SIGNAL TRANSDUCTION AND GENE EXPRESSION

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The Redox Modulation of Akt1 Activation in Mitochondria

Valeria Gabriela Antico Arciuch[1], *Daniela Paola Converso[1], María Cecilia Carreras[1], and Juan José Poderoso[1]*

[1]Laboratory of Oxygen Metabolism, Hospital de Clínicas, University of Buenos Aires

Akt (PKB) is a serine/threonine kinase, which regulates cellular responses such as proliferation, apoptosis and glucose metabolism. Akt becomes differentially activated by growth factors, cytokines, and oxidative stress. for complete activation, Akt requires to be sequentially phosphorylated in Ser473 by mTOR and in Thr308 by PDK1. Previously, we demonstrated that H2O2 induces Akt1 subcellular redistribution which correlates with a differential cell phenotype. Low H2O2 causes a rapid translocation to nucleus with a concomitant proliferative phenotype. High H2O2 promotes Akt1 retention in mitochondria and the cells exhibit an apoptotic behaviour. the aim of this work was to study mitochondrial contribution to sequential Akt1 activation by redox modulation in the NIH/3T3 cell line. Subcellular distribution of P-Akt was followed by transient transfection with pcDNA3-Akt1 Wt or T308A mutant and analyzed by western blot anti HA-tag. in vitro phosphorylation assay was carried out using inactive Akt1-HA and mTOR in kinase buffer. When appropriate, Akt1 was previously oxydized with H2O2. Translocation to mitochondria was then evaluated incubating these extracts with purified organelles in import buffer and total and phosphorylated Akt1 was assessed by Western blot. Akt1 activity was detected by an in **vitro** kinase assay using GSK-3 as a substrate. PDK1-Akt1 interaction was assesed by a pull down assay using previously oxydized Akt1. We herein demonstrate that a) Akt1 requires Ser473 phosphorylation to enter into mitochondria, where it is further phosphorylated at Thr308 by mitochondrial PDK1; b) differentially, low H_2O_2 favors and high H_2O_2 yield impedes the Akt1-PDK1 interaction in the organelles; c) in this context, Akt1 mitochondrial activity increases P-GSK-3 by 125% in cells incubated with low H_2O_2 while decreases by 43% at high H_2O_2 due to incomplete activation, and d) consequently, mutant Akt1 T308A is retained in mitochondria and cannot reach the nucleus. We conclude that mitochondria are important partners of Akt1 signaling and that, depending on redox status, they regulate Akt1 phosphorylation at Thr308 and furthermore contribute to drive cell fate to proliferation or apoptosis.

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Signaling Events in Apoptotic Photokilling of 5-Aminolevulinic Acid-Treated Tumor Cells: Inhibitory Effects of Nitric Oxide

Reshma Bhowmick[1], *and Albert W Girotti[1]*

[1]Medical College of Wisconsin

Photodynamic therapy (PDT) employs a photosensitizing agent, molecular oxygen, and visible light to produce reactive oxygen species that kill tumor and tumor vasculature cells in a site-specific manner. Nitric oxide (NO) produced by these cells could be pro-carcinogenic by inhibiting apoptosis and promoting angiogenesis and tumor growth. We recently showed that NO from spermine NONOate (SPNO) or activated macrophages make COH-BR1 breast tumor cells hyperresistant to photokilling sensitized by 5-aminolevulinic acid (ALA)-generated protoporphyrin IX (PpIX). Signaling events associated with this hyperresistance are examined here. ALA-treated COH-BR1 cells containing mitochondria-localized PpIX were irradiated with broad-band visible light in the absence and presence of SPNO. Apoptotic *vs.* necrotic cell death was assessed by fluorescence microscopy. Redox signaling associated with MAP kinase (ERK1/2, p38, JNK) phosphorylation-activation and heme oxygenase-1 (HO-1) upregulation was studied using immunoprecipitation and Western blot methodology. Irradiation of sensitized cells resulted in activation of pro-apoptotic p38α and JNK with concomitant deactivation of pro-survival p38β and ERK1/2. NO delivered during irradiation had an anti-apoptotic effect accompanied by substantially greater HO-1 induction and reversal of the effects seen without NO. Downstream of MAPK activation, we observed induction of pro-apoptotic Bax and repression of anti-apoptotic Bcl-xL, both responses being reversed by NO. These findings provide new insights into signaling activity associated with the intrinsic apoptotic pathway in ALA-PDT and how this activity can be modulated by NO. (Supported by NIH: CA70823)

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Mnsod and Cyclin D1 Facilitate Inter-Organeller Communication Coordinating Cellular Metabolism to DNA Synthesis in PCB Treated MCF10A Cells

Leena Chaudhuri[1], *Nùkhet Aykin Burns[1], Douglas R. Spitz[1], Amanda L. Kalen[1], Ehab H. Sarsour[1], and Prabhat C. Goswami[1]*

[1]University of Iowa

Polychlorinated biphenyls (PCBs) and their metabolites are environmental chemical contaminants which can produce reactive oxygen species (ROS) by autoxidation of dihydroxy PCBs or by reduction of quinones and redox-cycling. Manganese superoxide dismutase (MnSOD) is a nuclear encoded and mitochondria localized antioxidant enzyme that converts superoxide to hydrogen peroxide. This study investigates the hypothesis that *PCB-induced perturbation in MnSOD expression increases cellular ROS levels, which in turn delay G_0/G_1 to S progression*. Quiescent human non-malignant mammary epithelial cells (MCF10A) were incubated with PCBs (0-3 micromolar) for 4d, and analyzed for MnSOD expression (activity, protein, and mRNA) and flow cytometry measurements of ROS levels. MnSOD expression decreased (~2-fold) in 4-chloro-1, 4-benzoquinone (4-Cl-BQ) compared to PCB153, aroclor 1254 and un-treated control cells. Treatment of cells with PEG-SOD and PEG-CAT prior to the flow cytometry assay suppressed 4-Cl-BQ induced increases in DHE and DCFH fluorescence suggesting that the 4-Cl-BQ induced increase in ROS could be due to an increase in superoxide and hydrogen peroxide levels. Flow cytometry measurements of cumulative bromodeoxyuridine-incorporation showed that the 4-Cl-BQ treatment delayed quiescent cells' entry into S-phase following re-plating. This inhibition in S-entry was associated with suppression in cyclin D1 protein levels. Interestingly, a simultaneous treatment of quiescent cells with PEG-SOD, PEG-CAT and 4-Cl-BQ reversed 4-Cl-BQ -induced suppression in cyclin D1 protein levels and facilitated G0/G1 cells entry into S-phase. These results support the novel hypothesis that MnSOD and cyclin D1 regulate an inter-organeller communication coordinating cellular metabolism to DNA synthesis in PCB treated cells. (NIEHS P42 ES 013661 and NIH CA 111365)

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G6pd-Knockdown Exacerbates H_2o_2-Induced Cell Apoptosis by Inhibiting Protein Tyrosine Phosphatase Activity and Activating MAP Kinases

Chang-Jun Lin[1,2], Mei-Ling Cheng[1], Hung-Yao Ho[1], Jau-Song Yu[3], and ***Dainel Tsun-Yee Chiu[1]***

[1]Graduate Institute of Medical Biotechnology, Chang Gung Univ., [2]School of Life Sciences, Lanzhou Univ., [3]Graduate Institute of Biomedical Sciences, Chang Gung Univ.

G6PD plays a key role in the generation of NADPH and thus the cellular redox balance. However, it has not been clearly delineated how G6PD-knockdown may affect cellular response. to address this issue, we established G6PD- knockdown HepG2 cell line using RNAi. With their G6PD activity reduced by 90%, the G6PD-knockdown cells exhibited increased oxidative stress and enhanced susceptibility to proliferation arrest and apoptosis. G6PD-knockdown resulted in augmentation of H_2O_2-induced inhibition of protein tyrosine phosphatase (PTP) activity, and prolonged phosphorylation of MAPKs. Low concentrations of H_2O_2 (<500 μM) induced expression of MAPK phosphoatase-1 (MKP-1), while high concentration of H_2O_2 (1000 μM) inhibited its expression. G6PD-knockdown also inhibited/postponed H_2O_2 (500 μM)-induced MKP-1 expression. the kinetics of MKP-1 expression correlated with the dephosphorylation kinetics of MAPKs. Using pharmacological inhibitors, we further demonstrated that the prolonged activation of MAPKs was involved in enhanced apoptotic response of G6PD- knockdown cells to oxidative stress. Our data suggest that G6PD-knockdown may exacerbate H_2O_2-induced cell apoptosis by inhibiting PTP activity and activating MAPKs.

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Nox4 and Nox1 Enzymes Are Persistently Elevated in Human Hepatocytes Producing Infectious Hepatitis C Virus

Nabora Soledad Reyes deRogers[1], Scott Seronello[1], Chieri Ito[1], Mark Zern[2], Henry Jay for man[1], J. R. David Lambeth[3], and ***Jinah Choi[1]***

[1]University of California, Merced, [2]University of California, Davis, [3]Emory University

Hepatitis C virus (HCV) is an etiologic agent of severe liver diseases in humans. HCV infection has been associated with severe alterations of the host redox status, and oxidative stress has been identified as a key mechanism of HCV-induced pathogenesis. Nevertheless, the source of H2O2 and superoxide during HCV infection is still incompletely characterized. Recently, hepatocytes have been found to express Nox family enzymes, but whether these proteins participate in HCV-induced oxidative stress is unknown. We found that Huh7 hepatoma cells express Nox1, Nox2, Nox4, and Duox1/2 mRNAs. Most of all, Nox4 and Nox1 mRNA's were persistently elevated in Huh7 cells that produced infectious HCV particles, compared to mock-transfected controls. Duox2 mRNA was only transiently elevated with HCV. the same Nox/Duox mRNAs were also increased in telomerase-reconstituted primary human hepatocytes transfected with HCV RNA. in contrast, subgenomic HCV RNA did not increase Nox/Duox mRNA's, suggesting that the structural protein(s) or the production of infectious virions is necessary. in addition, Nox4 and Nox1 proteins were elevated in HCV RNA-transfected cells as well as HCV-infected cells and human liver. HCV increased the fluorescence of H2-dichlorofluorescein diacetate (DCF-DA) **in** Huh7 cells that was sensitive to diphenylene iodonium, an inhibitor of flavoenzymes including Nox enzymes. Finally, Nox4 was prominent in the nuclear compartment of these cells, particularly in the presence of HCV. Therefore, hepatocyte Nox proteins may act as a robust and regulated source of oxygen-centered reactive species during chronic HCV infection and are likely to contribute to the pathogenesis.

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(Pro)Renin Receptor Stimulation Induces Fibrosis Genes Via An Oxidant-Dependent Mechanism in Human Epithelial Kidney Cells

Nicolas Clavreul[1]*, Patricia Sansilvestri-morel[1], Delphine Magard[1], Alain Rupin[1], and Tony J. Verbeuren[1]*

[1]Servier Research Institute, Suresnes, France

The (pro)renin receptor has recently been demonstrated to bind equally well renin and its precursor, prorenin, leading to an intracellular signaling independently of angiotensin II production. in this study, we report that human epithelial kidney cells exposed to renin and prorenin (10 to 100 nM) for 24h in presence of an ACE inhibitor show an increase of Nox4 expression. Consequently, superoxide anion production is enhanced as measured by luminescence (lucigenin), and electron spin resonance spectroscopy (hydroxylamine radical transition). Also, both renin and prorenin stimulate transforming growth factor β (TGF-β), fibronectin and PAI-1 expression and therefore participate to an overall switch toward a pro-fibrotic state of the kidney cells. as suggested by others, the latter seems correlated to an increase of MAPK signaling that we measured via ERK phosphorylation. When the cells are transfected with a siRNA targeting the (pro)renin receptor, the (pro)renin-dependent increase of Nox4 expression is efficiently prevented as well as the increase of superoxide production. Interestingly, renin and prorenin are also no longer capable of inducing TGF-β, fibronectin and PAI-1 neither can they promote ERK phosphorylation. Finally, we demonstrated that transfection of the cells with a Nox4 specific siRNA also prevented fibrosis gene expression following treatment of renin and prorenin. We propose the new hypothesis that renin and prorenin, through a direct binding to their specific membrane receptor, promote fibrotic genes expression via a Nox4-dependent mechanism.

