the cascade, whereas several together have a combination of inhibitory mechanisms to enhance the efficacy.

In order to find new treatments, one must sort out the fact from the fiction in the literature and elaborate on the mechanisms involved in toxicity. In viruses, the combination of inhibitors of xanthine oxidase with protease inhibitors or reverse transcriptase inhibitors may help in HIV. Xanthine oxidase inhibitors may protect HIV positive patients from activation of HIV via xanthine oxidase, and combined

with other antioxidants, prolong the time before clinical symptoms manifest themselves. Encephalitis from Herpes simplex and Cytomegalovirus may be more effectively treated with a combination of acyclovir and a xanthine oxidase inhibitor. Ebstein Barr virus may also respond to combination treatments utilizing xanthine oxidase inhibitors.

It should be pointed out that allopurinol is not necessarily the best drug of choice to inhibit xanthine oxidase because it generates an oxygen radical during its metabolism to oxypurinol, which then irreversibly inhibits xanthine oxidase. The product oxypurinol is toxic to the kidney and therefore cannot be used. Lower dosages of allopurinol, however appear to be more effective than higher doses as they do not cause a generation of oxygen radicals.

Xanthine oxidase may be a local as well as systemic defense system that circulates and is capable of binding on the cells where oxygen radicals are generated either from a virus or from the breakdown products of ATP.

Further investigations will help to elaborate on the mechanism of xanthine oxidase in cell defense, in toxicity, in the method of the body to increase its synthesis, via endotoxin or interferon, its efficacy, via histamine, and its circulation and binding to other cells. In vivo and in vitro studies may introduce effective new treatment in many areas of medicine.

Nitric Oxide as Neurotransmitter in the Enteric Nervous System

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Abstract

NO has been identified as an important transmitter substrate in many regions of the body, especially in the vascular system, the immune system and the nervous system. In the gastrointestinal tract it seems to be an important mediator of the non-adrenergic non-cholinergic inhibition which has been observed in the intrinsic and extrinsic innervation throughout the gut. The inhibitory effect of NO is especially pronounced in sphincteric regions such as the lower esophageal sphincter, the pylorus, the sphincter of Oddi, the ileocecal junction and the internal anal sphincter. NO serves both as the putative final mediator of the non-adrenergic non-cholinergic inhibitory effect directly on the smooth muscle, but there is also evidence for a transmitter role of NO within the enteric nervous systems. NO is the putative inhibitory mediator, which causes relaxation of the pylorus in vivo and in vitro. Its action is independent of the action of VIP and in addition blockade of NO-synthesis seems to potentiate the excitatory effect of acetylcholine, suggesting a potential interaction with cholinergic mechanisms. Blockade of NO-synthesis also potentiates the contractile response of the ascending enteric reflex pathway in the small intestine and reduces the latency of neural transmission. This again suggest an additional neurotransmitter function of NO not only acting directly on smooth muscle but also within the enteric nervous system. Preliminary data suggest that NO can influence the transmitter release in the enteric nervous system.

Introduction

Throughout the gastrointestinal tract non-adrenergic, non-cholinergic (NANC) nerves are present which exert both inhibitory and excitatory effects. The putative excitatory mediator is thought to be substance P and/or neurokinin A. On the other hand there is good evidence that at least three different mechanisms are involved in the inhibitory NANC responses [1]. In sphincteric regions this NANC inhibitory

innervation seems to be of physiological importance for the relaxation of the sphincter. Vasoactive intestinal polypeptide (VIP) has been proposed as candidate mediator for this innervation in the various sphincter regions [2]. However, VIP did not mimic the actions of the final NANC electrophysiologically [3], biochemically [4], or functionally [5].

There is recent evidence that nitric oxide or a related metabolite of L-arginine, which causes the endothelium-dependent relaxation in the vascular bed [6-8], is involved in the enteric nervous system and is a putative NANC mediator [9]. Enzymatic blockade of nitric oxide production using arginine methyl-esters reduced or blocked the inhibitory NANC response in the lower esophageal sphincter [10, 11], the pylorus [2], the ileocecal sphincter [10, 12] and the internal anal sphincter [13]. Also in non-sphincteric regions of the gut such as the body of the esophagus, the fundus and corpus of the stomach, the descending inhibitory response of the peristaltic reflex in the small intestine and colon appears to be mediated by nitric oxide or a related compound [14].

So far there is a little information on the effect of NO on gut smooth muscle under in vivo conditions. We have established in previous studies an in vivo model which allows the assessment of pyloric motor activity in response to intra-arterial and intravenous stimuli as well as stimulation of extrinsic and intrinsic nerves [5, 15-19]. We and others have demonstrated that the canine pyloric muscle is characterized by a potent non-adrenergic non-cholinergic inhibitory innervation in vivo [5, 20, 21] and in vitro [5], which could be activated by electrical stimulation of the distal antrum and the cut ends of the cervical vagus. VIP mimicked the inhibitory response in the canine pylorus in vivo. However, VIP was almost ineffective in vitro although the NANC inhibitory response was present. It was suggested that a different mechanism not involving VIP must be present for eliciting the NANC response in the canine pylorus [5]. In addition we have characterized a system to study the ascending neural reflex in the small intestine in vitro and an isolated enriched preparation of enteric synaptosomes which were used to study the interaction of nitric oxide or related metabolites of the L-arginine-NO-pathway with neural elements [15].

Method

1. In vivo experiments

Mongrel dogs of either sex were anaesthetized with alpha-chloralose (60 mg/kg) and urethane (100 mg/kg) and maintained at stage III anaesthesia with 30 mg bolus injection of pentobarbital as required. The detailed method for the study of pyloric function has been describe elsewhere [15, 16, 19]. Briefly, the gastro-epiploic artery was cannulated in both directions, allowing a close intra-arterial injection either the pylorus or to the gastric antrum. Strain gauges were sutured to the serosa 3 cm proximal and 2 and 5 cm distal to the pylorus orientated to record circular muscle activity. Pairs of silver wire electrodes were inserted subserosally oral from the

pylorus around the proximal gastric strain gauge and around the duodenal strain gauges which allowed electrical stimulation (0.5 pps, 0.5 ms, 40V) of the antrum and of the duodenum.

Intraluminal pressure were recorded with a manometric assembly which incorporated a sleeve sensor, 3 cm long and 3.5 mm in diameter [15]. Two side holes, located at the upper and lower end of the sleeve, were used for recording the gastric or duodenal pressure, respectively. The assembly was inserted through a small fundic gastrostomy, passed through the pylorus, and brought out through a small duodenostomy approximately 15-20 cm abroad from the pylorus. The manometric channel were perfused by a low compliance pneumohydraulic perfusion system at a reservoir pressure of 7.5 PSI, giving a constant flow rate of 0.3 ml/min.

To investigate direct smooth muscle effects, neural blockade using intra-arterial application of tetrodotoxin (40 to 100 μ g i.a.) was used. The blockade of the excitatory and inhibitory effects of duodenal and antral field stimulation, respectively, was used to test if complete neural blockade had been achieved.

In vitro organ bath experiments

The gastro-duodenal junction from 8 mongrel dogs, sacrificed with 65 mg/kg i.v. pentobarbital, were collected in oxygenated Krebs-Ringer buffer. The gastro-duodenal junction was opened along the lesser curvature and the mucosa was carefully dissected off. The inner circular muscle ring of the pylorus was dissected out using a dissecting microscope. The circular muscle ring was subdivided into 4 strips 2 mm x 2 cm with a horizontal and a vertical cut parallel to the circular muscle fibers. One end of each strip was fixed to a hook on an electrode holder; the other end of the strip was passed through two circular platinum electrodes which were 1 cm apart. The electrode holder and the tissue were placed in a jacketed organ bath in 3 ml Krebs-Ringer bicarbonate solution (KRS) (NaCl 115.5 mM, MgSO₄ 1.16 mM, NaH₂PO₄ 1.16 mM, glucose 11.1 mM, NaHCO₃ 21.9 mM, CaCl₂ 2.5 mM, KCl 4.16 mM) gassed with 95% O₂, 5% CO₂ and maintained at 37 °C by heated water circulating through the jackets. The free end of the muscle strip was connected by a thread to an isometric force transducer (Grass force displacement transducer, model FTO3C, Grass Instrument Co., Quincy, MA). 10mN tension was applied to the muscle, and the preparation was allowed to equilibrate for at least 1 h. Changes in the tension were recorded on a Beckman R 611 dynograph. Field stimulation for neural responses was applied by a Grass S9 stimulator. Drugs and peptides were applied in microliter volumes and solvent controls were carried out for drugs and peptides.

Reflex model

Male wistar rats (400-500 g) were sacrificed using sodium pentobarbital intraperitoneally (100 mg/kg). The ileum was immediately removed and kept in oxygenated Krebs-Ringer bicarbonate solution (KRS). A segment of ileum (9-10 cm length) was carefully dissected and the mesenteric fat was removed. The gut

segment was placed in an 120 ml organ bath filled with Krebs-Ringer bicarbonate solution (KRS) gassed with 95% O₂, 5% CO₂ and maintained at 37 °C by a thermostatic water bath. The oral and anal end of the ileum were tied into polyvinyl tubing (outer diameter 1.8 mm) in such a way that the segment maintained its natural length. Two flat platinum electrodes (0.8 x 0.8 cm) which were 1 cm apart were placed around the anal end of the ileum. A double lumen manometric catheter was inserted through the oral end and placed in such a way that the side hole openings were located 2 and 4 cm from the stimulation electrode. Then the preparation was allowed to equilibrate for at least 30 minutes.

The manometric catheters were perfused with degassed saline using a low compliance pneumohydraulic perfusion system at a reservoir pressure of 10 PSI. Pressure changes were recording using spectramed pressure transducers, amplified and recorded on a Beckman R611 chart recorder. Field stimulation impulses for neural responses were applied using a Grass S9 stimulator (Grass, Quincy, Mass, USA), stimulating for 20 seconds at intervals of 2 minutes at standard parameters of 20 V pulse strength, 3 pps pulse frequency, 1 ms pulse width. The impulse signals were recorded simultaneously with the pressure using an Beckman A/C coupler.

Results

I. Role of endogenous NO for pyloric motor activity in vivo

1. Effect Blockade of NO-synthesis on pyloric activity in vivo

L-NG-Arginine-methyl-ester (L-NAME) given i.v. (10⁻⁸ to 10⁻⁵ mols/kg) significantly increased pyloric motor activity, suggesting the presence of an intrinsic inhibitory tone exerted by continuous release of NO on pyloric motility.

L-NAME given i.v. (10⁻⁸ to 10⁻⁵ mols/kg) also enhanced the pyloric motor response to duodenal field stimulation. Intra-arterial application of L-NAME (10⁻⁷ to 10⁻⁶ mols) also caused a dose dependent reduction of the inhibitory effect of both vagal stimulation and antral field stimulation. However, the effect of L-NAME was very short lasting when given intra-arterially and did not provide stable and consistent responses.

2. Effect of Blockade of endogenous NO-synthesis on the inhibitory effect of antral field stimulation

Field stimulation of the antrum caused a potent inhibition of the pyloric motor activity. Intravenous application (10⁻⁶ mols/kg to 10⁻⁵ mols/kg) of L-NAME dose dependently abolished the inhibitory effect of antral field stimulation on pyloric motor activity. A submaximal concentration (10⁻⁵ mols/kg) delayed the onset of the inhibitory response. When the inhibitory effect of antral field stimulation was completely blocked with L-NAME (5 x 10⁻⁵ mols/kg), a short inhibition following the cessation of the stimulus occurred (Figs 1, 2, 3A).

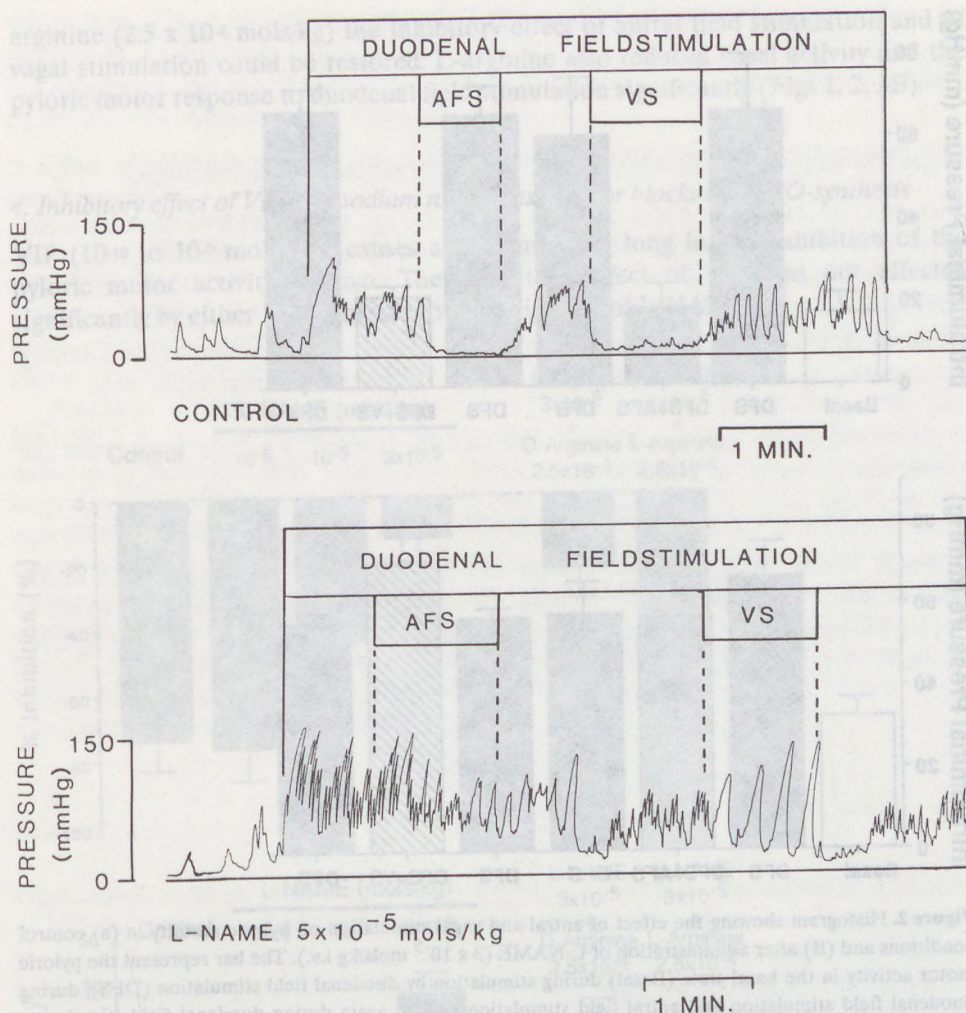


Figure 1. Representative tracings showing the pyloric motor response to duodenal field stimulation and simultaneous antral field stimulation (AFS) and vagal stimulation (VS). (A) Control (B) after L-NAME (5×10^{-5} mols/kg i.v.) (With permission of American Physiological Society)

3. Effect of intravenous L-NAME on the inhibitory effect of vagal stimulation

Stimulation of the distal cut ends of the vagal nerve with 1 pps, 1 ms and 20 V caused a potent inhibition of pyloric motor activity induced by duodenal field stimulation.

Intravenous application (10^{-6} mols/kg to 5×10^{-5} mols/kg) of L-NAME blocked the inhibitory effect of vagal stimulation on pyloric motor activity. Whereas 10^{-5} mols/kg L-NAME i.v. showed only an incomplete blockade of the inhibitory effect of vagal stimulation a complete inhibition of vagal stimulation was obtained at $5 \times$

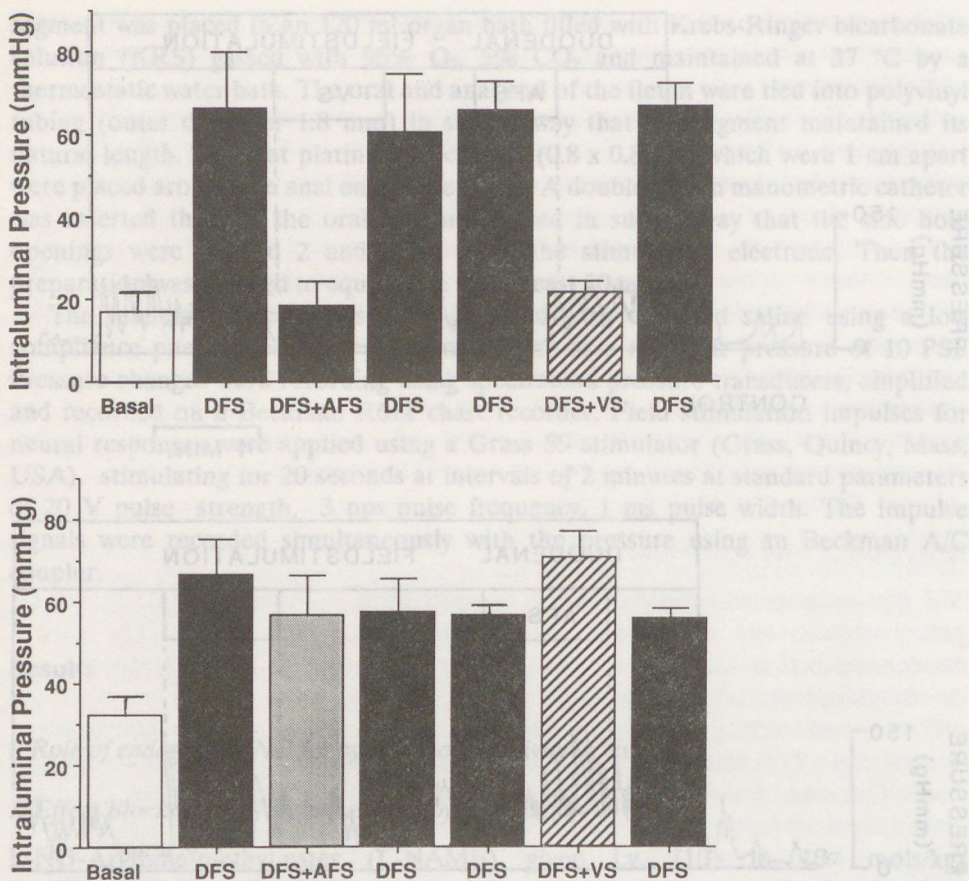


Figure 2. Histogram showing the effect of antral and vagal stimulation on pyloric motility in (a) control conditions and (B) after administration of L-NAME (3×10^{-5} mols/kg i.v.). The bar represent the pyloric motor activity in the basal state (Basal) during stimulation by duodenal field stimulation (DFS), during duodenal field stimulation and antral field stimulation (AFS), again during duodenal field stimulation alone (DFS) and during duodenal field stimulation and simultaneous vagal stimulation (DFS + VS). The values given represent mean $\pm$ SD of 5 individual experiments. Statistical comparison was carried out by comparing the respective responses with the control using one-way analysis of variance (With permission of American Physiological Society)

10^{-5} mols/kg L-NAME i.v. (Fig. 3). At this concentration vagal stimulation elicited even an excitatory response in the pylorus, consisting either of strong phasic contractions or of a persistent increase of pyloric tone. When vagal stimulation was continued strong propagated antral contractions involving the pyloric region occurred, which made a selective recording from the pylorus impossible.

An excess of D-arginine given i.v. (2.5×10^{-4} mols/kg) had no effect on the blockage on the inhibitory responses to vagal stimulation and antral field stimulation by L-NAME (5×10^{-5}). However, after intravenous administration of L-

arginine (2.5×10^{-4} mols/kg) the inhibitory effect of antral field stimulation and of vagal stimulation could be restored. L-arginine also reduced basal activity and the pyloric motor response to duodenal field stimulation significantly (Figs 1, 2, 3B).

4. Inhibitory effect of VIP and sodium nitroprusside after blockade of NO-synthesis

VIP (10^{-10} to 10^{-9} mols i.a.) causes a profound and long lasting inhibition of the pyloric motor activity in vivo. The inhibitory effect of VIP was not affected significantly by either i.a. or intravenous application of L-NAME.

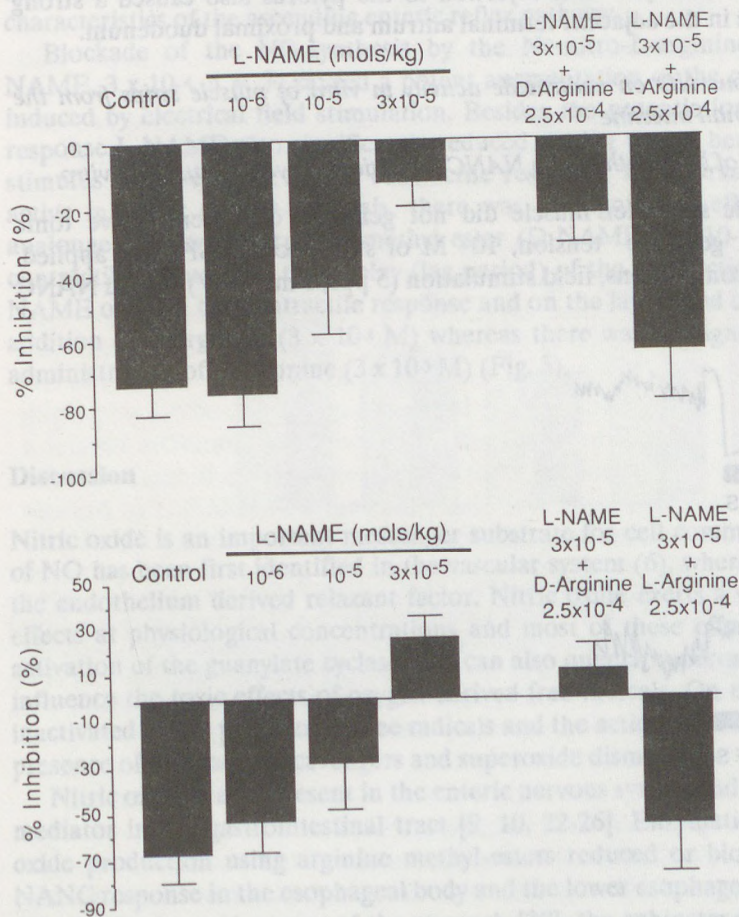


Figure 3. A. The histogram shows the effect of L-NAME in increasing concentrations, as well as additional administration of D-arginine and L-arginine on the inhibitory effect of antral field stimulation on pyloric motility. The values given represent mean $\pm$ SD of 5 individual experiments.

B. The histogram shows the effect of L-NAME in increasing concentrations, as well a additional administration of D-arginine and L-arginine on the inhibitory effect of vagal stimulation on pyloric motility. The values given represent mean $\pm$ SD of 5 individual experiments

Na⁺-nitroprusside (NaNP) (10^{-9} to 10^{-7} mols) given intra-arterially caused a dose dependent, TTX insensitive inhibition of the pyloric motor activity ($n = 4$). These inhibitory effect was not altered after application of L-NAME (5×10^{-5} mols/kg).

5. Effect of blockade of NO-synthesis on contractile responses to acetylcholine

Intra-arterial injection of acetylcholine (0.1 to $1.0 \mu\text{g}$) to the pylorus caused a dose-dependent contractile response in the pylorus. This contractile response was significantly enhanced after blockade of NO-synthesis with L-NAME (5×10^{-5} mols/kg i.v.). L-NAME had no significant effect on the blood pressure. In the presence of L-NAME acetylcholine injection to the pylorus also caused a strong contractile response in the adjacent terminal antrum and proximal duodenum.

II. Role of endogenous NO on contractile activity in vitro of muscle strips from the pylorus and the rat small intestine

1. Effect of blockade of NO-synthesis on NANC inhibition of pyloric muscle in vitro

Strips of the pyloric sphincter muscle did not generate consistent active tonic concentration. To generate tension, 10^{-6} M of substance P (SP) was applied. During SP-induced contractions, field stimulation (5 pps , 1 ms , 40 V) caused NANC

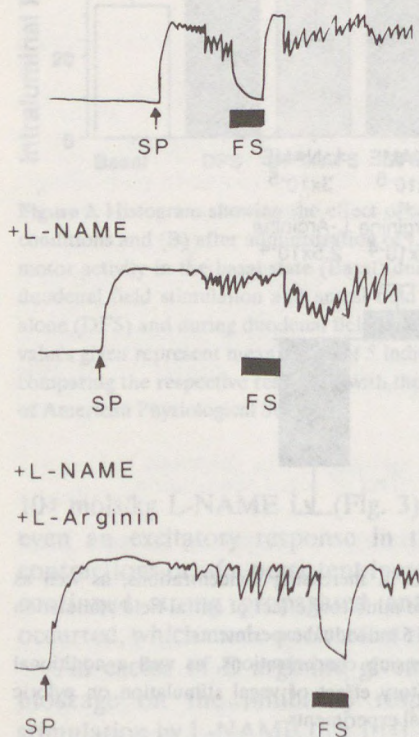


Figure 4. Representative tracing showing the effect of L-NAME on pyloric muscle strips in vitro. Effect of electrical field stimulation on SP prestimulated pyloric smooth muscle strips in the control state, in the presence of L-NAME (3×10^{-4} M) and in the presence of L-NAME + L-arginine (10^{-3} M)

relaxations. These inhibitory effect of electrical field stimulation was significantly reduced by 3×10^{-4} M of L-NAME (Fig. 7B) and restored by L-arginine (10^{-3} M) but not D-arginine (10^{-3} M). Similarly to previous experiments VIP had no relaxant effect on SP induced contractions in vitro (Fig. 4).

2. Effect of blockade of endogenous NO on the ascending neural pathway in the rat small intestine

Electrical stimulation (20 sec, 20 V, 1 ms, 3 pps) applied anally to the intraluminal recording sites of an isolated segment of rat small intestine elicited a contractile response 2 and 4 cm oral to the stimulation site. This contractile response was hexamethonium and atropine sensitive and showed all pharmacological characteristics of the ascending enteric reflex pathway.

Blockade of the NO-synthesis by the NG-nitro-L-arginine-methyl ester (L-NAME, 3×10^{-4} , $n = 7$) caused a potent augmentation of the contractile response induced by electrical field stimulation. Besides the potentiation of the contractile response L-NAME also significantly reduced the lag period between the electrical stimulus and the onset of the contractile response. In contrast to L-NAME, the active inhibitor of NO-synthesis, there was no significant effect of the inactive analogue NG-nitro-D-arginine-methyl ester (D-NAME, 3×10^{-4} , $n = 7$) on either contractile response or time delay (lag period) of the contraction. The effect of L-NAME on both the contractile response and on the lag period could be reversed by addition of L-arginine (3×10^{-3} M) whereas there was no significant change after administration of D-arginine (3×10^{-3} M) (Fig. 5).

Discussion

Nitric oxide is an important messenger substrate for cell communication. The role of NO has been first identified in the vascular system (6), where it is identical with the endothelium derived relaxant factor. Nitric oxide exerts a variety of biological effects at physiological concentrations and most of these effects are mediated by activation of the guanylate cyclase. NO can also quench superoxide radical and thus influence the toxic effects of oxygen derived free radicals. On the other side NO is inactivated by the presence of free radicals and the action of NO is prolonged in the presence of free radical scavengers and superoxide dismutase.

Nitric oxide is also present in the enteric nervous system and is a putative NANC mediator in the gastrointestinal tract [9, 10, 22-26]. Enzymatic blockade of nitric oxide production using arginine methyl-esters reduced or blocked the inhibitory NANC response in the esophageal body and the lower esophageal sphincter. [10, 26, 27], the fundus and corpus of the stomach [28], the sphincter of Oddi [29-31], the small intestine [25, 32, 33], the ileocecal sphincter [12, 22], the colon [1, 23, 34] and the internal anal sphincter [13].

A variety of actions and effects have been described and several physiological roles have been postulated in vitro, whereas little information is available to date on the role of nitric oxide in vivo. We have used a well characterized in vivo system to

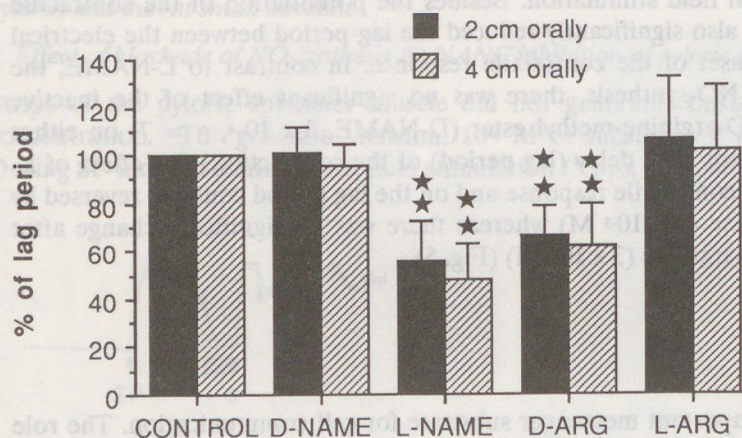
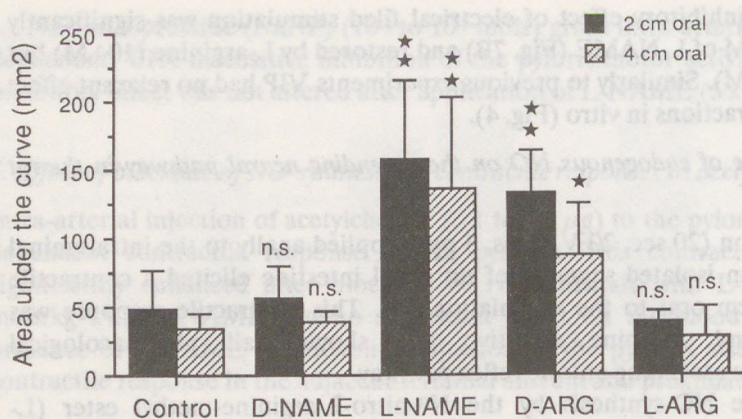


Figure 5. A) Histogram summarizing the effects of D-NAME, L-NAME, D-arginine and L-arginine on the ascending contractile response of the rat ileum. Statistical comparison of the mean values was carried out using analysis of variance in comparison to control state. B) Histogram summarizing the effects of D-NAME, L-NAME, D-arginine and L-arginine on the time delay (lag period) between the start of the electrical stimulation and the onset of the ascending contractile response. Statistical comparison of the mean values carried out using analysis of variance in comparison to control state.

establish the physiological role of endogenous NO for the regulation of antroduodenal motility *in vivo*. Furthermore we have used *in vitro* models to investigate the possible interaction of NO not only with smooth muscle but also with other neural elements in the gut.

