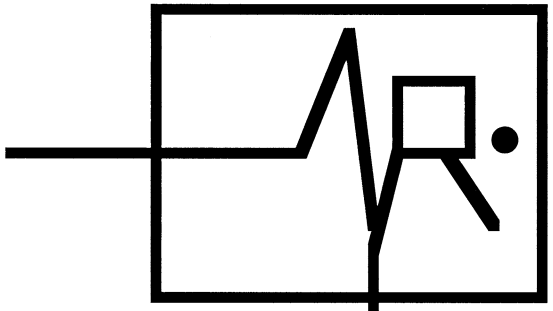

SIGNAL TRANSDUCTION AND GENE EXPRESSION

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PROTEIN KINASE B/AKT PROTECTS HUMAN LUNG MICROVASCULAR ENDOTHELIAL CELLS (HLMVEC) AGAINST HYPEROXIA INDUCED OXIDATIVE INJURY

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High oxygen concentrations (hyperoxia) often required in the treatment of preterm infants and critically ill patients also causes lung injury. Hypoxic preconditioning can protect against hyperoxia in animal models and lung cells. Protection by hypoxic preconditioning is mediated, at least in part, by PI 3-kinase both in primary lung microvascular endothelial cells and lung epithelial-like A549 cells. Since protein kinase-B(PKB)/Akt is a survival factor located downstream in the PI 3-kinase signaling cascade, we hypothesized that PKB/Akt protects lung-derived cells against hyperoxic injury and death. Exposure of primary human lung microvascular endothelial cells (HLMVEC) to hyperoxia or extreme hypoxia for 60-120 minutes caused increases in active Akt as determined by western blot analysis of phosphorylated Ser 473 residue of Akt. In further studies, adenoviral-mediated expression of myrAkt, the constitutively active form of Akt, protected HLMVEC against hyperoxic cell death assessed by YOYO-1 staining and analysis by flow cytometry. Akt kinase activity was elevated in the cells expressing myrAkt, but not in cells transduced with adenovirus vectors encoding dnAkt or lacZ. Cell death due to hyperoxia (8 days) was substantial in control (41.59±0.73), lacZ (40.49±1.48) and dnAkt (41.98±0.22) cells, but in myrAkt cells death in 95% O_2 (25.89±0.61) did not exceed that in 21% O_2 (28.74±1.27). Akt potently protects HLMVEC against hyperoxic stress.

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THE MITOCHONDRIAL PERMEABILITY TRANSITION IN MAMMALIAN CELLS AND ISOLATED MITOCHONDRIA: TWO DISTINCT PHENOMENA?

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The purpose of the present investigation was to compare the regulation of the mitochondrial permeability transition (MPT) in mammalian cells and isolated rat mitochondria. Direct manipulation of the glutathione (GSH) redox-status in HL60 cells with diethylmaleate (DEM) facilitated the generation of mitochondrial reactive oxygen species (ROS) from mitochondrial respiratory complex III (cytochrome bc_1) and caused a time dependent loss of mitochondrial membrane potential ($\Delta\psi_m$). The loss of $\Delta\psi_m$ was associated with mitochondrial swelling, and loss of the normal mitochondrial architecture and cell death. Although the loss of $\Delta\psi_m$ and cell death was effectively blocked with the adenine nucleotide translocator ligand bongkrekic acid (BA), it was not prevented with the MPT inhibitor cyclosporin A. These observations prompted us to determine whether DEM-dependent GSH redox modulation would activate the MPT in isolated mitochondria. Mitochondria were obtained from the liver of Sprague-Dawley rats, isolated using established methodologies and were treated with DEM at the same concentration and for the same duration used to activate the MPT in HL60 cells. DEM treatment failed to activate the classical Ca^{2+}dependent MPT measured by the swelling test; moreover, after DEM treatment the Ca^{2+}induced MPT was fully inhibited with cyclosporine A. These results suggest that the MPT in mammalian cells and isolated rat mitochondria represent two distinct phenomena and question the validity of extrapolating results obtained using isolated mitochondria to mechanistically explain cellular phenomena such as apoptosis and necrosis.

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OXIDANT SIGNALS LEADING TO PHOSPHORYLATION OF ERK AND CREB IN LUNG EPITHELIAL CELLS: DIFFERENT REQUIREMENTS FOR CA^{2+} INFLUX

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Oxidant stress-mediated ERK (extracellular-regulated kinase) phosphorylation is linked to pathologic signaling in lung epithelium. Little is known of a role for Ca^{2+}-mediated transcription in oxidant signaling pathways. Here, we tested the hypothesis that oxidants induce Ca^{2+} influx and phosphorylation of both ERK and the Ca^{2+}-regulated transcription factor, CREB (Ca^{2+}/cAMP-response element binding protein), in C10 lung epithelial cells. Ca^{2+} was imaged in single cells using a calmodulin-FRET construct. Phospho-ERK and phospho-CREB were visualized by immunofluorescence. H_2O_2 initiated a rise in Ca^{2+} that was prevented by reducing extracellular Ca^{2+}. H_2O_2 also increased levels of phospho-ERK and phospho-CREB. Reduction of extracellular Ca^{2+} did not affect ERK or CREB phosphorylation in response to H_2O_2, suggesting that H_2O_2 communicates to ERK and CREB independently of extracellular Ca^{2+}. The effect of oxidant-producing asbestos on ERK and CREB phosphorylation was also examined. Following exposure to asbestos, phospho-CREB was detected at 30 min, whereas phospho-ERK was detected at 4 h. Unlike results with H_2O_2, reducing extracellular Ca^{2+} prevented CREB phosphorylation in response to asbestos. These data reveal a role for the CREB transcription factor in oxidant-mediated signaling pathways and suggest disparate roles for extracellular Ca^{2+} influx in oxidant-induced activation of ERK and CREB. Further study of Ca^{2+} signaling pathways initiated by different oxidants will better define a role for Ca^{2+} in pathologies related to oxidant stress. Supported by P01 HL67004.

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IKK[BETA] DEFICIENCY CAUSES OXIDATIVE STRESS AND PROLONGED JNK ACTIVATION INDUCED BY ARSENIC

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Stress signals activate both IκB kinase (IKK) and c-Jun-N-terminal kinase (JNK). Recently, it was shown that IKK-dependent NF-κB activation results in attenuation of TNFα-induced JNK activation. How that negative cross-talk between NF-κB and JNK occurs is not well-understood. By using wild type (WT) and Ikkβ gene knockout ($Ikk\beta^{-/-}$) mouse embryo fibroblasts (MEFs), we found that IKKβ deficiency results in prolongation of arsenic-induced JNK activation, which was not due to the decreased expression of GADD45β or XIAP, as previously suggested for $RelA^{-/-}$ cells treated with TNFα. This enhanced JNK activation was largely associated with an oxidative stress response as indicated by elevated expression of heme oxygenase-1 and the accumulation of H_2O_2 in $Ikk\beta^{-/-}$ cells. Expression profiling experiments revealed an increased expression of p450 family CYP1B1 mRNA in $Ikk\beta^{-/-}$ cells compared to WT cells. Inhibition of CYP1B1 reduced both oxidative stress and arsenic-stimulated JNK activation. Thus,

increased CYP1B1 expression is central to and seems to be responsible for sensitizing $Ikk\beta^{-/-}$ cells to stress-induced JNK activation.

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REDOX-SENSITIVE GROWTH FACTOR RECEPTOR TRANSACTIVATION REQUIRES A FUNCTIONING MITOCHONDRIAL RESPIRATORY CHAIN

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Activation of mitogen activated protein kinases and Akt in cells governs vital functions such as growth, proliferation, and cell death. We have previously shown that activation of c-Jun N-terminal kinase (JNK) and Akt by H_2O_2 is mediated by EGF receptor transactivation. However, the mechanism(s) linking reactive oxygen species to receptor tyrosine kinase activation remain elusive. We examined the role of mitochondrial respiration in H_2O_2-mediated signal transduction using selective mitochondrial inhibitors and generating cells devoid of functioning mitochondria (ρ^0). Inhibition of mitochondrial function in cells abolished H_2O_2-induced transactivation of EGF receptor and its downstream targets JNK and Akt, indicating the involvement of functional mitochondrial respiration early in this process. Consistent with this notion, activation of the EGF receptor and its downstream targets in response to H_2O_2 was not detectable in ρ^0 cells. In addition to the EGF receptor, inhibition of mitochondrial respiration prevented H_2O_2-induced transactivation of the VEGF2 and PDGF receptors in endothelial cells and fibroblasts, respectively. Similarly, a mitochondrial targeted antioxidant MitoQ, but not non-targeted antioxidant Q1, inhibited transactivation of growth factor receptors and downstream signaling induced by H_2O_2. In contrast, mitochondrial respiration did not play a role in ligand-induced EGF, PDGF, or VEGF2 phosphorylation, or UV-induced activation of JNK, suggesting specificity of these responses for H_2O_2. These data indicate that intact mitochondria act as an important proximal redox sensor required for H_2O_2-induced growth factor receptor transactivation.

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ANTIOXIDANT INHIBITS DENDRITIC CELL DEPENDENT TH2 RESPONSES

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A SOD mimic, AEOL 10113 has been shown to modulate Th1 responses. In the presence of the antioxidant, antigen presenting cell (APC) dependent IFN gamma production by T lymphocytes was diminished. We hypothesize that AEOL 10113 can mediate APC dependent Th2 responses in the same manner. T cells that bear specific TCR for $OVA_{323-339}$ peptide were isolated from DO11.10 mice and cultured with IL-4 and IL-12 to develop the Th2 phenotype. Th2 cells were incubated with $OVA_{323-339}$ and bone marrow derived dendritic cells as APC in the presence or absence of AEOL 10113. T cell proliferation was measured by ^{3}H-thymidine uptake. Production of IL-4 and IL-5 was measured by ELISA. The results showed a dose dependent inhibition of T cell proliferation by AEOL 10113. The expressions of the costimulatory molecules, CD86 and MHC class II, on dendritic cells were reduced in AEOL 10113 treated cultures. In addition, T cell production of IL-4 and IL-5 was inhibited. These results show that AEOL 10113 inhibited T cell proliferation and Th2 cytokine production possibly by down regulation of costimulatory molecule expressions on dendritic cells. The ability of AEOL 10113 to modulate Th2 responses suggests a therapeutic potential of this antioxidant in the treatment of asthma. [3]

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MITOCHONDRIAL-DERIVED HYDROGEN PEROXIDE REGULATES PI3K SIGNALING VIA PTEN OXIDATION.

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Hydrogen peroxide (H_2O_2), is produced as a by-product of normal metabolism, and is a key regulator of signaling important to many normal cellular functions. Antioxidant enzymes such as catalase or small molecules like N-acetylcysteine can abrogate H_2O_2-dependent signaling. H_2O_2 has been shown to alter the phosphoinositide 3-kinase (PI3K) signaling cascade which maintains cellular metabolism, survival, proliferation and angiogenesis. Thus, we sought to examine the role of the PI3K in response to changes in the steady-state concentration of mitochondrial-derived H_2O_2. We have developed a number of redox-engineered cell lines through the expression of *Sod2* (manganese superoxide dismutase) and/or catalase that can be used to modulate the steady state production of mitochondrial H_2O_2. Our studies indicate that the overexpression of *Sod2* leads to oxidative inactivation of the tumor suppressor PTEN/MMAC (mutated in multiple advanced cancer), a negative regulator of the PI3K pathway, which is reversed by the coexpression of catalase. Coincident with the oxidative inactivation of PTEN is an increase in the phosphorylation status of Akt and its downstream target glycogen synthase kinase-3β. In addition, cells overexpressing *Sod2* exhibit increased survival, VEGF expression and cell cycle entry that is reversed upon H_2O_2 removal all of which have been shown to be regulated in an Akt-dependent fashion. These results suggest that effective and targeted H_2O_2 detoxification can inhibit the activation of PI3K/Akt signaling pathway by preserving functional PTEN.

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RECEPTOR-BOUND VERSUS INTRACELLULAR HIV-1 TAT HAS OPPOSITE EFFECTS ON SOD2 GENE EXPRESSION

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We previously reported on the downregulation of sod2 gene expression in HeLa cells stably-transfected with the HIV-1 Tat gene. We now have evidence from DNA footprinting, electrophoretic mobility and promoter-reporter studies that the Sp family of transcription factors is involved in this downregulation. In contrast, we recently observed that endothelial cells exposed to soluble Tat increase the levels of sod2 transcript and protein. Expression of a super-repressor form of IκBα inhibited the SOD2 up-regulation indicating that the Tat effect is mediated by NF-κB. We conclude the Tat will have opposing effects on cellular gene expression depending on whether it is located intra- or extracellularly. Exposure to soluble Tat involves an extracellular interaction that results in activation of signaling cascades whereas intracellular Tat by-passes the signaling cascades and activates transcription factors, such as the Sp family. In HIV infection Tat is secreted from infected cells and is found in the circulation. Thus, it is likely that Tat interacts with un-infected cells and activates receptor-mediated signaling cascades. Therefore, in HIV infection Tat is capable of modulating oxidative stress and other cellular responses in both infected and un-infected cells.

