2450 Ethanol Induces Fibronectin Expression through Redox Regulation of the Alpha-4 Nicotinic Acetylcholine Receptor (α4 nAChR)

W. Watson, Y. Zheng, J. Ritzenthaler, T. Burke, E. Torres-Gonzalez, and J. Roman. *University of Louisville, Louisville, KY.*

Chronic ethanol abuse increases susceptibility to acute lung injury. Previous studies suggested that ethanol acts through one or more nicotinic acetylcholine receptor (nAChR) subtypes to induce expression of fibronectin, an extracellular matrix protein implicated in lung injury and disrepair. Culturing fibroblasts in media with an oxidized cysteine/cystine redox potential (E_h Cys/CySS) mimics the effect of ethanol, suggesting a potential mechanism by which ethanol activates these receptors. The purpose of the present studies was to investigate redox regulation of the α4 nAChR subtype in response to ethanol. Primary lung fibroblasts were isolated from α4 nAChR knock out and wild type mice. NIH 3T3 fibroblasts in which wild type α4 nAChR was stably knocked down were used to express mutants of α4 nAChR. Fibronectin and CySS metabolizing genes were measured by real time-PCR. Intracellular and extracellular Cys, CySS, glutathione (GSH) and glutathione disulfide (GSSG) were measured by HPLC. Redox potentials (E_h) were calculated from the Nernst equation. Fibronectin mRNA levels were increased by 60 mM ethanol or 0 mV (oxidizing) redox media in wild type lung fibroblasts, but not in α4 nAChR knock out fibroblasts. Cells expressing mutants of α4 nAChR that lacked specific cysteine residues did not up-regulate fibronectin response to ethanol or 0 mV redox media. Lung fibroblasts from α4 nAChR knock out mice also had defects in CySS metabolism and distribution. Expression of the CySS transporter Slc7a11 was low, extracellular CySS accumulated, intracellular GSH levels dropped, and lower amounts of Cys and GSH were exported in cultures of α4 nAChR knock fibroblasts. Extracellular E_h Cys/CySS of knock out fibroblast cultures was more oxidized than wild types, and total GSH concentrations in the conditioned media were lower. These results showed Cys residues on the extracellular surface of α4 nAChR mediated cellular responses to ethanol in primary mouse lung fibroblasts. Furthermore, these studies provide evidence for a feedback loop, wherein activation of α4 nAChR by extracellular oxidation up-regulates intracellular production and export of Cys and GSH, providing a means to restore the redox state of α4 nAChR and limit signaling.

2451 Evaluation of Positive Controls for *In Vitro* Assays of Oxidative Stress

P. Dunlap, J. Rice, S. Ramaiahgari, S. Ferguson, and M. DeVito. *NIEHS, Research Triangle Park, NC.*

Increases in oxidative stress plays a critical role in the carcinogenic process as well as many other toxicities. The evaluation of this study compared the dose-response relationship and time course for effects on cell viability, mitochondrial membrane potential, and oxidative stress in in HepaRG and HaCaT cells. Diquat, antimycin, menadione, tertbutyl hydroperoxide, and etoposide were evaluated as positive controls in these assays. Potassium chloride and sucrose were used as negative controls. Cells were exposed for 1 or 24 hours, after which CellTiterGlo, ROSGLO, and JC-10 assays were performed and dose-response curves generated. These chemicals displayed unique dose- and time-response relationships. There was little difference in the response of the HepaRG and HaCaT cells to the test chemicals for all assays. Both antimycin and etoposide induced oxidative stress at one hour, but not at 24 hours of exposure. Induction of ROS by menadione was similar between the two-time points. Both diquat and TBHP induced greater ROS at 24 hours and at lower concentrations compared to one hour exposures. Decreases in cell viability, as measured by CellTiter-Glo occurred at one hour for antimycin and menadione but not with the other test chemicals. At 24 hours of exposure decreases in cell viability were observed for all chemicals except etopiside, KCl, and sucrose. At 1 and 24 hours antimycin, menadione, and diquat altered the MMP assay in both cell types, while it took 24 hours for TBHP to alter the MMP assay. While antimycin induced ROS, decreases in cell viability and alterations in MMP occurred at lower concentrations. Changes in viability, MMP, and ROS occurred at similar concentrations for menadione and diquat; however for diquat, the ROS and viability changes required 24 hours of exposure. These studies suggest that when evaluating ROS using *in vitro* assays, multiple time points and positive controls may be necessary due to the differences in potential mechanisms of oxidative stress induced by the chemicals under evaluation.