The canine pylorus has a potent inhibitory innervation which is neither adrenergic nor cholinergic. This inhibitory pathways can be activated using vagal stimulation with appropriate stimulus parameters and by activation of intrinsic nerves of the antrum by intramural field stimulation [15]. Endogenous opiates, alpha- and beta-adrenergic receptor mediated responses and galanin have been

ruled out in the canine pylorus as possible candidates for the inhibitory NANC innervation as the inhibitory responses, induced by vagal or antral field stimulation [5, 15-19]. VIP a post-synaptic inhibitory action, e.g. the inhibitory responses could not be blocked by tetrodotoxin. However, VIP failed to cause a consistent inhibitory response in vitro, suggesting that either VIP is not the final transmitter, or that there is an additional NANC transmitter different from VIP.

The data in the canine pylorus [2] demonstrate that the inhibitory effect of vagal stimulation and antral field stimulation on pyloric motor activity can be completely blocked by intravenous administration of N^G-nitro-L-arginine-methyl ester (L-NAME), a substance known to inhibit the generation of NO from L-arginine. The results clearly demonstrate that blockade of the synthesis of NO or other arginine metabolites will abolish the inhibitory effect of either vagal stimulation or antral field stimulation in vivo. This suggests an important role of NO or related compounds in the NANC inhibitory innervation of the canine pylorus also in the whole organism and that these responses are not only activated by rather artificial field stimulation but also in response to extrinsic nerve stimulation. In contrast to the blockage of these neural responses, the inhibitory effect of intra-arterial enjection of VIP was still preserved in vivo. Moreover, neural relaxation of the pylorus in vitro was also blocked by L-NAME and this effect was antagonized by L-arginine. In contrast VIP was ineffective in vitro before and after L-NAME. These data suggest that VIP, even so having an inhibitory effect in vivo similar to isoproterenol, might not be responsible for the NANC inhibitory response and that NO or a related compound is responsible. It can not be ruled out, however, that VIP might play a role through an additional neural site of action. Interestingly there was a delayed inhibition, which occurred after the vagal or antral stimulation was turned off. This inhibition was unmasked in the presence of L-NAME and could not be blocked by L-NAME. Furthermore, L-NAME increased both basal pyloric activity and the pyloric motor response to duodenal field stimulation suggesting that there might be a tonic release of NO in the pylorus providing a continuous inhibitory constraint. Such a tonic inhibitory input had also been demonstrated by excitation after neuronal blockade using intra-arterial TTX [15]. The removal of this inhibitory mechanism could explain the potentiation of the acetylcholine response seen after administration of L-NAME. Alternatively, it could be suggested that acetylcholine might normally release enteric nitric oxide, thus restraining responses to the drug. This has been shown to occur in the vascular epithelium.

To investigate the possible interaction of nitric oxide with neural mechanisms further we have used a different model looking at the ascending neural reflex in the rat small intestine [35]. The data demonstrate that specific blockade of endogenous NO-synthesis with L-NAME, but not with inactive analog D-NAME causes a potent augmentation of the ascending reflex. This augmentation of the ascending response is reversed by the L-form of arginine but not by the inactive D-form of arginine demonstrating the specificity of L-NAME on the NO-synthesis.

Blockade of NO-synthesis increased the contractile response and almost abolished the time delay between the onset of the electrical stimulus and the contractile response. The response to aboral electrical field stimulation showed a

characteristic time delay between onset of the stimulation and the increase of the intraluminal pressure. This delay could be explained either by a summation of neural responses or by simultaneous activation of inhibitory fibers by the electrical stimulation, or both. This indicates that NO might interfere with the transmission of the neural signal as well as the final motor pathway leading to the contraction.

These results confirm that NO has an important role as NANC inhibitory mediator to the smooth muscle and that it might also have an additional neuromodulatory effect. The interaction of NO within the nervous system needs to be further characterized.

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Significance of Superoxide Generation and Fc α -Receptor Bearing Polymorphonuclear Leukocyte in IgA Nephropathy

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Abstract

In this study, we investigated superoxide production (O_2^-) and Fc α receptor (Fc α R) molecule expression in circulating polymorphonuclear leukocytes (PMNL) isolated from patients with IgA nephropathy (IgAN) and non-IgA mesangial proliferative glomerulonephritis (PGN) and healthy volunteers, in order to establish their functional importance in the immunopathogenesis of mesangial proliferative glomerulonephritis with or without mesangial IgA deposits. PMNL from patient groups showed increased O_2^- generation when activated with N-formyl methionyl leucyl phenylalanine (FMLP) and phorbol myristate acetate (PMA) compared to control group. IgA aggregates induced O_2^- production significantly only in IgAN group. The increased O_2^- production demonstrated a positive correlation with the severity of proteinuria. Fc α R expression on circulating PMNL was higher in IgAN compared to those in PGN and control groups. PMNL Fc α R expression in IgAN group showed a positive correlation with the amount of PMNL O_2^- production to IgA aggregates and proteinuria. These observations suggest that polymorphs-derived superoxide may potentially be participating in the pathogenesis of glomerular injury in IgAN, augmenting Fc α R mediated activation by IgA aggregates/immune complexes.

Introduction

IgA nephropathy has become recognized worldwide as one of the most common primary glomerulonephritis [1] and is considered to be an immune mediated disease [2]. Since its original description in 1968 by Berger and Hinglais [3], an extensive effort to define the pathogenesis of this disease have been made. Despite such efforts the underlying pathophysiology and factors associated with the severity and progression of the disease mostly remain unclear. The relatively simple immunopathological patterns observed in the kidney biopsy, i.e. a mesangial

proliferation with mesangial deposits rich in IgA, is challenged by the complex blood immunological abnormalities. Deranged cellular immunoregulation apparently contributes an important pathogenetic role [4]. Prognosis of IgA nephropathy is not so benign as initially thought, and approximately 20% of patients with this disease will eventually shift to end stage renal disease [5].

Increasing evidence has been accumulated suggesting the participation of polymorphonuclear leukocytes (PMNL) in the pathogenesis of various types of nephritis [6-8]. Although the glomerular injury has been attributed to the release of enzymes from leukocytes [9], recent studies related to glomerular injury indicated that neutrophil-derived reactive oxygen species are also important in tissue injury [10]. Neutrophils and monocytes/macrophages exhibit a burst of oxidative metabolism in response to plasma membrane perturbation by a variety of soluble and particulate stimuli [11]. A recent study showed that aggregated IgG and IgA obtained from IgA nephropathy (IgAN) patients induced greater O_2^- production in PMNL of healthy controls than the immune aggregates obtained from other healthy controls [12]. Thereby, the potential role of circulating IgG and IgA immunoglobulins was indicated as an underlying mechanism for the activation of PMNL. It is gratefully accepted that leukocytes cause glomerular injury such as proteinuria by damaging the glomerular basement membrane (GMB), the major ultrafiltration barrier to restrict the entry of proteins into the urinary space. However, the correlation between the activities of PMNL and the severity of glomerular damage has never studied yet.

In this study, we measured O_2^- generation from PMNL of IgAN patients to demonstrate the primed state of PMNL in IgAN. A detailed study was performed to determine the relationship between O_2^- generation and receptor expression for IgA ($Fc\alpha$) as a causative factor for the activation of PMNL in IgAN. We measured O_2^- generation from PMNL of non-IgA mesangial proliferative glomerulonephritis (PGN) patients and healthy volunteers as a comparison. As a method of superoxide generation, a MCLA-dependent chemiluminescence system was used by which O_2^- production could be quantified with high specificity and reliability [13, 14]. The expression of $Fc\alpha R$ on PMNL was studied by flow cytometry using a monoclonal antibody, My-43 to evaluate whether specific IgA-receptor have any specific participation in the pathogenesis of IgAN. The peak O_2^- generation by PMNL was compared with the amount of urine protein to assess the involvement of PMNL in the inflammatory process of mesangial proliferative glomerulonephritis.

Materials and methods

Subjects

A total of forty-five patients with IgA nephropathy (35 males and 10 females; mean age 36 years) and forty patients with non-IgA mesangial proliferative glomerulonephritis (PGN, 25 males and 15 females; mean age 36 years) with clinical and renal immunopathogenic diagnosis were enrolled in the present study. Patients

with other systemic diseases as well as other proliferative glomerulonephritis (GN), such as membranoproliferative GN, endocapillary GN and crescentic GN were excluded from this study. Serum creatinine of all patients was below 2 mg%. None of the patients were on corticosteroid or any immunosuppressive therapy during this study. None of these patients showed any clinical signs of infection within two month prior to the study. Clinical status of the patients was assessed by estimating the urinary output of total protein in 24 hours (UTP), severity of hematuria, serum creatinine level, serum creatinine clearance (GFR) and serum immunoglobulins (Igs). Severity of renal tissue damage was scored histologically. Sixty age matched healthy volunteers (45 males and 15 females) were used as donors of control peripheral blood polymorphonuclear leukocytes (PMNL).

Preparation of polymorphonuclear leukocytes (PMNL)

The heparinized peripheral venous blood was subjected to simple Hemaceel[®] (3.5%) sedimentation for 30-45 minutes at 37 °C to hasten erythrocyte precipitation. Then PMNL were separated from mononuclear cells using Ficoll-Hypaque density centrifugation for thirty minutes at 1,500 revolutions per minute (rpm). The PMNL rich fluid at the interface of RBC sedimentation was aspirated and washed twice with calcium and magnesium free Hank's balanced salt solution (HBSS-). Erythrocyte contamination was removed by hypotonic shock. The final preparation was washed again with HBSS- and resuspended in the same solution on ice until superoxide production assay. The viability of PMNL was more than 95% as assessed by trypan blue exclusion. Final cell preparation contained 90-95% neutrophils, the rest of the cells being mononuclear cells and eosinophils.

Reagents

The following reagents were used in this study. Hemaceel[®] (3.5%) (Behring Inst. Erbehring, Germany), Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), Hank's balanced salt solution (Gibco, Grand Island, N.Y.), phorbol myristate acetate (PMA, 1 mg/ml), N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 3×10^{-8} M/ml), (Sigma Chemical, St. Louis, MO), 2-methyl-6[p-methoxyphenol]-3,7-dihydroimidazol[1,2- α]pyrazin-3-one (MCLA, 3 mM/ml), (Nihon Zoki, Osaka, Japan and cytochrome c (Boehringer Mannheim GmbH, Germany) were purchased. Recombinant human superoxide dismutase (SOD) was a donation from Nihon Kayaku Co. (Tokyo, Japam).

Immunoglobulins and monoclonal antibodies

Human monomeric IgA (IgA1, lambda) was prepared in our laboratory from serum of a myeloma patient using a Jacalin column [15]. Briefly, IgA rich fraction was precipitated by 50% ammonium sulphate saturation, and was applied to a Jacalin column (sepharose 4B coupled with extracted protein from the seeds of the jack fruit). After washing with borate buffered saline, IgA was eluted with 0.8 M

galactose solution. Purified IgA showed a single precipitating line against anti-whole human serum examined by immunoelectrophoresis, and found not to be contaminated with IgG or IgM when examined by ELISA. Human normal IgG (10 mg/ml) was purchased from Green Cross Co. (Osaka, Japan). Aggregates of immunoglobulins were made by heating IgG and IgA in PBS (pH 7.4) for 20 min and 90 min, respectively, at 63 °C which was found in a previous experiment to give an optimum amount of soluble aggregates [16]. Fluorescence isothiocyanated (FITC) goat F(ab')₂ antimouse Igs was purchased from Togo, Inc. (Burlingame, CA). A monoclonal antibody specific for IgA Fc receptor, My-43, was a gift from Dr. Li Shen (Dept. of Microbiology, Dartmouth Medical School, Hanover, NH) [17].

Superoxide assay

Superoxide generation was quantified by measuring O₂-dependent chemiluminescence (CL) using a Hamamatsu C1230 Photon counter with a H-R550 photomultiplier (Hamamatsu Photonics Co. Ltd., Japan; applied potential: -1.5 kV) as described previously [13, 14]. Briefly, 2.5-5.0 x 10⁴ cells were kept into a Petri dish (50 mm x 9 mm, Falcon, Lincoln Park, New Jersey) containing 10 µl (20 µM) of MCLA in warm HBBS+ on initial measurement. All procedures of the chemiluminescence method were conducted at 37 °C. After obtaining a basal photon count, 10 µl of either FMLP (6 x 10⁻¹⁰ M) or PMA (10 µg) or 10 µl of heat aggregated IgG (10 mg/ml)/IgA (10 mg/ml) was added as a stimulant at the 30th second of cell addition. Each stimulant was used separately to trigger PMNL O₂⁻ production. Changes in photon counts were monitored until it reached the plateau level, which was observed within 1.5-5 minutes after addition of a stimulant (depending on the nature of the stimulant) and terminated by adding 100 µl (236 µg/ml) of SOD. Photon counts caused by activated PMNL decreased to the level of basal counts by SOD at the concentration used in the experiment; indicating that photon counts in MCLA-chemiluminescence are O₂-dependent. The peak count of SOD-inhibitable chemiluminescence per cell were used to express the relative amount of O₂⁻ generation.

To determine the correspondent value of superoxide that was measured in MCLA-dependent method, cytochrome c reduction method as a parallel experiment was employed as previously described [18]. Briefly, in a sample tube, neutrophils (1 x 10⁶) were combined with 40 µl (10 mol) of horse ferricytochrome c and HBSS- was added to the final volume of one ml. Blank tube was prepared by adding SOD 10 µl (30 µl/ml) in addition to the above composition. After 10 min incubation at 37 °C, PMA (1 µg) stimulated O₂⁻ absorption by cytochrome c at 550 nm was determined in a spectrophotometer (Ubest-50, Japan Spectroscopic Co. Ltd.) and O₂⁻ production was calculated from the differences of the optical densities between the tubes with or without superoxide dismutase.

Flow cytometry

Flow cytometric analysis was performed using a FACScan analyzer (Becton Dickinson, USA) by forward light scattering (FSC) profile and side light scattering (SSC) profile. Five hundred thousand PMNL were stained firstly with monoclonal

antibody for Fc α R, My-43. After 30 minutes on ice, cells were washed with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and stained with FITC labeled goat antimouse Igs for another 30 minutes. After final washing cells were fixed with 2% paraformyldehyde (PFA) and kept at 4 °C until flow cytometric analysis. Mouse control immunoglobulin was used in the starting assays as a negative control to determine the background binding of mouse Ig.

Statistics

P values of all correlation assays were calculated by using the Sperman-Kendall test. The Mann Whitney U test and Fisher test were applied to calculate the p value among different groups. All results were expressed as mean $\pm$ SD.

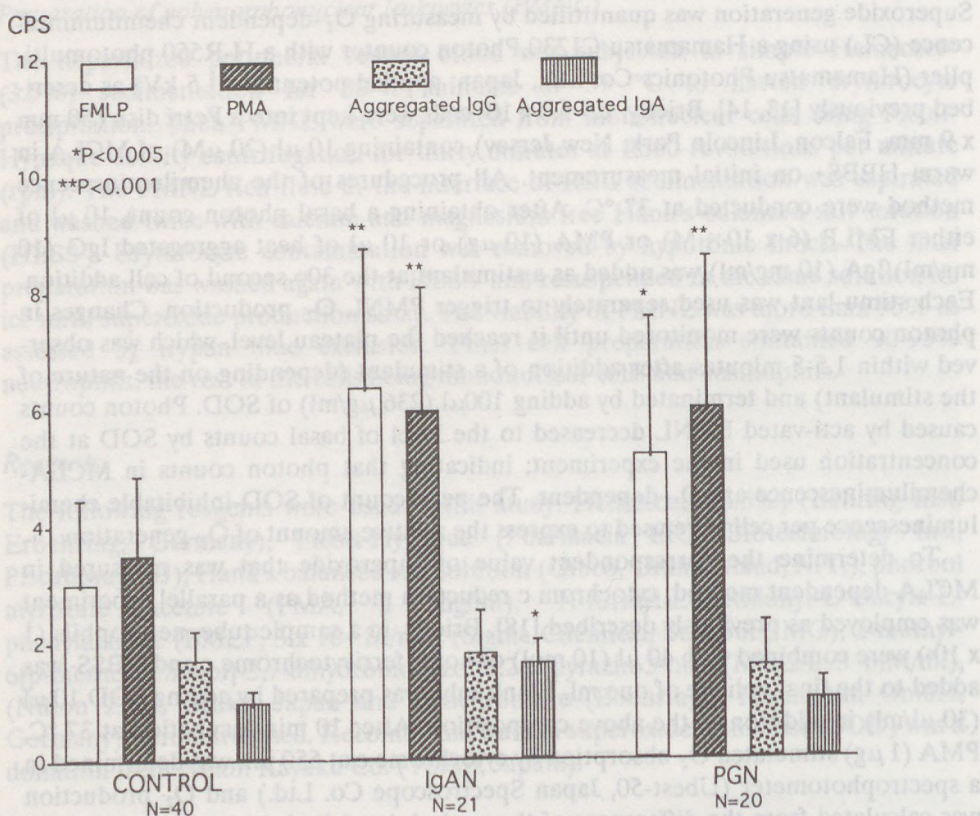


Figure 1. Polymorphs MCLA-dependent superoxide (O_2^-) generation stimulated by FMLP, PMA and immune aggregates IgG and IgA. IgAN; IgA nephropathy, PGN; non-IgA mesangial proliferative glomerulonephritis. Polymorphs O_2^- production ability was significantly higher in both IgAN and PGN patients in response to FMLP and PMA stimuli compared with that in control group. Amount of responses to FMLP and PMA did not differ significantly. IgA aggregates induced O_2^- production was significantly higher only in IgAN patients compared to PGN patients and controls. Responses to aggregated IgG showed no comparable variation among the three groups. FMLP and PMA showed a greater ability to induce O_2^- generation than immune aggregates. The error bars represent the m $\pm$ SD

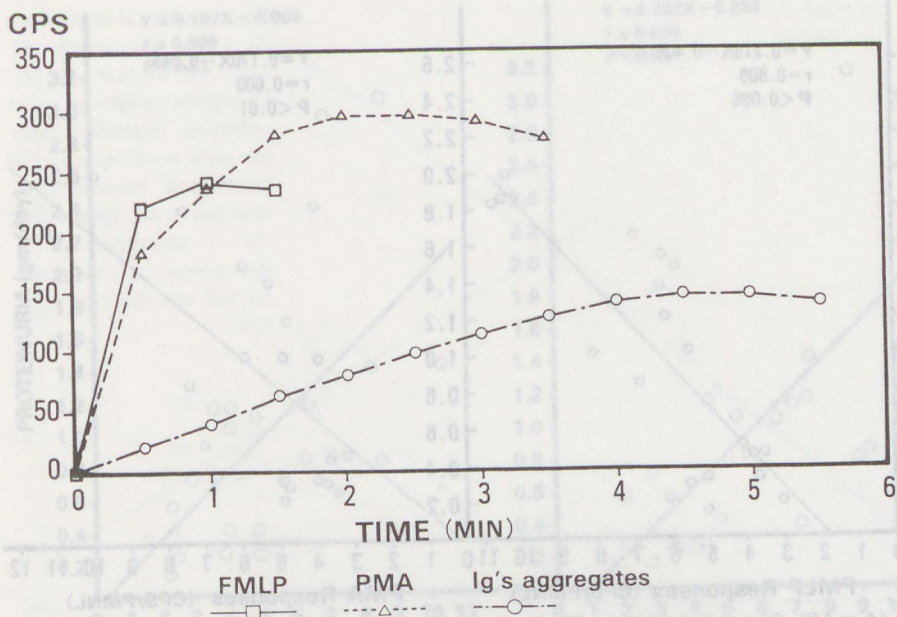


Figure 2. MCLA-dependent chemiluminescence (CL) of polymorphs showing the time units to attain the CL-peaks by different stimulants. FMLP induced CL-peak was much quicker (1.5 min) than induced PMA (2.5 min) and immune aggregates (5 min)

Results

The background chemiluminescence values of PMNL prior to stimulation were basically similar in controls and patients, suggesting that the PMNL was not activated during the isolation procedure.

In preliminary study the doses of different stimulants were determined to have optimal chemiluminescence (CL) responses (data not shown). Figure 1 shows that the mean values of CL-peaks of PMNL were significantly higher in patients with IgAN (6.23 ± 2.3 , 6.12 ± 2.0 CPS/cell for FMLP and PMA, respectively) and PGN (5.08 ± 2.4 , 5.90 ± 1.6 CPS/cell for FMLP and PMA, respectively) than in control group (2.83 ± 1.55 , 3.3 ± 1.5 CPS/cell for FMLP and PMA, respectively). IgA aggregates triggered a tendency of higher responses (1.67 ± 0.69 , 1.80 ± 0.57 and 1.58 ± 0.75) compared to those triggered by an equivalent dose of aggregated IgA (1.00 ± 0.34 , 1.77 ± 0.71 and 0.99 ± 0.39) in control, IgAN and PGN group, respectively. However, there was no significant difference in IgG-induced CL-peaks in three groups. IgA aggregates caused significantly higher CL-peaks (1.77 ± 0.71) only in IgAN group ($p < 0.01$) when compared with those in the PGN (0.99 ± 0.39) and control (1.00 ± 0.34) groups. The time of peak CL attained for FMLP, PMA and immune aggregates was shown in Fig. 2. CL-peaks with FMLP was sharp and fast (within 1.5 min of stimulant addition) compared to that of PMA (within 2.5 min).

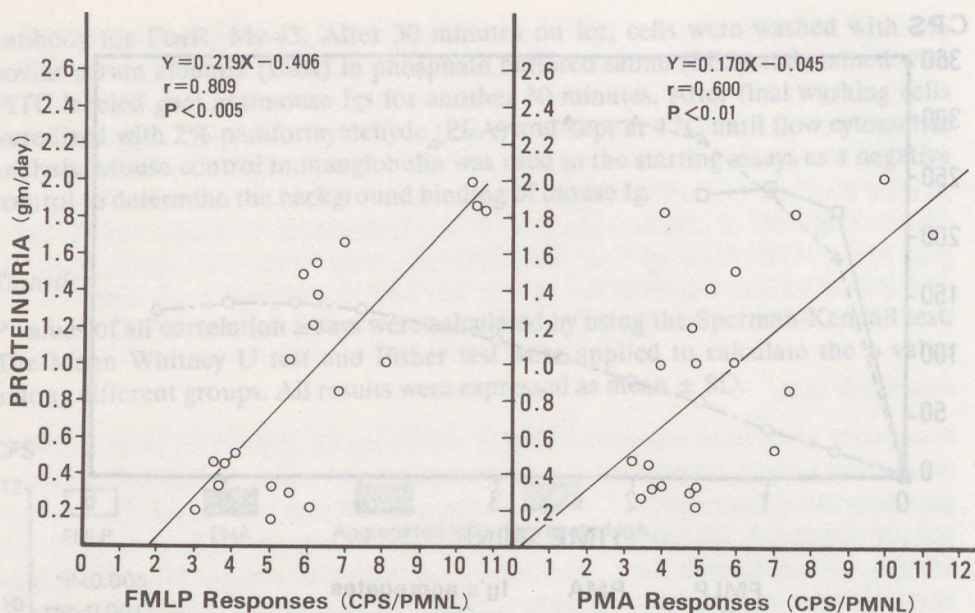


Figure 3. (A and B). Comparison between CL-peaks and the amount of proteinuria in IgAN patients. A positive correlation between these two data was observed ($p < 0.005$ for FMLP, Fig. 3A and $p < 0.01$ for PMA, Fig. 3B)

CL-peaks with immune aggregates were much slower (within 5 min) and lower in magnitude.

Correlation between PMNL CL-peaks to different stimuli and the severity of proteinuria was shown on Figs 3, 4 and 5. A positive correlation between these two parameters was observed in IgAN patients ($p < 0.005$ for FMLP, Fig. 3A; $p < 0.01$ for PMA, Fig. 3B) as well as in PGN patients ($p < 0.01$ of both FMLP and PMA) (Fig. 4A and B, respectively). Similar correlation was observed only in IgAN patients when triggered by IgA aggregates ($p < 0.01$) but not by IgG aggregates (Fig 5A and B). PGN patients did not show any such comparable data to either immune aggregate stimulation (data not shown).

In the comparative study (Fig. 6), mean value of O_2^- generation in MCLA system was 3.16 ± 0.54 CPS/cell as compared with that of cytochrome c monitored value, 0.42 ± 0.13 pm/min/cell at 550 nm. In other words, 1 nm/min/cell reduction of cytochrome c corresponded with 7.52 CPS/cell O_2^- production. Under the same condition a linear comparison of light emission in chemiluminescence system with O_2^- absorption by cytochrome c reduction method was observed; clearly indicating the participation of superoxide in MCLA-dependent chemiluminescence.

Figure 7 demonstrates the flow cytometric assessment of $Fc\alpha R$ expression on PMNL using a monoclonal antibody, My-43. The receptor expression was significantly increased in patients with IgAN compared to those in PGN patients and healthy volunteers. In order to evaluate the potential biological role of $Fc\alpha R$ in

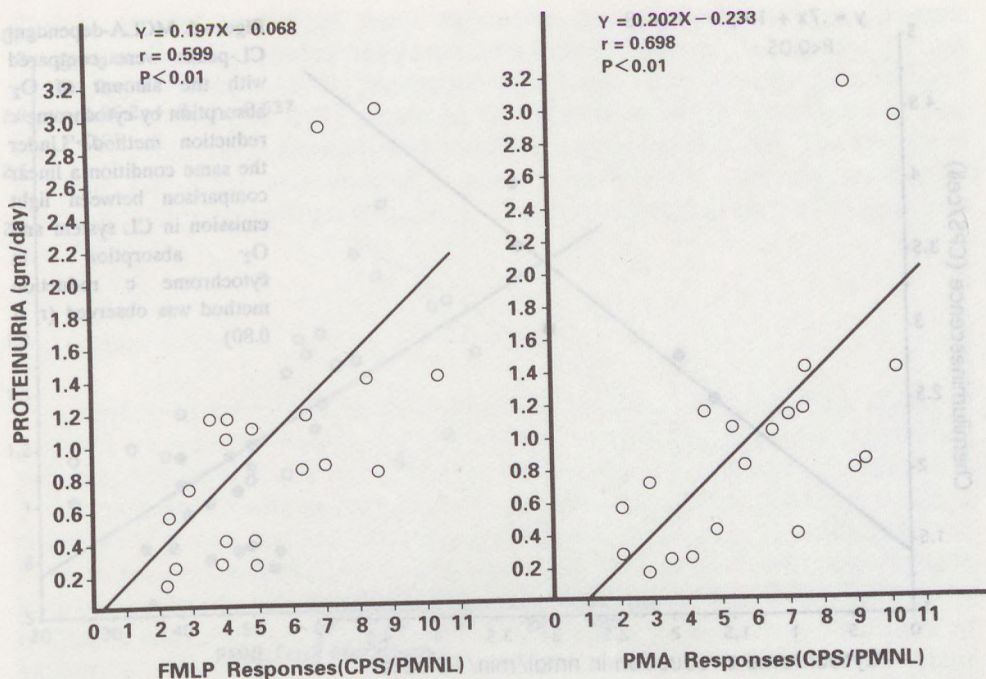


Figure 4. (A and B). Correlation between CL-peaks and proteinuria in patients. A significant correlation was noticed for both FMLP and PMA stimulants ($p < 0.01$)

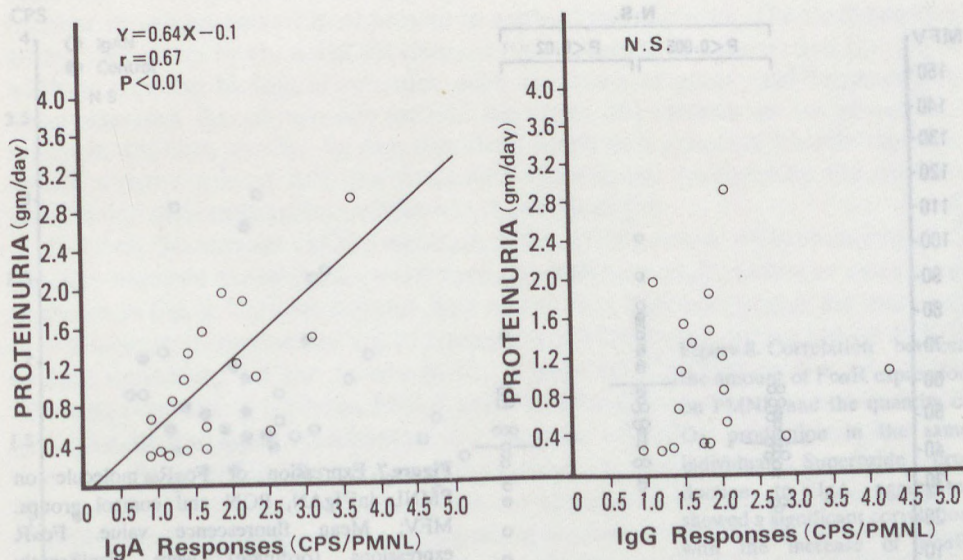


Figure 5. (A and B). Relationship between CL-peaks to immune aggregates and proteinuria. Aggregated IgA-induced responses showed a positive correlation with the severity of proteinuria in IgAN patients ($p < 0.01$, Fig. 5A). Responses to IgG aggregates did not show any correlation (Fig. 5B)

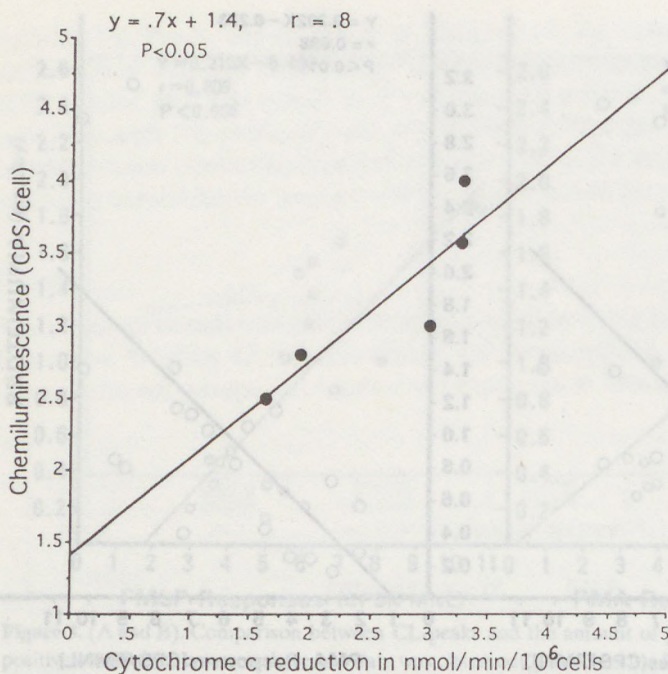


Figure 6. MCLA-dependent CL-peaks were compared with the amount of O₂⁻ absorption by cytochrome c reduction method. Under the same condition a linear comparison between light emission in CL system and O₂⁻ absorption in cytochrome c reduction method was observed ($r = 0.80$)