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MNSOD EXPRESSION PROTECTS AGAINST ADRIAMYCIN INDUCED CARDIAC INJURY

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MnSOD has been shown to have a cardioprotective effect. Tamoxifen (TAM), a synthetic nonsteroidal antiestrogen, is being widely used in the breast cancer treatment. In addition to its effectiveness for breast cancer, cardioprotective effect has been reported. To investigate whether the cardiovascular beneficial effect of TAM is due to an ability to induce MnSOD and this is related to the cardioprotective effect of TAM, adult mouse cardiomyocytes were used. Cardiomyocytes treated with TAM and adriamycin (ADR) showed an increase level of MnSOD mRNA. To determine the role of MnSOD in cardiomyocytes, cells from wild type (NTg), heterozygous MnSOD knock out (MnSOD+/-) and human MnSOD transgenic (TgH) mice were used. TUNEL assay indicated that the percentage of apoptotic cells after ADR treatment in MnSOD+/-cardiomyocytes is significantly greater than in NTg and TgH respectively. MTT assay showed that the basal level of mitochondrial function in MnSOD+/- cardiomyocytes is lower than NTg and TgH. Furthermore, MnSOD+/- is more sensitive to ADR induced mitochondrial function injury. ADR treatment increased caspase activity, which significantly higher in MnSOD+/- than NTg and TgH. Furthermore, Caspase-9 was increased after ADR treatment in MnSOD+/- and NTg cardiomyocytes but not in TgH suggesting that mitochondria are targets of ADR induced toxicity in cardiomyocytes and increase MnSOD level provides a protection. Taken together, these results suggest that TAM induced MnSOD expression may, at least in part, contribute to cardioprotective effect of TAM in patients receiving adriamycin for cancer treatment.

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REDOX REGULATION OF RECEPTOR PROTEIN-TYROSINE PHOSPHATASES

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[1]Hubrecht Laboratory, [2]McGill Cancer Center, [3]Ludwig Institute

The receptor protein-tyrosine phosphatases (RPTPs) form a subfamily of the classical protein-tyrosine phosphatases (PTPs). RPTPs are interesting because they have the potential to signal across the cell membrane due to their topology. Evidence is accumulating that RPTPs play important roles during embryonic development and in human disease. Dimerization is emerging as an important regulatory mechanism of RPTPs, and RPTPalpha - a proto-typical RPTP - dimerizes constitutively in living cells. Forced dimerization of RPTPalpha by introduction of a disulfide bond in the extracellular domain leads to inactivation of RPTPalpha, depending on the exact position of the disulfide bond, indicating that rotational coupling of the two monomers in the dimer determines the activity of the dimer. Dimerization-mediated inactivation is supported by the crystal structure of RPTPalpha-D1. Rotational coupling within RPTPalpha dimers is regulated by redox signalling. Oxidative stress leads to stabilization of RPTPalpha dimers, accompanied by inactivation, which is dependent on a conformational change in the membrane-distal PTP domain (D2) of RPTPalpha. Recently, we found that the conformational change in the cytoplasmic domain is reflected by a change in conformation in the ectodomain of RPTPalpha. These results suggest that RPTPalpha has the potential for inside-out signalling. Currently, we are investigating oxidation of RPTPalpha directly, using an antibody that specifically recognizes oxidized catalytic site cysteines of PTPs. We found differential oxidation of the catalytic site cysteines of D1 and D2. Taken together, we provide evidence that redox signalling regulates RPTPs through direct oxidation and through changes in rotational coupling of RPTP dimers.

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IDENTIFICATION OF NUCLEOPHOSMIN (NPM) AS AN NF-KB CO-ACTIVATOR FOR THE INDUCTION OF HUMAN SOD2 GENE

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Previously, we have cloned, sequenced and characterized the human MnSOD gene from various normal and tumor cell lines. We have discovered that several Sp1 and an intronic NF-κB binding element are essential for constitutive and induced expression of MnSOD. We have also found that NF-κB is essential but not sufficient for the synergistic induction of MnSOD by PMA and cytokines. A proteomic approach was used to identify transcription factors and co-activators that participate in the NF-κB mediated induction of MnSOD. Potential NF-κB interacting proteins were isolated from the nuclear extracts of HepG2 cells using an NF-κB affinity chromatography. Proteins eluted from the affinity column were separated using SDS-PAGE, in-gel digested, analyzed by MALDI/TOF, and tentatively identified by peptide mass fingerprinting using MASCOT. Identities were confirmed by LC electrospray tandem mass spectrometry. Among NF-κB interacting proteins, NPM was the most abundant single protein identified in the nuclear extract from PMA and cytokines treated HepG2 cells. Co-immunoprecipitation studies suggest a physical interaction between NPM and NF-κB proteins. To verify the role of NPM on MnSOD gene transcription, HepG2 cells were transfected with constructs expressing NPM in sense or anti-sense orientation. The data indicate that increase expression of NPM leads to increase of MnSOD gene transcription in a dose dependent manner. Consistent with this positive role of NPM on MnSOD, expression of anti-sense NPM leads to the inhibition of MnSOD gene transcription. These results identify NPM as a partner of NF-κB transcription complex to co-activate the induction of MnSOD by PMA and cytokines.

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H2O2 AND UVA MEDIATED OXIDATIVE STRESS INDUCES AP-2 ACTIVITY THAT IS ASSOCIATED WITH ALTERATIONS IN MNSOD EXPRESSION IN HACAT CELLS

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The AP-2 transcription factor family is a group of proteins that regulate a wide spectrum of genes that are involved in cell signaling processes including apoptosis, cell growth, and tissue differentiation during embryogenesis. AP-2 has been shown to be sensitive to redox conditions. Cellular redox states can be influenced by many environmental factors including UV radiation, especially longer wavelength UVA, in the 320-400 nm range. UV irradiation is a major risk factor for skin cancer, and UVA is a source of reactive oxygen species in the form of singlet oxygen (1O2). Previous work by others and us has demonstrated that 1O2 is a potent activating stimulus for AP-2 mediated signaling. We hypothesized that UVA and other oxidative stressors may be activating stimuli for AP-2 in keratinocytes. We treated HaCaT cells, an immortalized human keratinocyte cell line, with either UVA irradiation or H2O2 and measured their AP-2 response. After treatment with H2O2, both AP-2a mRNA and protein level were substantially increased over a time course of 3 h. During the same time course, the level of SOD2 mRNA displayed a marked

decrease. The AP-2 DNA binding activity reached its peak as early as 1 h and then returned to normal levels by 2 h. We also measured the changes of AP-2a and SOD2 mRNA levels after exposure of HaCaT cell to UVA. We observed two peaks of AP-2a mRNA at 4 h and 24 h after UV exposure. AP-2a protein levels also increased as a function of UVA dose. Interestingly, SOD2 mRNA levels displayed a similar biphasic increase in steady state mRNA with peaks at 3 h and 24 h. Taken together, our results suggest that AP-2a joins a growing list of redox sensitive transcription factors that govern cellular responses to oxidative stress.

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REGULATED EXPRESSION OF CATALASE AND ITS EFFECTS ON REACTIVE OXYGEN SPECIES AND GROWTH IN A MALIGNANT CELL LINE

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A previously developed malignant tumor progression model with benign papilloma producing mouse skin keratinocytes has shown that expression of catalase is repressed in malignant cell lines [Cancer Letters 173:115-125, 2000]. These cells lines had upregulated reactive oxygen species compared to the benign parent cell line and formed subcutaneous tumors in nude mice. Histological studies of chemically initiated tumors revealed catalase expression in benign tumors while squamous cell carcinomas showed reduced catalase staining. We hypothesize that catalase, an anti-oxidant defense enzyme, acts as a tumor suppressor by reducing cellular hydrogen peroxide radical, which may be utilized as a growth signal in malignant cells. The malignant 6M90 cell line was isolated from a nude mouse tumor from cells surviving 6 treatments of 90% toxicity with MNNG. We further adapted this cell line using the two-plasmid tet-on expression system T-Rex to express a myc-tagged catalase under the control of doxycycline. This cell line, MTOC2, demonstrates robust doxycycline-regulated overexpression of myc-tagged catalase transgene levels by western analysis. Consistent with this expression, the catalase enzyme assay demonstrated doxycyline-regulated overexpression of catalase in the MTOC2 cell line. Decreased hydrogen peroxide levels were shown in MTOC2 compared to 6M90 cells using a hydrogen peroxide sensitive assay that adds dichlorodihydro-fluorescein diacetate (DCFH-DA) directly to a growing monolayer of cells followed by detection of fluorescent oxidized dye by flow analysis. Doxycycline-regulated overexpression of transgene catalase inhibited in vitro cell growth compared to control, supporting the idea that catalase has growth repressing activity. This work is supported by NIH grant RO1 CA40584-18.

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IDENTIFICATION OF THE HUMAN SUCCINATE DEHYDROGENASE D (SDHD) TRANSCRIPT AS A TARGET OF RNA EDITING

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Complex II is an integral part of the enzyme complex of both the Kreb's cycle and electron transport chain (ETC). Complex II transfers electrons from succinate through FADH to CoQ. Germline mutations in SDHD cause familial neuroendocrine tumors suggesting a role for SDHD as a tumor suppressor. These findings led us to search for SDHD mutations in neuroblastoma (NB), another neural crest cell derived tumor. Among 5 human NBs we identified no mutations in the SDHD gene. However, upon examination of SDHD mRNA we identified in-frame stop codons in the cDNAs of 5/5 NBs. These resulted from a modification of cytidine to uridine, creating a change from an arginine (CGA) to a stop codon (UGA) at codon 22 in a subset of transcripts. These results suggest that SDHD mRNA is a target of posttranscriptional editing, and that the C to U conversion is the result of cytidine deamination. To confirm this C to U conversion we used the restriction enzyme SfuI that recognized the sequence of the stop codon. SfuI restriction of SDHD cDNA derived from NB mRNA showed a heterogeneous population of both the arginine and stop codons with about 10-40% stop codons. Thus SDHD joins fewer than ten known examples of edited mRNAs in humans. While the biological significance of SDHD RNA editing is not yet clear, aberrant RNA editing of SDHD could lead to stoichiometric mismatches of ETC components, increased electron leakage, superoxide production, and tumorigenesis. Clearly, more studies will be needed to determine the role and levels of SDHD editing in normal versus cancerous tissue. Supported by NIH CA66081

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GLUTATHIONE REGULATES ASBESTOS-INDUCED AP-1 PROTOONCOGENES

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Reactive oxygen species play an important role in asbestos-induced responses. Here we determined the role of oxidative stress in asbestos-induced upregulation of activator protein-1 (AP-1) family member genes in C10 lung epithelial cells. Cells were exposed to catalase, deferoxamine or N-acetyl cysteine (NAC) prior to exposure to crocidolite asbestos. Ribonuclease protection assays showed no effect of catalase or deferoxamine on asbestos-induced steady state mRNA levels of AP-1 family member genes. However, NAC or glutathione ester inhibited the upregulation of *c-jun*, *jun B* and *fra-1*, whereas buthionine sulfoxamine (BSO) increased mRNA levels. Asbestos-induced *fra-1* promoter activity was also increased by BSO. Moreover, asbestos-exposed cells showed depletion of reduced glutathione by HPLC. Both subunits of γ-glutamylcysteine synthetase (γ-GCS) were upregulated by asbestos at the transcriptional (TaqMan, quantitative PCR) as well as at the translational levels (Western blots). Asbestos-induced epidermal growth factor receptor (EGFR) kinase activation, which is causally associated with increased AP-1 family member expression, was also dependent on glutathione levels. Presently we are exploring whether overexpression of either or both subunits of γ-GCS can rescue cells from asbestos-induced injury. Supported by PO1 HL67004 from NHLBI.

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DISTINCT MOLECULAR MECHANISMS MEDIATE AS3+-INDUCED GLUTAMATE-CYSTEINE LIGASE SUBUNIT GENE EXPRESSION IN MURINE HEPATOCYTES

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Trivalent arsenite (As3+) is a known human carcinogen associated with increased incidence of skin, kidney, bladder, lung and liver cancers. While the molecular mechanism(s) mediating the cytotoxic and carcinogenic effects of As3+ are not completely understood, reactive oxygen species production have been implicated. Glutathione (GSH) constitutes a vital cellular defense mechanism against oxidative stress. The rate-limiting enzyme in GSH biosynthesis is glutamate-cysteine ligase (GCL), a heterodimeric holoenzyme composed of a catalytic (GCLC) and a modifier (GCLM) subunit. In this study, we demonstrate that As3+ coordinately upregulates the steady-state levels of gclc and gclm mRNA resulting in increased GCL subunit protein

expression and GCL enzymatic activity in the TAMH murine hepatocyte cell line. As3+ increases the rate of gene transcription of both the gclm and gclc genes. Interestingly, As3+ treatment also results in the post-transcriptional stabilization of gclm mRNA. Furthermore, the antioxidant N-acetylcysteine inhibits As3+-induced gclc, but not gclm, expression revealing a distinct redox-dependent induction of gclc. As3+ induction of gclc is dependent on p38 MAPK and ERK activity and is repressed by JNK activation, while gclm induction is partly mediated by p38 MAPK. Surprisingly, induction of both gclc and gclm occurs independent of Nrf1/2. These findings demonstrate that distinct transcriptional and post-transcriptional mechanisms mediate the coordinate induction of the GCL subunits in response to As3+ and highlight the potential importance of the GSH antioxidant defense system in regulating As3+-induced responses in hepatocytes.

