MOLECULAR BASIS OF OXIDATIVE DAMAGE BY LEUKOCYTES

Organizers: Algirdas Jesaitis, Edward Dratz and Frederick Van Kuijk

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Molecular Basis of Oxidative Damage by Leukocytes

Keynote Address

CC 001 THE CHEMISTRY AND BIOLOGY OF SUPEROXIDE: CENTRAL CONCEPTS AND RESIDUAL PROBLEMS, Irwin Fridovich, Department of Biochemistry, Duke University Medical Center, Durham, NC 27710. O_2^- is generated by spontaneous and by enzyme-catalyzed oxidations. It can exert deleterious effects directly by initiating the oxidation of sulfite, catecholamines, tetrahydropterins, leukoflavins and enediols; and by inactivating [4Fe-4S]-containing dihydroxy acid dehydratases such as those acting on 6-phosphogluconate, dihydroxyisovalerate, fumarate and citrate. It seems likely that additional targets, susceptible to direct attack by O_2^-, exist within cells. Its oxidizing capability is greatly augmented by association with cationic centers including H^+, Mn(II), and $V_{(V)}$. O_2^- can reduce Fe(III) to Fe(II), which then reacts with H_2O_2, generating potent oxidants such as FeO^{++} or HO·. This occurs both in vitro and in vivo. In the latter case, binding of the catalytic metal to membranes or DNA facilitates selective damage of these targets. Defensive enzymes such as superoxide dismutases, catalases and peroxidases lower steady-state levels of O_2^- and H_2O_2 and thus prevent much damage; while other enzymes minimize the consequences of such damage. The latter category includes alkyl hydroperoxide reductases, methionine sulfoxide reductases, proteinases specific for oxidatively modified proteins and enzyme systems which repair oxidized DNA. Antioxidants such as α-tocopherol which abort free radical chain reactions are also important.

Many interesting and potentially important problems remain. These include: How is the biosynthesis of the defensive enzymes regulated in both prokaryotes and eukaryotes? How does the pseudorevertant of the sodA sodB strain of *E. coli* manage without SODs? How does tumor necrosis factor (TNF) induce MnSOD and why does MnSOD protect against TNF? What is the source of O_2^- during reperfusion injury and how does exogenous SOD protect? Do activated phagocytic cells contribute to the lethality of ionizing radiation? What contribution do O_2^- and derived radicals make to senescence? And, why do a few species of bacteria contain a Cu,ZnSOD? It seems safe to predict that the excitement in the oxy-radical field will continue as enthusiastic, talented investigators explore these and other questions.

Superoxide: Generation and Targets

CC 002 MOLECULAR DISSECTION OF THE LEUKOCYTE SUPEROXIDE-GENERATING SYSTEM, Robert A. Clark, Bryan D. Volpp, Shankar Iyer and William M. Nauseef, Department of Medicine, University of Iowa and VA Medical Center, Iowa City, IA 52242 The stimulus-dependent burst of superoxide formation by phagocytic leukocytes is mediated by an activatable NADPH oxidase system comprised of membrane-associated as well as cytosolic components (1). Cytochrome b_{558}, a heterodimer of p22-*phox* and gp91-*phox*, is an integral membrane protein that appears to serve as the terminal electron carrier. Two cytosolic proteins, p47-*phox* and p67-*phox*, are essential for oxidase function (2), but whether they are involved in activation or catalysis is not clear. A putative flavoprotein component has not been fully characterized. Cell stimulation results in a protein kinase-mediated phosphorylation of multiple sites on p47-*phox* (3). The phosphoprotein then becomes associated with the cytoskeleton and with the plasma membrane (4). There is also secondary translocation of p67-*phox* to the membrane. The membrane docking site for p47-*phox* appears to be cytochrome b or a closely associated component (5). Additional sites on p47 are phosphorylated once it is membrane-bound. We hypothesize that cytoskeletal and membrane interactions of the cytosolic oxidase components lead to the assembly on the membrane of a catalytically competent electron transport system comprising the respiratory burst oxidase.

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Hemeproteins and Oxygen Radical Generation

CC 003 ELECTRONIC AND VIBRONIC CONTROL OF ELECTRON TRANSFER IN PROTEINS.
David N. Beratan, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 91109.

An introductory lecture will be presented describing the connection between protein structure and the rate of electron transfer. The strategy of separating the problem into an electronic two-state model with coupling between the two states and with nuclear modes will be reviewed and the generic structure-function predictions described. Recently, we presented a strategy for mapping specific covalent and noncovalent interactions in proteins (tunneling pathways) responsible for mediating the donor-acceptor coupling [1]. The electron transfer rate in proteins scales with the square of this coupling energy, which depends on details of the protein secondary structure between donor and acceptor. Mapping of the pathways responsible for the donor-acceptor coupling in specific electron transfer proteins such as cytochrome *c*, azurin, and cytochrome b_5 will be described.

1. D.N. Beratan, J.N. Onuchic, J.N. Betts. B.E. Bowler, and H.B. Gray, J. Am. Chem. Soc., to appear 10/90.

CC 004 RADICAL REACTIONS IN HEMEPROTEINS, Paul R. Ortiz de Montellano, Yearn S. Choe, and Angela Wilks, Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143-0446

The reduction of oxygen by a cytochrome in neutrophils produces superoxide and hydrogen peroxide. The hydrogen peroxide, in turn, is activated to reactive species that mediate protein and membrane damage. The conversion of hydrogen peroxide to the reactive species may be catalyzed by hemoproteins such as hemoglobin and myoglobin. We have recently explored the mechanisms of the reactions of hemoglobin and myoglobin with hydrogen peroxide and the reactions of the resulting activated hemoprotein species with lipids and small organic substrates. These two classes of hemoproteins have been found to catalyze three distinct oxidative processes (1): (a) cytochrome P450-like oxygen transfer, (b) peroxidase-like electron abstraction, and (c) a cooxidative process in which a protein radical binds and activates molecular oxygen. As a result of their participation in these reactions, hemoglobin and myoglobin undergo protein-protein and heme-protein cross-linking reactions (2,3) as well as heme and protein modification. The tyrosine residues of the hemoproteins play key roles in these reactions, as shown by differences in the behavior of the proteins lacking one or more of the tyrosine residues. The specific roles of the tyrosine residues are being explored by work with site specific mutants produced from a synthetic myoglobin gene. This work is supported by NIH grants DK 30297 and GM 34288.

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CC 005 THE STRUCTURE OF THE HUMAN NEUTROPHIL PLASMA MEMBRANE B-TYPE CYTOCHROME INVOLVED IN SUPEROXIDE PRODUCTION, C. Parkos and M. Quinn, Department of Pathology, Harvard Medical School, Boston, MA 02115 and Department of Chemistry, Montana State University, Bozeman, MT 59717.
The production of superoxide anion (O_2^-) in neutrophils is mediated through an unusual b-type cytochrome which is present in both the plasma membrane and specific granules. This cytochrome is crucial for the body's microbicidal defense, and its genetic absence leads to repeated, life-threatening infections (chronic granulomatous disease). Further evidence for its direct involvement in O_2^- production comes from its very low (-245 mV) electrochemical potential, co-purification with superoxide generating activity in detergent extracts of neutrophil membranes and high kinetic capacity to reduce molecular oxygen under aerobic conditions. Protein analysis of cytochrome b has revealed it to be a heterodimer composed of 91 and 22 kd subunits. The larger subunit is heavily glycosylated with N-linked carbohydrate, leaving a core polypeptide of 55-60 kd. Although it is unknown how this cytochrome functions to reduce molecular oxygen, the high heme-to-protein ratio of purified cytochrome b strongly suggests at least two heme prosthetic groups are present. Analysis of the complete polypeptide sequence of small subunit revealed conservation of a critical proline residue six amino acids beyond the histidine shown to be essential for up cytochrome function. This proline placement is conserved in other transmembrane b-type cytochromes. Because others have shown that the heme may be carried by the small subunit, and only one of the histidines in the small subunit is necessary for cytochrome function, we predict that the large subunit bears a noncovalently bound heme and the small and large subunits share another heme. Such a two heme system could then carry out a transmembrane electron transport function as has been postulated in several other b-cytochrome systems.

CC 006 MOLECULAR ASSOCIATIONS OF NEUTROPHIL CYTOCHROME B, M. Quinn, M. Mullen and J. Linner, Department of Chemistry, Montana State University, Bozeman, MT 59717 and Cryobiology Research Center, University of Texas, Houston, TX 77381.
Activation of the superoxide generating system in human neutrophils is thought to involve the interaction or assembly of cytochrome b with other cytosolic and membrane proteins. To determine the molecular and structural basis of this assembly, we have been analyzing the superoxide generating complex to determine components associated with cytochrome b, the putative terminal component of the system. Detergent solubilized superoxide activity fractionated by rate zonal ultracentrifugation resulted in the isolation of superoxide producing particles of the approximate size of 80S. These particles contained flavin and actin, as well as cytochrome b. Further analysis of detergent solubilized neutrophil membranes using immunoaffinity chromatography revealed the association of a ras-related GTP-binding protein, the rap1 protein. Treatment of detergent solubilized membranes with GTP-γ-S prior to the immunoaffinity purification procedure resulted in a loss of retention of the cytochrome and rap1 protein on anti-cytochrome immuno-affinity matrices, suggesting the GTP-binding protein may play a role in the structure and/or function of the cytochrome b and superoxide generating complex. Immunoelectron microscopic studies with rap1 protein specific antiserum revealed that the rap1 protein colocalized with cytochrome in both specific granules and plasma membranes in unstimulated neutrophils, suggesting that the two molecules may be preassociated prior to the assembly of the superoxide generating complex. The results suggest a possible regulatory role of the ras-related protein rap1 on the effector molecule, cytochrome b.

Regulatory Events in Oxygen Radical Production and Damage

CC 007 PERTURBATION OF ARACHIDONATE METABOLISM AND MEMBRANE FUNCTION BY OXIDANTS. Henry J.Forman,Timothy W. Robison, Michael J. Thomas, *Diane C. Skelton and Dennis P. Duncan, Cell Biology Group, Childrens Hospital Los Angeles, University of Southern of California, Los Angeles, CA 90027, and *Department of Biochemistry, Wake Forest University School of Medicine, Winston Salem, NC 27103.

The alveolar macrophage, like other phagocytes, produces oxidants as part of its microbicidal role. Nevertheless, the alveolar macrophage is itself functionally and metabolically altered during exposure to oxidant stress, such as hyperoxic or NO_2 exposure. The cellular activities that are affected include superoxide production, chemotaxis, phagocytosis, and synthesis of leukotrienes and prostanoids. Our recent investigations have focused upon identifying the products formed from arachidonic acid during exposure of alveolar macrophages to NO_2 and the mechanism whereby some of these products cause alteration of alveolar macrophage function. Exposure to NO_2 elicits of leukotrienes and prostaglandins through enzymatic pathways but also produces a large number of compounds particularly aldehydes, through non-enzymatic lipid peroxidation. The aldehydes generated after NO_2 exposure were converted to dinitrophenylhydrazones (DNP), which were separated by HPLC and compared with standards. Gas chromatography/mass spectrometry of DNP- and methoxyamine/trimethysiloxyl derivatives was used to determine structures. We found that significant amounts of acrolein and other aldehydes were produced. These aldehydes were probably formed from breakdown of hydroperoxides produced as a consequence of free radical processes initiated by NO_2 exposure. The effects of oxidants on superoxide production by stimulated alveolar macrophages were investigated with linoleic acid hydroperoxide, t-butyl hydroperoxide, and H_2O_2. These oxidants, at sublethal concentrations, produce transient alterations in intracellular Ca^{2+}, plasma membrane depolarization, and transient lowering of the NADPH and glutathione content of cells. The results suggest a complex mechanism in which oxidants do not themselves stimulate superoxide production but rather perturb the signal transduction processes and thereby affect normal cell function. This work was supported by grant No. HL37556 from the NHLBI.

CC 008 MOLECULAR MECHANISM OF ACTIVATION OF LEUKOCYTE SUPEROXIDE PRODUCTION, Linda C. McPhail, Susan L. Strum, Mary Ellenburg, Diane Qualliotine-Mann, David E. Agwu, Charles E. McCall, and Peter A. Leone, Departments of Biochemistry and Medicine, Wake Forest University Medical Center, Winston-Salem, NC 27103.

The enzyme responsible for leukocyte superoxide (O_2^-) production is an NADPH oxidase, consisting of several polypeptide components, which is inactive until the cell is exposed to any of a variety of agonists. Polypeptides implicated as either catalytic components and/or targets for regulation of enzyme activity include a heterodimeric cytochrome b, a membrane-associated ras-related protein rap-1, and two cytosolic proteins termed p47-phox and p67-phox. The mechanism(s) regulating activation of NADPH oxidase are still unknown. Potential mechanisms in human neutrophils (PMN) under active study in our laboratory include: 1) the generation of diacylglycerol (DG) and phosphatidic acid (PA) and their roles as activators of NADPH oxidase in a cell-free system; 2) the role of translocation of p47-phox from cytosolic to membrane fractions in the assembly and/or activation process; and 3) the role of phosphorylation of p47-phox and other oxidase components, including identification of protein kinases involved. We and others have found that most PMN stimuli induce the activation of specific phospholipases (A_2, C, and D), which act on either choline-containing phosphoglycerides or polyphosphoinositides to generate varying levels of PA, DG, and arachidonic acid (AA). Our recent data indicate that PA and DG synergize with each other for NADPH oxidase activation in a cell-free system. In addition, PA synergizes with either AA or sodium dodecyl sulfate (SDS) in the same system. These results suggest that PA is a direct activator of NADPH oxidase and that the level and combination of bioactive lipids in the cell are important for regulating NADPH oxidase activity. Our studies indicate that translocation of p47-phox also plays an important role in activation of NADPH oxidase. Translocation correlates closely with oxidase activation during stimulation of intact cells (by either fMet-Leu-Phe, calcium ionophore A23187, or phorbol myristate acetate) or during cell-free activation by SDS. In the cell-free system, translocation and activation require membrane-associated cytochome b and are blocked by the calmodulin antagonist W-7. Phosphorylation of p47-phox on Ser/Thr residues is not necessary for either process. In conclusion, the mechanism of NADPH oxidase activation may involve direct effects of bioactive lipids on cytosolic components to induce conformational changes (activation?), translocation and assembly of an active enzyme complex.