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Overexpression of Thioredoxin-1 in Mice Induced Changes in NFκB and Nrf2 Redox-Sensitive Signaling Pathways.

Wilson C. Y. Fok[1]*, Lisa Cortez[1], Celeste Webb[1], Nidia Garcia[1], Dean Jones[2], Junji Yodoi[3], Arlan Richardson[1], Yuji Ikeno[1], and Viviana Pérez[1]*

[1]The University of Texas Health Science Center at San Antonio, [2]Emory University School of Medicine, [3]Kyoto University School of Medicine

Thioredoxin 1 (Trx1) is a major antioxidant enzyme that plays an important role in the maintenance of the reduced environment in the cells through thiol-disulfide exchange reactions. Trx1 has been also shown to affect redox-sensitive signaling and the binding activity of transcription factors to DNA leading to changes in gene expression. in this work, we examined whether overexpression of Trx1 in Trx1 transgenic mice [Tg(hTRX1)$^{+/0}$] leads to changes in redox sensitive signaling such as NFκB, and Nrf2 in the liver of Tg(hTRX1)$^{+/0}$ and wild type mice. Our results indicate that Tg(hTRX1)$^{+/0}$mice have approximately 30% more reduced for m of Trx-1 in the cytosol than wild type mice, and 40% higher levels of total Trx-1 protein in the nucleus compared to wild type mice. Tg(hTRX1)$^{+/0}$mice showed higher levels of NFkB (p65 and P50) in the cytosol after diquat treatment compared to wild type mice. We also found that Nrf2 levels in the cytosol are lower in Tg(hTRX1)$^{+/0}$ compared to wild type mice,

which is associated with higher levels of Keap-1, an inhibitory protein for Nrf2. Our findings suggest that higher levels of Trx1 in the reduced for m in the cytosol, could inhibit the activation redox-sensitive signaling (NFκB and Nrf2) in the cytosol, and thereby Tg(hTRX1)$^{+/0}$mice would be expected to have lower expression of those target genes under these redox sensitive signaling pathways. Current experiments on the expression of genes regulated by these transcription factors are being conducted to determine the role of increased Trx-1 expression on cellular function. This work was supported by AFAR grant (VP), NIH Grants AG13319 (AR) and VA Merit Review Grant from Department of Veteran Affairs (YI).

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Variation of Nrf2 Activation in Differentiated and Undifferentiated Cells

***Jason M Hansen*[1]**

[1]*Emory University*

Development is hallmarked by the differentiation of cells from a uniform phenotype to various types that perform specific functions and display unique cellular characteristics. Cell models of differentiation and during organogenesis in vivo have shown changes in glutathione (GSH) redox status, where redox potentials (Eh) become increasingly oxidized as differentiation/development ensues. Interestingly, recent data has shown that GSH Eh is not in equilibrium with other thiol redox couples, such as the thioredoxins(Trx) and cysteine (Cys), but it is unknown how Trx and Cys are regulated during development and during periods of oxidative stress during differentiation. We propose that undifferentiated cells that are more reducing are more protected from oxidative insult as a result of their more reducing environment, but also do not induce redox-sensitive transcription factor activity as fully as in more oxidizing, differentiated cells. Using various in vitro models of differentiation, we showed that the GSH, Trx1 and Trx2 redox status in differentiated cells did not rebound as quickly over a 3 h period as they did in undifferentiated, following a single bolus dose with tert-butylhydroperoxide. the inability to quickly readjust redox imbalance may have also accounted for the observed increase in oxidant-induced cell death in differentiated vs. undifferentiated cells. Nrf2 is a redox-sensitive transcription factor that when activated up-regulates phase II detoxification and antioxidant enzymes, including those that regulate the GSH and Trx systems. Utilizing differentiated and undifferentiated cells, we showed that Nrf2 activation was much more easily stimulated in differentiated cells than in undifferentiated cells, suggesting that the control of redox-sensitive factors in differentiating cells that have a more oxidizing environment may be more easily stimulated that in undifferentiated cells. Since early organogenesis is the developmental stage where the majority of teratogens exert their effect, the inability to induce a normal antioxidant response via Nrf2 may lead to changes in cell function or ultimate differentiated phenotype and cause dysmorphogenesis.

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Mammalian Lanthionine Synthetase-Like Protein-1 (Lancl1) Catalyzes Glutathionylation of Phospho-Amino Acid Substrates in Vitro : Possible Implications to Redox Signaling

***Kenneth Hensley*[1]**, *Xuejun C. Zhang*[1], *Wenchi Zhang*[2], *Quentin N. Pye*[1], *and Nancy Wakeham*[1]

[1]*Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, United States,* [2]*Institute of Biophysics, Chinese Academy of Sciences, Beijing China*

Lanthionine synthetase (LanC) is a prokaryotic enzyme that catalyzes for mation of intramolecular thioether bonds between cysteine and serine or threonine residues, en route to synthesis of potent “lantibiotic” type antibiotic peptides. Recently we reported (Biochemistry 2007) that the mammalian homolog of LanC, lanthionine synthetase-like protein-1 (LanCL1), specifically binds glutathione (GSH) suggesting a role for LanCL1 in the catalysis of GSH conjugations. We now report the X-ray crystallographic structure of LanCL1 both alone and complexed with GSH. LanCL1 displays a double 7-helix barrel structure, with seven conserved GxxG motifs, for ming a Zn^{2+} binding site, similar to that of LanC. GSH binds to the zinc pocket, with its sulfhydryl as one ligand for the metal center. Recombinant human LanCL1 (rhLanCL1) neither catalyzed glutathione S-transferase-like alkylations, nor conjugated GSH to glutathionylation-prone proteins such as actin. rhLanCL1 did catalyze GSH conjugation to albumin-linked phosphoserine, phosphothreonine or phosphotyrosine but not to native albumin. This glutathionylation was partially but incompletely reversed with strong reducing agents, and could be accomplished using either GSH or to a lesser extent with GSSG. Weak glutathionylation was observed in vitro using the phosphoserine-rich egg yoke protein phosvitin, but not when the target substrate was phospho-casein or small phosphoserine-containing peptides modeled from phospho-MAPK or phospho-vimentin. Mass spectrometry studies using a synthetic FITC-labeled phosphoserine pseudo-substrate revealed LanCL1-catalyzed for mation of novel serine-O-SG linkage in vitro. These findings suggest that LanCL1 may play a role in redox signaling or in synthesis of small-molecule products from phosphoester substrates. Funded in part by the Oklahoma Center for Advancement of Science and Technology (OCAST).

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Ras Activity Is Modulated by Glutathione and Nitric Oxide Via One Electron But Not Two Electron Mediated Mechanisms

***Aaron Hobbs*[1]**, *and Marcelo Bonini*[2]

[1]*University of North Carolina at Chapel Hill,* [2]*NIH/NIEHS*

The Ras protein cycles between active GTP-bound and inactive GDP-bound states to regulate a host of cellular processes, including cell growth, differentiation and apoptosis. Single point mutations in Ras that increase Ras-GTP levels in cells result in deregulation of Ras activity, leading to cancer and developmental disorders. Ras activity can be regulated by protein modulatory agents and redox molecules. We uncovered a novel mechanism of Ras regulation by free radical-oxidants, and have demonstrated that superoxide and nitric oxide (NO) can react with C118, resulting in Ras activation via a radical mediated electron transfer mechanism. Intriguingly, stable nitrosation of Ras does not alter its activity. Although we anticipated that this radical-mediated mechanism could be generalized to other redox active molecules capable of reacting with Ras to generate a thiyl radical, recent reports indicate that glutathionylation of Ras at C118, alters its structure and activity. We conducted biochemical, NMR and EPR studies to characterize the effects of one-electron (NO) and two-electron (GSSG) oxidation on Ras activity and structure. Our results indicate that NO is able to modify Ras and cause guanine nucleotide dissociation (GND) via a radical-based mechanism. in contrast, NMR and kinetic data indicate that modification with glutathione via 2-electron oxidation does not alter Ras structure or activity. Using EPR spin trapping and kinetic measurements, we show that reaction of Ras with DEANO (an NO donor) promotes for mation of a radical at C118 in Ras, resulting in radical mediated transfer to the guanine nucleotide base and GND. the rate of GND is unaltered by treatment of Ras with oxidized glutathione under conditions that specifically modify C118 by a non-radical mechanism. in summary, our data suggest that free radicals induce GND through the intermediacy of a thiyl radical

centered at Ras C118 what constitutes an alternative route of Ras activation in the cell.

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Zinc Oxide Nanoparticles Induce Oxidative Stress and Alter Calcium Homeostasis in Human Bronchial Epithelial Cells (BEAS-2B)

***Chuan-Chin Huang*[1]**, *Robert S Aronstam*[1], *Da-Ren Chen*[2], *and Yue-Wern Huang*[1]

[1]*Missouri University of Science and Technology,* [2]*Washington University in St. Louis*

The influence of 20 nm zinc oxide particles on intracellular calcium levels and gene expression was studied in human bronchial epithelial cells (BEAS-2B). There was a steep decline in cell numbers at concentration between 6-10 μg/mL. Intracellular ROS levels and LDH concentrations in the cell culture media were increased in dose-dependent manners. Exposure to ZnO increased intracellular calcium levels. Treatment with an antioxidant *N*–acetylcysteine (NAC) prevented cell loss indicating oxidative stress mediated cytotoxicity. Ca2+ concentrations were elevated by four fold compared to control cells and the elevation was partially attenuated when treated with NAC. Exposure to a sublethal dose of ZnO (5 μg/mL) increased the expression of *BNIP*, *PRDX3*, *PRNP*, and *TXRND1* genes which were involved in apoptosis and OS responses. Thus, exposure of BEAS-2B cells to 20 nm ZnO results in 1) a dose- and time-dependent cytotoxicity reflected in cell viability reduction, elevated oxidative stress, and cell membrane damage, 2) a dose-dependent elevation of intracellular Ca2+ levels, and 3) several genes induced in apoptosis and OS responses.

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The Role of Cadmium-Induced Reactive Oxygen Species in Male Infertility

***Karen E Iles*[1]**, *Karen M Lewis*[1], *Corinne Zaragoza*[1], *Thomas Hock*[1], *and Dale a Dickinson*[1]

[1]*University of Alabama at Birmingham*

The incidence of male infertility and decreased fertility is increasing in industrialized countries. Cadmium (Cd) is an established male reproductive toxin in animals but its role in human infertility is poorly understood. Exposure to Cd comes from environmental contamination, occupational exposure and cigarette smoking. Cd bio-accumulates, with a half-life of around 20 years and can have impacts on human health long after exposure has ceased. Defining the potential role of Cd in male infertility is challenging as the development of functional spermatozoa is a complex process. Testicular Sertoli cells play essential roles in stem cell renewal and maturation. Environmental factors that negatively affect these cells may be critical in the development of male infertility. the specific aim of these studies is to define the mechanism of Cd-induced damage to Sertoli cells and the role that this may play in male infertility. It is hypothesized that Cd-mediated cellular damage increases ROS, induces stress response proteins, and depletes intracellular GSH. We have determined that Sertoli cells are acutely sensitive to Cd (0-100 μM; 2-48 h) versus other cell lines tested based on cell viability. Changes in the stress response protein heme oxygenase-1 (HO-1) were assayed in vitro and in vivo in mice exposed to Cd via Western blotting (protein) and Real-time PCR (mRNA) and found to increase significantly ($p<0.05$,0-100 μg, mice; 0-100, μM cells) indicating that Cd represents a significant stress for these cells. This was accompanied by visible testicular damage in vivo. a small induction (25%) of GSH was observed at low (5 μM) Cd, followed by a precipitous drop at all higher (25-100 μM) concentrations of Cd. Real-time PCR and Western blotting revealed an increase in both Gclc and Gclm mRNA and protein without a concomitant increase in GSH. Pretreatment or co-treatment of Sertoli cells with BSO (a GCL activity inhibitor) significantly reduced cell viability at high (25-100 μM) concentrations only suggesting a GSH-dependent mechanism of cell death. These data support an ROS-dependent mechanism for Cd-related damage to Sertoli cells and may provide mechanistic insights into the role of Cd in male infertility.