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OXIDATIVE STRESS IMPAIRS C-CBL MEDIATED UBIQUITINYLATION AND EGF RECEPTOR TRAFFICKING

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Activation of the epidermal growth factor receptor (EGFR) by its ligand EGF results in receptor endocytosis, degradation, and consequentially reduced receptor signaling. We have shown that under oxidative stress (in the form of H_2O_2) the EGFR lacks phosphorylation at tyrosine (Tyr) 1045, resulting in failure to recruit the ubiquitin ligase c-Cbl and inability to undergo ubiquitinylation and endocytosis. Since Tyr 1045 phosphorylation is essential for c-Cbl recruitment and ubiquitinylation of the EGFR, we used a mutant receptor in which a phenylalanine residue was substituted for Tyr 1045 (Y1045F). To compare the wild type (WT) and mutant receptor trafficking, we co-transfected CHO cells with c-Cbl and the respective receptor. Confocal microscopy showed internalization and the entry into endosomes of both receptors. However, biotinylation assays established that both WT-EGFR treated with H_2O_2 and Y1045F are internalized with significantly slower kinetics compared to WT-EGFR treated with the ligand EGF. Moreover, similar to WT-EGFR treated with H_2O_2, Y1045F fails to be degraded at extended time points and lacks the WT-EGFR levels of ubiquitinylation. Our data thus suggest a role for c-Cbl mediated ubiquitinylation in receptor degradation, but not in receptor internalization. H_2O_2 stimulation thus results in an activated receptor unable to undergo normal down regulation, which could lead to oxidant-mediated tumorigenesis.

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ROLE OF ROS IN IL-1B STIMULATED ERK1/2 ACTIVATION IN VASCULAR SMOOTH MUSCLE

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The proinflammatory cytokine interleukin 1β (IL-1β) stimulates production of nitric oxide (NO) in vascular smooth muscle cells (VSMC) through upregulation of inducible nitric oxide synthase (iNOS). While some studies suggest that activation of the MAPK family member ERK1/2 is necessary for iNOS expression by coordinated activation of NF-κB, others suggest that there are ERK1/2 independent pathways leading to iNOS expression. Since this pathway has been shown to be regulated by reactive oxygen species (ROS), it is concievable that ROS also regulate iNOS expression through ERK1/2 and NF-κB. The purpose of this study was to determine whether IL-1β induced ERK1/2 activation was involved in iNOS expression and to investigate a role for ROS in this regulation. To demonstrate the role of ERK in iNOS expression, cultured rat aortic VSMC were pretreated with U0126, a specific MEK inhibitor, followed by IL-1β stimulation. U0126 blocked ERK activity at 10 minutes, measured by immunoblotting (IB), iNOS expression, and the accumulation of nitrite in the extracellular media. Since ROS such as hydrogen peroxide (H_2O_2) are known to regulate ERK1/2, VSMC were loaded with catalase for 24 hours followed by IL-1β stimulation. Catalase potentiated the IL-1β induced ERK1/2 activation and iNOS expression, suggesting that H_2O_2 negatively regulates iNOS through ERK1/2. Catalase had no effect on p38 MAPK activation, suggesting that the effects of H_2O_2 production is specific to the ERK1/2 pathway. These results demonstrate that IL-1β induced ERK1/2 activation is required for iNOS expression, and H_2O_2 negatively regulates ERK1/2 activation and iNOS expression.

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THIOREDOXIN-1 BECOMES MORE OXIDIZED THAN GLUTATHIONE IN RESPONSE TO EPIDERMAL GROWTH FACTOR SIGNALING

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Epidermal growth factor (EGF) is involved in a ligand-induced signal transduction pathway. An increase in reactive oxygen species (ROS) accompanies EGF binding to its receptor and potentiates the phosphorylation cascade that leads to MAP kinase activation and cell proliferation. In order to investigate this oxidant signaling process we examined the redox state of glutathione (GSH) and thioredoxin (TRX1), two of the major cellular redox couples, after EGF treatment in human keratinocytes. GSH is the most abundant non-protein thiol while TRX1 is a 12kDa oxidoreductase involved in regulation of redox-dependent proteins. Using a dichlorofluorescein (DCF) assay, we observed a 22% increase in ROS in EGF (200 ng/ml) treated HaCaT cells after five minutes. Hydrogen peroxide (500 μM) caused a two fold increase in oxidation. GSH and the disulfide form (GSSG) were quantified by HPLC. EGF (200 ng/ml) did not cause significant oxidation of the GSH/GSSG redox couple compared to untreated controls whereas hydrogen peroxide (500 μM) treatment resulted in substantial oxidation of GSH. A Redox Western blot methodology for quantification of TRX1 redox state showed a 50% increase after two minute EGF (200 ng/ml) treatment. Maximal TRX1 oxidation occurred after two minutes and by ten minutes TRX1 redox state had returned to control levels. These results show that the GSH redox couple and the TRX1 redox couple respond differently to EGF stimulation. Given the abundance of GSH, it is surprising that EGF-induced ROS does not cause its oxidation. TRX1 oxidation during EGF signaling may be a ROS-independent process as is suggested by the differential redox states of TRX1 and GSH.

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INDEPENDENT REDOX REGULATION BY THIOREDOXIN AND GLUTATHIONE

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Glutathione (GSH) is the most abundant non-protein thiol pool and detoxifies free radicals and reactive intermediates. Thioredoxin (TRX1) is a small 12 kDa oxidoreductase that is not only implicated in detoxification but also in the regulation of several redox-sensitive transcription factors. However, the relationship and interaction between these two thiol pools, GSH and TRX1, has not been well defined. HT29 cells were treated with buthionine sulfoximine (BSO; 1-100 μM) for 0, 4, 24 hr to deplete intracellular GSH. GSH concentrations were determined via HPLC. GSH concentrations were significantly depleted. Interestingly, the remaining GSH pools were also significantly oxidized (-218 mV) compared to controls (-256 mV). Following BSO treatment, HT29 TRX1 status was determined by TRX Redox Western analysis. No significant changes in TRX1 redox status were observed, but at 24 hr, there was a significant

increase in total intracellular TRX1, suggestive of ARE involvement. Activation of the ARE results in the increased expression of numerous genes including glutamate cysteine ligase (GCL), the enzyme responsible for *de novo* GSH synthesis. The upregulation of TRX1 coupled with the preservation of TRX1 redox status during GSH depletion indicates that TRX1 may be a critical factor in the regulation of ARE-dependent gene expression. Using empty, wild-type TRX1, and active-site mutant (C35S) TRX1 expression vectors, cells were co-transfected with the reporter construct, GCL-ARE4-Luciferase. Co-transfection with wt TRX1 increased luminescence by 120%. Conversely, co-expression of the C35S TRX1 mutant decreased luminescence by nearly 50%. These findings show that GSH and TRX1 redox states are independently regulated and that TRX1 plays a role in regulation of ARE-mediated GCL gene expression.

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DIFFERENTIAL EXPRESSION OF DSCR1(ADAPT78) ISOFORMS IN ALZHEIMER?S DISEASE

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DSCR1(Adapt78) was identified by our laboratory, during differential display studies, as a gene strongly upregulated in mammalian cells during transient adaptation to oxidative stress. Previous work from our laboratory has shown there are significant levels of DSCR1(Adapt78) mRNA expressed in adult human brain, but levels were chronically upregulated in areas affected by Alzheimer's disease. This gene may be protective against transient oxidative stress, but may be damaging when chronically expressed, which now appears to occur in Alzheimer's disease. The *DSCR1 (Adapt78)* protein product, calcipressin 1, has been identified as an inhibitor of calcineurin, a major brain phosphatase. It is likely that adaptation to oxidative stress by calcipressin 1 occurs through regulation of this protein. This gene contains seven exons, that are alternatively spliced. We have found two isoforms, that are expressed in human brain, isoform 1 containing exons 1, 5,6 and 7 and isoform 4 containing exons 4, 5,6 and 7. We examined the expression these two major isoforms in brain and found that both isoform 1 and isoform 4 mRNAs are expressed predominantly in neuronal cells in both control and Alzheimer's disease post mortem samples; using combined in situ hybridization and immunocytochemistry. We further determined that both isoforms of calcipressin 1 are expressed in adult human brain using custom antibodies against exon 1 and exon 4, and expression of both isoforms of calcipressin 1 are greater in regions of the brain affected by Alzheimer's disease compared to age matched controls. The association between DSCR1(Adapt78) and Alzheimer's does not seem to be due to a shift in isoform expression, or due to a shift in cell type expression, but rather to increased expression of both major isoforms in regions affected by the disease.

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ANGIOTENSIN (A-II)-INDUCED CARDIOMYOCYTE HYPERTROPHY: ROLE OF REACTIVE OXYGEN SPECIES, NFKB, AND AKT/PROTEIN KINASE B

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A-II is implicated both in hypertrophy and in the pathogenesis of heart failure. Our laboratory recently showed A-II-induced cardiomyocyte hypertrophy is dependent on cytoplasmic superoxide generated from a Rac1-regulated NAD(P)H oxidase. Superoxide-mediated activation of the transcription factor NFκB is also involved. Here we tested the hypothesis that redox-sensitive regulation of Akt/Protein Kinase B (Akt) plays a central role in the activation of NFκB and in A-II-mediated cardiomyocyte hypertrophy. A-II (5μM) caused a marked increase in Akt activity in primary neonatal cardiomycoytes (2.6±0.26-fold, $p<0.01$ vs vehicle, n=4), which was abolished by overexpression of an adenovirus encoding cytoplasmic superoxide dismutase (AdSOD, 1000 MOI, $p<0.01$ vs A-II alone, n=3). AdSOD treatment also significantly reduced the increases in NFκB binding activity and transcriptional activation following A-II treatment (1000 MOI, $p<0.05$ vs. A-II alone, n=3). To explore the role of Akt in A-II-mediated NFκB activation, we treated cardiomyocytes with an adenovirus encoding a dominant-negative form of Akt for 24 hr (AdDNAkt, 500 MOI). A-II-stimulated increases in NFκB binding activity and transcriptional activation were significantly inhibited by AdDNAkt overexpression ($p<0.05$ vs. A-II alone, n=3). AdDNAkt treatment also reduced A-II-stimulated hypertrophy by 71.1±2.4% ($p<0.01$ vs. A-II alone, n=65 cells in each group), whereas a control vector had no effect. These results suggest that Akt is a central component of the redox-signaling cascade mediating A-II-induced activation of NFκB and hypertrophy in cultured cardiomyocytes, and may represent a novel therapeutic target for heart failure.

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DEFEROXAMINE SYNERGISTICALLY ENHANCES IRON-INDUCED STIMULATION OF ACTIVATOR PROTEIN-1: KINASE ACTIVATION VERSUS PHOSPHATASE INHIBITION

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Deferoxamine (DFO) is the only approved drug in the US for iron overload treatment. Studies have demonstrated the ability of DFO to remove iron bound to low molecular weight (LMW) chelators. Because of its ability to sequester LMW iron, DFO is known as an antioxidant. In the present study, we have shown in mouse epidermal JB6 cells that ferrous sulfate transactivated activator protein-1 (AP-1), an oxidative stress-responsive transcription factor. We have further shown that iron stimulated AP-1 through the phosphorylation of extracellular signal-regulated kinases (ERKs) and p38 mitogen-activated protein kinases (MAPK) but not c-jun NH2 terminal kinases (JNKs) after 15-90 min treatments. Interestingly, pretreatment of cells with DFO, followed by iron treatment, synergistically increased AP-1 activity over the control as compared to iron alone. DFO inhibited iron-induced ERKs and p38 MAPK phosphorylation within 1 h of treatment, whereas it further enhanced ERKs and p38 MAPK phosphorylstion in longer periods of treatment (over 6 h), which resulted in a synergistic stimulation of AP-1 by DFO and iron. Since the increased phosphorylation of ERKs and p38 MAPK may be due to an imbalance between phosphatases and kinases, we have measured the ability of cell extracts to remove phospho-ERKs and phospho-p38 MAPK. We have found that cell extracts treated with DFO or ferroxamine (a DFO-Fe complex) resulted in decreased dephosphorylation. These results suggest that DFO inhibits the tyrosine and serine/threonine phosphatases and iron activates ERKs and p38 MAPK, which lead to a synergistic increase in AP-1. The clinical implication of this observation may be important because many genes are regulated by AP-1.

