OXY-RADICALS IN MOLECULAR BIOLOGY AND PATHOLOGY

Organizers: Peter Cerutti, Irwin Fridovich and Joe McCord
January 24-30, 1988

Plenary Sessions Page

Poster Sessions

Cellular Oxidants

B 001 ASPECTS OF OXYGEN RADICAL PRODUCTION BY NEUTROPHILS, Bernard M. Babior, Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, CA 92037.

Neutrophils can be triggered by a wide variety of stimuli to manufacture a complex mixture of powerful oxidants for use as microbicidal agents. The starting material for these oxidants is O_2^-, which is produced from oxygen and NADPH by a membrane-bound oxidase that is dormant in resting neutrophils but comes to life when the cells are stimulated. Our studies with a fully soluble oxidase activating system suggest that the activation of the oxidase involves at least 2 cytosolic components plus Mg++, in addition to a membrane-associated element. In intact neutrophils, a family of 48K phosphoproteins participate in oxidase activation; these are not required, however, for oxidase activation in the cell-free system. In studies on mechanisms of killing, we found that neutrophils appear to do relatively little damage to the DNA of their targets. They do, however, induce an unusual sort of mutagenic event whose nature remains to be elucidated.

B 002 TRIGGER PHENOMENA FOR THE RELEASE OF OXYGEN RADICALS BY PHAGOCYTIC LEUKOCYTES. Manfred L. Karnovsky, John A. Badwey, Wilhelm Horn, and Lawrence K. Duffy, Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

A classical example of release of superoxide in biology is that by phagocytic leukocytes when stimulated. The particular cells involved include granulocytes, macrophages in certain states, and natural killer cells. The particular stimuli may be phagocytizable particles (a paradigm for control of infections); substances such as f-met-leu-phe (FMLP) and phorbol myristate acetate (TPA), which are recognized by specific receptors on the cell surface; or substances that perturb the cell membrane bilayer, e.g., polyenoic fatty acids, or retinoids. There are obviously differences between the various examples with respect to the details of signal transduction, but it appears that the phenomenon involves protein kinase C, and, in most cases, is accompanied by changes in the metabolism of inositol phospholipids. In such instances there is usually translocation of protein kinase C from cytoplasm to membrane. However, it is puzzling that this is not always so, presumably because the degree of translocation and the tightness of binding of the translocated protein kinase C to membrane must both be considered. For example, it had been reported that the stimulation of O_2^- release by f-met-leu-phe is not accompanied by translocation of PKC in granulocytes. Data will be presented to indicate that, in fact, it does occur. Of particular current interest is the fact that TPA or mezerein, at optimal concentrations will induce O_2^- release at maximal rates; at the same time PKC is massively translocated. Under conditions of synergy, e.g., mezerein plus calcium ionophore A23187 at levels of each agent that singly are ineffective, but together yield optimal rates of release of O_2^-, translocation is not observable. When "synergistic" concentrations of tumor promoter with 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) are examined, there is again massive release of O_2^- without detectable translocation of PKC, but with increased binding of tritiated phorbol-12,13-dibutyrate to membranes. Such conditions of synergism may be important in the release of oxygen radicals by granulocytes in vivo. A matter of some interest concerns the splitting of phospholipids other than inositol phospholipids during stimulation of granulocytes. Old observations suggested that the involvement of phosphatidylserine might have relevance to signal transduction. Preliminary evidence indicates the accumulation of serine phosphate when these cells are stimulated. Supported by USPHS Grants AI-23323, GM 35307, AI-03260 and AI-00672. We are indebted to Dr. R. Soberman who participated and supplied 5-HETE.

B 003 THE GENERATION AND USE OF HALOGENATED OXIDANTS BY HUMAN PHAGOCYTES, Stephen J. Weiss, M.D., Simpson Memorial Institute, Division of Hematology and Oncology, University of Michigan, Ann Arbor, MI 48109

Polymorphonuclear leukocytes (PMN's) are armed with a complex array of oxygen dependent and independent weapon systems. Although this offensive arsenal is normally reserved for antimicrobial defenses, host tissues are exposed to PMN derived toxins in inflammatory disease states. The most reactive oxygen metabolites generated by PMN's in large quantities are a group of halogenated oxidants which include hypochlorous acid (HOCl), hypobromous acid (HOBr), and a variety of haloamines. Both of these oxidants, as well as the derivative halogenated species, can exert powerful cytotoxic effects in vitro. However, their ability to directly damage cellular targets under physiological conditions is blunted because of both the relative non-selectivity of the highly reactive oxidants and the multiplicity of available biological targets. Instead, we find that halogenated oxidants can indirectly mediate toxicity by regulating the activities of a variety of tissue destructive proteinases including neutrophil elastase, collagenase and gelatinase. This is accomplished via processes wherein halogenated oxidants destroy plasma antiproteinases and activate latent metalloenzymes. In this manner, oxidative regulation of proteolytic activity allows phagocytes to transform short-lived, non-specific effects of halogenated oxidants into long-acting and relatively specific proteinase-mediated damage.

Antioxidant Defense

B 004 SUPEROXIDE DISMUTASE: AN ANTIOXIDANT DEFENSE ENZYME, Hosni M. Hassan - North Carolina State University, Raleigh, NC 27695-7624

Superoxide dismutases (SODs) are considered as the first line of defense against the deleterious effects of oxyradicals in cells. Superoxide dismutases are metalloproteins that specifically catalyze the dismutation of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen. There are three types of superoxide dismutases based on their prosthetic groups. The copper/zinc enzyme (CuZnSOD) is characteristic of eukaryotic cells and is found in the cytosol. The manganese-enzyme (MnSOD) is present in prokaryotes and in the mitochondria of eukaryotic cells. The iron-enzyme (FeSOD) is characteristic of prokaryotic cells. There are number of exceptions, however, to this generalized classification. Thus, CuZnSODs have been found in some bacteria and FeSODs have been reported to be present in some plants. At the present time, the evolutionary origins of these exceptions are not known. There is mounting evidence showing that superoxide radicals play a major role in cellular injury, cause single strand scission in DNA, act as tumor initiators and promoters and are involved in the pathogenesis of many disease. In all cases superoxide dismutases have been shown to protect against these deleterious effects. Most of the work that has led to our present understanding of the regulation and the physiological function of SOD has come from studies using the facultative anaerobe Escherichia coli. This organism exhibits a broad range of oxygen tolerance, and contains both MnSOD and FeSOD. The MnSOD gene (sodA) is located at 88.2 units of the E. coli chromosome, while the FeSOD gene (sodB) is located at 36.5 units. The sodB is expressed under both aerobic and anaerobic growth conditions, while the sodA is not expressed anaerobically. Exposure of anaerobically grown cells to air causes induction of the sodA gene. The sodA gene has been also shown to be induced by a variety of growth conditions and redox-active compounds that increase the intracellular flux of O_2^-. Recent studies have also demonstrated that ferrous iron plays a regulatory role over the expression of sodA. The validity of the superoxide theory of oxygen toxicity and the protective role of superoxide dismutase has been proven beyond any doubt by recent studies using mutants lacking SOD. Thus, oxygen and oxyradicals generator are highly toxic and mutagenic in the sodA sodB double mutant strain of E. coli.

B 005 EXTRACELLULAR-SUPEROXIDE DISMUTASE; MOLECULAR STRUCTURE AND LOCATION IN THE BODY, Stefan L Marklund*, Kurt Karlsson*, Thomas Edlund°, Åke Engström**, Karin Hjalmarsson°°, Gunnar Skogman°°, and Lena Tibell°°, Departments of *Clinical Chemistry and °Microbiology, Umeå University, S-901 87 Umeå, Sweden; **Department of Immunology, Biomedical Center, Box 582, 751 23 Uppsala Sweden, and °°SYN-TEK AB, Box 1451, S-901 24 Umeå Sweden.

Extracellular-superoxide dismutase (EC-SOD) is the major SOD isoenzyme in extracellular fluids like plasma, lymph and synovial fluid, but occurs also in tissues. EC-SOD is a tetrameric glycoprotein with an apparent subunit molecular weight of 30 kDa, and contains 1 Cu and 1 Zn atom per subunit. On chromatography on Heparin-Sepharose EC-SOD is divided into 3 fractions; A which does not bind, B which binds weakly, and C which binds relatively strongly.

A cDNA copy encoding human EC-SOD has been sequenced. It encodes a 240 aa long protein of which 18 aa represent a signal peptide. There is a strong homology with the part of the CuZn SOD's which defines the active site, there is a N-glycosylation site and a structure probably conferring the affinity for heparin. An expression vector containing the cDNA has been transfected into CHO cells. Recombinant EC-SOD secreted by the cells has been isolated and characterized. It is nearly identical to native EC-SOD.

Intravenous heparin leads in investigated mammals to a prompt release of EC-SOD C to plasma. The release is probably due to displacement from heparan sulfate on endothelial cell surfaces. Human EC-SOD C injected into rabbits is rapidly sequestered, but promptly released again to plasma by heparin. EC-SOD C binds to aorta endothelium in situ, and to most cell-types in culture. The binding is abolished by pretreatment of cells with heparitinase. EC-SOD C apparently forms an equilibrium between the fluid phase and the surface of cells in vessels and tissues. This surface-binding constitutes a new principle for the protection of cells against superoxide radicals.

B 006 ANTIOXIDANT DEFENSE: GLUTATHIONE, SELENIUM AND VITAMIN E, Helmut Sies, Institut für Physiologische Chemie I, Universität Düsseldorf, Moorenstrasse 5, D-4000-Düsseldorf, West Germany

Cellular antioxidant defense is compartmented, and different target sites require a variety of antioxidants. In intact cells, we have shown previously that a depletion of glutathione leads to an increased susceptibility to oxidative stress as exerted, for example, by the addition of a model organic hydroperoxide, t-butyl hydroperoxide. However, when the challenge was introduced by a compound capable of redox cycling, then apparently the depletion of glutathione afforded protection. This paradox was resolved in studies with menadione and mitomycin C, showing that the thioether generated with glutathione was also capable of redox cycling, like the parent compound. Further studies with GSH were carried out with isolated rat liver microsomes and with purified enzymes such as xanthine oxidase, horseradish peroxidase and soybean lipoxygenase. Results from these studies indicate that the glutathionyl radical may give rise to singlet molecular oxygen under aerobic conditions.

A selenoorganic compound, ebselen, was found to exhibit GSH peroxidase-like activity. The mechanism of action and the GSH requirement for the protection of cells against oxidative stress, and the metabolism of ebselen were studied recently. In the metabolism, a novel Se-glucuronide was detected.

In microsomes from vitamin E-deficient animals, the addition of vitamin E afforded a prolongation of the lagtime before the onset of lipid peroxidation. This effect was further enhanced by a number of compounds. For example, there is an interaction of vitamins E and C. Also, GSH can enhance the effect of tocopherol. Further thiol compounds were also investigated, and the potential role of enzymatic functions were examined.

For Refs., see: H.Sies(1986)Angewandte Chemie, Int.Ed.Engl.25,1058-1071
H.Sies,ed.(1985)Oxidative Stress, Academic Press, London

Biological Effects of Oxidants

B 007 FREE RADICALS IN MICROCIRCULATION, Karl-E. Arfors, Pharmacia Experimental Medicine, La Jolla, CA 92037

A variety of oxygen derived free radicals are produced as a consequence of normal cellular metabolism. In healthy tissue these potentially toxic metabolic by-products are removed by endogenous scavengers, thus avoiding serious cellular injury. Under conditions of ischemia and reperfusion a number of factors may combine to increase the extent of free radical production, initiate new sources of production (Xanthine oxidase) and consume natural scavenging mechanisms. The resulting increase in free radical activity may well represent an important component in the complex process of tissue injury and may play a significant role in the critical transition from reversible to irreversible cellular injury. The cellular and enzymatic sources of free radicals following reperfusion are however uncertain and somewhat controversial.

Experiments in our laboratory and in collaboration with other research groups have indicated the activated polymorphonuclear leukocyte (PMN) as another damaging entity after reperfusion. It is well known that activation of NADPH oxidase generates free radicals and the activated PMN's will adhere to the endothelial cells. Furthermore, activated PMN's will invade tissue, can release tissue degrading enzymes and induce lipid peroxidation. It has recently been found that PMN depletion or prevention of PMN adherence can protect tissue injury as effectively as free radical scavengers and enzyme inhibitors. A monoclonal antibody MoAb 60.3 totally prevents adherence and extravasation of neutrophils into tissue after reperfusion.

Modulation of PMN function may prove as advantageous as scavengers in organ protection after reperfusion and will definitely improve microcirculation during resuscitation.

B 008 THE ROLE OF PEROXYL FREE RADICALS IN METABOLIC ACTIVATION OF CHEMICAL CARCINOGENS *IN VITRO* AND *IN VIVO.*, Lawrence J. Marnett, Donna Pruess Schwartz, and Annette Nimesheim, Department of Chemistry, Wayne State University, Detroit, MI 48202.

Dihydrodiols are proximate carcinogenic metabolites of polycyclic hydrocarbons. They are oxidized to dihydrodiolepoxides that represent ultimate carcinogenic forms. Epoxidation of dihydrodiols is effected by mixed-function oxidases and by peroxyl free radicals. The stereochemistry of epoxidation provides a technique to differentiate the contribution of each pathway to epoxide formation. For example, the (+)-enantiomer of BP-7,8-diol is epoxidized to the (+)-enantiomer of the *syn*-diolepoxide by cytochrome P-450 but to the (-)-enantiomer of the *anti*-diolepoxide by peroxyl radicals. We have quantitated the formation of each diolepoxide by analyzing tetraol hydrolysis products or deoxynucleoside adducts following administration to mouse epidermal homogenates, freshly-isolated mouse epidermal cells, or mouse skin *in vivo*. The major products of metabolism of (+)-BP-7,8-diol by all three preparations are derived from the *anti*-diolepoxide when uninduced animals are used for the experiments. When animals are pretreated with ß-naphthoflavone 24 hr before the experiments, the major products are derived from the *syn*-diolepoxide. With control or induced animals the principal DNA adducts are derived from the *anti*-diolepoxide. Experiments with epidermal homogenates reveal that *syn*-diolepoxide formation requires NADPH, is abolished by mild heating , and is strongly enhanced by induction. NAPDH-dependent BP-7,8-diol oxidation is not inhibited by low concentrations of phenolic antioxidants. In contrast, *anti*-diolepoxide formation does not require NADPH, is not inhibited by mild heating, is not enhanced by induction, and is potently inhibited by low concentrations of phenolic antioxidants. These results indicate that two separate pethways exist for oxidation of dihydrodiols in mouse skin and that the quantitatively most important pathway for metabolic activation of BP-7,8-diol is one in which peroxyl free radicals are the epoxidizing agents.

Oxidants in Carcinogenesis and Xenobiotic-Induced Tissue

B 009 OXIDANT TUMOR PROMOTERS, Peter A. Cerutti, Swiss Institute for Experimental Cancer Research, 1066 Epalinges/Lausanne, Switzerland.

Oxidants are ubiquitous and may represent "natural" promoters. Our work with xanthine/xanthine-oxidase as an extracellular source of active oxygen (AO) and promotable (clone 41) and non-promotable (clone 30) mouse epidermal cells JB6 allows insights into the mechanism of action of oxidant promoters. We found that (1) AO stimulated the growth only of promotable Cl 41 after an initial period of moderate inhibition while it was strongly cytostatic for non-promotable Cl 30. (2) AO induced larger amounts of DNA strand breaks and poly ADP-ribosylation of chromosomal proteins in non-promotable cells. (3) AO was capable of inducing the growth- and differentiation-related proto-oncogenes c-fos and c-myc in promotable and non-promotable JB6 cells. We speculate that these genes can exert their functions only in the promotable clone 41 because the general cytostatic effects of AO are moderate. A possible explanation for the differences between these two clones was discovered when we compared the constitutive activities, protein concentrations and mRNA levels for the antioxidant enzymes catalase (CAT), Cu,Zn- superoxide dismutase (SOD) and glutathione-peroxidase (GPx). We found that CAT- and SOD- (but not GPx) levels were 2-3 fold higher in the promotable clone 41. We propose that promotable cells possess a superior antioxidant defence which protects them from excessive cytostatic effects of AO.

PMA is known to modulate the expression of numerous genes. We found that PMA induced a decrease in the mRNA and protein levels and enzymatic activities of CAT and SOD in JB6 cells probably contributing to a prooxidant state. These results support the notion that the decrease in CAT in SOD occurs at the mRNA level and is part of the program of modulation of gene expression which is set in motion by PMA.

B 010 PATHOBIOLOGICAL EFFECTS OF TOBACCO SMOKE, TUMOR PROMOTERS AND ASBESTOS IN HUMAN LUNG CELLS IN VITRO. Curtis C. Harris, Roland Grafstrom, James C. Willey, Edward Gabrielson, John Lechner, Angela Somers, Masao Miyashita and Benjamin F. Trump, Laboratory of Human Carcinogenesis, NCI, Bethesda, MD 20892, Karolinska Institute, Stockholm, Sweden, and Department of Pathology, University of Maryland, Baltimore, MD 21201

Data from epidemiological studies indicate that tobacco smoke is the major cause of bronchogenic carcinoma and that exposure to asbestos is the primary cause of mesothelioma and increases the risk of bronchogenic carcinoma in smokers. Therefore, we have been investigating the pathobiological effects of these agents in human bronchial epithelial cells and pleural mesothelial cells in vitro. Tobacco smoke, smoke condensate and smoke-related aldehydes (formaldehyde, acrolein and acetaldehyde) have both genotoxic and nongenotoxic effects in bronchial epithelial cells. For example, the aldehydes cause decreased colony forming efficiency, mobilization of intracellular calcium ion, induction of squamous differentiation, DNA damage, inhibition of certain DNA repair enzymes, and mutations. Exposure of human mesothelial cells to amosite asbestos did not cause a detectable increase in oxygen radicals measured by electron paramagnetic resonance. These and other results suggest that the fibers exert their effects by physically disrupting the spindle apparatus during mitosis.

B 011 MECHANISMS OF OXIDANT-INDUCED CELL DAMAGE, Sten Orrenius, Department of Toxicology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.