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HSF1-Dependent Induction of BAG3 by 4-Hydroxynonenal Stabilizes Bcl2-Family Members and Attenuates Cell Death

***Aaron Thomas Jacobs*[1]**, *and Lawrence Joseph Marnett*[1]

[1]*Vanderbilt University*

The oxidation of biological membranes is increasingly recognized as a contributing factor in a number of neurodegenerative and inflammatory diseases. 4-Hydroxynonenal (HNE), a cytotoxic and diffusible electrophile generated by the spontaneous decomposition of oxidized lipids, has a suspected role in disease processes. in addition to promoting cell death, elevated levels of HNE lead to the engagement of cytoprotective signaling pathways, including the heat shock, antioxidant, DNA damage, and ER stress responses. Activation of the heat shock response, mediated by the transcription factor heat shock factor 1 (HSF1), is critical for maintaining cellular viability in the presence of HNE. Accordingly, using siRNA to suppress HSF1 expression dramatically sensitizes cells to HNE-induced apoptosis. Silencing HSF1 is correlated with a marked reduction in the levels of the anti-apoptotic Bcl2-family proteins, Bcl-XL and Mcl-1. in contrast, Bcl-XL and Mcl-1 mRNA levels are not affected. Microarray analysis of gene expression from control and HSF1-silenced cells was used to identify anti-apoptotic HNE-inducible transcripts. Bcl-2 associated athanogene domain 3 (BAG3), a known Bcl-2 interacting protein and Hsp70 co-chaperone, was identified as an anti-apoptotic gene induced by HNE in an HSF1-dependent fashion. Using a FLAG-tagged BAG3 expression construct, we confirm that BAG3 binds to Hsp70, and demonstrate a novel interaction with endogenous Bcl-XL by co-immunoprecipitation. Similar to HSF1, silencing BAG3 using siRNA causes a dramatic reduction in Bcl-XL and Mcl-1 protein levels, but with no effect on mRNA expression. Furthermore, silencing BAG3 increases the sensitivity to HNE-induced apoptosis to a similar degree as silencing HSF1. BAG3 thus has a unique role in stabilizing Bcl-XL and Mcl-1 expression and is important in maintaining cellular viability in the presence of oxidized lipid stress.

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Nrf2-Dependent and Independent Responses to Nitro-Fatty Acids in Human Endothelial Cells: Identification of the Heat Shock Reponse As the Major Pathway Activated by Nitro-Oleic Acid

***Emilia Kansanen*[1]**, *Henna-Kaisa Jyrkkänen*[1], *Oscar L Volger*[2], *Hanna Leinonen*[1], *Annukka M Kivelä*[1], *Francisco Schopfer*[3], *Anton J Horrevoets*[2], *Seppo Ylä-Herttuala*[1], *Bruce a Freeman*[3], *and Anna-Liisa Levonen*[1]

[1]*University of Kuopio, Finland,* [2]*VU Medical Center, Amsterdam, the Netherlands,* [3]*University of Pittsburgh, USA*

Nitro-oleic acid ($OA\text{-}NO_2$) is an electrophilic fatty acid nitroalkene derivative that mediates anti-inflammatory signaling reactions. the

Nrf2-Keap1 pathway is activated by electrophilic fatty acids, including OA-NO_2, and the pathway has been shown to suppress redox sensitive pro-inflammatory gene expression and to protect against oxidant-mediated injury in endothelial cells. It was therefore postulated that Nrf2 in part accounts for anti-inflammatory actions of OA-NO_2, motivating the characterization of Nrf2-dependent and -independent effects of OA-NO_2 on gene expression using microarray analysis. Control and Nrf2-siRNA transfected human endothelial cells were treated with vehicle, oleic acid, or OA-NO_2 and the differential gene expression profiles were determined using an Affymetrix U133 Plus 2.0 Array. Consistent with the initial hypothesis, OA-NO_2 significantly induced the expression of Nrf2-dependent genes (e.g., HMOX1 and GCLM), but importantly, it was discovered that the majority of OA-NO_2 regulated genes were not Nrf2-dependent. Moreover, Gene Set Enrichment Analysis revealed that the heat shock response is the major pathway activated by OA-NO_2, with a robust induction of several heat shock genes such as HSPA6, DNAJ4 and HSPA1B. Inasmuch as the heat shock response mediates anti-inflammatory actions, this mechanism is proposed to contribute to the protective cell signaling actions of nitro-fatty acid derivatives.

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Activation of Endothelial Nitric Oxide Synthase and Heme Oxygenase 1 Expression in Vasculature by Nitro-Fatty Acids

Nicholas Khoo[1], *Franca Golin-Bisello*[1], *Rakesh P Patel*[2], *and Bruce a Freeman*[1]

[1]*University of Pittsburgh,* [2]*University of Alabama at Birmingham*

An early manifestation of vascular disease is endothelial dysfunction, often a consequence of a reduction in nitric oxide (NO) bioavailability. Reactive oxygen species mediate reduction of NO bioavailability, with secondary oxidized and nitrated byproducts of these reactions contributing to the pathogenesis of numerous cardiovascular diseases (CVDs). Herein we report that redox-derived nitro-fatty acids (NO2-FA) activate expression of endothelial nitric oxide synthase (eNOS) and heme oxygenase 1 (HO-1) in the vasculature, thus transducing vascular protective effects associated with enhanced NO production. NO2-FA treatment increased, in a dose-dependent manner, eNOS mRNA and protein expression (>3- and >2.5-fold, respectively) in cultured endothelial cells (ECs), 6 to 24 h after treatment. Cell viability was not significantly altered and HO-1 expression was also enhanced (>15-fold for mRNA and protein). Furthermore, HO-1 levels also significantly increased dose- and time-dependently in vascular smooth muscle cells (VSMCs). Appreciating that phosphorylation of eNOS at several regulatory sites plays an important role in enzyme activity and can result in a 2-3 fold increase in NO production, ECs were treated with 0.1-5 μM NO_2-FAs, which stimulated the phosphorylation of eNOS at Ser^{1179} and induced concomitant dephosphorylation at Thr^{497}. These post-translational modifications of eNOS, in concert with elevated eNOS gene expression contributed to an increase in cellular NO production as measured by ozone chemi-luminescence. Upstream stimulation of eNOS phosphorylation was dependent upon the phosphatidylinositol 3-kinase-Akt pathway and the p38 mitogen-activated protein kinase pathways. in aggregate, NO_2-FA-induced eNOS and HO-1 expression by ECs and HO-1 upregulation by VSMCs may have beneficial effects on endothelial dysfunction and provide a new strategy for therapy associated with CVD. While oxidized lipids and lipoproteins exacerbate inflammatory reactions in the vasculature, in stark contrast the nitration of polyunsaturated fatty acids and complex lipids yield electrophilic products that exhibit pluripotent anti-inflammatory signaling capabilities acting via both cGMP-dependent and -independent mechanisms.

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Overexpressed cyclophilin B suppresses apoptosis associated with ROS and calcium homeostasis after ER stress

Jin Hwan Kim[1], *Tae Kyu Choi*[1], *Young Hwan Kim*[1], *and* ***Sung soo Kim***[1]

[1]*Medical Science and Engineering Research Center, BK-21, Kyung Hee University, Seoul, Korea*

Prolonged accumulation of misfolded proteins in the endoplasmic reticulum (ER) results in ER stress-mediated apoptosis. Cyclophilins are protein chaperones that accelerate the rate of protein folding through their peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. in this study, we demonstrated that ER stress activates the expression of the ER-localized cyclophilin B (CypB) gene through a novel ER stress response element. Overexpression of wild type CypB attenuated ER stress-induced cell death, whereas overexpression of an isomerase activity defective mutant, CypB/R62A, not only increased calcium leakage from the ER and ROS generation, but also decreased mitochondrial membrane potential, resulting in cell death following exposure to ER stress-inducing agents. SiRNA-mediated inhibition of CypB expression rendered cells more vulnerable to ER stress. Finally, CypB interacted with the ER stress-related chaperones, Bip/Grp78 and Grp94. Taken together, we concluded that CypB performs a crucial function in protecting cells against ER stress via its PPIase activity.

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Proto-Oncogenic C-Ras Stimulates Muscle Differentiation

Jisun Lee[1], *and* ***Sung soo Kim***[1]

[1]*Medical Science and Engineering Research Center, BK-21, Kyung Hee University, Seoul, Korea*

Oncogenic H-Ras G12V and its variants with the mutations T35S, Y40C, or E37G have been shown to inhibit muscle differentiation. However, the role of proto-oncogenic c-Ras in muscle differentiation has not been reported. Therefore, we investigated if c-Ras stimulates muscle differentiation via PI 3-kinase and its downstream pathway. Inhibition of c-Ras by farnesyltransferase inhibitors and dominant negative Ras (RasS17N) suppressed muscle differentiation. Activity of c-Ras was maintained constant during myogenic stage as proliferation stage. However, the activated c-Ras enhanced PI 3-kinase activity with decrease in Erk activity in differentiating cells, compared with proliferating cells. PI 3-kinase and its downstream myogenic pathway, the p38 MAPK/Nox2/NF-κB/iNOS pathway, were all suppressed by inhibition of c-Ras activity. Interestingly, expression of constitutively active PI 3-kinase completely rescued differentiation block and suppression of p38 MAPK/Nox2/NF-κB/iNOS pathway both of which were induced by inhibition of c-Ras activity. in conclusion, c-Ras stimulates muscle differentiation by enhancing the activities of PI 3-kinase and its downstream pathway.

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Differential Modulation of Hepatic Drug Metabolizing Function by Ischemia/Reperfusion in Alcoholic Fatty Liver: Role of Kupffer Cells

Hyo-Yeon Kim[1], *Sang-Won Park*[1], *and Sun-Mee Lee*[1]

[1]*College of Pharmacy, Sungkyunkwan University*

Although fatty liver shows the higher incidence of primary graft non-function after transplantation, the mechanism responsible for

this remains unclear. Since Kupffer cells provide signals that regulate hepatic response in ischemia/reperfusion (I/R), the aim of this study was to investigate the role of Kupffer cells in hepatic cytochrome P450 (CYP) activity and its gene expression in fatty livers. Rats were fed alcohol liquid diet (ED) or isocaloric control diet (CD) for 5 weeks followed by 90 min of hepatic ischemia and 5 h of reperfusion. Kupffer cells were inactivated by gadolinium chloride ($GdCl_3$, 7.5 mg/kg i.v.) 24 h prior to ischemia. After I/R, alcoholic fatty liver showed significantly higher levels of serum aminotransferase and lipid peroxidation but lower levels of hepatic glutathione content. These changes attenuated by $GdCl_3$. CYP1A1 and 1A2 activities and the level of their protein and gene expression decreased in alcohol-treated ischemic groups, with no significant difference between saline and $GdCl_3$ groups. During I/R, the activity and levels of protein and gene expression for CYP2B1 decreased, whereas CYP2E1 activity and transcript increased in alcoholic fatty liver. These changes were attenuated by $GdCl_3$. in alcohol-treated ischemic rats, the increase in tumor necrosis factor-α, inducible nitric oxide synthase and cyclooxygenase-2 transcripts was attenuated by $GdCl_3$. We conclude that the activation of Kupffer cells plays an important role in abnormalities in microsomal drug metabolizing function induced by I/R in alcoholic fatty liver.