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HNE INDUCES HO-1 THROUGH ACTIVATION OF THE ERK AND JNK PATHWAYS IN ALVEOLAR TYPE II CELLS

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Heme oxygenase-1 (HO-1) is a cytoprotective enzyme and an established marker of oxidative stress. It has recently been shown that HO-1 expression is induced by the lipid peroxidation product 4-hydroxynonenal (HNE). HNE induces oxidative stress *in vivo* and also activates cell signaling. In some systems, HNE has been shown to activate the mitogen-activated protein kinases (MAPK). HO-1 induction by HNE has not been reported in the lung and the signaling pathways linking HNE to HO-1 induction are not well defined. Here we determine that HNE increases HO-1 mRNA and protein in alveolar type II cells, and that the ERK and JNK MAPK are the key pathways involved. L2 cells were treated with 20 μM HNE for various lengths of time. Western blotting revealed an increase in HO-1 protein at 5 hours post-incubation. Real-time PCR analysis confirmed that the increase in HO-1 protein was preceded by increased HO-1 mRNA. Treatment with HNE increased phospho-ERK, c-Fos protein, total and phospho-c-Jun. Gel shift analysis confirmed that HNE increased AP-1 binding, and supershift analysis identified that the binding complexes were c-Fos/c-Jun heterodimers. The ERK pathway inhibitor PD98059 significantly blocked HNE-mediated ERK phosphorylation, c-Fos protein induction, AP-1 binding, and HO-1 protein induction. Marked Nrf2 translocation to the nucleus was observed, which is consistent with the involvement of the stress response element (StRE). Future studies will investigate coordinate regulation of HO-1 gene expression by HNE via StRE and AP-1 in type II cells.

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ANTIOXIDANTS ATTENUATE DENDRITIC CELL MATURATION AND FUNCTIONS

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Dendritic cells (DC) are potent antigen presenting cells. Matured DC have enhanced expressions of major histocompatibility complex (MHC)and co-stimulatory molecule expressions that contribute to binding and activation of T-lymphocytes. We hypothesize that antioxidants may inhibit co-stimulatory molecule expression on DC that lead to reduced lymphocyte activation. We investigated the effects of N-acetyl-L-cysteine (NAC) and AEOL10113, a superoxide dismutase (SOD) mimetic, on bone marrow derived DC, focusing on the co-stimulatory molecule expressions and DC induced T cell proliferation. LPS was used to stimulate DC maturation. DC surface molecule expression was detected by flow cytometry. We found that NAC- and AEOL10113-treated DC exhibited lower surface expressions of CD11b, CD40, CD54 and CD86. However, NAC has a more potent effect on LPS stimulated expressions of co-stimulatory molecules on DC cell surface. On the other hand, AEOL10113, but not NAC, treated DC inhibited T cell proliferation in mixed lymphocyte reactions. These results suggest that antioxidants inhibit LPS induced DC maturation. In addition, NAC and AEOL10113 modulate different molecular signaling pathway in LPS-stimulated DC.

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MODULATION OF ENDOGENOUS MNSOD EXPRESSION BY INDUCIBLE OVEREXPRESSION OF EXOGENOUS MNSOD

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Previous studies from several laboratories have demonstrated an inverse correlation between the expression levels of manganese superoxide dismutase (MnSOD) and cell proliferation potential in nonmalignant and malignant cells both *in vitro* and in nude mice transplantation studies *in vivo*. Increased steady state levels of hydrogen peroxide (H_2O_2) due to unbalanced enzymatic function of MnSOD have been suggested to be involved in MnSOD-mediated cell growth inhibition. To study the possible mechanism(s) by which MnSOD inhibits cell proliferation, we developed an inducible MnSOD overexpression system in mouse fibroblast NIH/3T3 cells. Previous studies using inducible MnSOD overexpression clones demonstrated reversible and transient changes in cell cycle transition times, a transient increase of reactive oxygen species (ROS), and concomitant decrease in mitochondrial membrane potential following MnSOD induction. (*Antioxidants & Redox Signaling*, in press). Following induction, we demonstrated subsequent decrease in immunoreactive MnSOD protein levels in inducer-concentration and induction-time dependent manners. We postulated possible down-regulation of endogenous MnSOD expression following induction of exogenous MnSOD expression. In the current study, we demonstrated that modulation of endogenous MnSOD expression occurs at the mRNA level using quantitative real time PCR. Our studies suggest that the expression of MnSOD is tightly regulated to protect mitochondria from endogenously generated ROS as well as to maintain optimal mitochondrial redox state for cellular proliferation.

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ESCHERICHIA COLI THIOL PEROXIDASE (P20) AS A HEAT-SHOCK INDUCIBLE PROTEIN ACTS AS AN ANTIOXIDANT AGAINST A CELLULAR OXIDATIVE DAMAGE CAUSED BY HEAT SHOCK

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E. coli thiol peroxidase (p20) was characterized as a lipid hydroperoxidase. The null mutant strain showed the highest sensitivity toward various oxidative stresses among three types of null mutants (i.e., p20, AhpC, and bcp mutants). In sharply contrast to AhpC, p20 transcriptional activity was not changed upon various oxidative stresses. The transcriptional activity of p20 promoter was exponentially increased as a function of cell growth. The transcriptional activity was highly induced in the stationary-phased growth in aerobic condition, but in a strict anaerobic culture, the activity was decreased to a much lower level. P20 transcriptional activity was significantly induced upon the exposure of heat shock. Viability of p20 mutant against heat shock was considerably reduced compared to the isogenic strain. Collectively, these data suggest that p20 acts as an antioxidant against a cellular oxidative damage caused by heat shock.

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TRANSCRIPTIONAL MECHANISMS FOR ARSENIC-INDUCED VEGF EXPRESSION.

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Arsenite (As) exposure in drinking water increases incidence of cancer, diabetes and cardiovascular disease. Vascular remodeling is important in these diseases and As enhances neovascularization *in vivo*. To investigate the mechanisms for this neovascularization, As-induced expression of vascular endothelial growth factor (VEGF), a strong angiogenic factor, was examined in primary smooth muscle cells. As caused prolonged, dose-dependent increases in VEGF mRNA and protein levels. A concomitant increase in hypoxia-inducible factor-1α (HIF) protein occurred with a time course that was consistent with induction of VEGF transcription. However, the hypothesis that HIF mediated As-induced VEGF expression was disproved by the failure of HIF-specific siRNA to inhibit the response. Broad transcription factor arrays of nuclear proteins isolated from control or 24 hour, As-treated cells were conducted to identify transcription factors that support the sustained expression of VEGF. In addition, to examine *in vivo* changes in cardiovascular transcription factors, arrays were performed on nuclear proteins isolated from the hearts of mice that had been chronically exposed to 50–500 ppb of As in their drinking water. EMSAs were used to confirm the transcription factor changes identified in the arrays. Of the transcription factors that were known to drive VEGF expression, AP1 and Ets1/PEA3 increased in both models. Increases in smooth muscle Creb, AP-2, Ets, GATA, and NFAT were also observed. Notably absent were increases in NF-κB or proteins binding to heat shock elements. These studies demonstrate that As activates a complex, integrated program of transcriptional activation to increase expression of angiogenic factors that may contribute to As-induced vascular remodeling and cardiovascular diseases. *Supported by NIEHS grant ES07373.*

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EGF RECEPTOR IS A COMMON MEDIATOR OF QUINONE-INDUCED SIGNALING LEADING TO THE LOSS OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

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Doxorubicin, an anthraquinone derivative widely employed in cancer chemotherapy, was tested for its effects on gap junctional intercellular communication (GJC). Exposure of rat liver epithelial cells to doxorubicin led to a dose-dependent decrease in GJC, due to phosphorylation of connexin-43, the major connexin present. The effect was mediated by MEK 1,2 as well as by activation of ERK 1 and ERK 2. By contrast, ERK 5, another connexin kinase, was not involved. Ligand-independent activation of the EGF receptor (EGFR) was responsible for ERK activation, connexin phosphorylation and subsequent loss of GJC. Activation of this cascade was dependent on NAD(P)H:quinone oxidoreductase-1, pointing to a role of doxorubicin redox cycling. In line with this, an exclusively redox cycling quinone, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), activated this same pathway, concomitant with phosphorylation of connexin-43 at Ser^{279} and Ser^{282}. At least two further mechanisms for ligand-independent activation of the EGFR by quinones exist: (i) menadione activated the above cascade by blocking a tyrosine phosphatase which negatively regulates the EGFR, (ii) *p*-benzoquinone, a strongly alkylating quinone, led to a drastic decrease in cellular glutathione concentrations, sufficient for activation of the EGFR-ERK cascade; preincubation with N-acetyl cysteine prevented activation, and the mere depletion of GSH by application of diethyl maleate EGFR-dependently activated ERK, thus mimicking BQ. In summary, different quinones affect the same signaling pathway leading to downregulation of GJC, converging at the level of the EGFR.

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ACTIVATION OF ERK 1/2 AND AKT BY H2O2 PRODUCED BY GLUCOSE OXIDASE AND BY MONOAMINE OXIDASE A.

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Although H2O2 appears to be relatively long-lived in cells, it also acts as a localized mediator of signal transduction processes, a property expected of a short-lived species. To understand these apparently contrasting aspects of H2O2, we are developing systems to assess the influence of the location for H2O2 formation on specific cell processes. H2O2 was produced external to human keratinocytes from glucose oxidase (GO) and glucose in the medium and also by monoamine oxidase A (MAO-A), which resides on the cytoplasmic surface of the outer mitochondrial membrane. Production of H2O2 by GO /glucose was followed by the Amplex red fluorescence method. A steady state concentration of H2O2 was established between production by GO and consumption by the cells. Under these conditions, the level of pERK1/2 and pAkt increased. The phosphorylation was inhibited by AG1478, a specific inhibitor of EGFR kinase activity, and was enhanced by mercaptosuccinate (MS), an inhibitor of glutathione peroxidase. pERK1/2 was also enhanced by treatment of keratinocytes with tyramine, a substrate for MAO-A, in a dose dependent fashion, which was inhibited by AG1478 and enhanced by MS. These results suggest that H2O2 produced both externally and by a localized intracellular source can induce EGFR-mediated signaling.

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CARBONYLATION OF NF-KB BY OXIDIZED LOW-DENSITY LIPOPROTEIN IMPAIRS THE TRANSCRIPTIONAL ACTIVITY.

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An increase in the levels of oxidized low-density lipoprotein (oxLDL) in plasma from patients with hypercholesteremia is believed to be an atherogenic factor. It is considered that macrophages taking up oxLDL could modify the production of cytokines and other factors through oxidative-sensitive signaling pathways, and then initiate and promote atherosclerosis. However, it is not known how the functions of macrophages could be modified by chronic exposure to oxLDL. In the present study, we examined whether inflammatory stimulation of macrophages by lipopolysaccharide (LPS) is modified when the cells were exposed to oxLDL. We found that the nuclear factor-kB (NF-kB)-DNA binding activity by LPS (100 ng/ml) was suppressed by a 48-h pre-treatment with oxLDL (50 mg/ml), and this was due to a carbonylation of NF-kB subunit p65. With a-tocopherol, the oxLDL-induced carbonylation of proteins decreased with a restoration of DNA-binding activity of NF-kB. Histochemical analysis showed that carbonylated proteins were found in left mammalian artery from patients with ischemic heart disease, depended on the clinical stages. these indicate that oxidative modification of NF-kB suppresses LPS-induced expression of genes in ox-LDL-treated cells, suggesting an implication of oxLDL-induced modulation of NF-kB signaling. Together, chronic oxidative stress may modify protein structures in artery and promotes atherosclerosis.

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OXIDATIVE STRESS ELEVATES THE MOLAR RATIO OF GLUTAMATE CYSTEINE LIGASE (GCL) CATALYTIC TO MODULATORY SUBUNITS

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Exposure to reactive oxygen species (ROS) is known to increase cellular glutathione (GSH) content, primarily by increasing the rate of GSH synthesis. The enzyme that catalyzes the rate-limiting step of GSH biosynthesis is glutamate cysteine ligase (GCL); a heterodimeric protein composed of a catalytic (GCLC) and modulatory (GCLM) subunit. The molar ratio of GCLC:GCLM is unknown *in vivo* and it's unclear how ROS affect this ratio. We measured changes in cellular GSH, *Gcl* mRNA and GCL protein levels in response to the model oxidants 4-hydroxy-2-nonenal (4HNE), 2,3-dimethoxy-1,4-napthoquinone (DMNQ), and tert-butylhydroquinone (tBHQ). 4HNE and tBHQ increased cellular GSH levels, while DMNQ exposure depleted GSH. Interestingly, all treatments increased *Gclc* and *Gclm* mRNA, which was accompanied by a concomitant increase in GCLC protein content. In contrast, only 4HNE and DMNQ resulted in an increase in GCLM protein content that was consistent with the increase in *Gclm* message. While the changes in *Gcl* mRNA largely paralleled the changes in GCL protein, the magnitude of these changes clearly differed. Changes in GCL protein content in response to these treatments dramatically increased the molar ratio of GCLC:GCLM. These data demonstrate that alterations in cellular GSH are dependent on GCLC, suggesting that GCLM's principle role is indeed regulatory and not obligatory for GSH biosynthesis under physiologically relevant conditions of oxidative stress.