When hepatocytes or other mammalian cells are exposed to oxidative stress, their loss of viability is preceded by a depletion of intracellular glutathione (GSH) due to oxidation of GSH and subsequent excretion of glutathione disulfide. The disruption of normal glutathione homeostasis is followed by a modification of protein thiols; our findings with 2-methyl-1, 4-naphthoquinone (menadione) indicate that the toxicity of this redox active quinone is predominantly due to thiol oxidation.

Associated with menadione-induced protein thiol modification in hepatocytes is an impairment of various cell functions, including inhibition of enzyme activity, disruption of intracellular Ca^{2+} homeostasis, and perturbation of cytoskeletal organization. The latter effect appears to be responsible for the formation of the numerous plasma membrane blebs, typically seen in hepatocytes exposed to toxic levels of menadione. A recent study has shown that menadione-induced bleb formation in hepatocytes is caused predominantly by the oxidation of thiol groups in actin and the formation of large-molecular weight actin aggregates.

Following the disruption of thiol homeostasis in menadione-treated hepatocytes there is an impairment of Ca^{2+} transport and a subsequent perturbation of intracellular Ca^{2+} homeostasis, resulting in a sustained increase in cytosolic Ca^{2+} concentration. This Ca^{2+} increase can result in the activation of various Ca^{2+}-dependent degradative enzymes (phospholipases, proteases, endonucleases) which may contribute to cell killing. Thus, a previous study has shown that Ca^{2+}-activated proteases may play a critical role in the toxicity caused by extracellular ATP or cystamine to hepatocytes, whereas a more recent investigation has provided evidence for the activation of a Ca^{2+}-dependent endogenous endonuclease, previously shown to be involved in programmed cell death, in menadione-exposed hepatocytes. However, further work is required to establish the relative contribution of these Ca^{2+}-dependent processes to toxic cell killing.

Structure and Expression of Oxidant Stress Related Genes -I

B 012 MECHANISMS OF OXYGEN TOXICITY AS REVEALED BY STUDIES OF YEAST MUTANTS WITH CHANGED RESPONSE TO OXIDATIVE STRESS, **Tomasz Biliński,Jadwiga Litwińska,Zdzisława Krawiec and Mieczysław Błaszczyński,Zamość College of Agriculture,Agricultural Academy of Lublin, Pl 22-400 Zamość ul. H.Sawickiej 102 POLAND**

The vast majority of information as well as conclusions concerning the mechanisms of oxygen toxicity come from in vitro studies.Therefore it is obvious that hypotheses put forward to explain O_2^- toxicity,based on in vitro type studies must be verified in in vivo experiments.One classic approach , which was successfully applied to study other metabolic systems is the use of mutants.We have isolated a number of catalase/1/ and CuZnSOD deficient /scd/ mutants/2/. Their response to oxidative stress gave strong support to the opinion/3/ that O_2^- can exert deleterious effects on the cell directly. New data will be presented,which seem to explain why the protective role of catalases is hardly detectable under"physiological"conditions.Further studies on the mutants revealed, that anaerobically grown cells bearing scd mutations are hypersensitive to the products of autooxidation of polyunsaturated fatty acids.This character and the fact that anoxia leads to the lack of MnSOD activity/4/ suggest that superoxide dismutases are in some way involved in preventing such toxic effects.Hypersensitivity of various tumor cell lines to these products/5/and frequently observed deficiency of these cells in SOD activity/6/ make this observation worth further studies.The isolation of physiological suppressors of scd1 mutation represents another example of the use of mutants in studies on oxygen toxicity in vivo. This approach could be useful in studies concerning intracellular sources ofO_2^-, their cellular targets and possible protective mechanisms alternative to the activity of superoxide dismutases.Preliminary characteristics of such mutants will be presented.

/1/. A.Traczyk et al./1985/ Acta Microbiol.Polon.34.231. /2/.T.Biliński et al./1985/Biochem.Biophys.Res.Commun. 130,533. /3/. I.Fridovich / 1986/ Arch.Biochem.Biophys. 247,1. /4/. A.Autor /1982/ J.Biol.Chem. 257,2713. /5/. E.Schauenstein /1967/ J.Lipid Res. 8,417. /6/. L.W.Oberley and D.R. Spitz /1984/ in Methods in Enzymology 105 , 457. /L.Packer ed.Academic Press

B 013 A REGULON FOR DEFENSES AGAINST OXIDATIVE STRESS IN SALMONELLA AND E. COLI Michael F. Christman*, Gisela Storz#, and Bruce N. Ames#, *Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge MA 02142, and Department of Biochemistry, UC Berkeley, Berkeley, CA 94720.

Salmonella and E. coli become resistant to killing by hydrogen peroxide and other oxidants when pretreated with nonlethal levels of hydrogen peroxide. During adaptation to hydrogen peroxide, 30 proteins are induced. Nine are constitutively overexpressed in dominant hydrogen peroxide resistant oxyR mutants. Mutant oxyR1 is resistant to a variety of oxidizing agents and overexpresses at least five enzyme activities involved in defenses against oxidative damage. Deletions of oxyR are recessive and uninducible by hydrogen peroxide for the nine proteins overexpressed in oxyR1, demonstrating that oxyR is a positive regulatory element (1).

Five of the 30 hydrogen peroxide-inducible proteins have been identified, and their structural genes mapped (2). Other stresses such as heat-shock, naladixic acid, ethanol, and cumene hydroperoxide treatment also induce subsets of the 30 hydrogen peroxide-inducible proteins as well as additional proteins. Salmonella strains containing deletions of oxyR show 10-55 fold higher freqeuncies of spontaneous mutagenesis compared to otherwise isogenic oxyR+ controls (3). The largest increase in mutation frequency is observed for T:A to A:T transversions (40-146 fold), the base substitution mutation most frequently caused by chemical oxidants.

DNA sequence analysis of the oxyR gene (4) shows a single open reading frame of 305 amino acids with homology to LysR, another bacterial regulatory protein. The constitutive oxyR1 mutant carries a C:G to T:A transition mutation at codon 233 (Ala to Val). The precise mechanism by which oxidative stress activates the oxyR regulon is unknown.

1. Christman, M.F., Morgan, R.W., Jacobson, F.S., and Ames, B.N. (1985) Cell 43, 753-762.
2. Morgan, R.W., Christman, M.F., Jacobson, F.S., Storz, G., and Ames, B.N. (1986) P.N.A.S. 83, 8059-8063.
3. Storz, G., Christman, M.F., Sies, H., and Ames, B.N. (1987) P.N.A.S., in press.
4. Christman, M.F., Storz, G., and Ames, B.N., in preparation.

B 014 TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF MnSOD IN E. coli, Danièle TOUATI. Institut Jacques Monod, CNRS, Paris 7, 2 place jussieu 75251 PARIS CEDEX 05, FRANCE.

E. coli contains two superoxide dismutases. Whereas the level of iron containing enzyme is relatively constant, the level of the manganese containing enzyme (MnSOD) is modulated by numerous factors including oxygen , superoxide radicals, metal ions (Mn, Fe). We constructed tools in order to dissociate transcriptional, post-transcriptional and post-translational regulatory events. Protein fusions with the lacZ gene, in which ß-galactosidase is induced by MnSOD inducers, operator fusions in which the MnSOD is expressed from the foreigner "tac" promoter, plasmids carrying different fragments of the sodA structural or regultory region, and host cells devoid of SOD, were used to investigate the effects of various factors on MnSOD expression.

Our results show a multicontrol of MnSOD :

- the oxygen dependent induction mediated by superoxide radicals occurs at a transcriptional level, via a yet unidentified positive regulator. Although MnSOD has been shown to be induced by the oxidative stress produced by exposure to H_2O_2 or by heat shock, the induction of MnSOD by superoxide radicals is not dependent on the expression of the oxyR or htrpR gene which positively regulates induction of oxyR regulon or heat shock proteins respectively.
- ferrous ions inhibit MnSOD expression both at transcriptional and post-transcriptional level.
- in addition expression in trans of largely truncated MnSOD protein inhibits induction of ß-gal in protein fusions, strongly suggesting an autogenous regulation which do not imply the entirety of the apoenzyme.
- Finally Mn^{++} concentration can modulate MnSOD activity at the post-transcriptional stage of conversion from apoenzyme to holoenzyme.

Progress in identification and characterization of various regulation mutants will be presented.

Structure and Expression of Oxidant Stress Related Genes -II

B 015 CONTROL OF GLUTATHIONE PEROXIDASE GENE EXPRESSION, Ian Chambers and Paul R. Harrison, The Beatson Institute for Cancer Research, Glasgow, Scotland.

Our previous work has shown the selenocysteine (sec) in the active site of the mouse glutathione peroxidase (GSHPx) gene to be encoded by the "stop" codon, TGA(EMBO J.5,1221). A comparison of the sequences around the TGA codons of known selenoprotein mRNAs and mRNAs using TGA as a "stop" codon was conducted in an attempt to identify which other sequences in the mRNA are important in distinguishing between these two TGA functions and these results shall be presented. GSHPx mRNA is present in most cells at a low basal level, although in liver, kidney and erythroid cells this level of expression is elevated 20 -50 fold. To attempt to elucidate the molecular mechanisms underlying these differences in gene expression a segment of DNA extending 650 bp upstream from the major cap site was placed immediately upstream of the chloramphenicol acetyl transferase (CAT) gene. The resulting DNA was then introduced into cells expressing high (Friend) or low (L-) levels of GSHPx mRNA and the cell specificity of transcription assayed by measuring CAT enzyme activity. Similarly, a DNA segment from the 3' end of the gene shown by sequence analysis to contain regions homologous to the consensus binding sequences of known transcriptional regulatory protiens was assayed for its ability to modulate the level of transcription from a promoter linked in cis. These experiments show the GSHPx promoter to be equally active in both cell types. Moreover, the level of expression from either the GSHPx or a heterologous promoter is not affected in either cell type by the presence, in cis,of the 3' sequences. These results are discussed.

B 016 OVEREXPRESSION OF HUMAN CuZnSOD GENE IN TRANSFECTED CELLS AND TRANSGENIC MICE: IMPLICATION FOR DOWN'S SYNDROME PATHOLOGY, Yoram Groner, Orna Elroy-Stein, Karen B. Avraham, Galit Rotman, Yael Bernstein, Naomi Dafni and Michael Schickler, Dept. of Virology, Weizmann Institute of Science, Rehovot, Israel.

The "housekeeping enzyme CuZnSOD is encoded by a gene residing on human chromosome 21, at the region 21q22 known to be involved in Down's syndrome (DS). This disease is the most common genetic abnormality occurring once per 1000 live births and is the major known cause of mental retardation in the Western world. It results from the presence in the cells of an extra copy of chromosome 21, or a portion of it, the 21q22 segment. Overexpression of the CuZnSOD gene, due to gene dosage, may disturb the steady state equilibrium of active oxygen species within the cell, resulting in oxidative damage to biologically important molecules. Such a mechanism may in part be involved in the pathogenesis associated with the syndrome. To investigate the possible involvement of CuZnSOD overproduction in the etiology of DS we first cloned its cDNA, isolated the gene and constructed expression vectors containing the neo^R selectable marker. The recombinant plasmids were transfected into human HeLa cells, mouse L-cells and rat PC12 cells. Stable transformants were obtained, expressing elevated levels (up to six fold) of authentic, enzymatically active human CuZnSOD. HeLa and L-cell that overexpress the transfected CuZnSOD had altered properties; they were more resistant to paraquat than the parental cells and showed an increase in lipid peroxidation. Enhanced lipid peroxidation may alter the structure and function of cell membranes and thus contribute to clinical symptoms observed in DS. The PC12 transformants were analyzed for altered properties of their neurobiological functions e.g. membrane excitability after nerve growth factor treatment and neurotransmitter uptake. To relieve the symptoms caused by overexpression of CuZnSOD an "antisense" SOD plasmid was constructed. When introduced into L-cells expressing elevated levels of human CuZnSOD it caused a dramatic reduction in SOD expression. To study the influence exerted on development and morphogenesis by elevated levels of CuZnSOD we transferred the human gene into mouse embryos and prepared transgenic mice overexpressing the h-CuZnSOD gene. These animals synthesize the human enzyme in an active form capable of forming human-mouse enzyme heterodimers. Elevated human CuZnSOD activity was detected in nearly all tissues of the animals expressing the gene but the levels of activity varied from tissue to tissue. Expression in liver was quite low whereas high enzymatic activity was detected in the brain. Experiments are now under way to determine whether the biochemical effects which were observed in the transfected cells are detectable in the transgenic animals.

B 017 MOLECULAR STRUCTURE AND EXPRESSION OF THE HUMAN Mn-SOD GENE, Yaffa Beck, Rachel Oren, Carolina Abramovich, Boaz Amit, Avigdor Levanon, Marian Gorecki and Jacob R. Hartman, BioTechnology General Ltd, Kiryat Weizmann, Rehovot 76326, Israel.

Human Mn superoxide dismutase (MnSOD) encoded by chromosome 6 is a mitochondrial matrix enzyme positioned to scavenge oxygen radicals produced by the extensive oxidation-reduction and electron transport reactions occurring in that organelle. Nucleotide sequence analysis of human MnSOD cDNA clones suggests a mature protein of 198 amino acids preceded by a 24 amino acid prepeptide, in accordance with processing required for transport into mitochondria.
Southern blot hybridization to human DNA digested with several restriction endonucleases suggest a single copy gene. The gene for human MnSOD has been isolated and characterized. It spans about 15 Kb of chromosomal DNA and is composed of 6 exons interrupted by introns with typical splice junctions. The promoter region is highly rich in GC, contains 8 repeats of GGGCGG, consensus core for binding SP1 transcription factor, and includes several stem-loop structures as well as inverted repeats. However, it lacks TATA or CAT boxes.
Two classes of polyA RNA were identified in the cytoplasm of various human cells: A major fully processed transcript, 1000 nt long and a minor transcript, 4000 nt long. The longer transcript hybridizes to the fifth intron of MnSOD gene, downstream from the exon coding for the carboxy terminus of the enzyme. This partially spliced RNA is non-tissue specific and its significance is yet to be determined. Both mRNAs terminate at the same site about 10 nucleotides downstream from a single AATAAA polyadenylation signal at the 3' end of the gene.
SOD mRNA abundancy in human cells is in the order of 10^{-3}% of total polyA RNA. Both MnSOD and CuZnSOD are expressed simultaneously, suggesting the importance of each of the differentially compartmentalized enzymes for cellular survival.
Expression of active human MnSOD from its engineered cDNA has been achieved in *E. coli*. The isolation of gram quantities of pure enzyme should provide the means to elucidate the physical basis for superoxide dismutation and evaluate its broad therapeutic potential.

B 018 COMPLEMENTATION OF SUPEROXIDE DISMUTASE-DEFICIENT ESCHERICHIA COLI MUTANTS WITH CLONED EUKARYOTIC CU/ZN SUPEROXIDE DISMUTASE, Donald O. Natvig, Department of Biology, University of New Mexico, Albuquerque, NM 87131. The advent of E. coli cells that are completely lacking in Fe and Mn superoxide dismutases (SODs) (Carlioz and Touati 1986 EMBO J. 5:623-630) has provided an extremely valuable tool for studying the function and importance of proteins and genes believed to be involved in protecting organisms from the toxic byproducts of oxidative metabolism. The observed characteristics of these cells include (1) a failure to grow on minimal medium in the presence of atmospheric concentrations of oxygen; (2) hypersensitivity to superoxide-generating compounds, most notably paraquat; and (3) hypersensitivity to the toxic effects of hydrogen peroxide (op cit). By employing a recombinant plasmid carrying a cDNA-derived gene from human Cu/Zn superoxide dismutase (Hallewell et al. 1985 Nucleic Acids Res. 13:2017-2034), we have shown that expression of this gene in SOD-deficient E. coli cells reverses the oxygen-sensitivity characteristics mentioned above (Natvig et al. 1987 J. Biol. Chem. 262:14697-14701). These results provide additional strong evidence that the Cu/Zn and bacterial SOD proteins, though evolutionarily unrelated, have a common biological function. The results further suggest that SOD-deficient E. coli cells may provide a selection for cloned SOD genes. Studies in progress are investigating (1) the efficiency of complementation as a function of specific SOD activity and SOD type; and (2) the role of Cu/Zn SOD in reversing the hypersensitivity to oxygen of sodA sodB htpR triple mutants, which are sensitive to oxygen even on complex medium.

Oxidants in Atherogenesis

B 019 SUPEROXIDE-DEPENDENT OXIDATION OF LOW DENSITY LIPOPROTEIN, Jay W. Heinecke, Department of Biochemistry, SJ 70, University of Washington, Seattle WA 98195. Atherogenesis may be promoted by increased macrophage uptake of low density lipoprotein (LDL) modified by incubation with cells of the arterial wall. We have shown that LDL modification by arterial smooth muscle cells is accompanied by lipid peroxidation, apoprotein fragmentation, and requires transition metals. These results indicate that LDL modification by cells occurs by a free radical process. Loss of intact apoprotein B in oxidized LDL was accompanied by a parallel loss in LDL receptor mediated uptake by cells. In contrast, oxidized LDL was taken up to a greater degree than native LDL by macrophages, suggesting recognition of the oxidized lipoprotein by the scavenger receptor. Extracellular superoxide was detected in cultures of smooth muscle cells as measured by the superoxide dismutase inhibitable reduction of cytochrome c and acetylated cytochrome c. Superoxide dismutase and lipid soluble free radical scavengers inhibited LDL modification by cells, while mannitol and catalase were without effect. Thus, smooth muscle cells oxidize LDL by a superoxide dependent mechanism which does not require hydrogen peroxide or hydroxyl radical. Both LDL oxidation and superoxide production by cells required the disulfide L-cystine in the incubation medium. We proposed that smooth muscle cells take up L-cystine from the medium, reduce L-cystine to L-cysteine, which reoxidizes extracellularly with reduction of oxygen to superoxide. To test the proposed role of thiols in LDL modification by cells, LDL was incubated with L-cysteine and glutathione in a cell-free system. Under these conditions, both thiols were able to initiate oxidation of LDL in the presence of transition metals. However, only the L-cysteine dependent oxidation of LDL was inhibited by superoxide dismutase. Similarly, only L-cysteine oxidized LDL was taken up to a greater degree than native LDL by macrophages. These results indicate that oxidation alone is able to produce all of the changes in the properties of LDL modified by incubation with cells and strongly suggest that smooth muscle cells oxidize LDL by a superoxide mediated, L-cysteine dependent mechanism. Extracellular oxidation of proteins by cell-generated thiols may have broad biological significance.