CC 009 OXIDATIVE MODIFICATION OF LDL: MECHANISMS AND CONSEQUENCES, Daniel Steinberg, Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0613

Many converging lines of evidence indicate that much of the cholesteryl ester loading of macrophage foam cells in early atherosclerotic lesions results from the uptake of oxidatively modified LDL. The latter, but not native LDL, is recognized by the acetyl LDL receptor and by one or more receptors recognizing oxidatively modified LDL but not acetyl LDL. We have previously shown that LDL can be oxidatively modified by incubation with purified soybean lipoxygenase and that inhibitors of lipoxygenases block oxidative modification by endothelial cells. Recent studies show that macrophages also utilize lipoxygenases for oxidative modification of LDL. Using *in situ* hybridization and immunohistochemistry, we have demonstrated the presence in both rabbit and human arterial lesions of lipoxygenase mRNA and the enzyme protein. These were localized in the same areas as macrophage foam cells. These findings suggest a "vicious cycle" sequence of events in which oxidatively modified LDL, acting as a chemoattractant, recruits monocyte/macrophages. These cells are effective in oxidative modification of LDL, leading to the recruitment of more monocyte/macrophages, etc., etc. The possibility that administration of lipoxygenase inhibitors may slow the progression of atherosclerosis is under active investigation.

CC 010 INFLUENCE OF MYELOPEROXIDASE ON LIPID OXIDATION BY NEUTROPHILS,

Christine C Winterbourn and Anthony J Kettle, School of Medicine, Christchurch Hospital, Christchurch, New Zealand.

Production of O_2^- by neutrophils is accompanied by release of the azurophil granule enzyme myeloperoxidase (MPO), which converts most of the O_2^-, via H_2O_2, to HOCl. Other reactions of the H_2O_2 produced by neutrophils should, therefore, be minor. HOCl should be the major strong oxidant produced by neutrophils and play a key role in oxidative damage by these cells.

The activity of MPO depends on environmental constituents. In the presence of high H_2O_2 concentrations or appropriate reductants HOCl production is diminished, through conversion of Compound I of MPO to inactive Compound II. Activity can be restored by O_2^-, which converts Compound II back to the native enzyme. We have shown inhibition of HOCl production by superoxide dismutase both with the purified enzyme and with stimulated neutrophils. These results suggest that neutrophils may produce O_2^- rather than H_2O_2 directly so as to optimize production of HOCl. It also follows that superoxide dismutase could protect against neutrophil reactions mediated by HOCl.

Likely targets for neutrophil oxidants are membrane lipids. Stimulated neutrophils cause peroxidation of phospholipid liposomes, but only if exogenous iron or ferritin is added. We have found that peroxidation is enhanced by the MPO inhibitor, azide, and the HOCl scavenger, methionine, and is inhibited by exogenous MPO. Hence HOCl produced by MPO inhibits lipid peroxidation. This is not necessarily a protective effect. In fact, HOCl would not be expected to cause peroxidation but to add across double bonds to form chlorohydrins. In support of this we have reacted HOCl with phospholipids and shown a stoichiometric decrease in unsaturation. Hence lipid oxidation may be much more important than peroxidation as a reaction of neutrophils and functional effects of this process warrant investigation.

Molecular Basis of Oxidative Damage by Leukocytes

Role of Leukocyte and Target Surfaces in the Generation of Superoxide Anion

CC 011 INTRAVASCULAR REACTIONS OF SUPEROXIDE AND HYDROGEN PEROXIDE.

Bruce A. Freeman, Department of Anesthesiology and Biochemistry, University of Alabama at Birmingham, Birmingham, AL 35233.

Our recent studies have been directed toward understanding mechanisms of intravascular free radical reactions secondary to exposure to xenobiotics, hyperoxia and anoxia-reoxygenation. The extracellular release of intracellularly generated O_2^- and H_2O_2 occurs and accounts for a significant fraction of intracellularly produced O_2^- and H_2O_2. We have observed a reaction of O_2^- with ·NO (endothelial-derived relaxation factor generated by endothelium, inflammatory cells and some neuronal cells) to yield peroxynitrite anion ($ONOO^-$) which participates in hydroxyl radical-like reactions. Peroxynitrite will react with DMSO and deoxyribose similar to hydroxyl radical and oxidizes of sulfhydryls and initiates membrane lipid peroxidation. Another source of intravascular oxidant stress recently observed is circulating xanthine oxidase, which appears secondary to a number of pathophysiologic states including ischemia-reperfusion phenomena, acute viral infections, respiratory distress syndrome and acute thermal injury. While the source of the circulating xanthine oxidase has yet to be definitively established, its half-life is > 1 hr. This enzyme will bind to vascular endothelium in a heparin-reversible manner, exerting toxic effects at the cell surface. About 4×10^4 to 8×10^4 molecules of xanthine oxidase have been observed to reversibly bind per endothelial cell. These observations emphasize the importance of site-specific delivery of therapeutic scavengers of reactive oxygen species and represent oxidant reactions which can participate prior to or as a consequence of inflammatory responses.

CC 012 ORGANIZATION OF THE LEUKOCYTE PLASMA MEMBRANE COMPONENTS OF SUPEROXIDE PRODUCTION, A. Jesaitis and J. Linner, Department of Chemistry, Montana State University, Bozeman MT 59715 and Cryobiology Research Center, University of Texas, Houston TX 77381

The activation of superoxide production in human neutrophils involves spatial organization of the components of transduction and effector function in the plane of the plasma membrane. Subcellular fractionation of activated and resting neutrophils suggests that receptors for formyl peptides can be controlled by their restriction to a membrane domains enriched in either the cytoskeletal proteins, actin and fodrin or the transducing, G-proteins. A similar lateral heterogeneity is observed with respect to the components of superoxide production, which cosediment with the heavier, cytoskeletal-enriched domain. This fraction contains only a part (<25%) of the total plasma membrane b-type cytochrome, suggesting that not all of it is involved in superoxide production. Morphological evidence of such lateral heterogenity can also be observed as clusters of cytochrome b559 in the phagosomal membranes of cryofixed/molecular distillation dried human neutrophils, frozen while carrying out their phagocytic function on heat killed S. Aureus. We speculate that when such organization is perturbed, as may occur in certain pathological instances, control of activation of neutrophils could be lost, resulting in in appropriate superoxide generation.

CC 013 EFFECTS OF LEUKOCYTE ADHESION ON THE RESPIRATORY BURST. Carl Nathan, Department of Medicine, Cornell University Medical College, New York, NY 10021.

PMN adherent to extracellular matrix proteins react to peptide agonists differently than PMN in suspension. The respiratory burst of adherent PMN in response to tumor necrosis factor-α (TNF), other cytokines, N-formylated peptides, or complement component C5a is delayed in onset by 15-90 min, while suspended PMN respond to soluble stimuli in < 1 min. Thereafter, adherent PMN sustain the respiratory burst 10-100 times longer and produce 10-100 times more O_2 intermediates than PMN in suspension. The cytokine-induced PMN respiratory burst requires CD11/CD18 (β2) integrins. These serve as receptors for some matrix proteins and/or as mediators of responses to the ligation of others. The onset of the TNF-induced respiratory burst depends on the ability of actin to polymerize, in that cytochalasins abort the response if added during but not after the lag period.

The hypothesis was tested that changes in cAMP mediate the joint action of cytokines and integrins. When plated on FBS- or fibrinogen-coated surfaces, PMN responded to TNF with a sustained fall in intracellular cAMP. This did not occur without TNF; in suspended PMN; in PMN treated with anti-CD18 mAb; or in PMN genetically deficient in β2 integrins. A preceeding fall in cAMP appeared essential for TNF to induce a respiratory burst, because drugs that elevate cAMP blocked the burst if added any time before, but not after, its onset. Adenosine analogs and cytochalasins also block the TNF-induced respiratory burst if added before, but not after, its onset. Both also blocked the TNF-induced fall in cAMP.

The effect of cytochalasins prompted an examination the relationship between cAMP and actin reorganization. The same conditions that led to a sustained fall in cAMP led at the same time to cell spreading and the assembly of actin filaments. As with the respiratory burst, cAMP-elevating agents inhibited TNF-induced cell spreading and actin filament assembly if added before, but not after, spreading began.

Thus, occupation of TNF receptors and engagement of CD18 integrins interact synergistically in PMN to promote a fall in cAMP. The fall in cAMP is closely related to cell spreading and actin reorganization. These changes are necessary for TNF to induce a prolonged respiratory burst. These findings demonstrate that integrins can act jointly with cytokines to affect cell shape and function through alterations in the level of a second messenger, cAMP.

References: *J Clin Invest* 80:1550, 1987; *Blood* 73:301, 1989; *J Cell Biol* 109:1341, 1989; *J Cell Biol* In press, November 1990.

CC 014 POTENTIATION OF THE OXIDATIVE BURST IN POLYMORPHONUCLEAR LEUKOCYTES (PMN) BY OCCUPANCY OF LAMININ RECEPTORS, Marilyn C. Pike, Max S. Wicha, Laura Mayo and Laurence A. Boxer, Arthritis Unit, Massachusetts General Hospital, Boston, MA 02114 and Departments of Medicine and Pediatrics, University of Michigan Medical School, Ann Arbor, MI 48109.

Laminin is a high M.W. matrix protein (900,000 daltons) contained within basement membranes of many tissues including blood vessels. It promotes the spreading, adhesion and locomotion of many cell types in vitro, augments the chemotaxis of neutrophils in response to chemoattractants and by itself, stimulates the motility of peritoneal exudate PMN. The limited release of specific granules in PMN induced by chemoattractants or phorbol esters increases the expression of high affinity laminin receptors which are normally present in low numbers on the neutrophil surface. Based on these observations, we determined whether laminin acts in concert with chemoattractants to activate PMN. Laminin (5 to 100 µg/ml) stimulated lysozyme release and superoxide production in response to the chemoattractant, FMLP, by as much as 69%. These results could be explained by changes in cell surface chemoattractant receptor expression in that incubation of normal PMN with laminin (5 to 75 µg/ml) increased the binding of 19 nM FML[3H]P by 35 to 80%. This corresponded to as much as a 2.5-fold increase in the number of chemoattractant receptors/cells which had a lower average affinity. Laminin did not change the number or affinity of FML[3H]P receptors present on organelle-depleted PMN cytoplasts, and the laminin-induced increase in FML[3H]P receptors expressed on PMN from a patient with a specific granule deficiency was only 11 to 21% of that seen in normal PMN.

A nine amino acid peptide (CDPGYIGSR) derived from domain III of the laminin B1 chain which has been found to be one of the principle sites of the molecule mediating cell attachment, migration and receptor binding in other cell types, also increased the expression of chemoattractant receptors in human PMN. This peptide was more potent than intact laminin for producing this effect. These findings suggest that chemoattractants augment the expression of laminin receptors which mediate PMN attachment to basement membranes, followed by laminin-induced increases in the expression of cryptic chemoattractant receptors in intracellular granules, with resultant augmentation of the oxidative burst.

Toward Determination of Tissue Concentrations of Activated Oxygen Species

CC 015 MECHANISM OF INACTIVATION OF GLYCERALDEHYDE-3-PHOSHATE DEHYDROGENASE BY H_2O_2.

Hyslop, P.A.*, Halsey, W.A., Jr., Hinshaw, D.B.+, Schraufstatter, I.U. and Cochrane, C.G., *Lilly Research Laboratories, Indianapolis IN 46285, +University of Michigan, Ann Arbor MI 48105 and Scripps Clinic and Research Foundation, La Jolla, CA 92037. **(1)**. The reaction kinetics of purified porcine muscle GAPDH with H_2O_2 at 37°C, were determined. Complete oxidation of the protein in the presence of $3mol H_2O_2$/mol subunit was achieved by a maximum of $2mol\ H_2O_2$/subunit, with concomitant loss of 2 titratable cysteine thiols/subunit, identified as cys_{149} (active site thiol involved in catalysis) and cys_{153}. (a thiol located within a few Å of cys_{149}, not directly involved in catalysis) Second-order plots of the data at pH 7.8 yielded biphasic reaction kinetics. From qualitative analysis of the active site cysteine (cys_{149}) at various stages of the oxidation utilizing active site thiol-specific reagents, we assigned apparent bimolecular rate constants ($k_2\ M^{-1}min^{-1}$) of $1.0 \cdot 10^3$ and $0.35 \cdot 10^3$ to the oxidation of cys_{149} (k_2') and cys_{153} (k_2'') respectively. At pH 9.0; k_2' increased to $1.8 \cdot 10^3$, while k_2'' decreased to $0.1 \cdot 10^3$. At pH 7.0, both k_2' and $k_2'' = 0.52 \cdot 10^3$. As the pH dependance of thiolate oxidation by H_2O_2 almost certainly reflects the relative nucleophillicity/basicity of the cysteine thiols, these data identify the oxidation of the two cysteines by H_2O_2 as independent processes. **(2)** Incubation of the oxidized protein with DTT, resulted in recovery of enzyme activity when one, but not both cysteines had been oxidized by H_2O_2. **(3)** Incubation of the fully oxidized enzyme in a denaturing buffer (6M GuaHCl, pH 2.3) containing DTT resulted in restoration of enzyme activity following the spontaneous refolding and assembly of protein subunits after removal of the denaturant buffer. **(4)** Chemical analysis of the denatured, fully H_2O_2 oxidized protein yielded one sulfite-reducable group per enzyme subunit, indicating the presence of two sulfurs at the oxidation state of **at least** a disulfide. **(5)** We report, however, that a striking contrast exists between H_2O_2 oxidized GAPDH and GAPDH oxidized to the genuine disulfide at the active site by established methods. The activity of the 'disulfide' GAPDH is initially reactivatable by DTT, however, the enzyme undergoes a first-order conformational change ($k_1 = 1.08 \cdot 10^{-3}\ min^{-1}$@14°C) rendering the enzyme irreversibly inactive. The 'H_2O_2' GAPDH also undergoes a similar conformational change ($k_1' = 0.75 \cdot 10^{-3}\ min^{-1}$) but <u>both</u> states of the enzyme in this case are irreversibly inactive in the presence of DTT **(6)** In conclusion, independent nucleophillic attack on H_2O_2 by cys_{149} and cys_{153} results in heterolytic cleavage of the peroxide oxygens, yielding the corresponding sulfenic acids. We suggest from our data that disproportionation of $cys(SOH)_{149}$ and $cys(SOH)_{153}$ residues yields an interchain thiolsulfinate (RS(O)SR'), which would be expected to be reducable in the denatured enzyme by both sulfite and DTT. We hypothesize that the inability of DTT to reduce the putative thiolsulfinate in native GAPDH generated by the H_2O_2 double thiol oxidation (prior to the irreversible conformational change) may arise perhaps from the inaccessibility of the sulfide sulfur atom of the thiolsulfinate at the active site of the enzyme.