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ZINC SENSITIVE GENES IN THE MOUSE BRAIN: RESULTS FROM A HIGH-DENSITY OLIGONUCLEOTIDE MICROARRAY ANALYSIS

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Zinc (Zn) is an essential trace element required for normal brain development and function. Although the detrimental effects of Zn deficiency on brain function have been established, the underlying mechanisms remain unknown. We have hypothesized that the changes in brain function during Zn deficiency may result from alterations in gene expression directly related to Zn specific functions. We therefore sought to identify genes modulated by dietary Zn deficiency in vivo by comparing the mRNA expression profiles from the brain of Zn deficient compared to Zn adequate mice using a high-density oligonucleotide microarray. In this model using 6 wk old male mice and a dietary Zn deficiency protocol, we observed a significant depression in two parameters of Zn status (i.e. decreased serum Zn and pancreatic metallothionein level) between treatments. The results of the microarray analysis indicated that approximately 0.6% of expressed genes were differentially expressed in the brains of Zn deficient mice. Predominant among these were genes associated with RNA and protein biosynthesis (e.g. RNA polymerase I), embryogenesis and development (e.g. Hoxa-5) and cell adhesion and cell signaling (e.g. Ncam2). These observations provide evidence that Zn deficiency is associated with specific transcriptional alterations in brain tissue and that alterations in mRNA expression may account for some of the distinct phenotypic manifestations observed in Zn deficiency.

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DIFFERENTIAL EFFECTS OF H2O2 AND S-NITROSOCYSTEINE ON AKT AND MAP KINASE PATHWAY

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Biological oxidants play important signaling roles, in part, by altering the oxidation state of critical protein thiols. Through this mechanism, H2O2 has been found to increase proliferation and enhance survival of cells in culture. Activation of Akt (protein kinase B) and MAP kinases (ERK1/2) appear to be important in mediating oxidant signaling. In this regard, we have found that H2O2 increased the level of phosphorylation of Akt and ERK1/2 in A431 cells. Akt phosphorylation was inhibitable by AG1478 and LY294002 suggesting involvement of the EGF receptor and PI3 kinase. In addition, H2O2 inhibited protein tyrosine phosphatase 1B and increased the level of phosphorylation of EGFR. Since we had previously shown that S-nitrosothiols also potently inhibit PTP1B, we examined the effects of S-nitrosocysteine (CSNO) on the same pathways. CSNO was found to increase EGFR phosphorylation, but did not increase pAKT or pERK1/2 levels. In fact, CSNO was shown to block EGF-stimulated Akt and ERK1/2 phosphorylation. Furthermore CSNO but not H2O2 was found to inhibit EGF-stimulated ras activation. Since ras acting through raf1 is upstream of ERK1/2 and an activator of PI3 kinase it seems likely that the inhibitory effects of CSNO on EGF signaling are mediated through this mechanism. These data clearly show that two oxidants, which are well known to target protein thiols, differentially regulate downstream targets and thus may potentially produce different effects on growth and survival.

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THE ROLE OF NITRIC OXIDE IN THE PARTICULATE MATTER (PM2.5)-INDUCED NFKB ACTIVATION IN LUNG EPITHELIAL CELLS

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NFkB is one of key transcription factors involved in the inflammatory responses in the lung to the particulate matters (PM). To improve our understanding on its molecular mechanism, the effects of antioxidants and an inhibitor of inducible nitric oxide synthase (iNOS) were examined on the PM-induced NFkB activation in A549 lung epithelial cells. The NFkB activation by PM2.5 was evident from the degradation of an NFkB inhibitory protein, IkBa, and a luciferase reporter assay for the NFkB activity. In these experiments, pre-treatment of cells with antioxidants (N-acetyl-L-cysteine and dimethylthiourea) or an iNOS inhibitor, L-N6-1-iminoethyl-lysine (L-NIL) clearly inhibited the NFkB activation by PM2.5. The inhibitor effect of L-NIL was also seen on the PM2.5-induced interleukin 8 (IL-8) expression both at the transcriptional and protein levels. These results suggest that PM2.5 induces NFkB activity via pathways involving ROS and/or RNS generation. From the fact that NFkB also induces NO generation via iNOS expression, they might make a positive feedback loop to amplify the downstream responses. Supported by Korea Research Foundation(2003).

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EFFECTS OF DIESEL EXHAUST PARTICLES (DEP) ON THE ALVEOLAR MACROPHAGES FOR INOS INDUCTION AND NITRIC OXIDE WITH NITROTYROSILATED-PROTEIN FORMATION

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Alveolar macrophages (AM) and airway epithelial cells are known as important cellular targets in DEP-induced lung diseases. Other studies have shown that nitric oxide (NO) is involved in particle matter induced lung injury. The underlying precise mechanism has not been elucidated yet, however NO-induced structural modification of diverse functional proteins is believed as a one of the basic pathogenic mechanism.

The present study was undertaken to determine whether DEP has an synergistic effects on LPS-induced NO formation and iNOS with nitrotyrosilated-protein formation in cultured primary alveolar macrophages. The formation of NO was determined through the Griess reaction in the cultured medium and iNOS with nitrotyrosilated-proteins are analyzed by immunohistochemical staining and western analysis.

The results indicate that DEP exposure does not induce NO formation by itself, however DEP showed significant synergistic effects on LPS-induced NO formation. So, our results suggest that DEP inhalation could aggravate inflammatory lung disease through NO formation.

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PROTEASOME INHIBITORS AND OXIDATIVE STRESS: FRIEND OR FOE?

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The role of the proteasome in neurodegenerative diseases is controversial. On the one hand, there is evidence for an association between a dysfunction in proteasome activity and neurodegeneration. However, there is also data showing that proteasome inhibition can protect nerve cells from a variety of insults. In an attempt to clarify this question, we studied the effects of four different proteasome inhibitors in a well characterized model of oxidative stress-induced nerve cell death. Consistent with the hypothesis that proteasome inhibition can contribute to nerve cell death, we found that high concentrations of proteasome inhibitors are themselves toxic to nerve cells by a mechanism involving glutathione depletion and the production of reactive oxygen species (ROS). In contrast, 10 fold lower concentrations of the proteasome inhibitors induced only low levels of ROS and protected the nerve cells from oxidative stress-induced death. Surprisingly, this protection appeared to be at least partially mediated by the induction of NF-κB by the proteasome inhibitors since protection was significantly reduced in cells expressing a specific NF-κB repressor. Although classical induction of NF-κB involves the proteasome-mediated degradation of IκB, there is a second pathway for NF-κB activation which is activated by various types of stress and involves the release of IκB but not its degradation. Thus, low concentrations of proteasome inhibitors induce a low level of oxidative stress resulting in the activation of neuroprotective pathways. In contrast, higher levels of the inhibitors cause toxic levels of stress. However, in compromised cells, even these low levels of additional stress might induce death.

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ACTIVATION OF EARLY SIGNALING EVENTS IN ISCHEMIA/REPERFUSION-MEDIATED INJURY IN LUNG TRANSPLANTATION

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Lung transplantation (LT) is an accepted treatment for a wide range of advanced lung diseases. However, acute cellular rejection remain important cause of mortality after LT. Ischemia (I) /Reperfusion (R) injury represents a major cause of allograft dysfunction. Since I/R is the first and unavoidable event in LT, we examined the effects of cold and warm I time on activation of phosphoinositol 3 kinase (PI3K), mitogen activated protein kinase (MAPK-JNK, p38, and ERK1/2), and inducible nitric oxide synthase (iNOS) using a rat model of LT. Allogenic (Lewis donor to Sprague-Dawley recipient) orthotopic left LT was performed using the cuff technique. Donor lungs were subjected to 4 and 18 hr cold and 1-3 hr warm I. After 24 hr R, the rats were anesthetized, ventilated, and right (native) and left (transplanted) lungs were used to monitor PI3K, MAPK, and iNOS activities and/or phosphorylation/expression. In allografts subjected to 4 or 18 hr cold I/1 hr warm I/24 hr R, PI3K activity, but not JNK, p38, or ERK1/2 phosphorylation, was increased 4 fold. In contrast, increasing I time from 1 hr to 2 hr or 3 hr with similar cold I and R time, PI3K activities were reduced to comparable level to those in native lung whereas phosphorylation of ERK1/2 but not JNK or p38, was increased by 3-fold. The catalytic activity and expression of iNOS in allografts were comparable to those in native lungs irrespective of cold/warm I/24 hr R time. These results demonstrate that warm rather than cold I time is critical for early activation of PI3K in allograft transplants and that extended I time is required for activation down stream signaling events including phosphorylation of ERK1/2 in allograft transplants.

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RAS NITROSYLATION AND PROTEIN PHOSPHORYLATION IN NITRIC OXIDE-INDUCED APOPTOSIS

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The Ras signaling pathway can promote both, cell death and cell survival. Using the NO donor S-nitrosoglutathione(SNOG),and the monocytic THP-1 cell line, we investigated the participation of Ras in the NO-induced apoptosis of THP-1 cells. Cells dye(80%-MTT assay)upon exposure to SNOG 1mM. Apoptotic markers such as cell shrinkage and chromatin condensation (Hoechst 33258 positive staining) featured in SNOG-mediated cell death. Furthermore, externalization of phosphatidylserine was evidenced by using Annexin-V labeling. THP-1 cells were transfected with a Ras mutant (RasC118S) where the critical cysteine residue for S-nitrosylation (cys 118), was replaced by a serine, or with wild type(wt)Ras. After NO treatment, phosphatidylserine externalization in RasC118S transfected cells was not significant as compared to parental and wt Ras transfected cells. Contrasting, H2O2 1 mM equally induced apoptosis in parental, wt Ras, and RasC118S expressing cells. Early activation (30 min) of Ras was observed in parental and wt THP-1 cells exposed to SNOG. Ras remained inactive in RasC118S expressing cells. In parental cells, activation of both MAP kinases, ERK ½ and p38, was observed after 2 h incubation with the NO donor. Both kinases remained active for additional 2 h, and became inactive thereafter. Activation of JNK was observed after 2h incubation with the NO donor however de-activation occurred within 2h. Inhibition of ERK½ activation by the MEK inhibitor PD98059 prevented apoptosis. By promoting the S-nitrosylation of Ras and

stimulating the MAP kinase cascade, NO mediates a cooperation between protein phosphorylation and protein nitrosylation in a cell signaling event.

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INCREASE OF CYTOKINE PRODUCTION BY LYSOPHOSPHATIDYLCHOLINE THROUGH INDUCTION OF AMINO ACID TRANSPORTER IN ENDOTHELIAL CELL

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It has been accepted that oxidation of low-density lipoprotein (LDL) has important roles in atherogenesis. Oxidized LDL (Ox-LDL) induces not only foam cell formation but also several biological alterations in vascular cells, such as adhesion molecule expression, proliferation, cytokine release, and migration. Lysophosphatidylcholine (LysoPC) is one of the major oxidation products in Ox-LDL and possesses several biological properties. Genomic study using DNA microarray containing 8974 genes explored that the expression of genes encoding neutral amino acid transporters, SLC7A5 (LAT1) and SLC3A2 (4F2hc) was significantly induced by LysoPC in human umbilical vein endothelial cell (HUVEC). LysoPC increased the uptake of [14C]L-leucine in HUVEC, which was inhibited by an inhibitor for LAT1, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH). The expression of these amino acid transporters resulted in synthesis of IL-6 and IL-8, which was inhibited by BCH. Ox-LDL containing the similar concentration of LysoPC used in this study also induced these amino acid transporter genes. These results suggested that increase in uptake of neutral amino acids was a novel function of LysoPC by which it might contribute to development of atherosclerotic lesion.

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PROTEIN KINASE C REGULATES 15-LIPOXYGENASE OXIDATION OF MEMBRANE-BOUND ARACHIDONATE IN HUMAN MONOCYTES

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15-Lipoxygenase (15-LOX) is implicated in the development of inflammatory vascular disease. The mechanisms that acutely control 15-LOX intracellularly are poorly characterised, with enzyme activity usually measured as free 15-HETE formation, after addition of exogenous arachidonate to cells. Herein, signalling pathways that modulate 15-LOX turnover in IL-4-induced human monocytes were explored. Unstimulated cells contained a small amount of esterified 15-HETE, indicating basal enzymatic action on phospholipid-bound fatty acid as described (Conrad et al, PNAS, 1992). 15-HETE formation was stimulated by A23187 with most being formed in the membrane arachidonate pool (~90%). Formation of free or membrane-bound 15-HETE was enhanced 3-fold by co-stimulation with phorbol ester (PMA), and was partially inhibited by the protein kinase C (PKC) inhibitor bisindolymaleimide. In contrast, PMA alone didn't stimulate 15-LOX. Immunoprecipitation failed to demonstrate phosphorylation of 15-LOX, suggesting that the enzyme is not directly phosphorylated by PKC. This data indicates that PKC is a positive modulator of calcium-stimulated 15-LOX turnover and that the favoured 15-LOX substrate in IL-4-treated monocytes is membrane-bound. Formation of predominantly esterified 15-HETE suggests that phospholipid oxidation by 15-LOX may play a role in cellular functions independent of 15-HETE signalling.