B 020 CHANGES IN THE STRUCTURE AND FUNCTION OF LDL FOLLOWING INTERACTIONS WITH 4-HYDROXYNONENAL, Henry F. Hoff, Guy M. Chisolm III, Diane W. Morel, Günther Jürgens and Hermann Esterbauer, Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44106.

4-hydroxynonenal (HNE), a propagation product of lipid peroxidation, has been shown to be associated with both lipid and protein of low density lipoproteins (LDL) following in vitro oxidation. Oxidized LDL demonstrates two functional characteristics related to atherogenesis: unregulated uptake by tissue macrophages and toxicity to proliferating cells. These may explain foam cell formation in fatty streak lesions and the necrotic core observed at the base of atherosclerotic lesions. We wished to determine whether these two functional characteristics of oxidized LDL could be mimicked by interacting LDL directly with HNE. Incubation of LDL with 1 to 5 mM HNE led to 61 to 143 mol, respectively, of HNE covalently bound to each mol of apolipoprotein B. 39 to 99 mol, respectively, of HNE were associated with the corresponding lipid phase of LDL. These assessments were made using ^{14}C-HNE. Relative fluorescence of the apolipoprotein of LDL at 430 emission/360 excitation increased with the addition of HNE: from 22 units after incubation without HNE to 42 units after incubation with 5 mM HNE. Incubation of LDL with concentrations of HNE up to 4 mM led to a linear decrease in uptake of HNE-LDL by the macrophage cell line, J774. Incubation of LDL with 5 mM HNE, however, yielded a modified LDL which demonstrated an increase in cell uptake as measured both by stimulation of cholesterol esterification and by degradation of the ^{125}I-labeled lipoprotein. The concentration dependency of degradation showed saturation, but the affinity of the association was relatively low (half maximum degradation occurred at about 500 µg LDL-protein per ml). Degradation of labeled HNE-LDL (after incubation with 5 mM HNE) by macrophages was examined with 40-fold excesses of unlabeled acetyl LDL, LDL and HNE-LDL; only HNE-LDL inhibited degradation significantly. These results suggest that HNE-LDL is taken up by a mechanism independent of the scavenger receptor. When HNE-LDL was incubated with proliferating fibroblasts for 66 hr, the modified lipoprotein caused a concentration-dependent toxicity. This effect was similar to that seen with oxidized LDL and, as with oxidized LDL, the toxic factor was located in lipid extract of the lipoprotein. The toxicity of both oxidized LDL and HNE-LDL to proliferating cells was inhibited by coincubation with HDL. An LDL-like fraction extracted from human aortic atherosclerotic lesions (A-LDL) demonstrated similar functional properties as HNE-LDL, namely uptake by macrophages by a mechanism independent of scavenger and LDL receptors, and toxicity to proliferating cells in vitro. The similarities among HNE-LDL, oxidized LDL, and A-LDL suggest that association of HNE to LDL could be responsible for the enhanced uptake by macrophages, and increased toxicity to proliferating cells, of oxidized LDL and A-LDL. Lipid peroxidation of LDL, and its modification by alkenals like HNE, may occur when LDL accumulates in the artery wall. This may be the lipoprotein modification responsible for foam cell formation and the occurrence of necrotic regions in atherosclerotic lesions.

B 021 OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEIN (LDL) AND ITS POSSIBLE ROLE IN ATHEROGENESIS, Daniel Steinberg, Univ. of Calif. San Diego, La Jolla, CA 92093.

We have postulated that LDL only becomes fully atherogenic after it has undergone oxidative modification mediated by oxy-radicals: 1) Native LDL does not generate foam cells from macrophages in culture but oxidatively modified LDL (OX-LDL) does; 2) Native LDL is not chemotactic for circulating monocytes but OX-LDL is; 3) Native LDL has no effect on macrophage motility but OX-LDL inhibits it, an effect that could "trap" macrophages in the subendothelial space; 4) Native LDL is not cytotoxic but OX-LDL is, as shown in other laboratories. We have now tested this hypothesis in vivo and shown that an antioxidant can inhibit LDL degradation in arterial wall macrophages without inhibiting LDL degradation in the unaffected parts of the aorta. This is compatible with the proposed need to generate OX-LDL before its uptake becomes maximal in the macrophage; the unaffected areas contain no macrophages, thus serving as a negative control. The studies were done in LDL receptor-deficient rabbits and the antioxidant used was probucol, a drug used for treatment of hypercholesterolemia. The antioxidant effect of probucol discovered in our laboratory about a year ago, is probably unrelated to its cholesterol-lowering effects. The latter, however, required that we use a second control group treated with lovastatin, another cholesterol-lowering drug, so that the cholesterol levels in the two groups were matched. The animals treated with probucol showed 50% less extensive lesions in the aorta than the animals treated with lovastatin. We conclude that probucol, acting as an antioxidant, is antiatherogenic by inhibiting the generation of OX-LDL.

Oxidants in Pathologies of the Lung, Brain and Pancreas

B 022 BIOCHEMICAL MECHANISMS OF OXIDANT-INDUCED CELL INJURY, Charles G. Cochrane, Ingrid Schraufstatter, Janis H. Jackson, and Paul Hyslop, Research Institute of Scripps Clinic, La Jolla, CA 92037.

While oxidants have been detected by indirect means in inflammatory sites, knowledge of their participation in the development of injury has been limited by a lack of information about the biochemical mechanism of oxidant-induced damage of cells and tissues. In order to obtain an understanding of this problem, we have exposed several cell types (P388D1, GM1380, human lymphocytes, bovine endothelial cells etc.) to a variety of oxidants, including the spectrum of oxidants released by PMA-stimulated human leukocytes. Within 20 sec. following exposure of cells to oxidants, the glutathione (GSH) cycle was stimulated with a resulting 5-10 fold increase in the pentose pathway.[1] Ca^{2+} was translocated from intracellular stores within 60 sec., but only after 15-20 min was there a detectable increase in free intracellular Ca^{2+} which increased levels of intracellular Na^{+} and decreased levels of K^{+}.[2] Cellular energy systems were compromised within 3 min, leading to a dose (oxidant)-dependent fall in ATP.[3] Two principal mechanisms have emerged as being responsible for this fall in ATP: the glycolytic pathway is blocked at the position of glyceraldehyde-3-PO_4-dehydrogenase (GAPDH) and mitochondrial function is impaired.[4] In the first mechanism, the glycolytic pathway of ATP formation was inhibited within a few minutes' exposure to oxidants such as H_2O_2 or HOCl in uM concentration. Activity of the GAPDH-controlled steps in glycolysis resulted from direct inactivation of GAPDH by oxidation and an indirect impairment of GAPDH activity owing to diminished concentration of its essential coenzyme, nicotinamide adenine dinucleotide (NAD).[2,5] The fall in NAD levels in cells exposed to oxidants led us to a major target of oxidants:DNA. We found that the fall in NAD resulted from activation of an enzyme, poly ADP ribose polymerase (polymerase), which is known to function in DNA repair.[5,6] Studies of DNA in the target cells then revealed that within 20 seconds exposure to uM H_2O_2 (but not HOCl), DNA strand breaks could be measured.[5] In further studies of oxidant-induced DNA damage, H_2O_2 in the presence of 30 uM Fe induced strand breaks of isolated PM2 supercoiled DNA concomitant with the formation of $^{\cdot}OH$ (DMPO-OH adduct formation in EPR analysis).[7] Chelation of Fe or the addition of $^{\cdot}OH$ scavengers (DMSO,DMTV,etc), inhibited both $^{\cdot}OH$ formation and strand breaks.[7] The formation of 8-OH deoxyguanosine in whole cells exposed to stimulated human neutrophils (revealed by HPLC with electrochemical detection) confirmed the role of $^{\cdot}OH$ in DNA damage. These studies indicate DNA is an important primary target of oxidants.

Refs: 1. J.C.I. 76:1131, 1985. J.C.Phys. 129:356, 1986. 3. J.C.I. 76:1471, 1985. 4. J.B.C. Jan. 1988. 5. J.C.I. 77:1312, 1986. 6. PNAS. 83:4908, 1986. 7. J.C.I. 80:1090, 1987.

Oxidants in Infectious Disease

B 023 INTERPLAY OF REACTIVE OXYGEN SPECIES AND TUMOR NECROSIS FACTOR IN TISSUE INJURY, Ian A. Clark and Geeta Chaudhri, Australian National University, Canberra, ACT 2601, Australia.

Several years ago we provided in vivo evidence consistent with the proposal that reactive oxygen species were involved in host-induced damage to malarial parasites and, in higher concentrations, to the host itself(1). We had earlier suggested an important role for tumor necrosis factor (TNF) in these events(2), and with the advent of recombinant TNF began to test this proposal. Both systems proved to overlap functionally, with both antioxidants(3) and antibody to mouse TNF(4) able to inhibit experimental cerebral malaria. Another practical example of interaction between oxidant stress and TNF appears to be dyserythropoiesis. The anaemia of chronic infections, including malaria, is in part due to the breakdown of erythropoiesis, with multinucleated, pyknotic and karyorrhexic erythroid precursors being observed in bone marrow preparations. Such changes are induced by oxidant stress, being well-described in several species, including monkeys, fed diets deficient in vitamin E. We have recently reproduced these changes by injecting recombinant TNF into mice. Erythrophagocytosis, to which oxidantly-stressed red cells are particularly prone, is also common in bone marrows of TNF-treated mice.

We rationalized this through reports that TNF sensitizes neutrophils and monocytes to agents that induce them to generate reactive oxygen species, but we now report a further degree of complexity in this relationship: exogenous oxidant stress enhances, and radical scavengers and iron chelators inhibit, the release of TNF from macrophages. Thus TNF may provide a multiplier effect in oxidant damage, initial mild oxidant stress promoting release of TNF and this enhancing release of oxidants from leukocytes. Additionally, TNF can attract neutrophils and cause them to adhere to endothelial cells. This could explain their presence in oxidant damage to lungs, in ischaemia/reperfusion injury, and in infectious causes of shock lung such as malaria. Antibody to TNF warrants testing, in conjunction with antioxidants, in these circumstances.

1. Clark, I.A. and Hunt, N.H. (1983) Infect. Immun. 39: 1-6.
2. Clark, I.A., Virelizier, J.-L., Carswell, E.A. and Wood, P.R. (1981) Infect. Immun. 32: 1058-1066.
3. Clark, I.A., Thumwood, M., Chaudhri, G., Hunt, N.H. and Cowden, W.B. In: Free Radicals, Oxidant Stress and Drug Action. Ed. C. Rice-Evans, Richelieu Press, London, 1987, in press.
4. Grau, G.E., Fajardo, L.F., Piquet, P.F. et al. (1987) Science, in press.

B 024 OXIDANTS IN VIRAL DISEASE, Ernst Peterhans*, Thomas Burge and Marc Vandevelde**, *Institute of Veterinary Virology and **Animal Neurology, University of Berne, CH-3012 Berne, Switzerland.

The effector functions of phagocytic cells are focused on invading microorganisms by means of specific antibodies. This mechanism prevents the host from becoming the target of its own defense mechanisms. Several viruses are capable of bypassing the control of phagocyte triggering. On binding to the surface of polymorphonuclear leukocytes and monocytes, influenza- and several paramyxoviruses (e.g., Sendai and Newcastle Disease Viruses) stimulate in these cells the generation of a burst of reactive oxygen species (ROS). Viruses which in vitro trigger this phagocyte response are toxic in vivo by a mechanism involving the interaction between the viral particle and phagocytes.

We will describe one additional type of virus-related oxidant generation in cells of the monocyte-macrophage series. The replication of enveloped viruses in these cells results in the expression of viral surface antigens. Antiviral antibody can bind these antigens through Fab and via Fc interact with Fc receptors on the same infected mononuclear phagocyte, thus inducing in this cell a burst of ROS generation. We have observed this type of ROS generation in cultured microglial cells infected with a neurotropic virus, Canine Distemper Virus.

These two examples of virus-related ROS generation are complemented by several other mechanisms, such as complement activation and virus-induced TNF-production. In vitro evidence as well as observations made in vivo suggest that "autotoxicity" may contribute to, and in some cases essentially be responsible for, the symptoms and pathology of viral disease.

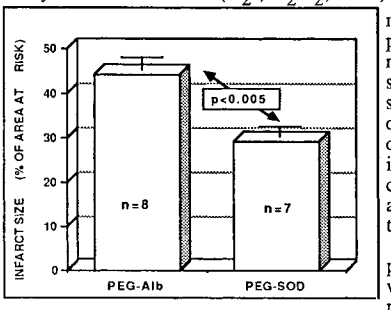
B 025 CYTOKINES AND OXYGEN RADICAL MEDIATED INJURY, Peter A. Ward, Jeffrey E. Warren, David Gannon, Kent J. Johnson, Sem H. Phan and James Varani, Department of Pathology, University of Michigan, Ann Arbor, MI 48104.

We have evaluated the ability of macrophage release products as well as recombinant human tumor necrosis factor (TNF) and interleukin 1 (IL-1) to influence oxygen radical responses of phagocytic cells and to alter neutrophil-dependent oxygen radical mediated injury of cultured endothelial cells. In a dose-dependent manner, exposure of rat alveolar macrophages to supernatant fluids from zymosan stimulated rat macrophages or to TNF or IL-1 does not initiate O_2^- responses in alveolar macrophages but "primes" these cells but not neutrophils for accentuated responses following addition IgG containing immune complexes. In additional experiments, rat pulmonary artery endothelial cells have been pretreated with TNF or IL-1 and then washed. When cytokine treated endothelial cells are then incubated with phorbol ester activated neutrophils, there is a substantial increase in oxygen radical mediated killing of endothelial cells. This killing is dependent on the concentration of cytokine as well as the duration of contact of endothelial cells with cytokine. Pretreatment of neutrophils with TNF or IL-1 does not enhance their ability to kill endothelial cells after addition of phorbol ester. The ability of the cytokines to increase susceptibility of endothelial cells to the cytotoxic effects of activated neutrophils can at least in part be related to an increased adherence of neutrophils to cytokine treated endothelial cells. These findings demonstrate that cytokines can enhance oxygen radial mediated injury by two different and independent mechanisms: alteration of either effector cells or target cells, or both.

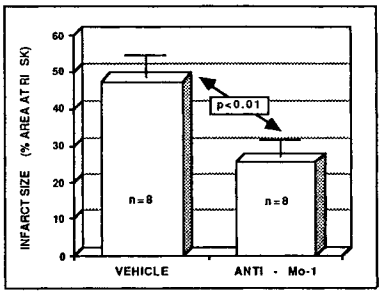
Ischemia - Reflow

B 026 THE ROLE OF OXY-RADICALS IN MYOCARDIAL REPERFUSION INJURY, Benedict R. Lucchesi, Yasuo Tamura and Paul Simpson. The University of Michigan Medical School, Ann Arbor, MI. 48109.

During the course of myocardial ischemia and especially upon reperfusion, the generation of oxygen derived free radical species is capable of producing both reversible and irreversible vascular endothelial and myocyte damage. In addition to intracellular sources of cytotoxic oxygen species such as the mitochondria, and the xanthine oxidase activity of the endothelial cell, the polymorphonuclear leukocyte represents the most significant extracellular source of superoxide anion and other derived reactive oxygen metabolites. Neutrophils which enter the ischemically injured myocardium under the influence of chemotactic attraction and activation of the complement system generate a number of cytotoxic substances (O_2^-, H_2O_2, OCl^-) in addition to releasing tissue destructive enzymes (elastase and metalloproteinases). Our studies have demonstrated that the pharmacologic suppression of neutrophil function or the induction of neutropenia can result in a significant salvage of heart muscle that is subjected to a period of ischemia followed by reperfusion. Recent studies with polyethylene conjugated superoxide dismutase have demonstrated that the reduction in infarct size is evident when quantitated 4 days after reperfusion and is accompanied by a prolonged increase in the circulating plasma SOD activity for periods of up to 7 days.



One of the most promising efforts to-date with respect to preventing myocardial reperfusion injury has been with the use of a monoclonal antibody directed against the leukocyte adhesion promoting CDw 18 receptor complex or Mo-1 receptor. Administration of the anti-Mo-1 antibody prevents leukocyte adherence to the endothelium as well as the production of cytotoxic oxygen radicals. The net result is a reduction in myocardial infarct size in the reperfused myocardium. Thus, myocardial reperfusion after a critical period of ischemia is associated with oxygen free radical mediated cell death of otherwise viable cardiac myocytes. The ultimate extension of the injury can be reduced by modulating one or more of the potential sites of formation of cytotoxic oxygen species.



B 027 **INACTIVATION OF CREATINE KINASE BY SUPEROXIDE DURING REPERFUSION INJURY**, *J.M. McCord and W.J. Russell, Univ. of South Alabama, Mobile, AL 36688.*
Isolated perfused rat hearts subjected to 80 min of complete ischemia followed by 10 min of oxygenated reperfusion, released creatine phosphokinase (CPK). The addition of superoxide dismutase (SOD) (20 ug/ml) to the perfusate resulted in a doubling of the amount of CPK activity present in the perfusate, suggesting that either SOD exacerbated tissue injury, or that SOD had protected the CPK activity from radical attack, resulting in the presence of more *active* CPK in the perfusate. Experiments with purified enzymes confirmed the latter possibility. CPK was quickly inactivated by subphysiologic concentrations of xanthine oxidase plus xanthine. SOD, catalase, and desferrioxamine protected against inactivation. The protection by catalase and desferrioxamine increased as pH increased from 6 to 8, suggesting an increasing contribution by the Haber-Weiss mechanism at the higher pH. SOD provided >95% protection throughout the pH range. This suggests that CPK can be inactivated by both superoxide and hydroxyl radicals, with inactivation by superoxide *per se* accounting for about half of the total inactivation occurring at pH 6. The rate constant for the reaction of CPK with superoxide is approximately 2×10^5 M^{-1} sec^{-1}. (This is equivalent to the rate constant for superoxide reacting with ferricytochrome *c*, and is about four orders of magnitude less than the rate constant for SOD). CPK is largely irreversibly inactivated, with the percentage of irreversibility increasing with time of exposure to xanthine oxidase/xanthine. Because CPK is an enzyme essential to normal energy balance in the myocardium, its inactivation during ischemia/reperfusion may account for some of the derangements of contractile function and high-energy phosphate balance observed following reperfusion.