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Protein Kinase C-Delta Mediated Ischemic Preconditioning Induced Hepatoprotection in Rat

***Tae-Hoon Kim*[1]**, *Seok-Joo Kim*[1], *Sung-Hwa Kim*[1], *and Sun-Mee Lee*[1]

[1]*College of Pharmacy, Sungkyunkwan University*The role of protein kinase C (PKC) in ischemic preconditioning (IPC) is still controversial, partly because of the multiple isozymes of PKC and the inability to directly measure PKC activity in vivo. to investigate which isoform is involved in ischemic preconditioning, we identified the PKC isoform that translocates to the membrane fraction by immunoblotting. IPC was achieved by 10 min of ischemia followed by 10 min of reperfusion prior to sustained ischemia. Western blot analysis demonstrated translocation of the PKC-delta isoform but not alpha, beta I, II and zeta isoforms from cytoplasm to the membrane fraction for IPC. Rats were subjected to 1.5 h of hepatic ischemia followed by 6 h reperfusion. We blocked PKC-delta with rottlerin (0.3 mg/kg, i.v.) 10 min before the IPC procedure to establish the effect of the PKC-delta in process of IPC. Increased serum aminotransferase activities and lipid peroxidation levels were markedly decreased by IPC during hepatic I/R. the rottlerin blunted the effect of IPC. IPC ameliorated multiple and extensive areas of portal inflammation, hepatocellular necrosis, which was randomly distributed throughout the parenchyma, and a moderate increase in inflammatory cell infiltration. These histological changes were reversed by the rottlerin treatment. the activation of caspase-3 was observed 6 h of reperfusion, which was attenuated by IPC. IPC showed markedly fewer apoptotic (TUNEL positive) cells compared with the I/R group. Anti-apoptotic effects of IPC were abolished when preconditioned rat liver was pretreated with rottlerin. Our data further support PKC as a critical part of the signal transduction pathway in IPC and indicate that PKC-delta alone is responsible for protection of rat hepatocyte during IPC.

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Cell-Type Specific Regulation of Responses to 4-Hydroxynonenal: Differential Mechanisms of Epre Activation of Phase II Genes in Two Human Cell Lines

***Smadar Levy*[1]**, *and Henry Jay for man*[1]

[1]*UCMerced*The transcription factors that bind to the EpRE elements play a key role in the regulation of phase II genes. in this work, we used chromatin immunoprecipitation assays (ChIP) to identify the recruitment of transcription factors to the EpRE following their translocation to the nucleus. Human bronchial epithelial (HBE1) and human hepatoma (HepG2) cells were exposed to 4-hydroxy-2-nonenal (HNE) and differences in regulation between the cell types were studied. We found significant translocation of Nrf2 to the nucleus of HBE1 cells following exposure to HNE; however, in HepG2 cells, only a moderate increment was observed although at an earlier time following exposure. We have shown by ChIP assays that marked increases in binding to EpRE sequences occurred subsequent to HNE exposure of c-Jun to nqo2, gclc and gclm in HepG2 cells; however, in HNE-exposed HBE1 cells, only phosphorylated c-Jun increased in binding to EpREs. the latter was accompanied by phosphorylation of c-Jun and JNK as determined by western blot analysis. in HepG2 cells, phosphorylation of c-Jun was not essential for binding to and activation of EpRE. Additionally, JNK activation was not essential in the induction of promoter activity, as determined by using the peptide JNK inhibitor (JNKi); however, expression of GCLM protein was inhibited by JNKi, suggesting that JNK exerted at post transcriptional regulation in HepG2 cells. in summary, we have shown that while HNE induces phase II genes in both cells, the mechanisms differ markedly with respect to recruitment of transcription factors and activation of the JNK pathway.

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Molecular Mechanisms of Nrf2-Mediated Antioxidative Defense

Ah-Ng Tony Kong[1], *and* ***Wenge Li*[1]**

[1]*Rutgers University*

Nrf2 plays the central role in the antioxidative defense of mammalian cells. Nrf2 regulates the transcription of diverse phase II detoxification enzymes such as GST, UGT and NQO1. Nrf2 also controls the expression of antioxidant enzymes such as SOD, Catalase, Trx, Gpx, and γGCS. in addition, Nrf2 also regulates the expression of phase III efflux transporters. Aberrant Nrf2 signaling and persistent overloading of reactive oxygen species are implied in the etiology of neurodegenerative disease, cancer and aging. Under unstressed conditions, Nrf2 is sequestered in the cytoplasm by a cytoskeleton anchoring protein Keap1. Keap1 is also a Cullin 3 dependent substrate adaptor protein for Nrf2 ubiquitination that mediates constant degradation of Nrf2. as a consequence, the pool of free Nrf2 is very small. When exposed to oxidative stress however, the Keap1-mediated Nrf2 degradation is impeded and Nrf2 translation is elevated. as a consequence, the pool of free Nrf2 expands. Nrf2 exhibits a graded nuclear translocation correlated with the dosage of oxidants. So Nrf2 can not only transmit the presence but also the intensity of oxidative signals into the nucleus. in the nucleus, Nrf2 for ms heterodimer with small Maf proteins to orchestrate the transcription of plethora of phase II detoxification enzymes/antioxidants and phase III efflux transporters. as a consequence, these cells can effectively neutralize and remove excess oxidants to restore redox homeostasis. Recent evidence

shows that Nrf2 signaling is regulated at the transcription, translation and post-translation levels. Collectively, the elaborate yet highly efficient cytoprotective machinery enables mammalian cells to adapt to a momentarily changing aerobic environment. (Supported by NIH R01-CA094828).

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Nrf2 and Antioxidant Response Element in the Rat Glutamate-Cysteine Ligase Catalytic Subunit

***Muyao Li*[1]**, *Jen-Fu Chiu*[2], *Anne T Kelsen*[1], *Shelly C Lu*[3], *and Naomi K Fukagawa*[1]

[1]*University of Vermont,* [2]*Hong Kong University,* [3]*Keck School of Medicine USC*

The antioxidant response element (ARE) is an essential component of upstream regulatory sequences present on genes for most phase II detoxification enzymes, including the glutamate cysteine ligase catalytic subunit (GCLC). NF-E2-related factor 2 (Nrf2) is a principal transcription factor that binds to the ARE and plays a key role in cellular responses to stress via the Keap1-Nrf2-ARE pathway. However, the ARE that mediates human GCLC gene expression has not been reported in the rat. Thus, how the ARE-mediated Keap1-Nrf2-ARE pathway regulates glutathione homeostasis in the rat remains a puzzle. in the current work, we identified a putative ARE sequence ~4kb upstream in the rat GCLC through a candidate sequence search. We further defined the rat GCLC-ARE in the category with the most ARE characters, i.e., this rat GCLC-ARE is a sequence-specific site that significantly enhances promoter activity in reporter genes. the rat GCLC-ARE is a Nrf2-mediated element to which binding has been demonstrated in nuclear extracts. the Nrf2-ARE binding activity can be induced by the antioxidant, tert-butylhydroquinone (TBH), or through over-expression of Nrf2. Therefore, discovery of the rat GCLC-ARE enhancer ***confirms the*** existence of identical mechanisms in both humans and the rat.

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Transforming Growth Factor Beta (TGF-β) Increases the Level of Nox4 and Induces Protein Thiol Modifications in MAPK Phosphatase-1 in Fibroblasts

***Rui-Ming Liu*[1]**, *Edward Postlethwait*[1], *Henry Jay for man*[2], *James Hagood*[1], *and Jinah Choi*[2]

[1]*University of Alabama at Birmingham,* [2]*University of California at Merced*

Transforming growth factor beta (TGF-β) stimulates ROS production in fibroblasts, which mediate many of TGF-β's fibrogenic effects. However, the molecular mechanisms whereby TGF-β increases ROS production and the mechanisms whereby ROS mediate TGF-β's signaling process remain equivocal. We have shown previously that TGF-β increases ROS production in murine embryonic fibroblasts (NIH3T3). Inhibition of NADPH oxidase with diphenyliodonium (DPI) or treating cells with MnTBAP, a superoxide dismutase and catalase mimetic, blocks TGF-β stimulation of JNK and p38 MAPK phosphorylation as well as induction of plasminogen activator inhibitor 1 (PAI-1), a protease inhibitor critical in many pathologic conditions including fibrogenesis. Our recent studies further demonstrate that TGF-β increases the level of Nox4 in fibroblasts in both cytosolic and nuclear fractions. Most interestingly, we show that TGF-β induces protein thiol modifications in MAPK phosphatase 1 (MKP-1), a JNK and p38 specific phosphatase, using biotin labeling-straptavidin pull down-western as well as immunoprecipitation techniques. the thiol modification of MKP-1 was associated with a decline in the activity of MKP-1. Taken together, the results suggest that TGF-β increases Nox4 expression/activity, which leads to oxidative modification of MAPK phosphatase-1 and therefore inhibition of its activity. Decreased activity of MKP-1 may then lead to a sustained activation of JNK and p38 MAPKs and induction of PAI-1 in TGF-β-induced fibrogenesis. Supported by NIH ES011831 and a grant from ALA (RM. Liu) and NIH HL54696 (EM. Postlethwait)

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FOXO3a Binding to the SOD2 Promoter Causes Chromatin Remodeling and Transcriptional Activation of Mnsod Expression

***Joshua MADSEN*[1]**, *and Frederick Domann*[1]

[1]*University of Iowa*

Despite extensive knowledge regarding aberrant expression of *SOD2* in human disease, little is known about the mechanisms that control *SOD2* expression in normal cells. We initially observed that *SOD2* mRNA levels were ~5-fold higher in post-confluent human mammary epithelial cell lines (MCF10A and HMEC-hTert) compared to cells in exponential growth phase. the induction of *SOD2* expression during conditions mimicking quiescence could be explained in part by the action of the cellular transcription factor FOXO3a whose DNA binding and transactivating activities are inhibited during proliferation by posttranslational modifications. to test the hypothesis that FOXO3a mediates the observed quiescence-associated increase in *SOD2* mRNA, we expressed a dominant active for m of FOXO3a in exponentially growing MCF7 mammary carcinoma cells. These cells displayed up to a 3-fold increase in *SOD2* expression by qRT-PCR accompanied by FOXO3a binding to its cognate site in the *SOD2* promoter in *vivo* as detected by chromatin immunoprecipitation (ChIP) followed by qPCR. to determine whether FOXO3a binding to the *SOD2* promoter resulted in epigenetic alterations affecting chromatin structure, we again used ChIP to query specific histone modifications in this region following FOXO3a binding. We observed increased acetylation of histone H3 and dimethylation of the H3 Lys4 residue, both indicative of active chromatin, in the regions surrounding both the FOXO3a binding site and the *SOD2* transcription start site. Finally, using a nuclease protection assay, we determined that these histone modifications were associated with chromatin remodeling of the *SOD2* locus and the acquisition of a more accessible chromatin structure. Taken together, these results suggest that FOXO3a binding to the *SOD2* promoter leads to a transcriptionally competent state as a result of epigenetic alterations, and that FOXO3a is sufficient for the induction of *SOD2* expression observed during quiescence in normal human cells. This work was supported by NIH grant CA73612 and a grant from the McCord Research Foundation.

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Lower Inos Induction in G6PD Deficient Cells Associated With Higher NFκB Activation

***Reiko Matsui*[1]**, *Ashley Decker*[1], *and Richard a Cohen*[1]

[1]*Boston University*

Inducible NO synthase (iNOS) can be responsible for both beneficial and deleterious effects in pathological conditions. Atherosclerosis is decreased by iNOS gene knockout (KO) in ApoE KO mice (Detmers 2000, Kuhlencordt 2001), suggesting iNOS-derived oxidants (superoxide, peroxynitrite) may accelerate atherogenesis in ApoE KO mice. We reported that G6PD deficiency decreased NADPH generation by the pentose

phosphate pathway, associated with lower oxidants and atherosclerosis in high-fat fed ApoE KO mice (Matsui 2006). This was associated with decreased iNOS expression in aorta. It has been reported that NFκB activation can be suppressed by *S*-glutathiolation (R-SSG) of its components (eg. p50, IKKβ). We hypothesized that G6PD deficiency may modulate R-SSG under oxidative stress and R-SSG causes inactivation of NFκB resulting in lower iNOS induction.