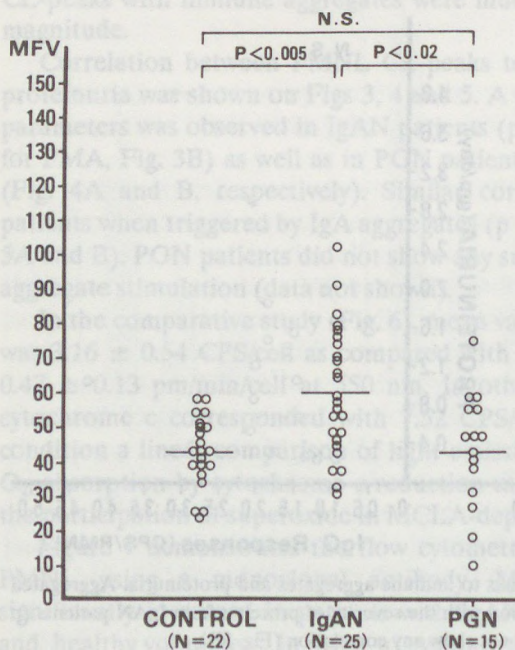


Figure 7. Expression of FcαR molecule on PMNL in IgAN, PGN and control groups. MFV; Mean fluorescence value. FcαR expressions (ordinate) were significantly increased in IgAN group compared with those in PGN and healthy volunteer groups. Horizontal bars indicate the mean $\pm$ SD

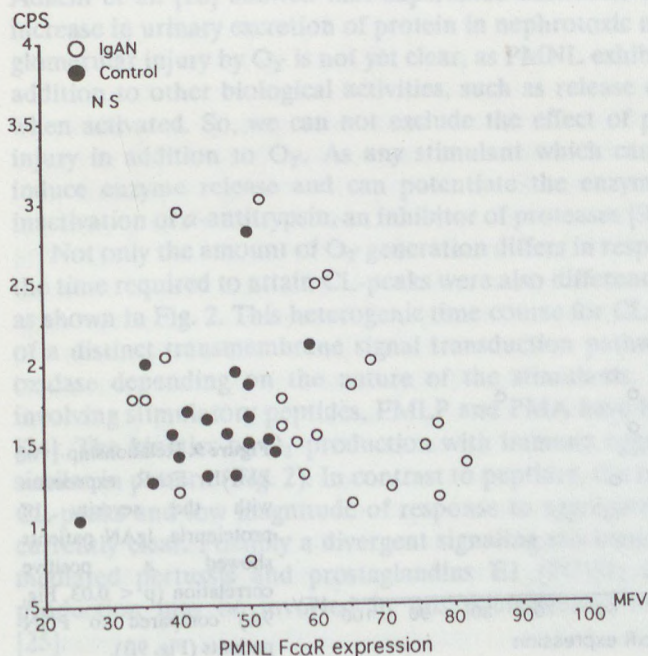
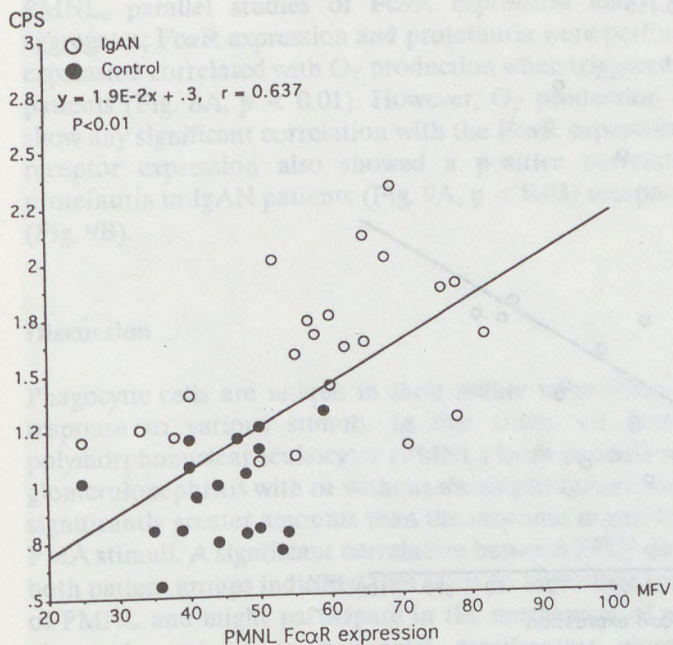


Figure 8. Correlation between the amount of FcαR expression on PMNL and the quantity of O₂ production in the same individual. Superoxide production to IgA aggregates showed a significant correlation with the increase of FcαR expression ($p < 0.01$, Fig. 8A) compared to IgG response (Fig. 8B)

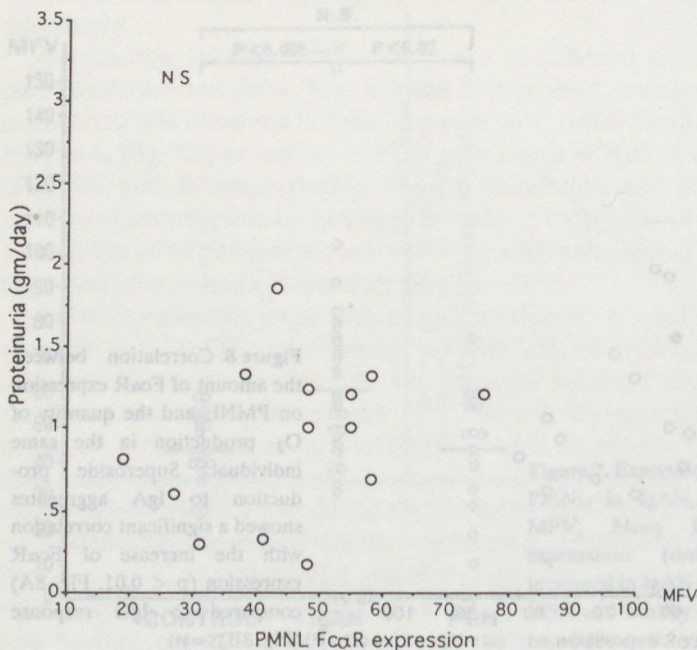
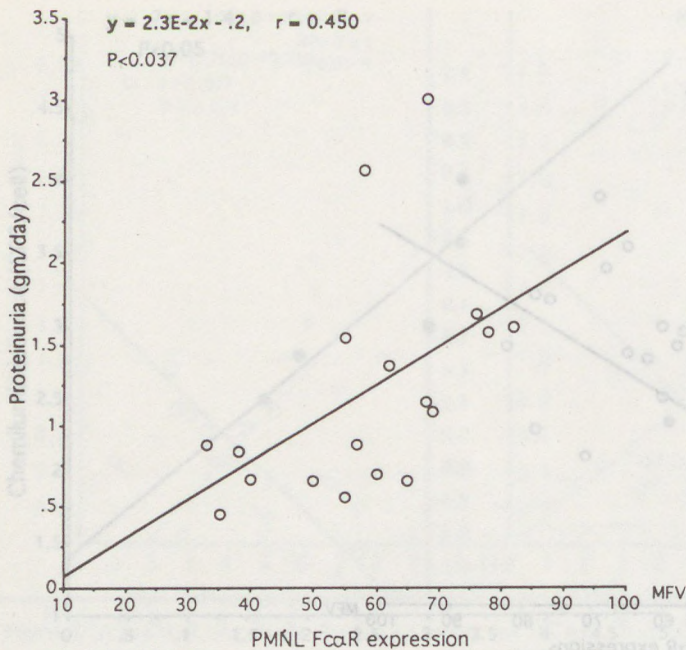


Figure 9. Relationship of PMNL FcαR expression with the severity of proteinuria. IgAN patients showed a positive correlation ($p < 0.03$, Fig. 9A) compared to PGN patients (Fig. 9B).

PMNL, parallel studies of Fc α R expression and O₂⁻ generation to immune aggregates; Fc α R expression and proteinuria were performed. The amount of Fc α R expression correlated with O₂⁻ production when triggered by IgA aggregates in IgAN patients (Fig. 8A, $p < 0.01$). However, O₂⁻ production to IgG aggregated did not show any significant correlation with the Fc α R expression (Fig 8B). The amount of receptor expression also showed a positive correlation with the severity of proteinuria in IgAN patients (Fig. 9A, $p < 0.03$) compared to that in PGN patients (Fig. 9B).

Discussion

Phagocytic cells are unique in their ability to produce potent oxidizing agents in response to various stimuli. In this study, we demonstrated that circulating polymorphonuclear leukocytes (PMNL) from patients with mesangial proliferative glomerulonephritis with or without mesangial IgA deposits generated superoxide in significantly greater amounts than the response in control group to either FMLP or PMA stimuli. A significant correlation between PMN-derived O₂⁻ and proteinuria in both patient groups indicated that O₂⁻ is an important tool for the functional activity of PMNL and might participate in the mechanism of proteinuria (an indicator of glomerular injury) in mesangial proliferative glomerulonephritis. No other glomerular histological changes related to PMNL O₂⁻ production were observed. A previous report suggested that O₂⁻ may be the most important mediator among reactive oxygen species released by activated PMNL, damaging the kidney [19]. Adachi et al. [20] showed that superoxide dismutase significantly suppressed the increase in urinary excretion of protein in nephrotoxic nephritis. The mechanism of glomerular injury by O₂⁻ is not yet clear, as PMNL exhibit O₂⁻ generation [21, 22] in addition to other biological activities, such as release of glomerular enzymes [23] when activated. So, we can not exclude the effect of proteases in the glomerular injury in addition to O₂⁻. As any stimulant which can generate PMNL O₂⁻, also induce enzyme release and can potentiate the enzyme activity by O₂⁻ induced inactivation of α -antitrypsin, an inhibitor of proteases [9].

Not only the amount of O₂⁻ generation differs in response to different stimuli but the time required to attain CL-peaks were also different on the nature of stimulants as shown in Fig. 2. This heterogenic time course for CL-peaks suggest the existence of a distinct transmembrane signal transduction pathway for activation of PMNL oxidase depending on the nature of the stimulants. The mechanism of PMNL involving stimulatory peptides, FMLP and PMA have been characterized in details [24]. The kinetics of O₂⁻ production with immune aggregates of IgG and IgA was similar in pattern (Fig. 2). In contrast to peptides, the reason for the delay to attain CL-peaks and low magnitude of response to aggregated immunoglobulins are not currently clear. Possibly a divergent signaling mechanism like Fc gamma- receptor mediated pertussis and prostaglandins E1 (PGE1) insensitive pathway for O₂⁻ production may be involved in this phenomenon for both immune aggregates [25].

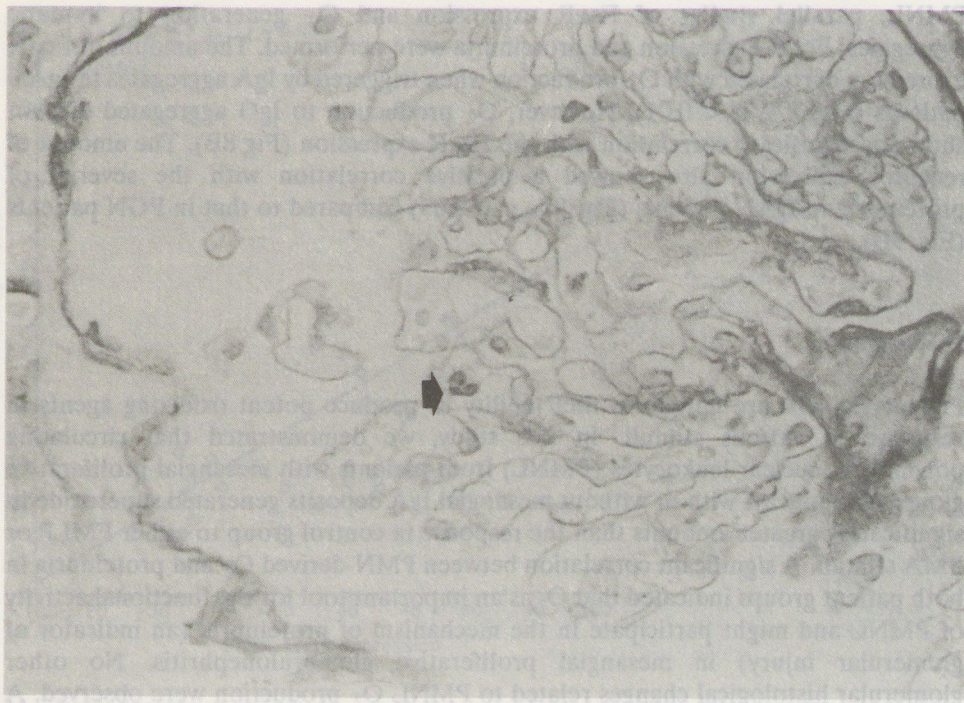


Figure 10. PMNL infiltration (light microscopy) in the glomerulus of IgA nephropathy (shown by arrow head).

The circulating PMNL could be relevant to the pathogenesis of IgAN which appears likely from their prominent infiltration in glomeruli in IgAN [8], increased expression of complement 3 receptors on neutrophils from IgAN patients [26]. Recent study showed a correlation between PMNL accumulation and glomerular damage in IgAN patients [27], support the participation of PMNL in glomerulonephritis. We usually observe infiltration of a few PMNL in some glomeruli of IgAN patients even in the clinically quiescent state of the disease (Fig. 10). The exact mechanism/s responsible for PMNL influx and activation in the inflamed glomeruli remain unclear, but it may be caused by chemoattractants released from both infiltrating and residual glomerular cells during glomerular inflammation [28]. Our results also indicated that PMNL in glomerulonephritis were in primed state which enable PMNL to produce more O_2^- upon activation with a second stimulant. Cytokines can prime phagocytic cells in addition to their chemoattractant effect [29, 30] and some of which like IL-2, INF-gamma has been reported to be raised in the serum of IgAN patients [31]. However, in our study IgA aggregates showed a priming effect only in IgAN patients and underlying mechanism of which is either an increase in the number of Fc α R or an increase in

the affinity of the receptors to the agonist. Our result showing a positive correlation between the amount of Fc α R expression and O₂⁻ production support former notion.

Unlike peptides FMLP and PMA, IgA immune aggregates caused higher O₂⁻ production only in IgAN patients (Fig. 1) which also showed correlation with the amount of proteinuria (Fig. 5A). Patients with PGN did not show such a correlation. Flow cytometric analysis revealed that Fc α R expression on PMNL from IgAN patients was significantly higher (Fig. 7). In a previous study an increased expression of these receptors on lymphocytes [32] and monocytes (own observation, data not shown) was observed. The precise mechanism of Fc α R expression and its functional significance in IgAN are not well clarified. Our observation of increased O₂⁻ production nad proteinuria, parallel to the increased expression of PMNL Fc α R in IgAN patients account the effector function of this receptor in IgAN. Previous in vitro studies indicated that Fc α R is an important effector molecule in IgA-mediated immune response, since binding immune complexes/antibody to Fc α R cells has been shown to trigger phagocytosis [33] and oxidative burst [17]. Increased Fc α R expression and their increased O₂⁻ production to IgA aggregates indicated that an overall increase in the responsiveness of the oxidase system which is probably related to the increased signaling from Fc α R. This isotype-specific activation of Fc α R by IgA aggregates is therefore a biological activity of human IgA, thus facilitating interactions between cells with Fc α R molecule and IgA immune complexes as a possible immunopathogenesis in IgAN.

Aggregated IgG-induced PMNL O₂⁻ production did not show any such comparable data in patients with IgAN. Although mesangial IgA deposits occasionally accompany with IgG [3] and some previous data supported the potentiality of IgA and IgG admixture to activate and fix the complement [34], but the individual role of IgG in IgAN is not clear as in lupus nephritis [35].

Although mean serum IgA level was significantly higher in IgAN patients (378 ± 103 mg%, $n = 40$) than in PGN patients (303 ± 128 mg%, $n = 40$) and healthy volunteers (290 ± 80 mg%, $n = 40$), we did not find any correlation between individual serum IgA level and PMNL Fc α R expression (data not shown). In our previous induction study we observed that IgA aggregates did induce Fc α R expression efficiently in neutrophils after overnight incubation, whereas monomeric IgA did not show a significant effect (unpublished data). Therefore, the molecules responsible for induction of Fc α R might be polymeric IgA immune complexes present in the IgAN-serum [36] and in the mesangium [37], but not monomeric form, since the major component of serum IgA is monomeric in nature [38]. Recently, Lai et al. [12] documented the ability of IgA aggregates obtained from IgAN patients to enhance O₂⁻ production and calcium mobilization in neutrophils of healthy controls.

Accounting all results, we can conclude that firstly, circulating PMNL may potentially be participating in the inflammatory process of glomerular and interstitial injury in mesangial proliferative glomerulonephritis and secondary, IgA immune complexes may also be involving in the immunopathogenesis of IgAN by augmenting PMNL Fc α R mediated generation of superoxide anion.

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Superoxide Generation of Phagocytes Estimated by ex vivo Monitoring Using Perfused Organs After Salmonella Infection

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Abstract

Ex vivo monitoring system using perfused heart or liver for detection of superoxide generated was established. A cypridina luciferin analog, MCLA (2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2- α]pyrazin-3-one and phorbol myristate acetate (PMA) were used as a superoxide (O_2^-)-specific chemiluminescence (CL) probe and a stimulant for superoxide generation, respectively). Markedly increased MCLA-CL was detected by the ex vivo monitoring system using perfused rat heart removed 8 and 14 hr after the infection with *Salmonella enteritidis*. On the other hand, so significant increase of MCLA-CL was observed from the surface the heart without *S. enteritidis* infection. Using perfused mouse liver, MCLA-CL was significantly increased after *Salmonella typhimurium* infection. In conventional (CV) mice infected with *S. typhimurium*, the maximum MCLA-CL was detected at 7 days after the infection in spite of that maximum cell number recovered was detected at 3 days after the infection. In contrast, in germfree (GF) mice infected with *S. typhimurium*, maximum MCLA-CL and cell number recovered were detected at 14 and 7 days after the infection, respectively. These results indicate that ex vivo monitoring system using perfused organs would be useful and reliable technique for estimation of superoxide generation in real-time manner.

Introduction

Attachement of microorganisms to phagocytes activates an oxidative system in the plasma membrane of the phagocytes that converts oxygen into superoxide - directly or indirectly - into hydrogen peroxide [1]. One of the adequate stimuli of phagocytosis is an invaded microorganism, as muramyl dipeptide and lipopolysaccharide, components of bacterial cell wall, are stimuli for generation of superoxide by phagocytes [2]. Therefore, it is well known that bacterial infection induces increased generation of superoxide.

However, several of reports on superoxide generation by phagocytes after bacterial infections have been done mainly using in vitro cells isolated from either peripharral leukocyte or peritoneal cells [3, 4]. In the present study, ex vivo monitoring system for estimation of superoxide generation by phagocytes in heart and liver after *Salmonella* infections was established. In addition, relationship between superoxide generation by phagocytes and bacterial growth was also examined in not only conventional (CV) but also germfree (GF) mice.

Materials and methods

Chemiluminescence apparatus

The ex vivo monitoring system consisted of a light-tight box, a photomultiplier, and a photon counter which were completely shielded from light. The heart isolated from rat was perfused with a modified K-H (Krebs-Henseleight) buffer containing NaCl 115 mM, KCl 4.0 mM, NaHCO₃ 23.8 mM, MgCl₂ 1.2 mM, CaCl₂ 1.8 mM, NaHPO₄ 1.2 mM, pyruvate 10 mM and glucose 10 mM. The filtered perfusate was bubbled with a gaseous mixture containing 95% O₂-5% CO₂, and temperature was maintained at 37 °C as described before [5]. For perfusion of mouse liver, inferior vena cava was ligated and the portal vein was connected with a catheter (Gelco 24G, Johnson and Johnson Ltd., USA). Hepatic artery was ligated, but superior vena cava was not. After the operation, the liver was set into an apparatus for a perfusion experiment, and perfusion with the modified K-H buffer. A cypridina luciferin analog, MCLA (2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2- α]pyrazin-3-one), which is a superoxide-specific chemiluminescence (CL) probe [6, 7], was introduced via a side arm at a final concentration of 3 μ M. In the experiment of occlusion and reperfusion using perfused heart, lucigenin was used as CL probe. The CL after stimulation by phorbtor myristate acetate (PMA) detected from the surface of either the heart or liver was counted every second (cps) through a window (diameter 7 mm). After maximum CL was detected in the ex vivo monitoring, superoxide dismutase (SOD) was added into the perfused organ. The entire perfusion system with heart or mouse liver was shielded from light. The perfusion flow was adjusted to 5 ml/min and the temperature was maintained at 37 °C. By these new method, the superoxide generation from heart or liver was monitored in real-time manner.

*Infection with *Salmonella enteritidis* and *Salmonella typhimurium**

For induction of perimyocarditis, *S. enteritidis* (1 x 10⁸ colony forming unit, cfu) was inoculated through the diaphragm into a pericardial space of rat heart. Eight or 24 hr later, the hearts were removed withdrawing of blood and placed in the dark box. For analysis of superoxide generation by liver phagocytes, 6-8 weeks old ICR strain of male either CV or GF mice were infected with LT-2 strain of *S. typhimurium*. Aliquot (8 x 10⁴ cfu/0.2 ml) of the culture was inoculated subcutaneously. At 3, 5, 7, 14, 21 and 28 days after the infection, superoxide generation was measured by the ex vivo method described above.

Quantification of *Salmonella* species

Quantitative assay for *S. enteritidis* and *S. typhimurium* was carried out using DHL (deoxycholate-hydrogen sulfide lactose) agar plate (Nissui, Tokyo, Japan) by aerobic incubation at 37 °C for 18 hr. For histological examination rat heart or mouse liver was fixed with formalin and stained with hematoxilin-eosin.

Results

Establishment of perfusion system

The ex vivo monitoring system using rat heart and mouse liver for superoxide generation is shown in Fig. 1. Prior to ex vivo analysis for superoxide generation, the specificity of MCLA-CL for O_2^- was examined using isolated human leukocytes as described before [8]. By addition of PMA, photon counts of MCLA-CL increased. The MCLA-CL was completely eliminated by addition of SOD (150 IU/ml), but by neither $NaNO_3$ nor catalase, indicating that MCLA is a specific CL probe for O_2^- (data not shown). On the other hand, it was shown that MCLA gives a significant intensity of CL from autoxidation; thus the heart was placed upside down

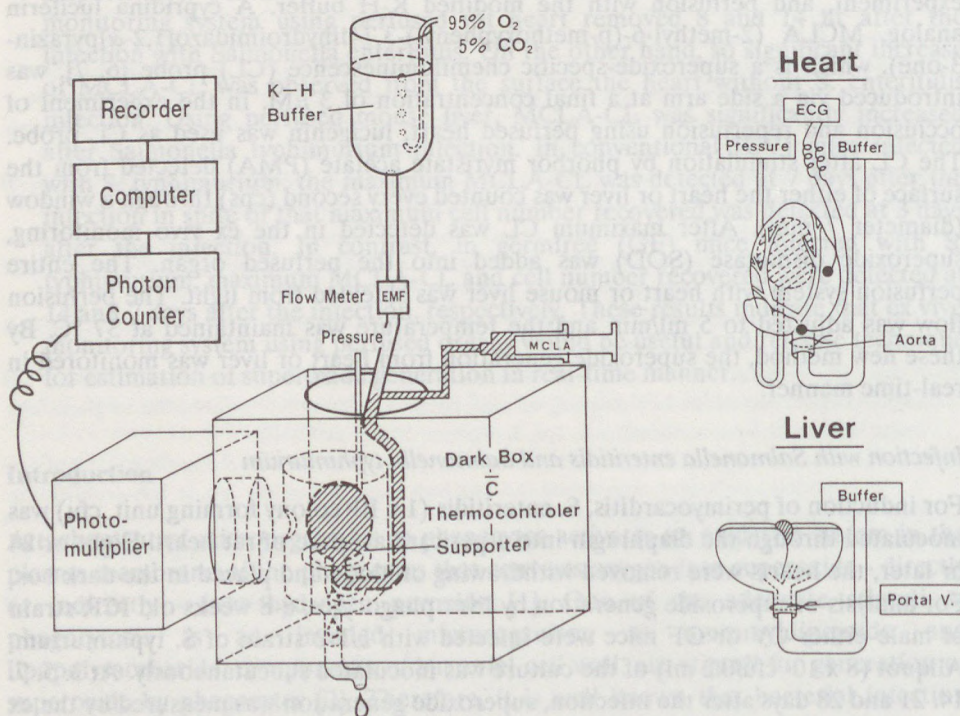


Figure 1. The ex vivo monitoring system for measurement of CL using perfused rat heart or mouse liver

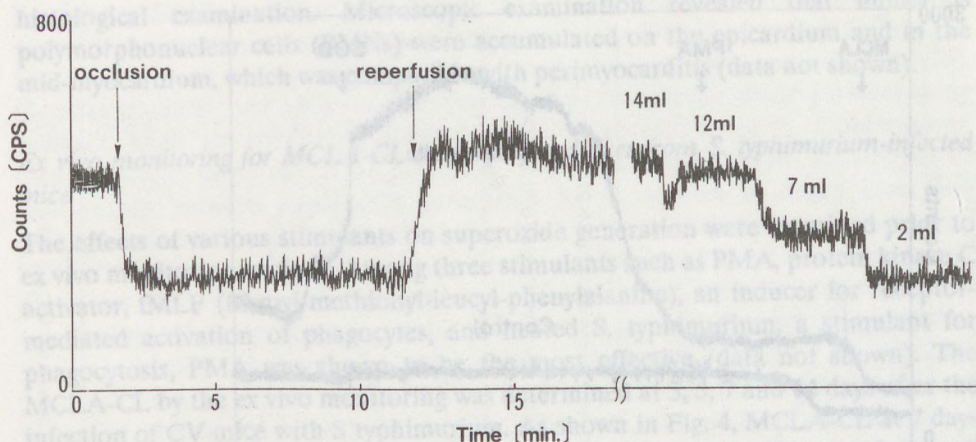


Figure 2. Lucigenin-CL using perfused rat heart. The heart isolated was occluded for induction of experimental ischemia (occlusion). After the occlusion of the heart for 10 min, the heart was reperfused with K-H buffer (reperfusion). Then, the perfusion pressure of coronary flow was decreased from 14 ml/min to 2 ml/min in a stepwise manner

to eliminate effluent CL since the perfusate must have high O_2 tension to maintain viability. It was also shown that MCLA did not produce CL through reactions with other substances myeloperoxidase, defferoxamine, hydrogen peroxide and tert-butylhydroperoxide (data not shown).

In the preliminary experiments for heat perfusion, decrease and increased lucigenin-CL were observed during occlusion of the artery (ischemia) and reperfusion, respectively (Fig. 2). In addition, the flow dependency of CL was examined. The photon counts decreased when the perfusion pressure was lowered and the coronary flow was decreased in a stepwise manner. These results suggest that decreased CL during ischemia was due to the absence of the CL probe (lucigenin) and the deficiency of oxygen.

*Induction of perimyocarditis by *S. enteritidis* and superoxide generation*

S. enteritidis grown on brain heart infusion agar plate was inoculated into a rat pericardial space through the diaphragm, resulting in an experimental perimyocarditis. Eight or 24 hr later, the heart was removed and used for estimation of superoxide generation. Photon counts of the hearts were monitored through the window in the control and *S. enteritidis*-infected heart (24 hr), before and after the addition of PMA (0.1 mg/min for 2 min) (Fig. 3). By addition of PMA, significant increase of MCLA-CL was induced to the level higher than 1500 cps which was SOD-inhibitable. On the other hand, no significant increase of MCLA-CL was observed in the control heart without *S. enteritidis* infection. It was shown that the maximum photon counts (2620 cps) detected from the heart removed 24 hr after the infection was significantly higher than that (1720 cps) 8 hr after the infection. At the end of ex vivo monitoring for MCLA-CL, the hearts were fixed with formalin for

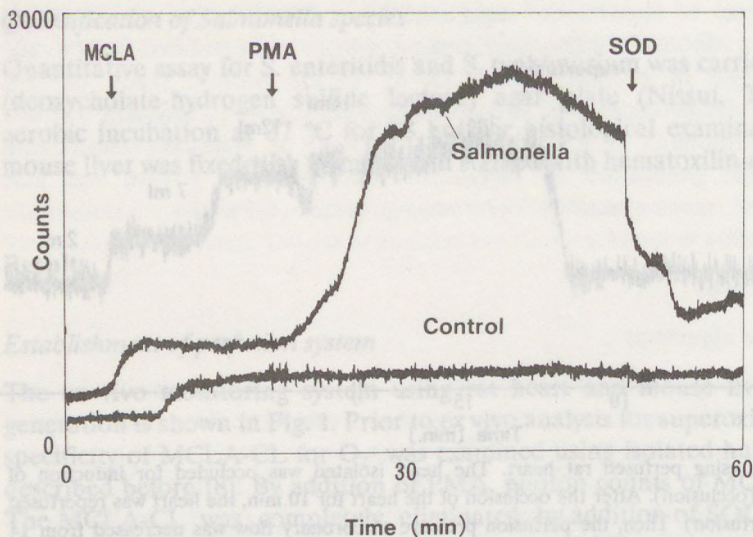


Figure 3. The time course of MCLA-CL detected from a control heart without *Salmonella enteritidis* infection and a heart 24 hr after the inoculation with *S. enteritidis*. The arrows in the figure indicate addition of MCLA ($5 \mu\text{M}$), PMA (0.1 mg/min for 2 min) and SOD (200 IU/min for 2 min)

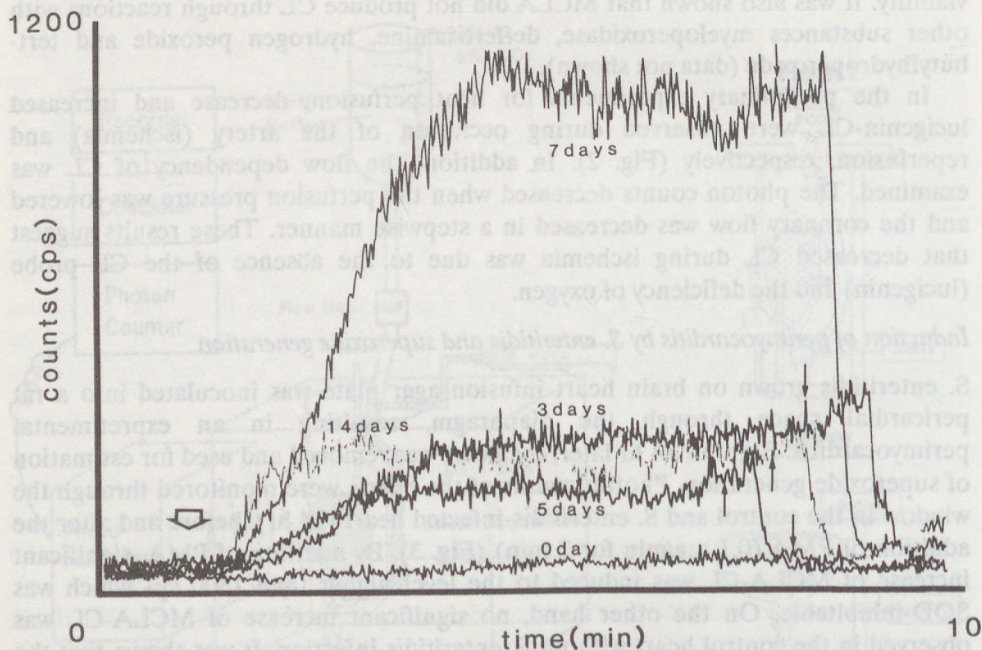


Figure 4. MCLA-CL detected from the perfused liver of the mice infected with *S. typhimurium*. At 0, 3, 5, 7 and 14 days after the infection, mouse liver was removed and used for ex vivo monitoring of MCLA-CL. ∇ , addition of PMA ($150 \mu\text{g}/1 \text{ ml/liver}$); $\downarrow$, addition of SOD ($1000 \text{ IU}/1 \text{ ml/liver}$)

histological examination. Microscopic examination revealed that numerous polymorphonuclear cells (PMNs) were accumulated on the epicardium and in the mid-myocardium, which was compatible with perimyocarditis (data not shown).

