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ISCHAEMIA-REPERFUSION INJURY; CURRENT RESEARCH STATUS

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During March 7th–9th 1988, scientists gathered in Point Clear, Alabama, for the second biannual meeting of Free Radicals in Biology and Medicine: Ischaemia/Reperfusion Injury. Thanks to the generous sponsorship of the Pharmacia-Chiron Partnership, approximately 180 delegates were able to exchange ideas and information in a warm and relaxed setting. The meeting included two full days of oral presentations by the faculty and two half-day sessions with free poster communications from the attendees. A high point of the meeting was the presentation of the Pharmacia-Chiron Partnership young investigator travel award. This award was given in recognition of outstanding and original work by a young investigator working in the field of ischaemia/reperfusion injury. The recipient of this award was Roberto Bolli of Baylor College of Medicine in Houston. The meeting was organized by James M. Downey, Aubrey E. Taylor, and Joe M. McCord with the invaluable assistance of Mrs. Penny Cook, all of the University of South Alabama in Mobile. In view of the importance of many of the papers presented at this meeting, it has been decided to publish the abstracts of the posters.

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DETECTION OF FREE RADICALS BY DIRECT ESR DURING MYOCARDIAL CELL INJURY – A CRITICAL EVALUATION

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Electron spin resonance (ESR) spectroscopy has recently been used by others to detect directly radical species in isolated perfused hearts. Sample processing in this study involved grinding of tissue which can artifactually generate radical species. We assessed in isolated perfused hearts the influence of tissue grinding on the identity of radical species detected by spectroscopy and evaluated whether exposure of the processed sample to room air resulted in decay of the radical species. Rabbit hearts (n = 5/group) were perfused aerobically for 10 min followed by 10 min of global normothermic ischaemia and freeze-clamped to 77 K. Frozen tissue was processed at 77 K for spectroscopic analysis by grinding vs chopping. ESR spectra were then recorded at 77 K at 9.2 GHz and 100 KHz field modulation at non-saturating microwave power. Ground aerobically perfused and ischaemic myocardium consisted of a multi-line spectrum. Spectra of ground tissue consisted of three components: a semiquinone (g = 2.004); a lipid peroxy radical ($g_{\parallel}$ = 2.04 and $g_{\perp}$ = 2.006) and a carbon-centered radical, possibly a lipid radical (g_{iso} = 2.002 and A_{zz}^{H} = 50 G). Chopped aerobically perfused myocardium consisted of a symmetrical one-line spectrum, a semiquinone (g = 2.004) with ischaemic myocardium composed of a two component spectrum: a second semiquinone (g = 2.004) and an iron-sulfur center (g = 2.024). Increasing the duration of exposure of the ground sample to room air from 1 to 120 sec did not result in the appearance of the chopped sample spectrum. We conclude (i) that the failure to obtain the multi-line spectrum using the chopping procedure is not due to sample warming and (ii) mechanical grinding artifactually generates lipid peroxy radical formation.

MECHANISMS OF OXYGEN ADAPTATION IN RABBITS

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We sought to determine whether rabbits develop tolerance to hyperoxia and, if so, to identify the mechanisms involved. New Zealand white rabbits were exposed to 100% O_2 at one atmosphere (1 ATA) for 64 hours and then returned to room air for seven days. At this time, they were either sacrificed or reexposed to 100% O_2. Prior to reexposure, arterial blood gases (Pa_{O_2}, Pa_{CO_2}), total lung capacity and alveolar permeability to solute were within normal limits. Preexposed rabbits (n = 7) survived significantly longer than controls (n = 3) in 100% O_2 (166 ± 26 vs. 89 ± 13 h) and had normal Pa_{O_2} and Pa_{CO_2} values for the first 120 hours of exposure. In control rabbits, Pa_{O_2} declined after 72 hours in 100% O_2, reaching values of 20–30 mm Hg just prior to death.

To investigate a possible mechanism that may account for the increased tolerance to hyperoxia, lung homogenates and isolated alveolar type II pneumocytes were assayed for CuZn and Mn superoxide dismutase (SOD) and catalase (CAT) activity. No significant increase in any of these values was demonstrated in O_2-tolerant rabbits either before or after 72 hours of 100% O_2 reexposure. However, total phospholipid levels from bronchoalveolar lavage were 217% higher than control prior to reexposure (26 ± 2 vs. 12 ± 2; $\bar{x}$ ± 1 SE; μmoles/kg) and 300% greater than control after reexposure to 100% O_2 for 72 hours (15 ± 2 vs. 5 ± 2; $\bar{x}$ ± 1 SE; μmoles/kg).

We concluded that rabbits may develop tolerance to hyperoxia without increases in lung SOD or catalase activity. Other factors, such as alveolar phospholipid levels may be important in the process of adaptation to oxidant stress. (supported by NIH HL 31197; a Career investigator Award by the American Lung Association of Southeast Florida; and a grant from the Toxicology Research Center, SUNYAB)

GRANULOCYTES AFFECT SURVIVAL AND CAPILLARY NO-REFLOW IN HEMORRHAGIC SHOCK.

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Recent evidence shows circulating granulocytes play an important role in capillary stasis and tissue injury. We investigated two aspects of the problem in a Wiggers hemorrhagic shock model in rats - the 24 hrs survival rate and the microvascular no-reflow phenomenon. Two groups were used to evaluate the effects of granulocytes: a conventional group (CONV) with normal blood cells, and a neutropenic group (NP) pretreated with intraperitoneal anti-granulocyte antibody. Two hemorrhagic shock protocols (HSP) were carried out: (a) HSP-1 (40 mmHg mean arterial pressure for 3 hrs), with a survival rate in the CONV group (n = 11) of 36% vs. 100% in the NP group (n = 6). (b) HSP-2 (30 mmHg mean arterial pressure for 7 hrs), with a survival rate of 0% in the CONV group (n = 8) vs. 100% in the NP group (n = 6).

In another series of rats the extent, location, and mechanism of the no-reflow phenomenon investigated by examining histological sections from several organs after infusion of a contrast medium to mark the flowing vessels in 3 groups; a control group without shock and, after 2 hrs of blood replacement in a CONV-Shock and NP-Shock group (7 hours of hypotension at 30 mmHg). All arterioles and venules were reperfused in all groups; only capillaries showed no-reflow.

Percent Capillaries with No-Reflow					
Group	Heart	Kidney	Pancreas	Abd. Muscle	T. Branchii
CONTROL	4.3 ± 0.3	6.8 ± 3.0	8.9 ± 1.4	6.3 ± 1.0	2.7 ± 0.6
CONV-Shock	14.4 ± 1.1	38.0 ± 3.2	32.7 ± 1.5	23.3 ± 4.4	16.0 ± 4.9
NP-Shock	5.4 ± 1.3	21.8 ± 5.0	15.5 ± 1.3	12.5 ± 3.6	2.7 ± 0.3

Abd. Muscle = Abdominal Muscle, T. Brachii = Triceps Brachii

$p < 0.005$ CONV-shock vs. NP-shock for all organs.

The obstruction of capillaries was largely due to trapped granulocytes suggesting that these leukocytes play a key role in the capillary no-reflow phenomenon and survival from hemorrhagic shock.

SOYBEAN FLOUR EFFECT ON SUPEROXIDE DISMUTASE IN ATHEROGENIC RABBITS

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Soybean polypeptides extracted from defatted soybean flour as well as crude soybean extract are known to possess superoxide dismutase activity. Soybean flour when added to the diet of rabbits has been found by others to counteract hypercholesterolemia induced by a casein rich diet and to inhibit atherosclerotic

development. In this study, eighteen male, New Zealand rabbits were maintained on an atherogenic diet for four weeks prior to dividing them into three equal groups for special dieting as follows: *Group 1.* Atherogenic control group fed 92% Purina Rabbit Chow, 2% cholesterol, 6% corn oil, *Group 2.* Atherogenic diet group with 10% soybean flour (Sigma Chem. Co.) added to the diet by weight, *Group 3.* Atherogenic diet group supplemented with 20% soybean flour. Rabbit blood platelets and plasma were isolated and analyzed twice weekly for six weeks during which these animals were fed atherogenic diet supplemented with soybean flour. In rabbits fed 10% soybean flour the blood plasma and platelet cholesterol levels were moderately lowered and were further reduced in rabbits given 20% soy flour diets as compared to control atherogenic dieted rabbits not given soy flour. Phospholipid levels in blood plasma and platelets were highest in the 20% soy flour supplemented rabbits and intermediate in the 10% supplement group with control rabbits showing the lowest levels. Plasminogen activator activity increased in the blood plasma and platelets of soy flour dieted rabbits as compared to atherogenic dieted controls. Soy flour added to the diet appears to have promoted plasminogen activator release from blood platelets into the plasma. In addition, feeding rabbits soy flour supplemented atherogenic diet resulted in a marked increase in the superoxide dismutase activity in rabbit red blood cells in comparison to the SOD activity in the red cells of atherogenic control rabbits.

XANTHINE OXIDASE AND OXYGEN FREE RADICALS IN CEREBRAL ISCHAEMIC INJURY.

