Annual Meeting
September 23, 2013

Louisville, KY
2013 Annual Meeting

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J. Christopher States, Ph.D.
Daniel Conklin, Ph.D.
Anne Noe
Ashley Ciszewski
**Keynote Speaker**

**B. Alex Merrick, Ph.D.** is a Molecular Toxicologist at the National Toxicology Program (NTP) in Research Triangle Park, NC in the Biomolecular Screening Branch (BSB). Merrick leads the Branch's Molecular Toxicology and Informatics Group. His responsibilities include identifying key signaling pathways altered by environmental toxicants and participating in the Tox21 Initiative - a collaboration between the NTP, the NIH Chemical Genomics Center (NCGC), the Environmental Protection Agency’s (USEPA) National Center for Computational Toxicology (NCCT) and the Food and Drug Administration (FDA). He is especially interested in performing molecular analysis in NTP archival tissues to discover gene expression and epigenetic signatures from chemical toxicity studies that further pathological insight and contribute to predictive models of toxicity and chemical prioritization to complement the Tox21 high throughput screening program. In addition, he conducts NextGen sequencing to better evaluate chemically exposed tissues for differential transcript profiles that include splice variants, low copy transcripts and non-coding mRNA’s. Merrick received his Ph.D. in 1984 at the University of Nebraska Medical Center in Omaha, performed postdoctoral work at Oak Ridge and was at the USEPA before joining NIEHS in 1989. He currently serves as an adjunct Associate Professor in the Department of Environmental and Molecular Toxicology at NC State University.
Program

8:00-9:00 am  Registration, continental breakfast, poster set-up
9:00-9:15 am  President’s Welcome
9:15-10:15 am  Ph.D. Student Platform Presentations
10:15-10:30 am  K-12 Presentation
10:30-noon  Poster Viewing & Judging
noon-1:30 pm  Lunch/Lunch with an Expert, Poster Viewing
1:30-2:30 pm  Post-Doctoral Platform Presentations
2:30-2:45 pm  Refreshment Break
2:45-3:45 pm  **Keynote Lecture**
   “High Throughput Screening for Chemical Toxicity Assessment”
   B. Alex Merrick, Ph.D.
   Group Leader
   Molecular Toxicology and Informatics Group
   Biomolecular Screening Branch / NIEHS
3:45-4:00 pm  Awards, Closing Remarks
4:30 pm  Student / Post-doc tour of Nucleus
Ph.D. Student Platform Session

Ruoyun Ma
Purdue University

*Brain GABA Levels Correlate with Manganese Exposure in Welders*

Veronica L. Massey
University of Louisville

*The prebiotic oligofructose protects against liver injury caused by arsenic in a model of NASH*

Marcus W. Stepp
University of Louisville

*Fischer 344 rat strains congenic at arylamine N-acetyltransferase differ in breast tumorigenesis induced by N-methylnitrourea*

Banrida Wahlang
University of Louisville

*Aroclor 1260 Exposure Worsens Hepatic and Systemic Inflammation in an Animal Model of Diet-Induced Obesity and Non-alcoholic Fatty Liver Disease*
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*Characterization of genetic susceptibility to PCB-induced developmental neurotoxicity*

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Purdue University

*Metabolic Vulnerability of Frontal Cortex to Occupational Mn Exposure*

Ying Xin
University of Louisville

*Prevention of Angiotensin II-induced Cardiomyopathy by Sulforaphane-activated Nrf2 Partially via AKT/GSK-3β/Fyn Pathway*
Abstracts

Keynote presentation

“High Throughput Screening for Chemical Toxicity Assessment”

B. Alex Merrick, Ph.D.

National Toxicology Program, Research Triangle Park, NC 27709

Thousands of chemicals to which humans are exposed have inadequate data on which to predict their potential for toxicological effects. However, dramatic technological advances in molecular and systems biology, computational toxicology, and bioinformatics can provide powerful new tools to improve public health. High throughput screening (HTS) techniques combined with computational methods and information technology can probe how chemicals interact with biological systems, both in vitro and in vivo. Progress is being made in recognizing the patterns of response in genes and pathways induced by certain chemicals or chemical classes that might be predictive of adverse human health outcomes. Tox21 began in 2008 as a collaboration between the National Institute of Environmental Health Sciences (NIEHS)/National Toxicology Program (NTP), the U.S. Environmental Protection Agency’s (EPA) National Center for Computational Toxicology (NCCT), and the National Human Genome Research Institute (NHGRI)/National Institutes of Health (NIH) Chemical Genomics Center (NCGC) (now located within the National Center for Advancing Translational Sciences) and in mid-2010, the U.S. Food and Drug Administration (FDA) to develop a vision and devise an implementation strategy to shift the assessment of chemical hazards from traditional experimental animal toxicology studies to target-specific, mechanism-based, biological observations largely obtained using in vitro assays. Tox21 goals, priorities, progress and challenges will be presented.
Development of molecular biomarkers for detection of gonadal sex reversal in Japanese medaka after exposure to synthetic estrogens and androgens

Ahmed Abdel-moneim, Cecon Mahapatra and Maria S. Sepúlveda

Department of Forestry and Natural Resources, Purdue University

Sex differentiation in teleosts is controlled by several proteins whose expression could be easily impacted by synthetic sex steroids in water causing complete sex reversal in extreme cases. Sewage waste-water treatment plants and concentrated animal feeding operations are a source of synthetic sex steroids to water bodies. In the Japanese medaka (Oryzias latipes), sex is chromosomally determined with gonads developing during the first month post hatch, and thus this model can be used to better understand the mechanisms of toxicity and gonadal effects of hormone-mimics. Our focus has been on early molecular events associated with gonadal differentiation in order to fill this research. To understand the molecular effects of hormone mimics on genes involved in sex differentiation, we first determined the baseline expression of three female sex specific genes during early development: ovarian specific protein 1, osp1; gonadal aromatase, cyp19a; and vitellogenin, vtg. Gene expression was quantified at 5, 8, 10, 12, 15, 25 and 30 days post fertilization (dpf) using the SK2MC strain in which males are easily distinguishable by the presence of leucophores. Genes were expressed at low levels in both genders as early as 5 dpf; however starting at 12 dpf, expression was several fold higher in females (osp1 ~ 17-fold; vtg ~10-fold; and cyp19a ~ 20-fold) compared to males. Our results are in agreement with reports that indicate oogenesis begins at ~15 dpf in female Japanese medaka. Next we exposed 15 dpf old male and female Japanese medaka larvae to 10 ng/L of 17α-ethynylestradiol (EE2) or 17β-trenbolone (TRB) for up to 10 days. We expect this exposure to cause an up-regulation in the expression of these female sex specific genes in males exposed to EE2 and a down-regulation in females exposed to androgens. Gonads are also being evaluated histologically in order to tie these gene changes with development of intersex or sex reversal.
Acrolein Induces Changes in Endothelial cell miRNA content and Cellular Function

Wesley Abplanalp, Timothy O’Toole, Xiaohong Li, Nigel Cooper, Daniel J. Conklin, and Aruni Bhatnagar

Diabetes and Obesity Center and Department of Anatomical Sciences and Neurobiology, University of Louisville, Louisville, KY 40202, USA

Epidemiological studies have demonstrated an association between the combustion product acrolein and endothelial dysfunction, which presents as atherosclerosis, coronary heart disease, myocardial infarction, and hypertension. However, little is known about the mechanisms whereby acrolein contributes to these pathologies. To determine if acrolein induces a change in miRNA levels we used microarray technology. We found that acrolein stimulated the upregulation of 12 miRNAs and the downregulation of 15 miRNAs by at least 1.5-fold in acrolein treated human umbilical vein endothelial cells (HUVEC) compared to untreated cells. Selected miRNA changes were confirmed by qPCR. Among the upregulated miRNAs were three members of the let-7 family that have roles in cellular differentiation, growth, and insulin signaling. Consistent with let-7a upregulation, acrolein treated HUVECs demonstrated a decrease in three of its protein targets: β3 integrin (57±8%), Cdc34 (73±8%), and K-Ras (55±4%). Exposure to acrolein also limited β3 integrin-dependent HUVEC migration (38% of control) compared to vehicle treated cells. Migration defects were reversed (87% of control) by expression of a let-7a inhibitor but not with a non-specific miRNA inhibitor (48% of control). Changes in let-7a expression and insulin signaling were also observed in the aortas of mice inhaling volatile acrolein (1ppm x 6h/day x 4 days). These studies suggest that endothelial miRNA repertoire, protein expression, and cellular function can be acutely regulated by acrolein. This mechanism may contribute to similar vascular pathologies resulting from exposure to related environmental pollutants.
2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) neurotoxicity in primary midbrain cultures

Zeynep Sena Agim¹, Amy Griggs², Vartika Mishra², Jean-Christophe Rochet², Jason R. Cannon¹

¹School of Health Sciences, Purdue University, West Lafayette, IN, 47907; ²Dept of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, 47907

Parkinson’s disease (PD) is characterized behaviorally by a combination of bradykinesia, resting tremor, postural instability, and rigidity. Motor symptoms are caused by a loss of dopamine neurons in the substantia nigra. While ~10% of cases can be attributed to genetics, the remainder arises from unknown causes (‘sporadic’). Much epidemiological research suggests that environmental factors have a role in PD etiology; pesticides, heavy metals and solvents have been repeatedly implicated, however, a single causative factor has not been identified. Dietary factors may be encountered more frequently and in higher doses than environmental exposures. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a heterocyclic amine produced during high-temperature cooking of meat products. PhIP bears structural semblance to dopamine and known dopaminergic toxicants, with a pyridine ring and aromatic hydroxyl groups. PhIP and its metabolites have been shown to induce carcinogenesis in rodents, however, PhIP-induced effects on nervous system function are not known. Notably, few compounds in this class have been examined for neurological effects, with limited data showing that certain heterocyclic amines inhibit dopamine catabolism and synthesis. Here, we tested the hypothesis that PhIP is toxic to dopaminergic neurons. Primary midbrain neuronal cultures containing dopaminergic and nondopaminergic neurons were treated with PhIP or select Phase I metabolites for 24 hours. Surviving dopaminergic and total neurons were quantified manually using immunofluorescence staining and fluorescence microscopy. Our data demonstrates, for the first time, that PhIP or N-OH-PhIP is selectively toxic to dopaminergic neurons, while 4’-OH-PhIP, another major metabolite, is not toxic at tested doses. We also tested whether a blueberry extract with antioxidant activity and the antioxidant molecule N-acetyl-cysteine (NAC) are protective. Pre- and co-treatments of the cultures with either antioxidant prevented PhIP- and N-OH-PhIP-induced toxicity to dopaminergic neurons, suggesting that dopaminergic toxicity by these agents is mediated by oxidative stress. Ongoing studies are being conducted to quantify lipid peroxidation and DNA oxidation as measures of oxidative damage. Finally, our future studies will assess the behavioral, neurochemical, and neuropathological effects of systemic PhIP treatment in vivo.

Acknowledgments: We thank Mitali A. Tambe for technical assistance. This work was funded by the Showalter Trust (J.R.C. and J.-C.R.) and NIEHS/NIH ES019879 (J.R.C.).
Identification Of Xenobiotic Receptor Agonists Which Could Contribute To Nonalcoholic Fatty Liver Disease

L. Al-Eryani¹, B. Wahlang¹, K.C. Falkner², H.B. Clair³, J.J. Guardiola², R.A. Prough³, J.C. States¹, M. Cave²

Departments of ¹Pharmacology and Toxicology, ²Medicine and ³Biochemistry and Molecular Biology University of Louisville, Louisville, KY

Introduction: Environmental chemical exposure is associated with nonalcoholic fatty liver disease (NAFLD) via xenobiotic receptors’ activation including pregnane-xenobiotic-receptor (PXR) and constitutive androstane receptor (CAR). In this study, we data-mined and validated environmental chemicals that activate these receptors.

Methods: EPA-ToxCast Phase I database (320 chemicals) was used to identify compounds associated with PXR activation. Human (h) and murine (m) PXR and CAR activation was evaluated for selected compounds from EPA screening assays and organochlorine pesticides (OCPs) associated with NAFLD (National Health and Nutritional Examination Survey study). HepG2 cells were transfected with plasmids expressing h or m PXR/CAR and receptor-responsive plasmids, pGL3-PXR/CAR-RE-luciferase then exposed to h or m PXR/CAR ligands or varying concentrations of OCPs.

Results: Nearly 2/3 of chemicals screened positive for PXR activation. 67 compounds and 102 compounds were found to activate hPXR by NGCG and Attagene assays respectively. CellzDirect assay identified 202 compounds that were found to change CYP3A4 (PXR target gene) expression. Among chemicals selected for validation (n=9) in cell-based reporter assays, trans-nonachlor, chlordane, DDE, DDT, lindane and alachlor activated h/m PXR. Dieldrin activated mPXR only. Dieldrin, trans-nonachlor, DDT, lindane and alachlor also activated hCAR but not mCAR.

Conclusion: Potential PXR effects were identified for nearly 2/3 of toxCast Phase I chemicals. Not all chemicals identified by the ToxCast screening assays proved to be xenobiotic receptor agonists. We postulate that the pesticides that activate PXR may contribute to NAFLD through the effect of this receptor on energy metabolism which may differ in humans and rodent models.
Vinyl Chloride And/Or Its Metabolites Induce Hepatic Necro-Inflammation In Mice And In Human Subjects With Chronic Low-Level Exposures

Lisanne C Anders1,2, Amanda N Douglas1,2, Nikole L Warner3,4, Irina A Kirpich2,5, Mohammad K Mohammad2,5, Keith C Falkner2,5, Matt Cave1,2,5, Craig J McClain1,2,5 and Juliane I Beier1,2,4

1Dep. of Pharmacology/Toxicology, 2UofL Alcohol Research Center 3Dep. of Microbiology/Immunology, 4Center for Predictive Medicine, 5Dep. of Medicine, University of Louisville Health Sciences Center, Louisville, KY 40292.

Background. Vinyl chloride (VC) is a ubiquitous environmental contaminant and ranks 4th on the ATSDR Hazardous Substances Priority List. We have previously reported increased hepatocellular necrosis in a highly exposed occupational cohort and in vitro models. The purpose of this study is to study hepatic necro-inflammation in vivo and assess potential liver injury in a residential cohort of human subjects living adjacent to a VC chemical plant.

Methods. C57Bl/6J mice were exposed to chloroethanol (ClEtOH; 50 mg/kg i.g.), a major metabolite of VC, and to lipopolysaccharide (LPS; 10 mg/kg i.p.) 24 h after the ClEtOH dose. Plasma and livers were harvested for determination of liver damage and inflammation. Serum cytokeratin 18 (CK18) M30 (apoptosis biomarker) and CK 18 M65 (necrosis biomarker) were measured by ELISA in 10 consenting residents living in “Rubbertown” adjacent to a VC plant and also in 10 consenting controls living elsewhere in Louisville, Kentucky.