Ex vivo monitoring for MCLA-CL in the perfused liver from S. typhimurium-infected mice

The effects of various stimulants on superoxide generation were examined prior to ex vivo monitoring analysis. Among three stimulants such as PMA, protein kinase C activator, fMLP (formyl-methionyl-leucyl-phenylalanine), an inducer for receptor-mediated activation of phagocytes, and heated *S. typhimurium*, a stimulant for phagocytosis, PMA was shown to be the most effective (data not shown). The MCLA-CL by the ex vivo monitoring was determined at 3, 5, 7 and 14 days after the infection of CV mice with *S. typhimurium*. As shown in Fig. 4, MCLA-CL at 7 days after the infection was the maximum, reaching 1200 cps in comparison to 12 cps at 0 day control. The MCLA-CL was rapidly decreased to the control level by additional of SOD.

Relationship between superoxide generation by liver phagocytes and bacterial number recovered from the liver

Using both CV and GF mice, a relationship between superoxide generation and bacterial number recovered was examined (Fig. 5). In CV mice infected with *S. typhimurium*, the maximum bacterial number of 9.0×10^3 cfu/g liver was obtained at 3 days after the infection (Fig. 5A). In contrast, the maximum MCLA-CL was detected at 7 days after the infection. In GF mice infected with *S. typhimurium*, 1.2×10^5 cfu/g liver of the maximum bacterial number was obtained at 7 days after the infection (Fig. 5B). MCLA-CL was the maximum at 14 days after the infection. Histological examination for the liver after *S. typhimurium* infection revealed that severity of the granuloma formation was the maximum at 7 and 14 days after the infection for *S. typhimurium*-infected CV and GF mice, respectively (data not shown).

Discussion

In the present study, ex vivo monitoring system for superoxide generation by phagocytes using perfused organs was established. A cypridina analog, MCLA, and PMA were used as CL probe and a stimulant of superoxide generation, respectively. It was shown that MCLA-CL was completely eliminated by SOD, indicating MCLA to be a superoxide-specific CL probe.

In the ex vivo monitoring system using rat heart, Henry et al. [9] reported the time course of CL from perfused rat hearts during ischemia and reperfusion using lucigenin as the CL probe. In their study, the CL decreased during ischemia and markedly increased during reperfusion, and this increase was attenuated with SOD.

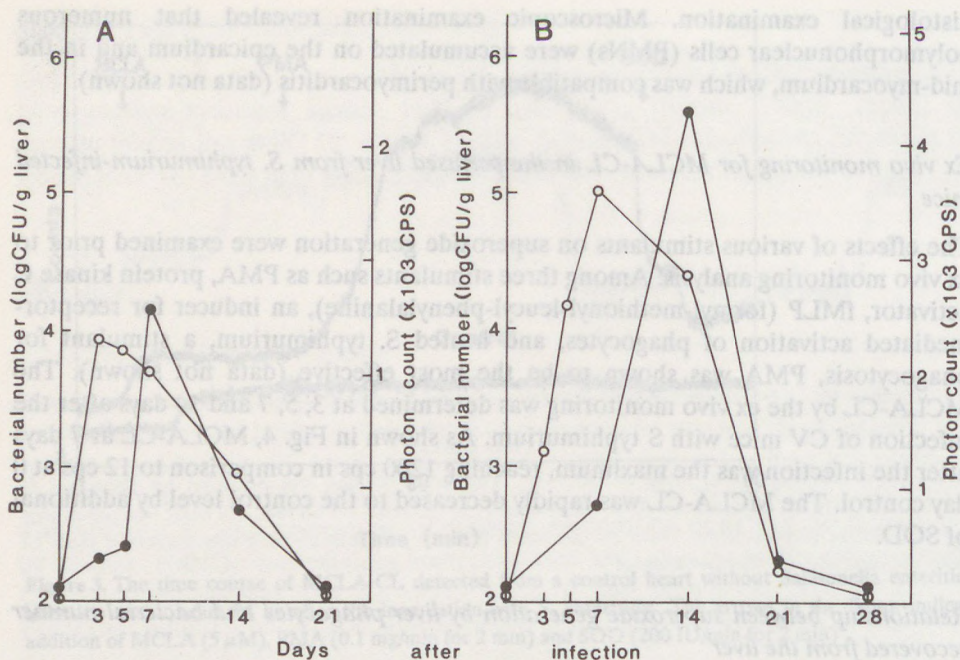


Figure 5. Relationship between MCLA-CL detected from liver surface and bacterial number recovered from the liver during *S. typhimurium* infection in CV (A) and GF (B) mice. O, cell number of *S. typhimurium*; ●, MCLA-CL after PMA stimulation.

It was assumed that the CL represented an acquisition of free radicals which were generated from the subepicardium. However, these observations should be evaluated with cautions since the CL method employed holds critical limitations, namely, the amount of oxygen and lucigenin are not constant during the occlusion and reperfusion process. It is possible that the reason for the lower CL during ischemia than that during control perfusion, is the absence of the CL probe and the deficiency of oxygen. The curve in Fig. 2 obtained in our laboratory shows a time course identical to that reported by Henry et al. [9]. It seems that the mechanism for the increase in CL is a consequence of the augmented flow after ischemia due to reactive hyperemia. It is also possible that lucigenin reacts with superoxide produced by the direct reaction between oxygen and lucigenin in the presence of a reductant such as glucose, contained in the perfusate, or a trace amount of some transition metals [10], in the myocardium and the effluent debris. Therefore, the CL obtained by the method of Henry et al. [9] might represent oxygen-related plus superoxide related CL which cannot be differentiated in a system in which the oxygen concentration changes.

The adequate stimulus of phagocytosis with generation of oxygen radicals in an invaded microorganism, and specific surface structure of microorganisms are recognizable as foreign [11-13]. It is also well known that muramyl dipeptide and

lipopolysaccharide are also stimulators of O_2^- formation in phagocytes. It was reported that treatment of neutrophil with either *S. typhimurium* or *S. typhimurium* LPS resulted in enhancement of generation of oxidative radicals [14, 15]. Significantly increased MCLA-CL was detected by ex vivo monitoring system using perfused heart or liver after infection with *S. enteritidis* or *S. typhimurium*, respectively, in this study. Both perfusion system seem to be useful for analysis of relationship between superoxide generation by phagocytes in organ level and pathogenesis by bacterial infection. Administration of nifedipine, Ca^{2+} -channel blocker, into mice prior to *S. typhimurium* infection induced both luminol-decreased CL by neutrophil and decreased intracellular killing of *S. typhimurium* by neutrophil [15], indicating that Ca^{2+} is a common regulator of neutrophil functions like superoxide generation, degranulation, chemotaxis effect. In contrast, administration of vitamin A into rats prior to *S. typhimurium* infection induced increased phagocytic activity of Kupffer cells and macrophages with decreased number of *S. typhimurium* isolated from liver and spleen [16], indicating a benefit of the host's response to infection by enhancing phagocytic cell function.

In the ex vivo system using perfused liver, it is not clear which cell population was responsible for the generation of superoxide. Nnalue et al [17] showed that most of the viable *S. typhimurium* recovered were associated with Kupffer cells prepared from rat liver after *S. typhimurium* infection. On the other hand, it was reported using perfused rat liver that PMNs and large Kupffer cells are predominantly responsible for the postischemic oxidant stress detected by increased superoxide generation [18].

In the present study, much more increased superoxide generation and decreased recovery of *S. typhimurium* were observed in GF mice than in CV mice. Billiar et al. [19] reported that much more strong response of Kupffer cells was demonstrated in CV mice than in GF mice, suggesting that intestinal bacterial overgrowth can modify Kupffer cell response to specific stimuli. The exact mechanism by which much more MCLA-CL was generated in GF mice than in CV mice after *S. typhimurium* infection remains to be determined.

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Cytotoxic Effect of Superoxide and its Interaction with Nitric Oxide in Reperfusion Injury

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Abstract

Superoxide ($\cdot\text{O}_2^-$) is known as the prime active oxygen which is generated upon reperfusion after an ischemic insult. Current interest of roles of $\cdot\text{O}_2^-$ involves the reaction to nitric oxide (NO), known as EDRF, since $\cdot\text{O}_2^-$ reacts with NO at diffusion-limited rate; thereby reduces NO but simultaneously yields its reaction product peroxynitrite (ONOO $\cdot$), a newly recognized molecule with important biological significance. This study showed 1. the generation of $\cdot\text{O}_2^-$ upon reperfusion, 2. the protective effect of $\cdot\text{O}_2^-$ scavenger, superoxide dismutase (SOD), against reperfusion injury and the effective delivery of exogenously administered SOD to the myocardium, 3. the demonstration of rapid quenching of $\cdot\text{O}_2^-$ by NO, production of ONOO $\cdot$ through the reaction of $\cdot\text{O}_2^-$ and NO, and NO-induced inactivation of xanthine oxidase, and 4. the injurious effect of ONOO $\cdot$ on cultured myocytes.

Introduction

Superoxide has been known as the most important free radical species in ischemia/reperfusion injury [1-3]. However the direct participation of superoxide ($\cdot\text{O}_2^-$) to myocardial injury can not be accounted since it has a limited reactivity with most of biological molecules. Therefore the secondary product, hydroxyl radical ($\cdot\text{OH}$) which is a far-more reactive species is proposed as an actual cytotoxic species. Formation pathway of $\cdot\text{OH}$ from $\cdot\text{O}_2^-$ is dismutation of $\cdot\text{O}_2^-$ to H_2O_2 with properly chelated transition metal (Haber-Weiss reaction). This $\cdot\text{O}_2^-$ -theory raises questions about the effect of SOD against $\cdot\text{O}_2^-$ -related damage since SOD increase the rate of H_2O_2 production from $\cdot\text{O}_2^-$. Thus, other mechanism(s) is/are necessary to explain the reason why the elimination of $\cdot\text{O}_2^-$ by SOD reduces the myocardial damage.

Recent advances in studies of endothelium-derived relaxing factor (EDRF) demonstrated that major from of EDRF activity is nitric oxide (NO) and that NO is released not only from endothelial cells but also from activated neutrophils and

macrophages [4-6]. In addition, it is shown that these NO producing cells can produce $\cdot\text{O}_2^-$ concomitantly. $\cdot\text{O}_2^-$ and NO react very rapidly with radical-radical reaction to form peroxynitrite (ONOO^-), which is a potent oxidizing agent shown to initiate lipid peroxidation and sulfhydryl oxidation. Thus the cytotoxic effect of $\cdot\text{O}_2^-$ has to be evaluated with a conjunction of the presence of NO and ONOO^- [7-9].

This article shows the cytotoxicity of $\cdot\text{O}_2^-$ in reperfusion injury and its possible interaction with NO using substantial data which have been performed in our laboratory in these 7 years. For these purpose we presented 1) the generation of $\cdot\text{O}_2^-$ upon reperfusion in the isolated perfused rat heart using ESR spin-trapping method, 2) the protective effect of $\cdot\text{O}_2^-$ SOD against reperfusion injury, 3) the demonstration of rapid quenching of $\cdot\text{O}_2^-$ by NO, production of ONOO^- through the reaction of $\cdot\text{O}_2^-$ and NO, and NO-induced inactivation of xanthine oxidase, and 4) the injurious effect of ONOO^- on cultured myocytes.

1. Generation of superoxide [10]

Method

Using anesthetized male-Wistar rats the hearts were excised and perfused from the aorta at 37 °C with physiological Krebs-Henseleit bicarbonate buffer containing 5.5 mM glucose, 5.5 mM pyruvate, 118 mM NaCl, 4.7 mM KCL, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 and 25 mM NaHCO_3 . The perfusate was aerated with a 95% O_2 /5% CO_2 to maintain a pH of 7.4; perfusion pressure was kept at 80 mmHg. Cardiac function was assessed through a latex balloon inserted into the left ventricle and left ventricular pressure was continuously monitored. Global ischemia was carried out by occluding the retro-perfusion line for 30 min and reperfusion was initiated by opening it. The spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), of 10 mM which passed through an ion-exchange column a Chelex-100 (Bio-Rad, Richmond, CA) was infused at a rate of 1.1 ml/min starting 30 sec prior to reperfusion and was continued for an additional 4 minutes. The experimental room was kept in darkness throughout the study to prevent photolytic degradation of DMPO. Coronary effluents were collected at specific intervals for ESR measurements and for enzyme release analyses. Creatine kinase (CPK), glutamic-oxaloacetic transaminase (GOT) and lactic dehydrogenase (LDH) activities were determined. For ESR measurements, in the presence of DMPO each collected coronary effluent was immediately frozen in liquid N_2 to prevent spin adduct decay and thawed just before their ESR spectra were obtained. ESR measurements were made on a JEOL (JES-FE 2X6, Tokyo, Japan) using a special quartz flat cells (volume 10 microliter). Condition of ESR spectrometry were as follows: magnetic field 335 ± 5 mT, power 0.8 mW, response 0.3 seconds, modulation 0.125 mT, temperature 23 ± 1 °C, amplitude 6.5×10^3 , sweep time 2 minutes, using a magnetic field modulation frequency of 1000 kHz. Hyperfine coupling constants were measured directly from the field scan using a manganese marker signal for calibration. DMPO was obtained from Shonan Analytic Center (Tokyo, Japan).

Results

Figure 1 shows a representative ESR signals obtained from effluents collected immediately after reperfusion. This poorly resolved spectrum were identified as three different ESR signals which overlap. By the best-resembling spectral simulation these are identified as DMPO-OOH adduct with hyperfine splitting constant of $a_N = 1.43$, $a_H = 1.15$, $a_H = 0.13$ mT, the spectrum of a 1:2:2:1 quartet with hyperfine coupling constants $a_N = a_H = 1.49$ mT attributable to DMPO-OH and a triplet with a hyperfine coupling constants $a_N = 1.9$ mT which is attributed to a nitroxide. This nitroxide is shown to be a decomposition product of DMPO in the preliminary study. The DMPO-OOH and DMPO-OH adducts could not be observed in effluents collected beyond 1 min after reperfusion. The formation of two radical adducts (DMPO-OOH and DMPO-OH) was completely inhibited by SOD. This indicates that peak generation of $\cdot O_2$ in the tissue is at the first minute of re-oxygenation.

Figure 2 illustrates the effect of DMPO on the developed pressure during ischemia and reperfusion. Arrhythmias were induced by reperfusion in both groups, but arrhythmias subsided more quickly in the heart with DMPO infusion and the recovery of the pressure was higher in the heart with DMPO infusion than in that without DMPO. Figure 3 illustrates the time course of the developed pressure of 10 hearts after 40 min of reperfusion. The LV developed pressure remained low (14% of the control value) in hearts without DMPO, but relatively high in hearts with DMPO (39%). Particularly significantly higher values were maintained until 4.5 min of reperfusion which coincided with the duration of DMPO infusion period. After that period, LV developed pressure in the DMPO group became similar to that without DMPO. Total values released enzymes (CPK, GOT and LDH) in the presence or absence of DMPO were 90 ± 13 , 64 ± 14 and 8.4 ± 1.4 vs 82 ± 12 , $63 \pm$

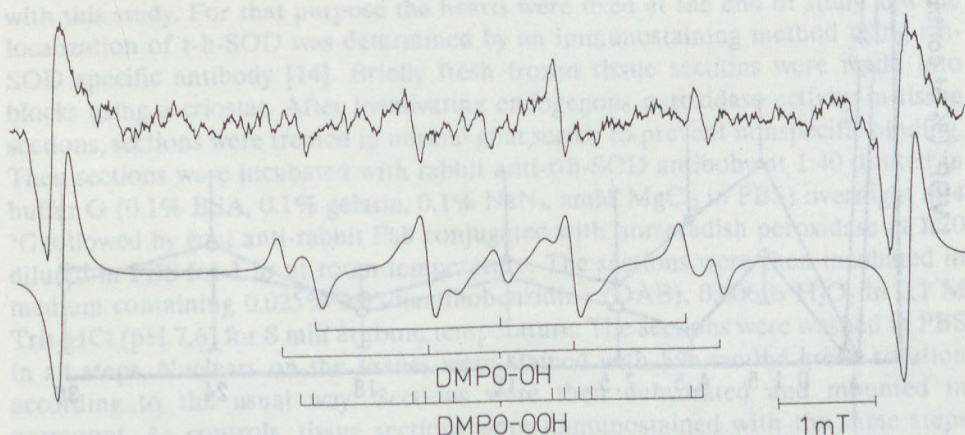
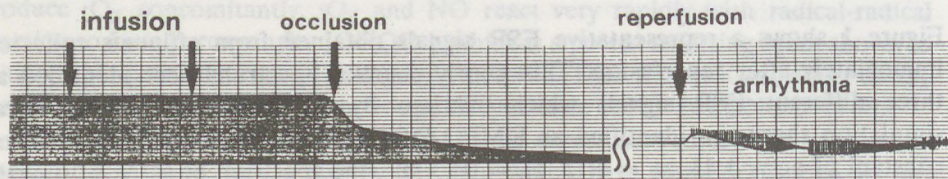


Figure 1. ESR spectrum of DMPO adducts in the effluent immediately after reperfusion

Mn: internal standard of MnO

DMPO (-)



DMPO (+)

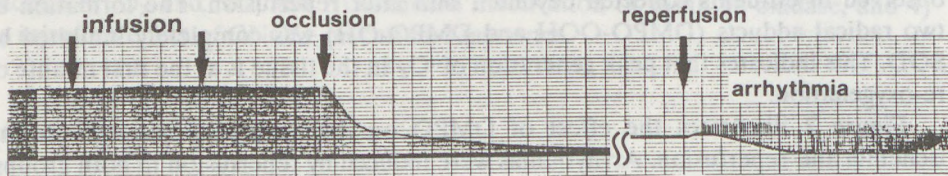


Figure 2. Record of the left ventricular pressure in the presence or absence of DMPO. DMPO: 10mM

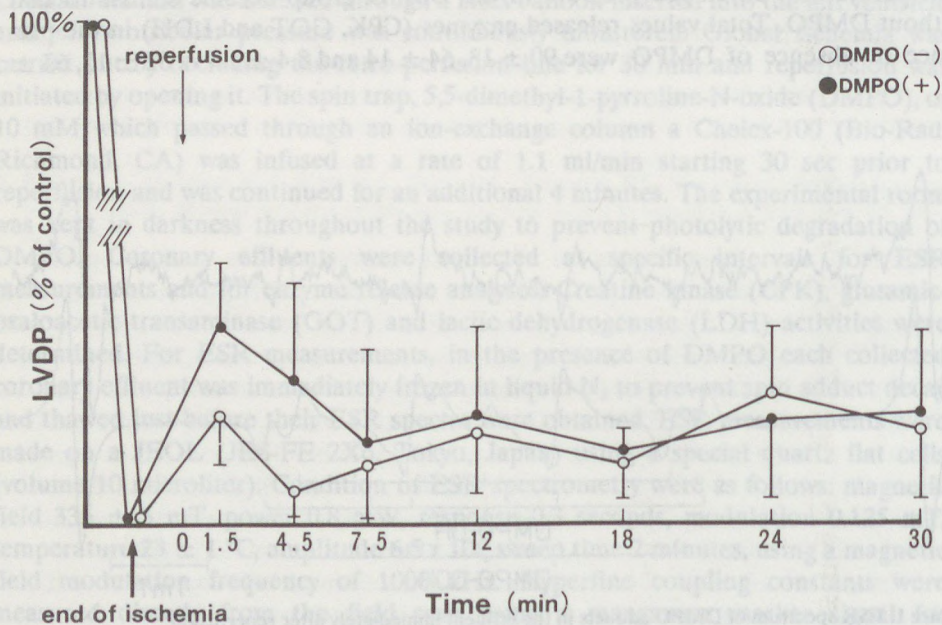


Figure 3. Left ventricular developed pressure after reperfusion. DMPO: 10mM

15 and $8.3 \pm 1.3 \times 10^4$ units/g wet wt). There was no significant difference in these values.

In 1987, one of our co-worker reported that high concentration of DMPO (100 mM) was essential in order to identify biologically generated $\cdot O_2^-$ in ischemia/reperfusion model [11]. However the possible cytotoxicity of DMPO and artifactual generation of $\cdot O_2^-$ by damaged tissue was claimed by Davies et al. [12]. Thus, we used low concentration (10 mM) of DMPO and we were able to show even low concentration DMPO can spin trap $\cdot O_2^-$ and related free radicals. In addition, we proved that DMPO in the concentration range of 10 mM did not produce significant functional damage or enzyme leakage in preliminary experiments. An explanation of the inability to show the protective effect of DMPO against reperfusion injury in this study might be that the effect of $\cdot O_2^-$ elimination by DMPO has been compromised by the toxic effect of DMPO even at this low concentration range.

2. Protective effect of SOD

Method

In experiment to demonstrate protective effect of SOD, we used hearts in a working heart preparation. In order to maintain the stable condition during an ischemic insult the heart was paced at 300 beats/min and regional ischemia model was utilized by occluding the left coronary artery instead of global ischemia model. Occlusion time varied from 5, 7, 15 to 60 min and reperfusion was followed for 30 min. Recombinant human-CuZn-SOD (r-h-SOD) was infused from 5 min prior to reperfusion. Cardiac output was used as the index of cardiac function and was expressed as the percent change of the pre-ischemic value. Since SOD was claimed not to enter tissue due to its relatively large molecular size (MW 31863), the distribution of exogenously administered SOD were also investigated in conjunction with this study. For that purpose the hearts were fixed at the end of study and the localization of r-h-SOD was determined by an immunostaining method using r-h-SOD specific antibody [14]. Briefly fresh frozen tissue sections were made into blocks using a criostat. After inactivating endogenous peroxidase activity in tissue sections, sections were treated in normal goat serum to prevent nonspecific binding. Then sections were incubated with rabbit anti-r-h-SOD antibody at 1:40 diluted in buffer G (0.1% BSA, 0.1% gelatin, 0.1% $NaNO_3$, mM $MgCl_2$ in PBS) overnight at 4 °C followed by goat anti-rabbit Fab conjugated with horseradish peroxidase at 1:20 diluted in PBS for 1 hr at room temperature. The sections were then incubated in medium containing 0.025% 3,3'-diaminobenzidine (DAB), 0.006% H_2O_2 in 0.1 M Tris-HCl (pH 7.6) for 8 min at room temperature. The sections were washed in PBS in all steps. Nuclears on the tissues were stained with 5% methyl green solution according to the usual way. Sections were then dehydrated and mounted in permount. As controls, tissue sections were immunostained with the same steps expect for the replacement of the rabbit anti-Cu-Zn-SOD with normal rabbit serum which didn't have anti-r-h-SOD antibody.

Result

The changes in percent cardiac output in groups with a 15 min ischemic period and 60 min ischemic period followed by reperfusion are shown in Fig. 4a and 4b. During ischemia, there were no significant differences in percent cardiac output between non-treatment and SOD-treatment groups. At 30 min of reperfusion in the 15 min-ischemia protocol, cardiac output in the SOD-treatment group showed a tendency

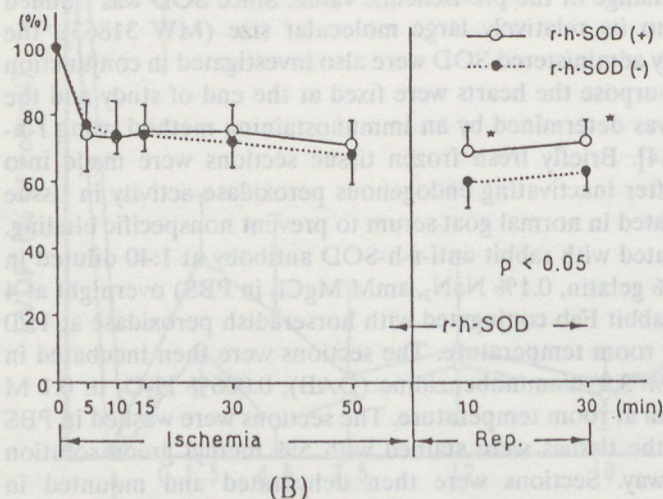
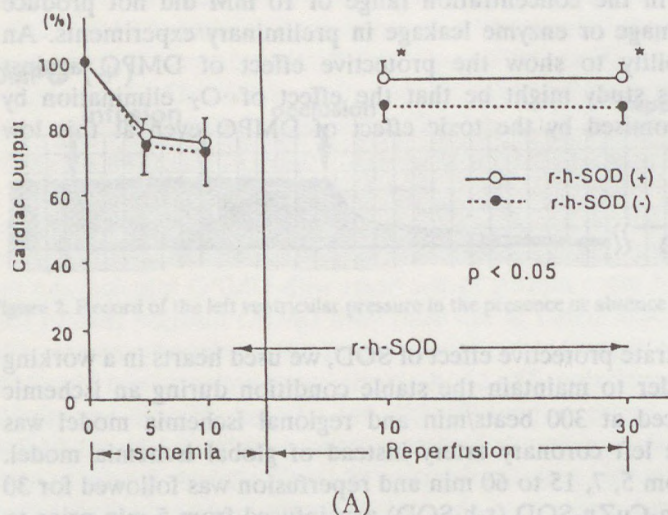


Figure 4. Cardiac output during ischemia-reperfusion period.

A) 15-min ischemia-reperfusion group;

B) 60-min ischemia-reperfusion group

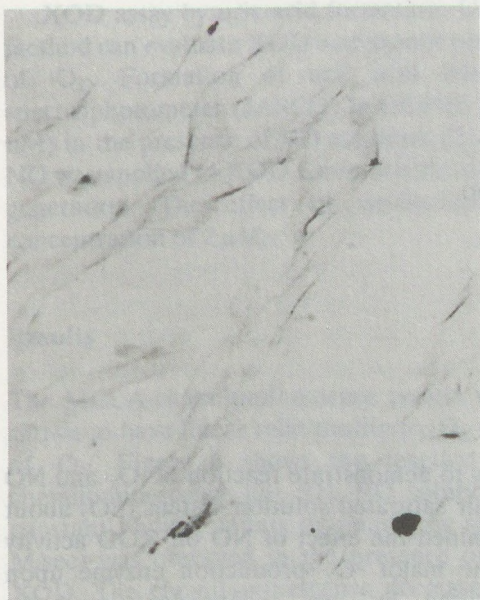


Figure 5. Light micrograph of non-ischemic region. X 200. The stained tissue depicts the presence of r-h-SOD

toward a higher value ($90 \pm 4\%$ vs $86 \pm 5\%$). In the 60 min-ischemia protocol, the percent cardiac output was apparently higher in the SOD-treatment group than in the non-treatment group ($70 \pm 7\%$ vs $60 \pm 8\%$) at 10 min reperfusion, and $74 \pm 7\%$ vs $63 \pm 6\%$, $p < 0.01$ at 30 min reperfusion. These results showed that SOD can effectively protect cardiac function against reperfusion injury.

The study to determine whether exogenously administered SOD can reach myocardium revealed the followings. In all areas of the control hearts in which no ischemia was produced, the r-h-SOD could be observed on the surface of the myocardium (Fig. 5). The myocyte were clearly demarcated by immunoperoxidase-indirect staining of SOD. By electron microscopy, electron dense compounds which are compatible with SOD-antibody conjugate were seen along the sarcolemma and in the endothelial cells. These results indicated that r-h-SOD can across the endothelial cells and reach the interstitial space and the surface of sarcolemma.

3. Interaction between superoxide and nitric oxide - production of ONOO- [20, 21]

In conditions where NO is simultaneously generated with $\cdot O_2$, $\cdot O_2$ decreases but simultaneously peroxynitrite (ONOO-) is produced (Table 1). ONOO- is unstable with a half-life of 1.9 sec at pH 7.4 [8] and decomposes to give hydroxyl radical and nitrogen oxide. In the presence of oxygen, NO also reacts with oxygen, yielding NO_2 , therefore influence of oxygen concentration has to be counted in the reactions. For example the rate of $\cdot O_2$ scavenging effect of NO depends the concentration of

Table 1. Reactions of NO, $\cdot\text{O}_2^-$ and O_2

$\text{NO} + 1/2 \text{O}_2$	$\rightarrow$	NO_2 ($\tau_{\text{NO}} = 6 \text{ sec}$)
$\text{NO} + \cdot\text{O}_2^-$	$\rightarrow$	ONOO^- ($3.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$)
$\text{ONOO}^- + \text{H}^+$	$\rightarrow$	ONOOH (diffusion constant)
ONOOH	$\rightarrow$	$\cdot\text{OH} + \text{NO}_2$ (1.4 s^{-1}) 20-30%
		self $\text{NO}_3^- + \text{H}^+$ rearrangement

oxygen. Thus, we first performed the study to demonstrate reaction of $\cdot\text{O}_2^-$ and NO by using chemiluminescence method in air saturated solution system ($p\text{O}_2$ about 150 mmHg). In the second study we examined the effect of NO on XOD activity since XOD is known [15] as one of the major $\cdot\text{O}_2^-$ production enzyme upon reperfusion. XOD activity was measured by two independent methods; chemiluminescence method and uric acid formation method.

Materials and methods

Saturated NO solution was made by the method of Feelish [16]. Briefly, N_2 was bubbled in 10 mM HEPES buffer (pH 7.4 NaOH) for 20 min to remove dissolved O_2 . Then authentic NO gas (99%, Nihon Sanso) that had passed through 1 M KOH to remove nitrogen dioxide (NO_2) was bubbled for 20 min. pH was checked to ascertain the complete removal of NO_2 since trace of NO_2 leads to low pH [16]. Saturated NO solution was diluted by N_2 -flushed HEPES buffer to desired concentration. Actual NO concentration of each solution was determined by the difference-spectrophotometric method [16, 17] based on NO-induced oxidation of oxyhemoglobin to methemoglobin. ONOO^- was synthesized by the reaction of acidified H_2O_2 and NaNO_2 with a subsequent stabilization by 1.5 M NaOH in a quenched flow reactor [18].