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The protective effects of the oxygen free radical scavengers, polyethylene glycolconjugated superoxide dismutase (PEG-SOD) and PEG-catalase, were investigated in a new rat stroke model. Ligation of the right middle cerebral artery (MCA) at the level of the rhinal fissure and temporary clamping of both carotid arteries for 90 min yields a focal infarct of 188 $\pm$ 28 mm^3 (mean $\pm$ SEM) restricted to the right cortex. In a randomized, blinded study, infarct volume was reduced by 30% (to 132 $\pm$ 15 mm^3) in rats pretreated with PEG-SOD plus PEG-catalase, compared to 187 $\pm$ 13 mm^3 with inactivated PEG-SOD plus PEG-catalase ($p < 0.01$; $n = 18$). Infarct volume in the inactive enzyme group was the same as historical controls, indicating that the protection obtained with active PEG-SOD plus PEG-catalase resulted from scavenging of O_2^- and/or H_2O_2 rather than a nonspecific protein artifact. Three hours after release of the carotid clamps, the fraction of xanthine dehydrogenase converted to the free radical-producing xanthine oxidase increased by 10% in the MCA territory of the right cortex but not in the left cortex. Using a sensitive fluorimetric assay, total xanthine dehydrogenase plus oxidase activity was 0.6 $\pm$ 0.2 nmol isoxanthopterin $min^{-1} g^{-1}$ cortex in control and ischaemic rat brain, but increased by 15% in right cortex after reperfusion for 3 hr. We found that rat plasma contained 6.5 $\pm$ 0.9 nmol isoxanthopterin $min^{-1} ml^{-1}$ of XDH + XO activity of which 90% was in the free radical-producing oxidase form. The increased XO activity in the right cortex may be due to plasma XO entrapped as the blood-brain barrier (BBB) becomes more permeable. Normally, plasma concentrations of hypoxanthine are too low to produce oxygen radicals but during cerebral ischaemia hypoxanthine formed by ATP degradation is released into microvessels. The amount of xanthine oxidase found in microvessels of the ischaemic right cortex can generate 10 and 24 μM/min of O_2^- and H_2O_2 respectively, more than sufficient to destroy barrier function in cultured endothelial cells. Thus, we hypothesize that free radical formation by xanthine oxidase contributes to the breakdown of the blood-brain barrier that occurs several hours after reperfusion of ischaemic brain tissue.

FREE RADICAL GENERATING SOLUTIONS INDUCE ALTERATIONS IN IONIC CALCIUM TRANSIENTS IN CULTURED NEONATAL RAT VENTRICULAR MYOCYTES.

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The ability of free radicals to initiate arrhythmias and also to cause severe membrane permeability alterations was evaluated by examining ionic calcium (Ca) transients associated with contraction in spontaneously beating cells using the fluorescent indicator, fura-2. Neonatal rat ventricular myocytes grown on glass coverslips for 3 days were preloaded with the acetoxymethylester of fura-2 in medium 199 for 1 hr followed by equilibration in fura-free medium for 1 hr. The coverslips were mounted in Sykes-Moore chambers and placed on a heated stage of an inverted Nikon Diaphot microscope with UV optics. The cells were superfused at 1 ml/min with control medium or with a free radical generating medium containing 2.3 mM purine, 0.01 U/ml xanthine oxidase and 2.4 μM iron-loaded transferrin (n = 6). A Tracor-Northern Fluoroplex III-1000 was used to measure the ionic calcium transients. The 340 and 380 nm excitation illumination was chopped at a rate of 40 revolutions/sec, and the emission measured using photon counting. Data were acquired for 3 sec intervals at various times during the control and experimental periods. The ionic concentration was reflected by the 340 nm (Ca^{++}-bound)/380 nm (Ca^{++}-free) ratio. The fura-2 ratio was 0.84 $\pm$ 0.14 (mean $\pm$ SE) during relaxation and 1.37 $\pm$ 0.15 at peak contraction in control states. Exposure of cells to free radical generating solutions resulted in both increases and decreases i the beating rates during the exposure period. Fura-2 ratios were 64.41 $\pm$ 19.86% of the control relaxation ratio and 79.90 $\pm$ 4.59% of the control peak contraction ratio during abnormally slow beating rates. After 70 to 90 min of treatment, the cell showed evidence of contracture and bleb formation. Ruptured blebs also were evident. Normal calcium transients ceased at this point and the cells became calcium loaded as evidenced by a fura ratio of 432.48 $\pm$ 83.10% of the control relaxation ratio. Returning the cells to normal medium did not result in a reversal of the calcium loading. Thus, free radical generating systems were found to alter the pattern of the normal ionic calcium transients in association with irregular beating activity. Prolonged exposure resulted in irreversible damage.

ADENINE NUCLEOTIDE CATABOLISM IN RAT SKELETAL MUSCLES DURING ISCHAEMIA AND REPERFUSION.

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The metabolic pathway for breakdown of ATP to uric acid was studied in an *in vivo* model of skeletal muscle ischaemia and reperfusion in the rat. Hind limb ischaemia was induced by a tourniquet applied tightly as proximal as possible around the limb. Two and 4 h of ischaemia followed by 1 h of reperfusion was studied. The soleus and tibialis muscles were excised for analysis of nucleotides by HPLC-technique. During ischaemia there was a loss in ATP and an increase in IMP, inosine, adenosine, hypoxanthine, xanthine and uric acid. The major loss in adenine nucleotides appeared as IMP and inosine. This pattern was amplified after 4 compared to 2 h of ischaemia and was also more pronounced in the tibialis compared

to the soleus muscle. The accumulation of hypoxanthine and xanthine corresponded to about 10% of the normal content of adenine nucleotides (TAN = ATP + ADP + AMP) after 2 h, and 20% after 4 h of ischaemia in both muscles. After 1 h of reperfusion the recovery of TAN was 87 and 49% in the soleus, and 55 and 22% in the tibialis, after 2 and 4 h of ischaemia, respectively. The IMP, inosine, hypoxanthine and xanthine levels were still above the control levels. The results demonstrate that adenine nucleotides are broken down mainly to IMP and inosine during skeletal muscle ischaemia and that a certain restoration of ATP occurs during reperfusion, depending on the length of the ischaemic period and also the fibre composition of the muscle. Precursors for oxygen radical damage in the form of hypoxanthine and xanthine are accumulating in muscle tissue during ischaemia.

RENAL HANDLING OF RECOMBINANT HUMAN SUPEROXIDE DISMUTASE (r-hSOD) IN NORMAL AND ISCHAEMIC KIDNEY.

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Plasma elimination kinetics of r-hSOD were studied in rats, rabbits and humans under normal and pathophysiological conditions. The initial volume of distribution corresponded to the plasma volume. The initial half-life, volume of distribution and the plasma clearance was independent of the dose. After a single intravenous dose most of the r-hSOD was distributed to and eliminated by the kidneys, as shown by whole body autoradiography.

Microautoradiography demonstrated that the enzyme was reabsorbed by the proximal tubular cells, which was also verified by immune histochemistry. The radioactivity not found in the kidney was recovered in the urine as intact enzyme. In micropuncture studies it was found that the sieving coefficient equaled the filtration fraction in steady state and was about 0.25. This shows that the renal extraction is solely by way of glomerular filtration, in agreement with the normal renal handling of a protein with a molecular weight of 32 000 and a valence of −4, showing some charge restriction to filtration.

In rats subjected to bilateral kidney clamping the plasma half-life was prolonged from 7 to 35 min. In rabbits subjected to various periods of kidney ischaemia and reperfusion the half-life of r-hSOD varied in proportion to the kidney function as assessed by the creatinine clearance.

URATE RELEASE BY HUMAN HEART REDUCED BY DILTIAZEM.

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The oxidase form of xanthine oxidoreductase generates free radicals, which could be atherogenic. In the heart of various species, the enzyme is present (1). However, its occurrence in human heart is controversial (2, 3). We measured the arterial-venous (A-V) difference in plasma urate, the end-product of the reaction catalyzed by xanthine oxidoreductase, across the heart of 27 catheterized patients. Before percutaneous transluminal coronary angioplasty, the arterial and venous urate concentrations did not differ. After

several dilation attempts, the hearts produced significant amounts of urate, indicating increased purine catabolism. The calcium entry blocker diltiazem (0.4 mg/kg i.v.) suppressed this sign of ischaemia completely. We conclude that xanthine oxidoreductase is presumably present in the heart of (a number of) cardiac patients. Diltiazem seems to inhibit ATP breakdown and thus diminish the production of substrate for the enzyme.