Results. In mice, ClEtOH alone caused no detectable liver damage, as determined by clinical chemistry and histology. LPS exposure caused inflammatory liver injury, characterized by an increase in monocytic inflammation and plasma transaminases. ClEtOH exacerbated liver damage caused by LPS; the activation of recruited (CD68+) and resident (F4/80+) monocytes was enhanced by ClEtOH. Neutrophil recruitment, as determined by staining and expression of LY6G, was also enhanced by ClEtOH. These inflammatory changes were coupled with a 2.5-fold increase in transaminases over LPS alone and an increase in the number of necroinflammatory foci. Whereas, ClEtOH enhanced liver injury caused by LPS, this combination decreased the number of TUNEL-positive cells, suggesting that the observed effects were not mediated via an increase in apoptosis, but rather necrosis. Human subjects exposed to VC had elevated CK18 M65>M30 (vs. controls) consistent with primarily necrotic rather than apoptotic hepatocellular death, similar to that observed in the animal model.

Conclusions. Taken together, these data suggest that ClEtOH (as a surrogate VC exposure) sensitizes the liver to necro-inflammation following a “second hit”. Chronic low-level environmental exposures appeared to increase hepatocellular necrosis in this small cohort of “Rubbertown” residents. These results serve as proof-of-concept for the possibility that the hepatotoxicity of VC may be modified by endotoxemia, which commonly occurs in diet-induced obesity and NAFLD. Furthermore, these data implicate exposure to volatile organic compounds in the development of liver disease in susceptible populations.
In Vitro Effects of Cytochrome P450 Isozyme Inhibitors on 3,5-Dichloroaniline Nephrotoxicity in Rat Renal Cortical Cells
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Chlorinated anilines are commonly used in the manufacture of agricultural chemicals, dyes, industrial compounds, and pharmaceuticals. Many of these compounds can induce nephrotoxicity in vivo and in vitro. 3,5-Dichloroaniline (3,5-DCA, 1.0 mM) induced nephrotoxicity in isolated rat renal cortical cells (IRCC) from male Fischer 344 rats following 90 min exposure. IRCC pretreated with non-selective cytochrome P450 (CYP) inhibitors partially attenuated 3,5-DCA toxicity, suggesting that CYPs may play a role in 3,5-DCA bioactivation. The purpose of the this study was to further explore the role of CYP mediated 3,5-DCA bioactivation. IRCC were obtained from male Fischer 344 rats (200 –275 g) and incubated (4 x10^6 cells/ml; 3mL) under a 95% oxygen/5% carbon dioxide atmosphere with shaking (90 min) with dimethyl sulfoxide or 3,5-DCA (1.0mM). IRCC were pretreated with various CYP inhibitors [isoniazid (1.0 mM), ketoconazole (0.1 mM), omeprazole (0.01 mM), diethyldithiocarbamate (DEDTCA; 0.1 mM), oleandomycin triacetate (0.5 mM), or sulfaphenazole (0.1 mM)] and cytotoxicity was determined by measuring lactate dehydrogenase release. Pretreatment with DEDTCA, omeprazole, or sulfaphenazole partially attenuated 3,5-DCA induced nephrotoxicity, while ketoconazole, isoniazid, and oleandomycin triacetate had no effect. These results suggest that 3,5-DCA is bioactivated via multiple pathways, including the CYP2C family. (Supported by NIH Grant 8P20GM103434 to West Virginia IDeA Network for Biomedical Research Excellence)
Transport of α-Synuclein at the Blood-Cerebrospinal Fluid Barrier and Effects of Heavy Metal Toxicities: Potential Role in Parkinson’s Disease Pathoetiology

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The blood-cerebrospinal fluid barrier (BCB) in the choroid plexus maintains the homeostasis of critical molecules in the brain by regulating their transport between the blood and cerebrospinal fluid (CSF). The protein α-synuclein (a-Syn) is integral in Parkinson’s disease (PD) pathoetiology and has been detected in the CSF. Little, however, is understood about how the BCB regulates a-Syn homeostasis in the brain. Previous findings in this lab show that the presence of manganese (Mn) or copper (Cu) in solution facilitates a-Syn aggregates in vitro and that exposure to toxic levels of Mn and Cu induces intracellular a-Syn aggregation in immortalized Z310 choroidal epithelial cells. The current studies were designed to test the hypotheses that concomitant exposure to both Mn and Cu accelerates the rate of a-Syn aggregation in solution and Mn toxicity may facilitate a-Syn transport across a monolayer of the modeled blood-CSF barrier. Our data revealed that contrarily to what we had expected, the combined exposure to Mn and Cu had a much slower rate of a-Syn aggregation in solution than did exposure to either metal individually (p<0.05). Our data also showed that in the primary culture of choroidal epithelial cells, Mn exposure not only increased a-Syn uptake but also increased its endogenous expression, notwithstanding the presence of excessive intracellular stress and cell death caused by over a 48-hr Mn exposure. Furthermore, the Two-Chamber Transwell study demonstrated that while there was a significant decrease in intracellular a-Syn levels at 48 hr compared to 24 hr within each control or Mn treated group, the pretreatment with Mn had no any significant effect on a-Syn transport across the primary rat choroidal epithelial monolayer at 24 or 48 hr. Overall, these findings suggest that Mn exposure increases a-Syn aggregation in the BCB; this increase, however, does not result in the increased efflux of a-Syn from the CSF to blood via the BCB. Mn or Cu alone participates in the process leading to a-Syn aggregation; yet their combined effect is not additive, but rather inhibitive. The exact role of Mn and Cu in a-Syn aggregation and the respective molecular interactions with a-Syn are currently under investigation. (Supported in part by NIH/NIEHS R01ES008146-14S2)
Use of ectoparasiticides on pets seems inevitable since the pets are commonly infested with fleas, ticks, and many other external parasites. Currently, pyrethroids are more commonly used than any other class of ectoparasiticides because they are considered relatively safe. But due to lack of safety data, pet owners have raised serious concerns for their use on dogs. Serious concerns have also been raised about transferable residues of ectoparasiticides to the owners, veterinarians, veterinary technologists, and dog handlers who come in contact with pesticide treated dogs on a daily basis. The present investigation was therefore undertaken with two specific objectives: (1) to determine the toxicity and safety of Bio Spot Defense Flea and Tick Spot On® applied to six dogs, and (2) to determine the residue of active ingredients of the product (etofenprox, s-methoprene, and piperonyl butoxide) in blood of dogs and gloves worn for five minutes to pet dogs at 24, 48, 72 hours, and 1, 2, 3, 4, and 5 weeks post-application. At these time intervals, dogs were evaluated for physical examination. Residues of active ingredients were confirmed and quantitated using GC/MS. In the blood, etofenprox was detected as early as after 48 hours (18.42±5.05 ppm) and the residue persisted until 1 week (0.80±0.35 ppm). S-methoprene and piperonyl butoxide were not detected in the blood at any time. In the gloves, the highest concentrations of etofenprox, s-methoprene, and piperonyl butoxide were determined at 24 hours (9,552.00±1551.83; 2,307.86±456.70; and 1286.13±0.49 ppm, respectively). Residues of all three compounds were detected in appreciable concentrations in the gloves until 1 week (294.86±27.22; 80.62±10.06; and 40.49±5.78 ppm, correspondingly). Of course, residues of these compounds in insignificant amounts were present in gloves until 5 weeks after application. In conclusion, findings of this investigation suggest that Bio Spot Defense product appears to be safe for dogs and their owners, but the veterinarians, veterinary technologists, and dog handlers can be exposed to significant levels of etofenprox, s-methoprene, and piperonyl butoxide following chronic exposure and may suffer from cumulative effects, if not properly protected.
Currently, every fourth or fifth dog out of 78 million dogs in the United States suffers from some form of arthritis. In dogs, osteoarthritis is more prevalent than rheumatoid or any other form of arthritis. Dogs develop arthritis due to aging, injury or genetic predisposition. In general, large breed dogs are more prone to develop osteoarthritis than are the smaller breeds. This investigation was undertaken to evaluate the antiarthritic efficacy, safety and tolerability of Naturally Preferred Holistic Frozen Dog Treats (a product of Henry Schein Animal Health) in moderately osteoarthritic dogs. Ten client-owned osteoarthritic dogs were divided into two groups, based on body weight (Group-1, <50 pounds each; and Group-2, > 50 pounds each), and were treated daily for a period of 28 days. Each dog in Group-1 received a half treat, while those in Group-2 received a full treat. Each dog was evaluated weekly for arthritis-associated pain (overall pain, pain upon limb manipulation, and pain after physical exertion) using different numeric scales. In addition, dogs were examined weekly for physical (body weight, heart rate, and body temperature), hepatic (bilirubin, ALT and AST), renal (BUN and creatinine), heart and muscle (CK) functions, as well as hematological parameters. Findings of this study revealed that pain level was reduced as early as day 7, but it was significantly (P<0.05) ameliorated in dogs of both groups with maximum improvement on day 28. No dogs in either group exhibited any untoward events, and the product (Naturally Preferred Holistic Frozen Dog Treats) was found to be safe and well tolerated over a 4 week period.
Spontaneous alternation behavior of $Ahr^dCyp1a2(-/-)$ and $Ahr^b1Cyp1a2(-/-)$ and $Ahr^b1Cyp1a2(+/+)$ mice exposed to polychlorinated biphenyls during gestation and lactation

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Cytochrome P450 1A2 (CYP1A2) has been shown to play an important role in metabolizing some toxicants including polychlorinated biphenyls (PCBs). CYP1A2 upregulation is dependent on the relative binding affinity of the aryl hydrocarbon receptor (AHR) to a ligand, in our case coplanar PCBs. Previous work in our lab found learning and memory deficits in PCB-exposed $Cyp1a2(-/-)$ mice compared to $Cyp1a2(+/-)$ wild type mice. Both poor-affinity $Ahr^dCyp1a2(-/-)$ and high-affinity $Ahr^b1Cyp1a2(-/-)$ mice had significant deficits in novel object recognition and Morris water maze tests compared with $Ahr^b1Cyp1a2(+/+)$ wild type mice. For this project, we modified our dosing protocol to provide an environmentally relevant mixture of PCBs in corn oil-soaked food daily to dams from gestational day 0 (GD0) to postnatal day 25 (PND25). We tested spatial learning and working memory in the offspring at PND60 using the Y-maze test of spontaneous alternation. We found no significant differences in latency to leave the original arm, total number of arms entered, or percent alternation based on genotype or treatment (P>0.05). This suggests that the Y-maze is not as sensitive as the Morris water maze in detecting deficits in spatial learning and memory following developmental PCB exposure.
The Effect of a Human Arylamine N-Acetyltransferase 1 Specific Inhibitor and Curcumin or Resveratrol on the Proliferation of Breast Cancer Cell Lines

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Human arylamine N-acetyltransferase 1 (NAT1) is a phase II xenobiotic-metabolizing enzyme that in recent years has been shown to play a role in breast cancer aggressiveness. Because of this, NAT1 has been proposed as a molecular target for small molecule inhibition. Compound 10, an effective inhibitor of human NAT1, was previously discovered using in silico screening and has been shown to decrease human NAT1 enzymatic activity, arylamine carcinogen-induced DNA adducts, cell proliferation, and cell invasion in human breast adenocarcinoma cells. Recently, there has been increased interest in using phytochemicals in combination with small molecule inhibitors to improve the effectiveness of cancer treatments. For this project we have chosen two of these phytochemicals, curcumin and resveratrol, to test on breast cancer cells in situ in combination with compound 10, to explore possible synergistic actions on compound 10’s aforementioned cancer prevention effects. Compound 10, curcumin, and resveratrol have all been tested in vitro using yeast lysates that recombinantly express human NAT1 to test inhibition of NAT1 activity independently and we are now investigating how each compound affects cell proliferation in multiple breast cancer cell lines. We first will investigate each compound’s effect on each cell line independently, and then once that effect is established we will test each phytochemical in combination with compound 10. The IC₅₀ for compound 10, curcumin, and resveratrol for in vitro NAT1 inhibition have been determined to be 1.14 μM, >4000 μM, and 887 μM, respectively. We have measured the independent effects of compound 10, curcumin, and resveratrol on proliferation of MDA-MB-231 cells. Each compound decreased proliferation of these cells with an IC₅₀ of approximately 45 μM, 7 μM, and 0.3 μM respectively. Comparing the IC₅₀ values for in vitro NAT1 inhibition and the IC₅₀ values for inhibition of the proliferation of MDA-MB-231 cells in situ, it appears that while compound 10 may be affecting proliferation through inhibition of NAT1, it is very unlikely that curcumin and resveratrol are affecting proliferation through NAT1 inhibition but through another mechanism of action. We will test all three compounds on other breast cancer cell lines and also on normal breast epithelial cells to investigate selectivity towards inhibiting proliferation of cancer cells.
In Silico prediction and in vitro validation of Daphnia pulex microRNAs

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Daphnia pulex, the crustacean with the first sequenced genome, is an important organism that has been widely used in ecological and toxicological research. MicroRNAs (miRNAs) are 21-25 nucleotide small non-coding RNAs that are involved in a myriad of physiological processes. In this research, we predicted 75 D. pulex miRNAs by sequence homology and secondary structure identification. To further test our predictions, we selected 14 predicted miRNAs for qRT-PCR validation. A total of 8 miRNAs have been validated which included mir-8, mir-9, mir-12, mir-92, mir-100, mir-133, mir-153 and mir-283. Expression levels of these validated miRNAs were quantified at three different life stages (days 4, 8 and 12 of age). RNU6 was selected as a reference gene and its expression stability at different life stages was tested by geNorm and Normfinder. The expression of mir-8, mir-9, mir-12, mir-92 and mir-100 were significantly different across time suggesting these miRNAs might play a critical role during D. pulex development. This is the first study to report data on miRNAs in D. pulex and may shed some light on the development and application of D. pulex miRNAs as biomarkers for environmental stressors including toxicants.
Acrolein, a reactive aldehyde metabolite, is a major mediator of alcohol-induced endoplasmic reticulum stress and liver injury

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Alcohol is the most socially accepted addictive drug, and it can cause alcoholic liver disease (ALD), which is a major cause of morbidity and mortality in the United States. Chronic alcohol consumption causes a pro-oxidant environment in the liver and increases hepatic lipid peroxidation. Acrolein is the most reactive and toxic aldehyde generated through lipid peroxidation. Also, acrolein is found in fried fatty foods and is a major component of cigarette smoke, which negatively impacts chronic liver diseases. Acrolein is known to form protein adducts, and we have demonstrated that acrolein triggers endoplasmic reticulum (ER) stress leading to cytotoxicity in hepatocytes. Notably, alcohol-induced ER stress is thought to be an etiologic factor in ALD. This study investigates the role of acrolein as a mediator of hepatic ER stress and injury during alcohol consumption.

Acrolein accumulation, activation of pro-apoptotic stress kinase-JNK, ER stress, and cell death was examined in alcohol exposed rat hepatic cells (H4IIEC), and in an in vivo mouse model of alcohol consumption. Exposure to alcohol led to the accumulation of acrolein adducts both in vitro and in vivo. This was accompanied by upregulation of ER stress proteins (ATF3, ATF4, and GADD153/CHOP) and phospho-activation of JNK. Alcohol exposure also induced apoptotic cell death in hepatocytes. This study demonstrates that acrolein is likely to be a major culprit in the ER stress and hepatotoxicity associated with alcohol consumption. Acrolein scavengers may have therapeutic potential in alleviating the adverse effects of alcohol consumption, and we are actively investigating this concept.
Association between Benzene Exposure, Circulating Angiogenic Cell Levels, and Cardiovascular Disease Risk in the Louisville Healthy Heart Study

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Background: Benzene is an aromatic hydrocarbon found in high amounts in vehicular exhaust and tobacco smoke. Exposure to traffic pollutants or chronic tobacco smoke exposure induces cardiovascular injury, suppresses circulating angiogenic cells (CACs), and increases thrombosis and atherogenesis. The purpose of this study was to examine whether benzene exposure is associated with CAC levels and cardiovascular injury in humans.