XOD assay by $\cdot\text{O}_2^-$ generation using the chemiluminescence method; chemiluminescence assay were conducted on a C1230 photon counter with a R550 photomultiplier using the Petri dish (45 mm diameter). Cypridina luciferin analog, MCLA, which has been known as the most sensitive and selective chemoluminescent probe for $\cdot\text{O}_2^-$ was used as a chemiluminescence probe [19]. Light emission per second was counted in the presence of MCLA (1 μM) XOD (335 pM) and xanthine (50 μM in 3.95 ml HEPES buffer). The rate of $\cdot\text{O}_2^-$ generation in this condition was determined as 285 pM/sec by comparing the value obtained by cytochrome c method [20, 22]. NO solutions (12.5 nM-1250 nM) were added by gas-tight syringe.

XOD assay by uric acid formation: Unlike the assay using $\cdot\text{O}_2^-$ generation, this method can evaluate XOD activity not being affected by the NO-induced quenching of $\cdot\text{O}_2^-$. Formation of uric acid was monitored at 295 nm by Ubest-50 spectrophotometer (JASCO) in HEPES buffer, xanthine (150 μM) and XOD (46.1 nM) in the presence of NO solutions (2.2-203 μM). To study the effect of ONOO $^-$, NO was applied to XOD 5 min before adding xanthine, prior to the initiation of $\cdot\text{O}_2^-$ generation. The effect of synthesized ONOO $^-$ was also examined at the concentration of 2 μM .

Results

The MCLA-chemiluminescence system which was utilized in this experiment was shown to have linear relationship to $\cdot\text{O}_2^-$ concentration and can detect 10^{-11} M order of $\cdot\text{O}_2^-$. Figure 6 shows the reaction of $\cdot\text{O}_2^-$ and NO using $\cdot\text{O}_2^-$ -dependent chemiluminescence [20, 21]. NO solution was added in the Petri dish while the constant photon counts originated by steady state generation of $\cdot\text{O}_2^-$ (4.25×10^{-6} M/sec) was observed in the presence of excess amount of xanthine and 335 pM XOD. The chemiluminescence decreased abruptly by NO addition but gradually recovered with an overshoot, and then returned to previous counts. The recovery

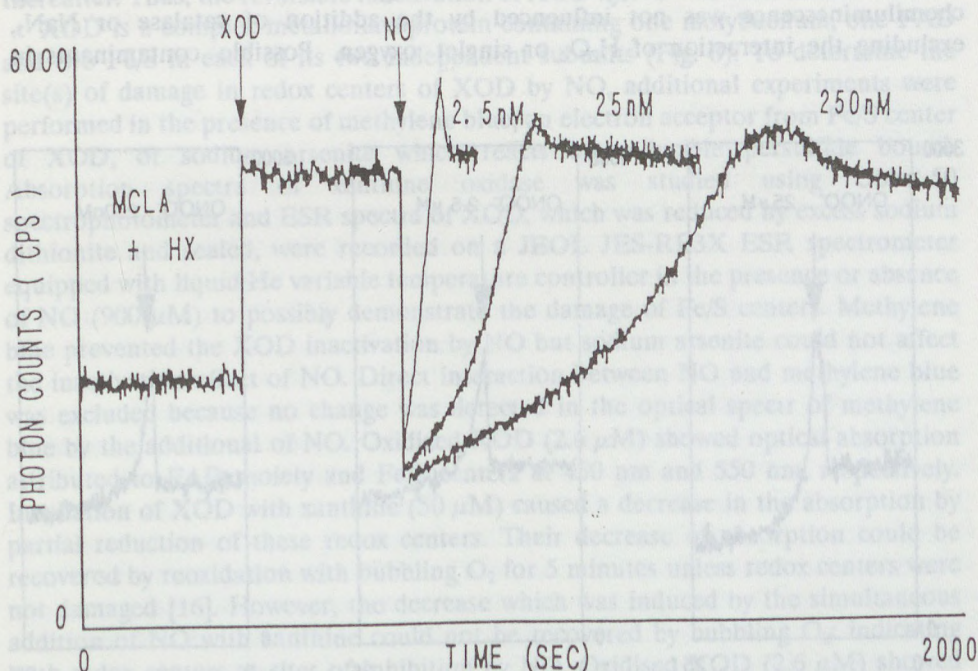


Figure 6. The MCLA-chemiluminescence in the reaction of NO and $\cdot\text{O}_2^-$. NO concentrations are denoted in figure

time showed a dose-dependent prolongation. Catalase (70 ng/ml), NO_2^- (100 μM) had no effect on the chemiluminescence; excluding the effect of dismutation product of O_2^- and decomposition products of NO in the reaction. This chemiluminescence time course suggested following three phenomena: firstly, the immediate and abrupt decrease is attributed to the direct quenching of $\cdot\text{O}_2^-$ by NO. Secondly, the overshoot after the initial decrease can be a reflection of ONOO $^-$ -dependent chemiluminescence. Thirdly, the prolonged attenuation which lasted beyond the half-life of NO (6 sec) and which can not be explained by the quenching effect of NO may due to that the interference of NO on $\cdot\text{O}_2^-$ -generating system i.e. XOD/xanthine system. The decomposition of MCLA by NO could be denied as the mechanism for the decrease because NO did not destroy MCLA at the concentration lower than 2 μM (data not shown) and the photon counts returned to the initial counts thereafter (in Fig. 6). Therefore it may implicate that the transient reduction in generation of $\cdot\text{O}_2^-$ occurred by the reversible suppression of XOD activity by NO.

Taken together with recent study which demonstrated the NO-induced suppression of biologically important enzymes [23], we performed the experiment to prove our assumption, NO-induced temporarily inactivation of XOD. To prove the second assumption, ONOO $^-$ solution (50 μl -200 μl , 2.3 mM) in 5 ml 1 M NaOH was mixed with 2 μM MCLA in a reaction chamber of stopped flow system (Fig. 7). ONOO $^-$ produce high photon counts which showed a dose-dependent increase. The chemiluminescence yield appeared to be higher than that of $\cdot\text{O}_2^-$. This chemiluminescence was not influenced by the addition of catalase or NaN_3 , excluding the interaction of H_2O_2 or singlet oxygen. Possible contaminants in

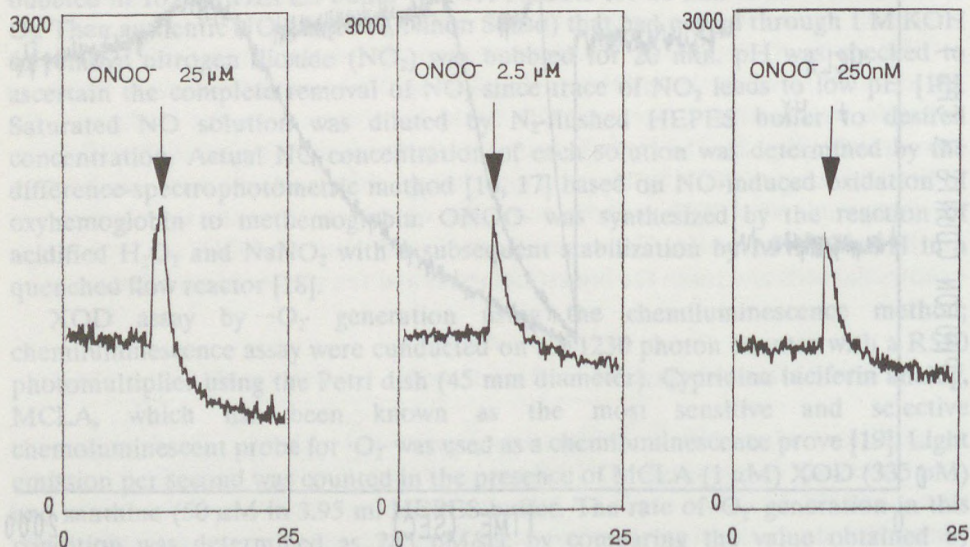
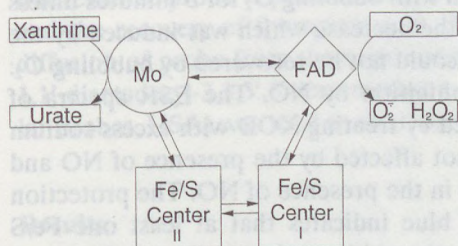


Figure 7. Transient MCLA-chemiluminescence by ONOO $^-$. ONOO $^-$ concentrations are denoted in the figure

Xanthine Oxidase

Figure 8. The active centers of xanthine oxidase



ONOO⁻ solution, such as NO₂⁻, NO₃⁻, NaOH and H₂O₂ showed no chemiluminescence when added alone. Therefore, we concluded that the observed chemiluminescence is exclusively due to ONOO⁻ or ONOO⁻-related substance.

To investigate the third assumption of transient inactivation of XOD by NO, we utilized another method for the measurement of XOD activity beside MCLA chemiluminescence method i.e. uric acid formation method which is free of the quenching effect of $\cdot\text{O}_2$ by NO. NO suppressed acid formation by XOD in a dose-dependent fashion. The suppression was transient i.e. the time-course of decreased uric acid formation demonstrated a nadir at 4 min with a gradual increase thereafter. Thus, the reversible inactivation of XOD by NO was established.

XOD is a complex metalloflavoprotein containing one molybdenum, one FAD and two Fe/S in each of its two independent subunits (Fig. 8). To determine the site(s) of damage in redox centers of XOD by NO, additional experiments were performed in the presence of methylene blue, an electron acceptor from Fe/S center of XOD, or sodium arsenite which reacts on reducible persulfide bound. Absorption spectra of xanthine oxidase was studied using Ubest-50 spectrophotometer and ESR spectra of XOD, which was reduced by excess sodium dithionite and sealed, were recorded on a JEOL JES-RE3X ESR spectrometer equipped with liquid He variable temperature controller in the presence or absence of NO (900 μM) to possibly demonstrate the damage of Fe/S centers. Methylene blue prevented the XOD inactivation by NO but sodium arsenite could not affect the inactivation effect of NO. Direct interaction between NO and methylene blue was excluded because no change was detected in the optical spectr of methylene blue by the additional of NO. Oxidized XOD (2.6 μM) showed optical absorption attributed to FAD moiety and Fe/S centers at 450 nm and 550 nm, respectively. Incubation of XOD with xanthine (50 μM) caused a decrease in the absorption by partial reduction of these redox centers. Their decrease of absorption could be recovered by reoxidation with bubbling O₂ for 5 minutes unless redox centers were not damaged [16]. However, the decrease which was induced by the simultaneous addition of NO with xanthine could not be recovered by bubbling O₂: indicating both redox centers as sites of inhibition by NO. Oxidised XOD (2.6 μM) showed optical absorption attributed to FAD moiety and Fe/S centers at 450 nm and 550 nm, respectively [15]. Incubation of XOD with xanthine (50 μM) caused a decrease

in the absorption by partial reduction of these redox centers. This decrease of absorption could be recovered by reoxidation with bubbling O_2 for 5 minutes unless redox centers were not damaged. However, the decrease which was induced by the simultaneous addition of NO with xanthine could not be recovered by bubbling O_2 ; indicating both redox centers as sites of inhibition by NO. The ESR spectra of reduced FAD and Fe/S centers were obtained by treating XOD with excess sodium dithionite. The spectra characteristic were not affected by the presence of NO and iron-nitrosyl complex could not be observed in the presence of NO. The protection against NO-induced damage by methylene blue indicates that at least one Fe/S center is spared from the damage. Taken together with persisted spectra of reduced FAD and Fe/S even after bubbling O_2 , the damage of Fe/S center and FAD are suggested although these studies using optical spectra and ESR spectra of XOD did not directly clarify damaged site(s). Although it remains to be clarified the detailed chemical mechanisms underlying the NO-induced inactivation and the conclusive evidence regarding to site(s) of damage in redox centers, the present observation demonstrated a new function of NO in O_2 -related injury.

4. Effect of ONOO⁻ on monocytes

In this series of experiments we used spontaneously contracting cultured myocytes of fetal mice hearts.

Methods

Cardiac cells in monolayer were prepared from mouse embryo hearts (15 to 20 ventricles of 17- to 19-day-old mouse embryos). The myocytes were diluted to 1×10^6 cells/ml and placed in plastic petri dishes containing 25 mm circular glass cover slips and incubated in a humidified 5% CO_2 -95% O_2 air at 37 °C for 2 days before use. A glass strip with adherent cells was placed in the chamber which was covered by a plastic box to maintain an atmosphere temperature of 37 °C. The contractile state of the myocytes was assessed by the use of a phase-contrast microscopic video motion detector system, using 40x objective lens, the image of cells was recorded by a low-light-level TV camera attached to the observation tube of the microscope using a 1x lens [24]. $[Ca^{2+}]_i$ was monitored by the use of the fluorescent calcium indicator, Calcium Green-1 after loading 10 μM in acetoxymethyl ester form. The fluorescent signal was measured with a fluorescence spectrophotometer (CAM 200 Japan Spectroscopic, Japan) attached to the microscope using a 40x fluor-objective lens. The excitation wavelength was obtained using a 75-W xenon lamp with a band-pass filter of 488 nm. The fluorescence was collected by the objective lens and passed to a photomultiplier tube through a band-pass filter of 530 nm. The fluorescence intensity was used to estimate changes in $[Ca^{2+}]_i$.

Membrane fluidity was evaluated by measuring the lateral diffusion coefficient using the method of fluorescence recovery after photobleaching (FRAP). The

principle of the FRAP method is that a uniformly labeled membrane with a fluorescence probe is photobleached in a specific spot by an intensified laser beam and the recovery of fluorescence at that spot is monitored to measure the lateral diffusion of probe from the surrounding membrane into the spot. The lipid analog 1,1'-diactadecyl-3,3,3',3'-tertamethylin-dodicarbocyanine perchlorate (DiI, Molecular Probes, USA) was used as the fluorescent probe.

Results

On exposure to 1 mM ONOO⁻, the spontaneous contraction of myocytes weakened at 3 min and rapidly fell into a complete arrest at 5 min. The arrested state was determined to be in diastole, which is an unusual phenomenon since arrested contraction induced by most of cytotoxic agents was associated with contracture of myocytes, namely systolic arrest. The concomitant measurement of intracellular Ca²⁺ ([Ca²⁺]_i) demonstrated that the increase in [Ca²⁺]_i coincided with the time when contraction started to decrease and marked elevation in [Ca²⁺]_i occurred at 5 min. Membrane fluidity was also measured concomitantly with contractility experiments and [Ca²⁺]_i measurement. Unlike other lipid peroxidation inducing agents, ONOO⁻ did not alter membrane fluidity at 5 min after its addition. Morphological evaluation by microscopy revealed that myocytes remained in adhered confluent shape, i.e. normal shape, and not rounded in shape even with a markedly increased [Ca²⁺]_i. NO itself, NO₂⁻, or NO₃⁻ did not produce any changes on myocytes when these were added at the same concentration (1 mM).

This myocytes study is still a preliminary experiment and more detailed experiments are necessary to clarify the underlying mechanism(s) for the ONOO⁻-induced myocytes arrest. However, the cytotoxic effect of ONOO⁻ became evident. Thus previous experimental results which demonstrated ·O₂⁻-related injury should be re-evaluated in the light of interaction of ·O₂⁻ and NO.

Conclusion

A tremendous work in past two decades has demonstrated that ·O₂⁻ plays the major role in ischemia/reperfusion injury. However ·O₂⁻ can not be a direct attacker of lipid membrane or proteins since ·O₂⁻ has a limited reactivity with most biological molecules despite of its excellent effect on reperfusion injury [1-3, 25]. The theory of Haber-Weiss reaction is not fully satisfactory to explain the role of ·O₂⁻ as a cytotoxic reaction. In successive experiments performed in our laboratory also suggested that other reactions may be important in understanding ·O₂⁻ toxicity to myocardium. We are convinced that further study on the reaction of ·O₂⁻ and NO can clarify the precise mechanism for that long-unsolved questions in active oxygen-related injury. The double edged role of NO in reperfusion injury is summarized in table 2 as a summary.

Table 2. Double-edged sword of nitric oxide

Protect effects

- 1) Scavenging superoxide
- 2) Attenuation of leukocyte adhesion
- 3) Inhibition of platelet aggregation
- 4) Maintaining circulation (EDRF)
- 5) Inactivation of superoxide generating enzymes

Deleterious Effects

- 1) Formation of ONOO⁻
 - lipid peroxidation
 - attack -SH groups
 - react with transition metal nitronium ion (NO₂⁺)
- 2) Attack iron-sulfur center
- 3) Lipid peroxidation through NO₂

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A Background of Hepatic Hypoxia in Alcoholic Liver Disease: Effects of Anemia and Erythrocyte Abnormalities

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Abstract

This study was conducted to observe whether or not abnormalities of peripheral red blood cells may affect an underlying pathological condition of hepatic hypoxia and microcirculatory disturbance in alcoholic liver disease. According to the severity of anemia associated with the disease, disturbance of liver functions, such as serum concentrations of bilirubin and liver-producing proteins, progressed stepwise. Erythrocytes of these patients were not only morphologically enlarged, but also revealed an increased solidity, namely decreased cell deformability and membrane fluidity. This physical alteration was partly explained by a change of membrane lipid composition (a decrease of unsaturated/saturated fatty acid ratio of esterified phospholipids). It was suggested that these quantitative and qualitative abnormalities of erythrocytes may play an important role in an exacerbation of hepatic hypoxia due to microcirculatory disturbance and its consequent increase of oxyradical generation.

Introduction

A number of studies have been reported about evidences that the liver is one of the most active organs which generate and disposes various free radicals. Pathophysiological significance of those in the liver were reviewed in detail in the previous papers [1, 2]. In recent experimental studies, it was elucidated that hepatic hypoxia may play important role in promoting the production of oxygen radicals and consequently producing hepatocyte damage [3-6]. The presence of heterogeneity of the damage observed in these studies also strongly suggested that microcirculatory disturbance in the liver may be an important factor of the exacerbation of this damaging mechanism.

The significance of oxygen radical effects in human liver disease, particularly in alcoholic liver disease, was also indicated strongly by the finding that lipid peroxide

levels were increased in the liver and serum under the pathologic condition [7]. Furthermore, hemoglobin and oxygen concentrations were found to be markedly reduced in alcoholic liver disease when they were measured directly from the surface of the liver [8]. As a mechanism of these abnormalities, it is known that hepatic circulation is disturbed from early of alcoholic liver disease by hepatocyte swelling due to intracellular accumulation of fat and protein (ballooning) and collagen deposition along sinusoidal space [9, 10].

On the other hand, abnormalities of circulating blood may have to be taken into account as another factor of microcirculatory disturbance and hypoxia, since various hematological changes are frequently associated with the disease.

Anemia due to various origins, including hemorrhage, hemolysis and hemopoietic disturbances, is a common complication of alcoholic liver disease and eventually reduces oxygen transport to the liver. It will be also conceivable that morphological enlargement and increased solidity of erythrocytes, which were not uncommon in the disease, may disturb sinusoidal circulation, since the sinusoidal spaces (15-30 μm in width in normal liver) become narrower than the diameter of erythrocyte (7 μm) by histological changes of the liver described above.

In spite of the possibility that such mechanism may be speculated, effects of circulating blood upon hepatocytes and liver functions have not been studied well, except for the effect of acute hypovolemic shock.

The aim of our study to clarify the effects of chronic anemia and erythrocyte abnormalities upon the liver functions in alcoholic liver disease.

1. Effects of anemia on liver function tests

Fifty patients with alcoholic liver disease were classified according to the degree of anemia (hemoglobin concentrations, Hb) into three groups as indicated in Table 1.

Table 1. Clinical background of patients of alcoholic liver disease associated with different degrees of anemia

Group (Cases)	I (no anemia) (20)	II (moderate) (20)	III (severe) (10)
Hemoglobin (g/dl)	≥ 13	$13 > \text{Hb} > 10$	≤ 10
Male:Female	18:2	15:5	10:0
Causes of anemia			
Hemolysis		7	4
Bleeding		0	2
Hemopoietic		13	4
Liver histology			
Fatty liver	9	2	3
Fibrosis	5	6	0
Cirrhosis	6	8	5
Alc. hepatitis	0	4	2

CASE : Alcoholic Fatty Liver S.S. 41 y.o.

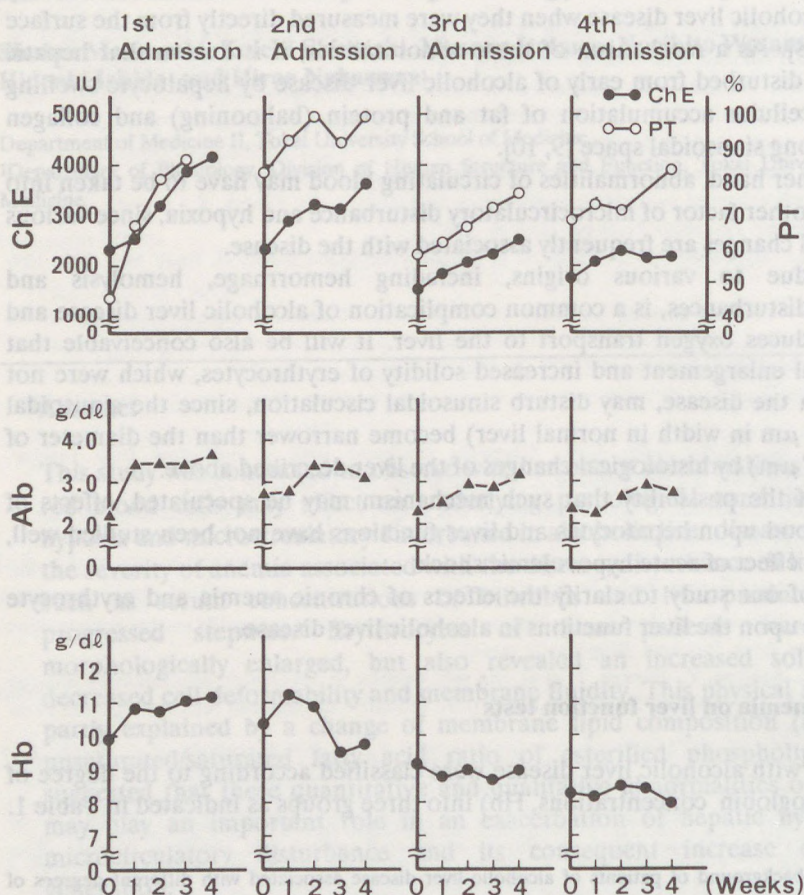


Figure 1. A clinical course of a patient with alcoholic fatty liver. Effects of repeated episodes of heavy drinking on liver functions and anemia

The origins of anemia and the histological diagnosis of the liver patients in respective groups were essentially similar.

Among them, a clinical course of a typical case, who revealed the effect of anemia on the liver function, was shown in Fig. 1. This forty one year male patient was admitted to our hospital four times during one year, because of severe exacerbations of his liver function by repeated heavy drinking. Every time, he developed marked anemia due to malnutrition and hemolysis, but it improved by medical treatment. Metabolic functions of the liver assessed by serum concentrations of liver-producing proteins, such as albumin (ALB), pseudocholinesterase (ChE) and prothrombin (PT), were found to be disturbed markedly on admission, but they recovered parallel to the improvement of hemoglobin (Hb).

However, by the repetition of the episodes, the degree of anemia and the

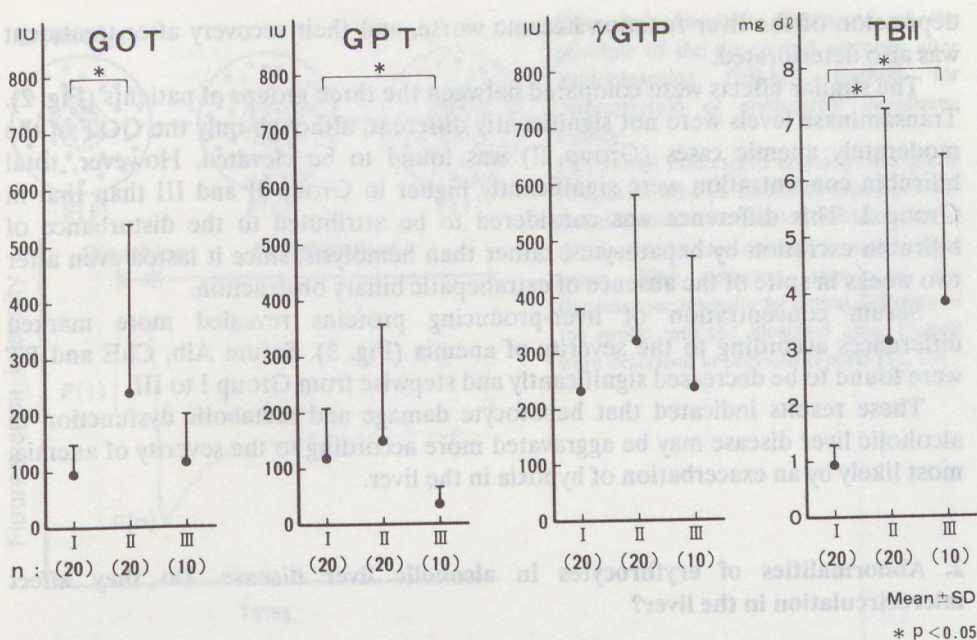


Figure 2. Effects of the severity of anemia on liver function tests in alcoholic liver disease

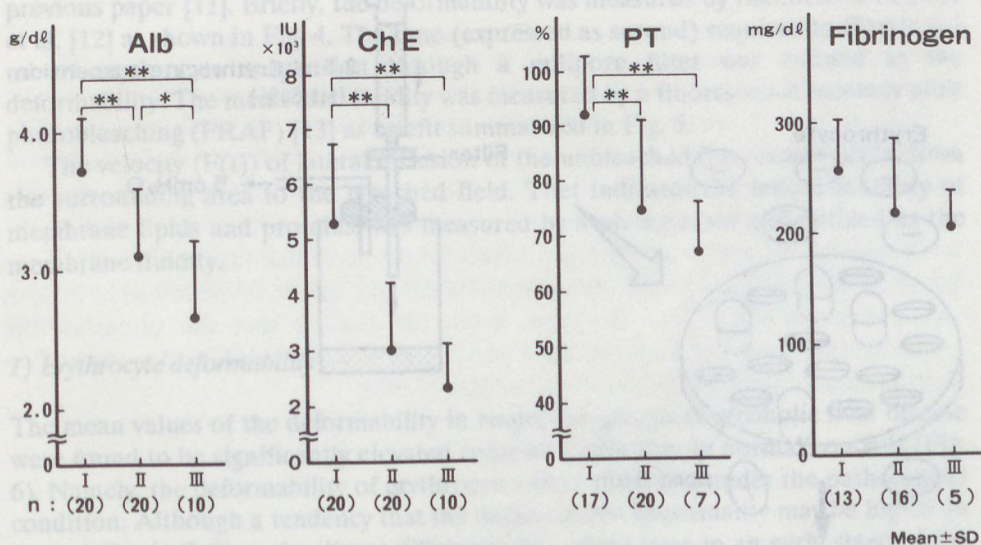


Figure 3. Effects of the severity of anemia on serum concentrations of liver-producing proteins

* and ** indicate p-values tested by Student's t-test

depression of the liver function became worse, and their recovery after treatment was also deteriorated.

The similar effects were compared between the three groups of patients (Fig. 2). Transaminase levels were not significantly different, although only the GOT of the moderately anemic cases (Group II) was found to be elevated. However, total bilirubin concentration were significantly higher in Group II and III than that in Group I. This difference was considered to be attributed to the disturbance of bilirubin excretion by hepatocytes, rather than hemolysis, since it lasted even after two weeks in spite of the absence of extrahepatic biliary obstruction.

Serum concentration of liver-producing proteins revealed more marked differences according to the severity of anemia (Fig. 3). Serum Alb, ChE and PT were found to be decreased significantly and stepwise from Group I to III.

These results indicated that hepatocyte damage and metabolic dysfunction in alcoholic liver disease may be aggravated more according to the severity of anemia, most likely by an exacerbation of hypoxia in the liver.

2. Abnormalities of erythrocytes in alcoholic liver disease. Do they affect microcirculation in the liver?

It has been known well that morphological changes of erythrocytes such as macrocytosis, spherocytosis and stomatocytosis, were frequently associated with alcoholic liver disease. A question whether or not such changes of red blood cells can

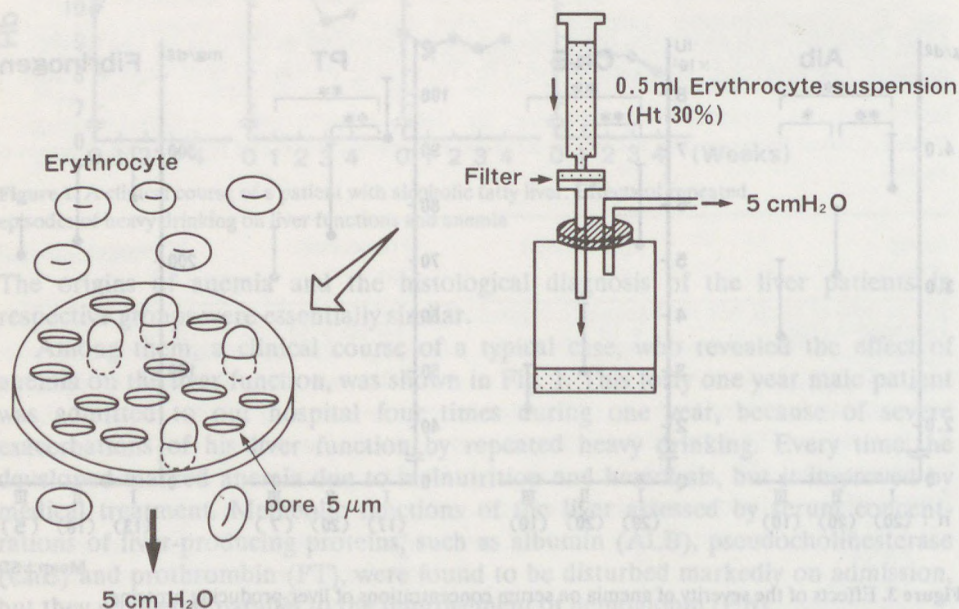


Figure 4. A schematic illustration of the method for determination of erythrocyte deformability

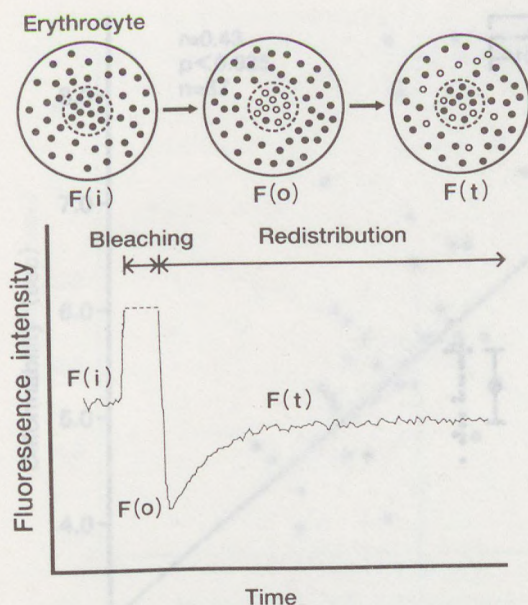


Figure 5. A schematic illustration of the principle of the fluorescent recovery after photobleaching (FRAP) method for determination of erythrocyte membrane fluidity.