References

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AGE RELATED DIFFERENCES IN ISCHAEMIA AND REPERFUSION INJURY

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The effect of ischaemia and reperfusion on adult and new born myocardium has been studied with differing results. We studied the effects of 30 minutes of normothermic ischaemic arrest and reperfusion in an isolated non-working heart model in newborn (3-5 day, n = 6), 2-3 wk old (n = 6) and adult (2-3 mth, n = 6) rabbits. We measured pre and post ischaemic (30 min. reperfusion) high energy phosphate content (ATP, ADP, AMP) and peak isovolumic developed pressure in all three groups and conjugated diene (product of lipid peroxidation) production pre and post ischaemia (10 min reperfusion) in the new born and adult groups. The results are tabulated below:

	PRE-ISCHAEMIA			END-ISCHAEMIA			REPERFUSION		
	ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP
Nb	17.1 ± 1.6	5.7 ± 1.3	1.4 ± 0.6	8.7 ± 1.7	6.1 ± 1.0	3.1 ± 0.5	15.5 ± 1.5	4.7 ± 0.7	1.5 ± 0.5
2 wk	16.5 ± 1.7 *	4.8 ± 0.9	2.0 ± 0.6	6.5 ± 1.1	5.7 ± 0.6 *	4.0 ± 0.5 *	13.4 ± 0.9 *	3.1 ± 0.4	0.7 ± 0.2
Ad	10.9 ± 1.1	3.9 ± 0.6	0.7 ± 0.1	5.3 ± 1.3	3.4 ± 0.7	1.6 ± 0.2	6.4 ± 1.1	3.4 ± 0.4	1.2 ± 0.3

Diene Content (pmol/mg wet wt)	Pre-Ischaemia		10 min Reperfusion
Newborn (n = 5)	1.1 ± 0.2	*	8.7 ± 2.6 *
Adult (n = 5)	1.4 ± 1.0		3.7 ± 1.0

* - $p < 0.05$ between adjacent values; ATP, ADP, AMP-mmol/kg dry wt.
all values mean ± SEM; Nb-newborn, Ad-adult

Peak developed pressure returned to 85% and 89% of control in newborn and 2 wk old hearts respectively, compared to 66% of control in adult hearts after 30 minutes of reperfusion. We conclude that immature hearts recover high energy phosphate content and ventricular function better than adult hearts despite a greater amount of lipid peroxidation products (free radical injury) in the newborn hearts.

A THREE STEP SYNTHESIS OF ^{14}C-TETRACYANOETHYLENE WITH A HIGH SPECIFIC ACTIVITY SUITABLE FOR QUANTITATION OF CONJUGATED DIENES IN BIOLOGICAL SYSTEMS.

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Oxygen mediated free radical injury is important in reperfusion injury of the heart, kidney and liver, in adult respiratory distress syndrome, cancer, aging and possibly in transplant rejection. Currently there is no simple or accurate method of quantitating the onset and intensity of free radical induced damage. Waller and Recknagel published a rapid and specific Diels-Alder condensation method using ^{14}C-tetracyanoethylene (^{14}TCNE) to measure conjugated dienes. Unfortunately their synthesis of ^{14}TCNE is costly, inefficient, and difficult, yielding a product of low specific activity.

We are reporting a new ^{14}C-TCNE synthetic technique, analytical method and some experimental and clinical results. ^{14}C-TCNE is synthesized in a three step procedure: 1) formation of ^{14}C-malononitrile from K^{14}CN and dichloromethane catalyzed by a phase transfer reaction; 2) bromination reaction of malononitrile in the presence of KBr to form dibromomalononitrile-^{14}C KBr complex; 3) elimination of Bromine from dibromomalononitrile-^{14}C complex with Cu to form a carbene intermediate which couples to form ^{14}C-TCNE. The overall yield is 20% with a specific activity of 2.1×10^6 dpm/umole. An analytical method using a calibration curve based on trans-2, trans-4-hexadiene-^{14}C-TCNE adduct formation to quantitate conjugated diene levels will be presented. In experimental studies, livers from CCl_4-treated rats had higher diene content than controls. By analyzing oxidized linoleic acid stereoisomers for conjugated diene content, a favourable comparison was made between the UV spectrophotometric and ^{14}C-TCNE radiochemical methods. Conjugated dienes can also be quantitated by ^{14}C-TCNE in myocyte cultures that have undergone free radical injury by hypoxanthine/xanthine oxidase and in cultures pre-treated with free radical scavengers prior to injury. Preliminary data indicates that diene conjugates can be quantitated in tissue and plasma samples. Further data will be presented that establishes the efficacy of the ^{14}C-TCNE technique in clinical and experimental models.

EFFECTS OF AUTOXIDATION OR PEROXIDATION OF PHOSPHOLIPID ON SUSCEPTIBILITY TO HYDROLYSIS BY ACID-ACTIVE PHOSPHOLIPASES OF CANINE CARDIAC SARCOPLASMIC RETICULUM.

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Susceptibility of partially peroxidized liposomes of 2-(1-^{14}C)-linoleoyl-phosphatidylethanolamine (PE) to hydrolysis by acid-active phospholipases associated with isolated canine cardiac sarcoplasmic reticulum (SR) was examined. PE was autoxidized by exposure to air at 37°C for 24 hours or peroxidized by addition

of NADPH (2 mM) plus ADP-Fe^{+3} (5 mM-20 uM). Both air and chemical oxidation of PE resulted in a comparable increase in thiobarbituric acid reactivity (OD_{532} = 0.04/nmole PE for each method). Autoxidation produced 31–40% polar lipid derivatives as determined by thin-layer chromatography. In contrast, chemical peroxidation did not alter subsequent chromatographic behavior despite an equivalent extent of thiobarbituric acid reactivity. The susceptibility of air oxidized and chemically peroxidized phospholipid to hydrolysis by acid-active phospholipases of SR was increased under these conditions, but differed quantitatively and qualitatively as a function of the mode and conditions of phospholipid oxidation. Specifically, autoxidation increased susceptibility to acid-active phospholipase C (420% of control) with no change in phospholipase A activities. SR phospholipase C preferentially degraded the polar lipid species which accumulated during autoxidation. Polar lipid generated by autoxidation at pH 7.0 was most susceptible to subsequent hydrolysis at acid pH. In contrast, chemical peroxidation of PE resulted in an increased susceptibility to acid-active phospholipase C (131% of control) and to phospholipases A_1 (250% of control) and A_2 (200% of control). These studies show that oxidative modification of phospholipid increases susceptibility to hydrolysis by acid-active (lysosomal) phospholipases. Further, expression of specific phospholipase activities is dependent on the nature of the altered lipids and thus, the conditions under which lipid is oxidatively modified.

USE OF METHANE SULPHINIC ACID PRODUCTION FROM DIMETHYL SULPHOXIDE AS A MARKER FOR HO* FORMATION.

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Development of a simple, direct, chemical means to detect and quantify oxygen centered free radicals in biologic systems would greatly benefit the study of free radical mechanisms in pathogenesis. This investigation was conducted to validate the use of dimethyl sulphoxide (DMSO) as a molecular probe for generation of hydroxyl radical(s) (HO*) under physiologic conditions. Reaction of HO* with DMSO produces methane sulphinic acid (MSA) as a primary non-radical product, in > 70% yield, which can be detected by a simple colorimetric assay. (Anal Biochem 163, 67–73, 1987). To determine favorable conditions for detection of .OH; we studied the iron catalyzed Haber-Weiss reaction *in vitro*, using xanthine oxidase as the source of superoxide. We also developed a computer model of reaction kinetics for this system. Measured MSA production *in vitro* was compared to that of the computer model for various concentrations of DMSO, mannitol, and EDTA-Fe. No sulphinic acid was detected in the absence of iron, enzyme, or hypoxanthine/xanthine substrate. The yield of MSA depended upon the concentrations of DMSO and the competing HO* trapping agent, mannitol, and approached 100 percent at DMSO concentrations of 1 to 5 percent (0.15 to 0.7 M). Experimental results were in good agreement with the model. Since DMSO is exceedingly non-toxic, distributes well to various tissues spaces, and yields an easily measured, stable, non-radical product – methane sulphinic acid – upon reaction with HO*, we believe that methane sulphinic acid production from DMSO holds promise as an easily measured marker for ·OH in DMSO pre-treated biologic systems.

ADENINE NUCLEOTIDE DEGRADATION AND RESYNTHESIS IN ISCHAEMIA REPERFUSION INJURY.

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The aims of this study are to determine [1] the effects of prolonged ischaemia on skeletal muscle cell adenine nucleotide content and rate of resynthesis during reperfusion, and [2] whether exogenous ATP-$MgCl_2$ during early reperfusion can reduce the extent of skeletal muscle necrosis.

Isolated canine gracilis muscles were exposed to 4 hours [n = 6] and 5 hours [n = 9] of ischaemia, biopsies were taken prior to, at end ischaemia, and during the first hour of reperfusion. Adenine nucleotide profiles were analyzed and quantified using HPLC.

Following 4 hours of bilateral muscle ischaemia [n = 5], the control muscle had normal reperfusion, the other muscle underwent controlled reperfusion with the addition of 2 mM ATP-$MgCl_2$ solution [treatment]. Necrosis was determined at 48 hours, using tetrazolium staining.

RESULTS

ISCH.	ATP μM/g.			ENERGY CHARGE POTENTIAL			AVG. NEC. %
	PRE-ISC.	END ISC.	45 min REP.	PRE-ISC.	END ISC.	45 min REP.	
4 HRS.	21.32 + 1.66	10.32 + 1.53	14.80 + 2.82	0.92 +0.01	-0.81 +0.02	0.89 +0.02	24.15 ± 7.57
5 HRS.	21.59 ± 0.74	3.90 ± 0.79	12.71 ± 3.31	0.92 ±0.02	0.67 ±0.01	0.79 ±0.04	51.15 ± 22.2

Reperfusion with 2 mM ATP-$MgCl_2$ solution resulted in an average reduction in necrosis from 59.23 ± 12.62% in the control muscle to 47.84 ± 13.76% in the treated muscle.