Methods: Benzene exposure was assessed in 240 participants of the Louisville Healthy Heart Study with cardiovascular disease (CVD) risk factors by measuring the urinary levels of the benzene metabolite – trans, trans-muconic acid (t,t-MA). Because benzene is both environmental pollutant and a tobacco smoke constituent, urine cotinine levels were also measured. Generalized linear models were used to assess the association between benzene exposure and parameters of CVD risk and injury and adjusted for potential confounders.

Results: The study population was 51±10 years old, 41% African American, 47% female, and 39% current smokers. As expected, urinary t,t-MA levels were higher in smokers than non-smokers and positively correlated with urinary cotinine levels. Urine t,t-MA level was inversely associated with residential proximity to roadways. Urinary t,t-MA levels are inversely related to both early (AC133+) and late (AC133–) CACs. However, no association was observed between t,t-MA and inflammation or thrombosis. In non-smokers, t,t-MA levels were positively associated with increased Framingham Risk Scores.

Conclusions: Regardless of the source of benzene exposure (e.g., tobacco smoke, or traffic emissions), benzene may increase cardiovascular disease risk in part through decreased levels of CACs and subsequent suppression of vascular repair.
Pathogenic Mechanisms in Highly Active Anti-retroviral Therapy (HAART) Induced Hepatotoxicity: Role of Hepatocyte Phosphodiesterase 4 (PDE4) Expression and ER-Stress

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HIV protease inhibitors (HIV-PIs) are the major components of the highly active anti-retroviral therapy (HAART) and have been successfully used in the treatment of HIV-1 infection in the past two decades. Importantly, HIV-PIs can induce endoplasmic reticulum (ER) stress response and subsequent activation of unfolded protein response (UPR) leading to dys-regulation of hepatic lipid metabolism and hepatotoxicity. The present study examined the potential mechanisms underlying HIV-PI induced hepatic ER stress and toxicity with a particular emphasis on the pathogenic role of phosphodiesterase 4 (PDE4) family of enzymes.

The effects of HIV-PIs [ritonavir (RIT) and lopinavir (LOP)] were examined both in a rat hepatoma cell line (H4IIEC3) as well as rat primary hepatocytes. The data obtained from these studies demonstrated that in comparison to individual HIV-PIs, the combinatorial treatment of RIT+LOP led to a significantly greater loss of hepatocyte survival. Notably, inhibition of PDE4 by a specific PDE4-inhibitor, rolipram, markedly attenuated hepatotoxicity induced by individual as well as combinatorial PI treatments. Examination of the mechanistic role of PDE4 showed that PDE4 inhibition significantly decreases the expression of the ER stress related proteins CHOP, ATF-4 and -3 induced by PIs. Additionally, in the context of attenuating PI induced hepatocyte death, inhibition of PDE4 significantly decreased the expression of FasL (death ligand) in response to PI treatment.

Overall, these studies show that PDE4 plays a major pathogenic role in the induction of ER stress and apoptotic gene expression in the HIV-PI mediated hepatocyte toxicity and is a relevant therapeutic target.
Elucidating the role of the polymorphic human hs1,2 enhancer in the effects of TCDD

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent environmental toxin known to inhibit immunoglobulin (Ig) gene expression in various animal studies. We have identified the mouse 3'Ig heavy chain regulatory region (3'IghRR) as a sensitive transcriptional target of TCDD, which may mediate the inhibitory effect of TCDD on Ig expression. Interestingly, the human hs1,2 enhancer is polymorphic and has been associated with a number of autoimmune diseases. However, previous studies have demonstrated a species difference in the effects of TCDD on hs1,2 enhancer activity. TCDD inhibits mouse hs1,2 enhancer activation, which correlates with the inhibitory effects of TCDD on mouse 3'IghRR and Ig. Whereas, TCDD activates the human hs1,2 enhancer. The objective of this study was to determine the effect of stimulation and TCDD on enhancer activity of the polymorphic human hs1,2 enhancer using a human B-cell line (CL-01) and luciferase reporter constructs regulated by each of the human hs1,2 alleles. Stimulation is an important component to these studies since B cells must be stimulated to produce antibody. Our results support that TCDD alone activates each of the human hs1,2 alleles. However, B-cell stimulation by R848, a ligand for Toll-Like Receptor 7 and 8 (TLR 7,8), and CpG, a ligand for TLR 9 inhibited basal activity of the hs1,2 alleles and TCDD reversed this inhibition. In contrast, R848 induced Ig secretion and activates a 3x NF-κB luciferase reporter, therefore confirming functional signaling through the TLRs and activation of the CL-01 B cells. R848 also induced class switch recombination from IgM to IgG. Furthermore, TCDD inhibited both IgM and IgG secretion in cells stimulated with R848. These results suggest that the human hs1,2 enhancer may be a negative regulator of 3'IghRR activity and Ig expression. Alternatively, the hs1,2 enhancer may behave differently when studied in isolation as compared to its function in the intact 3'IghRR. Future studies will evaluate the effect of TCDD in the absence or presence of stimulation on the activity of the human 3'IghRR and the hs3 and hs4 enhancers. Elucidating the role of the polymorphic hs1,2 enhancer in 3'IghRR activity and the effect of TCDD on the enhancers of the 3'IghRR may provide insights into the etiology of autoimmune diseases associated with the hs1,2 polymorphism and a greater understanding of Ig regulation in general. (Supported by NIEHS R01ES014676)
The Effects of Silver Nanoparticles Released from Commercial Products on Zebrafish (Danio rerio) Embryos

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Silver nanoparticles (AgNPs) have found practical applications in various consumer products due to their antimicrobial properties. However, the unintentional release of AgNPs could lead to harmful effects in non-target organisms. In this study, we used zebrafish (Danio rerio) embryos to assess the toxicity of AgNPs released from AgNP-coated commercial socks. Silver nanoparticles were extracted from socks (AgNP-Socks) after soaking in ultrapure water and gently agitating for 24 hr. Total silver content was quantified by inductively coupled plasma mass spectrometry (ICP-MS) and imaging of AgNPs was done by transmission electron microscopy (TEM). Zebrafish embryos were exposed to different concentrations of the AgNP-Socks solutions and were observed for 72 hrs for survival, hatching and development. The LC50 (72 hrs) was 0.1 ppm and ~50% of the embryos were deformed at this concentration with an EC50 for hatching of 0.04 ppm. Expression of genes commonly associated with mitochondrial function and oxidative stress were altered upon AgNP-Sock. Specifically we observed a marked up-regulation of superoxide dismutase (sod), catalase (cat), glutathione (gsh), B-cell lymphoma protein (bcl2) and bcl-2 associated protein (bax) genes with increasing AgNP-Sock concentration. These results suggest AgNP-Sock is causing mitochondria toxicity leading to oxidative stress and cellular toxicity. Currently, we are following-up these results by exposing embryos to pure AgNP as well as silver nitrate (AgNO3) to strengthen our observations and confirm that the effects are due to AgNPs and not due to silver ions or other elements present in the commercial socks.
FasL promoter histone modifications play a critical role in ethanol-mediated enhancement of FasL gene expression and cell death in CD4+ T lymphocytes.

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Excessive alcohol consumption is known to induce immunosuppression. Along with elevated serum levels of Fas Ligand (FasL), CD4+ T cell decline and immune dysfunction are clinical features associated with alcohol abuse; however the molecular mechanisms are poorly understood. FasL plays a critical role in the regulation of CD4+ T cell activation induced cell death (AICD) and the immune response. Although transcriptional regulation of FasL has been extensively studied, the chromatin remodeling involving promoter associated histones has not been elucidated. Accordingly, we investigated the immunotoxic effects of ethanol on promoter histone modifications involved in regulating FasL gene expression in activated CD4+ T lymphocytes. FasL promoter ChIP analysis of in vitro ethanol treated cells showed a significant increase in TCR-inducible transcriptionally permissive promoter histone modifications including histone H3-K4 trimethylation, S10 phosphorylation and K9 acetylation as compared to untreated cells. In correspondence with increased H3K9 acetylation, ethanol was observed to enhance the recruitment of p300 and relevant transcription factors which correlated with increased FasL expression and apoptotic death. The role of p300 in FasL gene expression was further confirmed by examining the effect of siRNA-mediated p300 knock-down or garcinol, a competitive inhibitor of p300. Notably, CD4+ T cells obtained from individuals with a history of heavy alcohol consumption showed significantly greater p300-dependent H3K9 acetylation and FasL expression. These data strongly suggest that histone modifications play a causal role in the regulation of FasL gene expression. These data also implicate histone modifications as potential targets for the development of therapy to prevent loss of CD4+ T lymphocytes and ensuing immune suppression in alcoholic patients.
STUDIES OF THE MECHANISM OF ZINC TOXICITY IN OLFATORY NEURONS

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Zinc has long been touted as a panacea for the common cold. However, there has been some controversy over whether an intranasal (IN) zinc gluconate gel (Zicam), purported to fight colds, causes anosmia, or the loss of the sense of smell, in humans. Previously, we had shown that zinc gluconate was as toxic as zinc sulfate in an in vitro olfactory neuron model, the rat Odora cell line. However, the mechanism of toxicity was not understood. Using RNA-seq analysis, we have shown that cationic zinc causes an up-regulation of oxidative stress-related genes, which have been associated with cell death. The zinc-mediated toxicity initially causes changes in genes involved with the pentose phosphate pathway, which is used to generate NADPH, a crucial co-factor for the generation of the antioxidant glutathione. Additionally, the cell also down-regulates expression of the ryanodine receptor, which is responsible for calcium release from the endoplasmic reticulum and other calcium stores. This may be a protective measure by the cell since increases in intracellular calcium have been linked to apoptosis and necrosis. Inevitably, the cell is overwhelmed by reactive oxygen species (ROS) and dies via a non-apoptotic mechanism, as evidenced by lack of DNA laddering and upregulation of several anti-apoptotic genes (e.g. Bcl2-XL, IAP). In conclusion, zinc toxicity in Odora cells appears to be mediated through oxidative stress in a non-apoptotic manner.
Characterization of genetic susceptibility to PCB-induced developmental neurotoxicity

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Polychlorinated biphenyls (PCB) are man-made organic pollutants that bioaccumulate in the environment. Gestational and early developmental exposure to PCB have been strongly linked to neurotoxicity. PCBs are found as a mixture of coplanar and noncoplanar congeners. Coplanar PCBs activate the aryl hydrocarbon receptor (AHR), which then leads to increased expression of cytochrome P450 enzymes (CYP), of which CYP1A2 sequesters PCB in the liver. We and others have shown that genetic variations in Ahr and Cyp1a2 loci affect the metabolism of PCB, and ultimately play a role in susceptibility to PCB-induced toxicity. Using a mouse model that models human variation in the AHR and CYP1A2, we extended our previous work on learning and memory deficits to examine genetic susceptibility to PCB-induced motor dysfunction. Pregnant dams were treated with a PCB mixture via oral gavage on gestational day 10 and postnatal day (PND) 5. We began motor testing at PND60. Tissues from littermates were harvested at PND30 for biochemical analyses of CYP1A1 expression and enzyme activity. Consistent with our previous findings, our motor battery test results indicate a significant gene by treatment interaction, with both AhrbCyp1a2(-/-) and AhrdCyp1a2(-/-) showing impaired performance after PCB exposure, when compared to wild type mice. We also found that corn oil-treated control Cyp1a2(-/-) mice showed significant motor coordination impairment when compared to wild type. Additionally, we report a significant gene x treatment interaction with higher EROD activity in liver of high-affinity Ahrb mice compared with poor-affinity Ahrd mice. We also found a trend for higher EROD activity in the cerebellum of AhrbCyp1a2(-/-) mice. Our results support our hypothesis that CYP1A enzymes may play a novel role in proper brain function and protection from PCB-induced neurotoxicity, potentially more so in cerebellar than nigrostriatal pathways. Our next steps are to characterize biochemical pathways in which CYP1A2 may be involved in the brain.
DHA Enhances Antioxidant Response Element (ARE)-Mediated Transcription in Lung Epithelial Cells

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Objectives: DHA is associated with decreased inflammatory responses but the mechanisms are poorly understood. We have previously observed decreases in neonatal hyperoxic lung injury with maternal DHA supplementation (Rogers et. al, 2010). To understand the anti-inflammatory effect of DHA on the lung epithelial cells, we developed a cell culture model using LPS treatment in MLE-12 cells (immortalized murine alveolar type II cells). Activation of the Antioxidant Response Element (ARE) has been shown to restore the barrier function of alveolar epithelium. We tested the hypothesis that DHA supplementation in MLE-12 cells will activate the ARE and this will reduce the inflammatory response to LPS-induced injury.

Methods: MLE-12 cells were grown in 25 uM DHA or control media and exposed to LPS. The optimal dose of LPS was determined by varying concentrations from 20 uM to 200 uM. A time-response curve was developed by treating the cells with 100 uM of LPS for 4 hours, then harvesting the cells at 0, 2, 4, 24, and 48 hours. Inflammation was assessed by measurement of the chemokines MCP-1 and KC by ELISA. In separate experiments, MLE-12 cells were transfected with a vector containing ARE-luciferase, treated with 100 uM LP, and harvested at 24 hours. Activity of the ARE was determined by measuring luciferase chemiluminescence.

Results: DHA treatment attenuated MCP-1 and KC protein levels at LPS doses of 100uM or lower. Chemokine protein levels were optimally measured at 24 hours post treatment. Luciferase assay revealed that DHA activated the ARE in response to LPS exposure (p=0.01).

Conclusions: The anti-inflammatory mechanisms associated with DHA treatment are not understood. Our data indicate that DHA enhances anti-oxidant responses through activation of ARE. Further studies are needed to determine the upstream effectors of this response and to identify the anti-oxidants specifically affected.
Enhanced Intranasal Delivery of Gemcitabine to the Central Nervous System

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Delivery of drugs from the nasal cavity to the brain is becoming more widely accepted, due to the non-invasive nature of this route and the ability to circumvent the blood brain barrier (BBB).

**Objective**- Because of similarities in the proteins comprising the olfactory epithelial tight junction (TJ) proteins and those of the BBB, we sought to determine whether papaverine, which is known to reversibly enhance BBB permeability, could increase the delivery of intranasally administered gemcitabine to the central nervous system in rats.

**Experimental methods** included intranasal administration of gemcitabine, fluorescein isothiocyanate-dextran beads and papaverine, histopathology, immunostaining, RT-PCR, western blot analysis, immunofluorescence localization, spectrofluorometric analysis, *in-vivo* brain microdialysis, HPLC analysis and *in-vitro* gemcitabine recovery.

**Results and discussion**- Similar to previous findings, papaverine transiently decreased the levels and altered immunolocalization of the TJ protein phosphorylated-occludin in the olfactory epithelium, while causing an approximately 4-fold increase in gemcitabine concentration reaching the brain. The enhanced delivery was not accompanied by nasal epithelial damage or toxicity to distant organs.