CC 016 **EFFECTS OF ACTIVATED OXYGEN SPECIES ON MEMBRANES AS DETECTED BY FLUORESCENCE AND BIOCHEMICAL TECHNIQUES,** **Alex Sevanian and MaryLou Wratten, University of Southern California, Institute for Toxicology, Los Angeles, CA 90033.**

The peroxidation of membrane lipids is associated with numerous pathological processes wherein activated oxygen species may be generated. A well documented source of such free radical species involves the activation and subsequent respiratory burst of inflammatory cells. Initiation of lipid peroxidation by activated oxygen species is kinetically sluggish and must overcome a range of primary antioxidant defenses before lipid peroxidation is promoted. Once initiated, propagation of lipid peroxidation is kinetically facile with rate constants five orders of magnitude greater than initiation reactions. Cells are endowed with highly effective antioxidant defenses to reduce or quench peroxyl radical reactions among polyunsaturated fatty acids (PUFA). Despite these defenses, there is ample evidence for the existence of lipid hydroperoxides in tissues indicating that the abatement processes are not perfect. One may posit that the high turnover of unsaturated phospholipids (PL) is accounted for, in part, by oxidative reactions. Such reactions can account for the "peroxide tone" in tissues and hence be functionally related to events that trigger release of specific fatty acids such as arachidonic acid (20:4). Several investigators have reported that the activation of phospholipase A2 (PLA2) is quite responsive to the peroxidation of membrane PL. PLA2 preferrentially hydrolyzes peroxidized PL's but such hydrolysis is accompanied by the selective release of intact PUFA, particularly 20:4. The sensitivity of PLA2 to disturbances in membrane structure was confirmed through analyses of model membranes using steady state and multi-frequency cross-correlation fluorometry techniques. Based on the photophysical behavior of DPH in membrane bilayers, the activation PLA2 was found to correlate with formation of structurally heterogeneous domains following lipid peroxidation, and PL-hydroperoxides appear to impart these structural transitions most effectively. Applying the techniques of angle-resolved fluorescence depolarization and electron spin resonance to probe molecules in oriented planar membranes we found that oxidized PL's markedly decrease the molecular orientational order and diffusional dynamics of the membrane. Heterogeneous domains are envisioned as structural imperfections which are susceptible to PLA2 attack. The propensity of membranes to display a homeoviscous response may account for the confluence of PUFA-PLs into saturated or oxidized PL domains, hence drawing 20:4 into the sites where PLA2 activity is high.

CC 017 MEASUREMENT OF LIPID PEROXIDATION IN BIOLOGICAL SYSTEMS

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A role for lipid peroxidation has been suggested in many human diseases, including atherosclerosis, arthritis, and cancer. A major problem remaining to prove this hypothesis is the direct detection of lipid peroxidation products in biological tissues. The most commonly used detection techniques, such as the thiobarbituric acid assay for detection of malondialdehyde and the ultraviolet absorption assay for conjugated dienes, work well in model systems, but are not reliable for measuring lipid peroxides in biological samples.

During recent years, sensitive and specific methods for detection of lipid peroxidation products in animal and human tissues have been developed. These include high performance liquid chromatography with chemiluminescense detection for hydroperoxides (1), and gas chromatography-mass spectrometry for lipid peroxides and aldehydic products of lipid peroxidation (2-4). An overview of various methods available and their limitations will be discussed.

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CC 018 ELECTRON PARAMAGNETIC RESONANCE MEASUREMENT OF LEUKOCYTE FREE RADICAL GENERATION IN ISOLATED CELLS AND WHOLE TISSUES

Jay L. Zweier, Suresh Shandelya, Periannan Kuppusamy, Myron L. Weisfeldt, Division of Cardiology, Johns Hopkins Medical Institutions, Baltimore, MD 21224

There is evidence that leukocytes are important mediators of postischemic injury. Electron Paramagnetic Resonance, EPR, spectroscopy in the presence of the spin trap 5,5'-dimethyl-1-pyrroline-N-oxide, DMPO, can be applied to measure and characterize free radical generation in isolated leukocytes and from leukocytes within whole tissues. Polymorphonuclear leukocytes, PMNs, were isolated from whole blood via dextran gradient centrifugation. When these cells were activated by the phorbol ester, TPA, prominent radical signals were observed corresponding to DMPO-OOH and DMPO-OH radical adducts. In a nonperfused cellular suspension the radical generation continued for only 10-15 minutes; however, when the cells were perfused with buffer containing oxygen and glucose, the radical generation persisted for over 8 hours. EPR detection of radical generation was found to be quite sensitive with prominent signals, S/N > 50, observed with only 10^5 cells using a conventional cavity resonator and as few as 10^3 cells using a dielectric or loop gap resonator. Experiments were performed to distinguish between superoxide and hydroxyl radical generation by the cells. A perfused heart model was developed in which the effects of PMNs on postischemic free radical generation and contractile function could be measured. Isolated rat hearts were subjected to 20 min global ischemia and then reperfused with perfusate alone, or with perfusate containing PMNs, plasma, PMNs + plasma, or PMNs + inactivated plasma (preheated to 56°C for 30 min to denature complement) (N=10 in each group). Left ventricular developed pressure (LVDP) was measured during 1 min preischemic control infusion and on reflow after 20 min of ischemia. During control infusion no significant alterations were observed. Plasma or PMNs alone did not alter postischemic LVDP while plasma and PMNs together caused marked injury. LVDP after 45 min reflow with PMN + plasma was 33±7% compared to 64±8% with plasma, 68±8% with PMN, and 75±4% with perfusate alone ($p<.01$). With plasma which was preheated inorder to inactivate complement this injury was not seen, LVDP 71±9%. It was observed that upon reperfusion in the absence of PMNs and plasma that there is only an early burst of radical generation peaking at 10-20 seconds of reflow and after 5 min no further radical generation is observed. When hearts were reperfused in the presence of PMNs and plasma radical generation persisted for more than 15 min . Thus, complement mediated leukocyte activation with subsequent free radical generation appears to be an important mechanism of postischemic injury in the heart.

Molecular Basis of Oxidative Damage by Leukocytes

Banquet Address

CC 019 SUPEROXIDE PRODUCTION AND HUMAN DISEASE, Joe M. McCord, Webb-Waring Lung Institute, University of Colorado Health Sciences Center, Denver, CO 80262

The reduction of molecular oxygen by healthy cells is a finely-tuned, tightly controlled process. When cells are sick or injured a common feature seems to be some modification of this process, resulting in the formation of increased amounts of superoxide radical and hydrogen peroxide. Sometimes the modification appears to be intentional, as when activated phagocytes turn on their NADPH oxidase to combat invading microbes. Sometimes the modification appears to be misdirected or misguided, as when activated phagocytes produce oxidants in the absence of infection (as in autoimmune disease). Sometimes the modification appears to be purely the result of injury, as when the mitochondria leak electrons to molecular oxygen from Site I or Site II in the electron transport chain, or when xanthine dehydrogenase is proteolytically or oxidatively transformed into xanthine oxidase. Hence, a few recurring basic mechanisms may be responsible for the free radical-mediated components of a broad spectrum of disease states. Furthermore, as a disease state progresses, there are several examples of how one free radical-producing mechanism may lead to another: 1) ischemic injury to the heart rapidly leads to infiltration by activated phagocytes; 2) inflammatory injury to the knee leads to increased synovial volume, which produces cyclical ischemia and reperfusion as the knee is alternately stood upon and rested; 3) superoxide releases iron from ferritin stores, which catalyzes the production of a yet more potent oxidizing species.

Pathology of Unregulated Oxygen Radical Production

CC 020 THE CHEMISTRY AND BIOLOGY OF SUPEROXIDE: CENTRAL CONCEPTS AND RESIDUAL PROBLEMS, Irwin Fridovich, Department of Biochemistry, Duke University Medical Center, Durham, NC 27710. O_2^- is generated by spontaneous and by enzyme-catalyzed oxidations. It can exert deleterious effects directly by initiating the oxidation of sulfite, catecholamines, tetrahydropterins, leukoflavins and enediols; and by inactivating [4Fe-4S]-containing dihydroxy acid dehydratases such as those acting on 6-phosphogluconate, dihydroxyisovalerate, fumarate and citrate. It seems likely that additional targets, susceptible to direct attack by O_2^-, exist within cells. Its oxidizing capability is greatly augmented by association with cationic centers including H^+, Mn(II), and $V_{(V)}$. O_2^- can reduce Fe(III) to Fe(II), which then reacts with H_2O_2, generating potent oxidants such as FeO^{++} or $HO\cdot$. This occurs both in vitro and in vivo. In the latter case, binding of the catalytic metal to membranes or DNA facilitates selective damage of these targets. Defensive enzymes such as superoxide dismutases, catalases and peroxidases lower steady-state levels of O_2^- and H_2O_2 and thus prevent much damage; while other enzymes minimize the consequences of such damage. The latter category includes alkyl hydroperoxide reductases, methionine sulfoxide reductases, proteinases specific for oxidatively modified proteins and enzyme systems which repair oxidized DNA. Antioxidants such as α-tocopherol which abort free radical chain reactions are also important.

Many interesting and potentially important problems remain. These include: How is the biosynthesis of the defensive enzymes regulated in both prokaryotes and eukaryotes? How does the pseudorevertant of the sodA sodB strain of E. coli manage without SODs? How does tumor necrosis factor (TNF) induce MnSOD and why does MnSOD protect against TNF? What is the source of O_2^- during reperfusion injury and how does exogenous SOD protect? Do activated phagocytic cells contribute to the lethality of ionizing radiation? What contribution do O_2^- and derived radicals make to senescence? And, why do a few species of bacteria contain a Cu,ZnSOD? It seems safe to predict that the excitement in the oxy-radical field will continue as enthusiastic, talented investigators explore these and other questions.

CC 021 LEUKOCYTE OXYGEN PRODUCTS AND TISSUE DAMAGE, Peter A. Ward, Dept. of Pathology, The University of Michigan Medical School, Ann Arbor, MI 48109

Injury occurring as a result of recruitment of leukocytes into inflammatory sites and the concomitant local activation of tissue macrophages results in the generation of oxygen radicals that are toxic to cells and tissues. Using the model of immune complex induced alveolitis, a complex array of steps appears to take place. Formation of immune complex deposits results both in complement activation as well as activation of lung macrophages which release TNFα and IL-1, as shown by the presence in bronchoalveolar lavage fluids of these cytokines. TNFα is essential for the full development of the lung inflammatory reaction, since its blocking by antibody abrogates recruitment of neutrophils This finding suggests that elaboration of TNFα results an up-regulation of leukocyte adhesion molecules on pulmonary vascular endothelial cells, following which C5a can exert its chemotactic function. Injury of lung cells by products of neutrophils (and macrophages) is probably the result of both generation of toxic oxygen products as well as release of proteases. Endothelial cell injury of lung microvasculature is catalase-sensitive and blocked by iron chelators and scavengers of $HO^{\bullet}$. To what extent generation of $ON^{\bullet}$ may also participate in the events leading to injury is unknown. In vitro injury of endothelial cells by H_2O_2 from neutrophils (or by reagent H_2O_2) is related not only to interaction of H_2O_2 with the endothelial cells but also to intracellular generation of O_2^- by xanthine oxidase of endothelial cells. The precise role of O_2^- in this reaction is not known but could relate to reduction of Fe^{3+} and its release, resulting in electron transfer to H_2O_2 with generation of $HO^{\bullet}$. These data emphasize the complex interactive steps in the inflammatory system, the result of which is tissue and organ injury.

CC 022 THE INTERPLAY OF OXIDANTS AND PROTEINASES IN NEUTROPHIL-MEDIATED TISSUE DAMAGE, Stephen J. Weiss, Department of Internal Medicine, Division of Hematology and Oncology, The University of Michigan Medical School, Ann Arbor, MI 48109

Neutrophils are armed with a complex array of oxygen dependent and independent weapon systems. Although this offensive arsenal has evolved in specific response to the threat of microbial invasion, host tissues are exposed to these destructive systems in inflammatory disease states. The most reactive oxygen metabolites generated by human neutrophils are a group of halogenated oxidants which include hypochlorous acid and N-chloramines. Although these species can exert cytocidal effects under in vitro conditions, their ability to damage cellular targets under physiological conditions is blunted because of both the relative non-selectivity of reactive oxidants and the multiplicity of competing targets found in biological fluids. Instead, chlorinated oxidants indirectly mediate injurious effects by regulating the activities of tissue-destructive proteinases including elastase, cathepsin G, collagenase and gelatinase. This is accomplished via a process wherein chlorinated oxidants mediate the inactivation of critical plasma proteinase inhibitors including $\alpha_1$proteinase inhibitor, $\alpha_1$antichymotrypsin, and $\alpha_2$macroglobulin while simultaneously activating the latent metalloproteinases, neutrophil collagenase and gelatinase. In this manner, the oxidative orchestration of proteolytic activities allows neutrophils to transform short-lived, non-specific effects of chlorinated oxidants into long-acting and specific proteinase-catalyzed damage.