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CHROMIUM(VI)-INDUCED PULMONARY SIGNALING AND PROLIFERATION *IN VIVO*

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Inhaled hexavalent chromium (Cr(VI)) activates alveolar macrophages, produces fibrotic changes in the alveoli, and induces apoptosis in lung. To investigate Cr(VI)-induced molecular and phenotypic changes, male C57BL6 mice were instilled either intra-nasally (IN) or intra-tracheally (IT) with either saline or potassium dichromate (0.75 mg/kg body weight) once per week for one week, two weeks or three weeks. In addition, mice were instilled IN with either saline or potassium dichromate (0.25 or 0.75 mg/kg) for three consecutive days and allowed to recover for three weeks following the last instillation. Animals in this latter study showed no significant changes in RNA levels of procollagen 1A1, procollagen 3A1, cjun, or cfos after a three week recovery period. In contrast, animals treated IN weekly showed decreases in RNA levels of these genes. Instead of apoptosis, epithelial cell proliferation was evident in this group as indicated by increased Ki-67 staining. Since activation of extracellular signal-regulated kinase (ERK) was associated with fibroproliferative diseases, tissue levels of phosphorylated ERK were measured. These levels were increased in animals instilled IN with 0.75 mg/kg of potassium dichromate once per week for three weeks. This study presents two models for examining the pulmonary toxicity from multiple exposures to Cr(VI). The data indicated that there was little difference between the IT and IN routes of administration and also suggested that at the doses used, which represent occupational exposures, Cr(VI) promoted proliferative responses in the lung that may represent localized activation of cell signaling and phenotypic change. These localized changes were both dose and time dependent and may have been mitigated following different recovery periods. *Supported by NIEHS grant ES10638.*

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HOMOCYSTEINE INDUCES GENES THAT REGULATE MITOCHONDRIAL BIOGENESIS IN ENDOTHELIAL CELLS. ROLE OF OXIDATIVE STRESS.

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Hyperhomocysteinemia (HH) has been associated with premature vascular damage (VD). It has been suggested that the mechanism of homocysteine (Hcy) induced VD is oxidative stress. Endothelial cells from rats with HH have mitochondrial (mt) damage. However, the mechanism by which Hcy affects mt function is not known. mt biogenesis is the result of the coordinated expression of nuclear and mitochondrial genes. mt transcription factor A (Tfam) and nuclear respiratory factor 1 (Nrf-1) are codified in the nucleus and regulate mt genes like CoxIII. We have shown that mt damage increases expression of these factors. In this work, we evaluate the hypothesis that homocysteine alters mitochondrial biogenesis by a ROS-mediated mechanism. Using RT-PCR we studied the expression of Nrf-1, Tfam and CoxIII in human umbilical vein endothelial cell (HUVEC) incubated with Hcy (100μM) and evaluated the protective effect of antioxidants cathechin (10μM) and Trolox (10μM). We found that Hcy-stimulated HUVEC increase ROS generation and induce Nrf-1 (1 vs 1.35±0.05 a.u) and Tfam (1 vs 1.12±0.01 a.u). These changes are followed by induction of Cox-III (1 vs 1.35±0.11 a.u). These effects were inhibited by antioxidants. The present study

support the role of ROS in mitochondrial biogenesis induced by homocysteine.

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ABSTRACT REMOVED FROM PRESENTATION

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THE MECHANISM OF NF-KAPPAB ACTIVATION BY OXIDATIVE STRESS IS CELL TYPE-DEPENDENT

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NF-κB is a redox-regulated transcription factor involved in many cellular processes, including regulation of immune and inflammatory genes, apoptosis, cell proliferation and development. Several reports have recently shown that the mechanism of NF-κB activation by an oxidative stress (including the tyrosine phosphatase inhibitor pervanadate and hypoxia/reoxygenation) was totally distinct from those triggered by proinflammatory cytokines or mitogens; it involves tyrosine phosphorylation of the inhibitor IκBα without activation of the IκB kinase (IKK) complex and phosphorylation of serine 32 and 36. In the present work, we provide several lines of evidence that NF-κB activation by an oxidative stress is not tyrosine phosphorylation-dependent in every cell lines. By using the T lymphocytic cell line CEM, we showed that pervanadate treatment activate NF-κB through tyrosine phosphorylation of IκBα without degradation of the protein. This phosphorylation does not implicate the IKK complex. In contrast, treatment with hydrogen peroxide does not involve tyrosine phosphorylation of IκBα, but induces a strong activation of the IKK complex, leading to phosphorylation of serine 32 and 36, degradation of IκBα and nuclear translocation of NF-κB. In another T lymphocytic cell line (Jurkat), hydrogen peroxide activate NF-κB via the same mechanism that pervanadate, *i.e.* tyrosine phosphorylation of IκBα without IκB kinase activation. Our findings suggest that the mechanism of NF-κB activation by oxidative stress is cell type-dependent, implicating either a tyrosine phosphorylation-dependent mechanism, or a classical mechanism involving IKK complex activation.

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THE EFFECTS OF OVER- AND UNDEREXPRESSION OF GLUTAMATE-CYSTEINE LIGASE ON DROSOPHILA S2 CELLS UNDER STRESS CONDITIONS

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Glutathione (GSH) is a vital intra- and extracellular protective antioxidant and regulator of the cellular redox potential. The rate-limiting enzyme in GSH synthesis is glutamate-cysteine ligase (GCL), which is composed of two subunits: a large catalytic subunit (GCLc) and a smaller modifying subunit (GCLm). GCLc and GCLm are encoded by two separate genes and exhibit differential expression patterns. In mice, the homozygous knockout for a catalytic subunit is embryonic lethal, whereas heterozygote is viable and fertile (Dalton et al., 2000). Knockout mice homozygous for a modifying subunit are viable and fertile, but have substantially reduced glutathione levels and more sensitive to oxidative stress caused by hydrogen peroxide (Yang et al., 2002). To define further the cellular function of GCL, we have generated Drosophila S2 cell lines under- and over-expressing the products of gclc and gclm genes. Over-expression was completed by transfection of the cells with the pMT/V5-His vector containing extra copies of the GCL genes. Gene knockdown (>95%) was achieved by RNAi techniques. We have examined cell viability under various stress conditions, including treatments with H2O2, paraquat and heavy metals. Cell lines under-expressing either the catalytic or the modifier subunits demonstrated substantially higher susceptibility to copper. Cytotoxicity caused by H2O2 and cadmium was more pronounced in cell lines under-expressing the catalytic subunit compared to cell lines under-expressing the modifying subunit. Furthermore, the reduced expression of the catalytic subunit through RNAi proved to be relatively stable and resulted in the demise of the cell culture by around day 40 in the absence of any stressor.

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INACTIVATION AND GLUTATHIONYLATION OF IKKB BY H_2O_2

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Previous work from our laboratory has demonstrated that H_2O_2 inhibits Inhibitory Kappa B kinase (IKK), the enzyme complex necessary for NF-κB activation in response to many stimuli, through direct oxidation of cysteine residues in the complex (Korn et al, JBC, 2001). In the present study we further investigated the mode of oxidation of IKK by H_2O_2 in lung alveolar type II epithelial cells (C10), and the critical cysteine that is the target of oxidation. An *in vitro* kinase assay showed that a cysteine 179 to alanine (c179a) mutant of IKKβ was refractory to inhibition by H_2O_2, indicating that H_2O_2inhibits the IKK complex through oxidation of cysteine 179. In order to investigate if glutathionylation is the mode of oxidation caused by H_2O_2, we utilized an antibody directed against against glutathione, or alternatively, preloaded the cells with biotinylated glutathione ethyl ester before treating the cells with H_2O_2. Both assays demonstrated that H_2O_2 induced glutathionylation of IKKβ. We furthermore demonstrated that incubation of immunopurified IKK complex with reduced or oxidized glutathione caused inhibition of its kinase acitivity. Our

data demonstrate that H_2O_2 inhibits IKK activity and causes glutathionylation.

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ACTIVATION OF NF KAPPA B IN CARBON INFUSION ACTIVATED LIVER MACROPHAGES

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NF-κB is a redox sensitive transcription factor, which regulates the expression of several effector molecules, involved in inflammatory and immune responses. Well known activators of NF-κB include LPS, IL-2, TNF- α and reactive oxygen species (ROS).

Kupffer cells (KC) function is significantly augmented in experimental hyperthyroidism, as evidenced by enhanced ROS-related phagocytosis and respiratory burst activity (RBA). NF-κB activation mediated by oxidative stress at KC level has been observed in this hormonal dysfunction.

The aim of this study was to evaluate NF-κB-DNA binding activity (EMSA) after colloidal carbon (CC) infusion to isolated liver samples which were isolated from Sprague Dawley rats 18 hours after treatment with 3, 3', 5 – Triiodothyronine (T_3).

Liver RBA (μmoles O_2/ g liver) and phagocytosis (nmoles CC/ g liver/ h) were significantly enhanced in hyperthyroid rats. However, NF-κB-DNA binding activity was not modified after CC infusion to liver samples from hyperthyroid rats. Enhancement of this parameter after CC infusion to control samples, suggest maximal activation of NF-κB in hyperthyroid rats due to exhaustion of transcriptional re-exchange system. Supershift analysis showed that both p50 and p65 NF-κB subunits are contributing to the NF-κB-DNA binding activity.

In conclusion, NF-κB-binding activity is enhanced after CC-activation KC.

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IS HYDROGEN PEROXIDE A SIGNAL FOR DORSOVENTRAL AXIS FORMATION IN DROSOPHILA?

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We found that a mutation that specifically disrupts CNC-C, a Drosophila Nrf2 homolog, causes a ventralized egg phenotype. CNC-C has an ETGE motif, a KEAP1-interacting domain. KEAP1 is a cytoplasmic protein that binds to vertebrate Nrf2, a protein in the same family as CNC. Hydrogen peroxide and other oxidants are thought to directly oxidize KEAP1, which allows Nrf2 to enter the nucleus and activate transcription of phase II anti-oxidant genes. We knocked out Drosophila KEAP1, dKEAP1, in the oocyte with inducible dsRNA, and these oocytes develop into dorsalized eggs. This suggests that germline CNC-C has a role in dorsal-ventral patterning. Feeding flies paraquat or tBHQ dorsalizs the eggs, and knocking out dKEAP1 sensitizes flies to this phenotype, suggesging that reactive oxygen is a developmental morphogen.

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ADVANCED GLYCATED PRODUCTS INDUCE THE EXPRESSION OF INOS IN VSMC

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Advanced Glycated End products (AGE) are the result of irreversible modifications of proteins by carbohydrates. AGE are associated to vascular complications in diabetes patients. The activation of its receptor (RAGE) by AGE has been implicated in inflammatory responses. Recently, we have described that AGE induces proatherogenic phenotypic changes in vascular smooth muscle cells (VSMC) through a mechanism that involves superoxide ($O_2^{\bullet-}$) generated mainly by NADPH-oxidase. $O_2^{\bullet-}$ reacts with $NO^{\bullet}$ to form peroxynitrite ($ONOO^-$) a highly reactive radical involved in ischemia-reperfusion and inflammation. Inducible nitric oxide synthase (iNOS) in VSMC is induced by cytokines and lipopolysacharides. We hypothesize that the induction of iNOS in VSMC is part of the response to AGE and explains, at least in part, the evidences for an increased $ONOO^-$ generation. We used VSMC from rat aorta in primary culture and AGE (200μg/mL) prepared with bovine albumin and glucose. We observed that AGE increases 3-nitrotyrosine formation, proteins oxidation product of $ONOO^-$. It is inhibited by the $O_2^{\bullet-}$ scavenger MnTBAP and the Flavin/NADPH-oxidases antagonist DPI. With regards to the source of $NO^{\bullet}$, we observe that AGE induces the transcription of iNOS. Effect not modified by MnTBAP or DPI. In addition, one of the main mechanisms that regulates the activity of eNOS, the phosphorylation of Akt was not modified by AGE incubation. In spite of the formation of $ONOO^-$ we found, by Griess method, that AGE increases the $NO^{\bullet}$ generated by VSMC(1vs 1,08±0,02 p<0,04u.a). In conclusion:AGE induces the formation of $ONOO^-$. This $ONOO^-$ is apparently formed by NADPH oxidase-derived $O_2^{\bullet-}$ and iNOS derived-$NO^{\bullet}$. AGE induces the expression of iNOS, by a $O_2^{\bullet-}$ not mediated mechanism. Fondecyt 1020486/ PBMEC.

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MECHANISM OF DRUG-INDUCED IL-10 OVERPRODUCTION IN VIVO: FUNCTIONAL INTERFERENCE OF NF-KB AND SP1

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NF-κB inhibitors such as phenyl N-t-butyl nitrone (PBN) and pyrrolidine dithiocarbamate (PDTC) have been shown to mediate IL-10 overproduction in LPS administered animals. PBN's anti-inflammatory activity was counteracted by anti-IL-10 antibody administration, suggesting that IL-10 plays a major role in PBN's protection. PBN and PDTC are known NF-κB inhibitors, thus we speculate that IL-10 overproduction may be an obligatory result of NF-κB inhibition. We show that other known NF-κB inhibitors, salicylate and arsenite promote IL-10 overproduction in a rat LPS model.

The binding of both Sp1 and STAT3 to the promoter region is required for IL-10 gene transcription. Sp1 is constitutively bound to IL-10 DNA, but STAT3 binding is activated by inflammatory stimulus. NF-κB shares the same binding sequence with Sp1 and displaces Sp1, which may decrease IL-10 expression. NF-κB inhibition could increase Sp-1 binding and IL-10 production. We conducted experiments to obtain supporting evidence for this postulate: 1) we were able to detect NF-κB:Sp1 complex in liver nuclear extract from LPS±PBN treated rats by immunoprecipitation with Sp1, which was subsequently detected with western blotting using NF-B antibody. DNA binding assay (TransAm assay, ActivMotif) for NF-κB, Sp1 and STAT3

indicated that in LPS livers NF-κB and STAT3 bindings were increased but Sp-1 binding was decreased. In LPS+PBN livers, however, NF-κB was decreased, Sp1 was recovered, and STAT3 was unchanged. These results support the postulated mechanism for IL-10 overproduction.