**Conclusion**- The ability to transiently and safely increase drug delivery from the nose to the brain represents a non-invasive way to improve treatment of patients with brain disorders.
Analysis of Motor Function in Cyp1a1 (-/-) Knockout Mice

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Cytochrome P450s CYP1A1 and CYP1A2 are detoxifying enzymes regulated by the aryl hydrocarbon receptor and are both upregulated following exposure to common pollutants such as cigarette smoke and polychlorinated biphenyls (PCBs). Typically these enzymes are expressed in the liver, but several studies report that they are also expressed in the cortex and cerebellum of the brain. Previous studies in our lab uncovered motor deficits in Cyp1a2 (-/-) knockout mice and Cyp1a1_1a2 (-/-) double knockout mice. To determine if the absence of CYP1A1 contributed to the observed deficits in the double knockouts, we are now testing Cyp1a1 (-/-) and Cyp1a1(+/+) wild type mice. We are using a battery of six tests of motor function, but are reporting here on two of those tests: Balance Beam and Rotarod. These tests can identify deficits in the major motor pathways of the brain. Rotarod primarily tests cerebellar function related to balance and motor coordination whereas the Balance Beam tests for coordination and alterations in nigrostriatal dopaminergic pathways. In our preliminary analysis, we found a significant difference in latency to fall off the rotator with Cyp1a1 (-/-) mice having shorter latencies on Days 1-3 (P< 0.001 Day 1, P<0.01 Day 2, P<0.05 Day 3), but no significant differences on Days 4-5 of testing. We also found a significant main effect of sex in the Balance Beam test with females traversing the beam significantly faster than male mice regardless of genotype (P<0.05). Overall, our results suggest Cyp1a1 genotype is less important than Cyp1a2 genotype in cerebellar function, but both appear to play a role in normal motor function.
Influence of environmental chemicals on epigenetic programming and its applicability in human health risk assessment.

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The field of epigenetics is rapidly evolving in response to the growing concern that heritable changes in gene expression may be involved in chemically-mediated adverse health outcomes, such as cancer. Although human and animal studies have shown a strong involvement of epigenetic dysregulation in the etiology of several toxicological conditions, applicability of epigenetic data across the current human health assessment paradigm is unclear. The objective of this study is to compare the sensitivity of epigenetic alterations with the development of tumors in animal toxicological studies and to explore the possibility of incorporating epigenetic information into the hazard identification process for human health risk assessments. Animal studies from various toxicological databases were mined for evidence of alterations in DNA methylation (a common epigenetic change) and tumor incidence data after exposure to the following probable and known human carcinogens: di(2-ethylhexyl) phthalate, bromodichloromethane, dibromochloromethane, chloroform, hydrazine, trichloroethylene, benzidine, and trichloroacetic acid. All comparative analyses involved conversion of animal doses to corresponding human equivalent doses (HEDs). Benchmark dose (BMD) modeling was performed using HEDs for tumor incidence. The resulting BMD values were compared to no-observed-adverse-effect levels (NOAELs) for changes in DNA methylation. In the absence of a NOAEL, a 10-fold uncertainty factor was applied to the lowest-observed-adverse-effect level (LOAEL) to approximate a NOAEL. Examination of these chemicals revealed that DNA methylation is 1.2- to 25-fold more sensitive than the corresponding tumor incidence for all chemicals, except for trichloroacetic acid, where there is no discernible difference in sensitivity. The predominant DNA methylation alteration in tumors, identified in the studies evaluated, was hypomethylation of either whole DNA or the promoter region of oncogenes (e.g., c-Myc, c-Jun, Ha-Ras, and Ki-Ras) in liver, kidney, and testes. Taken together, the analysis shows that DNA methylation changes are more sensitive than the corresponding tumor incidences; thus, DNA methylation could potentially be considered in determining a point of departure in human health risk assessment of potential carcinogens. In addition, the exposure duration in all of the epigenetic studies (except for hydrazine) was shorter suggesting that epigenetic studies may be more time- and cost-efficient compared to a 2-year carcinogenicity study. However, more research on epigenetic dose-response analysis and evaluation of additional epigenetic endpoints are needed to continue to ascertain how epigenetic data can be applied in human health risk assessment. [The views expressed in this abstract are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.]
Combination of Maternal Inflammation and Neonatal Hyperoxia is Associated with Microgliosis and Impaired Early Motor Development

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Background: Infants born prematurely are at high risk of neurodevelopmental impairment. Maternal inflammation is a risk factor for premature delivery, and those infants born prematurely often require supplemental oxygen therapy for survival. This combination of exposures occurs commonly in neonatal intensive care units and the effects are not clearly understood.

Objective: To develop a mouse model of these clinical conditions and investigate the effects on the developing brain.

Methods: Pregnant C3H/HeN mice were injected on embryonic day (E16) with 80μg/kg of lipopolysaccharide (LPS) or saline vehicle. Newborn pups were then exposed to room air (RA) or 85% oxygen (O2) for 14 days. Immunohistochemical analysis of the microglial population (Ionized binding calcium adaptor protein 1, Iba1) was performed in the cortex and hippocampal regions at 14 and 28 days of life. Early motor development was assessed by surface righting (day 1-4) and negative geotaxis testing (day 5-7).

Results: At postnatal day 14 pups in the combined LPS/O2 exposure group displayed an increase in microglial cell numbers when compared to Saline/RA (control), Saline/O2, and LPS/RA in both the cerebral cortex (p<0.02) and hippocampus (p<0.02). These differences did not persist at day 28, after a 14 day period of room air recovery. Functionally, behavioral analyses suggest that pups exposed to O2 (Saline/O2 and LPS/O2) were less able to successfully complete surface righting testing (p=0.03) and showed an increased latency to turn in negative geotaxis testing (p=0.03) without a significant effect of maternal LPS exposure.

Conclusions: Combined LPS/O2 exposure is associated with structural and functional changes in mouse brain development. This animal model provides a platform for further investigation of the mechanisms of preterm perinatal brain injury and a possible tool for identification of novel therapeutic strategies to improve neurodevelopmental outcomes for premature infants.
Sex-specific expression alterations of Alzheimer’s disease associated genes in young and aged zebrafish (Danio rerio) brains during the aging process and with a developmental lead exposure

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Environmental lead (Pb) exposure is a public health concern due to the adverse effects on neurological functions in adults and children. Recent studies also suggest a potential role of a developmental Pb exposure in the pathogenesis of Alzheimer’s disease (AD) with the presence of pathological hallmarks of AD and expression alterations in genes related to amyloid beta production in aged rodent and non-human primate brains. To identify the association between early-life Pb exposure and AD pathogenesis, zebrafish embryos at 1-2 hours post fertilization (hpf) were collected and exposed to a control treatment or 100 ppb of Pb through the end of embryogenesis (72 hpf). Larvae were then rinsed and reared until 3 months (young) or 12 months (aged) after fertilization for sex- and age-dependent quantitative gene expression analyses. Seven genes including amyloid beta precursor protein (appa and appb), presenilin (psen1 and psen2), apolipoprotein E (apoea and apoeb), and sortilin-related receptor L (sorl1) were analyzed using quantitative polymerase chain reaction. Sex-specific expression of the seven genes were characterized in brains from control young versus aged, control young versus Pb treated young, or control aged versus Pb treated aged zebrafish. There was a significant quantitative gene expression difference between young and aged control brains, exhibiting overexpression of sorl1 in both aged males and females as well as up-regulation of appa in aged females only. These results suggest that sex-specific and non-sex-specific alterations of AD-related gene expression occur during the natural aging process in zebrafish. In aged males, a significant down-regulation of apoeb was observed in Pb exposed groups compared to controls, while no quantitative differences were exhibited in other groups analyzed. Overall while a significant difference in expression was observed for sorl1 in both sexes and for appa in females during the natural aging process, a significant difference in expression was only observed for apoeb in aged males developmentally exposed to Pb. Further characterization of gene expression alterations induced by an early-life Pb exposure in the zebrafish brain will give insight into understanding impacts of developmental Pb exposure on later in life neurodegenerative disease pathogenesis.
Fine particulate matter-induced cardiac endothelium insulin resistance, ER-stress as a mechanism?

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Epidemiological studies have shown that exposure to airborne fine particulate matter (PM$_{2.5}$) increases the risk for cardiovascular disease (CVD) and Type-2 diabetes (T2D). However, the mechanism by which exposure to PM$_{2.5}$ increases the risk for the cardiometabolic syndrome remains unclear. Hence, we investigated PM$_{2.5}$-induced changes in cardiac insulin sensitivity and the induction of endoplasmic reticulum stress (ER stress) in a mouse model of diet induced obesity.

Adult C57BL/6 mice were fed low (LFD) - or high-fat diet (HFD) for 4 weeks prior to and during the 30 day exposure to air or fine concentrated ambient particulate matter (CAP). Systemic and cardiac-specific insulin resistance was measured, and the activation of the unfolded protein response (UPR) was investigated in the heart.

CAP exposure for 30 days exacerbated HFD-induced systemic insulin resistance without increasing adiposity; however, CAP-exposure alone had no effect on either outcome. CAP exposure for 30 days impaired endothelial insulin signaling in the heart of LFD-fed mice as measured by the phosphorylation of eNOS, while no changes in cardiac insulin-induced Akt phosphorylation were found. Impaired cardiac endothelium insulin signaling was accompanied by an increased activation of the unfolded protein response (UPR) indicating that the induction of ER stress may be potential mechanism.

Fine particulate air pollution is known to increase CVD risk especially ischemic heart disease, and our data indicate that CAP exposure selectively impairs insulin sensitivity in the cardiac endothelium – a change accompanied by the induction of ER stress, a pathway which may contribute to the development of the cardiometabolic syndrome.
FGF21 deficiency exacerbates chronic alcohol induced fatty liver disease

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Alcohol-induced hepatic fat accumulation, or alcoholic fatty liver disease (AFLD), is considered as the earliest pathological alteration in ALD from hepatic steatosis. AFLD could be developed to more harmful stages of liver injury, which include inflammation, fibrosis, cirrhosis and even malignancy. Clinical and experimental studies demonstrated that inhibition of fat accumulation (in situ lipogenesis) and promotion of fat clearance (β-oxidation) prevents hepatic steatosis and slows or halts the progression of ALD. Previous studies have identified extrahepatic hormones, such as pancreas-produced insulin and glucagon, are important in alcohol-induced alteration in liver lipid metabolism, little is known whether the paracrine and endocrine signal for metabolic regulation of hepatic itself participates in alcohol exposure associated lipid accumulation. Fibroblast growth factor 21 (FGF21) is a member of the endocrine FGF subfamily and a major metabolic regulator. Previous studies have demonstrated that FGF21 is hepatokine and plays a critical role in the glucose and lipid metabolism in obese and diabetic patients and experimental animals. Here we demonstrated that FGF21 deficiency exacerbated chronic alcohol-induced AFLD. Global FGF21 knockout mice and their controls were fed Lieber deCarli diet containing 5% alcohol or pair-fed isocaloric diet for 4 weeks. Alcohol feeding increased hepatic fatty acid and triglyceride contents and fat accumulation measured by H&E staining of liver section. There was no obvious alteration in hepatic fat accumulation in FGF21 deficiency mice under pair feeding. However, a markedly increased hepatic fat was detected in FGF21 knockout mice when fed chronic alcohol. Further studies showed that FGF21 deficiency exacerbated alcohol-induced liver injury assessed by liver enzyme and inflammation measurement. The FGF21 exacerbated-fat accumulation was associated with upregulation of the genes involved in fatty acid de novo synthesis, such as fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD1), and the decrease in the gene expression responsible for fatty acid β-oxidation, such as carnitine-palmitoyltransferase 1 (Cpt-1). Mechanistic studies showed that FGF21 knockout decreased SIRT1 which deacetylates SREBP-1c. In addition, we showed that FGF21 deficiency also decreased LKB1 which activates AMPK. The downregulation of SIRT1 and LKB1/AMPK in FGF21 deficient mice exposed to chronic alcohol leads to SREBP-1c- and PGC-1α -mediated changes of fatty acid synthesis and fatty acid β-oxidation. In conclusion, FGF21 seems to be required for cellular defense against hepatic lipogenesis and cellular capacity for fat clearance in subjects exposed to chronic alcohol.
Metabolic Vulnerability of Frontal Cortex to Occupational Mn Exposure

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Chronic manganese (Mn) exposure can lead to cognitive, psychiatric and motor deficits. Historically it is believed that the basal ganglia was the main target of Mn neurotoxicity. Recently, the frontal cortex has been shown to have increased α-synuclein aggregation and to be associated with impaired visuospatial learning in Mn-exposed non-human primates. However, there is a lack of studies investigating metabolic changes in frontal cortex compared to other brain regions in occupational Mn-exposure in humans.

To date 15 Mn-exposed welders and 11 healthy controls were recruited from a truck-trailer company in Indiana. Each subject underwent magnetic resonance imaging (MRI) and spectroscopy (MRS) exams, the motor part of the Unified Parkinson’s Disease Rating Scale and a series of neuropsychological and motor exams. Short-echo-time 1H MRS spectra were acquired from four volumes of interest: the frontal cortex, motor cortex, striatum and thalamus. A number of metabolites were quantified and compared from each region, including myo-inositol (mI, a glial marker), N-acetyl aspartate (NAA, a neuronal marker) and the sum of NAA and N-acetylaspartylglutamic acid (denoted as tNAA), the sum of the major excitatory neurotransmitter glutamate and its precursor glutamine (denoted as Glx), the sum of glycerophosphocholine and phosphocholine (denoted as tCho). All metabolites are expressed as ratio over creatine (Cr).

Our preliminary results revealed that in welders frontal cortex showed the most metabolic changes: a 22.1% decrease in mI/Cr (Mean±S.D.: 0.89±0.21 v.s. 1.14±0.11, p=0.09), an 11.8% decrease in tCho/Cr (Mean±S.D.: 0.31±0.04 v.s. 0.35±0.06, p=0.06), a 10.5% decrease in tNAA/Cr (Mean±S.D.: 1.31±0.26 v.s. 1.46±0.10) and a 7.1% decrease in Glx/Cr (Mean±S.D.: 2.48±0.46 v.s. 2.67±0.58), whereas no metabolite in other regions showed any changes to the same extents. Moreover, decreasing frontal Glx/Cr significantly correlated with increasing dominant-hand Grooved Pegboard test time (R=-0.803, p<0.01), indicating worse executive function and fine motor performance. In addition, decreasing Glx/Cr in the motor cortex significantly correlated with increasing Parallel Lines test score, indicating more severe tremor.

Overall our results suggest that frontal cortex may be especially vulnerable to Mn-caused neuronal and glial damage compared to the motor cortex, thalamus and striatum. The relationship between cortical Glx/Cr and motor tests may be due to impaired glutamate-glutamine cycling involved in the motor control pathway, which further indicates that cortical Glx/Cr may work as a biomarker of Mn-caused early motor deficits.
Brain GABA Levels Correlate with Manganese Exposure in Welders

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Introduction Excessive manganese (Mn) exposure has been associated with a movement disorder known as manganism, a neurodegenerative disease with similar symptoms as Parkinson’s disease. Our previous study in a cohort of highly Mn-exposed Chinese workers found that thalamic γ-aminobutyric acid (GABA) levels were significantly elevated (Dydak et al., 2011). The purpose of the current study is to a) verify whether similar changes in GABA levels can be found in a typical US occupational setting and b) to explore the relationship between brain GABA levels in vivo measured by magnetic resonance spectroscopy (MRS) and estimates of personal exposure to Mn exposure levels.