Energy Charge Potential [ECP] and ATP levels fell after both 4 and 5 hours of ischaemia, but ECP was restored on reperfusion after 4 hours of ischaemia only. ATP resynthesis failed to reach pre-ischaemic values in both 4 and 5 hour ischaemic muscles after 45 minutes of reperfusion. Reperfusion with exogenous ATP-$MgCl_2$ reduced necrosis following prolonged ischaemia.

EFFECT OF EXOGENOUS OXYGEN FREE RADICALS ON THE RELEASE OF CARDIAC LYSOSOMAL ENZYMES.

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Lysosomal enzymes play an important role in the cellular damage which occurs following ischaemia. It has been suggested that there are significant changes in the integrity of the myocardial lysosomal membrane during ischaemia which results in increased lysosomal enzyme release. Oxygen free radicals have been

implicated in the cardiac ischaemic and reperfusion injury. Several metabolic pathways may lead to an increase in the production of oxygen free radicals during cardiac ischaemia including an increase in xanthine and xanthine oxidase. The present investigation deals with the effect of exogenous oxygen free radicals on the release of lysosomal hydrolases from dog myocardium lysosomes. A lysosomal enriched subcellular fraction was prepared, using differential centrifugation techniques from homogenates of dog heart. Exogenous oxygen free radicals were generated by xanthine and xanthine oxidase reaction. The release of lysosomal hydrolases was measured from the lysosomal enriched fraction. There was a three to four fold increase in the release of Cathepsin D activity in those preparations treated with xanthine and xanthine oxidase as compared to that of control. The presence of superoxide dismutase, an oxygen free radical scavenger, prevented the release of Cathepsin D activity from lysosomes. Sonication and Lubrol treatment, which are well established to cause membrane disruption, also induced the release of Cathepsin D from lysosomal rich fraction. However, this release was not prevented by superoxide dismutase. Similar patterns in the release of β-N-acetylglucusaminidase were observed when lysosomal enriched fraction was treated with xanthine and xanthine oxidase. These studies suggest that oxygen free radicals generated *in vitro* cause the lysosomal membrane damage leading to the release of lysosomal hydrolases, which in turn, may be responsible for myocardial injury.

DELAY OF OCCURRENCE OF REPERFUSION-INDUCED VENTRICULAR FIBRILLATION IN THE ISOLATED RAT HEART WITH SUPEROXIDE DISMUTASE.

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Scavengers of oxygen-derived free radicals have been shown to reduce the incidence of reperfusion-induced ventricular fibrillation (R-VF) following acute regional myocardial ischaemia in the isolated, perfused rat heart. However, most studies were performed with reperfusion following a single time period of ischaemia, although a major determinant of R-VF is the duration of the preceding ischaemic period. Thus, scavengers of oxygen-derived free radicals could simply be delaying the occurrence of R-VF to longer periods of ischaemia. To test this hypothesis we examined the effect of superoxide dismutase (SOD) on R-VF in the isolated, perfused rat heart with reperfusion following periods of regional myocardial ischaemia ranging from 5 to 37.5 min. SOD was perfused at a concentration of 100 U/ml during both ischaemic and reperfusion periods. Regional myocardial ischaemia was produced by acute occlusion of the left anterior descending coronary artery. Our results indicate that SOD shifted the occurrence of R-VF to longer periods of ischaemia. Thus, with reperfusion following a short 8 min period of ischaemia, only 33% of SOD perfused hearts exhibited R-VF, compared with 100% of control hearts which exhibited R-VF ($p < 0.05$). In contrast, the incidence of R-VF was not affected by SOD with reperfusion following intermediate durations of 10, 15, and 22.5 min of ischaemia. Additionally, although following a relatively long 30 min period of ischaemia, reperfusion does not result in R-VF in control hearts, 87% of SOD treated hearts still exhibited R-VF ($p < 0.05$). No hearts exhibited R-VF with reperfusion following 37.5 min of ischaemia. These results suggest that scavengers of oxygen-derived free radicals delay the onset of electrophysiological derangements responsible for R-VF.

THE ROLE OF HYDROGEN PEROXIDE AND IRON IN ENDOTHELIAL CELL CYTOTOXICITY INDUCED BY ENZYMATICALLY GENERATED OXYGEN RADICALS.

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Ischaemia/reperfusion-induced injury to the microvasculature appears to be mediated by oxyradicals in accordance with following scheme. Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which are formed by the enzyme xanthine oxidase, interact in the presence of iron (Haber-Weiss chemistry) to produce the cytotoxic hydroxyl radical (·OH). In the present study we used calf pulmonary artery endothelial cells grown to confluence in M199 (with 10% FCS) to assess injury to the microvasculature induced by hypoxanthine (HX) and xanthine oxidase (XO) in the presence of chelated ferric iron (Fe). This oxyradical generating system follows Haber-Weiss chemistry, i.e., it produces O_2^-, H_2O_2 and ·OH. HX (0.5 mM) and XO (0.05 U/ml) in the presence of Fe (0.05 mM) resulted in the release of 70% of the previously incorporated ^{51}Cr from the endothelial cells. Superoxide dismutase (500 U/ml), a superoxide anion scavenger, failed to prevent the HX − XO − Fe induced ^{51}Cr release from endothelial cells. Similarly, the hydroxyl radical scavengers, Dimethylsulfoxide (25 and 50 mM), mannitol (50 mM), and benzoic acid (80 mM), did not prevent the HX-XO-Fe induced cytotoxicity. Catalase (1000 U/ml) abolished the HX-XO-Fe induced endothelial cytotoxicity, while addition of H_2O_2 alone to the cells induced ^{51}Cr-release. Pretreatment of the endothelial cells with 20 mM deferoxamine (an iron chelator) for three hours prior to the experiment attenuated the HX-XO-Fe and H_2O_2 induced ^{51}Cr-release by 75%. The results of this study indicate that H_2O_2 formed by HX + XO + Fe interacts with intracellular iron to form a cytotoxic specie that does not appear to be the hydroxyl radical.

ROLE OF NEUTROPHIL-DERIVED OXIDANTS IN CARDIAC SARCOPLASMIC RETICULUM DYSFUNCTION.

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An important aspect of myocardial injury is the role of neutrophils in post-ischaemic damage to the heart. Activated polymorphonuclear leucocytes (PMN) initiate a series of reactions that produce toxic oxidizing agents. $\cdot O_2^-$ rapidly dismutates to H_2O_2 and neutrophils contain myeloperoxidase which catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid (HOCl). The highly reactive HOCl combines non-enzymatically with nitrogenous compounds to generate long-lived, non-radical oxidants such as monochloramine (NH_2Cl) or Taurine monochloramine (TauNHCl). We investigated the role of oxygen radicals and long-lived oxidants on cardiac sarcoplasmic reticulum (SR) function, which plays a major role in regulation of intracellular Ca^{2+} and thereby in the generation of force. Incubation of SR with phorbol myristate acetate (PMA) activated neutrophils (4×10^6 cells/ml) significantly decreased calcium uptake rate (0.85 ± 0.013 to 0.113 ± 0.065 μmols/min mg) and Ca^{2+}-ATPase activity (1.67 ± 0.08 to 0.46 ± 0.10 μmols/min mg). Inclusion of myeloperoxidase inhibitors (cyanide, sodium azide and 3-amino-1,2,4 triazole), or oxygen radical scavengers (catalase, SOD plus catalase, and α-tocopherol) significantly protected ($P < 0.01$) calcium uptake rates and Ca^{2+}-ATPase activity of SR. The maximum inhibition in SR function was achieved by $3 - 4 \times 10^6$ cells/ml. Time course of preincubation of SR with PMA-

activated neutrophils showed that the calcium uptake rate and Ca^{2+}-ATPase activity was completely inhibited at 4–6 min. Pure HOCl, NH_2Cl and TauNHCl inhibited calcium uptake rate, Ca^{2+}-ATPase activity and induced – SH groups oxidation of SR in a dose dependent manner (2–20 μM) whereas H_2O_2 damaged SR function at non-physiological high levels (10–50 mM). $\cdot O_2^-$ and H_2O_2 generated by xanthine oxidase (0.05 U/ml) action on xanthine had no significant effect on SR function ($P > 0.01$). We therefore conclude that neutrophils damage cardiac SR mainly by long-lived oxidants and can be protected by myeloperoxidase inhibitors, catalase and α-tocopherol. Supported by grants from the American Heart Association and National Institutes of Health HL 24917.

TOXIC O_2 METABOLITES FROM NEUTROPHILS ACCENTUATE ISCHAEMIA-REPERFUSION INJURY IN ISOLATED PERFUSED RAT KIDNEYS.