$F(i)$: initial fluorescent intensity of a probe (DiI) in an area of $1.8 \mu\text{m}$ radius. $F(0)$: the intensity after irreversible photobleaching of the probe by an intensified argon laser beam. $F(t)$: recovery speed of the fluorescence intensity by lateral diffusion of the probe into the bleached area. Details were described in previous reports [11, 13]

disturb microcirculation in hepatic sinusoids, which were narrowed and hardened by histological alterations of the liver as described before, has not been investigated yet. To answer that, the deformability and the membrane fluidity of erythrocytes were examined by *in vitro* experiments as described in detail in our previous paper [11]. Briefly, the deformability was measured by the method of Reid et al. [12] as shown in Fig. 4. The time (expressed as second) required to filtrate 0.5 ml of erythrocyte suspension through a milipore filter was defined as the deformability. The membrane fluidity was measured by a fluorescence recovery after photobleaching (FRAP) [13] as briefly summarized in Fig. 5.

The velocity ($F(t)$) of lateral diffusion of the unbleached fluorescent probe from the surrounding area to the bleached field. That indicates the lateral mobility of membrane lipids and proteins, was measured by a photosensor and defined as the membrane fluidity.

1) Erythrocyte deformability

The mean values of the deformability in respective groups of alcoholic liver disease were found to be significantly elevated compared with that in normal controls (Fig. 6). Namely, the deformability of erythrocytes were disturbed under the pathological condition. Although a tendency that the degree of the abnormality may be higher in liver cirrhosis (LC), a significant difference was noted even in an early stage of the disease, such as fatty liver (FL).

When the values of respective patients were plotted against the size of

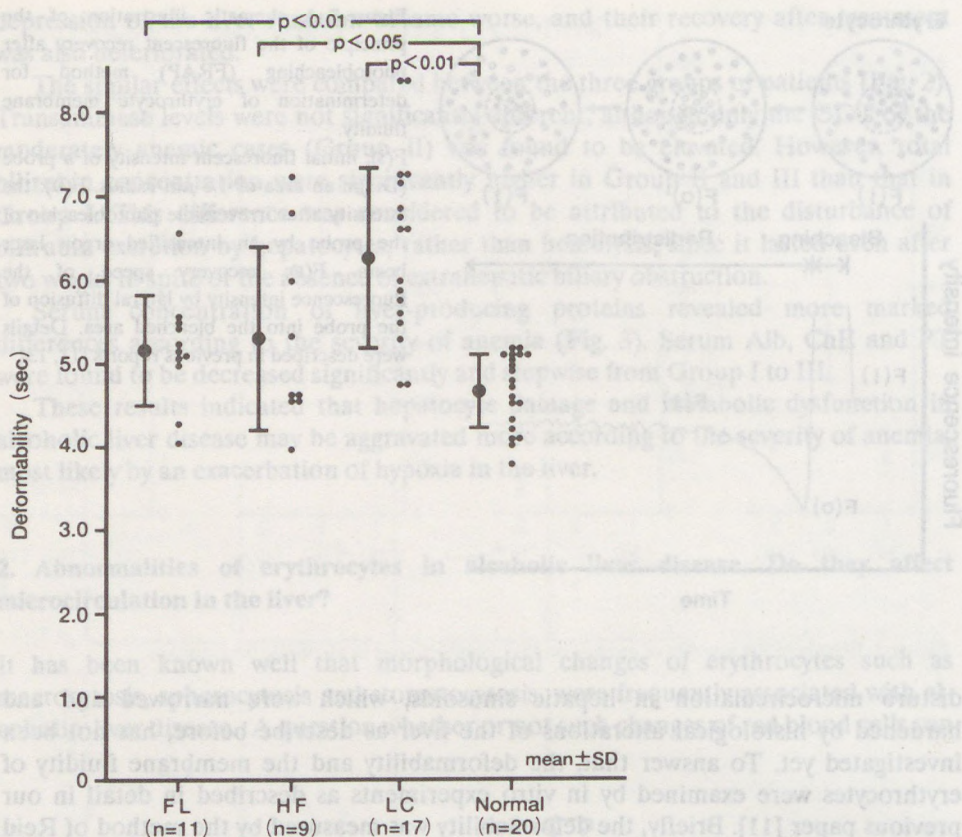


Figure 6. Erythrocyte deformability in alcoholic liver disease and normal controls

erythrocyte, determined as a mean corpuscular volume (MCV), a tendency of a linear relationship was observed between the two parameters (Fig. 7).

The result indicated that the disturbance of the deformability determined by the presence method may be partly due to the increased size of erythrocytes at least in the patients studied. On the other hand, the finding that the values of the deformability were widely distributed in spite of the same MCV may suggest an involvement of different factors from the size of cells.

2) Membrane fluidity of erythrocytes

The measurement was carried out in nine erythrocytes randomly selected in each patients and control subjects. The difference of the mean values between the two groups were tested by the nested analysis of variance [14].

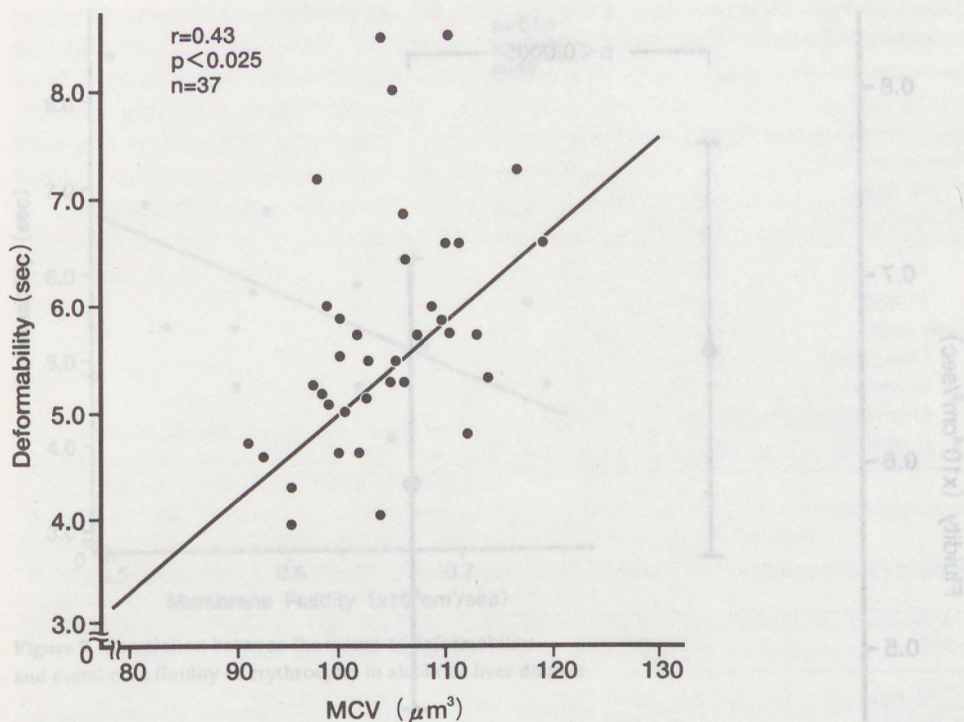


Figure 7. Correlation between deformability and mean corpuscular volumes (MCV) of erythrocytes in alcoholic liver disease

The membrane fluidity of erythrocytes in alcoholic liver disease was found to be significantly decreased compared with the value in controls (Fig. 8). When the mean value of the fluidity in each patient was plotted against the value of the deformability, a significant correlation was observed between the two parameters (Fig. 9). As a natural consequence, a linear relationship was also present between the values of the membrane fluidity and MCV.

From these results, it may be concluded that erythrocytes of alcoholic liver diseased are characterized by their disturbed deformability due to increased size and solidity.

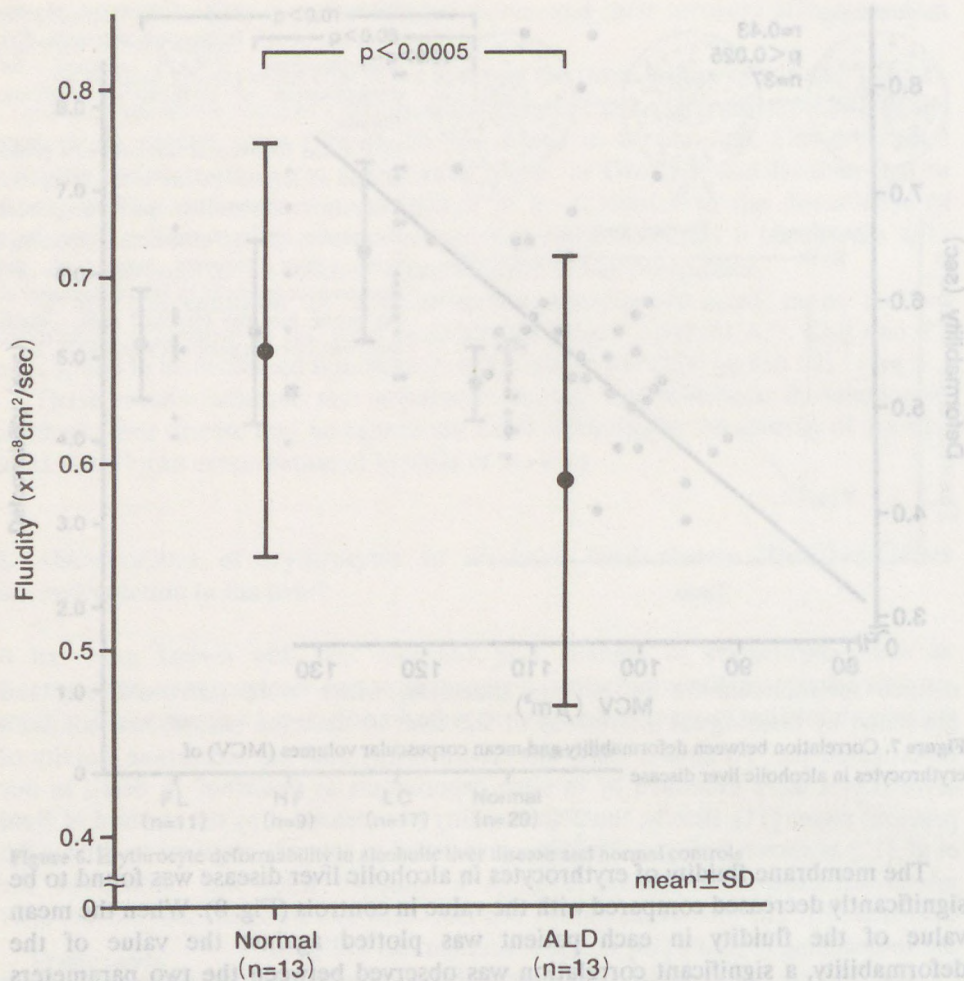


Figure 8. Erythrocyte membrane fluidity measured by FRAP method. A statistical difference (p value) between the means of the two groups was tested by the analysis of variance [14]

2) Membrane fluidity of erythrocytes

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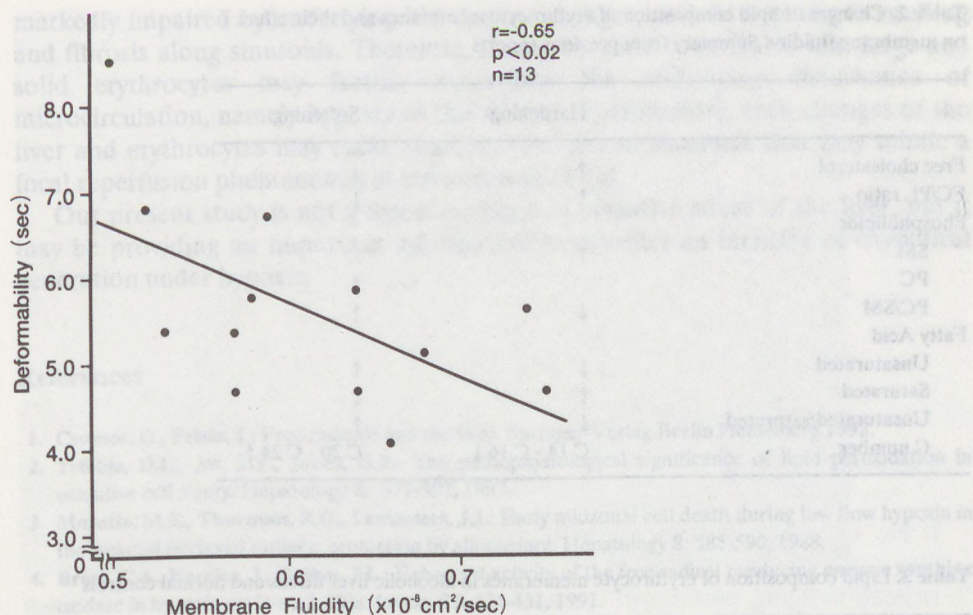


Figure 9. Correlation between the values of deformability and membrane fluidity of erythrocytes in alcoholic liver disease

3. Changes of membrane lipid compositions of erythrocytes; A case of the physical abnormality

Various factors have been reported as mechanisms of physical alteration of erythrocytes. It may be briefly summarized in the followings: changes of cell surface area/cell volume ratio, lipid and protein compositions of cell membrane and viscosity of intracellular fluid [15]. Among these, effects of lipids have been studied by many and may be summarized as shown in Table 2.

Changes of these in alcoholics also have been reported variously; an increase of free cholesterol/phospholipids ratio, a decrease of phosphatidyl choline (PC)/sphingomyelin (SM) ratio, and a decrease of unsaturated/saturated fatty acid ratio.

In our study, free cholesterol (FC) was not found to be increased, and PC/SM ratio appeared to be increased in alcoholic group, contrary to previous reports. As a hardening factor, only the ratio of unsaturated/saturated fatty acid ratio of esterified phospholipids was found to be decreased significantly (Table 3) [11]. These

Table 2. Changes of lipid composition of erythrocyte membranes and their effect on membrane fluidity: Summary from previous reports

	Hardening	Softening
Free cholesterol	↑	↓
FC/PL ratio	↑	↓
Phospholipids		
SM	↑	
PC		↑
PC/SM	↓	↑
Fatty Acid		
Unsaturated	↓	↑
Saturated	↑	↓
Unsaturated/saturated	↓	↑
C number	C 14 - C 16 ↓	C 20 - C 24 ↑

Table 3. Lipid composition of erythrocyte membranes in alcoholic liver diseases and normal controls

Lipids	Alcoholics (n)	Controls (n)	p
FC/PL ratio	0.42 ± 0.08 (42)	0.43 ± 0.04 (15)	ns
PC/SM ratio	1.22 ± 0.39 (40)	0.70 ± 0.07 (15)	< 0.001
Unsaturated/saturated fatty acid ratio	0.98 ± 0.34 (33)	1.36 ± 0.37 (15)	< 0.01

differences of lipid composition in respective reports may be partly attributable to differences of nutritions due to dietary habits of subjects studied in different countries, rather than the difference of pathological conditions of liver disease. In any event, such changes may be characteristic at least in Japanese patients, and may be reasonable to infer the increased solidity of erythrocyte membranes from a molecular biological aspect.

4. Significance of erythrocyte abnormalities in alcoholic liver disease

Our study suggested that anemia and pathophysiological changes of erythrocytes may play important roles in promoting hepatocyte damage and disturbances of liver functions in alcoholic liver disease.

It can be considered that anemia itself will be directly responsible for the exacerbation of underlying hypoxia of the liver by reducing oxygen transport.

It was also clarified that erythrocytes were not only enlarged in size, but also they were solid. Hepatic microcirculation in alcoholic liver disease is known to be

markedly impaired by underlying histological changes, such as hepatocyte swelling and fibrosis along sinusoids. Therefore, it may be quite conceivable that large and solid erythrocytes may further exacerbate the underlying disturbance of microcirculation, namely hypoxia of this disease. Furthermore, such changes of the liver and erythrocytes may cause sludging of blood in sinusoids that may mimic a focal reperfusion phenomenon at a microscopic level.

Our present study is not a direct evidence of oxidative stress of the liver, but it may be providing an important information to consider an increase of oxyradical generation under hypoxia.

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Oxygen Sensitive Mutants of the Nematode *Caenorhabditis elegans* and Aging

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Abstract

It has been proposed that free radicals, especially those of molecular oxygen, may accelerate aging in animals. To investigate the possible role of oxygen free radicals in aging, mutants of the nematode *Caenorhabditis elegans* were isolated which are hypersensitive to methyl viologen (paraquat) and oxyge. The free-living nematode is an attractive model system for aging research on metazons. The nematode offers the great advantages of genetic manipulability, short life span, cellular simplicity, and easy cultivation. Investigation of two genes in *C. elegans* should serve to illuminate the relationship between oxidative damage and aging. A mutation in one of these, *mev-1*, has been shown to reduce Cu/Zn superoxide dismutase activity by 30 to 50% relative to wild-type animals. The life spans of this mutant and a second mutant, *rad-8*, are significantly shortened in the presence of high oxygen concentration. We suggest that oxygen radicals may be involved in the normal aging in *C. elegans*.

Introduction

Even at present, the nature of aging process is unknown. Many theories have been advanced which seek to explain the mechanisms which underlie both cellular and organismal aging [1, 2]. As one of these theories, it has been attractively proposed that free radicals, especially those of molecular oxygen, may accelerate aging in animals [3, 4]. Cutler has provided a variety of data which support this view [5]. In particular, the superoxide dismutase (SOD) activity (E.C. 1.15.1.1) of several organs, a scavenger of oxygen free radicals [6], is positively correlated with the maximum life span for various animal species including primates. Similar correlations were observed for several other radical scavengers including plasma ureate, carotinoids, and vitamin E. Interestingly, the product of the standard metabolic rate and the maximum life span, called the LEP, is roughly constant for various animals, indicating that animals with lower standard metabolic rates can live

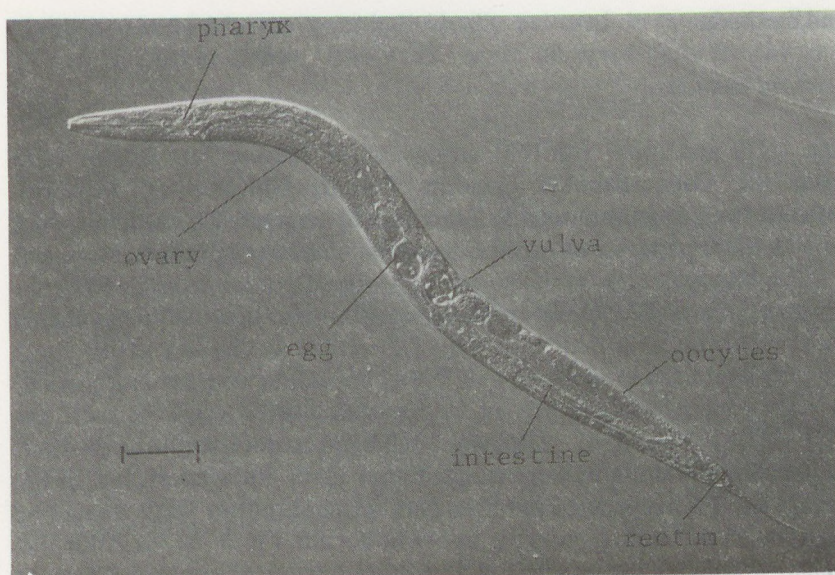


Figure 1. Photomicrographs showing major anatomical features of *Caenorhabditis elegans* adult hermaphrodite. Bar represents 100 μ m

for longer periods than animals with higher rate. Despite intense interest, however, it is hard to verify the role of oxygen free radicals in complex biological phenomena of aging. A variety of free radicals are generated in living organisms and damage biological materials such as DNA, proteins and lipids [3]. To reduce the damage induced by free radicals, organisms have been developed multiple defense system against these intermediates [7, 8]. To investigate the possible role of oxygen free radicals in aging, genetic approach is useful. In particular, the study of oxygen-sensitive mutants is valuable, as it illuminates the genetic mechanisms of these defense systems. It also provides insights into the relationship between oxygen toxicity and aging.

The small, free-living nematode *Caenorhabditis elegans* has received much attention as a model to dissect the genetics, and hence, the mechanisms which underline aging by its self-fertilizing, hermaphroditic mode of reproduction, which makes the isolation of mutants easy and allows rapid inbreeding, and by the occurrence of spontaneous males, which can be used to construct stocks and map new data (Fig. 1). They can grown in petri plates on a simple diet of *Escherichia coli*. The major advantage of using *C. elegans* for aging research is the 10- to 20-days mean life span. Associated with this short life span is a rapid life cycle of approximately 3.5 days at 20 °C [9, 10, 11].

We found two methyl viologen-sensitive mutants, *mev-1* and *rad-8*, in *C. elegans* and characterized. The toxic effects of methyl viologen as herbicidal drug on cells and animals believed to be mediated by superoxide anions [12, 13]. These mutants were also hypersensitive to oxygen.

Materials and methods

Strains and general methods

The wild-type (N2) and *rad-8* (nm162) strains of *C. elegans* var. Bristol were obtained from the Caenorhabditis Genetics Center, University of Missouri. Stock maintenance and handling were as described by Brenner [9]. Animals were cultured on NG agar plates seed with *Escherichia coli* strain OP50, an uracil auxotroph.

Ethylmethansulfonate (EMS) and methyl viologen were obtained from Sigma Chemical Co.

Isolation of mutants

Methyl viologen sensitive mutants were isolated after EMS mutagenesis [9]. In the initial screening, the F2 progeny of mutagenized hermaphrodites were treated as gravid adults with 30 mM methyl viologen in M9 buffer for 4 hr at 20 °C. After this treatment, inactive animals were individually cultured on small, seeded NG agar plates. Whereas wild-type animals survive this treatment, methyl viologen-sensitive animals may die. Because methyl viologen does not penetrate eggshells, viable embryos can still be recovered from the treated adults after transfer to drug-free plates.

To obtain other mutants, nine radiation-sensitive mutants were examined methyl viologen- and oxygen-sensitivities.

Sensitivity to methyl viologen or oxygen

Embryos (eggs) were collected from NG agar plates using sodium hypochlorite [14] and allowed to hatch by overnight incubation at 20 °C in S buffer [15]. The newly hatched L1 larvae were cultured on seeded NG agar plates. One set of NG agar plates contained various concentration of methyl viologen (0 - 0.6 mM) and another set was exposed to various oxygen concentrations in an airtight plastic chamber. The chamber was ventilated with one atmosphere of 100% oxygen at rate of 2 l/min until the desired concentration was achieved. For lower oxygen concentration than atmospheric, the gas in the chamber was partially replaced by nitrogen. The oxygen concentration was measured by an oxygen analyzer (Iijima Products Manufacturing Co. Model G-101-Y). Four to six days later, survival was determined by counting the number of late-stage larvae (L4 larvae) and adults. L4 larvae were scored as survivors because we have observed that such late-stage larvae usually attain adulthood.

Developmental rate

To determine the onset of sexual maturity, eggs were collected from NG agar plates using sodium hypochlorite and incubated overnight at 20 °C in S buffer.

Newly hatched L1 larvae were individually cultured in separate wells of 96-well or 24-well microtiter dishes. The wells were observed daily for the appearance of eggs.

Life span

L1 larvae were grown under atmospheric oxygen until they reached adulthood. The life span of individual adult hermaphrodites was then determined under various oxygen concentrations. The gases in the chamber were replaced daily. To prevent progeny production, 5-fluoro-2'-deoxyuridine (FudR; Sigma Chemical Company, St. Louis MO) was added to the NG agar in a final concentration of 40 μ M after animals had reached adulthood [16]. The animals were examined daily to determine the time of death. Death was defined by the absence of movement after either mechanical stimulation or immersion in water.

Superoxide dismutase activity

A xanthine/xanthine oxidase system [17] was used to produce superoxide anions while the hydroxylamine/N-1-naphthylethylene diamine/sulfanilic acid system was used for detection. The residual superoxide anion combines with the hydroxylamine to produce nitrite. The nitrate, in turn, reacts with the latter 2 compounds to create a dye with an extinction maximum at 550 nm. This method has been developed and described by Oyanagi [18].

For enzyme measurements, animals were grown at 20 °C with continuous shaking in a liquid medium prepared by adding Yeast Extract (Difco) and Bacto Soytone (Difco) to 360 ml of distilled water containing penicillin G, streptomycin sulfate, myoglobin, and steroid mixture (prepared by β -sitosterol, cholesterol, ergosterol and Tween 80) [19]. After 2 weeks the number of animals reached about 1.0×10^5 /ml. These were then harvested by centrifugation, washed, and stored frozen in liquid nitrogen. The frozen animals were powdered in a mortar cooled with dry ice, and extracted in S. buffer. Protein concentration was determined using the RioRad Protein Assay Reagent with bovine serum albumin as standard.

Results

Mutation isolation

From about 15,000 F2 progeny of EMS-mutanized hermaphrodites, 2 strains were proved to be highly sensitive to methyl viologen. One strain, *mev-1*, was back-crossed 5 times with wild-type males using methyl viologen sensitivity as a marker to identify mutant progeny [20]. It was found that the *mev-1* (kn-1) mutation behaves as a single, recessive Mendelian factor on linkage group III (Fig. 2).

In nine radiation-sensitive mutants, only *rad-8* was hypersensitive to oxygen and methyl viologen [21].

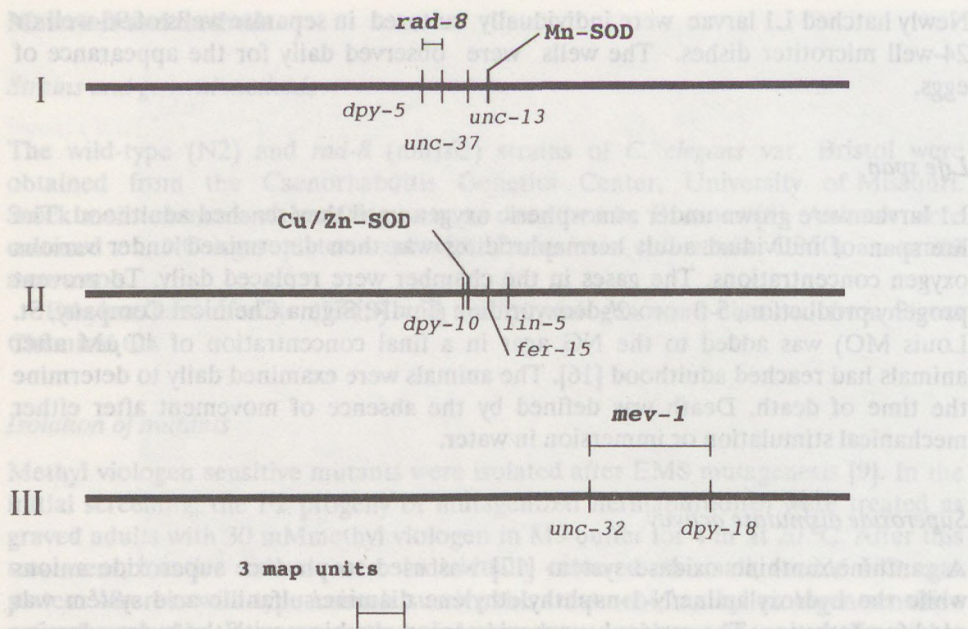


Figure 2. A simplified genetic map in *Caenorhabditis elegans*. Loci used in this work and several commonly used genetic markers (*dpy*: DumpY: shorter than wild type; *unc*: UNCoordinated; *lin*: abnormal cell LINEage; *fer*: FERtilization defective) are shown. The haploid set of *Caenorhabditis elegans* includes five autosomes and a sex chromosome. Autosome IV, V and sex chromosome were omitted

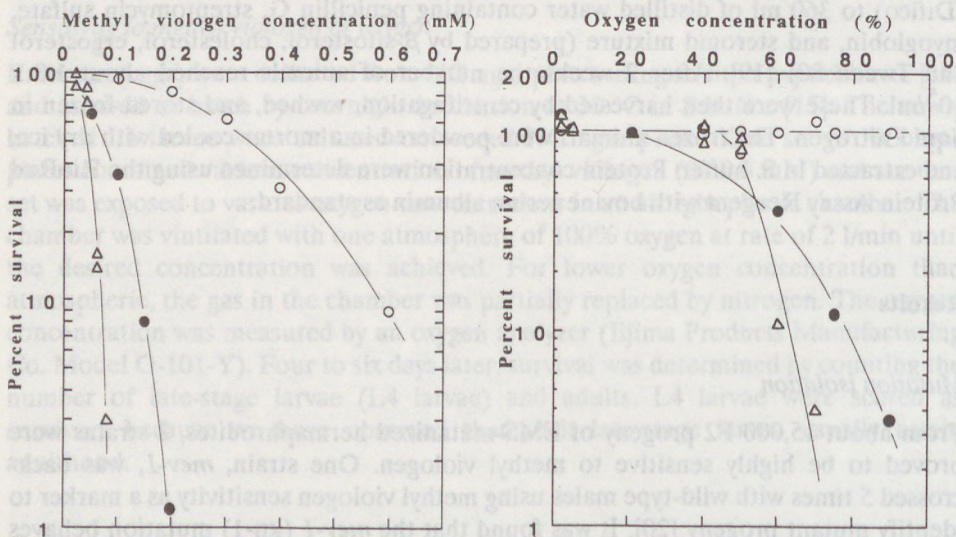


Figure 3. Sensitivities of wild type, *mev-1* and *rad-8* mutants to methyl viologen and oxygen. Approximately 100-300 animals were scored for each point. Open circles, wild type; closed *mev-1*; open triangles, *rad-8* [from Reference 20, 21]

Sensitivities to methyl viologen and oxygen

L1 larvae were cultured on plates containing various concentrations of methyl viologen. At the concentration of methyl viologen examined (0.2 mM), most wild-type animals develop into L4 larvae or adults within 4 days. However, *mev-1* mutants usually arrested as L1 or L2 larvae and were easily distinguished from the wild type [20]. Even at 0.1 mM of methyl viologen, *rad-8* La larvae arrested as L1 or L2 larvae even after 6 days [21]. Comparing the concentrations which caused inactivation to 37% survival, the *mev-1* and *rad-8* mutants were about 4 and 8.5 times more sensitive than wild type, respectively (Fig. 3).