Methods Thirteen welders and eleven controls were recruited from a local semi-truck trailer manufacturer in Indiana. The subjects underwent personal air sampling and filled out a questionnaire, which encompassed questions about their current job exposure, past job exposures, and exposures arising outside their job, including the amount of time spent welding, the type of welding, base metal type, exhaust use, respirator use, and the amount of space which they welded in. The responses to the questionnaire determined modifying factors which were multiplied with the results from the air sampling to calculate each subject’s personal exposure to respirable Mn (i.e. consisting of particles <4 μm in aerodynamic diameter). All subjects underwent an MRI examination on a 3T GE Signa MRI scanner. GABA MRS data was acquired by the MEGA-PRESS technique (TE = 68 ms, TR = 2 s, 196 averages). The volume of interest (25×30×25 mm³) was centered on the thalamus. Spectra were analyzed and GABA levels were quantified using a spectral fitting tool, LCModel.

Results The exposure level of recruited welders was determined to have an average airborne concentration of respirable Mn of 0.104 mg/m³. A significant difference in thalamic GABA levels between welders and controls was found [welders: 2.45±0.68 mM, controls: 1.40±0.45 mM, p<0.001], confirming our previous study in China. Increasing thalamic GABA levels significantly correlated with average exposure estimated for the past three months before the MRI exam (R=0.649, p<0.05), but not for longer past exposure periods (p>0.05).

Conclusion Even in occupational settings with low Mn exposure levels (<0.2 mg/m³), we confirmed our previous findings of increased thalamic GABA levels in Mn-exposed workers. The results further show that thalamic GABA levels reflect exposure to Mn during the last three months, rather than long-term cumulative exposure. These preliminary findings are of high interest for the evaluation of GABA as a biomarker of effect for Mn exposure.
The prebiotic oligofructose protects against liver injury caused by arsenic in a model of NASH

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Background. Arsenic (As), a ubiquitous drinking water contaminant, tops the ATSDR list of hazardous environmental chemicals and is known to cause liver injury. Although the concentrations of As found in the US water supply are generally too low to directly damage the liver, this group recently showed that subhepatotoxic doses of As sensitize the liver to experimental NAFLD caused by a high fat diet (HFD). It is now strongly suspected that an altered GI tract microbiome plays an important role in development of NALFD. Indeed, prebiotics (oligofructose; OFC), protect against experimental NAFLD, and correlate with repletion of commensal bacteria (e.g., Bifidobacteria spp.) in the GI tract. Arsenic has been shown to be bacteriostatic in vitro, and may therefore also affect GI tract bacteria. The purpose of the current study was to determine the effect of As exposure on key commensal bacteria in the GI tract, and to test the hypothesis that the OFC protects against enhanced liver injury caused by As in experimental NAFLD. Methods. Male C57BL6/J mice were fed low fat diet (LFD), high fat diet (HFD) or HFD containing oligofructose (OFC) during concomitant exposure to either tap water or As-containing water (4.9 ppm as sodium arsenite) for 10 wks. Abundance of select commensal bacteria in cecal content was determined by qPCR.

Results. HFD significantly increased body mass and caused fatty liver injury, as characterized by an increased liver weight-to-body weight ratio, histologic changes and transaminases. As synergistically enhanced HFD-induced liver damage, and was characterized by enhanced inflammation. In line with previous studies, HFD and As alone both altered content of cecal bacteria, and both decreased the abundance of Bifidobacterium spp. OFC supplementation protected against this effect of HFD and As on the abundance of Bifidobacterium spp. OFC supplementation also protected against the enhanced liver damage caused by the combination of HFD and As; indeed liver injury in this group was even improved compared to animals receiving HFD alone.

Conclusions. These data support previous findings that low levels of As enhance liver damage caused by high fat diet, suggesting that As exposure may be an underlying factor that increases the risk of obesity-induced liver disease. Furthermore, these results indicate that these effects of arsenic may be mediated, at least in part, by GI tract dysbiosis and that prebiotic supplementation may confer significant protective effects. Together, these data support our hypothesis that As-mediated changes in the GI tract flora play an important factor in sensitizing the liver to injury.
ALCOHOLIC LIVER CIRRHOSIS AND ABNORMAL IMMUNE RESPONSE TO ANTIGEN CHALLENGE: DATA FROM THE ZINC IN ALCOHOLIC CIRRHOSIS (ZAC) CLINICAL TRIAL

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Purpose: Immune dysfunction contributes to liver disease progression and infection risk in alcoholic cirrhosis (AC). The purpose of the study is to better characterize liver injury biomarkers, insulin resistance/adipokines, and immune function in subjects enrolled in an NIH-funded, placebo-controlled, clinical trial of zinc sulfate for alcoholic cirrhosis (ZAC).

Methods: Baseline data and fasting blood samples of 17 consenting subjects with (Child-Pugh class A or B) AC were evaluated and compared to 8 non-drinking, healthy controls. Plasma adipokines and whole blood ex vivo lipopolysaccharide-stimulated (LPS) and phytohemagglutinin-stimulated (PHA) cytokine production were measured by Luminex. Plasma cytokeratin 18 (CK18, M30 and M65) were measured by ELISA. Differences between the means (AC vs. controls) were evaluated by t-test using GraphPad-Prism and statistical significance was set at p<0.05.

Results: The mean age (55.0±10.1 years) and BMI (26.2±3.9 kg/m2) in AC were similar to controls. The mean Child-Pugh and MELD scores in AC were (6.0±1.4 and 9.0±3.5). 6 AC subjects were still drinking alcohol and 3 had type 2 diabetes. Mean plasma CK18 M30 and M65 were significantly increased in AC compared to controls (p<0.05). Mean insulin levels were significantly increased in AC (p<0.05) while mean glucose levels were similar. There were non-significant trends towards higher adiponectin, leptin, PAI-1, and resistin in AC. Un-stimulated whole blood ex vivo production of IL-6, IL-8, IL-10, and TNF-α were significantly increased in AC (p<0.05). Mean un-stimulated IL-1β, IL-2, IL-4, IL-17a, MCP-1, and IFN-γ were not significantly different. Mean LPS-stimulated cytokine production of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17a, TNF-α, MCP-1, and IFN-γ were not significantly different between groups. Mean PHA-stimulated cytokine production of IL-1β, IL-6, IL-10, and TNF-α were significantly decreased in AC (p<0.05). PHA-stimulated IL-2, IL-4, IL-17a, MCP-1, and IFN-γ were not different.

Conclusions: The first 17 subjects in the ZAC trial had increased CK18, insulin resistance, and immune dysfunction. Un-stimulated IL-6, 8, 10 and TNF-α were increased in AC. LPS stimulation induced cytokine production to a similar degree in AC and controls indicating an absence of priming in AC. PHA stimulation failed to induce production of IL-1β, IL-6, IL-10, and TNF-α in AC, but not controls, suggesting abnormal T-cell function. The potential of zinc therapy to correct these biomarkers will be evaluated in the ZAC Trial.

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Altered Neurotransmitter Levels in Rats Subchronically Exposed to Manganese: Relevance to Dopaminergic Dysfunction and Neurodegeneration.

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Manganese (Mn) is an essential nutrient, important to public health. Excessive occupational and environmental exposure to it can cause neurotoxicity, producing symptoms similar, but distinguishable from those observed in idiopathic Parkinson’s disease (IPD). Mn exposure may have direct effects on neurons and glia in the central nervous system, for instance by oxidizing dopamine (DA) and other catecholamines; it may also disrupt neurotransmitter metabolism, particularly in the nigrostriatal pathway, producing alterations in neurotransmitter levels. We used an *in vivo* rat model to examine changes in neurochemistry induced by subchronic Mn exposure. Adult male rats received IP injections of 6mg/kg or 15mg/kg Mn (as MnCl2) as the low and high dose groups, respectively, or saline as the control, 5 days per week for 4 weeks. Animals were sacrificed 24hr after the last injection and select brain regions, i.e., striatum (ST), hippocampus (HP) and substantia nigra (SN), were dissected and quantified for neurotransmitter contents of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), 5-hydroxyindolacetic acid (5-HIAA), and γ-aminobutyric acid (GABA) by HPLC with electrochemical detection. Data analysis by one-way ANOVA with Tukey’s post hoc comparisons revealed statistically significant differences in the neurotransmitter content of these three brain regions between control, low, and high dose groups. Most significantly, the level of DA and its metabolites were significantly higher in the ST and the DA turnover was significantly higher in SN in Mn-exposed animals than controls. GABA levels were significantly higher in HP in Mn-exposed animals than controls. Neurotransmitter differences seen in the striatum may be of particular relevance because alterations could lead to heightened sensitivity to additional insults, particularly due to oxidative modifications of DA. This study provides evidence that dopaminergic neurotransmission is especially sensitive to Mn intoxication. (Supported in part by NIEHS RO1-ES008164; ROO-ES019879)
Sinusoidal endothelial cell-derived extracellular matrix regulates basal and stimulated macrophage activation

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Background. Fatty liver disease, be it from alcohol (ALD), obesity (NAFLD) or other sources (e.g. viral infection), involves chronic inflammation, although the mechanism(s) are unclear. One potential mechanism of chronic hepatic inflammation is crosstalk between the extracellular matrix (ECM) of hepatic sinusoidal endothelial cells (SEC) and resident macrophages. Here, this hypothesis was tested in vitro using cultured SECs and macrophages.

Methods. Transformed hepatic sinusoidal endothelial cells (TSECs) were cultured for 72 hours. Culture plates were then washed with a solution that selectively removed the cells, but preserved the ECM. Cultured macrophages (RAW 264.7 cells) were seeded on the matrix and cultured for 24 hours; then stimulated with LPS for 0, 3, 6, 12, or 24 hours (10 or 100 ng/mL). Real time RT-PCR was used to measure mRNA expression of proinflammatory (IL-6, IL-1β, TNF-α, and INOS) and anti-inflammatory mediators (IL-10 and TGF-β).

Results. LPS stimulated production of all mediators by macrophages. With 100 ng/mL LPS, expression of IL-6, IL-1β, and IL-10 was attenuated by TSEC ECM, whereas expression of TNF-α, INOS, and TGF-β increased. Interestingly, TSEC ECM effect on the response to lower dose LPS (10 ng/ml) tended to be opposite to that observed with the higher dose. Experiments with the integrin inhibitor, CycloRGDfV, indicated some effects may be mediated via TSEC ECM binding to integrin receptors.

Conclusions. These data serve as first proof-of-concept that macrophage activation can be modulated by TSEC-derived ECM and identifies a new interaction between these cells that may contribute to inflammatory liver disease.

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Expression of glyoxylase 1 (glo1) throughout zebrafish embryonic development and alterations following atrazine exposure

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Atrazine, a commonly used herbicide in the Midwest, is an endocrine disruptor and a suspected carcinogen. Although atrazine was recently banned by the European Union for widespread contamination risks in potable water supplies, this herbicide is still commonly used in the United States with a current maximum contaminant level (MCL) of 3 ppb in drinking water. The health risks associated with this MCL are currently being reviewed by the Environmental Protection Agency; however, the mechanisms of atrazine toxicity are not well defined. In this study transcriptomics was first used to identify genes with altered expression following exposure to 0, 0.3, 3, or 30 ppb atrazine during embryogenesis with the zebrafish model system. This analysis showed that expression alterations were enriched with genes associated with neuroendocrine development and function, cell cycle regulation, and carcinogenesis. From this list of genes, glyoxylase 1 (glo1) was targeted for further study. glo1 is part of the glyoxylase system which converts methylglyoxal to S-D-lactoylglutathione. Upregulation of glo1 is linked to cell proliferation and is associated with various cancers in humans. To further our understanding of this genetic target, expression was analyzed at five developmental time points (24, 36, 48, 60, and 72 hpf). in situ hybridization was used to characterize spatial gene expression of glo1 throughout development under normal conditions and after exposure to 0, 0.3, 3, or 30 ppb atrazine. Quantitative PCR (qPCR) will then be applied to determine gene expression levels of glo1 throughout development. This data is furthering our understanding of glo1 expression during development and alterations induced by embryonic atrazine exposure.
The Effects of Developmental Atrazine Exposure on miRNA-126 Expression in Zebrafish

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Atrazine is a commonly used herbicide in the United States that is reported to frequently contaminate drinking water sources. Studies have indicated atrazine to adversely impact the neuroendocrine and reproductive systems and to be a potential carcinogen. The current maximum contaminant level for atrazine in drinking water set by the US Environmental Protection Agency is 3 parts per billion (ppb); however, levels higher than 3 ppb are often reported. Ongoing studies in our laboratory are investigating the immediate and latent adverse health outcomes associated with a developmental atrazine exposure and identifying the genetic and epigenetic mechanisms of atrazine toxicity using the zebrafish model system. MicroRNAs (miRNAs) are epigenetic regulators that post-transcriptionally control the translation of mRNA. To identify if an embryonic atrazine exposure would alter miRNA expression, a unique microarray platform containing all known zebrafish and human miRNAs was designed. Zebrafish embryos were exposed to environmentally relevant doses of atrazine (0, 0.3, 3, or 30 ppb) through 72 hours post fertilization (hpf), and miRNA expression was analyzed using the microarray platform. Expression of 18 zebrafish and 9 human miRNAs were significantly altered in response to atrazine exposure. One of the more robustly changed miRNAs was miR-126, a miRNA associated with angiogenesis and tumorigenesis. To further investigate the developmental expression of miR-126 and its deregulation by atrazine exposure, quantitative PCR (qPCR) was first used to profile expression throughout embryogenesis in control conditions at 12, 24, 36, 48, 60, and 72 hpf. Expression of miR-126 was developmental time point specific with an increase in expression after 12 hpf and peak in expression at 36 hpf. After a decrease in expression at 48 hpf, expression levels were significantly higher at 60 and 72 hpf. Deregulation of miR-126 following exposure to 0, 0.3, 3, or 30 ppb atrazine at all six developmental time points is now being analyzed to further our understanding on the impacts of atrazine exposure on miR-126 expression.
Preventive effect of non-mitogenic acidic fibroblast growth factor on diabetes-induced testicular cell death and atrophy

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Fibroblast growth factor (FGF) family members in previous studies were shown to prevent various oxidative stress-induced cardiac, neuronal and retinal inquires. Particularly, FGF-1 was found to protect the heart from ischemia-, doxorubicin- and diabetes-induced damage, suggesting the potential clinical application. However, long-term use of FGF-1 was restricted for its potential risk of carcinogenesis due to its non-specific proliferating activity. Thus a cluster of amino acids responsible for proliferation were deleted in the native FGF-1 to create a modified non-mitogenic FGF-1 (mFGF-1). The present study tested if the mFGF-1 protects male germ cells from diabetes-induced apoptotic cell death and testicular atrophy. Mice were treated with multiple low-doses of streptozotocin (STZ) to induce type-1 diabetes and then treated with mFGF-1 for 6 months. Diabetic mice showed a significant decrease in testicular weight and an increase in apoptotic cell death (detected by TUNEL staining). Treatment with mFGF-1 significantly alleviated diabetic effects on testicular weight and apoptotic cell death. Mechanistically, the diabetes induces, predominantly, the mitochondrial apoptotic pathway since there was a significant increase in BAX/Bcl-2 ratio, determined with Western blotting assay. Diabetes also induced mild increases in endoplasmic reticulum stress and associated cell death, reflected by increases in cleaved-caspase 12, CHOP, and GRP-78 expression, along with a significant increase in testicular TNF-alpha expression as an index of inflammation. All these effects induced by diabetes were significantly attenuated by treatment with mFGF-1. Therefore, these results suggest that mFGF-1 is able to prevent the apoptotic effect of diabetes on the testis via the mitochondrial pathway predominantly, resulting in a significant prevention of diabetes-induced testicular atrophy.
Assessing Genetic Susceptibility to Motor Function Deficits Following Developmental Exposure to Polychlorinated Biphenyls