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SINGLET OXYGEN-INDUCED ATTENUATION OF GROWTH FACTOR SIGNALING: POSSIBLE ROLE OF CERAMIDES

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Singlet oxygen, an electronically excited form of molecular oxygen, is generated either photochemically, such as in photodynamic therapy or during exposure to ultraviolet radiation, or metabolically in inflamed tissues. It has been shown to be a primary mediator of the activation of stress-activated protein kinases (JNK and p38-MAPK) elicited by ultraviolet A (UVA; 320-400 nm). In the present study the effects of singlet oxygen on the growth factor-induced activation of survival pathways, i.e. the extracellular signal-regulated kinase (ERK) 1/2 and protein kinase B (PKB)/Akt pathways, were analysed in human dermal fibroblasts. The activation of ERK 1/2 and Akt as induced by stimulation with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) was inhibited by singlet oxygen generated intracellularly upon photoexcitation of Rose Bengal (RB). Thus, singlet oxygen is capable of attenuating the activation of cellular survival pathways by growth factors such as EGF and PDGF. Since photodynamic therapy (PDT)-induced apoptosis is known to be associated with increased formation of ceramides we evaluated the role of ceramides as potential mediators of the survival pathway inhibition by singlet oxygen. Indeed, both UVA and singlet oxygen lead to ceramide generation in human skin cells, and the attenuation of EGF- and PDGF-induced activation of ERK 1/2- and Akt by singlet oxygen was mimicked by stimulation of fibroblasts with the cell-permeable C2-ceramide. This points to a role of ceramides as mediators of the singlet oxygen-induced inhibition of growth factor-induced activation of cellular survival pathways and to a novel mechanism of singlet oxygen toxicity.

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PROLONGED ACTIVATION OF AMP-ACTIVATED KINASE (AMPK) PROTECTS AGAINST HYDROGEN PEROXIDE MEDIATED ENDOTHELIAL CELL DEATH

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AMP-activated protein kinase (AMPK) is induced by oxidative stress. However, it is not known whether AMPK activation is protective or detrimental in this setting. Here we show that H_2O_2 time- and dose-dependently activates AMPK in porcine aortic endothelial cells (PAEC). To determine the impact of AMPK preactivation during oxidative stress, we used a model of H_2O_2 induced cell death. H_2O_2 at concentrations between 5 μM and 200 μM for 3-4 h lead to increased cell death in PAEC, as assessed by MTS and LDH release assays. Preincubation of PAEC with the AMPK activator 5-amino-imidazole carboxamide riboside (AICAR) for 20h lead to AMPK activation (assessed by Western blot and AMPK activity assay) and significantly reduced H_2O_2-mediated cell death, whereas shorter AICAR preincubation for 30 min or 3 h was ineffective. Metformin, an AMPK activating antidiabetic drug, showed similar protective effects when PAEC were pretreated for 20 h before H_2O_2 exposure. Concomitant infection with a AMPK dominant negative adenovirus (AdAMPK-dn) partially reversed the protective action of AICAR, suggesting a causal role for AMPK activation. Addition of the AdAMPK-dn to untreated control cells did not affect H_2O_2-mediated cell death. Cell death induction by H_2O_2 involves activation of several cellular signaling cascades, that might be altered by AMPK activation. We found that preincubation of PAEC with AICAR or Metformin abolished H_2O_2-mediated activation of the pro-death signal JNK as assessed by decreased c-jun phosphorylation. These results show that AMPK activation protects against oxidative stress mediated endothelial cell death, possibly via modulation of the JNK signaling pathway.

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EFFECTS OF ENHANCED GLUTATHIONE SYNTHESIS ON CARBON

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Glutamate-cysteine ligase (GCL) is the rate-limiting enzyme in the synthesis of glutathione (GSH), a major free radical scavenger and important factor in detoxification of reactive oxygen species and xenobiotics. Carbon tetrachloride (CCl4) is metabolized by cytochrome P450 to CCl3 radical, which causes lipid peroxidation and glutathione depletion in the

liver. To evaluate the effect of enhanced GSH synthesis on the toxicity of CCl4, we have created transgenic mice that conditionally overexpress the two subunits of GCL: GCLc and/or GLCm in the liver. Conditional expression is achieved in this model by administering the anti-progestigen RU486 (GeneSwitch system). We wished to determine the effects of increased GCL activity on the expression of other genes important for defense against toxicants in the liver. We used both cDNA and oligonucleotide microarrays to assess global gene expression in GCLm transgenic mice. There was little difference in gene expression between GCLm transgenic mice treated with vehicle (sesame oil) and non-transgenic litter-mate controls. After correcting for the effects of RU486 on global gene expression, comparisons of gene expression profiles reveal interactions between GCLm transgene expression and CCl4 treatment. The results indicate that these transgenic mice provide a useful model to study the role of GSH synthesis in defense against hepatotoxicants, and the mechanisms by which this protection occurs.

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ASBESTOS EXPOSURE UPREGULATES MATRIX METALLOPROTEINASES IN EPITHELIAL CELLS AND FIBROBLASTS

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Matrix metalloproteinases (MMPs), affect extracellular matrix proteins. As asbestos is known to cause fibrosis and cancer and no data are available regarding roles of MMPs and TIMPs (tissue inhibitor of metalloproteinases) in asbestos-induced responses, we explored the effects of asbestos on different MMPs and TIMPs in alveolar epithelial cells (C10) and primary fibroblasts from mouse lungs. Ribonuclease protection assays showed that in epithelial cells following crocidolite asbestos exposures, time-dependent, significant increases in MMP13 steady state mRNA levels occurred. MMP 3, 8, 9 and TIMP 1 were also slightly increased. Inhibition of PKC isoforms using rottlerin (PKC δ

specific inhibitor) or bisindolemaleimide (bis) (general PKC inhibitor) showed that MMPs and TIMPs were PKC δ dependent. Inhibition of MEK 1 using PD98059 had no effect on any of the MMPs or TIMPs. Confluent cells were more responsive to asbestos-induced effects as compared to log phase cells. Zymography showed no increase in activity of any MMP by asbestos, but presence of the active form of MMP13. In fibroblasts, time-dependent upregulation of different MMPs (13,12,9,8,3) occurred in response to asbestos. MMPs were inhibited by both rottlerin and bis whereas PD98059 had no effect. Zymography showed increased MMP2 activity in fibroblasts by asbestos. Novel studies here show that asbestos causes increased transcription of various MMPs (most importantly, MMP13 in both cell types), which appears to be modulated by PKC δ. Supported by PO1 grant 67004 from NHBLI.

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ALTERATIONS OF LUNG DEVELOPMENT IN NEWBORN MICE EXPOSED TO SUBLETHAL HYPEROXIA

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With improved survival of very premature infants, chronic lung disease of prematurity, also known as bronchopulmonary dysplasia (BPD), is characterized increasingly as arrested lung development. Newborn mice have lungs that are comparable developmentally to lungs in prematurely born human infants and exhibit similarly arrested lung development and alveolarization with exposure to sublethal hyperoxia. In the present studies, we tested the hypothesis that inhibition of normal alveolarization by exposure of newborn mice to hyperoxia would be paralleled by alterations of expressions of FGFR3 and 4, FGF7, and HIF-1a. Newborn FVB mice were exposed to 85% O2 (O2) within 12 h of birth or left in room air (AIR). No pup mortality was observed, and body weight gains were not affected by hyperoxia. Pups were sacrificed at 1, 3, 7, and 14 d. Lungs were fixed at 25 cm H2O for morphometry or were freeze-clamped for RNA and protein analyses. Levels of FGFR3 and 4, FGF7, and HIF-1a mRNA were assessed by real time PCR. At P14, lungs of O2 pups had fewer and larger alveoli and smaller alveolar surface-to-volume ratios than did AIR pups (14.3±0.4 vs 20.4±0.8 per HPF[640x]; 2038 vs 1402 mm2; and 0.111±0.002 vs 0.140±0.007 mm-1), respectively. Lung FGFR3 and 4 mRNA levels were lower at P3 and higher at P7 in O2 pups than in AIR pups. In AIR mice, lung FGF7 mRNA levels were greater at P14 than at P1, but this increase was not observed in O2 mice. Lung HIF-1a mRNA levels decreased to 20% of P1 levels by P14 in both AIR and O2 mice. The arrested alveolar development observed in newborn mice exposed to 85% O2 resembles BPD of human preterm infants, and the alterations in expressions of important growth regulatory genes, such as we observe, potentially contribute to this undesirable outcome.

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RESPONSE OF CELLULAR METABOLISM TO OVEREXPRESSION OF ANTIOXIDANT ENZYMES

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Overexpression of catalase, thioredoxin or Bcl-2 or selection of a population of WEHI7.2 cells resistant to 200 μM H_2O_2 results in WEHI7.2 variants that are more resistant to H_2O_2; however, the resistance is not proportional to catalase or glutathione peroxidase activity. To determine the source of this resistance we compared the cellular redox environment and surprisingly found that the variants are more oxidized. Most of the variants have lower glutathione levels and decreased antioxidant potential and all of them have a more oxidized NADP(H) pool. Baseline DCFH fluorescence is increased indicating increased intracellular ROS. All the variants also show an increase in glutathione S-transferase and NADP(H) quinone oxidoreductase activities, enzymes that contain antioxidant response elements. Interestingly, most of the variants show increased mitochondrial ATP production and all have increased total superoxide dismutase activity. These data suggest that multiple pathways that influence the cellular redox environment are coordinately regulated and that resistance to H_2O_2 may be more complex than simple H_2O_2 detoxification.

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REDOX-REGULATED ROTATIONAL COUPLING OF RPTP-ALPHA DIMERS

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Receptor Protein-Tyrosine Phosphatase alpha, RPTP-alpha, constitutively forms dimers in the membrane and activity studies with forced dimer-mutants of RPTP-alpha revealed that rotational coupling of the dimer defines its activity. Using constructs of RPTP-alpha with an haemagglutinin (HA)-tag on the N-terminal part of the extracellular domain of RPTP-alpha, we found that the conformation of RPTP dimers can be detected on living cells using the accessibility of the HA-tag as a read-out. The HA-tag of wildtype RPTP-alpha and of constitutively dimeric, active RPTP-alpha -F135C with a disulfide bond in the extracellular domain, was not accessible for antibody binding, while the HA-tag of constitutively dimeric, inactive RPTP-alpha-P137C was. All three proteins were expressed on the plasma membrane to a similar extent and the accessibility of their extracellular domains did not differ as was determined by biotinylation studies. Dimerization was required for masking the HA-tag and we identified a region in the N-terminus of RPTP-alpha that was essential for the effect. Oxidative stress has been shown to induce a conformational change of the membrane distal PTP domain (RPTP-alpha-D2). Here we report that H2O2 treatment of cells induced a dramatic, reversible inrease in accessibility of the HA-tag in the extracellular domain. The catalytic site Cys723 in RPTP-alpha-D2, which was required for the conformational change of RPTP-alpha-D2 upon H2O2 treatment, was essential for the H2O2-induced increase in accessibility. These results show for the first time that a conformational change in the intracellular domain of RPTP-alpha led to a change in conformation of the extracellular domains, indicating that RPTPs have the capacity for inside-out signaling.

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CYCLOOXYGENASE-2 IS INDUCED BY BRADYKININ IN VASCULAR SMOOTH MUSCLE CELLS

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Inflammation has an important role in the progression of atherosclerosis. Cyclooxygenase-2 (COX-2)which is involved in the inflammatory response via the production of prostanoids, is expressed in vascular smooth muscle cells (VSMC) in atherosclerotic lesions and has a potential role in the development of atherosclerosis. Considering bradykinin as a modulator of VSMC proliferation and migration, we hypothesized that bradykinin can induce COX-2 in VSMC from rat aorta through a mechanism mediated by Mitogen Activated Protein

Kinase(MAPK) activation and Reactive Oxygen Species (ROS) generation. VSMC from rat aorta were incubated with bradykinin (0.1 uM) in the absence or presence of a p42/p44 MAPK inhibitor (UO-126 1uM), a p38-MAPK inhibitor(SB-203580 1uM), or the antioxidants DPI (10uM), Lipoic Acid (0.1mM) or n-acetylcysteine (NAC, 20mM) for different times. COX-2 mRNA and protein were measured by RT-PCR and Western blot respectively. Bradykinin induced a time dependent increase in COX-2 mRNA and protein. The peak expression of RNA (4±1 fold over control, n= 4) and protein (2.6±0.6 folds over control; n= 4) were 2 and 24 hours respectively. The stimulatory effect of bradykinin in protein levels was abolished by UO-126 and NAC however SB-203580 or the antioxidants DPI and lipoic Acid did not affect bradykinin response. In conclusion our results demonstrate that bradykinin increases COX-2 in VSMC through a mechanism mediated by p42/p44-MAPK but not by p38-MAPK or ROS. This fenomenon is relevant in pathological situations where the direct contact of VSMC with bradykinin could increase COX-2 derived prostaglandins and modulate inflammation and VSMC function.