Molecular Basis of Oxidative Damage by Leukocytes

Strategies for The Control of Oxygen-Dependent Pathology

CC 023 **CLINICAL APPLICATION OF FREE RADICAL ABLATION FOR THE PREVENTION OF REPERFUSION INJURY.**

Gregory B. Bulkley, MD, FACS
The Johns Hopkins University School of Medicine.

Toxic metabolites of oxygen initially generated from activated xanthine oxidase at reperfusion have been found to trigger a major component of post-ischemic tissue damage in a wide variety of organs. The apparent ubiquity of this mechanism appears to be based upon the xanthine oxidase in the microvascular endothelium, the superoxide product of which secondarily activates circulating neutrophils. However, the quantitative importance of this mechanism, and therefore the clinical effectiveness of free radical ablation, is directly proportional to that component of the injury which is mediated by the above reperfusion mechanism, as compared to that proportion which is sustained during the time of the ischemia itself. That proportion is quite variable among different organs and after varying degrees and periods of ischemia. Studies in a porcine model of human cadaveric kidney transplantation have quantified that proportion under varying periods of complete warm and cold renal ischemia, in realistic simulations of the clinical transplant situation. Subsequently, two prospective, randomized, doubled-blind, placebo-controlled, paired, clinical trials of free radical ablation for the treatment of post-ischemic renal injury following human cadaveric transplantation with superoxide dismutase (SOD) in 100 patients, and with xanthine oxidase inhibition with allopurinol in 400 patients, respectively have revealed clinically substantial and statistically significant improvement in early post-transplant renal function, as well as a remarkable reduction in mortality. Moreover, these clinical results are quite consistent with the quantitative model of proportional injury developed in the earlier animal studies. These studies not only constitute the first clear demonstration of the clinical effectiveness of free radical ablation for the treatment of post-ischemic reperfusion injury, they also provide a model of study design for similar investigations in the future.

CC 024 INHIBITION OF SUPEROXIDE GENERATION BY CYTOCHROME B-DERIVED PEPTIDES.

H.L. Malech, T.L. Leto, M.E. Kleinberg, C. Kwong and D. Rotrosen, Bacterial Diseases Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892

Phagocyte superoxide generation requires assembly of a membrane NADPH oxidase complex from components initially present in cytoplasm and membrane. Known cytoplasmic oxidase components include p47-phox (p47), p67-phox (p67) and at least one additional component, while membrane components include the two subunits of cytochrome b558 (gp91-phox {gp91} and p22-phox) and possibly other elements. Using both a cell-free assay of oxidase activation or the more physiologic electropermeabilized cell system of Grinstein, we show that a 7 amino acid peptide, RGVHFIF, derived from gp91 carboxyterminus is a potent inhibitor of oxidase activation. The peptide must have access to the cytoplasm and must be present prior to activation to be inhibitory. We used chronic granulomatous disease (CGD) neutrophil cytosols deficient in either p47 or p67 for sequential addition to cell-free assays to show that oxidase activation can be separated functionally into a slow early phase requiring p47 and inhibitable by RGVHFIF, and a more rapid late phase reaction involving p67 and not inhibitable by this peptide. In studies of intact cells we examined p47 phosphorylation during phorbol ester activation of oxidase by 2-dimensional (NEPHGE-SDS) electrophoresis autoradiography examination of immunoprecipitated [32-P] phospho-p47 (pp47). In resting cells no pp47 was detected and by western blot p47 was only present in cytoplasm. At 30 seconds 8 discrete isoelectric forms of cytoplasmic pp47 were seen with hyperphosphorylated forms predominating. At the same time only hyperphosphorylated forms of p47 were associated with cell membrane including a single more acidic phosphoprotein than seen in cytosol. In neutrophils from CGD patients whose cells lack cytochrome b, activation was associated with only 6 pp47 isoelectric forms found exclusively in cytoplasm and hyperphosphorylated forms did not predominate. In related studies of normal cells pp47 could be immunoprecipitated from activated cell membranes by anti-cytochrome b558 antibodies, suggesting a physical association of p47 and cytochrome into the same molecular complex. In other studies we cloned cDNA encoding p47 and p67, and used the baculovirus vector to obtain highly active, purified recombinant proteins (rp47 and rp67). In the cell-free assay exposure of membranes to rp47 with arachidonate activator shortened the lag time to superoxide production following constitution of complete assay mix when compared to an initial incubation of either rp67 or normal cytosol with membranes. Also, cell-free activation of oxidase required phagocyte membranes, rp47, rp67 and an additional cytoplasmic component which appears to correspond both to our previously described highly anionic NCF-3 and the 37% ammonium sulfate supernate Sigma 1 fraction of Pick and colleagues. From these data a model of early events of oxidase activation indicate an initial multistep phosphorylation of cytoplasmic p47 where hyperphosphorylation requires the presence of gp91. Only hyperphosphorylated p47 translocates to the membrane, possibly associating directly with RGVHFIF on gp91 where additional phosphorylation occurs. Following these initial p47 reactions, p67 interacts to form active oxidase. The requirement of phosphorylation of p47 in these events and the stage of participation of the third required cytoplasmic oxidase component remains to be determined.

CC 025 XANTHINE OXIDASE INDUCED OXIDANT MEDIATED VASCULAR INJURY: CYTOKINE INITIATED PROTECTIVE MECHANISMS AGAINST OXIDANT DAMAGE,

John E. Repine, Webb-Waring Lung Institute, U. of Colorado, Denver, Colorado, 80262

We have been interested in the following hypotheses regarding the role of oxidant-antioxidant imbalance in the development of vascular injury. Specifically, we propose that following ischemia/ reperfusion (hypoxia/reoxygenation) that :

a. xanthine oxidase (XO) derived O_2 metabolites contribute directly to endothelial cell injury and indirectly cause injury by increasing neutrophil adherence and
b. that cytokine pretreatment increases endothelial cell antioxidants and decreases injury.

A summary of our work includes the following observations:

Prolonged (48h) hypoxic (5-10 torr) exposure increased total XO + xanthine dehydrogenase (XDH) enzyme activity 10 times compared to normoxic exposure without changing XO/XDH ratios. Second, EC exposed to hypoxia for 48 h and then to normoxia released more ($p < 0.05$) O_2^- than EC in normoxia (70±16 vs 3±3 pmol/min/g prot.). Release of O_2^- by EC was decreased by coaddition of allopurinol (XO inhibitor) or DIDS (anion channel blocker). Hypoxic preincubation also increased ($p < 0.05$) ^{51}Cr leak (38±2 vs 18±3%), neutrophil adherence (18±1 vs 6±1%), and albumin transit (36±4 vs 3±1%) across EC monolayers. Increases were attenuated ($p < 0.05$) by treatment with allopurinol and/or superoxide dismutase (SOD).

IL-1 pretreatment increased myocardial G6PD activity and decreased susceptibility of isolated rat hearts to ischemia-reperfusion damage (Ventricular Developed Pressure - 56±2 vs 28±4 mmHg). The protective effects of IL-1 pretreatment were associated with increased numbers of myocardial neutrophils (18 times) and both G6PD increases and protection against ischemia-reperfusion were abolished by neutrophil depleting (vinblastine) rats prior to IL-1 pretreatment.

We conclude that XO can increase after hypoxia and contribute to EC injury and inflammation. Cytokine pretreatment confers protection against a subsequent ischemia/reperfusion dependent oxidant injury by a neutrophil dependent mechanism. The latter is consistent with the possibility that a prior limited oxidant insult can decrease injury caused by a subsequent oxidant insult.

Poster Session I

CC 100 OXIDATION OF LDL BY STIMULATED NEUTROPHILS AND FERRITIN: EFFECT OF ANTIOXIDANTS. Dulcineia S.P. Abdalla, Ana Campa and Hugo P. Monteiro*, Department of Clinical and Toxicological Analysis and *Homocentro Foundation, University of Sao Paulo, S.P., Brasil CEP 05508. Stimulated neutrophils undergo a respiratory burst with production of oxyradicals ($O_2^{\cdot-}$, H_2O_2 and OH). $O_2^{\cdot-}$ is able to release iron from ferritin and these iron ions may interact further with $O_2^{\cdot-}$/H_2O_2 resulting oxidant species. e.g. OH and/or iron-oxygen complexes. Here we describe the oxidation of low density lipoprotein (LDL) by neutrophils stimulated with phorbol miristate acetate (PMA) and the peptide formyl-methly-leucyl-phenylalanine (FMLP) in the presence or absence of ferritin. In the presence of ferritin LDL oxidation by PMA or FMLP stimulated neutrophils was 60% higher, after 17 hours of incubation. Ascorbate (100uM), SOD (10ug/ml) and uric acid (70ug/ml) showed inhibitory effects of 10%, 70% and 50% in LDL oxidation, respectively. Ceruloplasmin (400 ug/ml) potentiated LDL oxidation both alone and in the presence of stimulated neutrophils plus ferritin. Catalase (30 ug/ml) and methionine (1mM) increased LDL oxidation in the presence of stimulated neutrophils plus ferritin. These data suggest that in the LDL oxidation induced by stimulated neutrophils plus ferritin the plasma antioxidant action should be due to uric acid and extracellular SOD.

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CC 101 FREE RADICALS AS MEDIATORS OF THE TUMORICIDAL ACTION OF CIS-UNSATURATED FATTY ACIDS. U.N.Das, Department of Medicine, The Nizam's Institute of Medical Sciences, Punjagutta, Hyderabad-500 482, India.

Tumor cells are known to have low rate of lipid peroxidation, be deficient in poly-unsaturated fatty acids, especially arachidonic acid (AA), and are extremely sensitive to the toxic action of free radicals. Since, some prostaglandins have anti-mitotic action both in normal and tumor cells, I studied the effect of prostaglandin-precursors on the rate of proliferation of normal and tumor cells in vitro.

Of all the fatty acids tested, gamma-linolenic acid (GLA, 18:3,n-6), arachidonic acid (AA, 20:4, n-6), and eicosapentaenoic acid (EPA, 20:5, n-3) were found to be toxic to human breast cancer cells (ZR-75-1) but not to human fibroblasts (41-SK) in vitro. These fatty acids initially induced decrease in the rate of proliferation of the cells followed by cytotoxic action when the tumor cells were grown in the presence of GLA, AA, and EPA for more than 72 hours. GLA, AA, and EPA were also toxic to normal human fibroblasts but only at 4 to 5 times higher concentrations of fatty acids compared to those that are necessary to kill tumor cells. In co-culture experiments where in both normal and tumor cells are grown together, GLA, AA, and EPA have shown selective tumoricidal action leaving normal cells untouched. Of these three fatty acids, GLA was found to be more selective in its tumoricidal action compared to AA and EPA. The tumoricidal action of GLA, AA, and EPA could be completely blocked by anti-oxidants and enhanced by copper and iron salts suggesting a role for free radicals and lipid peroxidation process.

CC 102 PRESENCE OF PEROXIDIZED LDL (pLDL) IN PLASMA OF CANCER PATIENTS AND ITS PREFERENTIAL CYTOTOXICITY TOWARD MALIGNANT CELLS, Eric T. Fossel[1], Kathleen K.S. Hui[1], Joel G. Fletcher[1], and Jan McDonagh[2], Departments of Radiology[1] and Pathology[2], Beth Israel Hospital and Harvard Medical School, Boston, MA 02215

Using C-13 NMR of plasma, we have found evidence for substantial peroxidation of plasma lipoprotein lipids. The C-13 NMR spectra show a reduction in the ratio of polyunsaturated to monounsaturated fatty acids. We have produced a similar change through treatment of normal control plasma with hydrogen peroxide and peroxidase. We have also induced these same changes in purified LDL by treatment with hydrogen peroxide and peroxidase, or irridation of LDL in the presence of methoxypsoralin by UV light type A, or by addition of micromolar copper ion in the absence of EDTA. The C-13 NMR spectrum of pLDL is essentially the same regardless of how peroxidation is accomplished.

We have demonstrated that pLDL produced by all of these methods are preferentially cytotoxic to malignant cells by treating malignant and normal cells of the same type with pLDL at the same concentration and cell density. For example, we treated normal lymphocytes and HUT-78 cells (a cell line derived from cutaneous T-cell lymphoma) with pLDL (0.14 mg protein/ml) produced by methoxypsoralin mediated photo-oxidation. Following 17 hours of incubation, 94% of normal lymphocytes and 24% of HUT-78 cells were viable by MTT assay. This result is typical of 7 cell pairs studied. We are currently testing the hypothesis that the preferential cytotoxicity occurs because of increased numbers of LDL receptors in malignant cells.

CC 103 EFFECT OF SPIN TRAPS ON THE OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEIN. B. Kalyanaraman[a], Joy Joseph[a], and Sampath Parthasarathy[b], [a]Department of Radiology, Medical College of Wisconsin, Milwaukee, WI 53226 and [b]Department Medicine, University of California at San Diego, La Jolla, CA 92093.

Results from studies using antioxidants have shown a definite involvement of free radical during the conversion of the "native" low density lipoprotein (LDL) to the more atherogenic "oxidatively-modified" LDL[1]. However, there exists very little direct evidence for the production of free radical during LDL oxidation. Recently, using electron spin resonance (ESR) technique, we have shown that one of the primary free radical events occurring during chemical and enzymatic oxidation of LDL is formation of the α-tocopheroxyl radical associated with LDL. The spin trap, α-phenyl-*t*-butylnitrone (PBN) inhibits formation of the α-tocopheroxyl radical[2]. Spin trapping data also suggest that the PBN adduct was derived from trapping of the LDL-lipid-derived radical. If free radicals are involved in the oxidative modification of LDL, then trapping them with spin traps should, in theory, decrease LDL modification and the subsequent degradation by macrophages. In accord, PBN inhibits macrophage degradation of oxidized LDL in a dose-dependent manner. PBN also deceased thiobarbituric acid-reactive substance (TBARS) formation as well as the electrophoretic mobility of endothelial cell- and Cu^{2+}-induced modification of LDL. Addition of PBN at the end of the incubation period did not affect LDL modification. Preliminary experiments have also shown that an analogous spin trap, α-pyridyl-N-t-butylnitrone (POBN) does not inhibit macrophage-mediated degradation of LDL. The corresponding ESR data have shown that POBN traps an alkyl-type radical. We conclude that spin traps capable of trapping LDL-lipid-derived radicals or their precursor radicals can potentially inhibit the chemical and biological modification of LDL.