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Polychlorinated biphenyls (PCBs) are ubiquitous industrial chemicals banned in the 1970s due to their wide-ranging toxicity. Children exposed in utero and lactationally have a higher risk of learning, memory and behavioral deficits. The goal of our research is to identify genes which affect susceptibility to these pollutants. The enzyme CYP1A2 can sequester toxicants such as dioxins and coplanar PCBs. The aryl hydrocarbon receptor (AHR) binds dioxins and coplanar PCBs, initiating transcription of several genes including CYP1A2. CYP1A2 is a member of the cytochrome P450 superfamily and a key detoxifying enzyme. Though it is also reportedly expressed in the cortex and cerebellum of the brain, its physiological function in the brain remains unknown. Previous work in our lab uncovered motor deficits in Cyp1a2(-/-) knockout mice. Our current work compares AhrbCyp1a2(-/-), AhrbCyp1a2(-/-), and AhrbCyp1a2(+/+) mice exposed during gestation and lactation to PCBs or the corn oil vehicle. Pregnant dams were treated from gestational day 0 (GD0) to postnatal day 25 (PND 25). We compared the three different genotypes of mice using a battery of six tests: rotarod, gait analysis, adhesive removal, pole climbing, balance beam, and grip strength. We used this comprehensive motor battery to pinpoint the brain regions affected, specifically the cerebellum and nigrostriatal pathways. Our preliminary analysis found a significant main effect of treatment on three of five days of rotarod acceleration testing (P< 0.05) and a significant gene x treatment interaction on Day 5 with PCB-treated AhrbCyp1a2(-/-) mice showing the greatest impairments. There was a significant main effect of genotype and treatment in gait analysis with PCB-treated animals and AhrbCyp1a2(-/-) mice having significantly longer stride lengths (P<0.05). There were no significant differences in the pole test (P>0.05). In the balance beam test, both Cyp1a2(-/-) knockouts had significantly shorter latencies to traverse the beam (P<0.001). Further analysis will be required to determine if this indicates a difference in motor function or greater motivation to reach the safety of the darkened goal box.
Role of Renal Biotransformation in 1,2,3-Trichloro-4-nitrobenzene Induced Nephrotoxicity In Vitro

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Chloronitrobenzenes (CNBs) have been used as chemical intermediates to manufacture a wide variety of compounds. Previous studies have shown that CNBs are directly nephrotoxic using a male Fischer 344 renal cortical slice model. Of the CNBs, 1,2,3-trichloro-4-nitrobenzene (1,2,3-TC4NB) proved to have the greatest nephrotoxic potential. This study was designed to explore the effects of antioxidants and inhibitors of renal biotransformation systems on 1,2,3-TC4NB induced nephrotoxicity using isolated renal cortical cells (IRCC) from male Fischer 344 rats. To determine 1,2,3-TC4NB nephrotoxic potential, IRCC (4 x 10^6 cells/ml) were treated with 1,2,3-TC4NB (0 or 1 mM) and incubated for 60 minutes. Lactate dehydrogenase release was used to measure cytotoxicity. In subsequent experiments, IRCCs were pretreated with antioxidants [glutathione (1.0mM), α-tocopherol (1.0mM), N-acetyl-L-cysteine (2.0mM) or ascorbate (2.0mM)], cytochrome P450 (CYP) inhibitor [piperonyl butoxide, isoniazid, or metyrapone (1.0mM)], a FMO inhibitor [N-octylamine (0.2mM) or methimazole (1.0mM)], a peroxidase inhibitor [mercaptosuccinate (0.1mM)], or a cyclooxygenase inhibitor [indomethacin (1.0mM)]. Following pretreatment with N-octylamine, methimazole, metyrapone, mercaptosuccinate, indomethacin, and all antioxidants significantly attenuated toxicity. These results suggest that free radical formation and oxidative stress play role in 1,2,3-TC4NB nephrotoxicity. These results also suggest that free radicals may result from multiple biotransformation pathways/intermediates. (Supported in part by NIH Grant 5P20RR016477 to the West Virginia IDeA Network for Biomedical Research Excellence)
Apelin regulation of potassium chloride cotransport (KCC) in vascular smooth muscle cells (VSMCs): Relation to Cardiovascular Disease (CVD)

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Apelin, a potent inodilator, uses the NO pathway to elicit its anti-atherogenic effect, and the PI3K/Akt and MAPK pathways to induce proliferation and migration of VSMCs. The NO pathway is predominant in healthy blood vessels and contractile VSMCs, whereas, the PI3K/Akt/MAPK pathways are prominent in diseased blood vessels and secretory VSMCs. VSMCs participate in atherosclerotic lesions due to their capacity to migrate and proliferate. Oxidized plasma cholesterol (oxLDL) is a detrimental factor in atheroma formation. It is known that KCC uses the same regulatory pathways (Adragna et al. 2006) as apelin and is implicated in CVD. Here, we tested the hypothesis that apelin through its APJ receptor signaling pathways regulates KCC. Expression and distribution patterns of contractile proteins were studied by Western blot and immunofluorescence. KCC activity was measured by atomic absorption spectrophotometry with Rb as K congener ± inhibitors of the aforementioned signaling pathways and by blocking other K⁺ transport mechanisms. The APJ receptor and key components of the signaling pathways were verified immunologically. VSMCs' identity was established by appropriate markers. Apelin stimulated KCC activity through the NO pathway by 335 % and by 142 % through the MAPK/PI3K pathways. In contrast, oxLDL inhibited baseline KCC in contractile VSMCs, and this inhibition was restored by apelin. These findings prove the hypothesis that apelin regulates KCC activity through the aforementioned pathways, are relevant to CVD and have potential as therapeutic targets. Supported by WSU Foundation.
Interactions between Nitrosamines and Cadmium Enhance Carcinogenic Potential of Cigarette Smoke

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Thousands of chemicals have been identified in cigarette smoke; many of these have been classified as human carcinogens. Cadmium (Cd) and nicotine-derived nitrosamine ketone (NNK) have been accredited, in part, to the development of lung cancer in smokers. Interactions between carcinogens present in cigarette smoke remain largely uncharacterized and represent a novel approach to understanding cancer initiation and promotion. A combination of mechanisms including altered signaling pathways, direct DNA damage, and dysfunctional DNA repair are likely to play a role in this transformation. Preliminary experiments confirm a synergistic induction of proliferation and anchorage independent growth assessed by tetrazolium-based (MTS) proliferation assays and the ability of 3T3 cells to form colonies in soft agar, respectively. Besides providing a novel mechanistic approach to understanding lung cancer initiation, we propose a unique solution in the lunasin peptide, which has proven chemopreventative properties. In this study, we confirm that pretreatment with lunasin will inhibit the transformation of 3T3 cells induced by NNK/Cd coexposure. Furthermore, we will use lunasin as a tool to probe the diverse genomic alterations stimulated by cadmium and nitrosamines. For a disease such as lung cancer, innovative and practical approaches are necessary to unlock key information about the illness as well as enhance its therapeutic options. This study provides a fundamental assessment of cancer initiation based on exposure to potent carcinogens present in tobacco smoke and how they interact to enhance each other's carcinogenic potential.
TCDD-induced activation of the human immunoglobulin hs1,2 enhancer is not altered by mutation of transcription factor binding sites within the polymorphic region

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The environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent suppressor of immunoglobulin (Ig) expression and antibody secretion in various animal models. This effect appears to be mediated through the aryl hydrocarbon receptor (AhR), a cytosolic receptor that when activated by ligand translocates to the nucleus and binds to dioxin response elements (DRE) leading to altered transcriptional activity. The expression of Ig is partially controlled by the 3' IgH regulatory region (3'IghRR), which contains four DNase I hypersensitive sites (i.e. hs3A; hs1,2; hs3B; hs4) that exhibit enhancer activity. Our previous results have identified a DRE site and TCDD-induced binding of the AhR to this site within both the hs1,2 and hs4 enhancers. Additionally, we demonstrated a sensitive inhibition of 3'IghRR activity by TCDD that correlated well with TCDD-induced inhibition of IgH expression and antibody secretion. Current efforts are focused on translating these results to the human IgH gene.

Although the 3'IghRR and many of the transcription factor binding sites including the DRE are fairly well conserved in the human IgH gene, there are some notable differences. For example the human hs1,2 (hu-hs1,2) is polymorphic resulting in a ~53 bp invariant sequence that can be repeated up to four times. Interestingly, an increased number of the invariant sequence, which contains a DRE, NF-κB and AP-1 site, has been associated with several autoimmune diseases. Since TCDD influences protein binding to these sites, it may impact disease states associated with the polymorphic hu-hs1,2. Indeed, we have identified TCDD-induced activation of the hu-hs1,2 enhancer and a marked decrease in hu-hs1,2 enhancer activity with deletion of the invariant sequence. These results contrasted with a markedly inhibitory effect of TCDD on mouse hs1,2 enhancer activation, suggesting species differences. The objective of the current study was to utilize mutational analysis and luciferase reporter constructs to evaluate the contribution of each transcription factor binding site to human hs1,2 activity and modulation by TCDD. Interestingly, TCDD induced a similar fold-induction in hu-hs1,2 reporter activity regardless of which transcription factor was mutated. Surprisingly, mutation of individual binding sites within the invariant sequence (i.e. NF-κB, AP-1, and DRE) increased hs1,2 activity.

Evaluation of the binding sites 5' of the invariant sequence demonstrated an increase in hs1,2 activity when the Oct site was mutated; whereas, mutation of the AP-1/ETS binding site resulted in a marked decrease in hs1,2 activity. These results suggest a complex interaction of proteins binding within the hs1,2 enhancer and a consistent increase in hs1,2 activity by TCDD, which represents a large class of AhR ligands. A better understanding regarding the role of the polymorphic hu-hs1,2 enhancer in IgH expression and how TCDD modulates hu-hs1,2 activity may lead to insights into the etiology of immune disorders with a significant antibody component and to new therapeutic options. (Supported by NIEHS R01ES014676)
Fischer 344 rat strains congenic at arylamine N-acetyltransferase differ in breast
tumorigenesis induced by N-methylnitrourea.

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Arylamine N-acetyltransferase 1 (NAT1) is a well-known phase II metabolic enzyme that has
been associated with carcinogenesis. Its role in the biotransformation of aromatic and
heterocyclic amine carcinogens has been investigated for many years, but more recent
investigations focus on a possible endogenous role of NAT1 in cancer progression. We
conducted in vivo studies using homozygous F344 rats, congenic at the rat Nat2 locus for high
(rapid) and low (slow) activity. The rat Nat2 gene is a functional ortholog for the human NAT1
gene, and rat Nat2 has shown similar substrate specificity to human NAT1. Chemically induced
breast tumors are produced in the rat following administration of 1-methyl-1-nitrourea (MNU), an
alkylating agent similar to others found in cigarette smoke. In this experiment, rapid and slow
acetylator female congenic rats were administrated a single dose of MNU (50mg/kg) by
intraperitoneal injection at three weeks of age to mimic exposure of pre-pubescent females to
cigarette smoke. Weekly measurements of weights and palpable tumors were recorded.
Palpable tumors showed a significantly lower latency in rapid compared to slow acetylator
congenic rats (p=0.040). At 23 weeks post administration, rats were euthanized, and tumor and
adjacent non-tumor tissue were collected. Tumors were found in over half (78%) of the rapid
acetylator congenic rats with an average of 1.78 ± 0.7 tumors per rat. In contrast, tumors were
found in less than half (30%) of slow acetylator congenic rats with an average of 0.5 ± 0.3
tumors per rat. Both tumor multiplicity and incidence differed between rapid and slow acetylator
rats just missing significance (p=0.073 and 0.069, respectively) in this initial pilot experiment.
Pathology on the tumors classified the majority of the tumors as intraductal papillomas that
were estrogen receptor positive by immunohistochemistry, with one treated rapid acetylator rat
having a poorly differentiated malignancy. The results suggest an important role for NAT1 in
tumorigenesis. Further studies are needed to confirm and understand the mechanisms of NAT1
involvement in cancer.
Assessing motor function in *Cyp1a1(-/-)* mice using gait and pole tests

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While trying to characterize genetic susceptibility to developmental polychlorinated biphenyl (PCB) exposure, we found that even corn oil-treated control *Cyp1a2(-/-)* knockout mice showed impairments in motor function compared with wild type *Cyp1a2(+/+)* wild type mice. To follow up on that study, we obtained *Cyp1a1_Cyp1a2(-/-)* double knockout mice that lack CYP1A2 and a related enzyme CYP1A1. Compared to wild-type *Cyp1a1_Cyp1a2(+/+)* mice, *Cyp1a1_Cyp1a2(-/-)* double knockout mice showed motor deficits in rotarod acceleration testing, which suggests that CYP1A2 has a key role in cerebellar development or function. To assess the role of CYP1A1, we followed up this study by *Cyp1a1(-/-)* knockouts and *Cyp1a1(+/+)* wild type mice to see if there is an underlying motor deficit similar to that found in the *Cyp1a2(-/-)* knockouts. We used the same comprehensive test battery from our previous studies and here report our findings from the gait and pole test. Gait test and pole test identify deficits in the dopaminergic nigrostriatal pathways. For gait test, we trained mice to walk down a narrow alley way, then tested them on the third day after painting their hind paws with non-toxic paint. In the pole test, mice are placed facing upward on a 50cm pole and need to turn and climb down the pole to return to their home cage. Our preliminary analysis found no significant difference in stride length, but a highly significant difference in stride width (*P*<0.001) with *Cyp1a1(-/-)* mice having wider strides than wild type mice. There were no significant differences in the pole climb test. Together, the data suggest that there are no serious motor deficits in the nigrostriatal pathways of *Cyp1a1(-/-)* mice.
Resveratrol Protects Renal Tissue from Reactive Oxygen Species (ROS) Cytotoxicity

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Cisplatin is a cancer chemotherapeutic agent used for treatment of cervical, ovarian and non-small cell lung cancer. Nephrotoxicity is a serious adverse effect associated with cisplatin. The mechanism for cisplatin nephrotoxicity is not known but includes oxidative stress and generation of Reactive Oxygen Species (ROS). Previous work in our laboratory has shown that Resveratrol (RES) reduced cisplatin nephrotoxicity in a rodent model. The purpose of this study was to evaluate whether RES could reduce renal toxicity induced by the ROS species, hydrogen peroxide (H$_2$O$_2$) and furthermore, did RES protection oxidative stress enzyme activity from H$_2$O$_2$. Renal cortical slices were prepared from male Fischer 344 rat kidneys (n=4/group). Renal slices were equilibrated in oxygenated Krebs buffer and incubated for 30 min at 37°C with 0, 50 or 150 uM RES. Renal slices were then incubated for 30-120 min with 0, 10 or 20 mM H$_2$O$_2$. In some experiments, RES was rinsed from the tissue to test the hypothesis that RES protection was not mediated by an extracellular reaction with H$_2$O$_2$. Loss of membrane integrity was assessed by lactate dehydrogenase (LDH) leakage. Oxidative stress enzyme activity was assessed on renal tissue. H$_2$O$_2$ induced a concentration dependent increase in LDH leakage within 30 min in the absence of RES (p<0.05). A final concentration of 50 or 150 uM RES prevented LDH leakage by 10 and 20 mM H$_2$O$_2$. A 30 min incubation with RES (50 uM) followed by further incubation with H$_2$O$_2$ in the absence of RES also prevented a rise in LDH by H$_2$O$_2$. Catalase enzyme activity was not depressed by H$_2$O$_2$ or RES during the exposure period. In conclusion, RES can protect renal cortical tissue from ROS cellular toxicity. Further studies need to evaluate the mechanism for protection. (Supported by NIH Grants P20GM103434 and 5P20RR016477 to the West Virginia IDeA Network for Biomedical Research Excellence).
A reverse genetic screen identifies the putative multidrug resistance protein MRP-7 as an inhibitor of MeHg-associated animal toxicity and dopamine neurodegeneration

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Background: Methylmercury (MeHg) exposure from occupational, environmental, and food sources is a significant threat to public health. Recent epidemiological and vertebrate studies suggest that MeHg exposure may contribute to dopamine (DA) neuron vulnerability and the propensity to develop Parkinson’s disease (PD). We have developed a novel Caenorhabditis elegans (C. elegans) model of MeHg toxicity and have shown that low, chronic exposure confers embryonic defects, developmental delays, and DA neuron degeneration, and that the toxicity is partially dependent on the phase II antioxidant transcription factor SKN-1/Nrf2.