Methods: Mouse smooth muscle cells (mSMC) isolated from wild type (WT) and G6PD deficient (Hemi) mice were used to study 1) R-SSG for mation by Western blot with anti-GSH antibody, 2) iNOS induction by real-time qPCR, 3) Tumor necrosis factor (TNFα) or Interleukin-1 (IL-1) induced NFκB activation by Western blot (phospho-IκBα) and by infection of adenoviral construct Ad.NFκB-luc followed by luciferase assay. **Results:** H2O2 induced R-SSG was detected by anti-GSH antibody in 10 min in cultured mSMC, and was enhanced with more positive bands in G6PD deficient (Hemi) cells. IL-1 (20 hrs) induced iNOS expression by qPCR showed the ratio of iNOS/GAPDH in Hemi cells was 10% of WT cells with IL-1 10 ng/ml, and 40% of WT cells with IL-1 100 ng/ml. in contrast, COX-2/GAPDH expression was over 7-fold higher in Hemi cells with IL-1. Luciferase activity in NFκB-luc infected cells was significantly higher in Hemi cells with IL-1 (100 ng/ml, 6 hrs, WT 543 ±30 vs Hemi 1073 ±67 RLU/μg protein). the same effect was observed also with LPS or TNFα stimulation. Pretreatment with H2O2 (supposed to induce R-SSG for mation) further enhanced NFκB activation in both cell types. in addition, TNFα-induced phosphorylation and degradation of IκBα were more prominent in Hemi cells, suggesting NFκB activation was enhanced in Hemi cells. **Conclusion:** Hemi cells showed enhanced R-SSG for mation under H2O2 exposure, and lower iNOS induction similar to Hemi Apo E KO mouse aorta. However, cytokine-induced NFκB activity was not inhibited, but rather enhanced in Hemi cells shown by luciferase and increased COX-2. Further study is required to explain the inhibition of iNOS, but enhanced COX2 and NFkB in G6PD deficiency.

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HNF1α Regulation of Sodium-Dependent Vitamin C Transporter 1 (SVCT1)

***Alexander J MIchels*[1,2], *Balz Frei*[1,2], *and Tory M Hagen*[1,2]**

[1]*Oregon State University,* [2]*Linus Pauling Institute*

Vitamin C is an important vitamin and antioxidant critical to normal cell function. Levels of vitamin C in tissues and plasma are tightly controlled. Sodium-dependent vitamin C transporter 1 (SVCT1), the vitamin C transporter found in intestine, kidney and liver, appears to control plasma vitamin C status and, thus, regulates the availability of this vitamin to all cells. the transcriptional regulation of SVCT1 gene expression has not been explored previously. We characterized the transcriptional control mechanisms of Slc23a1, the gene encoding for SVCT1, using a firefly luciferase reporter gene assay in HepG2 cells. Truncation deletions of this construct identified a small segment of the Slc23a1 promoter critical for transcriptional activity. Furthermore, mutations in this region identified a Hepatic Nuclear Factor 1 (HNF-1) binding site that is critical for transcription of SVCT1. Binding to this specific SVCT1/HNF-1 sequence was confirmed by the use of EMSA. as expected, siRNA knockdown of HNF-1α and the upstream regulator of HNF-1α transcription, HNF-4α, resulted in a significant decline in HNF-1α expression within 48-72 hours post-transfection. This decline in HNF-1α corresponded to a similar decline in SVCT1 transcription as measured by RT-qPCR. Treatment with HNF-1α or HNF-4α siRNA, respectively, resulted in a 91% and 69% decrease in SVCT1 mRNA. This decrease in SVCT1 was confirmed by analysis of the vitamin C transport of siRNA transfected cells, where lowered levels of HNF-1α and HNF-4α resulted in a decreased capacity for vitamin C uptake. Stimulation of HNF-4α and HNF-1α by insulin resulted in an 85% increase in SVCT1 reporter gene expression. Overall, these novel findings suggest that HNF-1α is an important transcription factor for the control of vitamin C transport through modulating SVCT1 levels, a process that involves HNF-4α and insulin signaling pathways.

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Cooperation of Cyclin B1 and P53 in Radioadaptive Tumor Resistance

***Danupon Nantajit*[1], *Ming Fan*[1], *Haijuan Gao*[1], *and Jian Jian Li*[1,2]**

[1]*Purdue University,* [2]*Purdue Cancer Center*

It is widely accepted that activation of tumor suppressor gene p53 plays a significant role for cell adaptive responses to ionizing radiation including the decision of cell fate. Several stress sensor genes have been identified to be able to activate p53 by phosphorylation at different residue of p53. Cyclin B and cdk1 complex is considered to be able to phosphorylate p53 at its Serine-315 residue and increase its transcriptional activity. This phosphorylation is elevated after exposure to ionizing radiation. Moreover, cells that undergo cell cycle are arrested at the G2 phase along with elevated expression of both p53 and cyclin B after irradiation. the interaction between p53 and cyclin B1 and their activation are involved for the cell fate decision. We have aimed to demonstrate that in the radiation dose dependent manner, phosphorylation at Ser-315 of p53 is increased and that is due to the induction of cyclin B1 expression by radiation. We will also confirm this result by observing phosphorylation of p53 at Ser-315 in naturally progressed cell in the G2 phase when cyclin B1 expression is maximized. These results will provide significant information about how p53 is activated under both normal and stress conditions.

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Adaptive radioresistance study in immortalized prostate versus transformed prostate cells

Danupon Nantajit[1], Ming Fan[1], Jeffrey S. Murley[2], David J. Grdina[2], and Jian Jian Li[1,3]

[1]Purdue University, [2]The University of Chicago, [3]Purdue Cancer Center

We have reported that cultured normal and tumor cells surviving long-term, low or high, fractionated radiation exposure express different radiation sensitivity. the purpose of this research is to identify the function of mitochondrial antioxidant enzyme MnSOD in normal and transformed human prostate cell lines. a profile of clones has been established with the radiation regimen of long-term exposure with different daily doses. Our current results suggest that both non-transformed 267B1 and ras-transformed 267B1Ki-ras cells show reduced MnSOD expressions. FIR derived cells further down-regulated MnSOD expression and mitochondria membrane potentials reflect the level of MnSOD expressions. We are currently measuring the expression level of MnSOD and mitochondrial functions to determine radiosensitivity of the clones from both FIR-derived cell lines. We also aim to identify their expression of cyclin B1, cyclin D1, p53 and p21 which are involved in regulation of mitochondrial functions and apoptosis in the selected radioresistant and radiosensitive

clones. These results may generate specific targets to enhance the efficacy of radiation therapy of prostate cancer.

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ICAM-1 Cytoplasmic Tail Inhibits PTEN Activity Through Changes in Endothelial Cell Redox Status

Sibile Pardue[1], *Christopher B Pattillo*[1], *and Christopher G Kevil*[1]

[1]*Louisiana State University Health Sciences Center - Shreveport*

Recently a link has been established between ICAM-1 and regulation of endothelial cell redox status. Among the molecules affected by altered redox status is the lipid and protein *phosphatase and tensin homologue deleted on chromosome 10* (PTEN). Active PTEN serves as an endogenous PI3 kinase antagonist by dephosphorylating phosphotidylinositol-3,4,5-triphosphate (PIP_3) back to phosphoitidylinositol-4,5-diphosphate (PIP_2), thereby disrupting the Akt signaling pathway. Previously ICAM-1 has been shown to increase levels of glutathione, total PTEN, phosphorylated PTEN, and decrease PTEN activity. to elucidate the relationship between ICAM-1, PTEN and GSH, peptides containing the antennapedia cell permeable sequence (AP) or AP peptide linked to the transmembrane and cytosolic tail of ICAM-1 were synthesized. Cell and molecular techniques were used to explore the redox status and activity of PTEN in cultured endothelial cells treated with the ICAM-1 peptide. Treatment of cell cultures with the cytoplasmic tail of ICAM-1 resulted in increased levels of phosphorylated PTEN and therefore decreased activity compared to AP treated cultures. Immunoprecipitation revealed that PTEN was also glutathiolated in the presence of ICAM-1. in conclusion we found that PTEN activity is reduced in the presence of the ICAM-1 peptide which may be mediated by glutathiolation.

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Sulfiredoxin Specifically Catalyzes the Deglutathionylation of 2 Cys-Peroxiredoxin: An in Vitro and in Vivo Study

JI WON PARK[1,2], *Sue Goo Rhee*[2], *and P. Boon Chock*[1]

[1]*NHLBI/NIH, Bethesda, US,* [2]*Ewha Womans University, Seoul, Korea*

Reversible protein glutathionylation, a redox-mediated regulatory mechanism, plays a key role in cellular regulation and cell signaling. It can also protect protein thiol from hyperoxidation to its irreversible sulfinic derivative. Sulfiredoxin (Srx), an enzyme catalyzing the reduction of Cys-sulfinic derivatives of peroxiredoxin (Prx), has been shown to catalyze the deglutathionylation of actin and protein tyrosine phosphatase 1B. in this study, we reveal that Srx specifically catalyzes the deglutathionylation of 2 Cys-Prx, a family of peroxidases that involves in removing H2O2 and organic hydroperoxides. Prx I is known to exist in various oligomeric for ms. the decamer or higher multimer of Prx I can function as a molecular chaperone while its dimer possesses high peroxidase activity. Using the abundant and ubiquitously expressed member of 2 Cys Prx, Prx I, we revealed that (i) among its four Cys residues, C52,83,173 can be glutathionylated in *vitro*. to avoid complexity, double Cys mutants were used to study the deglutathionylation by Srx. the results showed that C^{83} and C^{173} were specifically deglutathionylated by Srx, with glutathionylated Srx as intermediate, while glutaredoxin (Grx) was more specific for deglutathionylating C^{52}. (ii) Studies using site-directed mutagenesis coupled with binding and deglutathionylation activities showed that P^{174} and P^{179} of Prx I and Y^{92} of Srx are essential for both activities. Furthermore, relative to Grx, Srx exhibited negligible deglutathionylation activity for CSSG and BSA-SSG. These results indicate that Srx is specific for deglutathionylating Prx I, due to its favorable affinity for Prx I. (iii) Glutathionylation shifted Prx I from its decameric structure to a population consisting mainly of dimer. Thus, glutathionylation appears to convert Prx I from a molecular chaperone to a peroxidase enzyme. to assess the biological relevance of these observations, we investigated the effects due to Srx knockdown using the siRNA technique and overexpressing Srx in A549 cells. the results show that glutathionylated Prx I was substantially elevated in siRNA treated cells while the reverse was observed in Srx overexpressing cells. the physiological implications of these findings will be discussed.

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Modulation of Novel Genes in Apoptosis Pathways in Human Islets Exposed to Oxidative Stress and Hypoxia

Kalpana Velmurugan[1], *and* ***Subbiah Pugazhenthi***[1]

[1]*University of Colorado Denver*

Following the successful pancreatic islet transplantation in diabetic patients eight years ago, more than 500 transplantations have been performed around the world. the limitations of this cell-based therapy include the need for transplantation of islets from multiple donors because a significant number of islets from cadaveric donors die during isolation and after transplantation. Multiple stresses including oxidative stress, hypoxia and cytokines play major roles in the death of insulin-producing beta cells in islets. These beta cells are particularly vulnerable to oxidative stress due to the low-level expression of antioxidant enzymes and thus during injury to islets, beta cells are affected more than other cell types. **Methods:** in an attempt to further understand the loss of beta cells in the transplantation setting, we carried out transcriptional profiling of islets exposed to oxidative stress (peroxynitrite) or hypoxia. This PCR-based expression array contains 84 genes in the pathways of apoptosis, grouped under the following families: TNF Ligand, TNF Receptor, Bcl-2, Caspase, IAP, CARD, Death Domain, Death Effector Domain, and CIDE Domain. the expression of genes showing more than 2-fold changes were further analyzed by real-time RT-PCR using Taqman probe for confirmation. **Results:** Peroxynitrite and hypoxia decreased the expression of bifunctional apoptosis regulator (**BFAR**) that links extrinsic and intrinsic pathways of apoptosis and **CARD8,** an inhibitor of caspase 9. the expression of **CIDEa,** which belongs to the family of death activators, increased under these conditions. in addition, hypoxia increased the expression of **BNIP3,** an inducer of autophagy, a pathway by which cells degrade and recycle long lived proteins and defective organelles. Examination of islets exposed to peroxynitrite or hypoxia by a multiplex assay using xMAP technology revealed activation of caspase-3 and accumulation of cleaved PARP, markers of apoptosis. Our findings suggest that careful modulation of these novel genes in apoptosis pathways can lead to improved success in islet transplantation.

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Macrophage Differentiation Increases Expression of the Ascorbate Transporter SVCT2

Huan Qiao[1]

[1]*Vanderbilt University*

To determine whether vitamin C, or ascorbic acid, is required for macrophage function, we assessed the expression and function of its transporter SVCT2 during phorbol ester-induced differentiation of human-derived THP-1 mononuclear cells.