B 028 MEASUREMENT OF FREE RADICAL GENERATION IN THE POST-ISCHEMIC HEART
Jay L. Zweier, Johns Hopkins University, Baltimore, MD 21224

It has been hypothesized that oxygen free radicals are important mediators of cell damage in the post-ischemic heart. Electron paramagnetic resonance techniques have been developed and applied to measure this myocardial free radical generation. Direct measurements performed on hearts freeze clamped at 77 K demonstrate that a signal $g_{\parallel}=2.033$ $g_{\perp}=2.005$ with properties indicative of the alkyl peroxy radical, ROO·, is generated in ischemic myocardium and greatly increases during reperfusion. Studies performed perfusing the spin trap DMPO (10-50 mM) demonstrate no detectable radicals during control perfusion while on reperfusion the highly reactive ·OH is trapped along with the R· or RO· derived from ·OH, as evidenced by the appearance of prominent DMPO-OH ($a_N=a_H=14.9$ G) and DMPO-R or DMPO-OR ($a_N=15.8$ G, $a_H=22.8$ G) signals in the coronary effluent. Both direct and spin trap measurements demonstrate a burst of radical generation peaking in the first min of reperfusion. Reperfusion with enzymatically active superoxide dismutase 1,500 U/ml eliminated the reperfusion free radical burst and improved contractile function while the inactivated enzyme had no effect. Thus, superoxide derived ·OH, R·, and ROO· radicals are generated in the post-ischemic heart and are important mediators of reperfusion injury.

Superoxide Dismutases: Isolation, Characterization and Mechanisms; Assay Methods for Superoxide Hydroxy Radical and Superoxide Dismutase

B 100 OXYGEN FREE RADICALS AND XANTHINE OXIDASE IN CEREBRAL ISCHEMIC INJURY, J.S. Beckman[1], T.H. Liu[2], E.L. Hogan[2], C.Y. Hsu[2], and B.A. Freeman[1], Depts of Anesthesiology and Biochemistry, University of Alabama at Birmingham, Birmingham, AL 35233 and Dept of Neurolology, Medical College of South Carolina, Charleston, SC 29425.

The protective effects of polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) and PEG-catalase, were investigated in a rat stroke model. Ligation of the right middle cerebral artery (MCA) and temporary clamping of both carotid arteries for 90 min yields a sub-maximal but highly reproducible focal infarct of 188 ± 28 mm^3 (mean ± SEM). In a randomized, blinded study, infarct volume was reduced by 30% (to 132 ± 15 mm^3) in rats pretreated IV with 10,000 units/kg each of PEG-SOD plus PEG-catalase, whereas infarct volume with inactivated PEG-enzymes was 187 ± 13 mm^3 ($p<0.01$; $n=18$) indicating that the protection obtained resulted from scavenging of superoxide and/or H_2O_2 rather than a nonspecific protein artifact. Superoxide and H_2O_2 may be produced by the ischemia-induced conversion of xanthine dehydrogenase (XDH) to the free radical-producing form, xanthine oxidase (XO). Three hours after release of the carotid clamps, the fraction of XDH present as XO increased by 17% in the MCA territory of the right cortex but not in the left cortex. Because 40% of brain XDH is localized within the microvasculature and endothelium constitutes only 0.5% of brain volume, the amount of XO formed in right cortex can generate 70 and 170 μM/min of superoxide and H_2O_2 respectively within the endothelium. Equivalent concentrations of XO applied to cultured endothelial cells will rapidly destroy barrier function and cause cell lysis. Thus, we hypothesize that free radical formation by XO contributes to the breakdown of the blood-brain barrier that occurs several hours after reperfusion of ischemic brain tissue.

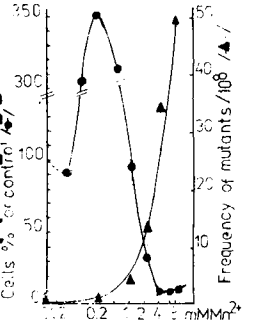
B 100 EXAMINATION OF THE ROLE OF ARGININE-143 IN THE HUMAN COPPER AND ZINC SUPEROXIDE DISMUTASE BY SITE-SPECIFIC MUTAGENESIS, Wayne F. Beyer, Jr., Irwin Fridovich (Duke University Medical Center, Durham N.C. 27710) Guy T. Mullenbach and Robert Hallewell (The Chiron Corporation, Emeryville Ca. 94608).

Superoxide dismutases are metalloenzymes which play a defensive role by catalyzing the dismutation of O_2^- into $H_2O_2 + O_2$. X-ray crystallographic studies on the bovine enzyme indicate an arginine reside (Arg-141) is found in the active site crevice, within 5 A of the Cu(II) and has been supposed to play an important role in the catalytic cycle both by electrostatic attraction of O_2^- and by providing a docking site for O_2^-, which would then be within electron-conductive reach of the Cu(II). The active site arginine-143 of human Cu,Zn superoxide dismutase has been replaced by lysine or by isoleucine. The mutant proteins were expressed at high levels in yeast, purified, and the amino acid substitution explored through the use of group specific reagents. The specific activities of these enzymes, measured by the xanthine oxidase/cytochrome c method and by using dry weight determination to establish protein concentration, were: native enzyme, 6570 U/mg; Lys-substituted enzyme, 2840 U/mg, Ile-substituted enzyme, 708 U/mg. The active site arginine thus plays an important, but not an essential role in the catalytic process.

B 102 **SIMULATION OF SOD ACTIVITY BY MANGANESE AND COPPER SALTS IN VIVO. Tomasz Biliński and Adam Liczmański, Zamość College of Agriculture Agricultural Academy of Lublin, Pl 22-400 Zamość ul H.Sawickiej 102 POLAND**

Yeast strains bearing scd mutations leading to CuZnSOD deficiency respond to supplementation of growth medium with manganese and cupric salts by increased rate of growth and higher tolerance of paraquat. However complete restoration of wild type phenotype was never observed. Figure 1 shows typical response of aerobically grown scd mutant to manganese salt administration. It is clear that although the observed log phase yield of cells is higher on Mn containing medium frequency of mitochondrial mutations is rised even at metal concentrations optimal for growth of the mutant. Frequency of rho- mutation is rised after Mn and Cu administration. Presented data may explain why the stratedy of preventeing deleterious effects of O_2^- by accumulation of Mn cations within the cell is restricted to several prokaryotic species only. Detailed data concerning described character will be presented.

Figure 1 presents frequency of mitochondrial mutants resistant to erythromycin .



B 103 IMMUNOCYTOCHEMISTRY TO HUMAN SOD, F.J. Denaro, Dept of Peds/Inf Dis, UCSD Medical Center, J.S. Schneider, Dept of Neurology, UCLA Medical Center

Antisera to human CuZn-SOD was produced in rabbits. This antibody was used to examine human post-mortem tissue for the presence of SOD. Tissue was obtained at the time of autopsy and was fixed in buffered formalin. Paraffin or frozen sections were produced. The sections were stained by immunocytochemistry for SOD by use of the PAP or ABC techniques. At this time brain, lung and muscle have been examined by SOD immunocytochemistry. Briefly, the results are: 1) In the brain, neurons were found to stain in a number of locations. The cortex, hippocampus and sub.nigra are among the location where positive SOD neurons were found. 2) Cells positive for SOD were found in the lungs. 3) SOD immunoreactivity in the muscle produced a checkerboard pattern. This pattern may correspond to muscle fiber types. Currently, we are continuing to survey human tissue in both control and disease states for SOD immunoreactivity.

B 104 CONTROLS ON THE BIOSYNTHESIS OF THE MANGANESE-CONTAINING SUPEROXIDE DISMUTASE OF ESCHERICHIA COLI: EFFECTS OF THIOLS, Paul R. Gardner and Irwin Fridovich, Duke University Medical Center, Durham, NC 27710

In vitro synthesis of Escherichia coli manganese-containing superoxide dismutase (MnSOD), directed by the plasmid pDT1-5, has been achieved. The MnSOD polypeptide was identified by electrophoresis on polyacrylamide gels, immunoprecipitation and the competitive immunoprecipitation effect of pure, active E. coli MnSOD. Dithiothreitol and glutathione, but not cysteine, suppressed in vitro synthesis of MnSOD. The parallel syntheses of β-lactamase and of another unidentified polypeptide were not suppressed by thiols. In vitro transcription of the E. coli MnSOD gene was similarly suppressed by glutathione, dithiothreitol and β-mercaptoethanol; but not by L-cysteine or thioglycolate. Compounds, such as diamide, 1-chloro-2,4-dinitrobenzene, potassium ferricyanide and methylene blue, which are expected to deplete intracellular glutathione, caused the induction of MnSOD in anaerobic E. coli.

B 105 COPPER, ZINC SUPEROXIDE DISMUTASE GENE OF Saccharomyces cerevisiae: CLONING, SEQUENCE, AND BIOLOGICAL ACTIVITY. Edith Butler Gralla, Olivia Bermingham-McDonogh, and Joan Selverstone Valentine, Dept. of Chemistry and Biochemistry, UCLA, Los Angeles, CA 90024.

The gene for Copper, Zinc Superoxide Dismutase (Cu,Zn-SOD) from the yeast Saccharomyces cerevisiae has been cloned, sequenced and shown to have biological activity. The gene was isolated from a lambda gt11 library using a long unique deoxyoligonucleotide probe whose sequence was deduced from the known amino acid sequence using a computer generated yeast codon preference table. The sequence of the coding and flanking regions has been determined. The cloned gene is expressed and active in vivo. A 3.2 kb fragment containing the coding region and 160 upstream bases, subcloned in a yeast/E. coli shuttle vector, was used to transform a yeast strain lacking Cu,Zn-SOD activity (from T. Bilinski). The presence of the Cu,Zn-SOD gene-containing plasmid corrected the characteristic oxygen sensitivity of this strain. Western blots with antibody to yeast Cu,Zn-SOD showed the presence of the protein in transformants and wild type yeast, but not in the mutant. The role of Cu,Zn-SOD in dioxygen toxicity will be discussed in light of these findings. Data on the effect of the absence of the Cu,Zn-SOD gene on the basal mutation rate will be included.

B 106 CLONING AND CHARACTERIZATION OF HUMAN EC-SOD. COMPARISON OF NATIVE AND RECOMBINANT FORMS.
Lena Tibell, Karin Hjalmarsson, Thomas Edlund, Gunnar Skogman, Åke Engström, and Stefan Marklund.
SYN-TEK AB, Box 1451, S-901 24 UMEÅ, Sweden.

Extracellular superoxide dismutase (EC-SOD) is the major SOD isoenzyme found in extracellular fluids. Human EC-SOD is heterogenous with regard to binding to Heparin-Sepharose and can be separated into three fractions: A, B, and C with no, weak or relatively high affinity, respectively. In the vascular system EC-SOD has been shown to bind to endothelial cell surfaces where membranebound heparan sulphate is a likely receptor for the enzyme. The type C is the form that binds.

A cDNA clone encoding EC-SOD has been isolated from a human placenta cDNA library and the nucleotide sequence determined. As deduced from the DNA sequence EC-SOD is synthesized with a 18 amino acid signal peptide, preceding the 222 amino acids in the mature enzyme, indicating that the enzyme is a secretory protein. The enzyme contains one possible N-glycosylation site. A sequence in the carboxy-terminal end is strongly hydrophilic and is probably responsible for the heparin binding.

An expression plasmid, based on the EC-SOD cDNA, was transfected into CHO-K1 cells. The transfected cells secreted human EC-SOD into the culture medium. The secreted recombinant (r-) EC-SOD was isolated in high yield and its properties were compared with native (n-) EC-SOD isolated from umbilical cords. The specific activities and amino-terminal amino acid sequences were identical. The amino acid composition were virtually identical and very similar to the composition deduced from the cDNA sequence. Both r-EC-SOD and n-EC-SOD was shown to contain 4 Cu atoms and 4 Zn atoms per molecule and to have identical CuEPR signals. The r-EC-SOD produced is of the type C, since its affinity for Heparin-Sepharose is identical to that of n-EC-SOD type C. Both enzymes was shown to be glycoproteins and seem to have the same subunit structure and composition as analysed by polyacrylamide gel electrophoresis and gel chromatography.

B 107 USE OF SPECIFIC COPPER CHELATORS TO INACTIVATE INTRACELLULAR SUPEROXIDE DISMUTASE WITHOUT DEPLETING ENDOGENOUS GLUTATHIONE OR PRODUCING METHEMOGLOBIN
Michael J. Kelner, Richard Bagnell, and Nicholas M. Alexander, University of California, San Diego, CA 92103. Attempts to inactivate intracellular superoxide dismutase generally use the copper chelator N,N-diethyldithiocarbamate (DDC). However, we found that DDC interacts with hemeproteins, such as hemoglobin, to produce large quantities of hydrogen peroxide and superoxide. This is a cyclic oxidation-reduction reaction and in the process endogenous glutathione is depleted and high concentrations of lipid peroxides and methemoglobin are produced. Thus, it is difficult to attribute cell damage solely to SOD inactivation as the DDC itself induces glutathione depletion and production of oxygen radicals, lipid peroxidation, and methemoglobin. Thirty copper chelators were examined for their ability to rapidly inactivate intracellular superoxide dismutase, without other adverse effects, in erythrocytes or immortalized human cells. Catechol, tetraethylenepentamine, and triethylenetetraamine were effective compounds under appropriate conditions. Other compounds either failed to adequately inhibit intracellular SOD, or like DDC, induced methemoglobin production or GSH depletion. Examination of the effect of chelators on purified SOD indicate that Cu/Zn SOD has a binding affinity (log K_1) between 12.64 and 13.76 for copper.

B 108 OXYGEN SENSITIVITY OF SULFITE REDUCTASE IN CU,ZN SUPEROXIDE DISMUTASE-DEFICIENT *SACCHAROMYCES cerevisae*.
Eric Chang, Thomas Krueger, Daniel Kosman, Edith Gralla, and Joan Valentine, SUNY at Buffalo, Buffalo, NY 14214, and UCLA, Los Angeles, CA 90024.

A number of Cu,Zn superoxide dismutase (SOD-1) deficient mutants of *S. cerevisiae* have been isolated by Tomasz Bilinski, Zamosc, Poland. These mutants lack SOD-1 activity although they contain normal levels of an SOD-1 mRNA and 5 polypeptides (16-20 kDa) which cross-react with an SOD-1 antisera. These mutants exhibit an O_2-dependent requirement for specific amino acids. One of these mutants, DSCD2-4A, exhibits an absolute requirement for Met when grown under air. This requirement is eliminated by transformation of the mutant with a CEN plasmid carrying the SOD-1 gene. Another of these mutants, DSCD1-1C, also requires Met when grown under air. However, Met can be replaced by Cys, homoCys, reduced or oxidized glutathione, thiosulfate, or sulfide. Ser, O-AcSer, β-mercaptoethanol, or sulfite do not support aerobic growth. This O_2-dependent auxotrophy is eliminated by growth of the mutant in media containing Mn. The Mn-grown cells exhibit an O_2^- dismutation activity attributable to the metal ion, as indicated by dialysis and metal chelation experiments. The origin of the auxotrophy in DSCD1-1C appears to be an O_2(radical) sensitivity of sulfite reductase(SRase). Wild-type strains contain *ca* 10-14 u/mg protein while aerobically-grown DSCD1-1C exhibits no SRase activity. On the other hand, cells grown under N_2 or in the presence of Mn exhibit wild-type levels of this enzymic activity, consistent with the lack of requirement for a reduced form of sulfur under these growth conditions.

B 109 ANAEROBIC BIOSYNTHESIS OF AN INACTIVE MANGANESE-CONTAINING SUPEROXIDE DISMUTASE IN ESCHERICHIA COLI, Christopher T. Privalle and Irwin Fridovich, Duke University Medical Center, Durham, NC 27710.

Escherichia coli growing anaerobically respond to NO_3^- with a 3-fold induction of the iron-containing superoxide dismutase (FeSOD). Mutants lacking a functional nitrate reductase do not show this response, suggesting that anaerobic induction of active FeSOD is due to electron flow to nitrate. Anaerobically-grown cells also contain an inactive form of the manganese-containing superoxide dismutase (MnSOD) which can be activated by addition of Mn(II) salts in the presence of acidic guanidinium chloride, followed by dialysis against neutral buffer. Direct addition of Mn(II) to a neutral solution of the inactive MnSOD does not impart activity. This is consistent with the behavior expected of the MnSOD apoenzyme or the enzyme bearing a metal other than Mn(II) at the active sites. Terminal electron acceptors, such as NO_3^- or trimethylamine-N-oxide (TMAO), increase the amount of inactive MnSOD produced by anaerobic E. coli. Paraquat, which is itself ineffective in this regard, markedly augments the effect of these terminal electron acceptors. Anaerobic induction of the biosynthesis of the MnSOD polypeptide by a variety of electron sinks eliminates the possibility that oxygen, or O_2^-, plays a role in this process. However, oxygenation and concomitant O_2^- production do appear important for the insertion of manganese into the growing MnSOD polypeptide, possibly because O_2^- oxidizes Mn(II) to Mn(III) and the latter is the valence state most effective in binding to the apoenzyme.

B 110 PRODUCTION OF AN ANTIBODY TO HUMAN COPPER-ZINC SUPEROXIDE DISMUTASE AND AN EVALUATION OF SPECIES CROSS REACTIVITY, J.S. Schneider, Dept of Neurology, UCEA School of Medicine, F.J. Denaro, Dept of Peds/Inf Dis, UCSD Medical Center

Human CuZn SOD was obtained from erythrocytes and the purity was assessed by gel electrophoresis. Control sera was obtained from rabbits prior to any immunizations. Antisera to CuZn-SOD was then produced by injection of 2 mgs of the enzyme emulsified in Freund's complete adjuvant into New Zealand white rabbits. Injections were given in 8 intracutaneous sites along the back. At 5-week intervals, blood was drawn from an ear vein and rabbits were then boosted with additional antigen. Immunodiffusion assays were performed to determine the cross reactivity of the SOD antibodies produced. The types of SOD tested were human SOD from erythrocytes, bovine SOD from erythrocytes, liver, and kidney, canine SOD from erythrocytes, SOD from horseradish, and Mg-SOD and Fe-SOD from E coli. Strong reactivity was found with the control human SOD. Slight reaction was noted with the bovine SOD and no reaction was noted with the other forms. We are continuing at this time to test crude isolates from other species.