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[2] Kalyanaraman, B., *et al.* (1990) *Biochim. Biophys. Acta* **1035**, 286-292.

CC 104 ELECTRON MICROSCOPIC IMMUNOCYTOCHEMICAL CO-LOCALIZATION OF PEROXIDE GENERATING COMPONENTS AND TARGETS IN RESTING AND STIMULATED HUMAN NEUTROPHILS, J. Linner*, D. Harrison*, E.Buscher*, D. Siemsen, E. Dratz, E. Van Kuijk, M. Quinn, and A. Jesaitis, *Cryobiological Research Center of the University of Texas, Houston, Texas 77381 and Department of Chemistry, Montana State University, Bozeman, MT 59715.

Human neutrophils produce superoxide anion when stimulated by a variety of agents, including phorbol myristate acetate (PMA) and heat-killed S aureus. Although a number of components of the superoxide generating system have been identified, their spatial relationships in the membrane and their proximity to microbial or injury targets have not been identified. To analyze these relationships, specific rabbit antibodies were produced that recognized human neutrophil cytochrome b, the putative terminal component of the superoxide generating system; rap1, a GTP-binding protein associated with the cytochrome; and protein adducts of 4-hydroxynonenal (4-HNE), a marker of lipid peroxidation. The antisera were then used to label these components in cryofixed/molecular distillation dried thin sections of resting and stimulated human neutrophils. We found that the rap1 protein proportionally colocalized with cytochrome b in the specific granules and on the plasma membrane in unstimulated cells. This was supported by subcellular fractionation studies which also showed that the rap1 protein was translocated to the plasma membrane in PMA stimulated cells. Antisera produced against Keyhole Limpet Hemocyanin (KLH) adducts of 4-HNE specifically recognized 4-HNE adducts of bovine serum albumin. These 4-HNE antibodies also specifically labelled as yet undefined species that colocalize with cytochrome b in the phagosome of phagocytosing human neutrophils. Additional studies are in progress to further identify local sites of activity of superoxide generation in neutrophils and their adherent targets.

CC 105 PEROXYNITRITE-INDUCED MEMBRANE LIPID PEROXIDATION, Rafael Radi, Joseph S. Beckman, Kenneth Bush, and Bruce A. Freeman, Department of Anesthesiology, University of Alabama at Birmingham, Birmingham, AL 35233

Endothelial cells, macrophages, neutrophils and neuronal cells can generate $O_2^{\cdot-}$ and ·NO (endothelial-derived relaxation factor) which can lead to the production of peroxynitrite anion ($ONOO^-$) in vivo. We have previously shown that peroxynitrite induced oxidation of protein and non-protein sulfhydryls and yielded products from deoxyribose and dimethylsulfoxide indicative of ·OH reaction. Herein we report that peroxynitrite causes membrane lipid peroxidation. Peroxynitrite addition to soybean PC liposomes resulted in TBARS and conjugated diene formation, as well as oxygen consumption. Lipid peroxidation yields were higher at acidic and neutral pH with no significant lipid peroxidation occurring at pH 9.5. Addition of Fe^{+2} or Fe^{+3} did not enhance lipid peroxide formation. Diethylentetraminepentacetic acid (DTPA) decreased conjugated diene formation by 30-60% while desferrioxamine provided a stronger and concentration-dependent effect, completely abolishing lipid peroxidation at 200 μM. In view of these and previous results we postulate that peroxynitrite-induced lipid peroxidation depends on formation of hydroxyl radical-like species coming from the spontaneous decomposition of the conjugate acid of peroxynitrite anion, peroxynitrous acid ($ONOOH$, $t_{\frac{1}{2}}$ = 1s at 37°C, pH 7.4). Iron did not play an essential role in initiating or propagating lipid peroxidation, since DTPA was only partially inhibitory and the effect of desferrioxamine was due to direct reaction with peroxynitrous acid, in addition to iron chelation. These studies suggest a new mechanism for $O_2^{\cdot-}$ and/or ·NO-mediated cytotoxicity and emphasizes the strong oxidant properties of peroxynitrite.

CC 106 PHAGOCYTES, GONOCOCCI, AND OXIDANT STRESS, Myron S. Cohen and Daniel Hassett, Departments of Medicine, Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27599

N. gonorrhoeae is an obligate pathogen of man which causes an intense neutrophilic inflammatory response. The organism can be killed in vitro by relatively low concentrations of H_2O_2 or O_2^- independent bactericidal mechanisms. We have studied gonococcal responses to redox stress at the level of antioxidant defenses, DNA repair processes, and as part of a dynamic competition for molecular O_2. First, we examined the ability of a variety of DNA repair genes to hybridize with gonococcal DNA under different stringencies. uvrA and B, oxyR and recA demonstrated homology. Using Northern blot analysis constituitive mRNA was observed for these genes, and response to H_2O_2 is being studied. Among DNA repair genes, the only gonococcal mutant available is recA. The recA system affords E. coli some resistance to H_2O_2. Although recA gonococci demonstrate increased UV sensitivity to H_2O_2, they are no more sensitive to the latter oxidant than wild-type organisms. Gonococci generate no SOD, but express catalase in concentration 100 times that of E. coli. However, gonococci are more sensitive to H_2O_2 than E. coli. We have shown that gonococcal use of L(+)-lactate, through the action of a membrane bound L(+)-lactate dehydrogenase, promotes O_2 consumption so as to prevent PMN formation of reactive O_2 species. Gonococcal LDH (lct) mutants cannot compete with PMNs and allow formation of reactive O_2 species. We predict that isogenic lct mutants of N. gonorrhoeae will be affectively killed by PMN and O_2 dependent metabolism and prove incapable of human disease.

CC 107 INCREASED NEUTROPHIL RADICAL PRODUCTION, INCREASED LIPID PEROXIDATION AND INCREASED CONSUMPTION OF ANTIOXIDANTS IN HIV-INFECTED PATIENTS, Connie Jarstrand, Börje Åkerlund and Björn Lindeke, Department of Microbiology, Huddinge Hospital, Roslagstull Hospital, ACO AB Stockholm, Sweden

We have found an increased oxidative metabolism of unstimulated neutrophils with an increased production of oxygen free radicals from these cells (Scand J Infect Dis 86). Further, we found increased plasma malondialdehyde levels (MDA) indicating enhanced lipidperoxidation (Scand J Infect Dis 88) caused by the radicals in HIV patients.

The plasma cysteine level in 47 HIV positive patients and in 16 controls have been measured. In the same patients the oxygen radical production of the neutrophils was estimated simultaneously using the nitroblue tetrazolium (NBT) test. The reduction of NBT to dark blue formazan by the superoxide anion formed by the neutrophils, was measured quantitatively and expressed as optical densities (OD). In the patients the means $\pm$ SD of the amount of cystein per ml plasma was 7.55 $\pm$ 1.84 ng and the NBT reduction 0.66 $\pm$ 0.32 OD whereas in the controls the corresponding values were 9.36 $\pm$ 1.46 ng and 0.35 $\pm$ 0.20 OD, giving the p-values <0.01 and <0.001 respectively.

In conclusion, the increased radical production and lipid peroxidation noted in the HIV positive patients might contribute to the tissue damage, cancer development and premature ageing (Dorian Grey Syndrome) seen in AIDS patients. Cystein has an anti-oxidant role and is likely to be consumed in this disease.

CC 108 LOSS OF DNA-MEMBRANE INTERACTIONS AND CESSATION OF DNA SYNTHESIS IN MYELOPEROXIDASE-TREATED *ESCHERICHIA COLI*, Henry Rosen, Jill Orman, Robert M. Rakita, Bryce R. Michel, Donald R. VanDevanter, Department of Medicine, University of Washington, and Tumor Institute, Swedish Hospital Medical Center, Seattle, WA 98195

Microbicidal activity by a neutrophil-derived antimicrobial system, consisting of myeloperoxidase (MPO), enzymatically generated H_2O_2, and Cl^-, is accompanied by prompt cessation of DNA synthesis in *E. coli*, as determined by markedly reduced incorporation of [^{3}H]-thymidine into trichloracetic acid precipitable material. Simultaneously and proportionately, the MPO system mediates a decline in the ability of *E. coli* membranes to bind hemimethylated DNA sequences containing the *E. coli* chromosomal origin of replication (ori C). Binding of ori C to the *E. coli* membrane is an essential element of orderly chromosomal DNA replication. Comparable early changes in DNA synthesis and DNA-membrane interactions were not observed with alternative microbicidal oxidants (acetaldehyde + xanthine oxidase + Fe/EDTA), or with antibiotic-mediated microbicidal systems. It is proposed that oxidants generated by the MPO system modify the *E. coli* membrane in such a fashion that ori C binding is markedly impaired. As a consequence chromosomal DNA replication is impaired and organisms can no longer replicate.

CC 109 EXPRESSION OF SOD DURING ENDOTHELIAL CELL STRESS.
N.V. Ketis, Michelle Mehra and Julia Jones. Department of Anatomy, Queen's University, Kingston, Ontario, CANADA, K7L 3N6.

Intracellular antioxidant enzyme activity was determined for endothelial cells in culture exposed to anoxia (95% N_2: 5% CO_2) (± serum) for up to 24 hours, ethanol (2 hours, 4%) and hyperthermia (2 hours, 42°C). Superoxide dismutase (SOD) activity was measured in cell lysates of rat aortic endothelial cells. The amount of intracellular SOD increased during anoxia (15-30%) and ethanol (18-20%) treatment. The corresponding non-denaturing polyacrylamide gels demonstrated that all four achromatic zones in the cell lysates appeared to be due to cuprozinc SOD. Marked increase in SOD activity was noted during anoxia and ethanol treatments. The data obtained by using colorimetric assay are supported by those from non-denaturing gel system. Polyacrylamide gels stained with silver nitrate bearing sonicated cell lysates revealed that the amount of at least 4-5 polypeptides is modulated during anoxia, hyperthermia and ethanol treatment when normalized to the level of corresponding polypeptides in "unstressed" cells. Cell viability was assessed by trypan blue exclusion and ^{51}Cr-release. No effect of anoxia, hyperthermia and ethanol was observed as cell viability. The values represented the mean ± SD taken from three separate experiments. These data suggest that oxygen free radicals are generated during anoxia and ethanol treatments. N.V. Ketis is a Queen's National Scholar supported by MRC and ARC/PDF grants.

CC 110 CATALYTIC EFFICACIES OF AGENTS THAT DISMUTATE SUPEROXIDE
Randy H. Weiss, Dennis P. Riley, Willie R. Rivers, Donald J. Fretland†, and Timothy S. Gaginella†, Monsanto Corporate Research, St. Louis, MO 63167, U.S.A. and Gastrointestinal Diseases Research†, G. D. Searle, Skokie, IL 60077, U.S.A.

Agents that can catalytically dismutate superoxide and reduce tissue levels of superoxide may be useful in regulating the oxidant-induced pathology of a number of inflammatory disease states, such as rheumatoid arthritis and inflammatory bowel disease. We have been utilizing the technique of stopped flow kinetic analysis to directly assess the ability of agents to catalytically dismutate superoxide. From the assay, we determine an efficacy ratio (e.r.) which is defined as {k_{cat} (the rate constant for the catalytic dismutation of superoxide by the agent)/M.W. (the molecular weight of the agent)} x 10^{-4}. The ratio is useful for comparing the superoxide dismutate activity of agents on a weight basis. In 50 mM HEPES buffer, pH 8.1 at 21°C, bovine erythrocyte Cu/Zn superoxide dismutase (SOD) and recombinant human placental Cu/Zn SOD were determined to have an e.r. of 6.6 and 1.7, respectively; the apoenzyme of bovine Cu/Zn SOD had an e.r. of 0.34. Accordingly, in a mouse acetic acid-induced colitis model, intra-rectal administration of the bovine Cu/Zn SOD or human Cu/Zn SOD reduced neutrophil-mediated inflammation as assessed by myeloperoxidase activity, whereas the apoenzyme was ineffective demonstrating a role for superoxide in tissue inflammation in this model. A Mn SOD from *E. coli* (e.r.=0.058) was much less active than the Cu/Zn SOD enzymes. Aquo Cu^{2+} was the most active superoxide dismutase tested with an e.r. of 52; Mn^{2+} was inactive (e.r.=0.0) as a catalyst. We conclude that stopped flow kinetic analysis is an effective technique to directly evaluate the ability of an agent to catalytically dismutate superoxide and recommend that the kinetic activity of such agents be compared on a weight basis.

CC 111 COMPARISON OF OXIDATIVE DAMAGE AND INHIBITION OF FE UPTAKE IN HL60 CELLS FROM A MONO OR BIS THIOSEMICARBAZONE CU COMPLEX, William E. Antholine[1], Jana Narasimhan[1], Christopher R. Chitambar[2], and David H. Petering[3], [1]National Biomedical ESR Center, [2]Division of Hematology /Oncology, Medical College of Wisconsin, Milwaukee, WI 53226 and [3]University of Wisconsin—Milwaukee, WI 53201.

Previous work by us has shown that CuL, 2-formylpyridinemonothiosemicarbazonato Cu(II), inhibits the activity of ribonucleoside diphosphate reductase (RDR) and cell proliferation, oxidizes thiols and generates oxy radicals, causes strand scission, and inhibits Fe uptake. The uncharged complex CuKTS, 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato) Cu(II), is more soluble in lipids (octanol to water ratio of 20:1) compared to CuL, and more inert with respect to adduct formation (tetradentate versus tridentate). These properties are used to help explain the greater toxicity, the increase in membrane damage, and the inhibition of uptake of ^{59}Fe from $^{59}FeTf$ after 24 h.