Induction of THP-1 cell differentiation by phorbol 12-myristate 13-acetate (PMA) caused a marked increase in SVCT2 mRNA, protein, and function. When ascorbate was present during PMA-induced differentiation, the increase in SVCT2 protein expression was inhibited, but differentiation was enhanced. PMA-induced SVCT2 protein expression was blocked by inhibitors of protein kinase C (PKC), with most of the affect due to the PKCβI and βII isoforms. Inhibitors of MEK/ERK completely blocked PMA-stimulated SVCT2 protein expression, indicating an exclusive role for the classical MAP kinase pathway. Further, PMA was found to induce SVCT2 expression through c-Raf-dependent B-Raf activation, exclusive of Ras. Inhibitors of NF-κB activation also strongly inhibited SVCT2 induction, mostly through the transcriptional activity of the P2 promoter, but partially also through the P1 promoter. in conclusion, monocyte-macrophage differentiation is enhanced by ascorbate and associated with increased expression and function of the SVCT2 protein through a pathway involving activation of PKCβI/II, MAP kinase, and NF-κB.

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H_2o_2 Modulates Igh Transcriptional Activity Via a 3′ Regulatory Region

Eric J Romer[1], *and Courtney E.W. Sulentic[1]*

[1]Wright State University

The transcription of the immunoglobulin heavy chain (IgH) gene in B lymphocytes is governed by the 3'IgH Regulatory Region (3'IgHRR) and is an essential step in antibody production and humoral immunity. the activity of lymphocytes has been shown to be effected by changes in the redox state of the cell as determined by fluctuations in the levels of intracellular reactive oxygen species (ROS). the response of lymphocyte activity to changes in the redox state of the lymphocyte appears to be mediated by oxidative modulation of the NFκB signal transduction pathway. the stability of the Inhibitory κB (IκBα) protein appears to play an important role in the redox regulation of the NFκB pathway. This study proposes that transiently shifting the redox state of the cell to a more oxidizing state by exposure to H2O2 can modulate, in an IκBα dependent manner, IgH transcriptional activity regulated by the 3'IgHRR. We utilized the CH12 IκBαAA mouse B-cell lymphoma cell line containing a stably integrated IκBαAA transgene which inducibly expresses an IκBα protein that is resistant to degradation. the transient expression of luciferase reporter constructs driven by a variable heavy chain promoter under the regulation of the 3'IgHRR in B cells exposed to μM concentrations of H2O2 demonstrated an enhanced reporter activity at lower H2O2 concentrations and suppressed activity at higher concentrations. the expression of the IκBα AA transgene inhibited the enhanced reporter activity. These observations suggest that transient oxidative shifts in the redox state of B lymphocytes can modulate IgH transcriptional activity in a concentration-dependent manner via the 3'IgHRR and the effects are in part dependent on IκBα expression. (Supported by NIEHS and Boonshoft School of Medicine)

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Cytoprotective Effects of Lipid Oxidation Products: Induction of Adaptive Response Through Different Mechanisms

Yoshiro Saito[1,2]

[1]Doshisha University, [2]National Institute of Advanced Industrial Science and Technology (AIST)

"Oxidative stress" was defined as a disturbance in the prooxidant-antioxidant balance in favor of the prooxidant. However, even if the level of stress is less than that of antioxidant capacity, stress functions as a signal to which the body responds irrespective of its level– namely, the low level stress may stimulate defense network and acts as a good stress, "eustress." Recent studies have revealed that low levels of ROS and certain lipid oxidation products may play essential roles in the cell signal transduction and can induce adaptive response. in this presentation, I would like to summarize the molecular mechanism of adaptive response induced by toxic lipid oxidation products such as 15-deoxy-delta12,14-prostaglandin J2 (15d-PGJ2) and arylating γ-tocopheryl quinone (γ-TQ). 15d-PGJ2 is electrophilic lipids containing the reactive α,β-unsaturated carbonyl functional group, while γ-TQ is oxidized metabolite of γ-tocopherol, which has some characteristics of an electrophilic arylating quinone. These lipid oxidation products induced adaptive response through up-regulation of cellular antioxidant system via different mechanisms.

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Induction of Adaptive Response and Enhancement of PC12 Cell Tolerance by Lipopolysaccharide Primarily Through the Upregulation of Glutathione S-Transferase A3 Via Nrf2 Activation

Yoshiro Saito[1,2], *Yo Omata[2], Yasukazu Yoshida[2], Noriko Noguchi[1], and Etsuo Niki[2]*

[1]Doshisha University, [2]National Institute of Advanced Industrial Science and Technology (AIST)

In the present study, we focused on adaptive cytoprotection induced by lipopolysaccharide (LPS), which induces oxidative stress and inflammatory cytokines, in PC12 cells, a model of neuronal cell. After treating PC12 cells with LPS at sublethal concentrations, we found that they developed resistance to subsequent oxidative stress. LPS treatment resulted in the increase of the gene expression of glutathione S-transferase A3 (GST-A3) by up to 60-fold as well as GST enzyme activity. GST A3-small interfering RNA and Nrf2-small interfering RNA effectively attenuated the adaptive response induced by LPS. in addition, peripheral injection of LPS at sublethal concentrations increased GST enzyme activity in mouse brain. These findings, taken together, indicate that stimulation with LPS at sublethal concentrations induces an adaptive response and enhances PC12 cell tolerance, primarily through the induction of GST A3 via the transcriptional activation of the Nrf2 signaling pathway.

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In Vivo Activation of Pparγ by Nitro-Fatty Acids Involves Nitroalkylation of Pparγ

Francisco Jose Schopfer[1], *Alison Groeger[1], Gustavo Bonacci[1], Chen-Shan Chen[1], Marsha Cole[1], Carlos Batthyany[2], Paul Baker[1], Jifeng Zhang[3], Tina Hallis[4], Eugene Chen[3], and Bruce Freeman[1]*

[1]University of Pittsburgh, [2]Institut Pasteur de Montevideo, [3]University of Michigan, [4]Invitrogen

Nitro-fatty acids (NO2-FA) are endogenous derivatives that exert signaling actions through mechanisms that include serving as for peroxisome proliferator-activated receptor gamma (PPARg) ligands and via electrophilic reactivity. Activation of PPAR by ligand binding induces conformational changes that facilitate co-repressor release and co-activator recruitment resulting in specific cellular responses. Mass spectrometric analysis of the NO2-FA treated PPARg ligand binding domain (LBD) revealed covalent adduction of Cys285 by NO2-FA. This electrophilic nitroalkylation reaction is fast, concentration-dependent and detectable by MS analysis using NO2-FA:PPARg-LBD at mol ratios as low as 1:60.

When nitro-oleic acid (OA-NO2) was derivatized to an allyl ester, no PPARg nitroalkylation was detectable, indicating the relevance of the carboxylic acid for ligand activity. the identity of the NO_2-FA modified tryptic peptide containing Cys285 was confirmed by comparison with nitroalkylated synthetic LBD peptide. in addition, we detected in-cell nitroalkylation of Cys285 by OA-NO_2 wherein the 10- and not the 9-OA-NO_2 isomer alkylated PPARg, supporting the stereospecific reaction of OA-NO_2 with PPARg. Time-resolved Förster resonance energy transfer (TR-FRET) showed NO_2-FA induced unique patterns of coregulator protein interactions compared with thiazolidinediones (TZDs). Importantly, TRAP220/DRIP2, a key mediator of triglyceride accumulation in adipocytes, was poorly recruited by PPARg upon NO_2-FA activation. in concordance, less triglyceride accumulation occurred in adipocytes differentiated with NO_2-FA versus TZDs. in summary, activation of PPARg by NO_2-FA is region-specific, involves covalent reaction with Cys285 and is a potential therapeutic strategy for treating inflammation and metabolic disorders.

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Hypoxia-Induced Vascular Repair by Progenitor Cells Requires the NADPH Oxidase Nox2

Katrin Schröder[1], *Andreas Kohen*[1], *Alexandra Aicher*[1], *and Ralf P. Brandes*[1]

[1]*Goethe-Universität*

Endothelial progenitor cells (EPC) promote vascular repair but the impact of systemic hypoxia on EPC function is unclear. Hypoxia is a strong stimulus for angiogenesis. and affects reactive-oxygen species (ROS) production and signalling. We studied the role of hypoxia for EPC mobilization and vascular repair and the contribution of the NADPH oxidase Nox2, the most important source of ROS in endothelial cells, in this process.

Murine EPCs were defined as linage negative and sca-1/flk-1 positive and measured by FACS from whole blood. Vascular repair was induced by the carotid artery wire injury model. Hypoxia (10%, 96hours) induced mobilisation of EPC and improved vascular repair after wire injury in wild-type mice but not in NADPH oxidase knockout mice ($Nox2^{y/-}$). Hypoxia induces the for mation of erythropoietin (EPO), which might contribute to EPC mobilization. EPO production in response to hypoxia however was enhanced in $Nox2^{y/-}$ as compared to WT mice. Importantly, EPO, which induced pronounced EPC mobilization in WT mice, failed to do so in $Nox2^{-/-}$ mice. These data suggest that EPO signalling is defective in $Nox2^{y/-}$ mice. Indeed, in EPCs from WT- mice, EPO induced ROS production and proliferation and both responses were missing in EPCs from $Nox2^{y/-}$ mice. Transplantation of bone marrow from $Nox2^{y/-}$ mice into WT- mice resulted in an attenuated mobilisation in response to hypoxia and EPO, whereas the transplantation of WT bone marrow into $Nox2^{y/-}$ mice restored the normal phenotype . This demonstrates that a failure of bone marrow stem cells to generate ROS in response to EPO underly the mobilization defect.

We conclude that Nox2-derived ROS are required for EPO-signalling in stem cells and that hypoxia-induced EPC mobilisation and vascular repair requires the NADPH oxidase Nox2.

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Hypoxia Impediments Ovarian Cancer Treatment Through Modulation of ROS and STAT3 Activation

Karuppaiyah Selvendiran[1], *Anna Bratasz*[1], *M Lakshmi Kuppusamy*[1], *Brian K Rivera*[1], *and Periannan Kuppusamy*[1]

[1]*The Ohio State University, Columbus,*

STAT3 is overexpressed in a variety of human cancers, including ovarian cancer. However, the molecular mechanism by which STAT3 is upregulated in tumor cells is poorly understood. Blocking of STAT3 expression results in apoptosis of tumor cells; thus, STAT3 is considered to be an attractive target for anticancer therapy. We found that pSTAT3, the active for m of STAT3, is significantly upregulated by hypoxia, and is accompanied by an increase in reactive oxygen species (ROS) generation in human ovarian cancer. More importantly, we observed that anticancer drugs cisplatin and Taxol were far less effective in ovarian cancer cells cultured under hypoxic (1% O_2) conditions when compared to normoxic (20% O_2) conditions. We further observed that pSTAT3 (Tyr705) expression was O_2-dependent, and significantly increased at O_2 levels less than 2%, without any changes in total STAT3 or other pSTAT (Ser727) levels. a correlation, therefore, may exist between the expression of pSTAT3 and cellular resistance to chemotherapy. We found that increased pSTAT3 expression under hypoxia could be reversed within 12 hours after returning the cells to a 20% O_2 environment. the overexpression of STAT3 in hypoxia-cultured cells could also be inhibited by N-acetylcysteine (NAC). Inhibition of STAT3 by AG490, followed by treatment with cisplatin or Taxol resulted in a >2-fold increase in apoptosis when compared to cells not treated with AG490. Taken together, these results indicated that STAT3 activation is upregulated by hypoxia via induction of ROS levels, and the hypoxia-induced chemoresistance is at least partially due to STAT3 upregulation. We show that this chemoresistance may be overcome through the use of STAT3-specific inhibitors and antioxidants, or by increasing cellular or tissue oxygen levels.