Aims/Objectives: In this study we asked what genes and molecular pathways are involved in MeHg-induced whole animal and DA neuron pathology. Methods: We utilized a reverse genetic screen, biochemical assays, immunofluorescence, transgenic C. elegans, RT-PCR, ICP-MS, Western analysis, and neuronal morphology analysis to characterize expression, localization and the role that SKN-1, MRP-7, and post-translational modifications play in MeHg-induced whole animal and DA neuronal death. Results: Over 18,500 genes were screened for whole animal sensitivity to MeHg, and 92 genes were identified (93% have strong human homologues) that affect whole animal and/or DA neuron pathology. These genes are strongly biased towards mechanisms that affect the mitochondria, transcription, apoptosis and calcium signaling. Here we report detailed analysis of a putative transporter. Specifically, genetic knockdown of MRP-7 results in 40% of animals showing DA neurodegeneration relative to 0% in WT animals following 500 nM MeHg exposure for 4 days. Genetic knockdown of transporter MRP-7 following high levels of MeHg exposure (10 μM) results in 0% viability, compared to 100% in WT animals. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) studies show that knockdown of MRP-7 results in 2-fold higher Hg levels relative to WT. We also provide evidence for cellular localization. Conclusions: This study describes a novel whole genome reverse genetic screen that has identified a number of molecular transporters and proteins involved in MeHg resistance that will likely prove useful in identifying therapeutic targets to inhibit MeHg-induced cellular toxicity in humans. Support: NIEHS ES014459 and ES003299 to RN; and EPA STAR Graduate Fellowship to NVD.
Aroclor 1260 Exposure Worsens Hepatic and Systemic Inflammation in an Animal Model of Diet-Induced Obesity and Non-alcoholic Fatty Liver Disease

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Purpose: Polychlorinated biphenyls (PCBs) are persistent organic pollutants associated with non-alcoholic fatty liver disease (NAFLD) in epidemiologic studies. Our previous work demonstrated that PCB 153 worsened diet-induced obesity (DIO) and hepatic steatosis in mice fed high fat diet (HFD) (PMID: 23618531). Because highly chlorinated PCB mixtures, rather than single congeners, have bio-accumulated in humans, the purpose of this study is to evaluate the effects of the commercial PCB mixture, Aroclor 1260 (Ar), in a mouse model of DIO and NAFLD.

Methods: Male C57BL/6 mice (8 weeks old, n=10) were fed either a control diet (CD, 10% kCal fat) or HFD (42% kCal fat). Ar (20 mg/kg or 200 mg/kg in corn oil) was administered by oral gavage. Body fat composition was measured by dексасаnning. Serum, liver and fat samples were taken for immunohistochemistry, RT-PCR, lipid analysis and adipocytokine measurements.

Results: In mice fed HFD, Ar co-exposures were associated with increased lean mass (20 mg/kg) and decreased body fat mass (200 mg/kg). Blood glucose/lipid levels and insulin resistance were higher in HFD groups, but this was not affected by Ar exposure. Mice fed HFD developed hepatic steatosis by histology and hepatic triglyceride measurement, but the degree of steatosis was not affected by Ar co-exposures. However, Ar worsened liver necro-inflammation in mice fed HFD. Serum AST and ALT levels were increased in HFD+Ar (20 mg/kg); and more inflammatory foci and hepatocellular death were observed histologically in HFD+Ar animals than HFD. In contrast, hepatocyte hypertrophy and karyomegaly without steatosis or necro-inflammation were noted in CD+Ar treated mice. Expression of several pro-inflammatory hepatic TLR4 target genes, including IL-6 and TNFα, were increased in HFD+Ar (20 mg/kg). Likewise, serum IL-6 and tPAI-1 levels were increased in HFD+Ar (20 mg/kg). Hepatic gene expression of cytochrome P450s including Cyp3a11 (PXR target gene) and Cyp2b10 (CAR target gene) was upregulated by Ar exposure in both CD and HFD-fed mice. Interestingly, Cyp1a2 (AhR target gene) was upregulated only in groups exposed to Ar (200 mg/kg) suggesting dose-dependent activation of this receptor. Hepatic expression of PPARα and LXR target genes was not affected by Ar exposure.

Conclusion: Aroclor 1260 worsened hepatic and systemic inflammation in the DIO model of NAFLD consistent with a transition from steatosis to steatohepatitis. While Aroclor 1260 did not worsen insulin resistance or hepatic steatosis, it was paradoxically associated with an increase in lean body mass at low-dose exposure. These effects may be driven by dose and congener-dependent receptor activation. Environmental pollution may be a relevant "second hit" in the transformation of steatosis to steatohepatitis.

Funding sources: This work is supported by the NIEHS grants 1R01ES021375 and T35ES14559 and the NIH grant K23AA018399.
Epidemiological and experimental animals studies suggest that exposure to arsenic exacerbates atherosclerosis; however the mechanisms by which arsenic exerts its atherogenic effects are not known. We observed that in cultured murine and human endothelial cells, sodium arsenite increased the surface expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin by 1.2-1.5-fold; leukocyte adhesion by 1.3-2.5-fold; leukocyte transmigration by 2.5-4.0-fold; and cytokine production by 27-150-fold. Sodium arsenite also activated the IRE-1 and ATF-6 arms of unfolded protein response (UPR). Knockdown of IRE-1 by siRNA and adenoviral transfection with ATF-6 prevented sodium arsenite -induced endothelial activation. Similarly, phenyl butyric acid (PBA), a chemical chaperone of protein folding, also prevented sodium arsenite -induced endothelial activation. Feeding PBA to apoE-null mice for 16 weeks also inhibited sodium arsenite -induced unfolded protein response in atherosclerotic lesions; expression of adhesion molecules on endothelial cells lining the atherosclerotic lesions; lesional and systemic inflammation and prevented the arsenic-induced exacerbation of lesion formation in the aortic valve by 80%. Together, these data suggest that arsenic causes endothelial activation and exacerbation of atherosclerosis by triggering UPR and chemical chaperones of protein folding could prevent arsenic-induced exacerbation of atherogenesis and inflammation.
Cellular Tracking of Chelerythrine by MALDI-TOF Mass Spectrometry

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Chelerythrine (CET), a crystalline alkaloid isolated from Chelidonium majus, a papaveraceous plant, forms a yellow salt with a violet fluorescence. Chemically, CET is a benzophenanthridine with 4 benzene rings, 1 dioxanol ring, and 2 methoxy groups. CET is a known inhibitor of protein kinase C (PKC) and recent discoveries show that it also inhibits the Na/K ATPase (NKA). The research objective of this study is to track CET into the cellular sub-compartments of human lens epithelial cells (HLECs). Here the hypothesis is proposed that CET crosses the plasma membrane as a pseudobase monomer and acts positively charged within the more acidic cytosolic micro-environment to inhibit the NKA through specific binding sites recently proposed. To test this hypothesis MALDI-TOF and ION Trap Mass Spec were employed to first analyze the monomer, dimer and trimer peaks of CET, and establish a calibration curve. Laser induced dissociation ion fragmentation shows a fingerprint for CET with ions at 332 and 319 for the monomer, and at 665 and 649 for the dimer. These peaks show characteristics of CET minus either or both methyl groups. Additional results suggest that CET ion peak intensity of the monomer and dimer is dependent upon the pH of the solution. A low acidic pH yields a higher peak intensity of CET while a higher, alkaline pH yields a decreased peak intensity of CET. The pKa for the transition between the high and low peak intensity was found to be ~8.0. Further tests will be conducted to track the sub-cellular location of CET in HLEC’s to elucidate the mechanism underlying the inhibition of NKA.
Dose-dependent alterations in excitatory GABA during embryonic development associated with lead (Pb) neurotoxicity

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Lead (Pb) is a physiologically non-essential toxic heavy metal. The use of Pb in industrial applications is widespread and has resulted in an increased risk of human environmental exposure. Multiple organs are targets of Pb toxicity; however, the central nervous system (CNS) is most sensitive during early development due to rapid cell proliferation and migration, axonal growth, and synaptogenesis. One of the primary components of CNS development is the GABAergic system. Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the adult brain. However, during early development GABA functions as an excitatory neurotrophic factor which contributes to cell proliferation, neuronal differentiation and migration, and synaptogenesis. Studies report the effects of Pb on GABA in the mature brain, but little is known regarding the adverse effects of Pb exposure on the GABAergic system during embryonic development. To characterize the effects of developmental Pb exposure on the GABAergic system, zebrafish embryos were exposed to 10, 50, or 100 ppb (mg/L) Pb or a control treatment shortly after fertilization through 24, 48, 60, or 72 hours post fertilization (hpf). A dose-response was observed between Pb exposure and tissue up-take in zebrafish larvae. Genetic analysis showed that developmental Pb exposure caused both an increase and a decrease in mRNA expression of seven genes (gad2, gad1b, gabra1, gabbr1a, gat-1, gat-3, vgat) throughout the GABAergic pathway that was developmental time point specific. GABA levels were analyzed and also revealed dose and time point specific alterations. These data provide evidence indicating that changes in gene expression throughout the GABAergic pathway do not cause similar immediate alterations in GABA levels and that the compensatory response of the GABAergic system is highly dependent upon dose and developmental time point. These data provide a framework for further analysis of the effects of Pb on the GABAergic system during the excitatory phase and transition to an inhibitory neurotransmitter during development.
Levels of Early Circulating Angiogenic Cells Associated with Geographic Metrics of Roadway Exposure

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Background

Multiple epidemiological investigations have established that proximity to roadways is associated with adverse cardiovascular conditions. Recent literature indicates that circulating angiogenic cells (CAC), a type of vascular progenitor cell, may be indicators of exposure to roadway-generated pollutants. Our study advances cardiovascular science by describing associations between CACs and metrics of roadway exposure.

Methods

Participants in the Louisville, KY Healthy Heart Study, (n=240), were recruited from University of Louisville Cardiology clinics (2009 –2011). Peripheral blood CAC levels were quantified using flow cytometry. Roadway was assessed by measuring distance from subject residences to the nearest roadway, total length of major roadways, total length of all roadways, and vehicle distance travelled, all within buffer areas between 50 and 300 meter intervals from subject residential locations. Generalized Linear Modeling techniques were used to assess the associations between roadway exposure and CAC levels.

Results

CACs positive for the early progenitor cell marker, AC133+, were elevated in blood of individuals residing closer to major roadways (p<0.05). Associations of all early CACs were significantly higher in subjects living within 50m of a major roadway. Levels of some AC133+ positive cells also were significantly associated with total roadway length and vehicle distance travelled within buffer areas. Associations were found with CACs in all exposure metrics. The strength of the associations was dependent on the size of the assessed buffer areas.

Discussion

These findings suggest that early CACs are increased as residential distance to major roadways decreases. The distance at which specific CAC associations become significant may be an indicator of what types of exposures from roadways may be leading to associations. CACs are mobilized by some environmental insults as a means of vascular protection.
Cortical Manganese Accumulation in the Non-Human Primate Brain Measured by MRI

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Introduction: Overexposure to manganese (Mn) can cause Parkinson’s disease-like neurological impairment with motor and cognitive deficiencies. Magnetic resonance imaging (MRI) allows to assess brain Mn concentrations in vivo. Several studies have shown that the primary targets of Mn deposition in the brain are basal ganglia structures such as the globus pallidus, substantia nigra and striatum. However, there is increasing evidence that the cerebral cortex is also involved in Mn neurotoxicity and that Mn accumulates throughout the whole brain. In this study, we present a whole-brain high-resolution MRI approach to automatically quantify Mn deposition in cerebrum, mid-brain and cerebellum of the non-human primate.

Methods: A total of 7 (reference group=3, Mn-treated group=4) adult male cynomolgus monkeys underwent MRI on a 3 T Philips Achieva MRI scanner at baseline and after eight months of Mn/saline exposure (1.66-2.5 mg Mn/kg per injection, 2x/wk for the Mn-treated group; same dose of saline for the reference group). A series of high resolution inversion recovery images were acquired (resolution: 0.5x0.5x2.2 mm\textsuperscript{3}, TR = 4000 ms) using seven different inversion times (TI=100, 300, 500, 700, 1000, 1500, 3000 ms). T1 relaxation time was calculated for each pixel throughout the whole brain as a quantitative inverse indicator of increased Mn deposition. All brain images were co-registered and normalized to the Montreal Neurological Institute (MNI) template using the Statistical Parametric Mapping package in Matlab. The Mn-treated group was compared to the control group pixel by pixel with a nonparametric Kruskal-Wallis test.

Results: High Mn deposition (>25% decrease in T1 relaxation time) was found in the globus pallidus (GP, p<0.01), putamen (p<0.05), caudate (p<0.01) and frontal cortex (p<0.01) in the Mn group. Reduced T1 values were also found in the occipital lobe and temporal lobe, especially in the visual cortex (V1), and parietal-occipital cortex in the Mn group (all with p<0.05). This finding suggests increased Mn accumulation in the cortical visual pathway, which may be related to impaired visuospatial learning as a result of Mn treatment. The statistically higher Mn accumulation in insula (p<0.01), which contributes to hand and eye motor control and cognitive function suggest that these domains are likely to be impaired by Mn.