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GENE EXPRESSION PROFILE AND HISTOPATHOLOGY OF EXPERIMENTAL BRONCHOPULMONARY DYSPLASIA INDUCED BY PROLONGED OXIDATIVE STRESS.

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Oxidative stress is an important factor in the pathogenesis of bronchopulmonary dysplasia (BPD), a chronic lung disease of premature infants characterized by arrested alveolar and vascular development of the immature lung. We investigated differential gene expression with DNA micro-array analysis in premature rat lungs exposed to prolonged hyperoxia during the saccular stage of development, which closely resembles the development of the lungs of premature infants receiving neonatal intensive care. Expression profiles were largely confirmed by real-time RT-PCR (27 genes) and in line with histopathology and Western blotting. Oxidative stress affected a complex orchestra of genes involved in inflammation, coagulation, fibrinolysis, extracellular matrix turnover, cell cycle, signal transduction and alveolar enlargement and explains, at least in part, the pathological alterations that occur in lungs developing BPD. Exciting findings were the magnitude of fibrin deposition, the upregulation of growth related oncogene-1, monocyte chemoattractant protein-1, amphiregulin, plasminogen activator inhibitor-1, secretory leukocyte proteinase inhibitor, matrix metalloproteinase-12, p21, metallothionein and heme oxygenase, and the downregulation of fibroblast growth factor receptor-4 and vascular endothelial growth factor receptor-2 (Flk-1). These findings are not only of fundamental importance in the understanding of the pathophysiology of BPD, but also essential for the development of new therapeutic strategies.

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UPREGULATION OF HO-1 AND HSP90 EXPRESSION INDUCED BY PEROXYNITRITE IN HUMAN ENDOTHELIAL CELLS.

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Peroxynitrite, a marker of oxidative stress, is elevated in conditions associated with vascular endothelial cell dysfunction. Heme-oxygenase 1 (HO-1) and heat shock protein-90 (Hsp90) are molecular chaperones and play protective roles against oxidative injury. In this study, we tested effects of peroxynitrite on HO-1 and Hsp90 expression in human endothelial cells. Endothelial cells isolated from umbilical cords were treated with 3-morpholinosydnonimine (SIN-1, a peroxynitrite donor) at a concentration of 1.0μM for 30 min, 1, 2, 4, and 6 hours. Total cellular protein and RNA were extracted. mRNA and protein expressions for HO-1 and Hsp90 were determined by RT-PCR and Western blot analysis. Our results showed that the baseline level for Hsp90 protein and mRNA expression was higher than those for HO-1. Although mRNA and protein levels for HO-1 and Hsp90 were increased after SIN-1 challenge, differential patterns for HO-1 and Hsp90 expressions were observed. We conclude that upregulation of HO-1 and Hsp90 may play an important role in defense mechanisms against peroxynitrite-induced oxidative injury in vascular endothelial cells.

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ETS-1: A TARGET OF MITOCHONDRIAL SIGNALING VIA REACTIVE OXYGEN SPECIES

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The role of mitochondrial signaling in tumorigenesis is a topic of growing interest. There is evidence for mutations to mitochondrial DNA in almost every cancer cell type, often these changes lead to a more aggressive phenotype. Signals generated by the altered mitochondria, in the form of reactive oxygen species (ROS), may be responsible for these downstream effects. A model used to study ROS signaling makes use of a pair of ovarian cancer cells-the parental 2008 cells and derived C13 variants, displaying altered mitochondria and greater production of ROS. It was previously observed that the transcription factor Ets-1 is up-regulated in C13 cells and is a likely target of ROS, as expression of this transcription factor increases with treatment by hydrogen peroxide and mitochondrial inhibitors. It is of interest to study the downstream effects of increased Ets-1 expression that relate to its regulation by mitochondrial signaling. To this end, 2008 cells were stably transfected with an inducible Ets-1 expression vector and microarray analysis was performed. It was observed that with increased expression of Ets-1, numerous components of the electron transport chain were down-regulated, whereas genes involved in antioxidant defense and metabolism were up-regulated. These observations indicate that increased expression of Ets-1 results in an inhibition of electron transport chain activity, increased tolerance to ROS and a preference for glycolysis as an energy source. Decreased oxygen consumption and increased production of lactate was confirmed in cells overexpressing Ets-1 indicating that they are more glycolytic than parental, untransfected, cells. This data indicates that the transcription factor Ets-1 is an important target of mitochondrial signaling in cancer cells, sensing changes to the organelle and inducing the cell to compensate.

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8-OXOGTP RELAYS INTRACELLULAR OXIDATIVE MESSAGES THROUGH RAS, A SMALL GTP-BINDING PROTEIN

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Oxidative stress leads to substantial activation of the Ras-Extracellular signal regulated kinase (ERK) pathway. However, the identity of the direct mediator of oxidative stress-induced signaling remains elusive. In this study, we hypothesized that the oxidative by-product itself serves as an intracellular oxidative signaling molecule, and we investigated that 8-oxoGTP, an oxidized form of GTP, differs from GTP in terms of the activation of small GTP-binding proteins, such as Ras. Interestingly, we found that 8-oxoGTP is significantly more potent than GTP at activating Ras by in vitro binding assay. Moreover, Raf-1, the downstream effector of Ras in the ERK pathway was also more

highly activated by 8-oxoGTP than by GTP in a kinase assay using MEK-1 as a substrate. These results suggest that Ras, an intracellular molecular switch, senses oxidative stress through 8-oxoGTP and transmits this to the oxidative signaling pathway.

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HYPOXIA-INDUCIBLE FACTOR IN RINGED SEAL TISSUES

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Tissue hypoxia and ischemia-reperfusion pose a dangerous situation for oxidative stress. However, diving mammals and birds show pronounced resistance to oxidative injury under such conditions, which are a consequence of selective vasoconstriction during a dive. As the function of Hypoxia-Inducible Factor-1α (HIF-1α) in protection against and adaptation to hypoxia has been recognized in terrestial animals, we have investigated the genomics and expression of this protein in ringed seal (*Phoca hispida*) in order to determine if it may play a protective role in this diving mammal. PCR studies using primers based on sequences from mouse HIF-1α exons 3, 4, 5, 6, 9, 10, 11 and 12 showed that DNA from seal lung generated PCR products similar to those from mouse DNA. These studies have established that a putative HIF-1α gene exists in the seal genome that appears to have a similar but not identical sequence to the mouse gene. Seal lung and skeletal muscle tissues showed the highest relative levels of expression, with heart muscle showing significantly lower levels. Analysis of oxidized cellular protein levels indicated that seal lung and heart muscle had the lowest levels of oxidized proteins. Seal lung tissue had the highest level of HIF-1α expression and the lowest level of protein oxidation; this suggests that HIF-1α expression may have an important protective effect in this tissue in diving mammals. Our results support the hypothesis that HIF-1α expression is dependent on both tissue-specific energy requirements and adequate metabolic supply-to-demand ratio. Combined, the evidence available suggests that diving mammals have an overall anticipatory response to avoid the ill effects of dive-associated ischemia/reperfusion which may involve the HIF system.

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4HNE INCREASES GGT EXPRESSION THROUGH MAP KINASE PATHWAYS IN RAT ALVEOLAR TYPE II CELLS

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γ-glutamyl transpeptidase (GGT) plays key roles in the metabolism of glutathione (GSH) S-conjugates, and in de novo synthesis of GSH. GGT is an essential component of the cellular adaptive response, and its expression is increased during oxidative stress, but the mechanism of this up-regulation remains unclear. In rats, GGT is a single copy gene regulated by 5 tandemly-arranged promoters; alternative splicing allows for 7 different transcripts. Our previous studies have shown that 4-hydroxy-2-nonenal, an end product of lipid peroxidation, upregulated GGT transcription in rat lung alveolar type II (L2) cells, and that mRNA subtypes I and V were involved. Previously we demonstrated that 4HNE activated the ERK, p38 and JNK MAPK signaling pathways in L2 cells. In the present study, we investigated which MAPK signaling pathways are involved in GGT up regulation by 4HNE in L2 cells. PD98059 and SB203580, specific inhibitors of the ERK and p38 pathways, respectively, blocked the 4HNE-mediated up-regulation of both GGT activity and mRNA; surprisingly, SP600125, which specifically inhibits JNK1/2, synergistically increased the induction of GGT by 4HNE. In conclusion, our results demonstrate that the signaling for increasing GGT expression in response to 4HNE is mediated through MAP kinase pathways in rat alveolar type II cells.

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ALPHA-LIPOIC ACID INHIBITS LIPOPOLYSACCHARIDE-INDUCED NF-KB ACTIVATION AND ADHESION MOLECULE EXPRESSION *IN VIVO*

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Endothelial expression of adhesion molecules and chemoattractants such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) are important initiating events in atherosclerosis. We have shown previously that α-lipoic acid (LA) inhibits TNFα- and lipopolysaccharide (LPS)-induced expression of adhesion molecules and MCP-1 in cultured human aortic endothelial cells. In this study, we investigated whether LA acts as an anti-inflammatory agent and inhibits LPS-induced endothelial activation *in vivo*. Male C57BL/6N mice were divided into three groups: control (saline, i.p. injection); LPS (50 μg/animal, i.p.); and LA plus LPS (LA 100 mg/kg body weight, i.p., 1 h before LPS challenge). We found that LA strongly inhibited LPS-induced increases in plasma concentrations of soluble VCAM-1 (sVCAM-1) and soluble ICAM-1 (sICAM-1). For sVCAM-1, the data were as follows: control, 1227±60 ng/ml; LPS, 2001±236 ng/ml; and LA plus LPS, 1180±141 ng/ml; and for sICAM-1: control, 42.5±3.5 μg/ml; LPS, 118.9±26.0 μg/ml; and LA plus LPS, 56.9±8.4 μg/ml ($P<0.01$ *vs.* LPS without LA; n=5-8). LA also attenuated LPS-induced up-regulation of VCAM-1 and MCP-1 mRNA levels and NF-κB activation in aorta and heart ($P<0.05$; n=5). Finally, pretreatment with LA significantly increased survival of mice injected with a lethal dose of LPS (1.5 mg/animal) ($P<0.01$ *vs.* LPS without LA; n=6). These data indicate that the anti-inflammatory activity of LA *in vivo* is mediated by inhibition of NF-κB activation and adhesion molecule expression, suggesting a possible role for LA in inhibiting atherosclerosis and other inflammatory conditions.

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VITAMIN E MODULATES ANDROGEN INDUCED OXIDATIVE STRESS AND POTENTIATES CELL DEATH IN LNCAP HUMAN PROSTATE CARCINOMA CELLS

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The antioxidant vitamin E has been proposed as a prostate cancer preventive agent. Previous studies have shown that growth inhibitory doses of androgen produce elevated levels of reactive oxygen species (ROS) activity in the androgen-sensitive LNCaP prostate carcinoma cell line. Here we show that vitamin E induces cell death in androgen-treated LNCaP cells. Our goal is to explore the possible mechanism(s) of cell death in LNCaP cells co-treated with vitamin E and androgens. We found that LNCaP cells co-treated with α-tocopherol succinate (ATS) and R1881, a synthetic androgen, resulted in a dramatic time-dependent decrease in the GSH/GSSG ratio. We also observed that co-treatment of LNCaP cells with ATS and R1881 caused a significant G0/G1 cell cycle arrest and a concomitant increase in p21 expression, a protein that is known to be redox sensitive and to have cell cycle regulatory functions. Furthermore, ATS exposure decreased androgen receptor and prostate specific

antigen levels in a time and dose-dependent manner in LNCaP cells. Our data suggests that vitamin E is a potential augmentor of cell death by altering cell cycle progression, inhibiting androgen receptor function and the expression of downstream target genes, and most importantly, alters the redox status in androgen treated LNCaP cells. Thus, vitamin E may play a role as an adjuvant in prostate cancer prevention through cell cycle inhibition and induction of cell death.

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NADPH-CYTOCHROME P450 REDUCTASE MODULATES THE ACTIVATION OF HYPOXIA-INDUCIBLE FACTOR 1

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Hypoxia induces a group of physiologically important genes that include erythropoietin (EPO) and vascular endothelial growth factor (VEGF). Hypoxia inducible factor-1 (HIF-1)_ is identified as a hypoxia-activated transcription factor and plays a critical role in induction of many genes under hypoxia. We found that a flavoprotein, NADPH-P450 reductase (NPR), regulated the induction of EPO mRNA under hypoxic conditions. NPR-knockdown hepatocyte cells (NPR$^-$ cells) were established and lacked EPO induction under hypoxia. In NPR$^-$ cells, HIF-1_ responded to neither transcriptional activation nor translocation to the nucleus based on electrophoretic mobility shift assays and reporter gene assay including hypoxia response element. In contrast, NPR overexpression in hepatocyte cells enhanced the DNA binding activity of HIF-1_. We further studied the expression of NPR and VEGF mRNA in human tumor tissues and found that the NPR mRNA levels were correlated with the VEGF mRNA levels, suggesting that NPR might be an important factor in the hypoxic induction of gene such as VEGF *in vivo*.