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Transforming Growth Factor B-1 Elicits Nrf2-Mediated Heme Oxygenase-1 Induction in Aortic Smooth Muscle Cells

Richard CM Siow[1], *Adrian T Churchman*[1], *Tetsuro Ishii*[2], *and Giovanni E Mann*[1]

[1]*King's College London, UK,* [2]*University of Tsukuba, Japan*

The anti-inflammatory properties of transforming growth factor β-1 (TGFβ-1) account for its protection against atherosclerotic plaque rupture. This study investigates whether activation of the Nrf2 transcription pathway is involved in TGFβ-1 mediated induction of the antioxidant enzyme heme oxygenase-1 (HO-1) in smooth muscle cells (SMC). Human (HAoSMC) or wild-type and Nrf2 deficient mouse (MAoSMC) aortic SMC were treated with TGFβ-1 (2.5-10 ng/ml, 0-24 h). We report the first evidence that TGFβ-1 induces Nrf2 mediated HO-1 expression and antioxidant response element activity, which was paralleled by enhanced superoxide production and expression of the NAD(P)H oxidase subunit $p22^{phox}$. TGFβ-1 failed to induce HO-1 expression in MAoSMC derived from Nrf2 deficient mice, and HO-1 induction by TGFβ-1 in HAoSMC was attenuated by inhibition of extracellular signal regulated kinase or c-jun-N-terminal kinase but not p38 mitogen activated protein kinase. Inhibition of NAD(P)H oxidase or scavenging of superoxide diminished HO-1 induction in response to TGFβ-1. the oxidative stress agents glucose oxidase and diethylmaleate enhanced TGFβ-1 generation and HO-1 expression in HAoSMC, while antagonism of TGFβ-1 signaling by adenoviral Smad7 overexpression attenuated their induction of HO-1. Pretreatment of HAoSMC with TGFβ-1 reduced nuclear

translocation of the pro-apoptotic mediator p53 elicited by glucose oxidase. Our findings demonstrate that Nrf2 is a new target of TGFβ-1 signaling in the vasculature which may contribute to the atheroprotective properties attributed to this growth factor. **Supported by the British Heart Foundation**

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Redox Control of Co-Activators and Repressors of MMP-1 Transcription

***Sita Subbaram*[1]**, *and J Andres Melendez*[1]

[1]*Albany Medical College*

Matrix metalloproteinase-1 (MMP-1) is an important extracellular matrix remodeling enzyme whose transcription has been shown to be oxidant responsive. Aberrant overexpression of MMP-1 has been observed in disease conditions such as atherosclerosis, arthritis and tumor metastasis. We have previously shown that MMP-1 transcription is sensitive to alterations in the steady state production of hydrogen peroxide. in the present study we define the oxidant sensitive chromatin remodeling proteins and transcription factors that contribute to the H_2O_2 sensitivity of the MMP-1 promoter. Using a series of redox-engineered HT1080 fibrosarcoma cell lines we have been able to monitor the effects of altering the steady state production of H_2O_2 on MMP-1 transcription in real time. Chromatin immunoprecipitation (ChIP) followed by real time PCR analysis shows an enhanced recruitment of Ets-1, c-Jun, c-Fos as well as P/CAF (histone acetyl transferase) at specific sites on the MMP-1 promoter in response to increased H_2O_2 levels. We also observe increased acetylation of histone H3 indicating an increase in transcriptional activation. in addition to increased recruitment of co-activators of transcription, there is also a decrease in the recruitment of HDAC2 (histone deacetylase2), a negative regulator of transcriptional activation to the MMP-1 promoter. HDAC2 is post-transcriptionally modified at tyrosine residues by nitration. This oxidative modification potentially targets the protein for degradation by the ubiquitin proteosome pathway. Western blot analysis shows enhanced nitration and ubiquitination of HDAC2 in our Sod2 overexpressing cells. Treatment with TSA, a HDAC inhibitor increases MMP-1 transcription and histone H3 acetylation in a redox dependent manner These results indicate that the regulation of MMP-1 by H_2O_2 occurs by both enhancing the recruitment of transcription activators as well decreasing the recruitment of negative regulators. This study defines the precise redox responsive molecular triggers that control MMP-1 expression and may lead to the development of new targeted antioxidant based therapies for degenerative disease processes.

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Oxidized Messenger RNA Induces Endoplasmic Reticulum Stress in HEK293 Cells

***Mikiei Tanaka*[1]**, *Yoko Hirata*[2], *Duck-Yeon Lee*[1], *Boon Chock*[1], *and Earl Stadtman*[1]

[1]*NHLBI,* [2]*Gifu university*

RNA oxidation has been implicated in several age-related diseases. Recently we reported that moderately oxidized mRNA leads to generation of dysfunctional polypeptides accounting for more than 15% of total products due to translation errors. in this study, mRNA encoding bovine rhodopsin, a cytoplasmic membrane protein, was used as a model to investigate the biological impact of oxidized mRNA-induced translation errors on protein quality control. Our results demonstrate that (i) transfection of the in vitro moderately oxidized mRNA into HEK293 cells caused accumulation of aberrant high molecular weight oligomeric species, suggesting that the translate product was qualitatively different from the native for m; and (ii) translation of the oxidized rhodopsin mRNA up-regulated the ER stress transducers, such as ATF6 activation monitored by the luciferase reporter assay, and elevation of CHOP transcription factor, phosphorylated eIF2 and ATF4 expression, as well as a moderate increase in caspase-3 activity, in a dose-dependent manner with respect to Fenton reagents. These results suggest that oxidized mRNA increases the risk of ER protein quality control due to the accumulation of aberrant proteins. in addition, GC/MS analysis revealed that treatment of HEK293 cells with thapsigargin caused a transient increase in cytosolic Ca(II) and induced cellular RNA oxidation, suggesting that ER stress via Ca(II) deregulation is, in part mediated by translation errors due to oxidation of RNA. Thus our novel pathophysiologically relevant ER stress model indicates the direct connection between ER stress induction and oxidative stress through oxidation of mRNA.

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Translational Regulation of Glutathione Peroxidase 4 Expression Through Guanine-Rich Sequence Binding Factor 1 Is Essential for Embryonic Brain Development

***Christoph Ufer*[1]**, *Chi Chiu Wang*[2], *Michael Fahling*[1], *Heike Schiebel*[1], *Bernd J Thiele*[1], *E Ellen Billett*[3], *Astrid Borchert*[1], *and Hartmut Kuhn*[1]

[1]*University Medicine Charite Berlin,* [2]*The Chinese University of Hong Kong,* [3]*Nottingham Trent University*

The phospholipid hydroperoxide glutathione peroxidase (GPx4) is a moonlighting selenoprotein, which has been implicated in basic cell functions such as anti-oxidative defense, apoptosis, redox signaling, eicosanoid biosynthesis and testicular sperm maturation. GPx4-null mice die in utero at midgestation and siRNA-dependent knockdown of GPx4 expression induced developmental retardation of the brain. Transcriptional control of GPx4 expression has been studied before but little is known about post-transcriptional regulatory mechanisms. Applying the yeast three-hybrid system we screened a testis expression library to search for regulatory proteins capable of interacting with the 5'-untranslated region (5'-UTR) of the GPx4 mRNA. Exploring some 1×10^7 transformants with a highly stringent screening strategy we isolated a single yeast clone and identified the guanine-rich sequence-binding factor 1 (Grsf1) as GPx4 mRNA binding protein. Grsf1 binds to a specific target sequence (AGGGGA) in the 5'-UTR of the GPx4 mRNA that adopts a defined secondary structure. This interaction proceeded with rather high affinity (K_d=40 nM) and upregulated GPx4 mRNA expression as indicated by UTR-dependent reporter gene expression in cellular translation assays. Differential polysome profiles indicated that Grsf1 recruits GPx4 mRNA to translationally active polysome fractions and immunoprecipitation of transient Grsf1 transfectants revealed coprecipitaion of Grsf1 and GPx4 mRNA. During murine embryo development, Grsf1 and mGPx4 are coexpressed in the brain as indicated by quantitative RT-PCR and immunohistochemical staining and siRNA-induced Grsf1 knockdown impaired cerebral GPx4 expression during in vitro embryogenesis. When compared with mock controls, Grsf1 deficient embryos showed significant signs of developmental retardations that are paralleled by apoptotic alterations and enhanced lipid peroxidation. Overexpression of m-GPx4 prevented the apoptotic alterations in Grsf1-deficient embryos and rescued them from retardations of brain maturation. These data indicate that Grsf1 upregulates translation of GPx4 mRNA and implicate the two proteins in cerebral embryogenesis.

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Regulation of Alphattp in the Liver

***Lynn M Ulatowski*[1]**, *and Danny Manor*[1]

[1]*Case Western Reserve University*

Vitamin E is a family of neutral plant lipids of which *RRR*-α-tocopherol (tocopherol) is selectively retained in the body and is thus considered the most biologically active for m. Tocopherol's protective effect as the major lipid-soluble chain-breaking antioxidant has been implicated in many oxidative-stress related diseases, including Alzheimer's disease, cardiovascular disease, diabetes, cancer and cataracts. the tocopherol transfer protein (alphaTTP) regulates vitamin E status in humans and its critical importance is exemplified in the fact that heritable mutations in the *ttpA* gene cause the devastating neurodegenerative syndrome ataxia with vitamin E deficiency (AVED), characterized by low tocopherol levels and spinocerebellar degeneration. AlphaTTP is highly expressed in the liver, as well as to a lesser extent in the kidney, the Bergman glia and Purkinje neurons of the brain, the lung and at various gestational stages in the placenta. the tissue-specific and temporal pattern of alphaTTP expression, combined with its critical function in regulating whole body vitamin E raises the question of how is the expression of alphaTTP regulated. Despite the obvious importance of this question, the mechanisms that regulate alphaTTP expression are completely unknown. We analyzed the sequence of the alphaTTP promoter and identified the following sequences that could mediate liver-specific transcription factor binding and that are phylogenetically conserved: HNF3b, C/EBPβ, MDS, FOXL1 and CDX1, MEF2, SRF, NR2F2, and MyT1. Using deletion constructs of the alphaTTP promoter to drive expression of the luciferase reporter gene we identified the promoter elements that regulate the hepatic-specific expression of human alphaTTP.

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Thioredoxin Reductase Knock Down Inhibits NF-κB Activity in the Nucleus

Jacqueline M Heilman[1], *and* ***Walter H Watson***[1]

[1]*Johns Hopkins Bloomberg School of Public Health*

The redox-sensitive transcription factor nuclear factor-κB (NF-κB) controls cell survival, proliferation and inflammation. Some inhibitors of NF-κB have been shown to also inhibit thioredoxin reductase (TR1). One of the major substrates of TR1 is thioredoxin (Trx1), and it has been assumed that the effects of TR1 inhibition are mediated through a buildup of oxidized Trx1. the purpose of the present study was to test the hypothesis that TR1 regulates NF-κB activity through effects on the redox state of Trx1. to do this, the function of the NF-κB pathway was assessed in HeLa cells depleted of TR1 using siRNA. TNF-stimulated activity of an NF-κB luciferase reporter and expression of an endogenous NF-κB-dependent gene (IκB-α) were 40% lower in TR1 knock down cells than in control cells. in contrast, TNF-stimulated degradation of cytoplasmic IκB-alpha (measured by western blot) and nuclear translocation of p50 and p65 (measured by western blot and gel shift assay) were unaffected by TR1 knock down, suggesting that TR1 affects NF-κB at the level of the nucleus. the redox state of Trx1 (as measured by the Redox Western blot) was unaffected by 90% depletion of TR1 protein levels and activity. From these data we conclude that TR1 activity is required for maximal NF-κB transcriptional activity in the nuclear compartment and that loss of NF-κB activity upon TR1 depletion does not depend on Trx1 oxidation.