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The contribution of neutrophils to reperfusion injury after ischaemia is not known. To determine the effect of neutrophils on the function of ischaemic kidneys, we added purified human neutrophils during perfusion of isolated ischaemic or non-ischaemic rat kidneys. Perfusates from kidneys made ischaemic for 20 min *in situ* and reperfused for 60 min caused more neutrophil locomotion than perfusates from non-ischaemic kidneys: non-ischaemic -3.2 ± 1.2; ischaemic 12.8 ± 4.7 cells/hpf, $p < 0.25$. Reperfusion of ischaemic kidneys with neutrophils caused more morphological and functional injury than reperfusion with buffered albumin alone: with neutrophils, glomerular filtration rate (GFR) 113 ± 7 μl/min/g, tubular sodium reabsorption (T_{Na}) $72 \pm 2\%$; without neutrophils, GFR 222 ± 18 μl/min/gm; T_{Na} $90 \pm 2\%$; both $p <).01$ vs reperfusion with neutrophils. In contrast, addition of neutrophils did not injure control kidneys, unless the neutrophil activator, phorbol myristate acetate (PMA) was also added: with neutrophils, GFR 616 ± 41 μl/min/g, T_{Na} $97 \pm 1\%$; with neutrophils +PMA, GFR 254 ± 25 μl/min/g, T_{Na} $72 \pm 4\%$; both $p < 0.01$ vs neutrophils. Two experiments suggested that O_2 metabolites contributed to neutrophil-mediated injury to ischaemic kidneys. First, reperfusion of ischaemic kidneys with O_2 metabolite-deficient neutrophils from a patient with chronic granulomatous disease did not cause more injury than reperfusion with buffered albumin alone. Second, simultaneous addition of the O_2 metabolite scavenger, catalase, prevented the decrease in GFR and T_{Na} caused by neutrophils but did not decrease injury in the absence of neutrophils. We conclude that neutrophils contribute to renal ischaemia-reperfusion injury in the isolated perfused kidney by an O_2 metabolite-dependent mechanism.

PREVENTION OF REPERFUSION INJURY OF HYPOXICALLY STORED RABBIT HEARTS BY OXYPURINOL AND ALLOPURINOL.

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To evaluate their potential benefit in heart transplantation, the xanthine oxidase inhibitor allopurinol (ALLO) and its metabolite oxypurinol (OXY) were investigated. Using Langendorff techniques with a perfusion pressure of 80 cm H_2O, isolated rabbit hearts were perfused at 37°C with Krebs-Henseleit solution. A latex balloon was used to measure LV volume, peak systolic pressure (PSP), and LVEDP.

Hearts were held globally ischaemic for 3 hrs. at 15°C after KCL cardioplegic arrest. Coronary circulation (CC), PSP, and LVEDP were measured prior to ischaemia and following reperfusion. Hearts were paced at 260 bts/min and received drug treatment during the first 5 min. of reperfusion. Four groups were studied. Group 1 and 2 received ALLO 1 mM or OXY 1 mM, respectively. Group 3 received superoxide dismutase (SOD) 90 IU/ml plus catalase (CAT) 8085 IU/ml. Group 4 served as control. Data was expressed as a function of LV volume. Post-ischaemic were compared to pre-ischaemic values and the differences compared between groups. Multivariant analysis and Tukey tests were used to identify significant differences. All drug treated groups significantly recovered end diastolic function compared to control. PSP decreased significantly in the SOD/CAT group when compared to all other groups. CC decreased significantly in both the SOD/CAT group and control group when compared to the ALLO or OXY groups. LV isovolumetric work showed significantly bigger decreases in the SOD/CAT group and control group than in the OXY group.

It has recently been demonstrated that rabbit as well as human myocardium lack xanthine oxidase. Our results in rabbit hearts however, shows a significant improvement of function when oxypurinol and allopurinol are administered at the time of reperfusion.

The beneficial effects of these drugs may not be dependent on the presence of xanthine oxidase, but rather on direct scavenging of hydroxyl radicals.

BENOXAPROFEN: REDUCTION OF REPERFUSION-INDUCED INFARCT SIZE AND MYELOPEROXIDASE ACTIVITY IN ANAESTHETIZED RATS

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Inhibition of activated neutrophil (PMN) accumulation has been suggested to reduce reperfusion-induced tissue injury. We have developed a small animal preparation with temporary coronary artery occlusion (CAL) followed by reperfusion and have studied (a) the time course of PMN accumulation as measured by myeloperoxidase activity (MPO) and (b) the effects of benoxaprofen (an agent known to inhibit PMN chemotaxis) on PMN accumulation and ultimate infarct size. Hearts from anaesthetized rats with either 30 or 60 min CAL followed by 30, 60, or 120 min reperfusion were used for the initial study. At the end of the reperfusion period, the artery was re-occluded and methylene blue dye was injected iv to delineate non-ischaemic from ischaemic tissue. Hearts were frozen and later, tissue was dissected from both regions and assayed for MPO activity spectrophotometrically. A large time-dependent increase in MPO activity within the previously ischaemic zone was shown to occur, with the greatest increases occurring after the longest durations of both CAL and reperfusion. For studies with benoxaprofen, a time of 60 min CAL followed by 60 min reperfusion was used. Benoxaprofen was given orally (30 mg/kg) for 3 days, with the final dosage given 1 h prior to CAL. Infarct size was assessed using a single blind procedure where hearts were sliced, stained with NBT and the necrotic area dissected and weighed. Benoxaprofen reduced the necrotic zone from 33.4 $\pm$ 4.1% ventricular tissue to 17.0 $\pm$ 3.3 ($P < 0.01$) and eliminated mortality (0/9 compared to 4/16 in the control group). Benoxaprofen reduced PMN accumulation within the necrotic zone by over 70% ($P < 0.01$). Thus pretreatment with benoxaprofen simultaneously reduces both reperfusion-induced infarct size and also MPO activity. This suggests that neutrophil-induced tissue injury does occur in this preparation and can be prevented by pretreatment with benoxaprofen.

BINDING OF HUMAN EXTRACELLULAR-SUPEROXIDE DISMUTASE C TO PIG AORTA ENDOTHELIUM IN SITU, TO CULTURED CELL LINES AND TO BLOOD CELLS

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The secretory enzyme extracellular-superoxide dismutase (EC-SOD) occurs in at least three forms, differing with regard to heparin-affinity; A without affinity, B with weak affinity and C with relatively strong affinity. The binding of human EC-SOD C to pig aorta endothelium in situ, to cultured cells and to blood cells was investigated. The endothelium and the cultured anchorage-dependant cell lines bound EC-SOD C avidly. The suspension growing cell lines investigated bound clearly less EC-SOD C. Blood monomorphonuclear leucocytes bound only little EC-SOD C whereas no significant binding to erythrocytes and to polymorphonuclear neutrophil leucocytes (PMNL's) could be demonstrated. Nor could any significant binding to several investigated E coli strains be demonstrated. EC-SOD C bound to the surface of the cells, forming an equilibrium between the medium and the cell surfaces. The presence of heparin in the medium abolished binding. The maximal binding capacity was very high, and at physiological concentrations, the binding of EC-SOD C is very far from saturation. The principal binding substance is apparently heparin sulfate in the glycocalyx of the cells, since pretreatment with heparitinase abolished subsequent binding. The concentration of EC-SOD C in the glycocalyx of endothelial cells and cultured anchorage-dependent cells is at equilibrium several thousand-fold higher than in the medium. The findings suggest that in vivo the surface of tissue cells and endothelial cells is coated with a protective sheet of EC-SOD C. Since EC-SOD C did not bind to PMNL's, the enzyme will not interfere with superoxide produced by the cells. Invading microorganisms lacking affinity for EC-SOD C will not be protected.

MYOCARDIAL ISCHAEMIA AND REPERFUSION-INDUCED MEMBRANE DAMAGE IN ISOLATED PERFUSED RAT HEARTS: ATTENUATION BY α-TOCOPHEROL.

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Free radical mediated damage to the plasma membrane is proposed as a critical component in the development of ischaemic injury. α-Tocopherol was evaluated as an agent which attenuates free radical-induced membrane damage in a model of ischaemia/reperfusion injury. Isolated perfused Langendorff rat hearts (n = 97) were either used as control perfused hearts or made globally ischaemic (I) for 25 min and reperfused (R) for varying time periods. α-Tocopherol treated animals were implanted with a subcutaneous time-release pellet 14 days prior to utilization resulting in a 50% increase in myocardial tissue tocopherol levels. Left ventricular contractile function was continuously monitored, accumulation of unesterified fatty acids was characterized and quantitated by HPLC as a marker of membrane perturbation, increases in total tissue calcium and release of lactate dehydrogenase (LDH) were measured as indicators of severe myocardial cell damage. Following ischaemia, recovery of contractile function at 30 min R was found to be enhanced in α-tocopherol treated hearts when compared to non-treated hearts I + R resulted in significant increases in unesterified fatty acids. Tissue levels of arachidonic acid were (in nmol/mg protein, mean ± SEM, *$p < 0.05$) 137.8 ± 24.4, 249 ± 45.4* and 102.7 ± 9.2 for control perfused, non-treated ischaemic

reperfused and tocopherol-treated ischaemic reperfused hearts, respectively. The tocopherol treatment offset the large alterations in the fatty acid profile. Tocopherol treatment was also found to reduce the 2.7-fold accumulation in total tissue calcium measured by atomic absorption spectrophotometry found in non-treated hearts. Total LDH activity was found to increase 2.4-fold in I + R hearts compared to control perfused hearts. The most significant increases in LDH release occurred between 3 and 5 min of reperfusion. Hearts from animals pretreated with α-tocopherol demonstrated significant attenuation in LDH release. Radical-lipid interactions produce lipid peroxide radicals, lipid peroxides, and lipid hydroperoxides. In current work, detection of peroxidized lipid products, conjugated dienes (CD), is being evaluated. The levels of CD were (in O.D./g wet weight) 3.01 ± 0.20, 4.65 ± 0.64*, 3.02 ± 0.19, 2.69 ± 0.13, and 3.04 ± 0.38 for control perfused, 2 min R, 3 min R, 5 min R, and 10 min R, respectively. Work is continuing to evaluate the ability of α-tocopherol to reduce the levels of CD following I + R. These results demonstrate a protective role of α-tocopherol and support the concept that free radicals produced during ischaemia and reperfusion may play a role in the resulting myocardial injury. The ability of α-tocopherol to partition within the membrane and prevent lipid peroxidative events may protect membrane integrity allowing enhanced functional recovery of the cell upon reperfusion.