B 111 GLYCATION AND INACTIVATION OF Cu-Zn-SUPEROXIDE DISMUTASE; IDENTIFICATION OF THE GLYCATED SITES, Naoyuki Taniguchi, Katsura Arai, Noriaki Kinoshita, and Yoshihiko Tada, Department of Biochemistry, Osaka University Medical School, Osaka 530, Japan.

Human erythrocytes contain glycated and nonglycated Cu-Zn-superoxide dismutases which can be separated by boronate affinity chromatography. The percentage of the glycated form is significantly increased in the erythrocytes of patients with diabetes as compared to normal erythrocytes. The nonglycated form of Cu-Zn-superoxide dismutase, which was washed through the boronate column, was glycated in vitro upon exposure to radioactive or non-radioactive D-glucose. Incorporation of D-glucose into the protein was observed, and with the increase in glycation, the enzyme activity decreased, indicating that the glycation of the enzyme led to a low active form. The glycated sites were identified by amino acid analysis after reverse-phase high performance liquid chromatography of the trypsin-treated peptides. Lysin residues, i.e., Lys 3, 9, 30, 36, 122 and 128, were found to be glycated. Three of the glycated sites lie in Lys-Gly, two in Lys-Ala and one in Lys-Val. The inactivation of the superoxide dismutase on the glycation is due mainly to the glycation of Lys 122 and Lys 128, which are supposed to be located in an active liganding loop. The remaining 5 sites, such as Lys-Glu, Lys-Asp, Lys-His, and Lys-Thr, are relatively inactive as to the formation of either a Schiff-base or an Amadori adduct.

B 112 CLONING AND CHARACTERIZATION OF HUMAN EC-SOD. COMPARISON OF NATIVE AND RECOMBINANT FORMS.
Lena Tibell, Karin Hjalmarsson, Thomas Edlund, Gunnar Skogman, Åke Engström, and Stefan Marklund.
SYN-TEK AB, Box 1451, S-901 24 UMEÅ, Sweden.

Extracellular superoxide dismutase (EC-SOD) is the major SOD isoenzyme found in extracellular fluids. Human EC-SOD is heterogenous with regard to binding to Heparin-Sepharose and can be separated into three fractions: A, B, and C with no, weak or relatively high affinity, respectively. In the vascular system EC-SOD has been shown to bind to endothelial cell surfaces where membranebound heparan sulphate is a likely receptor for the enzyme. The type C is the form that binds.

A cDNA clone encoding EC-SOD has been isolated from a human placenta cDNA library and the nucleotide sequence determined. As deduced from the DNA sequence EC-SOD is synthesized with a 18 amino acid signal peptide, preceding the 222 amino acids in the mature enzyme, indicating that the enzyme is a secretory protein. The enzyme contains one possible N-glycosylation site. A sequence in the carboxy-terminal end is strongly hydrophilic and is probably responsible for the heparin binding.

An expression plasmid, based on the EC-SOD cDNA, was transfected into CHO-K1 cells. The transfected cells secreted human EC-SOD into the culture medium. The secreted recombinant (r-) EC-SOD was isolated in high yield and its properties were compared with native (n-) EC-SOD isolated from umbilical cords. The specific activities and amino-terminal amino acid sequences were identical. The amino acid composition were virtually identical and very similar to the composition deduced from the cDNA sequence. Both r-EC-SOD and n-EC-SOD was shown to contain 4 Cu atoms and 4 Zn atoms per molecule and to have identical CuEPR signals. The r-EC-SOD produced is of the type C, since its affinity for Heparin-Sepharose is identical to that of n-EC-SOD type C. Both enzymes was shown to be glycoproteins and seem to have the same subunit structure and composition as analysed by polyacrylamide gel electrophoresis and gel chromatography.

Reperfusion Injury
Oxy-Radicals in Disease Processes

B 200 OXYGEN RADICALS STIMULATE SECRETION OF MUCIN BY RODENT AIRWAY EPITHELIAL CELLS IN ORGANOTYPIC CULTURE. Kenneth B. Adler, North Carolina State University, Raleigh, NC 27606.
Oxidant injury is associated with development of several pulmonary lesions, but pathogenetic mechanisms at the cellular level have not been elucidated. We have reported previously that chemically-generated (purine + xanthine oxidase) oxidants stimulate secretion of mucus from explants of rodent airway tissue, suggesting a possible pathogenetic mechanism (excess mucus secretion) linking oxidant injury with development of obstructive pulmonary disorders (Am Rev Resp Dis 135:A164, 1987). In studies utilizing a primary **epithelial cell culture** methodology developed in this laboratory, purine + xanthine oxidase increased secretion of mucin by guinea pig epithelial cells in a dose-dependent manner. Increased secretion was inhibited by superoxide dismutase, but not by catalase nor mannitol. However, when catalase was coupled to polyethylene glycol, allowing penetration into the epithelial cells, it inhibited stimulated secretion, as did dimethlythiourea (DMTU) a permeable hydroxyl radical scavenger. These results suggest exposure of airway epithelial cells to chemically-generated oxidants stimulates secretion of mucin, an event which can be inhibited by exogenous SOD, or by scavengers of H_2O_2 or hydroxyl radical which can gain entry into cells. Thus, extracellularly-generated oxidants appear to provoke production of oxygen radicals within the epithelial cells themselves, and these intracellularly-generated oxidants appear capable of stimulating mucin secretion.

B 201 ALTERATIONS IN ENDOTHELIAL CELL ATP METABOLISM AND EFFLUX OF ATP METABOLITES FOLLOWING OXIDANT INJURY, Sharon P. Andreoli, IUMC, Indianapolis, IN.
To investigate mechanisms responsible for oxidant injury in human umbilical vein endothelial cells (EC), we studied alterations in ATP metabolism following injury generated with hypoxanthine-xanthine oxidase (H-XO) and glucose-glucose oxidase (G-GO). EC ATP levels fell significantly ($P < .05$) from 3.6 $\pm$ .62 pmol/ug protein in control cells to 0.8 $\pm$ .26, 0.1 $\pm$ .04, and 0.14 $\pm$ .12 pmol/ug protein in cells exposed to 10, 25 and 50 mU/ml H-XO and from 5.07 $\pm$ .90 in control cells to 0.66 $\pm$ .43, 0.15 $\pm$ .03, 0.21 $\pm$ .3 pmol/ug protein in cells exposed to 10, 25 and 50 mU/ml G-GO for 90 min. ATP levels were also reduced in EC exposed to H-XO and G-GO for shorter periods of time. The decline in ATP levels was inversely proportional to efflux of ^{3}H-adenine from labeled cells while ^{51}Cr release occurred considerably later. NAD levels fell simultaneously with the fall in ATP levels. DNA damage occurred in each system; the percent residual double stranded DNA was 73.2 $\pm$ 2.4, 35.0 $\pm$ 1.5 and 9.9 $\pm$ 7.7% for EC exposed to 0, 25 and 50 mU/ml GO for 60 min. and 75.7 $\pm$ 3.9, 15.2 $\pm$ 1.2 and 4.6 $\pm$ 0.5% for EC exposed to 0, 25 and 50 mU/ml XO for 60 minutes. Catalase but not SOD or DMSO prevented the decline in ATP levels and efflux of hypoxanthine. In cells exposed to G-GO, ATP, ADP, AMP, adenosine, xanthine and uric acid were not detected in the extracellular fluid while inosine and hypoxanthine were detected and correlated with the decline in ATP levels. These results demonstrate that EC levels of ATP and NAD fall rapidly following exposure to H-XO and G-GO, that oxidant stress results in DNA damage, that hydrogen peroxide is the oxygen species responsible and that ATP is rapidly metabolized to inosine and hypoxanthine which is detected in the extracellular fluid.

B 202 IRON AND OH· IN LIPID OXIDATION: FENTON REACTIONS IN LIPID PHASES, Karen M. Schaich and Donald C. Borg, Brookhaven National Laboratory, Upton, NY 11973.

Metal catalysis of membrane lipid oxidation has been thought to occur only at cell surfaces. However, conflicting reports on the activities of ferric versus ferrous chelates led us to question this dogma. This work shows that the solubilities of iron complexes in lipid phases and their abilities to initiate lipid oxidation there, either directly or via Fenton-like production of OH·, are critical determinants of catalytic efficacy.

Fe^{3+} and Fe^{2+} complexes and chelates were partitioned into bulk phases of purified lipids and were quantified by atomic absorption spectroscopy. Solutions of iron salts (mM) partitioned into oleic acid at levels of about micromolar. EDTA and DTPA chelates were significantly less soluble, while ADP-iron and ferrioxamine were soluble as chelates at greater than 10^{-5}M. Less protic methyl linoleate is a better surrogate for hydrophobic membrane interiors, and all iron compounds were 10-100 times less soluble in it.

To determine whether Fenton-like reactions occur in lipid phases, H_2O_2, complexes of either Fe^{2+} or Fe^{3+}, and a reducing agent (hydroxylamine), when needed, were partitioned into the lipid along with the spin trap DMPO, and free radical adducts were recorded by EPR. A spin adduct attributed to OH· was seen in oleic acid, but only changing levels of secondary radicals were detected in lipid esters, including both alkoxyl and peroxyl species. These findings imply that similar OH· induction of lipid oxidation might occur in vivo within membrane bilayers. In that locus Fenton chemistry would not be expected to show the sensitivity to hydrophilic scavengers expected of aqueous Fenton reactions.

B 203 ANTI-OXIDANT ENZYMES IN TWO FILARIAL NEMATODES, Heather L. Callahan, Eric James, and Rosalie K. Crouch, Medical University of South Carolina, Charleston, SC 29425.

Despite elliciting a host response, many parasites, including *Dirofilaria immitis* and *Onchocerca cervicalis*, survive and reproduce causing host morbidity and death. Since the host defense includes the release of hydrogen peroxide and superoxide radicals by phagocytes, the presence of endogenous parasite anti-oxidant enzymes and the *in vitro* toxicity of oxidants were investigated. *D. immitis* adult homogenate contains 125 U/mg superoxide dismutase (SOD), and threshhold levels of glutathione peroxidase (GPO) and catalase, while microfilariae contain 148.1 U/mg SOD, threshhold levels of GPO, and 7.03 mU/mg catalase. *O. cervicalis* adult homogenate contains 112.4 U/mg SOD, 0.152 mU/mg GPO, and 3.37 mU/mg catalase, while microfilariae contain equivalent levels of SOD, 0.114 mU/mg GPO, and 0.411 mU/mg catalase. All species and stages showed similar levels of SOD, but *D. immitis* appears to have no GPO in either stage and no catalase in the microfilariae, while *O. cervicalis* has low levels of both enzymes in both stages.

The SODs from adult worms of both species have been partially purified and characterized as copper/zinc based enzymes that migrate similarly on a non-denaturing gel, but have different molecular weights by SDS-PAGE. *D. immitis* SOD has a molecular weight of 16,800 by SDS-PAGE, but 35,000 MW by Sephadex G-100 chromatography indicating a dimeric active enzyme.

Sensitivity of *O. cervicalis* microfilariae to oxidant killing was shown by preliminary *in vitro* cytotoxicity assays using hydrogen peroxide and chemically generating hydrogen peroxide and superoxide radicals by xanthine-xanthine oxidase reactions. Therefore, oxidants may mediate killing of microfilariae *in vivo* indicating that endogenous anti-oxidants may protect microfilariae from the host response. Supported by NIH Grants EY05757 and EY06462.

B 204 IS THE REOXYGENATION DAMAGE OF THE HYPOXIC RAT HEART MYOCARDIUM DUE TO OXIDATIVE STRESS ? V. M. Darley-Usmar & V. O'Leary, Department of Biochemistry, Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS, U.K.

Reoxygenation of the hypoxic rat heart results in greater cell damage than occurs in the preceding hypoxic period. It has been suggested that this may be due to production of superoxide and H202 at the time of reoxygenation by proteins such as xanthine oxidase. This form of oxidative stress would cause oxidative modification of proteins and lipids and so damage the cell. It is a characteristic of oxidative stress that the levels of the important cellular antioxidant reduced glutathione (GSH) are decreased. This occurs with a concomitant rise and export of GSSG (the oxidised form of GSH) and an increase in protein-GSH mixed disulphides (PS-SG). We have confirmed that in Langendorff perfused hearts glutathione is present predominantly as GSH with a small amount of GSSG and an even smaller amount of PS-SG. After hearts were exposed to oxidative stress in the form of H202, GSH decreased, intracellular GSSG remained unchanged (although some was exported) and PS-SG increased. The H202 treatment resulted in loss of cell cytosol to the perfusate similar to that which occurs on reoxygenation of hypoxic myocardium. In comparison hearts subjected to 30 min hypoxia and 20 min reoxygenation showed a small decrease in GSH, no change in GSSG (and no export) and also no change in PS-SG compared to normoxic control hearts. The degree of cell damage under these conditions was however greater than that caused by treatment of hearts with H202. This data suggests that oxidative stress is not the underlying cause of reoxygenation damage and that it is not therefore responsible for the oxygen sensitivity of the hypoxic heart.

B 205 COPPER-MEDIATED CHANGES IN MEMBRANE PERMEABILITY.

C.H. Ric de Vos, Henk Schat and Wilfried H.O. Ernst, Department of Ecology and Ecotoxicology, Free University, P.O. Box 7161, 1007 MC Amsterdam, The Netherlands.

The effect of Cu^{2+} on membrane permeability of root cells was studied in a copper-sensitive and a copper-tolerant population of *Silene cucubalus*.
In sensitive plants, K^+-leakage could be detected within 15 minutes after addition of 20 or 100 µM Cu^{2+} to the whole root system. A significant increase in MDA-content, indicating lipid peroxidation, and inclusion of trypan blue could be observed. Addition of N-ethylmaleimide also caused K^+-leakage, but the MDA-content did not increase. PCMB, Mg^{2+} or Cd^{2+} had no effect on either K^+-leakage or MDA-content. In tolerant plants, the effect of Cu^{2+} on K^+-leakage and MDA-content of the roots was significant less then in sensitive plants, and trypan blue inclusion could not be observed.
The results show that copper-toxicity in *Silene cucubalus* is connected with changes in membrane permeability, caused by depletion of cellular thiol groups and Cu^{2+}-induced lipid peroxidation.
Further work on processes related to copper-mediated oxidative stress in *Silene cucubalus* is planned.

B 206 ROLE OF SUPEROXIDE DISMUTASE IN THE PHAGOCYTIC PROCESS,

H. Brian Dunford, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

The phagocytic process by neutrophils in which invading microorganisms are destroyed has been described as analogous to an attack by Attila the Hun. Superoxide dismutase has been regarded as part of the defence mechanism against self-injury in leukocytes. However there are two reasons for regarding it as part of the attack mechanism. First, removal of $O_2^{\cdot-}$ prevents the tying up of myeloperoxidase as its rather inactive compound III or oxymyeloperoxidase. Second, the myeloperoxidase in a stimulated neutrophil wants hydrogen peroxide in a hurry. Even at pH 4.6, where the uncatalyzed dismutation of superoxide and hydroperoxyl radical has its optimum rate, superoxide dismutase accelerates the reaction rate. Therefore a completely logical role can be defined for superoxide dismutase in the phagocytic process. The killing action is a result of the obligatory sequence of reactions by NADPH oxidase, superoxide dismutase and myeloperoxidase which catalyze the sequence of reaction $O_2 \rightarrow O_2^{\cdot-} \rightarrow H_2O_2 \rightarrow HOCl$ or enzyme-activated hypochlorous acid. Finally, the NADPH oxidase reaction protects myeloperoxidase from being tied up in reactions with NADPH analogous to those of NADH with peroxidase. These also result in compound III formation. Neutrophils in their exuberance spill hydrogen peroxide into their environment which may be responsible for the inflammatory response. The correlation between inflammation and cancer has been known for a long time.

B 207 A GENETIC SYSTEM FOR STUDYING RESISTANCE TO OXIDATIVE ATTACK IN CRYPTOCOCCUS,

Herschell S. Emery and Eric S. Jacobson, Veterans Administration Medical Center, and Medical College of Virginia, Richmond, VA 23249

Oxidative killing by white cells has been shown important in resistance to many infections. Thus microbial defenses against oxidative stress constitute a potential virulence factor. Several studies have shown a correlation between virulence of clinical isolates and levels of enzymes able to scavenge oxygen radicals. We wish to test genetically the role of such scavengers in the pathogenesis of cryptococcosis and to establish a genetic model system for oxidative stress in eukaryotes. We have developed an assay to estimate sensitivity to hyperbaric oxygen (hyperoxia) of colonies of Cryptococcus. Using this assay, we have isolated UV-induced mutants abnormally sensitive to hyperoxia, yet able to grow well under ambient conditions. Mutant Ox-219 shows a substantial lag in growth, relative to the wild type, following hyperoxia treatment but appears to be only minimally more sensitive in a killing assay. When Ox-219 was crossed to the wild type, random-spore analysis showed progeny displaying the sensitivity of either parent in a 1:1 ratio, suggestive of a single-gene mutation. If redundant genes protect Cryptococcus against oxidative stress, mutagenesis of wild-type cells might generate mutants of only limited sensitivity. To allow for this possibility, we isolated in the Ox-219 background mutants showing an even longer lag following hyperoxia treatment. Five of the new mutants carry a single additional mutant gene conferring enhanced sensitivity.

B 208 INDUCTION OF CERULOPLASMIN GENE EXPRESSION IN PULMONARY TISSUE AND ALVEOLAR MACROPHAGES DURING INFLAMMATION, Robert Fleming and Jonathan D. Gitlin, Washington University School of Medicine, St. Louis, Mo 63130.

Pulmonary parenchymal damage from inflammation is mediated in part by a balance between toxic oxygen products generated during acute inflammation and the available antioxidants in the lung. Ceruloplasmin is a liver-derived copper binding plasma protein which can scavange free radicals and prevent lipid peroxidation in vitro. In addition, ceruloplasmin appears to play a role in tissue copper metabolism and may, therefore, regulate copper availability to cellular and extracellular superoxide dismutases. In order to study the role of ceruloplasmin in pulmonary antioxidant defense, we elicited an inflammatory response in syrian hamsters using a variety of mediators including endotoxin, turpentine, and interleukin-1 and examined the possibility of extra-hepatic pulmonary ceruloplasmin gene expression. Both systemic and pulmonary inflammation resulted in a five-fold induction of ceruloplasmin specific mRNA in pulmonary tissues. Isolation of alveolar macrophages from the lungs of inflammed animals revealed that these cells are in part the source of extra-hepatic pulmonary ceruloplasmin gene expression. Furthermore, ceruloplasmin gene expression could be induced with endotoxin in vitro in alveolar macrophages from normal animals, and preliminary studies suggest that this response can be modulated by ambient oxygen tensions. These data suggest that the local induction of ceruloplasmin gene expression in alveolar macrophages within sites of pulmonary inflammation may be an important determinant of pulmonary antioxidant host defense.