Survival of wild type was unaffected by oxygen concentration between 5 and 90%, with essentially all L1 larvae maturing within 4 days. Conversely, few *mev-1* larvae survive a 3-days exposure under 90% oxygen [20]. On the other hand, while many *rad-8* animals reached adulthood within 6 days under oxygen concentration between 5 and 50%, most mutant animals arrested as L1 or L2 larvae under more than 60% oxygen [21] (Fig. 3).

Developmental rate

Egg-laying in the wild type began on the third day after hatching at 20 °C, while the onset of egg production was delayed for only about half day in *mev-1* mutants. On the other hand, *rad-8* animals started to lay eggs on the sixth days after hatching

Table 1. Life spans of wild type and *mev-1* when grown under various oxygen concentration

Strain	Oxygen concentration (%)	Maximum life span (days after hatch)	Mean life span	R (mean)*
N2	1	41	30.3	1.15
	2	33	26.3	1.00
	21	33	26.3	1.00
	40	33	26.5	1.01
	60	28	22.6	0.86
<i>mev-1</i>	1	35	25.6	0.97
	2	31	24.6	0.94
	21	26	21.2	0.81
	40	19	16.6	0.63
	60	10	7.96	0.30

$\text{*R (mean) = } \frac{\text{mean life span under various oxygen}}{\text{mean life span of wild type under 21\% oxygen}}$
Animals were grown at 20 °C [from Reference 20].

Table 2. The effects of the timing and length of 1% oxygen exposure on life span extension in the *mev-1* mutants

Period of 1% oxygen exposure@	Maximum life span (days)	R (max)*	Mean life span (days)	R (mean)**
none#	24	1.00	21.3	1.00
day 0 - day 30#	30	1.25	25.2	1.18
day 0 - day 7	24	1.00	21.0	0.98
day 7 - day 14	26	1.08	22.6	1.06
day 7 - day 21	30	1.25	24.9	1.17
day 7 - day 28#	28	1.17	24.7	1.16

$$*R(\text{max}) = \frac{\text{maximum life span of various period of 1\% oxygen exposure}}{\text{maximum life span of none period of 1\% oxygen exposure}}$$

$$*R(\text{mean}) = \frac{\text{mean life span of various period of 1\% oxygen exposure}}{\text{mean life span of none period of 1\% oxygen exposure}}$$

@dates from hatching

#until the end of life span

[from Reference 22].

when grown under atmospheric oxygen. However under lower oxygen concentration (e.g. 5%) the time to maturity of the mutant was accelerated to 5 days [21] (Table 1).

Life span

Life spans of the wild type, *mev-1* and *rad-8* mutants under various concentrations of oxygen are shown in Table 1 and 3 [20, 22]. The life span of wild type under 1% oxygen were extended significantly, while those under 60% oxygen were shortened considerably. Those under oxygen concentrations within a range between 2 and 40% remained unchanged. On the other hand, the life span of *mev-1* under 1% oxygen was also longer, while that under 60% oxygen was much shorter. The life spans were observed to vary over a wide range of oxygen concentration [23] (Table 1). We examined the effects of the timing and length of a 1% oxygen exposure on life span extension in the mutant, in order to see at what time point and how long the mutant needs exposure to 1% oxygen for life span extension. The life span of mutants exposed to 1% oxygen various phases of life span and for various periods of time are

shown in Table 2. A 1% oxygen exposure for 7 days from day 0 after hatching to day 7 had no effect on life span. Exposure for 7 days from day 7 to day 14 has a slightly positive effect, whereas the exposures for 14 days from day 7 to day 21 and for 18 days from day 7 to day 28 had the most positive effects. For life span extension, a 1% oxygen exposure for more than 14 days after the middle of life span was the most effective [22].

The life span of *rad-8* was almost same as the wild type under atmospheric oxygen at 22 °C. The life span of wild type remained constant over the range of atmospheric to 60% oxygen. Conversely, the life span of *rad-8* animals was substantially reduced when oxygen concentrations were elevated [21] (Table 3).

SOD activity

The SOD activity in *mev-1* was 30 to 50% of that in the wild type. Most wild-type activity was inhibited by KCN, indicating that there was relatively little, if any, Mn-SOD. In contrast, 14-21% of the residual activity in *mev-1* could be attributed to the Mn-SOD [20].

Table 3. Time to maturity and life spans of wild type and *rad-8* when grown under various oxygen concentrations

Strain	Oxygen concentration (%)	Time to maturity (days after hatch)	Maximum life span (days after hatch)	Mean life span	R (mean)*
N2	5	3.5			
	21	3.7	23	16.4	1.00
	40	4	22	16.0	0.98
	50	4	21	16.6	1.00
	60		23	16.5	1.00
<i>rad-8</i>	5	5			
	21	6	22	16.9	1.03
	40	6	22	12.5	0.76
	50	7	21	10.2	0.62
	60		15	8.9	0.54

$$*R(\text{mean}) = \frac{\text{mean life span under various oxygen}}{\text{mean life span of wild type under 21\% oxygen}}$$

Animals were grown at 20 °C for time to maturity and at 22 °C for life span [from Reference 21].

Discussion

Much attention has focused on the role of oxygen damage in aging [4]. To investigate the possible role of oxygen free radicals in aging, mutants of the nematode *C. elegans* were isolated which are hypersensitive to oxygen. In particular, investigation of two genes should serve to illuminate the relationship between oxidative damage and aging. A mutation in one of these, *mev-1*, has been shown to reduce Cu/Zn superoxide dismutase activity by 30 to 50% relative to wild-type animals. The life span of the wild type remained constant under the range of 2% to 40% oxygen, while the life span of the *mev-1* mutants varied with dependence on the concentration of oxygen. From the Gompertz analysis indicated that changes in oxygen concentration perturb aging rate of the nematode. For both the wild type and *mev-1* mutant, the Gompertz component under 1% oxygen were smaller than under the other oxygen concentration. For the mutant, the Gompertz component increased significantly with an increase in oxygen concentration [22]. These effects of oxygen on the perturbation of life span and aging rate seems to be enhanced by a genetic defects of the mutant in antioxidant defense.

A second gene, *rad-8*, was originally isolated on the basis of its hypersensitivity to UV radiation [23]. Development of the mutants was hypersensitive to high oxygen concentration. The maximum and mean life spans of *rad-8* were almost the same as the wild type under atmospheric oxygen. When oxygen concentrations were elevated, however, the mean life span of *rad-8* animals was substantially reduced.

For life span extension in the *mev-1* mutant, a 1% oxygen exposure after the early phase of life span was the most effective. A 1% oxygen exposure at the early phase of life span was ineffective, suggesting that the effect of oxygen concentrations on life span is not secondary to the effects of development and maturation. Johnson and associates reported that the development, maturation and life span of *C. elegans* each were under independent control [24].

These results suggest that oxygen is involved in the specification of life span of the nematode.

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Glutamate Neurotoxicity and Free Radicals in Cerebral Ischemia

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Abstract

Numerous studies have demonstrated the importance of glutamate neurotoxicity in cerebral ischemia. We studied the effects of a comparative NMDA antagonist (CGS-19755) on local cerebral pH and cerebral blood flow using a double-label autoradiographic technique in rats with focal ischaemia. The NMDA antagonist, in addition to its antagonistic effect on the NMDA receptor, increased cerebral blood flow and corrected pH in the moderately ischemic regions. On the other hand, oxygen free radicals also act as toxic intermediates in ischemia. However there is no report concerning the causal relation between glutamate neurotoxicity and oxygen free radicals in ischemia. We, therefore, evaluated the effects of superoxide dismutase (SOD) on extracellular glutamate using an in-vivo brain microdialysis technique and on cerebral blood flow using a hydrogen clearance method in rats with focal ischemia. SOD significantly decreased extracellular glutamate from 44.8 ± 16.9 to 21.9 ± 17.0 μM (mean $\pm$ SD, $p < 0.01$) in the infarcted cortex at 50 min after occlusion. Infarct volume was significantly smaller in rats receiving SOD than in the vehicle-treated group ($p < 0.05$). In this paper, we suggest that oxygen free radicals may contribute to a release of glutamate from presynaptic sites as a neurotoxic effect in addition to causing direct damage to the membranes, and we review recent papers concerning glutamate neurotoxicity in cerebral ischemia.

1. The mechanisms of neuronal damage in ischemia

Recently, numerous studies have confirmed the importance of glutamate neurotoxicity in cerebral ischemia. Figure 1 schematically illustrates pre- and postsynaptic voltage sensitive calcium channels (VSCC) and antagonist-operated calcium channels (AOCC) in cerebral ischemic status. In the presynaptic site, excitatory amino acids including glutamate are released from synaptosomes in a Ca^{2+} -dependent manner on depolarization of the plasma membrane and from

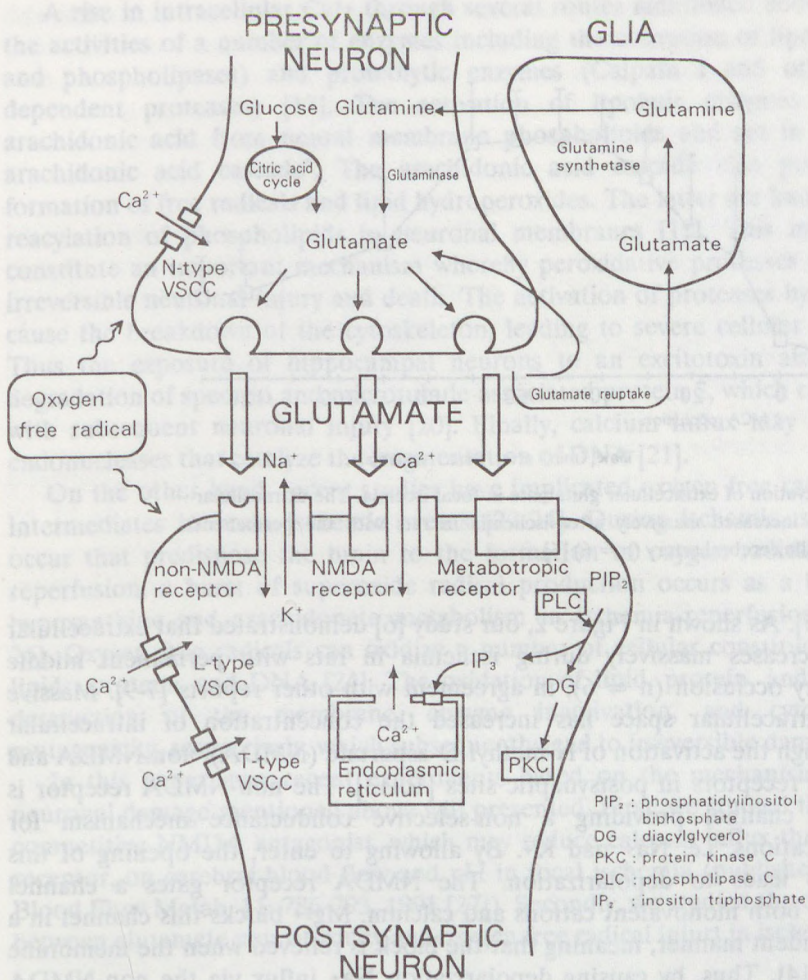


Figure 1. Calcium fluxes in pre- and postsynaptic structures. Presynaptically, glutamate release is triggered by the activation of N type VSCC. Post synaptically, the major contributors to postsynaptic calcium entry are AOCC, gated by NMDA and non-NMDA receptors. Both receptor types are linked to channels allowing monovalent cations to pass, while the NMDA-gated channel is permeable to calcium as well. The activation of metabotropic receptors leads to increased cellular levels of diacylglycerol and inositol 1,4,5-triphosphate with the mobilization of Ca²⁺ from intracellular stores. Furthermore, the L and T types of VSCC also mediate calcium influx. A rise in intracellular Ca²⁺ may affect the activities of a number of enzymes, including the activation of lipolytic and proteolytic enzymes, and subsequently lead to irreversible damage [6]

cytoplasm in a Ca²⁺-independent manner on depolarization by reversal of the uptake pathway [1-3]. The calcium channels at presynaptic nerve terminals mediating calcium-dependent transmitter release are predominantly N-type

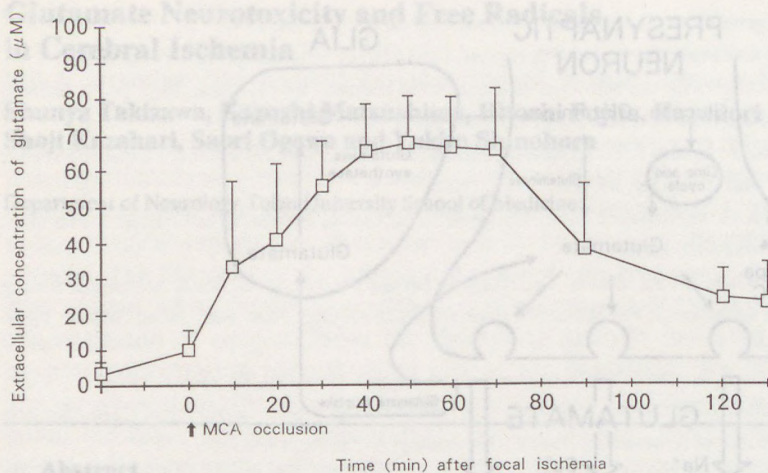


Figure 2. The elevation of extracellular glutamate in focal ischemia. The extracellular glutamate levels increased massively after ischemia in rats with the permanent occlusion of middle cerebral artery ($n = 6$) [6]

channels [4, 5]. As shown in Figure 2, our study [6] demonstrated that extracellular glutamate increases massively during ischemia in rats with permanent middle cerebral artery occlusion ($n = 6$), in agreement with other reports [7-9]. Massive glutamate extracellular space has increased the concentration of intracellular calcium through the activation of N-methyl-D-aspartate (NMDA), non-NMDA and metabotropic receptors in postsynaptic sites [10-13]. The non-NMDA receptor is linked to a channel providing a non-selective conductance mechanism for monovalent cations, i.e. Na^+ and K^+ . By allowing to enter, the opening of this channel thus leads to depolarization. The NMDA receptor gates a channel permeable to both monovalent cations and calcium. Mg^{2+} blocks this channel in a voltage-dependent manner, meaning that the block is relieved when the membrane depolarizes [14]. Thus, by causing depolarization Na^+ influx via the non-NMDA receptor-linked channel sets the stage for massive calcium influx via the NMDA-gated channel. By causing depolarization, non-NMDA receptor activation also allows Ca^{2+} influx through VSCC of L and T types, and could be one of the factors leading to reversed $\text{Na}^+/\text{Ca}^{2+}$ exchange. Furthermore, activation of NMDA receptor leads to an influx of Ca^{2+} , which binds to calmodulin and activates nitric oxide (NO) synthase, converting molecular oxygen and arginine to citrulline and NO [15]. NO acts as an inhibitor of platelet aggregation, a vasodilator, and a neurotoxin [15]. Metabotropic receptors act through a GTP-binding protein-dependent mechanism to elicit polyphosphoinositide hydrolysis [16]. The activation of metabotropic receptors leads to increased cellular levels of diacylglycerol and inositol 1,4,5-triphosphate. Diacylglycerol activates protein kinase C that catalyzes phosphorylation of many synaptic membrane proteins, and inositol 1,4,5-triphosphate mobilizes Ca^{2+} from intracellular stores, such as endoplasmic reticulum, to cytoplasm.

A rise in intracellular Ca^{2+} through several routes mentioned above may affect the activities of a number of enzymes including the activation of lipolytic (lipases and phospholipases) and proteolytic enzymes (Calpain I and other calcium-dependent proteases) [17]. The activation of lipolytic enzymes will release arachidonic acid from neural membrane phospholipids and set in motion "the arachidonic acid cascade". The arachidonic acid cascade also potentiates the formation of free radicals and lipid hydroperoxides. The latter are known to inhibit reacylation of phospholipids in neuronal membranes [18]. This inhibition may constitute an important mechanism whereby peroxidative processes contribute to irreversible neuronal injury and death. The activation of proteases by calcium may cause the breakdown of the cytoskeleton, leading to severe cellular damage [19]. Thus the exposure of hippocampal neurons to an excitotoxin also causes the degradation of spectrin and microtubule-associated protein 2, which correlates well with subsequent neuronal injury [20]. Finally, calcium influx may even activate endonucleases that catalyze the fragmentation of DNA [21].

On the other hand, recent studies have implicated oxygen free radicals as toxic intermediates in brain ischemic events [22-24]. During ischemia, several events occur that predispose the brain to the formation of oxygen radicals [25]. After reperfusion, a burst of superoxide radical production occurs as a by-product of hypoxanthine and arachidonate metabolism in ischemia-reperfusion models [25, 26]. Oxygen free radicals can oxidize a number of cellular constituents including lipids, proteins and DNA [24]. The oxidation of lipid, protein, and DNA causes destruction of the membrane, enzyme inactivation, and cytotoxicity and mutagenicity, respectively, which subsequently lead to irreversible damage.

In this paper, two recent experiments based on the mechanism of ischemic neuronal damage mentioned above will be presented. First, we studied the effects of a competitive NMDA antagonist, which may reduce calcium influx through NMDA receptor, on cerebral blood flow and pH in focal ischemia (published in *J. Cereb. Blood Flow Metab.* 11: 786-793, 1991 [27]). Secondly, we studied the causal relation between glutamate neurotoxicity and oxygen free radical injury in ischemia.

II. The effects of a NMDA antagonist on cerebral blood flow and pH in focal ischemia [27]

1. Introduction

Although comparative antagonists of the NMDA receptor penetrate the blood-brain barrier relatively poorly [28, 29], several researches have evaluated their efficacy by systemic administration [29-32]. We therefore decided to investigate CGS-19755 (cis-4-phosphonomethyl-2-piperidine carboxyl acid) a highly potent and selective competitive NMDA antagonist [33], to see if in cerebral ischemia it increased tissue perfusion or brought about other potentially neuroprotective mechanisms. We utilized a double-label autoradiographic technique to measure

local cerebral pH (LCpH) and local cerebral blood flow (LCBF) in rats with occluded middle cerebral artery (MCA) and ipsilateral common carotid artery (CCA).

2. Materials and methods

Male Sprague-Dawley rats, weighing approximately 250 grams, were fasted overnight before the experiment. On the day of the experiment, each rat was anesthetized with 1.5% halothane, and polyethylene catheters were placed in both femoral arteries and both femoral veins for physiological monitorings and CGS-19755 administration. The left MCA was occluded over a 2-3 mm segment proximal to the lateral olfactory tract using a method of Tamura et al. [34] as modified by Bederson et al. [35]. This was immediately followed by ligation of the ipsilateral CCA. Prior to occlusion of the MCA, a thermocouple probe (52 K/J thermometer, John Fluke, WA, U.S.A.) was placed in the ventral frontoparietal cortex through a burr hole. The brain and rectal temperatures were maintained at 36-37 °C throughout the experiment with a warming lamp. Arterial blood pressure was monitored continuously. Arterial blood gases were examined at 0.3 l and 3 hours, and plasma glucose was measured at 3 and 4 hours after surgery.

Animals with MCA and CCA occlusions were divided into 3 experimental groups: Group 1 (n = 6) was given CGS-19755 (Ciba-Geigy Co., New Jersey, U.S.A.) by intravenous bolus injection of 10 mg/kg given immediately after the occlusions, followed by continuous infusion at 5 mg/kg/hr for four hours. CGS-19755 was dissolved in 10 mM isotonic Sorensen's buffer, adjusted to pH 7.3 with sodium hydroxide. The rate of infusion was 4.1 μ l/min, started just after CCA occlusion and continued until decapitation 4 hour post-occlusion. CGS-19755 solution was protected from light during preparation and infusion. Group 2 (n = 6) was the carrier group given isotonic Sorensen's buffer alone, starting immediately after the occlusions, at the same infusion rate as group 1. Group 3 (n = 7) was another control group not infused with any drug. All animals were decapited 4 hours after CCA occlusion.

Autoradiographic measurement of LPpH and LCBF

The method for the simultaneous measurement of LCpH and LCBF has been described elsewhere [36]. Briefly, three hours after the occlusion, 85 μ Ci of [14 C]dimethyloxazolidinedione ([14 C]DMO, specific activity 56 mCi/mmol; Amersham, Ontario, Canada) dissolved in saline at 100 μ Ci/ml was injected intravenously. Blood samples for measurement of [14 C]DMO concentration were drawn at 30, 55, 56, and 60 min after [14 C]DMO injection. At 60 min after [14 C]DMO injection, 30 μ Ci of [14 C]iodoantipyrine [14 C]IAP, specific activity 58 mCi/mmol; Amersham) was injected intravenously in 1.8 ml of saline. The animal was decapited 1 min later, and the brain was removed, immediately frozen in 2-methylbutane, and sectioned coronally into consecutive 20- μ m sections.

Approximately 90 sections were exposed to x-ray film (Kodak SB-5, Rochester, NY, U.S.A.) for 3 days. After the first exposure, which yielded the first film reflecting both [^{14}C]DMO and [^{14}C]IAP, the brain sections were left exposed to air in a fume hood for at least 10 days. This resulted in total sublimation of [^{14}C]DMO. The sections were then reexposed as above for an additional 3 days, resulting in a second film showing only [^{14}C]IAP. Autoradiographic concentration of [^{14}C]DMO was then calculated by subtraction. Densitometric measurements were made with a computer-assisted image analysis system (MCID, Imaging Research, Ontario, Canada). Five coronal levels were chosen using a rat brain and subdivided into symmetric regions of interest (ROI). In each rat, the autoradiographic brain sections most closely matching these templates then selected, the templates were modified to fit the individual anatomy and the activity in the different ROI's was read and compared to standards exposed with the brain.

Histological studies

In each rat, after every 7th section obtained for autoradiography, a section was removed for histologic assessment. The brain sections were soaked for at least 1 hour in a fresh solution made up of 0.4 M sodium cacodylate (25 ml), 50% glutaraldehyde (4 ml), and distilled water (70 ml). The slides were then transferred into cacodylate buffer before staining with cresyl violet. In each rat, the volumes of infarct in the cerebral cortex and basal ganglia were determined by the method of Swanson et al. [37] using the MCID system. The volumes of normal cortex and caudate were then calculated by multiplying each area by the distance between sections. The infarct volume of the structure was then determined as a percentage of the difference between the left and right volumes normalized to the volume of the structure in the control hemisphere. The measurement of infarct size was carried out with the examines blinded as to the animal's experimental status.

Statistical analysis

LCpH, LCBF and infarct size in the controls and the animals receiving carrier and CGS-19755 were submitted to a one-way analysis of variance in both the occluded and the non-occluded hemispheres. An independent two-tailed Student's *t* test was used to determine the statistical significance of the difference between the groups' means. All values were expressed as mean $\pm$ SD.

3. Results

Physiological parameters

There were no significant differences in any of mean brain temperature, rectal temperature, arterial blood pressure, arterial blood gases, and plasma glucose levels among the three groups of rats. No significant fall occurred in brain or rectal temperatures during CGS-19755 infusion.

Table 1. Local cerebral blood flow (mean $\pm$ SD, ml/100 g/min) in rats with MCA + CCA occlusions receiving no treatment, carrier and CGS-19755 [27]

Level/ ROI	Ischemic hemisphere			Non-ischemic hemisphere		
	Control	Carrier	CGS-19755	Control	Carrier	CGS-19755
1/1	87 $\pm$ 21	66 $\pm$ 14	136 $\pm$ 72	127 $\pm$ 28	128 $\pm$ 19	240 $\pm$ 93
1/2	40 $\pm$ 14 ⁺	40 $\pm$ 8	80 $\pm$ 42	139 $\pm$ 36	139 $\pm$ 23	262 $\pm$ 86*
1/3	23 $\pm$ 13 ⁺	22 $\pm$ 13	49 $\pm$ 26	121 $\pm$ 35	116 $\pm$ 16	218 $\pm$ 83
1/4	19 $\pm$ 9 ⁺	41 $\pm$ 16 [#]	53 $\pm$ 48	146 $\pm$ 58	133 $\pm$ 29	227 $\pm$ 103
1/5	16 $\pm$ 14 ⁺	56 $\pm$ 27	78 $\pm$ 98	102 $\pm$ 35	113 $\pm$ 26	218 $\pm$ 71*
1/6	46 $\pm$ 28 ⁺	74 $\pm$ 12	107 $\pm$ 76	120 $\pm$ 28	137 $\pm$ 26	239 $\pm$ 56*
2/1	89 $\pm$ 30	65 $\pm$ 14	129 $\pm$ 63	126 $\pm$ 23	138 $\pm$ 20	204 $\pm$ 78
2/2	45 $\pm$ 14 ⁺	41 $\pm$ 9	80 $\pm$ 45	163 $\pm$ 35	172 $\pm$ 20	275 $\pm$ 87
2/3	31 $\pm$ 19 ⁺	21 $\pm$ 9	63 $\pm$ 29*	158 $\pm$ 34	146 $\pm$ 20	249 $\pm$ 99
2/4	13 $\pm$ 12 ⁺	14 $\pm$ 14	38 $\pm$ 25	143 $\pm$ 35	110 $\pm$ 16	237 $\pm$ 114
2/5	10 $\pm$ 9 ⁺	7 $\pm$ 7	21 $\pm$ 24	138 $\pm$ 28	106 $\pm$ 18	271 $\pm$ 127*
2/6	6 $\pm$ 5 ⁺	8 $\pm$ 8	27 $\pm$ 40	122 $\pm$ 23	104 $\pm$ 18	207 $\pm$ 91*
2/7	7 $\pm$ 5 ⁺	12 $\pm$ 11	22 $\pm$ 22	134 $\pm$ 28	101 $\pm$ 16	200 $\pm$ 94
2/8	23 $\pm$ 15 ⁺	24 $\pm$ 10	52 $\pm$ 37	126 $\pm$ 29	96 $\pm$ 11	200 $\pm$ 93
2/9	25 $\pm$ 16 ⁺	24 $\pm$ 11	32 $\pm$ 21	64 $\pm$ 16	57 $\pm$ 6	80 $\pm$ 10*
3/1	102 $\pm$ 41	78 $\pm$ 14	149 $\pm$ 57	116 $\pm$ 21	127 $\pm$ 23	211 $\pm$ 75
3/2	44 $\pm$ 16 ⁺	43 $\pm$ 13	104 $\pm$ 90	138 $\pm$ 28	156 $\pm$ 22	253 $\pm$ 78
3/3	28 $\pm$ 12 ⁺	26 $\pm$ 12	91 $\pm$ 79	164 $\pm$ 30	158 $\pm$ 23	268 $\pm$ 63*
3/4	30 $\pm$ 22 ⁺	24 $\pm$ 17	82 $\pm$ 70	171 $\pm$ 39	149 $\pm$ 33	278 $\pm$ 75*
3/5	13 $\pm$ 11 ⁺	15 $\pm$ 11	45 $\pm$ 50	116 $\pm$ 25	88 $\pm$ 22	226 $\pm$ 81*
3/6	78 $\pm$ 35 ⁺	44 $\pm$ 21	94 $\pm$ 55	99 $\pm$ 22	86 $\pm$ 18	138 $\pm$ 24*
3/7	51 $\pm$ 9 ⁺	45 $\pm$ 19	82 $\pm$ 45	109 $\pm$ 35	72 $\pm$ 13	131 $\pm$ 39*
3/8	67 $\pm$ 18	63 $\pm$ 9	134 $\pm$ 47	88 $\pm$ 22	76 $\pm$ 11	162 $\pm$ 55*
3/9	74 $\pm$ 43	47 $\pm$ 11	83 $\pm$ 40	83 $\pm$ 34	57 $\pm$ 8	89 $\pm$ 25
4/1	88 $\pm$ 35	84 $\pm$ 16	177 $\pm$ 56*	103 $\pm$ 16	112 $\pm$ 20	205 $\pm$ 58*
4/2	49 $\pm$ 18 ⁺	54 $\pm$ 16	115 $\pm$ 75	115 $\pm$ 23	138 $\pm$ 19	198 $\pm$ 45
4/3	32 $\pm$ 7 ⁺	36 $\pm$ 12	90 $\pm$ 58	158 $\pm$ 54	171 $\pm$ 34	271 $\pm$ 110
4/4	38 $\pm$ 6 ⁺	40 $\pm$ 17	101 $\pm$ 71	170 $\pm$ 49	177 $\pm$ 59	281 $\pm$ 121
4/5	27 $\pm$ 10 ⁺	30 $\pm$ 14	49 $\pm$ 31	109 $\pm$ 29	93 $\pm$ 22	256 $\pm$ 91*
4/6	76 $\pm$ 15 ⁺	64 $\pm$ 17	160 $\pm$ 63*	110 $\pm$ 23	95 $\pm$ 23	215 $\pm$ 69*
5/1	66 $\pm$ 31 ⁺	82 $\pm$ 38	208 $\pm$ 95*	120 $\pm$ 32	146 $\pm$ 27	238 $\pm$ 96*
5/2	42 $\pm$ 23 ⁺	53 $\pm$ 17	142 $\pm$ 85	126 $\pm$ 26	165 $\pm$ 31	249 $\pm$ 81
5/3	40 $\pm$ 23 ⁺	46 $\pm$ 17	111 $\pm$ 67	128 $\pm$ 15	152 $\pm$ 36	271 $\pm$ 124
5/4	50 $\pm$ 13 ⁺	60 $\pm$ 24	135 $\pm$ 78	111 $\pm$ 11	127 $\pm$ 29	234 $\pm$ 131
5/5	48 $\pm$ 6 ⁺	57 $\pm$ 14	114 $\pm$ 89	109 $\pm$ 25	111 $\pm$ 24	245 $\pm$ 109*

The level/ROI locations are shown in Figure 1.

+ indicates $p < 0.01$ for comparison between ischemic and non-ischemic hemispheres in normal rats.

indicates $p < 0.01$ for comparison between untreated and carrier-receiving control rats.

* indicates $p < 0.01$ for comparison between rats receiving carrier and CGS-19755 in each hemisphere.