HYPOCHLORITE- AND HYDROGEN PEROXIDE-INDUCED INHIBITION OF Na^+K^+ ATPASE.

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In this study, effects of oxidants, H_2O_2 and HOCl, postulated to be involved in ischaemia/reperfusion injury, on a membrane model were investigated. Enzyme activity, ouabain binding and SH content of Na^+K^+-ATPase prepared from porcine kidney medulla were decreased in proportion to the concentration of H_2O_2 (0.01–5 mM). Enzyme inhibition by H_2O_2 plus Fe^{3+} was time-dependent, took place under anerobic conditions and was influenced by the kind of buffer in the incubation medium. The inhibition was antagonized by deferoxamine or EDTA, and was ameliorated by superoxide dismutase, but not by dimethylsulfoxide or benzoate. The actions of the oxidant and its antagonists depended on the presence of Fe ions; inhibition was more severe in the presence of Fe^{2+} than Fe^{3+}. DTT potentiated the action of H_2O_2 plus Fe^{3+} on the enzyme, whereas the enzyme inhibition in the presence of H_2O_2 and Fe^{2+} was prevented by a combination of DTT and deferoxamine. HOCl also inhibited Na^+K^+ ATPase activity in a time- and concentration-dependent manner. This inhibition was antagonized by DTT. However, when Fe^{2+} or Fe^{3+} was present in addition to HOCl, a combined action of deferoxamine and DTT was required to block the enzyme inhibition. Ouabain binding to Na^+K^+ ATPase was also inhibited by H_2O_2 plus Fe^{3+} or by HOCl, which was ameliorated by deferoxamine or DTT, respectively. Deferoxamine could not reverse the H_2O_2-induced enzyme inhibition, nor could DTT reverse the HOCl-induced inhibition. Malondialdehyde formation was increased by incubation with H_2O_2 plus Fe^{3+}. DTT and N-(2-mercaptopropionyl)-glycine potentiated free radical-induced malondialdehyde formation. BHT and some other antioxidants inhibited free radical-induced malondialdehyde formation but did not correct inhibition of ATPase activity. These results indicate that free radical effects on enzyme protein are linked to the iron-catalyzed Fenton reaction and their prevention requires pretreatment with a combination of metal-chelating and thiol-reducing agents. The results also suggest that oxidative modification of proteins is site-specific, progressive and irreversible.

SUPEROXIDE DISMUTASE DOES NOT AFFECT THE RELATIONSHIP BETWEEN MYOCARDIAL INFARCT SIZE AND DURATION OF ISCHAEMIA IN THE RABBIT ISCHAEMIA/REPERFUSION MODEL.

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In our previous study, superoxide dismutase (SOD) failed to limit myocardial infarct size following 45 min ischaemia and 72 h reperfusion in the rabbit model. SOD, however, reportedly limited infarct size in some studies. To test whether this discrepancy is attributable to duration of ischaemia, we examined the relationship between myocardial infarct size and the duration of ischaemia in our model, and the effect of SOD on the infarct size following 20 min ischaemia. Under anaesthesia, a coronary branch was occluded for 10, 15, 20, 30 or 60 min (n = 4, 3, 9, 3, 3, respectively), then reperfused and the operative wounds were repaired. In another 4 rabbits, the coronary was permanently ligated. Five of the 20 min occlusion group received an IV bolus of SOD (45,000 U/kg) 5 min prior to reperfusion. After 3 days, the heart was removed, the coronary reoccluded, and irrigated with 3–30 μ fluorescent particles to mark the ischaemic zone (risk zone). The heart was sectioned in 3 mm slices and the infarct was delineated by H-E staining. The plot of the percentage of risk zone infarcted (I/R) was converted to probits, the plot against Log T was linear and the correlation was significant ($I/R = 2.7 \times \text{Log}\,T + .78$, $r = .814$, $p < .01$). This regression suggests that 36 min of ishcemia causes 50% of myocytes to infarct in our model. The I/R following 20 min ischaemia was 20.2 ± 11.7% in control group and 16.6 ± 9.7% in SOD group, which were not significantly different. Taking our previous 45 min ischaemia study also into account, it is unlikely that SOD affects myocardial infarct size in rabbit ischaemia/reperfusion model.

EFFECTS OF ACUTE MYOCARDIAL ISCHAEMIA AND REPERFUSION ON REACTIVE HYPERAEMIA AND CORONARY VASCULAR RESERVE.

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Thrombolytic agents restore blood flow to ischaemic regions of the myocardium. To determine the effects of acute myocardial ischaemia on regional coronary vascular reserve, peak reactive hyperaemic (RH) blood flow (F) was obtained in 8 dogs subjected to circumflex (Cx) artery occlusion for one hour followed by reperfusion for one hour (O-R).

RESULTS: (n = 4)	Time O	After Cx O-R	P
CxF (ml/min)	27 ± 4	30 ± 5	NS
RH CxF	85 ± 12	56 ± 9	0.001
LADF (ml/min)	21 ± 4	24 ± 5	NS
RH LADF	70 ± 11	69 ± 10	NS

Myocardial ischaemia resulting from Cx O-R attenuated peak RH CxF (from 215 ± 27% to 87 ± 13%. $P \leqslant 0.001$) as well as i.c. acetylcholine (ACh, 2 ug) induced increase in CxF (from 133 ± 15% to 63 ± 9%, $P \leqslant 0.001$). These alterations were not observed in the LAD region. O-R had no effect on heart rate or arterial pressure. Pretreatment of 4 dogs with indomethacin (4 mg/kg) inhibited prostaglandin release ($P \leqslant 0.01$), but did not affect peak RH CxF or ACh induced increase in CxF before or after O-R. Histopathology revealed extensive myocardial neutrophil infiltration and capillary plugging by neutrophils in the Cx-supplied region compared to the LAD-supplied region. In conclusion, impairment of microvascular endothelium and/or capillary plugging by neutrophils may account for the observed attenuation in stimulated CxF and depression of regional coronary vascular reserve.

XANTHINE DEHYDROGENASE (XD) IS CONVERTED TO XANTHINE OXIDASE (XO) BY EXTRACELLULAR OXIDANT STRESS IN CULTURED BOVINE, PULMONARY ARTERY ENDOTHELIAL CELLS (BPAEC).

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Ischaemia-reperfusion injury is the result of both XO^- catalyzed intracellular oxidants as well as extracellular oxidant production from inflammatory cells. Sulfhydryl group oxidation converts XD to XO, then XO catalyzed oxidation of hypoxanthine produces superoxide anion. Since toxic oxygen species from neutrophils injure lung endothelium, we hypothesize that extracellular oxidant stress results in intracellular conversion of XD to XO, amplifying oxidant injury. A sensitive fluorimetric method was used to measure XO content and total (XO + XD) activity in cultured BPAEC. The rate of isoxanthopterin production from pterin (Exc. 340 nm; Em. 310 nm) is proportional to XO activity and, after addition of methylene blue, to total enzyme activity. The change in fluorescence per unit time was converted to μmol product using a standard curve of isoxanthopterin concentration vs. Fluorescence (units = μmol product per min per g protein). Confluent BPAEC (n = 9) contained 965.8 ± 205.1 (m ± SE) units total activity (XD + XO) which was 29.3 ± 5.7% XO (XO/XO + XD). Cells exposed to H_2O_2 (n = 8) had similar total activity (1055.6 ± 356.9) but all of the enzyme was present as XO (98.8 ± 1.6%). The addition of dithiothreitol to lysates of H_2O_2-treated cells decreased % XO to 39.5 (n = 3). We conclude that BPAEC contain measurable amounts of XD/XO and that XD is the predominant intracellular form. Furthermore, extracellular oxidant stress can cause intracellular conversion of XD to XO probably by thiol group oxidation. This reaction may amplify oxidant injury to cells by creating an intracellular source of toxic oxygen species. (Supported by HL 34208, HL 19153, and ES 03272).

IRON AND POST-ISCHAEMIC RENAL INJURY: NEPHRON SITE OF PROTECTION BY IRON CHELATORS.