B 209 CHANGES IN ANTIOXIDANT ENZYME ACTIVITIES WITH DIETARY FAT AND COPPER DEFICIENCY IN THE RAT. S.M. Lynch, D.G.M. Carville, J.J. Strain and B.M. Hannigan. Biomedical Sciences Research Centre, University of Ulster at Jordanstown, Newtownabbey, Co Antrim, BT37 0QB, Northern Ireland.

Dietary fat-type and a copper (Cu) deficiency have been independently identified, inter alia, as potentially important factors in the aetiology of ischaemic heart disease (IHD); a disease which has been linked to inflammation and oxygen free radical-mediated damage. In this study, groups (n=6) of male, weanling, Wistar rats were provided ad libitum with deionised water and one of four diets containing 200g/kg saturated or polyunsaturated fatty acid (SFA or PUFA) with either control (11.5 ug/g) or deficient (0.4 ug/g) quantities of Cu. After 56 days blood was obtained by cardiac puncture and the liver removed. Measurement of several indices of Cu status indicated that both groups fed the Cu-deficient diets had experienced a mild form of Cu deficiency. SFA consumption resulted in significantly increased hepatic iron and increased hepatic xanthine oxidase activity. Erythrocyte glutathione peroxidase (GSH-Px) activity, although significantly increased in the Cu-deficient PUFA groups, was significantly decreased in the Cu-deficient SFA group compared to the corresponding controls. Interestingly, the hepatic activity of glucose-6-phosphate dehydrogenase paralleled these changes in erythrocyte GSH-Px activity. These results suggest a possible interaction between fat-type and Cu deficiency and may have implications for the degradation of potentially harmful lipid peroxides via glutathione pathways. Both Cu-deficient groups had significantly ($p<0.05$) higher hepatic malondialdehyde levels than the corresponding control groups but no significant effect due to fat-type was recorded.

B 210 MORPHOLOGICAL AND QUANTITATIVE CHANGES OF CYTOSKELETAL FILAMENTOUS (F)- ACTIN DURING EXPOSURE OF ENDOTHELIAL CELLS TO 95% 02. S. Harding, T. Howard, R. Jackson, University of Alabama at Birmingham and VAMC, Birmingham, AL.

Endothelial stress fibers (microfilaments composed of F-actin) influence vascular permeability by affecting cell surface area, shape, interendothelial junctions and may undergo oxidative or proteolytic degradation in hyperoxia. This study investigated the effects of 95% 02 exposure on porcine aortic endothelial cells. Endothelial stress fiber morphology, F-actin content, and cell numbers were assessed after 0(contol), 48, 72, and 84 h oxygen exposure. Control cells displayed linear fluorescence typical of endothelial stress fibers after staining with NBD-phallacidin. Peripheral staining decreased after 48 h and after 72-84 h the stress fibers had aggregated, shortened, and decreased in number. Cytolysis was prominent at 84 h. The following changes in F-actin content occurred prior to cytolysis:

F-actin (units x 10^7)	Control	48 h 0_2	P
	10.52 + .95	6.35 + 2.01	<.05
cells ($\times 10^{-5}$)	5.24 ± .41	4.90 ± .57	>.05

Endothelial cell F-actin, the major protein component of stress fibers, changes in configuration and decreases in quantity during 95% 0_2 exposure. These findings imply that degradation of endothelial cytoskeletal proteins may be relevant to increased lung microvascular permeability in pulmonary oxygen toxicity.

B 211 THE ROLE OF OXYGEN METABOLITES IN THE KILLING OF MYCOBACTERIUM TUBERCULOSIS. G.S. Jones, B.R. Andersen, University of Illinois, Chicago, Ill 60612

Killing of Mycobacterium tuberculosis (Mtb) by neutrophils (PMN) has previously been demonstrated. In order to determine the role of oxygen metabolites in mycobacterial killing, PMNs from patients with Chronic Granulatomas Disease (CGD) were used. CGD patients have a defect in the NADPH oxidase pathway, consequently PMNs from these patients are unable to generate the bactericidal species O_2^-, -OH, H_2O_2 and other radicals. If these metabolites are involved in killing, then CGD PMNs should be less effective killers of Mtb than the normal PMNs. Changes in the viability of Mtb were determined by agar plate quantitation, and by using a radiometric system which measures the release of $^{14}CO_2$ from ^{14}C-labeled fatty acid. As measured by agar plate quantitation CGD PMNs were not distinguishable from normal PMNs with 44% and 45% killing respectively. Loss of Mtb viability was 73% for CGD PMNs and 63% for normal PMNs as measured by release of $^{14}CO_2$. These data indicate that the killing of Mtb by PMNs is not impaired in the absence of oxygen metabolites. Normal PMNs were examined in conjunction with free radical inhibitors: catalase (CAT), superoxide dismutase (SOD), CAT +SOD, mannitol, and histidine. No impairment of killing was seen with any of these inhibitors. Killing was also effective in the absence of serum, and in the absence of complement. We conclude that the mechanisms by which Mtb are killed by PMNs are independent of the oxygen metabolic burst.

B 212 PROTECTION AGAINST LIPID PEROXIDATION: THE ROLE OF GLUTATHIONE REDUCTASE, Karl J. Kunert, Philip M. Mullineaux, John Innes Institute, Norwich NR4 7UH, United Kingdom.

Air pollutants, certain herbicides, and natural ageing can cause oxidation of plant cell compounds, such as lipids and proteins. Specifically the peroxidation of lipids has been identified as one of the most deteriorative reactions basic to the mechanism of cellular damage. Protection against these harmful events is, however, provided by antioxidative systems including antioxidants, such as glutathione and the vitamins C and E, and a variety of enzymes. In plants, we have strong evidence that a specific response to herbicide-induced peroxidation is an increase of activity of enzymes either involved in biosynthesis of an antioxidant, such as the vitamin C-producing enzyme galactonolactone oxidase, or reduction of an antioxidant, such as glutathione reductase (gor). Increase in gor activity is further directly related to decrease of lipid peroxidation and increase of SH-compounds.

Since our future research is specifically focused on the manipulation of the content of enzymes that will limit cellular oxidation, we investigated the consequences of amplification of the gor gene in E. coli. Amplification of the cloned gor gene results in a general growth stimulation, protection against the organic peroxide methyl ethyl ketone peroxide, and expression of stress proteins after induction of lipid peroxidation.

B 213 PURIFICATION AND CHARACTERIZATION OF THE MAJOR HYDROPEROXIDE-REDUCING ACTIVITY OF HUMAN PLASMA, Krishna Rao Maddipati and Lawrence J. Marnett, Wayne State University, Detroit MI 48202.

The major hydroperoxide-reducing activity of human plasma was identified as a glutathione peroxidase using a novel peroxidase assay. The peroxidase was purified from human plasma to electrophoretic homogeneity in about 20% yield using hydrophobic interaction, ion-exchange, and gel filtration chromatography. The purified human plasma glutathione peroxidase appears to be a tetramer of identical, 22 kDa subunits and is different from the cellular enzyme, human erythrocyte glutathione peroxidase. The plasma peroxidase is a selenoprotein containing one mol of selenium per mol of subunit. Selenium dependency of the peroxidase is also shown by its inhibition with β-mercaptosuccinic acid. The human plasma glutathione peroxidase can be saturated with glutathione unlike many other selenium-dependent glutathione peroxidases from other mammalian sources. The K_m value for glutathione is 4.3 μM. The plasma glutathione peroxidase efficiently reduces hydrogen peroxide as well as organic hydroperoxides at near diffusion controlled rates. The K_m values for hydroperoxides at 5 mM glutathione are H_2O_2, 13.2 μM; 5-phenyl-4-pentenyl hydroperoxide, 11.2 μM; 15-OOH-20:4, 4.5 μM; and 13-OOH-18:2, 2.3 μM. (Supported by research grant CA43209 from the NIH).

B 214 THE ROLE OF OXYGEN IN DYE MEDIATED PHOTODYNAMIC EFFECTS IN E. COLI. B.J.P. Martin, Jr. and N. Logsdon, Biology Department, Rice University, Houston, Texas 77005.
Photosensitive dyes representative of the thiazines, xanthenes, acridines and phenazines mediate photoxicity in E. *coli* B. The observed phototoxicity is a photodynamic effect. Hydroxyl radical scavengers confer protection against the photodynamic action of all of the representative dyes. The extent of protection is dependent on the concentration of scavenger and on the *in vitro* reactivity of the scavenger with the hydroxyl radical. Exogenous superoxide dismutase and catalase partially protect the cells against the dye mediated phototoxicity, and prior induction of intracellular SOD and catalase by growth in glucose minimal medium containing manganese and paraquat substantially protects E. *coli* B against the photodynamic action of all of the dyes examined. Combinations of protective treatments against the phototoxicity of all four classes of dyes, including SOD and catalase preinduction and addition of extracellular SOD and catalase or the addition of hydroxyl radical scavengers, provide nearly complete protection against the oxygen-dependent component of dye mediated lethality. E. *coli* B, grown in glucose minimal medium containing maganese and photosensitive dyes, induces MnSOD. The extent of induction is correlated with the dye's ability to photooxidize NADH *in vitro*. Thus, oxygen radicals are primarily responsible for the oxygen-dependent toxicity of the photosensitive dyes examined, and one adaptive response of E. *coli* B to a dye mediated oxidative threat is to induce superoxide dismutase. This work was supported by NIH grant #AI-19695, grant #C900 of the Robert A. Welch Foundation and the American Heart Association, Texas Affiliate.

B 215 QUANTITATION OF CONJUGATED DIENES IN PLASMA AS INDICATOR OF LIVER TRANSPLANT REJECTION, Tai-Wing Wu and Donald A.G. Mickle, Univ. of Toronto and Toronto General Hospital, Canada M5G 2C4.
Using HPLC, we have observed the presence of elevated peroxidized phospholipids (PPP) in the plasma of liver transplant recipients who rejected their grafts, but not in those who recovered uneventfully from allograft transplantation, nor from healthy volunteers. The nominal amounts of PPP rose with the intensity of graft rejection, and mirrored the response to immunosuppressive therapy, but increased minimally in transplant patients who had cytomegalovirus or bacterial infections which can be easily confused clinically and histologically with transplant rejection. Using a more specific radiochemical method for PPP, in which conjugated dienes are reacted with ^{14}C-tetracyano-ethyelene, we have quantitated for the first time the ranges of plasma PPP in the above patient categories. The rejecting patients had plasma conjugated dienes per umole of phospholipids of 9-29 times higher than those seen in non-rejecting patients or healthy volunteers. Molecular identifications of the putative peroxidized phospholipids will be presented.

B 216 SPECIFIC PLASMALOGEN BREAKDOWN INDUCED BY PHOTOSENSITIZATION: A ROLE IN PROTECTING ANIMAL CELL MEMBRANES AGAINST SINGLET OXYGEN DAMAGE. Olivier H. MORAND, Raphael A. ZOELLER and Christian R.H. RAETZ. Department of Biochemistry, University of Wisconsin, Madison, WI 53706.
An attempt has been made to study the biological functions of plasmalogens, a unique class of glycerophospholipids containing a vinyl-ether substituent at the *sn*-1 position of the glycerol backbone. We have isolated Chinese Hamster Ovary cells defective in peroxisome assembly, also showing reduced plasmalogen content. Cells were labelled with 12-(1-pyrene) dodecanoic acid (P12), a photosensitizer, and then irradiated with long wavelength UV light. Plasmalogen-deficient mutants appeared to be much more sensitive to UV/P12 treatment than wild-type cells. Mutants treated with 1-*O*-hexadecyl-*sn*-glycerol regained normal plasmalogen levels and were also more resistant to P12/UV. The relative resistance of plasmalogen-containing cells to photodynamic damage indicated that the vinyl ether linkage of plasmalogens might protect membranes from reactive oxygen species. We therefore hypothesized that the vinyl ether structure could play a direct role in scavenging singlet oxygen, leading to specific chemical destruction of the plasmalogen. Exposure of wild-type cells to P12/UV resulted in a selective turnover of the plasmalogen form of phosphatidylethanolamine, accompanied by an accumulation of 2-monoacyl-glycerophosphoethanolamine. When wild-type cells were labelled with radioactive hexadecanol and P12, exposure of the cells to UV was followed by the formation a 15 carbon fatty aldehyde and the excretion of formic acid. These data provide strong evidence for the remarkable reactivity of the vinyl ether structure of plasmalogens toward singlet oxygen, accompanied by the formation of three expected breakdown products that can be recycled or eliminated by cells.

B 217 IN VITRO DEPENDENT CHANGES IN ENZYMES OF REACTIVE OXYGEN METABOLISM IN TYPE II PNEUMOCYTES, Peter C. Panus, Sadis Matalon and Bruce A. Freeman, Depts. of Anesthesiol., Biochem., and Physiol., University of Alabama, Birmingham AL 35233.

To increase understanding of cellular responses from oxidant-induced pulmonary damage, various cell types have been isolated and cultured. The purpose of this study was to quantify the effects of normoxia (95% air, 5% CO_2) and hyperoxia (95% O_2, 5% CO_2) on phenotypic changes of type II pneumocytes (T2), in vitro. Cells were isolated from rabbit lungs by trypsin-elastase digestion of alveolar cells, followed by tissue dissociation, density gradient centrifugation and differential adherence. Cell yield was approximately 4.38×10^7/lung with an average of 83% T2 cells. Cells were then adhered (39 hrs) to fibronectin pretreated dishes (2.1 $\mu g/cm^2$) with a plating efficiency of 23%. After adherence, cell media was changed before experimentation. During 0-96 hrs of normoxia, cell division occurred as demonstrated by increased cellular DNA, protein, and lactate dehydrogenase (LDH) activity. However, activities of cellular antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) decreased over time in normoxia. Hyperoxia depressed cell division, and was toxic to T2 cells as demonstrated by increased release of cellular DNA, LDH, and preincorporated ^{14}C adenine. Antioxidant enzymes responded differently to hyperoxia, with cellular CAT and GPx activities remaining unchanged from normoxic controls, whereas SOD activity was increased. Unlike in vivo, in vitro T2 cells do not respond to hyperoxia by increasing protein synthesis. Furthermore, compared to freshly isolated cells, in vitro culture results in an overall loss with time of cellular antioxidant enzymes during both normoxia and hyperoxia, with the exception of SOD activity which increased in response to hyperoxia.

B 218 OXYGEN-INDUCED RETINOPATHY IN THE RAT. John S. Penn, Lisa A. Thum and Robert E. Anderson,Cullen Eye Institute, Baylor College of Medicine, Houston, TX 77030

Oxygen-induced retinopathy (OIR) was produced by subjecting newborn albino rats to 60% oxygen for 14 days before sacrifice and retinal analysis. The extent of OIR was measured in India ink-perfused retinal whole mounts as the percent of total retinal area containing intact blood vessels. The values were 59% for exposed rats and 94% for room air controls. This reduction in retinal vasculature is manifested by a complete loss of the deep capillary net and a partial loss of the superficial capillary net. Retinal levels of the antioxidants vitamin E and ascorbic acid were reduced in oxygen-exposed rats by 34% and 20%, respectively. No differences were found in the levels of glutathione peroxidase, S-transferase, or reductase. Results indicate that we can successfully raise and lower -tocopherol in the retinas of both groups by dietary manipulation. Mothers are fed diets containing either 1.0 gm -tocopherol acetate/kg food, or none, from 21-25 days prior to birth of litters, throughout the exposure. This treatment results in -tocopherol levels in the ratling retinas which are 1.5 and 0.5 mmole/mole phospholipid, respectively, for E+ and E- room air control ratlings and 1.5 and 0.6, respectively, for the two hyperoxic groups. Further, this combination of dietary and atmospheric oxygen manipulations results in significant differences in glutathione peroxidase activity, particularly in the E- ratlings raised in hyperoxic conditions whose activities are 63% increased over control levels. Effects of manipulation of antioxidants on retinal vasculature will be reported, and the general role of peroxidation in the pathogenicity of OIR will be discussed.

B 219 THE EFFECTS OF ENDOTOXIN, DEXAMETHASONE AND MEDRYSONE ON SUPEROXIDE DISMUTASE ACTIVITY IN THE RABBIT IRIS, Jaime F. Recasens, Anastasios Costarides and Keith Green, Department of Ophthalmology and Department of Physiology & Endocrinology, Medical College of Georgia, Augusta, Georgia 30912.

Superoxide dismutase (SOD) activity was measured in the irides of control animals and 24 hours after the intravitreal administration of 1 µg of E. coli endotoxin. Over a ten-fold increase in SOD activity was noted in the endotoxin-treated animals versus controls ($P<0.001$). In order to assess the induction of SOD activity while protecting against the inflammatory process, topical dexamethasone was administered t.i.d. for 2 days before and 1 day after endotoxin injection. Although dexamethasone prevented the conjunctival hyperemia, vascular injection and iritis seen with endotoxin treatment alone, a two-fold increase in SOD activity was measured ($P<0.05$). However, administration of dexamethasone alone t.i.d. for 3 days increased SOD activity compared to controls ($P<0.05$) giving values approximating those found after dexamethasone and endotoxin combined. Similar medrysone application failed to prevent the visible signs of endotoxin-induced ocular inflammation yet produced a similar induction of SOD activity when given before endotoxin ($P<0.01$) or by itself ($P<0.001$). It appears that anti-inflammatory steroids such as dexamethasone and medrysone may play a role in augmenting antioxidant enzyme activity possibly independently of the inflammatory process.