Table: Effects of Two Copper Complexes on HL60 Cells

	CuKTS	CuL
50% Growth Inhibition	0.2 μM	5 μM
Trypan Blue Exclusion	60% with 0.2 μM	80% with 10 μM
Inhibition of ^{59}Fe Uptake	2-fold decrease with 0.2 μM	6-fold decrease with 10 μM
Tryosyl Radical Signal from RDR	not determined*	at least 4-fold decrease with 10 μM

* Cells not viable under conditions for which Fe-uptake completely inhibited

It is hypothesized that the inhibition of Fe uptake contributes to a depletion of RDR (M_2) and inhibition of cell growth. Given this hypothesis, it appears that CuL is more selective for inhibition of Fe uptake while CuKTS is equally potent with respect to iron uptake and destruction of membrane integrity. Supported by NIH grants CA41740, CA23184 and RR01008.

CC 112 TRANSGENIC EXPRESSION OF GLUTATHIONE PEROXIDASE IN HUMAN BREAST CELLS: INCREASED RESISTANCE TO CLASTOGENIC OXIDANTS,
Marc-Edouard Mirault, Alain Tremblay, Lyne Lavoie, Martine Tremblay and Nicole Baudoin.
Department of Medicine, Laval University, CHUL Research Center, Québec, Qc, GIV 4G2.

The production of toxic oxidants and arachidonate metabolites by activated inflammatory cells have been implicated in a large number of degenerative diseases, including cancer. We are investigating the role of specific antioxidant enzymes and oxidative stress responses in the cellular defence against clastogenic oxidants including H_2O_2, peroxides and generators of oxyradicals. Novel models were developed to evaluate the relative importance of glutathione peroxidases in the cellular protection against these oxidants. Expression vectors were used to generate human breast cell T47D transfectants that overproduce a seleno-glutathione peroxidase (Se-GSHPx) or a rat glutathione *S*-transferase (*Yc*) which has an associated glutathione peroxidase activity toward organic hydroperoxides but not H_2O_2. Transfectant clones overexpressing these enzymes constitutively, or following hormone induction, were investigated and compared to control cells regarding their resistance, growth properties and oxidative stress responses to various types of oxidants. Results to be presented on the relative protection afforded by each enzyme indicate that both types of glutathione peroxidases can play a major role in the cellular protection against oxidative damage. Moreover, under severe oxidative stress conditions, high levels of glutathione peroxidase prevent the inhibition of protein synthesis and permit the vigourous induction of heat shock proteins (hsps). Their correlation with increased cellular growth resistance suggests a synergic protection by glutathione peroxidase and hsps.

CC 113 CD11b/CD18-MEDIATED POLYMORPHONUCLEAR LEUKOCYTE (PMN)-INTESTINAL EPITHELIAL INTERACTIONS, Charles A. Parkos, M. Amin Arnaout and James L. Madara, Departments of Pathology and Medicine, Brigham and Women's Hospital and Massachusetts General Hospital, Boston, MA

We developed an *in vitro* model of active intestinal inflammation. Isolated human PMN are driven, by n-formyl peptide (FMLP) gradients, across monolayers composed of T84 cells – a cell line with both differentiated phenotype and electrical characteristics. When PMN are applied to the apical surface of these monolayers at a density of $6x10^6/cm^2$ in the presence of a 10^{-6}M FMLP gradient, monolayer resistance to passive ion flow falls from 1063 ± 218 to 223 ± 126 ohm·cm^2 with a t1/2 of 40 minutes. This resistance fall correlates with the degree of PMN transmigration, as assessed by myeloperoxidase activity in the opposite compartment. As many as 20% of applied PMN transmigrate in 100 minutes under these conditions. Antibodies to the common β subunit (CD18) or to the αM subunit (CD11b) of the PMN surface CD11/18 adhesion complex totally inhibit both transmigration and the resistance fall. Antibodies to the remaining two α subunits which associate with CD18 (CD11a, CD11c) do not substantially inhibit transmigration, nor do antibodies to CD54; a suspected ligand for CD11b/18. These data indicate that CD11b/18 mediated PMN-epithelial adhesive interactions are required for PMN transmigration across T84 monolayers.

CC 114 MYELOPEROXIDASE-DEPENDENT FORMATION OF BENZO(a)PYRENE-7,8-DIOL DNA ADDUCTS BY STIMULATED PULMONARY NEUTROPHILS. Janet M. Petruska, George Jakab, and Michael A. Trush, Department of Environmental Health Sciences, Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205

Several observations have suggested a relationship between the presence of inflammation and the induction of cancer. Pulmonary infection has been shown to effect a rise in lung tumor incidence in rats (JNCI 49: 1107, 1972). The aim of our study was to determine if neutrophils could contribute to the initiation of carcinogenesis via a myeloperoxidase (MPO)-dependent transformation of benzo(a)pyrene-7,8-diol (BP-7,8-diol), a respiratory carcinogen. To test this hypothesis, virus-free male DBA/2 mice were exposed by inhalation to the Gram negative bacteria Proteus mirabilis for 1 hour. For various time points post-exposure, bronchoalveolar lavage (BAL) was performed to determine total cellular MPO, total and differential cell counts, and superoxide ($O_2^{\cdot-}$) generation. Twelve hours after the exposure, cellular levels of MPO as well as percentage and total number of polymorphonuclear leukocytes (PMNs) peaked and began to decrease thereafter. In addition, cells from BAL exhibited increased release of $O_2^{\cdot-}$, as measured by reduction of cytochrome *c*, after addition of soluble or particulate stimuli, phorbol myristate acetate (PMA) or opsonized zymosan, respectively. These cells also elicited biotransformation of BP-7,8-diol as evidenced by enhanced diol chemiluminescence, tetraol production, and covalently bound adduct formation to exogenous DNA upon addition of PMA or opsonized zymosan. Unlike previous work emphasizing the role of oxygen free radicals produced by inflammatory cells in the promotion stage of carcinogenesis, our results indicate an important role of PMNs, due to their content of MPO, in the initiation stage of carcinogenesis. Supported by ES03760, ES07141, and Amer. Cancer Soc. SIG3.

CC 115 CARDIOVASCULAR EFFECTS OF ACTIVATED POLYMORPHONUCLEAR LEUKOCYTES, Kailash Prasad, Jawahar Kalra and Debjani Debnath, Departments of Physiology and Pathology, College of Medicine, University of Saskatchewan and Royal University Hospital, Saskatoon, Saskatchewan, Canada. S7N OWO

Many clinical situations are associated with activation of Complement (C_5). Activated Complements are known to stimulate polymorphonuclear leukocytes (PMNL) to produce oxygen free radicals (OFR) and hypochlorous acid (HOCl). The effects of OFR and HOCl on the cardiovascular function are not clearly understood. We studied the effects of zymosan-activated PMNL on the cardiovascular function in anaesthetized dogs for a period of 2 hours. Zymosan produced a decrease in the cardiac function and myocardial contractility, and an increase in systemic and pulmonary vascular resistance. There was a decrease in the PMNL chemiluminescence (OFR producing activity of PMNL) and an increase in the blood malondialdehyde (MDA), a lipid peroxidation product. Scavengers of oxygen free radicals (superoxide dismutase [SOD] and catalase) reduced the effects of zymosan on cardiac function and contractility and blood MDA. Methionine, a quencher of HOCl also reduced the zymosan induced changes in the cardiovascular function and blood MDA. Cardiac tissue MDA content were lower in those animals treated with scavengers of OFR and methionine as compared to those with zymosan treatment only. These results indicate that OFR and HOCl are cardiac depressant and increase peripheral vascular resistance.

CC 116 PREVENTION OF TYPE I DIABETES WITH A FREE RADICAL SCAVENGER, Yi Wang, Jill A. Panetta* and Kevin J. Lafferty, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Denver, CO 80262, *Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285

Type I diabetes in man and non-obese diabetic (NOD) mice is an immunologically-mediated disease. Recent studies from our laboratory show that the disease process in NOD mice is CD4 T cell dependent and is not restricted by the MHC antigen of the islets. We have also shown that polyethylene glycol-modified superoxide dismutase can protect islet tissue from disease recurrence when islets are transplanted to spontaneously diabetic animals. On the basis of these evidences we hypothesize that the destruction of islet β cells results from inflammatory tissue damage, in which oxygen free radicals are directly involved. In the present study, we further tested the possibility of controlling type I diabetes with a biologically stable free radical scavenger, which can be administered orally.

The experimental models we are using in this study are a disease recurrence model, in which cultured islets are acutely destroyed after grafting to spontaneously diabetic NOD mice and an accelerated model, in which cyclophosphamide treatment accelerates diabetes in 110 days old male NOD mice. Cyclophosphamide increases the incidence of diabetes from <10% to 66% in 12 days. These two models allow us to study the effect of our free radical scavenger on both the acute stage of islet graft damage and the development of diabetes. The scavenger is mixed into the diet (0.1% or 0.05% wt / wt) and administered one day prior to islet transplantation or cyclophosphamide injection. In the disease recurrence model, the administration of the scavenger prolongs the islet graft survival. The mean graft survival time ± 95% confidence are: 0.9 ± 1.1 days (n=12) in the control group; 18.0 ± 9.1 days (n=11) in the 0.1% scavenger-treated group; 13.6 ± 14.6 days (n=10) in the 0.05% scavenger-treated group. In the accelerated model, the administration of the scavenger reduces the incidence of diabetes and prolongs the onset. The incidence of diabetes and the mean time to onset ± 95% confidence are: 66%(n=50), 12.0 ± 1.3 days in the control group; 29% (n=17), 15.0 ± 2.2 days in the 0.1% scavenger-treated group. These data are consistent with our previous hypothesis that free radicals are involved in the destruction of islet β cells and demonstrate that oral administration of radical scavengers can inhibit the development of type I diabetes.

CC 117 H_2O_2-INDUCED INCREASES IN CELLULAR F-ACTIN OCCUR WITHOUT INCREASES IN ACTIN NUCLEATION ACTIVITY. Geneva M. Omann, Josephine M. Harter, Jeanne M. Burger, Daniel B. Hinshaw, Departments of Surgery and Biological Chemistry, University of Michigan Medical School and VA Medical Center, Ann Arbor, MI 48105.

Previous work has shown that H_2O_2 causes an increase in polymerized actin (F-actin) inside cells. To test the hypothesis that increased polymerization resulted from a mechanism involving increased actin nucleation activity, we employed methods utilizing pyrene-labeled actin to quantify the actin nucleation activity of cell lysates and NBD-phallacidin binding assays to quantify the amount of F-actin in P388D1 cells. H_2O_2 increased polymerized actin (NBD-assay) in a dose-dependent manner with an ED_{50} ~ 1 mM. Five mM H_2O_2 caused a 1.6-fold increase in NBD-phallacidin staining. In contrast, actin nucleation activity decreased in a dose-dependent manner with a similar ED_{50}. Five mM H_2O_2 caused a 40% decrease in actin nucleation activity. The effect was rapid, occurring within five minutes of H_2O_2 addition. The results indicate that H_2O_2 causes cytoskeletal changes that enhance NBD-phallacidin binding without increasing actin nucleation activity.

CC 118 **INHIBITION OF THE HUMAN NEUTROPHIL RESPIRATORY BURST BY THE CELL ACTIVATION INHIBITOR CI-959: EFFECT ON INTRACELLULAR BACTERICIDAL ACTIVITY.** Clifford D. Wright, Larry J. Devall, and Mary Carol Conroy, Department of Immunopathology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co., Ann Arbor, MI 48105

CI-959(5-methoxy-3-(1-methylethoxy)-N-1H-tetrazol-5-ylbenzo[b]-thiophene-2-carboxamide, sodium salt) is a potent inhibitor of oxygen radical production and myeloperoxidase release from human neutrophils[1]. The compound acts distal to the generation of phosphoinositide-derived second messengers to inhibit cell activation in response to stimuli which promote intracellular calcium mobilization or calcium influx[2]. CI-959 was further evaluated for its effect on the intracellular bactericidal activity of human neutrophils. Human neutrophils (1×10^7) and bacteria (5×10^7) were coincubated on a glass slide for 60 minutes at 37° C in 5% CO_2. The slides were then washed and stained with 10% acridine orange. Bacterial viability was determined by fluorescence microscopy. When neutrophils were incubated with CI-959, intracellular killing of gram (+) and gram (-) bacteria was inhibited with IC_{50}s ranging from 3.1 to 13.9 μM. Washing the neutrophils free of drug prior to addition of bacteria restored the bactericidal activity to control levels. In contrast, CI-949, a 1-phenyl indole analogue of CI-959 which inhibits myeloperoxidase release but not superoxide production, had no effect on intracellular bactericidal activity at concentrations up to 100 μM. These results highlight the important role of the respiratory burst in host defense.

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Poster Session II

CC 200 IN VITRO AND IN VIVO MODULATION OF THE ACUTE INFLAMMATORY RESPONSE BY ALL-TRANS RETINOIC ACID. Kent J. Johnson, Judie Jones, Michael Dame, Douglas F. Gibbs and James Varani, Department of Pathology, University of Michigan, Ann Arbor, Michigan 49109-0602

Retinoids have been used for many years in the treatment of inflammatory skin diseases such as acute acne. The molecular and cellular basis for these anti-inflammatory effects are unknown. In order to begin to delineate the mechanisms by which retinoids modulate the inflammatory response, we have conducted a series of studies to determine the effects of all-trans retinoic acid (RA) on neutrophil-dependent acute inflammation. Pretreatment of rats with RA leads to a marked inhibition of immune complex-induced dermal as well as lung injury. This anti-inflammatory effect by RA is maximal at four hours. In correlative in vitro studies we have observed that RA inhibits both the generation of oxygen radicals and the release of proteolytic enzymes by stimulated neutrophils. RA does not inhibit the adhesion of activated neutrophils to monolayers of rat pulmonary artery endothelial cells but markedly inhibits endothelial cell killing by neutrophils. In addition to inhibiting the cytotoxic activity of neutrophils we found that pretreatment of the endothelial cells with RA reduces their sensitivity to the cytotoxic effects of activated neutrophils. These data suggest that RA has an effect on both the effector cells (neutrophils) and the target cells (endothelial cells) which together may contribute to the anti-inflammatory effects observed in vivo.