Conclusion: This is the first MRI study that systematically studies Mn accumulation in the whole brain on a pixel by pixel basis. In conclusion, this study reveals Mn accumulation in many cortical brain regions besides the midbrain with high spatial resolution, involving in particular frontal cortex and visual and insula cortex. Supported by NIEHS grant #ES010975
Sulforaphane prevents angiotensin II-induced male germ cell death probably via up-regulation of Nrf2

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Patients with diabetes and/or obesity often exhibit reproductive reduction because of germ cell death. Since in these patients, angiotensin II (Ang II) seems acting as an important pathogenic factor for the cell death via induction of oxidative stress and damage, we speculate that Ang II may induce germ cell death without requirement for increased blood pressure, rather than through its direct induction of oxidative stress. Transcription factor NE-F2 (Nrf2) has been recognized as important anti-oxidative systems to prevent various oxidative injuries while sulforaphane (SFN) is a known Nrf2 activator. In the present project, therefore, we treated male C57 mice with low doses of Ang II every other day for two months without increasing blood pressure to define Ang II direct induction of testicular germ cell death. These Ang II-treated and age-matched control mice were treated with and without SFN for 3 months to define the protection from Ang II-induced effects. Results showed that Ang II induced significant increases in testicular oxidative damage, apoptotic cell death and endoplasmic reticulum (ER) stress. SFN significantly prevented Ang II-induced testicular cell death and oxidative damage. Furthermore, SFN up-regulated Nrf2 expression and transcription activity that was reflected by increased Nrf2 nuclear accumulation and phosphorylation as well as the protein expression of Nrf2 downstream antioxidants. These results suggest that Ang II is able to induce testicular cell death predominantly via ER stress and mitochondrial pathways. SFN can prevent Ang II-induced testicular cell death, which is associated with the up-regulated Nrf2 expression and transcription function.
Protective effect of a novel curcumin analog C66 on the aortic pathological damage in Type 1 Diabetic Mice: role of nuclear factor E2-related factor-2

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Cardiovascular diseases remain a leading cause of the mortality worldwide and also contribute to the predominant diseases of diabetic complications. Endothelial dysfunction is the essential initiation of diabetic vascular complications that is mainly due to diabetes-induced over expression of inflammation and oxidative stress. Nrf2, as a transcription factor in regulation of various antioxidative and cytoprotective responses, plays an important role in cellular prevention against damage. This study thus was designed to investigate whether C66 can protective the diabetes-induced aortic pathogenic changes via induction of Nrf2 function. Diabetes was induced in male C57BL/6 mice with a single intraperitoneal injection of streptozotocin. Diabetic and age-matched control mice were randomly divided into three groups treated with saline, C66 or JNK inhibitor (JNKi, sp600125) once every 2 days for 3 months. Aortas from these mice were morphologically and immunohistochemically examined. Significant increases in the wall thickness and structural derangement (HE) of aorta, along with significant increases in aortic inflammation (PAI-1, TNF-α and p-JNK), oxidative and nitrative stress (3-NT), apoptosis (TUNEL staining), proliferation (PCNA), and fibrosis (Sirius red and mast cell staining) were found in diabetic group, but not in C66- or JNKi-treated diabetic mice. C66 and JNKi treatment significantly increased Nrf2 expression and phosphorylation with increased expression of its down-stream antioxidant mRNA and protein levels. These results suggest that C66 prevents aortic pathological damage in Type 1 Diabetic Mice, probably via inhibition of JNK function to up-regulate Nrf2 function, and may be potential medication for diabetic patients to prevent their cardiovascular complications.
Prevention of Angiotensin II-induced Cardiomyopathy by Sulforaphane-activated Nrf2 Partially via AKT/GSK-3β/Fyn Pathway

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Angiotensin II (Ang II) is an important causative of diabetic cardiomyopathy. Sulforaphane (SFN) is anti-oxidative health supplement because of its activation of KEAP1/Nrf2 pathway. The present study examined whether SFN could protect from Ang II-induced cardiomyopathy through activation of Nrf2 and the underlying mechanism. FVB mice were given subcutaneous injection of Ang II (0.5 mg/kg) every other day for 2 months with or without SFN treatment (0.5 mg/kg) in five days of each week for 3 months and then kept until 6 months. At 3 and 6 months, blood pressure and cardiac function were assessed. Cardiac fibrosis, inflammation, and oxidative damage were detected by Western blotting, real-time qPCR, and immunohistochemical staining. SFN significantly prevented Ang II-induced high blood pressure at 6 months and cardiac dysfunction at both 3 and 6 months. Ang II significantly changed cardiac pathology, including increased myocardial hypertrophy or degeneration and interstitial and perivascular collagen accumulation, along with significant increases in cardiac oxidative damage (3-NT and 4-HNE), inflammation (TNF-α and PAI-1), and fibrotic response (TGF-β1 and CTGF). Those changes and damages were almost completely prevented by 3-month SFN treatment that significantly up-regulated Nrf2 transcription function, reflected by increased Nrf2 phosphorylation and nuclear accumulation and also increased expression of Nrf2 downstream antioxidants. To define the direct role of SFN-activated Nrf2 in preventing Ang II-induced cardiomyopathy, in vitro cultured H9c2 cells were treated with Ang II in the absence or presence of Nrf2 siRNA to silence Nrf2 expression. SFN significantly up-regulated Nrf2 and also prevented Ang II-induced CTGF and PAI-1 expression, which were completely abolished by Nrf2 silence. Furthermore, cardiac-overexpressing Nrf2 gene (Nrf2-TG) and wild-type (WT) mice were treated with Ang II (0.5 mg/kg) for 2 months. Ang II-induced cardiomyopathy was seen in WT mice, but not in Nrf2-TG mice. To dissect the mechanism for SFN activation of Nrf2, H9c2 cells were given SFN (10 µM) simultaneously with and without Akt inhibitor (LY294002, 10 µM) for 24 hours. SFN’s activation of Nrf2 was partially inhibited by Akt inhibition that also induced GSK-3β activation and Fyn nuclear accumulation. These results suggest that Ang II-induced cardiomyopathy can be prevented by SFN via Akt/GSK-3β/Fyn-mediated activation of Nrf2 antioxidant pathway.
Liver Toxicity of Munition Compounds 2,4-, 2,6-, and Technical Grade Dinitrotoluene

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Munitions compounds 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) are the two of the six most common isomers of dinitrotoluene (DNT). Technical grade dinitrotoluene (tgDNT) is a mixture of the six DNT isomers and is comprised of 76% 2,4-DNT and 19% 2,6-DNT with the remaining 5% a combination of four other DNT isomers (2,3-, 2,5-, 3,4-, and 3,5-DNT). Toxicity studies and health assessments on 2,4-, 2,6-, and tgDNT have reported distinct liver effects. In order to compare liver toxicity potencies of these three DNTs, we reviewed available studies in dogs, rats, and mice and compared non cancer and cancer liver effects following oral subchronic- and chronic-duration exposure to 2,4-, 2,6- and tgDNT. Potential points of departure for liver effects were identified/modelled and used to compare their toxicity potencies. We then examined the potential mechanisms that could account for the differences in hepatotoxicity. Our results indicated that similar pathologic liver lesions were induced after chronic-duration exposure to the three DNTs and their progression can be summarized in four stages: liver degenerative effects and/or cell death, hyperplastic effects, neoplastic nodules, and hepatocellular carcinoma. While the three DNTs have similar non cancer liver toxicity potencies, 2,6-DNT is the most potent liver carcinogen followed by tgDNT; 2,4-DNT is the least potent. Based on available biotransformation, toxicogenomics and genotoxicity information, imbalanced detoxification due to covalent binding with proteins and/or production of reactive oxygen species, inhibition of oxygen supply and covalent binding of DNA may all contribute to DNTs-induced non cancer liver toxicity and carcinogenesis. The differences of liver cancer potencies are consistent with their potencies of DNTs-induced hepatocyte genotoxicity. However, due to limited information, other putative modes of action of DNTs-induced hepatotoxicity are unclear. The views expressed in this abstract are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.
**Lactobacillus rhamnosus GG culture supernatant treatment attenuates alcohol-induced fat accumulation in the liver by enhancing AMPK-mediated fatty acid oxidation**

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Probiotic species produce biologically active compounds that can modulate mucosal integrity and prevent pathogen-induced intestine leakage. Our previous study showed that *Lactobacillus rhamnosus* GG (LGG) culture supernatant (LGGs) ameliorated acute alcohol-induced intestinal permeability and liver injury. However, whether LGGs administration prevents chronic alcohol exposure-induced liver injury is unknown. The purpose of this study was to examine whether LGGs provides a protective effect and explore the underlying mechanism in chronic-induced liver injury in mice. Mice were fed Lieber DeCarli diet containing 5% alcohol for 4 weeks, and LGGs was administered at a dose of equivalent to $10^9$ CFU/mouse with alcohol. LGGs decreased chronic alcohol-induced liver injury evaluated by measuring the activity of alanine aminotransferase and aspartate aminotransferase in plasma. Alcohol-induced hepatic steatosis was prevented by LGGs treatment as evaluated by triglyceride content measurement and Oil Red O staining of the liver sections. In addition, alcohol exposure also increased circulation and hepatic free fatty acid concentration, which is attenuated by LGGs treatment. Further study demonstrated that alcohol induced a significant decrease in AMPK and ACC phosphorylation, and these effects were prevented by LGGs administration in animals, which is correlated with the increase of the expression of adiponectin receptors. We also found that alcohol exposure increased hepatic SREBP-1c protein expression and LGGs treatment inhibited this increase. Further studies showed that LGGs prevented alcohol-induced hepatic apoptosis as evaluated by TUNEL assay. In vitro studies confirmed the positive effects of LGGs in promoting AMPK phosphorylation. These results suggest that LGGs is effective in preventing chronic alcohol-induced liver injury by suppression of alcohol-induced hepatic apoptosis and steatosis through AMPK phosphorylation. This finding could lead to developing new strategies for reducing chronic alcohol-induced liver injury by secreted factors of LGG culture. The identification of active ingredients of LGG culture secreted factors deserves further investigation.
Role of epithelial to mesenchymal transition in the hepatic fibrosis of OVE26 type 1 diabetic mice

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Diabetes is a global health issue. Diabetic liver injury is one of common complications in diabetic patients. Epithelial to mesenchymal transition (EMT) has been considered as an important mechanism in hepatic fibrosis. In the present study, therefore, we utilized OVE26 transgenic type 1 diabetes mouse model to investigate diabetic effect on EMT and its association with hepatic fibrosis. Serum glucose, aminotransferase and triglyceride levels of OVE26 and their wild-type (FVB) mice at 1, 3, 5 and 8 months old were measured. Hepatic pathology and fibrotic response were examined with H&E, oil-red, and Sirius-red staining as well as Western blotting along with immunohistochemical staining for TGF$\beta$1, CTGF, E-Cadherin, and $\alpha$SMA protein expression. Compared to FVB mice, OVE26 mice exhibited significant increases in serum levels of glucose, aminotransferase and triglyceride. At ages of 5 and 8 months, OVE26 mice developed steatohepatitis in varying degrees, reflected by significant increases in oil-red staining, collagenous fiber hyperplasia, and Sirius-red staining, compared to age-matched FVB mice. The protein expression of TGF$\beta$1, CTGF, $\alpha$SMA, and fibronectin as index of EMT, detected by Western blotting and/or immunohistochemical staining, was significantly increased in 5- or 8-month old OVE26 mice compared to age-matched FVB mice. There was no significant difference between OVE26 and FVB for E-Cadherin expressions, examined with Western blotting. These results suggest that steatohepatitis is an early manifestation in the liver of diabetic mice, and developed into hepatic fibrosis at late stage, which may be related to the EMT.
Serum and hepatic FGF-21 levels are increased in subjects with alcoholic hepatitis and in mice exposed to chronic-binge alcohol by decreased transcriptional suppression

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Purpose: Fibroblast growth factor 21 (FGF-21) is a novel metabolic regulator of glucose and lipid metabolism and has excellent potential in the treatment of obesity and type 2 diabetes in rodents and monkeys. Alcohol exposure affects lipid metabolism by increasing lipogenesis and decreasing fatty acid beta-oxidation. However, it is currently unknown whether alcohol exposure affects FGF-21 expression, which is the purpose of this study.

Methods: Serum FGF-21 levels were measured in 25 consenting human subjects with severe acute alcoholic hepatitis and were compared to 17 healthy, non-drinking controls by ELISA. C57BL/6 mice were fed Lieber DeCarli diet containing 5% alcohol or maltose dextrin for 12 days, and were given one dose of alcohol at 6 g/kg by gavage 6 hours before sacrificing. Serum and hepatic tissues from alcohol-exposed and control mice were harvested. Serum and hepatic FGF-21 levels were measured by ELISA, and hepatic FGF-21 mRNA levels were measured by real-time PCR. Liver triglyceride and serum free fatty acids were also measured. H4IIE cells were cultured and exposed to ethanol for various times and at different concentrations. mRNA levels of FGF-21 were measured. The data were analyzed by one-way analysis of variance and Newman-Keuls multiple-comparison test. Differences between groups were considered significant at P < 0.05.

Results: Serum levels of FGF-21 were markedly increased in both human subjects with alcoholic hepatitis and in mice exposed to alcohol administrated in chronic-binge pattern vs. non drinking controls. In ethanol-treated mice, the hepatic and adipose expression of FGF-21 were increased by both mRNA and protein levels. The increased FGF-21 expression was positively correlated with increased hepatic levels of triglyceride and serum levels of free fatty acids. Alcohol increased FGF-21 expression in hepatocytes in a time- and dose-dependent manner. The expression of PGC-1α and Rev-Erbα, which are important transcription suppressors of FGF-21, were decreased in mouse livers exposed to alcohol.

Conclusions: Alcohol exposure increased hepatic and circulating FGF-21 expression likely through an inhibition of transcriptional suppression mediated by the PGC-1α-Rev-Erbα pathway. The regulation of FGF-21 expression may be associated with hepatic lipid metabolism in alcoholic steatohepatitis. The observed increase in circulating FGF-21 was conserved between animal models and human subjects with alcoholic hepatitis. Altered FGF-21 metabolism plays an etiologic role in the development/progression of alcoholic liver disease.
Metallothionein as a compensatory component prevents intermittent hypoxia-induced cardiomyopathy in mice

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Obstructive sleep apnea (OSA) causes chronic intermittent hypoxia (IH) to induce cardiovascular disease, which may be related to oxidative damage. Metallothionein (MT) is a potent and highly inducible antioxidant protein that is expressed in the heart. The present study was to test the hypotheses that MT as a potent antioxidant protects the heart from OSA derived IH-induced cardiomyopathy. Mice were exposed to IH for 3 days to 8 weeks, which is consisted of alternating cycles of 20.9% $O_2$ /8% $O_2$ $F_{O_2}$ (30 episodes per h) with 20 seconds at the nadir $F_{O_2}$ for 12 h a day during daylight. IH significantly increased the ratio of heart weight to tibia length at 4 weeks with a decrease in cardiac function from 4 to 8 weeks, shown by decreased left ventricular ejection fraction (EF) and fractional shortening (FS). Cardiac oxidative damage and fibrosis were observed after 4 and 8 weeks of IH exposures. Endogenous MT expression was up-regulated in response to 3-day IH, but significantly decreased at 4 and 8 weeks of IH. In support of MT as a major compensatory component, mice with cardiac overexpression of MT gene and mice with global MT gene deletion were completely resistant and highly sensitive, respectively, to chronic IH-induced cardiac effects. These findings show that chronic IH induces cardiomyopathy characterized by oxidative stress and cardiac damage. The antioxidant MT protects the heart from such pathological changes.