B 220 A NEW ROUTE FOR THE SYNTHESIS OF 14-C-TETRACYANOETHYLENE AND ITS APPLICATION TO THE MEASUREMENT OF LIPID PEROXIDATION PRODUCTS FROM MYOCARDIUM AND LUNG, Alexander D. Romaschin, Paul M. Walker, University of Toronto, Toronto, Ontario, Canada M5G 2C4. Previous studies by Rechnagel (lipids, 12:914-921, 1977) demonstrated the feasibility of using 14-C-tetracyanoethylene as a dienophile in the classical Diels-Alder 4+2 cycloaddition reaction to detect phospholipid peroxidation products in the livers of rats poisoned with CCl_4. This radiochemical assay which is highly specific for the conjugated diene moeity was limited in sensitivity by the difficulty of synthesizing a high specific activity 14-C-tetracyanoethylene using an inefficient exchange reaction. We have devised a new synthetic route which easily allows the synthesis of gram amounts of this reagent to specific activities of 10^7 dpm/mg or more. This reagent is extremely useful for the screening of phospholipid extracts for peroxidation products containing the conjugated diene chromophore. When used in conjunction with our HPLC assay for phospholipid oxidation products it gives definitive structural evidence for the presence of the conjugated diene chromophore in the oxidation products of polyenoic fatty acyl groups. We have used this reagent to map out the subcellular topology of membrane oxidation in globally ischemic and reperfused canine myocardium (which we have previously characterized with respect to free radical mediated injury, J. Molec. Cell. Cardiol., 19:289-302, 1987) and to detect lung free radical mediated injury in ICU patients suffering from Adult Respiratory Distress Syndrome. Our results indicate that the sarcolemma is the primary cellular membrane target site for oxidative injury although both mitochondrial and sarcoplasmic reticulum membranes also show evidence of significant phospholipid peroxidation in our model of reversible myocardial injury.

B 221 THE ROLE OF GLUTATHIONE (GSH) IN HAEMORRHAGIC SHOCK (HS) INDUCED GASTRIC MUCOSAL LESIONS IN RATS. H.J. Stein, M.M.J. Oosthuizen, R.A. Hinder, Dept of Surgery, University of the Witwatersrand, Johannesburg. Oxygen derived free radicals (FR) are beleived to be a major mediator of gastric mucosal lesion formation. This study was performed to investigate the role of GSH which thought to play an important role in the detoxification of FR. Methods: There were three groups of rats: a control group (A), a group undergoing HS followed by retransfusion (B), and a group undergoing HS and retransfusion but pretreated with 500 mg/KG GSH s.c. (C). At the end of the experiments gastric mucosal damage was assessed macroscopically, histologically and by scanning electron microscopy using a gastric lesion score (0-3) and mucosal GSH content was determined. In an additional set of experiments mucosal samples and gastric content were obtained to assess GSH levels prior to HS and at 10 min intervalls during HS and retransfusion. Results: There was an increase in gastric lesion formation and a decrease in mucosal GSH content in group B compared to the control (group A) ($p<0.01$, table), the decrease in GSH levels taking place during the first minutes of the hypotensive period accompanied by an threefold increase of GSH concentration in the gastric content. Pretreatment with GSH partly prevented gastric lesion formation and mucosal GSH depletion ($p<0.01$).

	group A	group B	group C
lesion score	0.18±0.02	2.18±0.31	1.20±0.18
GSH mol/g	4.10±0.47	2.03±0.28	3.99±0.60

Conclusions: Since the adminstration of exogenous GSH can prevent mucosal damage the decrease of cellular GSH in HS in probably more than simply a marker of cell damage. The protective effect of GSH pretreatment might be due to an increase in anti-oxidative capacity thus supporting the hypothesis of FR as a mediator of gastric mucosal damage.

B 222 AIRWAY RESPONSE AND HYPERREACTIVITY AFTER ACTIVE OXYGEN EXPOSURE, U.Katsumata, M.Ichinose, M.Miura, H.Inoue, T.Takishima, Tohoku University Medicine, Sendai, Japan.

To examine whether superoxide produce bronchoconstriction and airway hyperresponsiveness in vivo, we performed the successive inhalation of xanthine(0.1%) and xanthine oxidase(1U/ml) using 16 cats. Pulmonary resistance(R_L) instantaneously increased from 15.9±1.4 to 101.4±37.4 $cmH_2O/L/s$, however after vagotomy the increase was significantly depressed (n=6). Superoxide dismutase also inhibited the increase of R_L (n=6).

In the other group (n=5), acetylcholine (ACh, 0.015-1.0 %) was inhaled before and after the inhalation of xanthine and xanthine oxidase to examine the changes of airway responsiveness. After their inhalation, there persisted the increase of airway responsiveness about for 60min.. At 30min. after inhalation, the airway became 6 fold hyperreactive to ACh, so that ACh provocative concentration decreased from 0.25±0.03% to 0.045±0.03%. These resulrs suggest our hypothesis that superoxide produced by airway inflammation could cause airway constriction and successive airway hyperresponsiveness.

B 223 QUANTITATION OF CONJUGATED DIENES IN PLASMA AS INDICATOR OF LIVER TRANSPLANT REJECTION, Tai-Wing Wu and Donald A.G. Mickle, Univ. of Toronto and Toronto General Hospital, Canada M5G 2C4.
Using HPLC, we have observed the presence of elevated peroxidized phospholipids (PPP) in the plasma of liver transplant recipients who rejected their grafts, but not in those who recovered uneventfully from allograft transplantation, nor from healthy volunteers. The nominal amounts of PPP rose with the intensity of graft rejection, and mirrored the response to immunosuppressive therapy, but increased minimally in transplant patients who had cytomegalovirus or bacterial infections which can be easily confused clinically and histologically with transplant rejection. Using a more specific radiochemical method for PPP, in which conjugated dienes are reacted with ^{14}C-tetracyano-ethyelene, we have quantitated for the first time the ranges of plasma PPP in the above patient categories. The rejecting patients had plasma conjugated dienes per umole of phospholipids of 9-29 times higher than those seen in non-rejecting patients or healthy volunteers. Molecular identifications of the putative peroxidized phospholipids will be presented.

B 224 Free Radicals are Involved in the Mechanism of Action of Recombinant Human Tumor Necrosis Factor, Robert J. Zimmerman, CETUS Corporation, Emeryville, CA 94608, Steven A. Leadon, University of California, Berkeley, CA 94720
The intracellular glutathione levels of 3 human and 7 mouse tumor lines were determined in vitro in order to investigate the role of free radicals in tumor cell sensitivity to recombinant human TNF (rhTNF). The response of the tumors to rhTNF therapy in vivo correlated with their radical scavenging capacity (r=0.95): resistant tumors contained high levels of glutathione, whereas sensitive tumors had low levels. The transplantable murine fibrosarcoma Meth A, a rhTNF-sensitive tumor in vivo, was less sensitive and host toxicity was reduced when hosts were pretreated with uric acid, a major radical scavenger in humans. Conversely, pretreatment of the hosts with buthionine sulfoximine, an inhibitor of glutathione biosynthesis, increased the sensitivity of Meth A to rhTNF. In in vitro studies, the intracellular levels of total and oxidized glutathione were determined after rhTNF addition to tumor cells. An increase in the amount of both GSH and GSSG was observed in 3 of 4 cell lines exposed to rhTNF. In the Meth A cell line there was no effect on these levels, which indicated that the mechanism(s) which produced the oxidative damage was not functioning in these cells. A monoclonal antibody directed against thymine glycols (TG, a form of oxidative base damage) was used in a competitive ELISA assay to look for evidence of radical attack at the genomic level in the same 4 cell lines. The highest levels of TG's were found in L929 cells, while once again no evidence of radical attack was found in the Meth A cells. We hypothesize that rhTNF's direct cytotoxicity mechanism, as well as its indirect, immunomodulatory activity, involve the production of free radical damage to target cells.

Oxy-Radicals and Aging
Oxy-Radicals in Mutagenesis and Cancer

B 300 **EFFECTS OF SUPPLEMENTATION OF THE ELDERLY ON PLASMA AND CELLULAR CONCENTRATIONS OF TRACE ELEMENTS REQUIRED BY ANTIOXIDANT ENZYMES, John D. Bogden, Francis W. Kemp, Kay Bruening, and Kimberly Holding, UMDNJ-New Jersey Medical School, Newark, NJ**
Some diseases associated with oxy-radicals are more prevalent in the elderly. Inadequate nutriture or altered metabolism of Se, Cu, or Zn in older subjects could contribute to cellular damage by oxy-radicals, since glutathione peroxidase (GTP) is a Se metalloenzyme, and superoxide dismutase (SOD) has a Cu/Zn form. A study was conducted to determine if plasma Se, Cu, and Zn and cellular Zn decline with age in the elderly, and if they are influenced by safe doses of these nutrients. Male and female subjects (n=103), aged 60-89, were studied before and after daily supplementation for 3 months with 10 µg Se, 2 mg Cu, and 0, 15, or 100 mg Zn. Intake from food of Se, Cu, and Zn was below recommended levels in many subjects. Mean zinc and copper intakes were 54% and 60% of recommended values despite adequate consumption of protein. Only the 100 mg dose of Zn significantly increased plasma Zn, from 86±13 to 110±23 µg/dl, but cellular (erythrocyte, platelet, and mononuclear leukocyte) Zn concentrations were not significantly increased. Plasma Se and Cu were not influenced by supplementation. There was no significant decline in plasma concentrations of Se, Cu, or Zn with age, and values found were similar to normal laboratory concentrations of young healthy adults. Cellular Zn was also not influenced by age. Though plasma Zn concentrations can be increased by Zn supplementation of 100 mg/day, Zn concentrations in circulating cells are resistant to the influence of supplementation. Altered GTP and SOD status in elderly subjects cannot be explained by changes in plasma Se, Cu, or Zn or cellular Zn with aging, but low dietary intakes of these elements might be a factor. (Supported by NIH grant AG04612)

B 301 MIXED FUNCTION OXIDATION OF LENS PROTEINS INDUCES CHANGES FOUND WITH AGING, Donita Garland, Paul Russell, and J. Samuel Zigler, Jr., National Eye Institute, Bethesda, Maryland 20892.

Protein modifications that accumulate in the lens with aging are thought to be the result of oxidative processes. Recent studies indicate that many of these modifications are induced by treatment of lens proteins with mixed function oxidation systems in vitro. The modifications include non-disulfide crosslinks, degradation, formation of non-tryptophan fluorescence, charge changes and the introduction of carbonyl groups. Studies are now being done on a human 21 kDa gamma crystallin that is expressed in mouse L cells [(Meakin et al., Mol. Cell. Biochem. 5, 1408 (1987)]. Treatment with ascorbate, ferric ion, and oxygen or hydrogen peroxide and ferrous ion induced the formation of modified species that comigrate on polyacrylamide gel electrophoresis and isoelectric focusing with modified forms of this protein isolated from 4 month-old and 15 year-old lenses. When analyzed on SDS polyacrylamide gel electrophoresis, the molecular weight of this protein was not decreased. Thus, these modifications represent charge alterations and not degradation of this protein. Studies are in progress to correlate the amino acid changes with the modified protein species. These results indicate that mixed function oxidation may contribute to the changes seen with aging in lens.

B 302 EVIDENCE FOR AND IMPLICATIONS OF HYPERMUTATION OF YEAST CYTOCHROME C PEROXIDASE, Jim Kaput, Wesley Miyazaki, and Marietta Piattoni, Department of Biochemistry, University of Illinois College of Medicine, Urbana, Illinois 61801.

Cytochrome c peroxidase is a nuclearly-encoded protein that catalyzes the degradation of H_2O_2 in vitro. This enzyme is located in the intermembrane space of yeast mitochondria and its exact role in cellular physiology is unknown. We have constructed a yeast strain which is deficient in CCP using gene disruption techniques. Since the haploid mutant is viable, CCP is not essential for cell growth. In addition, the strain grows on nonfermentable carbon sources at normal oxygen tensions indicating that CCP is not directly involved in oxidative metabolism. We have initiated experiments that may determine the growth conditions under which CCP is expressed or required for growth. As a part of this study, the CCP gene and its 5' regulatory sequences have been cloned from the yeast library YEp24. Analyses revealed that there were at least 3 differences in the amino acid sequence compared to the protein sequence published by Takio et al. (Arch. Biochem., Biophys. 203, 615-629) and 5 differences compared to the preCCP gene isolated from the YEp13 yeast library (Kaput et al., J. Biol. Chem. 257, 15054-15058). In addition, there were 5 silent changes in the nucleic acid sequence of the mature protein coding region. Two amino acid changes were found in the presequence that directs CCP to the intermembrane space. We are currently investigating whether these mutations were generated by propagation of the gene in E. coli or resulted from genetic drift in Saccharomyces cerevisiae. In either case, the lack of selective growth conditions may allow rapid mutations to accumulate in "nonessential" genes.

This research was supported by a grant from the NIH (GM35425) and a gift from the Eastman Kodak Company.

B 303 HEMOGLOBIN: A SOURCE OF SUPEROXIDE RADICAL UNDER HYPOXIC CONDITIONS, Abraham Levy, Lu Zhang, and Joseph M. Rifkind, NIH, NIA, Gerontology Research Center, Balto., MD 21224. It has previously[1,2] been shown that autoxidation of hemoglobin coincides with the formation of superoxide radicals. We have recently reported that at low oxygen pressure there is an enhanced rate of superoxide leakage from the erythrocyte. It has been suggested that these superoxide radicals are a factor in cellular and tissue damage under hypoxic conditions. Such a mechanism would contribute to oxyradical damage in aging and various disease processes. In order to understand this process, the autoxidation of hemoglobin has been studied as a function of oxygen pressure under various conditions. By combining this data with oxygen binding data, it has been possible to determine the relationship between the state of oxygenation and the enhanced rate of autoxidation. Studies with the closely related monomeric protein, myoglobin, which does not undergo the same conformational changes as hemoglobin, imply a relationship between structural changes responsible for cooperative oxygen binding and the enhanced rate of autoxidation at low oxygen pressure.

1. Misra, H.P., and Fridovich, I. J. Biol. Chem. 247: 6960-6962 (1972).
2. Wever, R., Oudega, B., and Van Gelder, B.F. Biochim. Biophys. Acta 302: 475-478 (1973).

B 304 PROTEOLYSIS OF OXIDIZED PROTEINS DURING OXIDATIVE STRESS AND AGING, P.E. Starke-Reed & C.N. Oliver, Laboratory of Biochemistry, NHLBI, NIH, Bethesda, MD 20892.
We have previously demonstrated that a variety of enzymic and nonenzymic mixed function oxidation systems catalyze the oxidative inactivation of E. coli glutamine synthetase and other key metabolic enzymes in vitro. Considerable evidence indicates that inactivation is mediated by Fe^{2+} and H_2O_2 via a site-directed mechanism involving the loss of a single histidine in each GS subunit. Further oxidation leads to increased proteolytic susceptibility by known proteases as well as by cytosolic proteases which exhibit selectivity for oxidized proteins suggesting that oxidative inactivation is a marking step for selective degradation of enzymes and proteins. In order to study the fate of specific enzymes in vivo we have compared the oxidation and proteolysis of G-6-PDH and GS in isolated hepatocytes from rats exposed to 100% oxygen for 54 hours and from rats of various ages, 3 to 24 months. Oxidized proteins accumulate as a function of oxygen exposure and also during normal aging. The patterns of proteolysis are different in the two model systems. Although the levels of acid proteases remain relatively constant, alkaline protease activities increase after 48 hours of oxygen treatment. During aging there is a precipitous drop in alkaline protease activity in 24 month old rats. Preliminary studies indicate that at least nine alkaline protease activities can be fractionated from hepatocytes derived from oxygen toxicity model but only two or three show the time-dependent increase observed earlier. Other alkaline proteases decrease or remain unchanged. These data suggest that some proteases may be activated or induced during oxygen exposure and similar proteases may be deficient or defective in old animals.

B 305 ACTIVATION OF DAUNOMYCIN BY ENZYMATICALLY NITROXIDE LABELED NUCLEIC ACIDS IN THE PRESENCE OF MOLECULAR OXYGEN, Albert M. Bobst, John C. Ireland and Elizabeth V. Bobst, University of Cincinnati, Cincinnati, OH 45221.
Daunomycin (DM) is an anthracycline quinone used to treat certain solid tumors and leukemias in humans. There is no concensus as to the mechanism by which DM exhibits its antitumor effect. One mode of action of DM is believed to involve the DNA intercalation properties of the drug chromophore. However, there is also evidence for mechanisms involving the redox cycling properties of the drug's quinone moiety. Electron spin resonance (ESR) studies have been carried out with enzymatically nitroxide labeled nucleic acids and DM in the presence of molecular oxygen. It was observed that the nitroxide radical can under certain conditions serve as a localized electron source in the DNA. The ESR data indicate that the oxidation of the nitroxide is an irreversible step. It is proposed that the nitroxide serves as an activator of DM by first reducing the quinone moiety to the unstable radical anion, which then is oxidized in the presence of molecular oxygen to form O_2^-. Experiments are in progress to show that the proposed mechanism is responsible for causing DNA strand breaks (supported in part with NIH CA-39396).

B 306 **ISOLATION OF THE HUMAN GLUTATHIONE PEROXIDASE GENE - IDENTIFICATION OF AN OPAL SUPPRESSOR tRNA ACTIVITY IN HUMAN CELLS. Sunil Chada and Peter Newburger, University of Massachusetts Medical Center, Worcester, MA 01605.**

Selenium is an essential element in the mammalian diet, yet the only well characterised selenoprotein is Glutathione Peroxidase (GSH-Px), which contains a selenocysteine at amino-acid 47. This enzyme protects cells against peroxide-induced damage to cellular membranes and possibly also DNA.

Using oligonucleotides synthesised against regions of the bovine amino-acid sequence, we probed a cDNA library constructed from human (HL60) mRNA, and isolated cDNA clones encoding the entire Glutathione Peroxidase mRNA. Upon sequence analysis, it was found that the selenocysteine residue in the active site of the enzyme was encoded by an opal terminator TGA. This is the first example of a terminator codon being supressed in human cells, and confirms the sequence analysis of Mullenbach et. al., (1987) NAR 15:5484. Studies on the mechanism of this supression shall be presented.

B 307 **REGULATION OF HUMAN GLUTATHIONE PEROXIDASE GENE EXPRESSION BY SELENIUM.**
Sunil Chada, Jocyndra Wright, Constance Whitney and Peter Newburger, University of Massachusetts Medical Center, Worcester, MA 01605.