Table 2. Local cerebral pH (mean $\pm$ SD) in rats with MCA + CCA occlusions receiving no treatment, carrier and CGS-19755 [27]

Level/ ROI	Ischemic hemisphere			Non-ischemic hemisphere		
	Control	Carrier	CGS-19755	Control	Carrier	CGS-19755
1/1	6.99 $\pm$ 0.02	7.01 $\pm$ 0.04	7.05 $\pm$ 0.03	7.01 $\pm$ 0.04	7.04 $\pm$ 0.04	7.06 $\pm$ 0.04
1/2	6.88 $\pm$ 0.10+	6.94 $\pm$ 0.06	7.02 $\pm$ 0.06	7.02 $\pm$ 0.03	7.05 $\pm$ 0.04	7.05 $\pm$ 0.03
1/3	6.85 $\pm$ 0.11+	6.92 $\pm$ 0.05	7.02 $\pm$ 0.08	7.03 $\pm$ 0.04	7.06 $\pm$ 0.04	7.05 $\pm$ 0.04
1/4	6.91 $\pm$ 0.15	6.98 $\pm$ 0.07	6.98 $\pm$ 0.10	7.02 $\pm$ 0.05	7.08 $\pm$ 0.04	7.03 $\pm$ 0.03
1/5	6.79 $\pm$ 0.14+	6.94 $\pm$ 0.07	6.95 $\pm$ 0.09	7.02 $\pm$ 0.04	7.05 $\pm$ 0.03	7.05 $\pm$ 0.04
1/6	6.90 $\pm$ 0.08+	7.00 $\pm$ 0.05	7.00 $\pm$ 0.06	7.01 $\pm$ 0.04	7.06 $\pm$ 0.04	7.05 $\pm$ 0.04
2/1	7.01 $\pm$ 0.03	7.01 $\pm$ 0.04	7.03 $\pm$ 0.04	7.03 $\pm$ 0.02	7.09 $\pm$ 0.04#	7.07 $\pm$ 0.03
2/2	6.90 $\pm$ 0.09+	6.94 $\pm$ 0.03	7.01 $\pm$ 0.06	7.04 $\pm$ 0.03	7.06 $\pm$ 0.04	7.04 $\pm$ 0.03
2/3	6.83 $\pm$ 0.13+	6.82 $\pm$ 0.08	7.00 $\pm$ 0.09*	7.05 $\pm$ 0.04	7.04 $\pm$ 0.03	7.05 $\pm$ 0.04
2/4	6.83 $\pm$ 0.07+	6.81 $\pm$ 0.12	6.94 $\pm$ 0.15	7.06 $\pm$ 0.03	7.03 $\pm$ 0.04	7.05 $\pm$ 0.04
2/5	6.91 $\pm$ 0.09+	6.91 $\pm$ 0.09	6.91 $\pm$ 0.14	7.06 $\pm$ 0.04	7.05 $\pm$ 0.04	7.06 $\pm$ 0.02
2/6	6.80 $\pm$ 0.06+	6.80 $\pm$ 0.03	6.84 $\pm$ 0.12	7.06 $\pm$ 0.04	7.04 $\pm$ 0.04	7.04 $\pm$ 0.05
2/7	6.70 $\pm$ 0.10+	6.79 $\pm$ 0.05	6.79 $\pm$ 0.15	7.07 $\pm$ 0.04	7.02 $\pm$ 0.05	7.04 $\pm$ 0.04
2/8	6.85 $\pm$ 0.06+	6.86 $\pm$ 0.03	6.91 $\pm$ 0.09	7.06 $\pm$ 0.04	7.02 $\pm$ 0.03	7.04 $\pm$ 0.04
2/9	6.92 $\pm$ 0.08+	6.92 $\pm$ 0.04	6.91 $\pm$ 0.05	7.03 $\pm$ 0.04	6.98 $\pm$ 0.03	6.98 $\pm$ 0.04
3/1	7.00 $\pm$ 0.03	7.02 $\pm$ 0.04	7.05 $\pm$ 0.04	7.01 $\pm$ 0.03	7.05 $\pm$ 0.05	7.04 $\pm$ 0.04
3/2	6.92 $\pm$ 0.07+	6.96 $\pm$ 0.03	7.04 $\pm$ 0.06*	7.03 $\pm$ 0.03	7.07 $\pm$ 0.05	7.05 $\pm$ 0.04
3/3	6.93 $\pm$ 0.12+	6.96 $\pm$ 0.14	7.13 $\pm$ 0.07	7.11 $\pm$ 0.06	7.15 $\pm$ 0.04	7.13 $\pm$ 0.04
3/4	6.97 $\pm$ 0.10+	6.98 $\pm$ 0.12	7.15 $\pm$ 0.07	7.13 $\pm$ 0.06	7.14 $\pm$ 0.06	7.14 $\pm$ 0.04
3/5	7.10 $\pm$ 0.10	7.05 $\pm$ 0.05	7.15 $\pm$ 0.10	7.15 $\pm$ 0.05	7.12 $\pm$ 0.07	7.14 $\pm$ 0.04
3/6	7.10 $\pm$ 0.07	7.03 $\pm$ 0.11	7.13 $\pm$ 0.06	7.12 $\pm$ 0.04	7.11 $\pm$ 0.06	7.12 $\pm$ 0.04
3/7	7.11 $\pm$ 0.07	7.07 $\pm$ 0.05	7.13 $\pm$ 0.07	7.12 $\pm$ 0.04	7.10 $\pm$ 0.06	7.13 $\pm$ 0.07
3/8	7.14 $\pm$ 0.03	7.13 $\pm$ 0.03	7.13 $\pm$ 0.05	7.14 $\pm$ 0.03	7.14 $\pm$ 0.03	7.14 $\pm$ 0.04
3/9	7.06 $\pm$ 0.05	7.06 $\pm$ 0.05	7.07 $\pm$ 0.05	7.08 $\pm$ 0.04	7.06 $\pm$ 0.05	7.08 $\pm$ 0.07
4/1	7.08 $\pm$ 0.04	7.11 $\pm$ 0.04	7.13 $\pm$ 0.05	7.10 $\pm$ 0.04	7.13 $\pm$ 0.05	7.14 $\pm$ 0.03
4/2	7.01 $\pm$ 0.04+	7.05 $\pm$ 0.03	7.13 $\pm$ 0.03*	7.10 $\pm$ 0.03	7.15 $\pm$ 0.05	7.14 $\pm$ 0.03
4/3	6.99 $\pm$ 0.07+	6.98 $\pm$ 0.04	7.14 $\pm$ 0.03	7.12 $\pm$ 0.04	7.15 $\pm$ 0.05	7.15 $\pm$ 0.02
4/4	7.02 $\pm$ 0.08	7.01 $\pm$ 0.06	7.13 $\pm$ 0.04	7.11 $\pm$ 0.05	7.14 $\pm$ 0.06	7.15 $\pm$ 0.02
4/5	7.10 $\pm$ 0.04	7.10 $\pm$ 0.07	7.20 $\pm$ 0.05	7.13 $\pm$ 0.08	7.13 $\pm$ 0.07	7.18 $\pm$ 0.02
4/6	7.14 $\pm$ 0.05	7.12 $\pm$ 0.09	7.17 $\pm$ 0.03	7.14 $\pm$ 0.05	7.13 $\pm$ 0.08	7.16 $\pm$ 0.02
5/1	7.04 $\pm$ 0.04+	7.10 $\pm$ 0.03#	7.13 $\pm$ 0.06	7.11 $\pm$ 0.04	7.16 $\pm$ 0.05	7.13 $\pm$ 0.04
5/2	6.97 $\pm$ 0.07+	7.05 $\pm$ 0.04	7.13 $\pm$ 0.05*	7.11 $\pm$ 0.05	7.16 $\pm$ 0.05	7.13 $\pm$ 0.05
5/3	6.98 $\pm$ 0.07+	7.04 $\pm$ 0.03	7.15 $\pm$ 0.06*	7.12 $\pm$ 0.05	7.14 $\pm$ 0.04	7.14 $\pm$ 0.04
5/4	7.02 $\pm$ 0.06	7.09 $\pm$ 0.04	7.15 $\pm$ 0.05	7.11 $\pm$ 0.05	7.15 $\pm$ 0.05	7.14 $\pm$ 0.04
5/5	7.10 $\pm$ 0.05	7.13 $\pm$ 0.04	7.13 $\pm$ 0.08	7.12 $\pm$ 0.06	7.15 $\pm$ 0.07	7.13 $\pm$ 0.02

The level/ROI locations are shown in Figure 1.

+ indicates $p < 0.01$ for comparison between ischemic and non-ischemic hemispheres in normal rats.

indicates $p < 0.01$ for comparison between untreated and carrier-receiving control rats.

* indicates $p < 0.01$ for comparison between rats receiving carrier and CGS-19755 in each hemisphere.

Cerebral pH and blood flow

Tables 1 and 2 show LCBF and LCpH values respectively in rats with MCA and CCA occlusions receiving no treatment, carrier or CGS-19755 infusion. LCpH and LCBF values in the no-treatment and carrier control groups were identical except in three structures, where significant differences ($p < 0.01$) were noted: 5/1 on the occluded side and 2/1 on the opposite hemisphere, where pH was 7.04 ± 0.04 and 7.03 ± 0.02 respectively in the no-treatment group and increased to 7.10 ± 0.03 and 7.09 ± 0.04 in the carrier group. LCBF was different in only one structure between the no-treatment carrier groups, increasing in 1/4 from 19 ± 9 to 41 ± 16 ml/100 g/min, respectively.

LCBF was significantly increased in 18 structures within the ischemic and non-ischemic hemispheres of rats receiving CGS-19755 compared to those receiving carrier. It can be seen that in response to CGS-19755, the average blood flow increased in all structures in both the ischemic and non-ischemic hemispheres. CGS-19755 significantly increased the mean LCBF of the entire non-occluded hemisphere from 122 ± 17 ml/100 g/min in the carrier group to 221 ± 64 ml/100 g/min ($p < 0.01$). The drug did not affect LCpH in the non-ischemic hemisphere structures but on the ischemic side, LCpH was significantly raised to non-ischemic values in 7 of the 35 structures.

Histological studies

The percent volume of the gray matter areas in the non-ischemic hemisphere, defined as cortex basal ganglia, which were infarcted on the ischemic hemisphere are shown in Table 3. Compared to those rats receiving carrier, infusion of CGS-19755 significantly reduced the cortical volume of infarction but did not affect the volume of the basal ganglia infarct.

4. Discussion

Our data suggest that CGS-19755, in addition to any NMDA receptor blockade it may cause, increased LCBF and corrected LCpH in the moderately ischemic regions of the brain. The marked impairment of perfusion in the caudate putamen probably prevents sufficient delivery of CGS-19755 [31] explaining the lack of any effect of this drug on LCBF or LCpH in this region. It is not possible from these data to decide categorically if the change in LCpH we report is only secondary to improvement in LCBF or if the drug has an additional selective influence on hydrogen ion homeostasis. Several factors probably contribute to the observed effects of CGS-19755 on LCpH and LCBF. Increasing perfusion may decrease production of lactate by alleviating the oligemia or may increase its washout. Ischemic thresholds for ionic homeostasis may be such that only small increases in CBF could bring about complete correction of pH. However, since LCpH also improved in regions where LCBF did not change significantly, additional

Table 3. The percent of control basal ganglia and cortex volume that is infarcted [27]

	N	Cortex	Basal ganglia
Control group	7	28.6 ± 3.1	36.4 ± 2.6
Carrier group	6	24.0 ± 4.6	38.4 ± 3.1
CGS-19755 group	6	13.2 ± 1.88*	33.6 ± 3.9

mechanisms may involved. It has been reported that non-competitive NMDA antagonists, such as MK-801, have a hypothermic effect in rats with global ischemia [38], but we have not observed in our model any change in brain temperature with this drug. Unlike MK-801, most competitive NMDA antagonists penetrate the blood-brain barrier relatively poorly [28, 29]. We have reported preliminary data showing that CGS-19755, infused into normal and MCA + CCA occluded rats, had very low plasma clearance and low volumes of distribution in all brain regions sampled [39]. Despite this, a number of researchers have reported this drug's metabolic and therapeutic effects. Boast et al. [40] demonstrated that CGS-19755, given intra-peritoneally in gerbils, had more potent neuroprotective effect than CPP. This drug also had a tenfold greater potency than 2-amino-7-phosphonoheptanoate (AP-7) in inhibiting [3 H]CPP binding to NMDA receptors and in blocking NMDA induced convulsions [33]. CGS-19755 has also been reported to attenuate postischemic cerebral glucose hypermetabolism [31]. Thus, CGS-19755 brings about metabolic effects which may secondarily improve LCBF and LCpH. We must conclude that the small amounts of CGS-19755 that pass through the blood-brain barrier protect against ischemic damage by decreasing ischemic acidosis in addition to any blockade of NMDA-linked ion channels. Our findings do suggest that in addition CGS-19755 may protect against ischemic damage by potentially increasing perfusion.

Finally, our data show a statistically significant reduction of infarct volume in the cortex with CGS-19755. Simon and Siraishi [31] have shown in a histological study that treatment with CGS-19755 resulted in reduced infarct size at 72 hours following MCA occlusion, which is in agreement with our results. The reduction in infarct volume, which was limited to the cortex, suggest that this drug is neuroprotective primarily in areas suffering moderate ischemia.

III. The relationship between glutamate neurotoxicity and oxygen free radicals in focal ischemia

1. Introduction

As mentioned in part I of this paper, both oxygen-derived free radicals [22-26] and excitatory amino acid [9-12] have been implicated as important mechanisms of neuronal damage in ischemia. These mechanisms, however, have been

independently studied, and the relationship between them is still unclear. Recent studies suggest that oxygen free radicals generated from xanthine/xanthine-oxidase stimulated the release of excitatory amino acids and free radical scavengers prevented the release in in-vitro hippocampal slices of rats [41, 42]. To clarify the causal relation between glutamate neurotoxicity and oxygen free radicals, we evaluated the effects of recombinant Cu,Zn superoxide dismutase (SOD) on the extracellular glutamate using a microdialysis technique and on cerebral blood flow using a hydrogen clearance method in in-vivo ischemic models.

2. Materials and methods

Male Sprague-Dawley rats, Weighing approximately 350 grams, were used in this study. On the day prior to the experiment, a microdialysis probe (Eicom Co., Ltd., Kyoto, Japan) with a tubular dialysis membrane (3 mm long and 0.2 mm in diameter) and a platinum electrode (UHE-100, Unique Medical Co. Ltd., Tokyo, Japan; 0.1 mm in diameter, insulated to within 1 mm of the tip) were assembled with a tip-to-tip distance of 1 mm. A probe/electrode assembly was placed stereotaxically through a burr hole in the cortex in right MCA territory (anteroposterior bregma -3.5 lateral 4.0, depth 5.0 mm at 30 ° laterally from the sagittal plane). This probe/electrode position corresponds to the core of the eventual infarction as determined by TTC staining. Simultaneously, the thermocouple probe (52 K/J thermometer; John Fluke, WA, U.S.A.) was placed into the subdural space through the same burr hole. On the day of the experiment, a rat was anesthetized, and polyethylene catheters were placed in both femoral arteries for physiological monitorings and one femoral vein for drug administrations. A rat was then tracheostomized, paralyzed with pancuronium bromide (0.6 mg/kg intravenously), and ventilated with a mixture of 30% oxygen, 70% nitrous oxide and 0.5% halothane. The focal cerebral ischemia was produced using method described by Chen et al. [43]. Briefly, the right distal portion of MCA above the rhinal fissure was ligated with a 10-0 silk suture. This was immediately followed by the permanent ligation of the right CCA and temporary clip compression of the left CCA for 60 minutes. During the experiment, brain and rectal temperatures were controlled at about 37 °C with an external heating lamp and a heating pad, respectively. These temperatures and MABP were continuously monitored. Arterial gases, blood glucose level and hematocrit were examined pre-occlusion, and at 30 and 90 min after occlusion.

Experimental design

Animals were divided into three experimental groups: Group 1 (n = 7) was administered recombinant SOD (Nihonkayaku Co., Ltd., Japan). Each rat was given a 0.2 ml bolus dose of 24000 U/kg of SOD intravenously 15 min prior to occlusion following continuous infusion of 1600 U/kg/min in a volume of 10 ml/min until release of left CCA occlusion. Group 2 (n = 8) was the untreated group with the

same occlusion as group 1. Group 3 ($n = 6$) was the sham-operated group. Using a warming pad, rectal temperature was maintained at about 37.5°C during the experiment.

In-vivo microdialysis and cerebral blood flow measurement

The cortex was perfused with Ringer solution (155 mM NaCl; 5.5 mM KCl; 2.3 mM CaCl_2), which was warmed to approximately 36°C . The dialysates were collected at 10-min intervals into a plastic tube, and then frozen at -70°C . Analysis of glutamate concentration in the dialysates was performed by HPLC with fluorescence detection after precolumn derivatization with o-phthalaldehyde [44]. The response of glutamate was calibrated with an external standard.

The regional CBF was measured by a hydrogen clearance method with a digital tissue blood flow meter (digital UH-meter model NMG-D1; Unique Medical Co. Ltd., Tokyo, Japan). After a 10% hydrogen gas mixture had been administered for 2 min, a hydrogen clearance curve was charted. The CBF was automatically calculated from the clearance curve according to the formula of Aukland et al. [45].

Infarcted volume

Four hours after occlusions a rat was perfused with 0.01 M phosphate-buffered saline (pH 7.2) through the descending aorta under anesthesia. The brain was then removed and dissected in the coronal plane at 2-mm intervals started at 3 mm from the frontal tip, including the infarcted lesion. The brain slices were incubated in 2% TTC in phosphate-buffered saline at 37°C for 15 min and then stored in 10% neutral-buffered formalin. Both surfaces of the 2-mm thick TTC-stained sections were photographed using color slide film. The volume of infarct in the cerebral cortex was determined by the method of Swanson et al. [37] using a computer-assisted image analysis system (MCID, Imaging Research, Canada).

Statistical analysis

The statistical significance of differences in physiological parameters, CBF, extracellular glutamate concentration and infarct volume was examined by one-way ANOVA followed by Scheffe's multiple comparison. All values were expressed as mean $\pm$ SD.

3. Results

Physiological parameters

There were no significant differences in physiological parameters including brain and rectal temperatures, arterial gas analysis, plasma glucose, and hematocrit among the three experimental groups.

Cerebral blood flow

Figure 3 shows CBF values in the pre-ischemic state, and at 50 and 240 min after MCA + both CCAs occlusions in the three experimental groups. In the pre-ischemic state, there were no significant differences in CBF among the three groups. The CBF values at 50 and 240 min after focal ischemia less than 10 ml/100 g/min in groups 1 and 2, with no significant differences.

Extracellular glutamate concentration

Figure 4 shows the extracellular glutamate concentration in the three groups. In the sham-operated group, extracellular glutamate concentration did not change throughout the experiment. After ischemia, extracellular glutamate concentrations in the non-treated group increase immediately and reached a peak level of $44.8 \pm 16.9 \mu\text{M}$ at 50 min of ischemia. In contrast, SOD administration significantly inhibited the increase in extracellular glutamate concentrations and reached a peak

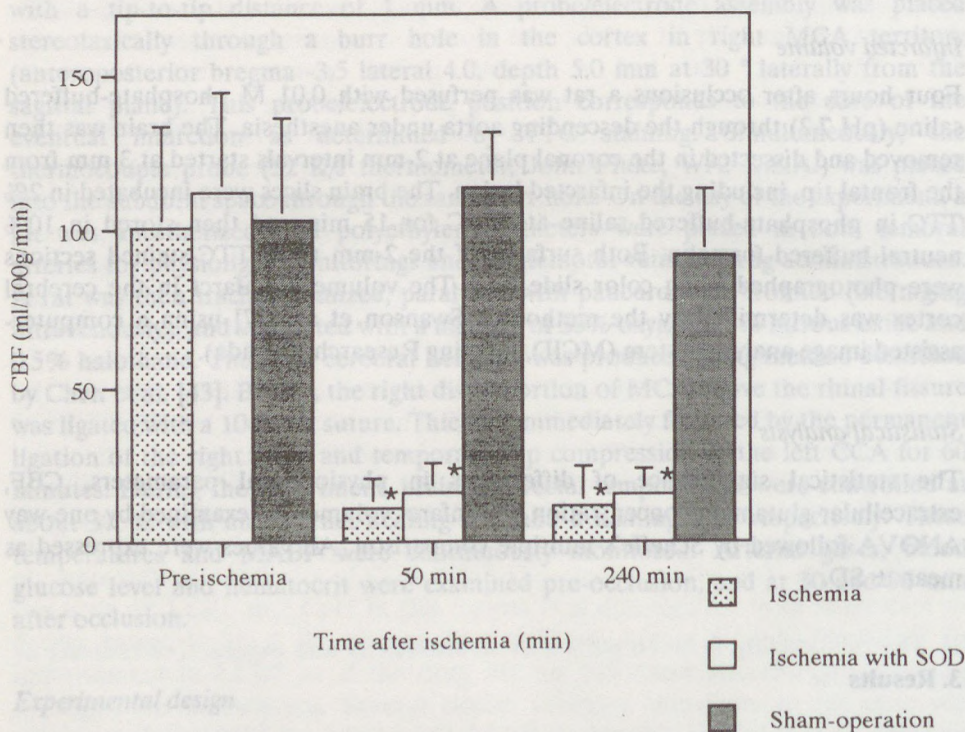


Figure 3. The changes in CBF values in the pre-ischemic state, and at 50 and 240 min after MCA + both CCAs occlusions in the three experimental groups. In the pre-ischemic state, there were no significant differences in CBF among the three groups. The CBFs at 50 and 240 min after focal ischemia were less than 10 ml/100 g/min in group 1 and 2 with no significant differences, although these values are significantly reduced compared with those in the sham-operated group

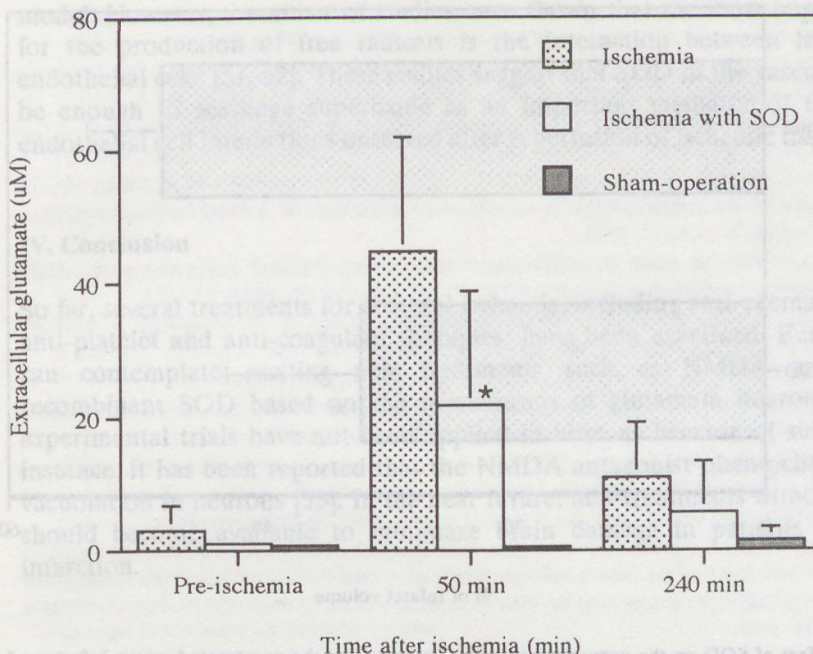


Figure 4. The extracellular glutamate concentrations in the three groups. In the sham-operated group, extracellular glutamate concentration did not change throughout the experiment. The extracellular glutamate concentrations in the non-treated group began to increase immediately and reached a peak level of $44.8 \pm 16.9 \mu\text{M}$ at 50 min of ischemia. In contrast, SOD administration significantly inhibited the increase in extracellular glutamate concentrations, which reached a peak level of $21.9 \pm 17.0 \mu\text{M}$ at 50 min of ischemia. * indicates $p < 0.01$ for comparison between the untreated group and the SOD-treated group

level of $21.9 \pm 17.0 \mu\text{M}$ at 50 min of ischemia ($p < 0.01$). Elevated glutamate concentrations in non-treated and SOD treated groups declined gradually after unclipping of the right CCA. At 240 min after occlusion, extracellular glutamate concentrations in non-treated and SOD treated groups were 10.8 ± 8.3 , and $5.6 \pm 7.6 \mu\text{M}$, respectively, the difference being without statistical significance.

Infarct volume

The percentage volume of cortex in the non-ischemic hemisphere is shown in Figure 5. Compared to the non-treated group, infusion of SOD significantly reduced the total volume of cortical infarction from $47.8 \pm 5.9\%$ to $32.2 \pm 10.6\%$.

4. Discussion

Our results suggest that oxygen free radicals may contribute to release of glutamate from presynaptic sites as a neurotoxic effect, in addition to causing direct damage to the membranes in neurons.

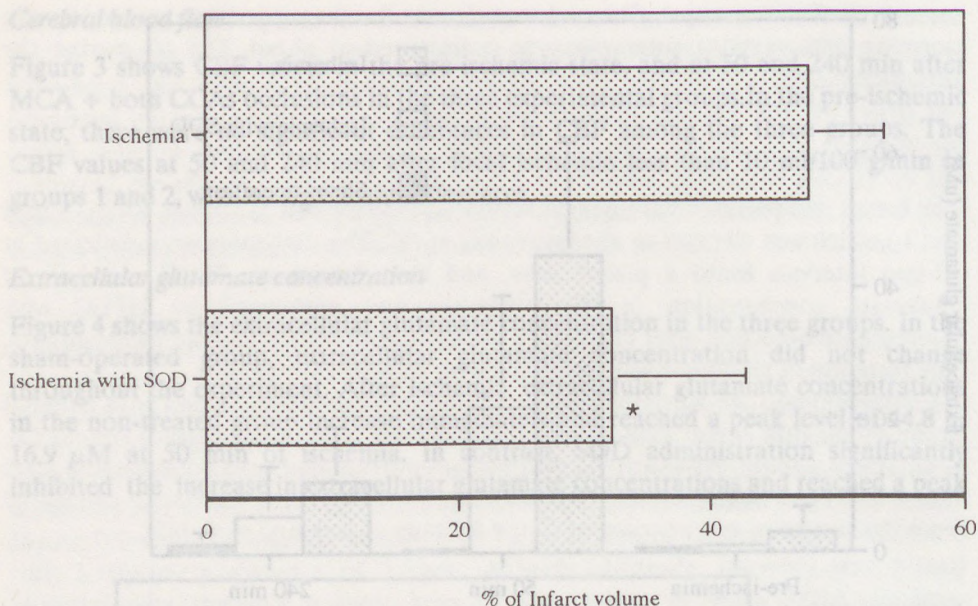


Figure 5. The effect of SOD on the percentage volume. Compared to the non-treated group, infusion of SOD significantly reduced the total volume of cortical infarction from $47.8 \pm 5.9\%$ to $32.2 \pm 10.6\%$. * indicates $p < 0.01$ for comparison between the untreated group and the SOD-treated group

The precise mechanism of the relationship between glutamate neurotoxicity and free radicals is not clear, although three possibilities should be considered. First, free radicals may inactivate the Ca^{2+} -ATPase as a major calcium transport system. It has been shown that activated oxygen can produce a decrease in Ca^{2+} -ATPase in the erythrocyte [46] as well as in the cardiac sarcoplasmic reticulum [47]. Second, free radicals may inhibit the Na^{+} - Ca^{2+} exchange system, which has been shown to be influenced by free radicals [48]. A third possibility concerns the damage to Na^{+} , K^{+} -ATPase by oxygen free radicals. Kim and Akera [49] have suggested that oxygen radicals play a crucial role in the injury of sarcolemmal Na^{+} , K^{+} -ATPase during reperfusion of ischemic cardiac tissue. If the Na^{+} pump is disturbed, intracellular Na^{+} will increase, leading to depolarization. Once depolarization occurs, the electrogenic $3 \text{ Na}^{+}/\text{Ca}^{2+}$ antiporter may be reversed [24, 28], VSCC will open, and the cytosolic free calcium will increase.

The effectiveness of free SOD was carefully evaluated in this study. The half-life of free SOD in-vivo is less than 10 min [50]. In addition, free SOD can poorly penetrate into brain tissue through the blood-brain barrier under normal conditions. Since SOD was continuously infused from 15 min prior to occlusions until release of left CCA occlusion following an initial bolus infusion of SOD in this study, SOD might reach the ischemic lesion and be effective. We did not verify whether free SOD injected intravenously could penetrate into the brain in this

model. However, a number of studies have shown that the most important source for the production of free radicals is the interaction between leukocytes and endothelial cells [51, 52]. These studies suggest that SOD in the vascular space may be enough to scavenge superoxide as an important mediator of the leukocyte-endothelial cell interactions observed after reperfusion of ischemic tissues.

IV. Conclusion

So far, several treatments for cerebral ischemia, including anti-edema, vasodilating, anti-platelet and anti-coagulant therapies, have been examined. Furthermore, we can contemplate exciting new treatments such as NMDA antagonist and recombinant SOD based on the mechanisms of glutamate neurotoxicity. These experimental trials have not been applied in humans because of side effects. For instance, it has been reported that the NMDA antagonist phencyclidine produced vacuolation in neurons [53]. In the near future, new treatments without side effects should become available to minimize brain damage in patients with cerebral infarction.

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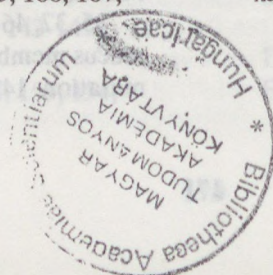
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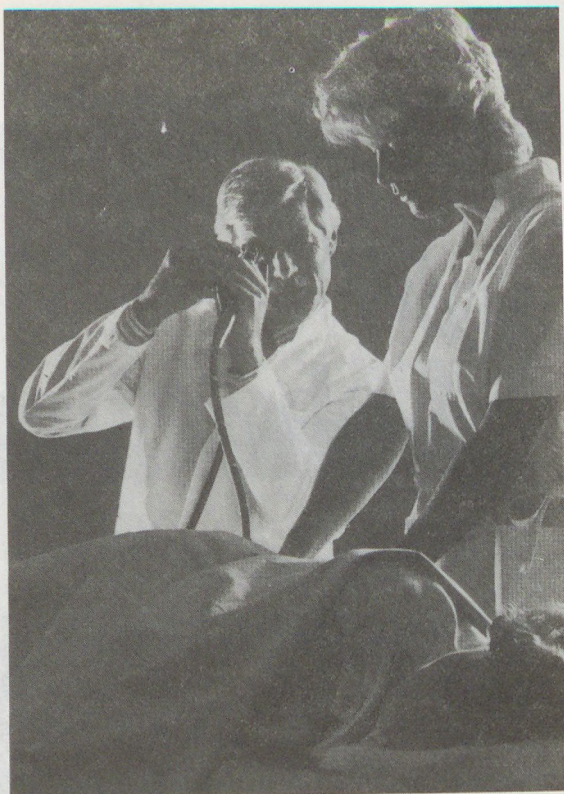
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