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ROS Mediate Hypoxia-Induced Cyclin D1 and FGF R1 Expression

Stephen Wedgwood[1], *Gregory Waypa*[1], *and Paul Schumacker*[1]

[1]*Northwestern University*

Hypoxia-induced pulmonary hypertension has been studied extensively in rats and mice. Exposure to 10% oxygen for 3 weeks results in increases in right ventricular hypertrophy, mean pulmonary arterial pressure, and pulmonary arterial muscularization. Expression of hypoxia-inducible factor-1α (HIF-1α) is increased under these conditions, and HIF-1$\alpha^{+/-}$ mice demonstrated a significantly delayed development of right ventricular hypertrophy, pulmonary hypertension and pulmonary vascular remodeling relative to wild-type littermates. Reactive oxygen species (ROS) are known to stabilize HIF-1α protein, and superoxide levels were demonstrated to be increased in the pulmonary arteries of mice exposed to 10% oxygen for 3 weeks. the purpose of this study was to identify ROS-mediated signaling pathways that may contribute to hypoxia-induced pulmonary vascular remodeling. Pulmonary artery smooth muscle cells (PASMC) were isolated from rat lungs and exposed to 1.5% O_2 in a Coy chamber for 0-72h. Our previous studies demonstrated an increase in the oxidation of a redox-sensitive FRET probe in PASMC exposed to hypoxia that was attenuated by over-expression of mitochondrial catalase or by treatment with the mitochondrial inhibitor myxothiazol. Here Western blotting detected time-dependent increases in HIF-1α and cyclin D1 protein levels in PASMC exposed to hypoxia. in addition, increases in cyclin D1 protein and promoter activity were attenuated by myxothiazol and by the antioxidant N-acetyl cysteine (NAC). Fibroblast growth factor receptor 1 (FGF R1) expression is stimulated by the cyclin D1/pRb/E2F pathway, and the FGF R1 promoter also contains a putative binding site for HIF-1α. Hypoxia increased FGF R1 protein levels in PASMC in a NAC-sensitive fashion, while the HIF-1α stabilizing compound deferoxamine increased FGF R1 expression under normoxic conditions. From these data we hypothesize that hypoxia triggers mitochondrial ROS generation resulting in HIF-1α stabilization and increased cyclin D1 expression. Subsequent activation of target genes including FGF R1 may contribute to PASMC proliferation associated with hypoxia-induced pulmonary vascular remodeling.

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"Click" Chemistry – Based Identification of Cellular Targets of Electrophilic Nitro-Fatty Acids and Binding of Ppar-γ Receptor

Steven R Woodcock[1], *Chen-Shan Chen*[1], *Emilia Kansanen*[1,2], *Y Eugene Chen*[3], *Alessandro Bisello*[1], *Francisco J Schopfer*[1], *and Bruce a Freeman*[1]

[1]*University of Pittsburgh,* [2]*University of Kuopio, Finland,* [3]*University of Michigan*

Nitro-fatty acids are a class of inflammatory-derived cell signaling mediators that display anti-inflammatory signaling actions, in part via potent PPAR-γ receptor/ligand activity. Vinyl nitro regioisomers are strongly electrophilic, for m covalent adducts via Michael addition with nucleophilic amino acids, and regulate cell signaling via post-translational nitroalkylation to alter protein structure and function, resulting in the modulation of enzyme activity and protein translocation. the extent and specificity of biomolecule nitroalkylation reactions remain undefined, thus the systematic detection and identification of target proteins are important to explore. We applied bioorthogonal labeling to nitrated lipids using a "Click" chemistry approach of highly specific and

complementary molecular "handles" for the copper-catalyzed 1,3-Huisgen dipolar cycloaddition reaction that permits unobtrusive molecular tags to be incorporated into known pharmacophores containing all characteristic molecular interactions. These labeled molecules can be introduced into biological milieu, reacted in situ with an orthogonal fluorescent molecule or affinity marker (such as biotin or HA-tag) and subsequently used to identify and isolate native binding sites. We synthesized an azido derivative of nitro-octadecenoic acid (N3-OA-NO2) and evaluated binding of this derivative with the peroxisome proliferator-activated receptor-γ (PPARγ). the covalent modification of PPARγ of intact cells treated with N_3-OA-NO_2 was detected by HPLC-MS/MS analysis and occurred specifically at the Cys285 residue in the ligand-binding domain of treated cells, representing a novel mode of nitro-fatty acid-nuclear lipid receptor binding. in conclusion, Click chemistry is a powerful and sensitive tool to study the interaction of nitro-fatty acids with cellular targets.

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Interaction of Dermal Fibroblasts With Extracellular Matrix Attenuates Oxidative Stress-Induced Toxicity

Irene E. Kochevar[1], Robert W. Redmond[1], and ***Min Yao[1]***

[1]Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA

We are developing a light-activated, sutureless method for sealing surgical wounds that relies on dye-photosensitized crosslinking of tissue proteins. Phototoxicity is a potential side effect because the dye used, Rose Bengal (RB), generates singlet oxygen, which initiates toxicity through oxidative stress. Surprisingly, RB was not phototoxic to dermal fibroblasts (FB) in skin being sealed with our light-activated process. We hypothesized that the interaction of the FB with extracellular matrix in the dermis increases their resistant to oxidative stress. We tested this hypothesis by comparing the LD_{50} for RB photosensitization of FB grown in monolayers on plastic versus FB grown in 3D collagen gels. the LD_{50} light dose for FB grown in 3D was >1000-fold greater than that for FB grown in a monolayer with the same intracellular RB concentration. Reduced diffusion of oxygen through the collagen gel matrix inhibited phototoxicity by <2-fold. Western blots indicated that FB grown in collagen matrices contained higher levels of phospho-Akt, a pro-survival signaling protein, than FB in monolayers and that phospho-Akt increased after photosensitization. Inhibiting PI3K with LY294002 increased the RB phototoxicity to FB in 3D gels by ~8-fold. When receptor tyrosine kinase activity, which is also associated with cell survival, was blocked with suramin, the protective effect of the collagen matrix against oxidative stress was also substantially (~10-fold) inhibited. FB in 3D culture also expressed higher catalase (~4-fold) and had greater glutathione peroxidase activity (~2-fold) after RB photosensitization than monolayer FB. These results indicate that interaction of FB with extracellular matrix increases pro-survival signaling and antioxidant levels, thus protecting the cells from oxidative stress and cell death and suggest that cells in monolayer cultures may not accurately represent in vivo oxidative stress responses.

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Inhibition of Erk Signaling Pathway Induced by Mnsod Contributes to the Decrease of Mobility in Bladder Cancer UM-UC-3 Cells

Hanqing Ye[1]*, and J. Andres Melendez[1]*

[1]Albany Medical College

There are a lot of studies suggesting that Manganese superoxide dismutase (MnSOD) could act as a latent tumor suppressor gene and overexpressing MnSOD in cancer cells could suppress tumor for mation and reverse malignant growth. However, the molecular mechanisms of MnSOD anti-tumor effects remain unclear. Here we examined whether MnSOD could influence the signaling pathway of extracellular signal regulated kinase (Erk) to exert its anti-tumor activity. Analysis of wound healing activity indicated that overexpression of MnSOD decreased migration significantly compared with the control cell line (UM-UC-3/EGFP). According to ***matrix*** metalloproteinase (MMP) gelatin zymography and MMP-1 real-time quantitative reverse transcription PCR (qRT-PCR) assays, it was observed that the mRNA level of MMP-1 and the activities of MMP-2 and MMP-9 were decreased in response to MnSOD overexpression. Due to the well-known roles of PI3K/Akt and Ras/MEK/ERK signaling pathways in cell mobility, the phosphorylation of ***Akt*** *and* ***Erk*** after MnSOD overexpression was determined by western blot. in UM-UC-3/MnSODGFP cells, remarkable decrease of phophorylated Erk but not phosphorylated Akt was observed in comparison with UM-UC-3/EGFP cells. in addition, treatment with the inhibitor of Erk, U0126 in the control cell line UM-UC-3/EGFP could mimic the effect of MnSOD, exhibiting the decrease of wound healing ability. Our findings identify a mechanism underlying MnSOD anti-tumor effects and provide evidence to support MnSOD as a genetic therapy in the treatment of human bladder cancer.

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The Role of Protein Kinase C in Early and Late Hepatic Ischemic Preconditioning

Nari Yun[1]*, Woo Cheol Lee[1], Sung-Hwa Kim[1], and Sun-Mee Lee[1]*

[1]College of Pharmacy, Sungkyunkwan University

Activation of protein kinase C (PKC) has been implicated in hepatoprotective mechanisms of ischemic preconditioning (IPC). However, the exact role of these events in early and late IPC remains unclear. Rats were subjected to 90 min of hepatic ischemia following 3, 6 (early phase) and 24 hr (late phase) reperfusion. IPC was achieved by clamping blood supply to the left and median lobes for 10 min followed by 10 min of reperfusion prior to sustained ischemia. PKC inhibitor chelerythrine (5 mg/kg) was injected intravenously 10 min before the IPC procedure. Pretreatment of chelerythrine abrogated the IPC-induced hepatoprotection and reduced oxidative damage in both 3 and 6 hr of reperfusion, which was indicated by the increased serum aminotransferase activities and lipid peroxidation levels and the decreased hepatic glutathione content. At 24 hr of reperfusion, IPC increased the levels of heme oxygenase-1 and inducible nitric oxide synthase protein expression, which was attenuated by chelerythrine. the TUNEL positive hepatocytes, cytochrome C release and caspase-3 activity increased 3 hr of reperfusion, peaked at 6 hr and remained elevated throughout 24 hr of reperfusion, which were attenuated by IPC. These effects of IPC were reversed with chelerythrine. in conclusion, our results suggest that modulation of protein kinase C is essential for early and late ischemic preconditioning responses in hepatic I/R.

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Epigenetic Regulation of Extracellular Oxidative Stress in the Lung

Rodney J. Folz[1], *and* ***Igor N. Zelko***[1]

[1]*University of Louisville, Division of Pulmonary, Critical Care and Sleep Disorders Medicine*

Molecular mechanisms which govern cell-specific expression of human extracellular superoxide dismutase (EC-SOD), the major extracellular antioxidant enzyme, are mostly unknown. We localized multiple transcription start sites to a finite region located 3.9 kb upstream of the ATG initiation codon and subsequently identified a functional Sp1/Sp3 binding site in close proximity. Expression of a promoter/reporter construct containing the human EC-SOD promoter was comparably high in both MRC5 (high levels of native EC-SOD expression) and in A549 (non-expressing EC-SOD cell line) cells, suggesting other, possibly epigenetic mechanism are involved in its transcriptional regulation. When analyzed for EC-SOD promoter methylation, we found high levels in A549 cells and correspondingly low levels of methylation in MRC5 cells. Inhibition of DNA methyltransferae activity by 5-azacytidine in A549 cells reactivated EC-SOD transcription demonstrating the importance of methylation in repression of EC-SOD expression. Furthermore, methylation of cytosines in the promoter/reporter constructs, in *vitro*, markedly decreased Sp1/Sp3 driven promoter activity. This attenuation of transcription was, at least in part, attributed to the binding of methyl-binding protein MeCP2 to the methylated EC-SOD promoter. We also found marked differences in the chromatin organization of the EC-SOD promoter between these two cell lines further supporting the important role epigenetic modifications play in the regulation of human EC-SOD expression.

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Epre Sequence and Function: Nucleotides Essential for the Function of Epres in Human GCLC Promoter

Hongqiao Zhang[1], *and Henry Jay for man*[1]

[1]*University of California at Merced*

Glutamate cysteine ligase (GCL) is the rate-limiting enzyme for the synthesis of glutathione, a major antioxidant that plays key roles in maintaining the redox homeostasis and detoxifying oxidants. Studies have shown that the expression of the catalytic subunit of GCL (GCLC) can be induced in response to oxidative stress through electrophile responsive elements (EpRE). Searching the 5'-untranslated region (5'-UTR) of GCLC identified 13 putative EpRE motifs. in this study we studied the relationship between the sequence of these EpREs and their function. Using reporter assay, we demonstrated that only two of the 13 putative EpREs were responsive to 4-hydroxynonenal (HNE), a well established GCLC inducer, and only the distal EpRE is critical for the GCLC induction by HNE. After changing the location of the core sequence to the distal region of GCL promoter, another EpRE became responsive to HNE treatment at the whole promoter level, suggesting that EpRE location in the promoter region is important for its function in gene regulation. the nucleotides essential for EpRE function are then analyzed by mutating nucleotides in the core and flanking region of EpRE. Finally, results from electrophoresis mobility shift assay (EMSA) indicate that the protein profiles binding to EpREs varied with EpRE sequences. in summary, we examined the sequence and function of the 13 putative EpREs in human GCLC promoter and found that the EpRE function in gene regulation was related to its sequence and location in the promoter. It is also found that the protein profiles binding to the functional EpRE might vary with the EpRE sequence.