We have developed a human model system with which to examine Glutathione Peroxidase (GSH-Px) activity and gene expression in the presence or absence of selenium. The HL60 cell line is derived from a human promyelocytic leukemia, and may be grown in a defined medium containing insulin and transferrin. Selenium may be added to this medium as an optional supplement.

We assayed glutathione peroxidase activity in cells grown in selenium-containing and selenium-depleted media. A time-dependent decrease in GSH-Px activity was observed when selenium was removed from the media, with negligible activity attained when cells were deprived of selenium for 7 days. Upon return of these cells to selenium-containing media, a rapid increase in GSH-Px activity was observed. Approximately 30-fold more enzymatic activity was observed from cells grown in selenium than those without. This fall in enzymatic activity was compared to steady-state levels of GSH-Px mRNA in selenium-supplemented or depleted cells, and it was found that mRNA levels did not vary significantly with selenium depletion or replenishment. Cellular GSH-Px protein levels were monitored using a polyclonal antibody raised against human erythrocyte GSH-Px. Total GSH-Px protein levels paralleled enzymatic activity during selenium depletion and/or replenishment.

Thus GSH-Px gene expression is regulated post-transcriptionally by selenium.

B 308 **DEVELOPMENTAL REGULATION OF GLUTATHIONE PEROXIDASE GENE EXPRESSION.**
Sunil Chada, Constance Whitney, Jocyndra Wright and Peter Newburger. University of Massachusetts Medical Center, Worcester, MA 01605.

We recently isolated the human gene encoding the major selenoprotein glutathione peroxidase, and have examined its expression during differentiation of the human promyelocytic leukaemia cell line, HL60. This cell line is unique in that it has the ability to differentiate to form neutrophils, macrophages or eosinophils. These end-point cells share many morphological and functional characteristics with their in vivo counterparts. Upon treatment with phorbol esters to induce macrophagic differentiation or with dimethylformamide to induce neutrophilic differentiation, a rapid increase is observed in the steady-state levels of glutathione peroxidase mRNA, with levels approximately 20-fold greater than those found in uninduced promyelocytes being observed. However, nuclear run-on studies indicate that the rate of transcription of this gene decreases more than 10-fold during differentiation.

These results suggests a complex mode of pretranslational regulation for the glutathione peroxidase gene during differentiation.

B 309 BENZO(A)PYRENE [B(A)P]-INDUCED FORMATION OF H_2O_2 AND OXIDATIVE DNA DAMAGE, Krystyna Frenkel and Jean Donahue, New York University Medical Center, New York, NY 10016.
B(a)P, an ubiquitous environmental pollutant, is a complete carcinogen. Its tumor-initiating properties correlate with formation of DNA adducts. However, it is not clear what constitutes its tumor-promoting activities. In this work we show that B(a)P induces rat liver microsomes to form H_2O_2, whereas pyrene, a non-carcinogen does not. H_2O_2 has been shown to be a first-stage tumor promoter. Therefore, its formation by B(a)P-treated microsomes may be a part of tumor-promoting activity exerted by this carcinogen. We show that H_2O_2 is generated in a dose-dependent manner by B(a)P-treated liver microsomes obtained from Fisher 344 and Sprague-Dawley rats. Its formation is similar in both strains of rats and about the same in uninduced as well as 3-methylcholanthrene-induced rats. Incubation of DNA with B(a)P-treated microsomes results in formation of oxidized thymidines 5-hydroxymetyl-2'-deoxyuridine (HMdU) and thymidine glycol (dTG). In the presence of catalase, HMdU formation decreases well below that of the control incubated without B(a)P. This last finding proves that H_2O_2 is necessary for the formation of oxidized bases in co-incubated DNA. Since H_2O_2 can cross membranes like water, it may cause similar damage in DNA of neighboring cells as well as in the cells where it is generated. We previously have shown that formation of H_2O_2 and of HMdU and dTG by tumor promoter-stimulated human neutrophils correlates well with the first-stage tumor-promoting activity of the promoters used. Therefore, the ability of B(a)P to cause formation of H_2O_2 and oxidized bases in DNA points to these processes as B(a)P equivalents of first-stage tumor promotion.
(Supported by grants CA 37858, CA 13343, ES 00260 and ES 07081.)

B 310 MACROPHAGE INDUCED DNA STRAND BREAKS IN TUMOR TARGET CELLS, Amy Fulton, Leslie Paul, Yen Chong and Gloria Heppner, Michigan Cancer Foundation, Detroit, MI 48201

We have shown that macrophages isolated from murine mammary tumors can induce mutation in the Ames assay. Activated macrophages can also induce the appearance of drug resistant variants of mammary tumor cells. We have proposed that tumor-infiltrating macrophages can, by acting on genetically unstable tumor cells, induce tumor cell diversity that may fuel progression. We now report that macrophages can induce DNA strand breaks in tumor cell targets as detected by fluorometric analysis of DNA unwinding. These breaks occur after brief co-incubation (60 min.), at macrophage-target cell ratios of 1:1, and cause the equivalent damage of 900-1800 rads of irradiation. We found, to our surprise, that the level of macrophage activation correlated negatively with the ability to induce DNA strand breaks. That is, unstimulated resident peritoneal macrophages induced the greatest number of strand breaks, primed (MVE-2) macrophages expressed an intermediate level of activity while fully activated macrophages were least active. Using inhibitors of reactive oxygen species or of arachidonate metabolism, divergent results were again seen for the different macrophage populations. Superoxide dismutase (SOD) or catalase alone each reduced the number of strand breaks induced by resident macrophages. The combination of the two was somewhat more protective. Indomethacin or NDGA were moderately protective in all experiments. In contrast, when MVE-2 macrophages were examined, SOD was either not protective in 2/9 experiments, or led to more strand breaks in 4/9 cases. Catalase was protective in half the tests. Thus, in the case of MVE-2 cells, treatment with SOD may have led to more H_2O_2 production. With these cells NDGA, which inhibits both cyclooxygenase and lipoxygenase metabolism of arachidonate, was never protective. These studies suggest that both oxygen-centered species and arachidonate metabolites may contribute to macrophage-induced DNA strand breaks in tumor target cells.

B 311 CYTOPATHIC EFFECTS OF CIGARRETTE SMOKE FRACTIONS AND COMPONENTS IN CULTURED HUMAN BRONCHIAL CELLS, R.C. Grafström, K. Sundqvist, J.M. Dypbukt, C.E. Edman, P. Moldéus and L. Nilsson, Karolinska Institutet, Stockholm, Sweden.

The effects of cigarrette smoke fractions, aldehydes and oxidants have been investigated in cultured human bronchial epithelial cells. Exposure of cells to whole cigarrette smoke causes decreased survival, depletion of cellular thiols and formation of DNA single strand breaks. Smoke condensate and different subfractions decrease both colony forming efficiency and growth rate of epithelial cells at 1 to 10 ug/ml concentrations. When effects of smoke condensate, a semivolatile and a nonvolatile fraction are compared, total condensate is the most cytotoxic, whereas the semivolatile fraction is the most potent to decrease cellular thiols. Further fractionation of the semivolatile fraction indicate that a neutral subfraction is more thiol-reactive than the basic, acidic or phenolic subfractions. Gaseous phase aldehydes and oxidants cause several effects in bronchial cells, including: decreased survival, squamous differentiation, DNA damage, thiol depletion, inhibition of DNA repair and mutations. Exposure to uM concentrations of N-acetylcysteine significantly protects against cytopathic effects caused by smoke, subfractions, oxidants and aldehydes. The results show that whole cigarrette smoke, as well as many subfractions and gaseous phase components in vitro cause many pathobiological effects that relate tobacco smoking to multistage carcinogenesis in human bronchial epithelium. Furthermore, the protective action of N-acetylcysteine against such adverse effects of tobacco smoke in human bronchial epithelial cells may be clinically relevant.

B 312 ROLE OF METABOLIC COOPERATION IN MODULATING SENSITIVITY OF V79 CELLS TO SUPEROXIDE ANION. TJ Kavanagh, G Raghu, SE Masta, and GM Martin. Departments of Pathology and Medicine, University of Washington, Seattle, WA

Cell-cell contact is an important modulator of cellular sensitivity to hyperoxia and radiation. This phenomenon could be explained by sharing of cellular defense molecules through gap junctions (metabolic cooperation (MC)). A candidate molecule for mediating such protection is glutathione (GSH) because of its known role in free radical scavenging and its low molecular weight. MC proficient (MC^+) or deficient (MC^-) thioguanine resistant (TG^r) strains of Chinese hamster V79 cells were depleted of their GSH with buthionine sulfoximine (BSO). They were then cocultured at various densities with nondepleted, MC^+, thioguanine sensitive (TG^s) cells. Alternatively, TG^s,MC^+ cells were depleted of their GSH and cocultured at various densities with either TG^r,MC^+ cells or TG^r,MC^- cells. Cocultures were exposed to xanthine oxidase/hypoxanthine generated superoxide anion for 1 hr., and then replated in thioguanine or, for the alternative cocultures in HAT medium to assess the survival of the BSO pretreated cells. In both sets of experiments, the restoration of GSH content that occured in GSH-depleted cells in cocultures of MC^+ and MC^+ cells resulted in a higher density dependent survival than in the cocultures containing MC^+ and MC^- cells. These results strongly suggest a role for MC of GSH among cells as an attributing factor in cellular sensitivity to toxic oxygen species.

B 313 ANTHRAPYRAZOLES AS PHOTOSENSITIZERS IN OXIDATION REACTIONS. K. Reszka, P. Kolodziejczyk, P. G. Tsoungas and J. W. Lown, Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2 Canada.

The photosensitizing properties of the novel anticancer agents, anthrapyrazoles (**1**), have been studied using electron paramagnetic resonance (EPR) and measurement of oxygen consumption. Several derivatives, both hydroxylated and non-hydroxylated in ring A, were tested for their ability to generate singlet oxygen upon irradiation with visible light in ethanolic solutions. DMFu (2,5-dimethylfuran) and sodium azide were used as singlet oxygen acceptor and quencher respectively. The highest rate of oxygen consumption was found for the derivative **1**, lacking OH groups in ring A. Further studies with **1** showed that it readily sensitized photo-oxidation of ascorbic acid (AH^-), 3,4-dihydroxyphenylalanine (DOPA) and NADH in aqueous solutions. The effects of the enzymes superoxide dismutase and catalase indicated production of superoxide radical with AH^- and NADH and hydrogen peroxide with $AH^-$and DOPA respectively. The rates of these reactions were independent on the presence or absence of sodium azide. EPR measurements showed that with **1** and AH^- the ascorbyl radical $A^{\cdot -}$, is also produced during the photo-oxidation reaction. Thus,results indicate that a mixed mechanism (Type I and Type II) may operate .

B 314 INHIBITION OF LUNG INJURY, INFLAMMATION AND FIBROSIS BY POLYETHYLENE GLYCOL (PEG)-CONJUGATED CATALASE IN RATS EXPOSED TO ASBESTOS. J.P.Marsh, R.Gilbert, D.Hardwick, A.Sesko, K.B.Adler, D.Hemenway, R.Mickey, E. Kagan*, B.T.Mossman, University of Vermont, Burlington, VT. and Georgetown University*, Washington, D.C.

Inhibition of lung injury and asbestosis were assessed using administration of varying dosages of PEG-conjugated catalase, delivered continuously via osmotic pumps, to rats exposed over a 28 day period (6/hr/day, 5 days/wk for 4 weeks) to crocidolite asbestos (~10mg/m^3). Exposure to asbestos without catalase administration caused a significant increase in the amount of hydroxyproline in lung, and fibrosis as determined by histopathology, as well as elevations in protein content, enzymes (LDH, alkaline phosphatase), and total cell count in lavage fluid. All above parameters of lung injury were inhibited significantly ($p<.005$) in rats receiving the highest concentration of PEG-catalase tested (8000 IU/day). These results emphasize the importance of active oxygen species in the mediation of asbestosis, and implicate a valuable therapeutic role for scavenger enzymes as well.

In related studies, we assayed the levels of scavenger enzymes [superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX)] in lungs of control and crocidolite-exposed rats, and found all 3 enzymes to be elevated significantly after inhalation of asbestos for 10 days, suggesting an adaptive response of endogenous lung enzymes in response to a free radical insult. Supported by SCOR grant PHS 14212 from N.H.L.B.I., grant ROl ES03878 from N.I.E.H.S., and grant ROl HL39469 from N.H.L.B.I.

B 315 ROLE OF METABOLIC COOPERATION IN MODULATING SENSITIVITY OF V79 CELLS TO SUPEROXIDE ANION. TJ Kavanagh, G Raghu, SE Masta, and GM Martin. Departments of Pathology and Medicine, University of Washington, Seattle, WA

Cell-cell contact is an important modulator of cellular sensitivity to hyperoxia and radiation. This phenomenon could be explained by sharing of cellular defense molecules through gap junctions (metabolic cooperation (MC)). A candidate molecule for mediating such protection is glutathione (GSH) because of its known role in free radical scavenging and its low molecular weight. MC proficient (MC^+) or deficient (MC^-) thioguanine resistant (TG^r) strains of Chinese hamster V79 cells were depleted of their GSH with buthionine sulfoximine (BSO). They were then cocultured at various densities with nondepleted, MC^+, thioguanine sensitive (TG^s) cells. Alternatively, TG^s,MC^+ cells were depleted of their GSH and cocultured at various densities with either TG^r,MC^+ cells or TG^r,MC^- cells. Cocultures were exposed to xanthine oxidase/hypoxanthine generated superoxide anion for 1 hr., and then replated in thioguanine or, for the alternative cocultures in HAT medium to assess the survival of the BSO pretreated cells. In both sets of experiments, the restoration of GSH content that occured in GSH-depleted cells in cocultures of MC^+ and MC^+ cells resulted in a higher density dependent survival than in the cocultures containing MC^+ and MC^- cells. These results strongly suggest a role for MC of GSH among cells as an attributing factor in cellular sensitivity to toxic oxygen species.

B 316 POSSIBILITIES OF USING COMPUTER TO MAKE UP REVERSORY, ANTIOXIDANT, CHEMOPREVENTIVE DIETS. Nister C. (1); Harke, T. (1); Szabe Susana (2); Dinea, Diana (3); Taranu, C. (3); Chira, Dana (1); Balan, Maria (1); Nister, Vectoria (1); Nister, C.C. (2); Faur, Virginia (2); Hebedean, Mieara (2); Pana, Deina (2); Oniser, Maria (1); Timis, M. (1); (1). Oncological Institute Cluj Napeca, Cluj Napeca, Romania (2). Interdisciplinary Laborator, Univ. Cluj Napeca, Cluj Napeca, Romania (3). Electronic Computer Center Cluj Napeca, Cluj Napeca, Romania. Alimentation represents a fundamental support in prophilaxis and therapy of cancer. Technologically the most up to date solutions for formulating natural reversory, antioxidant, revitaminizing, chemopreventive diets, are informational techniques. We created a series of programs which may be circulated in a personal computer (PRAE-M) and which may offer diets with natural feeds, characterized by a rich content of A,C,D vitamins, proteins, amino acids, and a reduced lipid content. An open data bank stores 95 parameters of chemical composition, active principles, number of calories, etc., for 140 natural dishes. According to same combinatory algorithms, the computer programs establish for every subject separately (in accordance to profession, life environment, age, sex, nutritional necessities) constant and effective levels of vitamins A and C, in accordance with the literature, suitable to protect against development of cancer, to prevent malignisation of pre-cancer lesions, and to secure an immunologically active status. The most important advantages of these programs are their applicability on a large scale, at the level of large scale populations, speed and accuracy.

B 317 ROLE OF METABOLIC COOPERATION IN MODULATING SENSITIVITY OF V79 CELLS TO SUPEROXIDE ANION. TJ Kavanagh, G Raghu, SE Masta, and GM Martin. Departments of Pathology and Medicine, University of Washington, Seattle, WA

Cell-cell contact is an important modulator of cellular sensitivity to hyperoxia and radiation. This phenomenon could be explained by sharing of cellular defense molecules through gap junctions (metabolic cooperation (MC)). A candidate molecule for mediating such protection is glutathione (GSH) because of its known role in free radical scavenging and its low molecular weight. MC proficient (MC^+) or deficient (MC^-) thioguanine resistant (TG^r) strains of Chinese hamster V79 cells were depleted of their GSH with buthionine sulfoximine (BSO). They were then cocultured at various densities with nondepleted, MC^+, thioguanine sensitive (TG^s) cells. Alternatively, TG^s,MC^+ cells were depleted of their GSH and cocultured at various densities with either TG^r,MC^+ cells or TG^r,MC^- cells. Cocultures were exposed to xanthine oxidase/hypoxanthine generated superoxide anion for 1 hr., and then replated in thioguanine or, for the alternative cocultures in HAT medium to assess the survival of the BSO pretreated cells. In both sets of experiments, the restoration of GSH content that occured in GSH-depleted cells in cocultures of MC^+ and MC^+ cells resulted in a higher density dependent survival than in the cocultures containing MC^+ and MC^- cells. These results strongly suggest a role for MC of GSH among cells as an attributing factor in cellular sensitivity to toxic oxygen species.

B 318 OXYGEN RADICAL-INDUCED DAMAGE TO RAT LIVER INTRACELLULAR MEMBRANES AFTER IRRADIATION, Osami Yukawa, Natl. Inst. Radiol. Sci., Chiba-shi 260, Japan.

Changes in intracellular membrane-bound enzymes and phospholipids in rat liver were investigated after in vivo or in vitro irradiation. Phospholipids of intracellular membranes were peroxidized through oxygen radicals produced by irradiation. The level of lipid peroxidation was different in each intracellular membrane. Microsomal and plasma membrane lipids were strongly peroxidized, but nuclear and mitochondrial membrane lipids showed very low peroxidation. The level of lysosomal lipid peroxidation was between them. In the case of microsomes, G-6-Pase and P-450 which is one of the microsomal drug metabolizing enzymes were inactivated as a result of the membrane lipid peroxidation. On the other hand, plasma membrane 5'-nucleotidase and microsomal ATPase activities were not affected in spite of higher lipid peroxidation of these membranes. In addition, G-6-Pase activity in nuclear membranes was significantly inactivated, though little peroxidation was seen in the membrane lipids. These relationships between membrane-bound enzymes and membrane lipids were confirmed using two different types of reconstitution system of membranes. These results are discussed with reference to oxygen radical-induced membrane damage after irradiation.