CC 201 IS THERE A ROLE FOR PHOSPHOLIPASE A_2 IN SUPEROXIDE PRODUCTION IN HUMAN NEUTROPHILS AND HL 60 CELLS?, Edward D. Mihelich, Richard M. Schultz, C. H. Chang, Gary A. Hite, Tulio Suarez and David L. Saussy, Jr., Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46285

A variety of studies have proposed the involvement of phospholipase A_2 in the production of superoxide anion by human neutrophils when stimulated with formyl peptide (f-MLP),[1,2] arachidonic acid,[3] or phorbol myristyl acetate (PMA).[4] We sought to address this question by using phospholipase A_2 inhibitors, manoalogue and its congeners, in the study of both receptor activated (GM-CSF, f-MLP) and PKC activated (PMA) neutrophils. Furthermore, we sought to establish a link between superoxide inhibition and inhibition of arachidonate metabolite production in the neutrophil-like cell line, HL 60, using A23187 as stimulant. These studies failed to show a correlation between the two events and suggest that PLA_2 is not a necessary component of the signal transduction pathways leading to superoxide production.

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4) L. M. Henderson, J. B. Chappell, O. T. G. Jones, *Biochem. J.* **264**, 249 (1989).

CC 202 IN VITRO NEUTROPHIL-MEDIATED LIPID PEROXIDATION OF NEONATAL PULMONARY PHOSPHOLIPIDS, Jerry J. Zimmerman and June R. Lewandoski, Department of Pediatrics, Division of Critical Care Medicine, University of Wisconsin, Madison, WI 53792.

Activated neutrophils (PMNs) have been implicated as a cause of inflammatory pathology in acute lung injury. Obvious tissue targets for PMN-mediated pulmonary host autoinjury are the various pools of lung phospholipid (PL). This investigation examined in vitro PMN-mediated oxyradical injury towards pulmonary parenchyma (PP), surfactant (PS) and erythrocyte (RBC) PL. PL were extracted using 2:1 $CHCl_3$:MeOH; quantitated by phosphorus analysis; and qualitated by gas chromatography. Peroxidation was facilitated with 10e6 PMNs, 0.10 mM $Fe_4(P_2O_7)_3$, and 0.1 mg PL in physiologic buffer; initiated with phorbol myristate acetate (1 µg/ml final); and quantitated with thiobarbituric acid and HPLC-fractionated phosphatidylcholine conjugated dienes (PC-CD). PP PL and RBC PL displayed similar fatty acid profiles with roughly 4 times the polyunsaturated fatty acid content of PS. However, PP PL peroxidation was 13 times that of RBC PL (21.0 ± 1.4 vs 1.6 ± 1.6 nmol TBARS/mg PL). 13.8 ± 1.8 nmol (n=3) of PC-CD per mg of PP PL were generated employing the above noted conditions. PP PL peroxidation was significantly enhanced (↑361%) by chelated iron and inhibited by superoxide dismutase (↓~85%) and catalase, (↓~50%) but not azide. Minimal peroxidation products were noted when either PL or PMNs were omitted from the incubation mixture. Preliminary data suggest that perhaps the enhanced susceptibility of PP PL to PMN-mediated peroxidation compared to RBC PL may relate to its lower concentration of α-tocopherol (0.0435 vs .0730 µg/mg PL).

CC 203 SECRETION OF ENZYMATICALLY ACTIVE HUMAN RECOMBINANT MYELOPEROXIDASE BY CHINESE HAMSTER OVARY CELLS IN CULTURE. N. Moguilevsky, L. Garcia-Quintana, A. Jacquet, C. Tournay, L. Fabry*, L. Piérard* and A. Bollen, Department of Applied Genetics, Université Libre de Bruxelles, 1400 Nivelles, Belgium, and *SmithKline Biologicals, 1330 Rixensart, Belgium.

The cDNA encoding human myeloperoxidase carries three ATG codons in frame, respectively 144, 111 and 66 bp upstream from the proprotein DNA sequence. In order to determine the most efficient signal sequence, three cDNA modules starting at each of the ATG were cloned into an eukaryotic expression vector and stably expressed in Chinese Hamster Ovary cell lines. In all three cases, recombinant MPO was secreted into the culture medium of transfected cells, indicating that each of the signal peptides function efficiently. One of the recombinant cell line,which was amplified using methotrexate, overexpresses enzymatically active recMPO up to 6 µg/ml/day. The recombinant product was purified by a combination of ion exchange and metal chelate chromatography and characterized in terms of molecular weight, N-terminal amino acid analysis, glycosylation, activity and physico-chemical properties. The data show that recMPO is secreted essentially as a heme-containing single chain precursor of 84 kDa which functions as a monomer. N-terminal analysis indicated that cleavage of the signal peptide occurs between amino acids 48 and 49. In addition, recMPO appeared glycosylated up to the last stage of syalylation, to an extent similar to the one of the natural enzyme. At last, specific activity measurements as well as stability data, in various pH, T°, ionic strength and reducing conditions, indicated that the recombinant single chain enzyme behaves essentially as the natural two chains molecule.

CC 204 THE ROLE OF OXIDANTS IN INFLUENZA-INDUCED AIRWAY HYPERREACTIVITY IN RATS, J.S. Tepper, J.R. Lehmann, J.R. Hoidal[1], D.L. Costa[2] , G.R. Burleson[2] and T.P. Kennedy[3], NSI Environmental Sciences, Research Triangle Park, NC, 27709, [1]University of Utah, Salt Lake City, UT, 84132, [2]US EPA, Research Triangle Park, NC, 27711, [3]Richmond, VA

Airway hyperreactivity (AHR) to intravenous acetylcholine has been demonstrated 3d after F-344 rats were intranasally instilled with a rat-adapted influenza virus (ARRD, A657:1990). N-acetyl cysteine (NAC), an oxidant scavenger, was able to block AHR if orally administered (1% in tap water) for 2d prior to virus infection and continued for 3d until AHR was examined. To evaluate if these oxygen radicals derived from altered adenosine metabolism were responsible for AHR, oxypurinol (OXY) was administered (50 mg/kg, i.p.) for 4d prior to virus infection and AHR challenge. Virus increased xanthine oxidase (XO) activity and OXY inhibited this effect, but AHR was not blocked. However, virus-infected OXY-pretreated animals had less protein in the bronchoalveolar lavage suggesting that OXY had reduced a portion of the virus-induced lung damage. Experiments are currently in progress to see if oxidative damage from leukocytes, rather than altered adenosine metabolism, contribute to AHR. (This abstract does not reflect EPA policy).

CC 205 SOME BASIC STUDIES INTO THE CHEMISTRY OF HYPOCHLORITE IN THE LEUKOCYTE PHAGOLYSOSOME Michael J. Thomas and Jian Pang, Department of Biochemistry, The Wake Forest University Medical Center, Winston-Salem, NC 27103

Leukocytes secrete large amounts HOCl when they kill bacteria. Hypochlorite oxidizes a variety of organic compounds, but a major reaction is the N-chlorination of nitrogen containing compounds like proteins and nucleic acids. To examine the chlorinating reactions of human neutrophils we treated neutrophils with latex beads coated with the chlorine trap ethyl 6-(3',5'-dimethoxyphenoxyl)hexanoate (**1**). The phenyl ring of **1** is readily chlorinated by HOCl giving two products: one product (**2**) chlorinated at the 2' or 6' position and the second (**3**) chlorinated at the 4' position of the aromatic ring. The product distribution (**2**/**3**) from neutrophils is different from that obtained by treating **1** with N-chloroHEPES, but similar to that obtained with HOCl. Therefore, mechanistic studies of chlorination performed with amine buffers must be interpreted with caution. A second interesting phenomena was observed in the course of these investigations. The N-chloroamines react with Fe(II) by a one electron transfer to the N-chloroamine forming Fe(III) and unknown products. We are studying the reaction between Fe(II) salts and N-chloro derivatives of several amino acids and proteins under physiological conditions. The results of these studies will be presented.

CC 206 MANGANESE SUPEROXIDE DISMUTASE IS ASSOCIATED WITH THE NEUTROPHIL PLASMA MEMBRANE, B.E. Britigan, M.L. McCormick, T.D. Oberley, H.P. Cihla and L.W. Oberley, Research Service, VA Medical Center, Iowa City, IA 52246, Dept. of Internal Medicine and Radiation Research Lab, Univ. of Iowa, Iowa City, IA 52242, and Pathology Service, VA Medical Center, Madison, WI 53705.

Neutrophils (PMN) function in the presence of damaging reactive oxygen species, yet little is known about their antioxidant mechanisms. Previous assays have shown low CuZn superoxide dismutase (SOD), negligible MnSOD, and moderate catalase (CAT) and glutathione peroxidase (GPX) enzyme activity. To further assess human PMN antioxidant defenses, immunoblot analysis for each antioxidant enzyme was performed on whole cell lysates as well as subcellular fractions of N_2-cavitated PMNs. Primary or secondary granules had no antioxidant enzyme immunoreactivity. As expected, CuZnSOD, CAT, and GPX were almost exclusively cytosolic. Similar amounts of MnSOD and CuZnSOD were detected in whole cell lysates. This was surprising since MnSOD is a mitochondrial matrix enzyme and PMNs are nearly devoid of mitochondria. More surprising, however, >95% of the immunoreactive MnSOD was in the plasma membrane fraction. Immunoblots with antisera to medium chain acyl dehydrogenase, another mitochondrial matrix protein, did not suggest contamination of membrane fractions by mitochondria. Furthermore, electron microscopy of immunogold labeled PMN confirmed the presence of plasma membrane MnSOD. There was good correlation between PMN CuZnSOD and CAT immunoreactive protein and enzymatic activity (activity gel). However, little MnSOD enzymatic activity was detectable in spite of considerable MnSOD immunoreactivity. PMN stimulation with PMA or 2h incubation with priming doses of TNF-α and IL-1β did not alter PMN anti-oxidant enzyme activity, immunoreactivity, or subcellular location (PMA only). These data suggest that the PMN plasma membrane contains immunoreactive MnSOD. Further work is required to assess its potential role in protection of the PMN membrane from oxidant injury and/or modulation of PMN superoxide production.

CC 207 LUNG OXIDATIVE STRESS PRODUCED BY HYPERBARIC OXYGEN AND PARAQUAT (PQ), Cecilia Giulivi, Carla Lavagno, Jorge Pisarello and Alberto Boveris, Department of Physical Chemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, Argentina.

PQ produces superoxide anion (O_2-) by intracellular redox cycling, while hyperbaric O_2 increases free radical production from intracellular sources by increasing the O_2 partial pressure. Lung chemiluminescence (CL, measurement of the steady state concentration of O_2 free radicals), malondialdehyde (MDA, final product of lipid peroxidation) and lung wet/ dry wt. ratio (lung edema) were determined in 3 groups of male adult Wistar rats under different treatments: a) Control, b) Exposure to 2 ATA O_2, c) Administration of *i.v.* 30 mg PQ/ kg b.wt. Lung CL values showed a maximal 2-fold increase at 30 h of PQ treatment while CL values as high as 7-times those of the control (100 $\pm$ 5 cps/lungs) were obtained at 15 h of O_2 exposure. MDA formation was increased in 60 and 200% while lung edema increase was 50 and 30 % for PQ and O_2 treatment, respectively. Both treatments showed a maximal CL preceded by an increase in the polymorphonuclear (PMN) cell migration to the lungs, determined enzymatically and by light microscopy (r: 0.99). The results suggested that PQ and O_2 are responsible of oxidative lung stress by a mechanism in common, where PMN cell migration to the lungs and activation would enhance the damage already started by PQ and O_2.

CC 208 **THE ROLE OF NEUTROPHILS IN HEPATIC ISCHEMIA/REPERFUSION INJURY**, Hartmut Jaeschke, C. Wayne Smith, Helen Hughes and Anwar Farhood, Departments of Medicine, Pediatrics and Pathology, Baylor College of Medicine, Houston, Texas 77030

The pathogenesis of hepatic ischemia/reperfusion injury was investigated in a model of normothermic no-flow ischemia for 45 min (median and left lateral hepatic lobe) and reperfusion in male Fischer rats. Two phases of liver injury were identified, an initial phase during the first hour of reperfusion and a later progression phase with 80 $\pm$ 3% hepatocyte necrosis at 24 h of reperfusion. The number of neutrophils in the liver (basal value: 14 $\pm$ 3 PMNs per 50 high power fields) started to increase at the end of the first hour of reperfusion (61 $\pm$ 13) reaching values of 889 $\pm$ 145 after 24 h. Plasma levels of glutathione disulfide (GSSG) increased immediately after initiation of reflow, indicating a significant oxidant stress at that time. Activation of Kupffer cells with retinol, galactosamine, or *Proprionibacterium acnes* prior to ischemia enhanced the postischemic GSSG formation while inactivation of Kupffer cells with methyl palmitate or gadolinium chloride attenuated the increase of plasma GSSG conc. and partially protected against the initial ischemia/reperfusion injury. Pretreatment with high doses of an antineutrophil antibody that caused consistent neutropenia for 24 h significantly reduced liver injury after 24 h of reperfusion. Hepatic leukotriene B_4 (LTB_4) content as determined by GC/MS (basal value: < 20 pg/g liver wt.) did not change during the initial injury phase and early accumulation of neutrophils but was significantly increased during the second phase of reperfusion injury (809 $\pm$ 310 pg/g). It is concluded that Kupffer cell activity is at least partially responsible for the initial injury; however, the later injury phase seems to be mainly neutrophil-dependent. LTB_4 appears to be involved in neutrophil activation during the later phase of reperfusion injury.