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Oxygen free radical scavengers have been demonstrated to limit post-ischaemic renal injury. However, there is little information as to their site of action. Free radicals might be generated by vascular en-

dothelium, neutrophils, glomerular, tubular epithelial or interstitial cells. Protective agents might, therefore, act at an intravascular, intracellular or intralumenal site. We previously showed that the iron chelator deferoxamine (DFO) limited post-ischaemic injury, probably by preventing OH· formation and lipid peroxidation. To determine whether "free" iron was released after ischaemia we used the bleomycin assay of Gutteridge et al. Rats underwent right nephrectomy and left renal artery occlusion for 60 min. No free iron was detectable in plasma from the renal artery or vein after ischaemia or after reperfusion. On the other hand there was a 10 to 20-fold increase in urinary free iron during reperfusion compared to base line values. To determine whether this urinary iron was the critical source of iron in reperfusion injury, we compared the ability of free DFO (MW 657), which undergoes glomerular filtration and may enter cells, to DFO conjugated to inulin (MW ~ 5000; also undergoes glomerular filtration) or to dextran T40 (MW ~ 36,000; retained in vascular space) to protect against post-ischaemic renal injury. After renal artery occlusion for 60 min, DFO or DFO-inulin were infused during the first 60 min of reperfusion to deliver 50 mg/kg DFO. DFO-dextran was infused to attain plasma DFO levels equivalent to those with free DFO. Inulin clearance (C_{In}) was measured 24 h after ischaemia:

	C_{In} (*ul/min*)	
Inulin (n = 4)	220 ± 39	
Free DFO (n = 6)	490 ± 102†	(ANOVA $p < 0.01$;
DFO-inulin (n = 7)	563 ± 73†	†$p < 0.05$ vs other groups)
Dextran (n = 5)	247 ± 63	
DFO-dextran (n = 6)	225 ± 60	

Glomerular filtration appears to be a requirement for protection since DFO-dextran did not protect. Ability to enter intact cells is not necessary since the protective effects of DFO and DFO-inulin were comparable. We conclude that after ischaemia free iron is released into the urinary space. The iron chelator DFO protects against post-ischaemic injury from within the urinary space, probably at a site adjacent to the tubular epithelial membrane.

DETECTION AND CHARACTERIZATION OF XANTHINE DEHYDROGENASE/OXIDASE IN FRESHLY ISOLATED AND CULTURED TYPE II PNEUMOCYTES FROM SEVERAL SPECIES.

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An important intracellular source of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) is the oxidase form of xanthine dehydrogenase/oxidase (XDH/XO). XDH/XO was detected in isolated type II pneumocytes (T_2) from several species and the activity was followed in culture. Cells were isolated from rabbit and rat lungs by trypsin-elastase digestion to free alveolar cells, followed by tissue dissociation, density gradient centrifugation, and differential adherence. Cells were then adhered (39 hr) to fibronectin pretreated dishes with a plating efficiency of 25%. Cells were lysed by sonication in a buffer designed to inhibit artifactual conversion of XDH to XO by proteolysis and/or sulfydryl oxidation. XDH/OX activity was detected by conversion of pterin to isoxanthopterin, a fluorescent product λex = 345 nm and λem = 390 nm), using methylene blue as the electron acceptor for XDH. Isolated rat (n = 3) and rabbit (n = 5) T_2 cells contained 243 ± 197 and 33 ± 24 μU/mg protein, respectively, of which 33 ± 14% and 25 ± 10%, respectively, was in the XO form. A 50% XDH to XO conversion would result in intracellular H_2O_2 and O_2^- production of 73 and 49 pmol/min/mg protein respectively, in rat T_2 cells, and 10 and 7 pmol/min mg protein, respectively, in rabbit T_2 cells. Production of H_2O_2 and O_2^- at these rates would be cytotoxic. Culture of rat T_2 cells results in a continuous loss of XDH/XO activity becoming 8% of freshly isolated cell values after 135 hr of culture. In rabbit T_2 cells, 39 hr of culture resulted in loss of XDH/XO activity to levels below detectability. Thus, XDH to XO conversion in vivo and subsequent XO-derived oxidants may play an important role in pulmonary damage, but culture-induced loss of XDH/XO activity may alter the in vivo relevance of information gained during in vitro experimentation.

INCREASED HYDRAULIC CONDUCTIVITY IN PULMONARY AND SUBCUTANEOUS INTERSTITIUM INDUCED BY FREE RADICAL INJURY AND OEDEMA.

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The normally hydrated interstitial gel matrix has a very high resistance to fluid and protein movement. However, during inflammation the large proteins, cells, and fluid pass easily through the tissues. We compared the relative effects of oedema alone to oedema with free radical damage on interstitial hydraulic conductivities (k/η) measured in subcutaneous (SC) and lung hilar interstitium (HI) in excised canine tissues. The flow rate of Tyrode's between implanted porous catheters was measured at near normal hydration and after graded increases in interstitial fluid volume. Groups of each tissue were perfused with Tyrode's alone (TY) and with 10 units/l xanthine oxidase (XO) and 100 mg/l purine added to the Tyrode's for generation of oxygen free radicals. Baseline values of K/η in $cm^4\ dyne^{-1}\ s^{-1}$ were: 3.7×10^{-11} for SC; 20.0×10^{-11} for SC + XO; 56.3×10^{-11} for HI and 217.2×10^{11} for HI + XO. K/η increased over a range of about 10^3 with increased hydration in all groups and obeyed Darcy's law for flow through a homogenous porous matrix over a wide range of hydrations. K/η for both XO groups were significantly higher for comparable hydration states than TY groups. These data indicate that the interstitial spaces in normally hydrated lung and skin offer a very high resistance to fluid movement, but that this resistance decreases with edema formation because of spreading of gel matrix elements. Oxygen free radical damage increased K/η for any given hydration state by 15.2-fold for skin and 10.9-fold for lung interstitium presumably due to depolymerization of glycosaminoglycans and proteoglycans within the interstitial matrix. Supported by NIH HL-24571.

ALLOPURINOL ENHANCES ATP PRODUCTION BY ISOLATED MITOCHONDRIA IN A REPERFUSION MODEL.

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Studies from several laboratories have shown that allopurinol has a beneficial effect on recovery of function following myocardial ischaemia. While the mechanism of this was initially felt to be due to the ability of allopurinol to inhibit xanthine oxidase, recent studies suggest that it might work by a different mechanism. Shlafer found allopurinol to be effective in rabbits, whose myocardium lacks xanthine oxidase, in enhancing recovery of function. The reason for this is currently under investigation. We have suggested that allopurinol might act as an electron transfer agent similar to coenzyme Q_{10} which has similar salutary effects on the recovery of myocardial function. It has also been shown that allopurinol is a hydroxyl redical scavenger. Recently, Godin and Bhimi showed that allopurinol elevated cellular ATP levels and increased mitochondrial ATP production in reperfused rabbit myocardium. We have used isolated rat mitochondria and a model of reperfusion involving bubbling N_2 followed by O_2 through the mitochondrial suspension. ATP generation by these mitochondria is significantly increased in the presence of allopurinol (37%), but not by superoxide dismutase. This suggests that the beneficial action of allopurinol is related to improved mitochondrial function and not to the inhibition of xanthine oxidase.

DOES SUPEROXIDE DISMUTASE PLUS CATALASE ENHANCE RECOVERY OF REGIONAL CONTRACTILE FUNCTION FOLLOWING PROLONGED CORONARY ARTERY OCCLUSION?

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Previous studies from our laboratory and by others have shown that pretreatment with superoxide dismutase (SOD) + catalase (CAT) significantly attenuates post-ischaemic contractile dysfunction produced by a brief (15 min) period of coronary occlusion not associated with myocyte necrosis. The effect of these free scavenging enzymes on regional contractile function following a *prolonged* period of transient occlusion, however, are poorly defined. To address this issue, 23 dogs underwent 2 h of LAD occlusion and 4 h of reperfusion. Immediately prior to reflow, each dog was randomized to receive either SOD + CAT or saline. Segment shortening (SS: by sonomicrometry) was measured in the superficial subepicardial layer of the LAD bed and expressed as a % of baseline, preocclusion values. Area of necrosis (AN) was assessed by triphenyltetrazolium staining and expressed as a % of the in vivo area at risk (AR). Both groups were equally ischaemic during LAD occlusion: mean regional myocardial blood flow (RMBF: by microspheres in 22/23 dogs) to the ischaemic midmyocardium was 0.06 ml/min/g tissue. When all animals were considered, SOD + CAT had no apparent effect on either SS or AN/AR:

			Occlusion TREAT:		*Reperfusion*		
	AN/AR	SS:Pre	30'	120'	30'	120'	240'
Control (n = 11)	46 ± 6%	100%	−27 ±8%	−27 ±9%	−8.8%	−18 ± 5%	−23 ± 5%
SOD + CAT (n = 12)	39 ± 6%	100%	−20 ± 8%	−23 ± 8%	+7 ± 12%	−2 ± 11%	−24 ± 9%

In the subgroup of dogs with *higher than average* mid-RMBF during occlusion (> 0.06 ml/min/g), SOD + CAT reduced infarct size (*$p < 0.05$), but produced only shortlived improvement in SS (**$p < 0.02$):

Control (n = 4)	40 ± 7%	100%	−20 ± 6%	−19 ± 6%	−4 ±15%	−13.5%	−18 ± 5%
SOD + CAT (n = 4)	18 ± 6%*	100%	5 ± 6%	−3 ± 4%	+35 ± 4%	+21 ± 7%**	−11 ± 9%

In animals with *low than average* collateral blood flow (< 0.06 ml/min/g), no beneficial effects were observed:

Control (n = 5)	57 ± 10%	100%	−41 ± 16%	−43 ± 16%	−20 ± 4%	−27 ± 7%	−36 ± 5%
SOD + CAT (n = 8)	49 ± 6%	100%	−32 ± 6%	−34 ± 6%	−8 ± 13%	−14 ± 11%	−31 ± 10%

Thus, in contrast to previous results obtained in models of brief, transient coronary occlusion, SOD + CAT did not produce a sustained improvement in contractile function of myocardium salvaged by reflow after 2 hours of coronary occlusion.