CC 209 THE CATECHOL PROTOCATECHUIC ACID IS ABLE TO INHIBIT ENDOTOXIN INDUCED, EMPHYSEMA IN THE HAMSTER, JAN STOLK and JOHANNES KRAMPS, Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands.

Protocatechuic acid (PA) is an inhibitor of NADPH-oxidase activity after metabolic activation by reactive oxidant species (ROS). This mode of action was concluded from experiments in which oxygen consumption was determined in a cell free system for NADPH-oxidase assemblage from lipid membranes and cytosol. Moreover, PA was able to inhibit cheminuliscense from lucigenin after stimulation of human PMN with PMA and after ROS generation from xanthine by xanthine-oxidase in a dose dependent way.
To investigate the in vivo potency of PA, hamsters were treated with different concentrations of PA in their drinkwater. Two weeks later, the hamsters were instilled with endotoxin in the trachea twice a week for 4 weeks. A dose of 40 ug/ml in drinkwater reduced the development of pulmonary emphysema by 60%. We conclude that protocatechuic acid is a potent inhibitor of tissue destruction by inflammatory cells.

CC 210 HYDROGEN PEROXIDE-INDUCED CELL AND TISSUE INJURY: PROTECTIVE EFFECTS OF MANGANESE, James Varani, Douglas F. Gibbs, Partha S. Mukhopadhyay, Chris Sulavik, Kent J. Johnson, Joel M. Weinberg, Isaac Ginsburg and Peter A. Ward, Department of Pathology, University of Michigan, Ann Arbor, Michigan 48109-0602

Recent evidence indicates that under in vitro conditions, superoxide anion and hydrogen peroxide (H_2O_2) are unstable in the presence of manganese ion (Mn2+). The current studies show that in the presence of Mn2+, H_2O_2-mediated injury of endothelial cells is greatly attenuated. A source of bicarbonate ion and an amino acid source are required for Mn2+ to exert its protective effects. Injury induced by phorbol ester-activated human neutrophils (previously shown to be dependent on H_2O_2 production) is also attenuated under the same conditions. EDTA reverses the protective effects of Mn2+ under conditions that do not result in Ca2+ chelation or disruption of endothelial cell physiology. Acute lung injury produced in vivo in Long Evans rats by intratracheal instillation of glucose/glucose oxidase is almost completely blocked in rats treated intraperitoneally with Mn2+ and glycine. Treatment with either Mn2+ or glycine alone is partially protective. Conversely, treatment of rats with EDTA markedly accentuates lung injury mediated by glucose/glucose oxidase under conditions in which EDTA is not toxic by itself. These data suggest that of the several oxidant-limiting reactions that Mn2+ is known to participate in, it is the direct oxidation of amino acids with concomitant H_2O_2 disproportionation that is responsible for much of its protective effects. This could form the basis of a new therapeutic approach against oxygen radical-mediated tissue injury.

CC 211 SUPEROXIDE PRODUCTION BY MURINE TUMOUR-ASSOCIATED MACROPHAGES IS INHIBITED BY A SPECIES ACTING ON THE SIGNAL TRANSDUCTION PATHWAY.

Stephen J. Eason and Bernadette M. Hannigan, Biomedical Sciences Research Centre, University of Ulster at Coleraine, Coleraine, Co.Londonderry, N.Ireland. BT52 1SA.

Oxygen radicals produced by macrophages, dependant on the membrane-bound enzyme NADPH Oxidase, have been shown in vitro to be cytotoxic to tumour cells. We have shown that macrophage populations isolated from within solid tumours (TAM) are deficient in their capability to produce superoxide. This inhibition would appear to be occurring at the level of signal transduction, since NADPH Oxidase isolated from TAM, has normal activity. In this study, a comparison was made between several activators of NADPH Oxidase, utilising different points in the signal transduction pathway, and their effect on superoxide production by intact macrophages and isolated enzyme was determined.

CC 212 DIFFERENTIAL REGULATION OF REACTIVE NITROGEN AND REACTIVE OXYGEN INTERMEDIATE PRODUCTION BY HEPATIC MACROPHAGES (MP) AND ENDOTHELIAL CELLS (EC), Carol R. Gardner, Lisa S. Feder, Diane E. Heck, Thomas W. McCloskey, Jeffrey D. Laskin and Debra L. Laskin, Rutgers University and UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854.
Nitric oxide is a highly reactive molecule produced by both MP and EC via an L-arginine-dependent nitric oxide synthase. In the present studies we compared production of nitric oxide and H_2O_2 by liver MP and EC. Cells were isolated from livers of female Sprague-Dawley rats by in situ perfusion followed by centrifugal elutriation and differential centrifugation. We found that both MP and EC produced nitric oxide as measured by the accumulation of nitrites in the cell culture medium, in response to lipopolysaccharide (LPS). Formation of nitrites was dependent on both the dose of LPS and cell number. Hepatic MP were found to produce significantly more nitrite than EC. Using dichlorofluorescin diacetate and flow cytometry, we also found that hepatic MP produced significantly more H_2O_2 than EC. Phorbol myristate acetate (PMA) was found to be a potent inducer of H_2O_2 production by EC and MP. EC were more responsive to PMA than were the MP. In contrast PMA had no effect on nitrite production by the cells. Treatment of rats with LPS resulted in increased production of H_2O_2 by EC and MP with no effects on nitrite production. These data demonstrate that nitric oxide and H_2O_2 production by MP and EC are regulated by distinct mechanisms. Supported by NIH GM34310.

CC 213 GENERATION OF REACTIVE OXYGEN INTERMEDIATES BY LEUKOCYTES TREATED WITH PSORALENS AND ULTRAVIOLET LIGHT, Diane E. Heck, Emil Bisaccia, Steven Armus, and Jeffrey D. Laskin, UMDNJ-R.W. Johnson Med. School Piscataway, NJ 08854 and Morristown Memorial Hospital, Morristown, NJ 07962
Systemically disseminated cutaneous T-cell lymphoma (CTCL) consists of a group of T-lymphocyte disorders usually of a mature T-helper/inducer phenotype. Recently, treatments with the combination of 8-methoxypsoralen (8-MOP) and ultraviolet light, a procedure referred to as PUVA, has proven beneficial in the management of the disease. The mechanism by which PUVA causes regression of the tumor is not well understood and may be due to an immunologic response to the treated tumor cells. In the present studies we used a highly sensitive flow cytometric assay in conjunction with the hydroperoxide sensitive dye 2',7'-dichlorofluorescein diacetate to measure intracellular hydrogen peroxide in normal lymphocytes and CTCL following PUVA treatment. We found that ultraviolet light alone caused a dramatic increase in the intracellular hydrogen peroxide content of the cells. The cellular response was dose-dependent in the range of 0.07-1.4 J/cm^2. Pretreatment of the cells with 8-MOP (50 ng/ml) was found augment responses of the cells to ultraviolet light. Hydrogen peroxide is known to be important in cytotoxicity as well as in modulating mitogen-stimulated T cell proliferation. The generation of this reactive oxygen intermediate in CTCL may be an important mechanism by which PUVA suppresses the growth of these leukemic cells.

CC 214 EFFECTS OF THE RESPIRATORY BURST ON POLYMORPHONUCLEAR LEUKOCYTES PLASMA MEMBRANE: A FLUORESCENCE STUDY, Ahmad Kantar, Pier Luigi Giorgi, Giovanna Curatola*, and Rosamaria Fiorini*. Departments of Pediatrics and Biochemistry*, University of Ancona, (I) 60123 Ancona-Italy.
Experimental evidences suggest the existence of plasma membrane "domains" of different composition that are involved in the regulation of the activation of the respiratory burst (RB) of human polymorhonuclear leukocytes (PMNs). Steady-state fluorescence anisotropy and fluorescence decay of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) incorporated into PMNs plasma membranes were studied (1,2). The activity of the RB was monitored using chemiluminescence assay (3). PMNs were activated with phorbol myristate acetate, f-Met-Leu-Phe, and PAF. The effect of Diphenyl iodonium, adenosine, SOD, and catalase were also evaluated. Our results showed that the RB activation is accompanied by a significant increase of TMA-DPH steady-state anisotropy and with changes of fluorescence lifetime values and distributions. These perturbations of PMNs plasma membrane phospholipid order and changes in membrane heterogeneity indicate modifications of membrane architecture during the RB.
1. R. Fiorini et al. Biochim Biophys Acta 939,485-492 (1988)
2. R. Fiorini et al. Biochem Int 20,715-724 (1990)
3. A. Kantar et al. Acta Paediatr Scand 79,535-541 (1990)

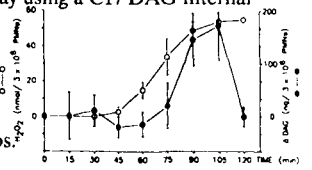
CC 215 B LYMPHOCYTE SUPEROXIDE GENERATION CAPACITY IS UP-REGULATED BY BLAST TRANSFORMATION AND TRIGGERED BY CROSS-LINKING OF SURFACE IG OR HLA-DR

*Friedrich E. Maly, *Kazuhito Furukawa, oMichio Nakamura, oShiro Kanegasaki and *Alain L. de Weck *Institute of Clinical Immunology, Bern, Switzerland and oInstitute of Medical Science, Tokyo, Japan

As phagocytes, human B lymphocytes possess a NADPH oxidase-type superoxide generating system triggerable by cross-linking of surface immunoglobulin. We investigated oxidase activity of tonsil B cells during in vitro blast transformation by measuring Lucigenin-dependent chemiluminescence responses (CL) to anti-IgM, control antibodies, phorbol ester PMA and opsonized zymosan (OPZ). Already for freshly purified tonsil B cells, anti-IgM was the strongest stimulus of CL. After 3 days of culture with 0.4 ng/ml PMA + 1 ug/ml ionomycin, CL responses to anti-IgM were increased 4-5 fold while responses to OPZ and PMA were lost. B blast oxidase activity was also triggered by anti-HLA-DR. Compared with resting B cells, markedly fewer B blasts expressed cytochrome b559 surface antigen (16 versus 95 %) and reduced nitroblue tetrazolium in an SOD-inhibitable fashion (10 versus 50 %). Thus, a subpopulation of B blasts exhibits strongly upregulated oxidase activity which may be physiologically triggered by encounter with antigen or interaction with T lymphocytes.

CC 216 NEUTROPHIL (PMN) CD18-MEDIATED ADHERENCE-DEPENDENT H_2O_2 PRODUCTION IS ACCOMPANIED BY DIACYLGLYCEROL (DAG) ACCUMULATION, S.B. Shappell, A.A. Taylor, H. Hughes, C.W. Smith, Baylor College of Medicine, Houston, TX 77030.

Beta2 (CD11/CD18) integrins mediate adherence-dependent production of H_2O_2 by human and canine PMNs on protein-coated surfaces, cultured endothelium, and isolated cardiac myocytes in response to low levels of cytokines or chemotactic factors (Shappell *et al.*, J Immunol 144: 2702; Nathan *et al.*, J Cell Biol 109: 1341; Entman *et al.*, J Clin Invest 85: 1497). In all cases, this production of H_2O_2 is prolonged and proceeds after a distinct lag period. Adhesion and H_2O_2 production (40.0 ± 10.2 nmol/10^6 PMNs, lag = 50.5 ± 8.8 min, n = 12) by 10 nM fMLP-stimulated human PMNs on keyhole limpet hemocyanin (KLH)-coated surfaces is Mac-1 (CD11b/CD18)-dependent and is blocked by protein kinase C (PKC) inhibitors H7 and staurosporine (Shappell *et al.*, FASEB J 4:2179a, 1990). That these compounds do not block fMLP-induced adhesion or increased Mac-1 surface expression and inhibit H_2O_2 production when added any time during the lag phase suggests that Mac-1-dependent adhesion may result in a delayed PKC-dependent activation of the NADPH oxidase. This was explored by quantitating DAG (endogenous PKC activator) in adherent PMNs by a gas chromatography-mass spectrometry (GCMS) assay using a C17 DAG internal standard. Following lipid extraction from 10 nM fMLP-stimulated PMNs on KLH, the DAG fraction is separated by TLC and transesterified with sodium methoxide. The resulting methyl esters are quantified by GCMS. fMLP-stimulated adhesion to the Mac-1 substrate KLH resulted in delayed accumulation of DAG (method 1) that correlates temporally with the production of H_2O_2 (Figure, n = 3). Adhesion dependence will be verified by studies with CD18-deficient PMNs and anti-CD11/CD18 MAbs.



CC 217 A STRUCTURAL AND FUNCTIONAL ASSOCIATION OF THE RESPIRATORY BURST OXIDASE WITH THE NEUTROPHIL CYTOSKELETON. Richard C. Woodman, Julie M. Ruedi, Naoki Okamura and Bernard M. Babior. Dept. of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, CA. 92037.

Earlier evidence had suggested an important relationship between the respiratory burst oxidase and the cytoskeleton of human neutrophils. To study the oxidase-cytoskeletal association further, Triton X-100 treatment of resting and phorbal-activated human neutrophils was performed, followed by measurements of oxidase activity (O_2^- production) and determination of the presence of cytochrome b_{558}, p47-*phox* and p67-*phox* in the cytoskeleton and detergent-extract fractions. After two minutes of phorbal activation, most of the detectable oxidase activity (>95%) was restricted to the cytoskeleton. In contrast, only about 10-15% of cytochrome b_{558} localized to the cytoskeleton as determined by dithionite difference spectroscopy and immunoblotting with anti-p22-*phox* and anti-gp91-*phox*. A substantial amount of p67-*phox* was also associated with the cytoskeleton. However, the cytoskeletal distribution for p67-*phox* or either of the cytochrome b_{558} subunits was unaffected by phorbal activation. The entire p47-*phox* phosphoprotein family was exclusively confined to the detergent-extract fraction as determined by both immunoblotting and autoradiography of two dimensional gels of resting and activated $^{32}P_i$-labelled neutrophils. In conclusion, these results provide direct evidence for an important functional and structural relationship between the active form of the respiratory burst oxidase and the cytoskeleton of human neutrophils.