ROLE OF IRON IN MORTALITY FROM INTESTINAL ISCHAEMIA

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Free iron may aggravate the tissue injury which follows ischaemia and reperfusion. The origins of this reactive iron are presently unknown but it is clear that, in many circumstances, iron chelators such as deferoxamine (DFO) prevent the damage. Unfortunately DFO has a short vascular survival time and is therefore of limited clinical utility. We have recently developed new forms of DFO conjugated to high

molecular weight colloids such as dextran and hydroxyethyl starch (HES). These DFO derivatives have greatly increased vascular retention times and do not manifest the acute toxicity associated with DFO alone. To invesigate the role of reactive iron in death following ischaemia-reperfusion we employed an *in-vivo* rat model which produced high (> 80%) mortality following 90-minute periods of clamping of the root of the mesentery. To determine if reperfusion was associated with detectable increases in plasma iron, transferrin-bound plasma iron and free plasma iron were measured in rats subjected to intestinal clamping. Results showed no changes in plasma iron during 90-minutes of intestinal ischaemia but significant increases in (transferrin-bound) iron 1-3 hours following clamp release and reperfusion. Thus, reperfusion of ischaemic intestines was accompanied by sufficient delocalization of iron to significantly alter degree of transferrin saturation, but the precise source of iron remains undefined. Further experiments suggested iron may be involved in the lethal consequences of intestinal ischaemia-reperfusion. Four groups of rats ($n = 9$) with intestinal ischaemia were treated intravenously with 3 ml/100 g bodyweight (BW) of, (1) saline, (2) 5% HES in saline, (3) free DFO (100 mg/kg BW) and HES in saline, or (4) DFO:HES conjugate (equivalent to free DFO 100 mg/kg BW) in saline. Mortality within 24 hours was; Group 1 67%, Group 2 67%, Group 3 89%*, Group 4 44%* (* sig. diff., $p < 0.05$). the possible protective role of an iron chelator was demonstrated by increased survival of ischaemic rats given fluid therapy containing high molecular weight DFO conjugate.

CARDIAC ANTIOXIDANTS AND THE PROTECTION AGAINST HYPOXIA-REOXYGENATION INJURIES IN EXERCISE-TRAINED RATS.

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There is evidence that regular physical exercise has a preventive effect on ischaemic cardiac disease. The purpose of this study was to find whether endurance training induces protection against hypoxia-reperfusion injuries in rat heart and whether the protection correlates with changes occurring in cardiac antioxidant capacity. Wistar rats were exposed to 200 h swimming protocol which caused a significant increase in cardiac weight. Langendorff-perfusion of isolated hearts consisted of 45 min hypoxic phase and 20 min reperfusion phase. Perfusion flow strikingly decreased between 4–15 min during hypoxic perfusion both in the hearts of control and trained rats, slightly later in the trained hearts. The start of reoxygenation phase caused a prominent release of creatine kinase to the perfusate in untrained control rats. The release was highly significantly less in trained hearts showing a significant protection against reperfusion injuries. Antioxidant levels were registered from right ventricle and from left ventricle (subendo- and subepimyocardium) of rats without hypoxic-reoxygenation perfusion. The activities of catalase, Cu-Zn superoxide dismutase, glutathione reductase and thioredoxin reductase as well the concentration of vitamin E were lower in the hearts of trained rats. Most of the differences were statistically significant. The activity of glutathione peroxidase (Se-dependent) as well the total concentrations of sulfhydryl groups and anserine and carnosine were unaffected by training. The results suggest that training-induced protection against reperfusion injuries is not due to adaptation in the cardiac antioxidant capacity.

ALTERATIONS IN FREE RADICAL TISSUE DEFENCE MECHANISMS AND NEUTROPHIL INFILTRATION IN POST-ISCHAEMIC CANINE SKELETAL MUSCLE.

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A growing body of experimental data indicates that reactive oxygen metabolites such as superoxide, hydrogen peroxide, and hydroxyl radical may mediate the microvascular endothelial injury produced by reperfusion of ischaemic skeletal muscle. One potential source of these reactive oxygen metabolites is the inflammatory neutrophil. To assess neutrophil accumulation we measured tissue myeloperoxidase activities in skeletal muscle biopsies taken during control, after four hours of ischaemia, and after one hour of reperfusion. Tissue levels of reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) were measured in the same samples to identify alterations in tissue free radical defence mechanisms due to ischaemia-reperfusion.

	Control	4 h Ischemia	15" Reperfusion	1 h Rep
MPO U/g wet wt.	0.4 ± 0.1	0.7 ± 0.5	9.9 ± 2.5*	10.3 ± 2.2*
GSH uM/g dry wt.	2.1 ± 0.2	2.2 ± 0.3	1.7 ± 0.3	1.1 ± 0.2*
SOD U/mg protein	22.5 ± 1.8	28.1 ± 2.9	22.7 ± 2.4	20.0 ± 1.9
CAT U/mg protein	5.1 ± 0.6	5.1 ± 0.6	5.0 ± 0.8	4.8 ± 0.9

The data indicate that skeletal muscle contains relatively low levels of GSH, SOD, and Catalase when compared to other tissues. They also indicate that reperfusion of ischaemic skeletal muscle causes a dramatic increase in tissue neutrophil content and a concurrent decrease in GSH content. Catalase and SOD activities were unaffected by ischaemia-reperfusion. These results suggest a possible relationship among ischaemia-reperfusion induced neutrophil infiltration, and the reduction in tissue GSH. This study was supported by NIH grants HL-36069 and HL-07710.

STIMULATION OF HEART MITOCHONDRIAL SUPEROXIDE FORMATION BY ADENINE NUCLEOTIDE TRANSLOCASE INHIBITION.

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In previous studies we found that long-chain acyl CoA esters (LCACAE), which accumulate during myocardial ischaemia, inhibit adenine nucleotide translocase (ANT) activity of heart mitochondria. However, it was unclear if LCACAE-ANT inhibition persisted during reperfusion and how this might affect recovery from ischaemia. Recent studies by others have suggested that several types of amphiphiles potentiate free radical formation in sarcolemmal membrane preparations, and a cell-free system from

macrophages. Because of these findings, we have examined the effects of carboxyatractyloside (C. atrac), a known specific ANT inhibitor, and palmitoyl CoA (PCoA) upon free radical formation by heart mitochondria. H_2O_2 production was measured spectrophotometrically at OD 402–418 with horseradish peroxidase. The results are shown below:

Substrate	Addition	H_2O_2 (nmoles/min/mg prot)	n
Succ(2 mM)	–	0.0398 ± 0.003	8
same	Antimycin A(0.2 μM)	0.1093 ± 0.006*	8
same	PCoA(1 μM)	0.0925 ± 0.004*	8
same	C. atrac(1 μM)	0.1583 ± 0.007*	7

Values are mean ± S.E. Succ = Succinate. *$p < 0.01$, vs Succ.

Lesions of inner mitochondrial membrane may contribute to myocardial damage during prolonged ischaemia/reperfusion. The presence of elevated levels of long-chain fatty acids also decreases recovery from ischaemia. Our results support the hypothesis that LCACAE-ANT inhibition may lead to increased free radical formation and mitochondrial damage during ischaemia/reperfusion.

ROLE OF SUPEROXIDE AND VITAMIN C IN OXIDATIVE TISSUE INJURY.

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Inflammatory cells such as PMN leukocytes contribute to the tissue injury associated with inflammatory diseases and ischaemia-reperfusion. These cells produce superoxide ions (O_2^-) and release these free-radicals into the extracellular medium. O_2^- in the extracellular medium is not highly toxic to tissue cells, but O_2^- oxidizes vitamin C (ascorbate ion), and ascorbate is the major low-molecular weight reducing agent in plasma and tissue interstitial fluid. Each O_2^- that reacts with ascorbate is reduced to hydrogen peroxide (H_2O_2), whereas in the absence of ascorbate two O_2^- react with each other to produce one H_2O_2. Therefore, ascorbate promotes the production of H_2O_2 and other toxic oxidants that are derived from H_2O_2. Oxidation by O_2^- also depletes ascorbate, so that less ascorbate is available to block toxicity by reacting with toxic oxidants. The enzyme superoxide dismutase (SOD) inhibits ascorbate oxidation by catalyzing the conversion of two O_2^- to one H_2O_2. Therefore, SOD lowers the production of oxidants and allows ascorbate to detoxify the oxidants.

Experimental support for this proposed interaction of O_2^-, ascorbate, and SOD was obtained by incubating stimulated PMN leukocytes with red blood cells (RBCs). Low concentrations of ascorbate promoted oxidant production and increased the oxidative damage to hemoglobin in RBCs. High ascorbate concentrations blocked toxicity by reducing the oxidants. SOD alone had no effect, but low concentrations of ascorbate blocked toxicity when SOD was added. The results indicate that ascorbate modulates the production and toxicity of inflammatory-cell oxidants. In the presence of ascorbate, SOD in the extracellular medium can inhibit oxidative damage to cells even though the cytotoxic oxidant is H_2O_2 or a product of the myeloperoxidase/H_2O_2/Cl^- system rather than O_2^-.

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