2452 Effects of Oxidants on Human Superoxide Dismutase (SOD)

J. J. Tao, and V. Mishin. *Rutgers University School of Public Health, Piscataway, NJ.*

Amyotrophic lateral sclerosis (ALS) is a progressive neurological disease, characterized by the gradual degeneration and death of motor neurons. In some dominant familial amyotrophic lateral sclerosis (FALS) pedigrees, mutations have been linked to a genetic defect for Cu, Zn-binding in superoxide dismutase (SOD). SOD catalyzes the dismutation of superoxide radical anion to hydrogen peroxide (H_2O_2) and oxygen (O_2). The modern view is that mutations in *SOD1*, the gene for Cu, Zn superoxide dismutase (Cu, Zn-SOD), may be implicated in FALS. It is thought that the free radical generating activity by Cu, Zn-SOD in the FALS mutant is enhanced relative wild-type human SOD possibly due to a decrease in the K_m value for H_2O_2. Previous reports also indicate that Cu, Zn-SOD is capable of using high concentrations of H_2O_2 as a substrate to form hydroxyl radicals. In the present studies, we determined if following treatment of human SOD with low concentrations of H_2O_2 (1-100 μM) in the absence of superoxide anion, SOD catalyzes the formation of hydroxyl radicals from H_2O_2. For these studies, we used a highly sensitive probe, disodium terephthalate (TPT), as a hydroxyl radical trap to quantify the formation of the free radical. TPT reacts with hydroxyl radicals to form 2-hydroxyterephthalate (2-OHTA) in a 1:1 ratio. 2-OHTA can be detected and quantified using a microplate reader with fluorescence detection (excitation and emission wavelengths, 310 and 425 um). We found that SOD readily generates hydroxyl radicals from H_2O_2. Hydroxyl radical formation was time and concentration-dependent, concentrations of H_2O_2 used ranged from 1-100 μM. Hydroxyl radical formation was inhibited by the antioxidant, dimethyl sulfoxide. Based on these results, we conclude that human SOD is capable of catalyzing the formation of hydroxyl radicals from H_2O_2. In future experiments, we will use high performance liquid chromatography to determine if treatment of human SOD with lower, physiologically relevant concentrations of H_2O_2, also catalyzes the formation of hydroxyl radicals. This may be an area of further study to better understand the progressive nature of FALS and how SOD may be implicated in the disease process.

2453 Fracking Sand Dust Elicits ROS and Pro-Inflammatory Cytokines from Murine Macrophage Cells

A. Morris, N. Olgun, K. Russ, J. Fedan, and S. Leonard. *NIOSH, Morgantown, WV.*

Hydraulic fracturing is used in the majority of natural gas wells across the United States. Water, sand, and chemicals are delivered at high pressure to drilled wells to cause fractures in the shale formations, allowing for the release of natural gas. Fracking sand, comprised mainly of silica dioxide (SiO_2), along with water and chemicals, is used to keep these fissures open. Silicosis is a pulmonary disease that affects workers exposed to inhaled silica and is characterized by inflammation and fibrosis, causing a decrease in lung capacity. Fracking sand dust (FSD) is generated during preparation of fracking fluid for injection. In this study, murine macrophage cells (RAW 264.7) were used to investigate whether pro-inflammatory signals are associated with inhaled FSD (<10 μm). We hypothesized that soluble and insoluble components in the FSD would each play a unique role in initiating pro-inflammatory responses and cytotoxicity. FSD was washed in PBS two separate times, 5 d each time, allowing for any soluble material to be released. On the 10th day of washing, sand that was twice washed was re-suspended in PBS (10 mg/ml) so that comparisons could be made to a freshly prepared, unwashed mixture. Production of the hydroxyl radical (·OH), measured with electron paramagnetic resonance (EPR), was the highest in unwashed sand, followed by PBS from the 5 d and 10 d washes. Unwashed FSD sand also generated the most intracellular reactive oxygen species and the response was significantly larger than that obtained from FSD re-suspended after two consecutive washes. Cells were treated with a 1:2 and 1:10 dilution of stock solution. Compared to PBS controls, the viability of RAW 264.7 cells decreased by 40% following exposure to FSD that was washed and re-suspended after 10 days, whereas unwashed sand decreased viability by 30% over a 24 h period. Finally, production of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 were measured using ELISA. While IL-1β and IL-6 production decreased with washing, TNFα production remained elevated. Our results indicate that FSD is cytotoxic to RAW 264.7 cells, as evidenced by decreases in viability, and stimulates intracellular ROS and ·OH production. The substantial differences in the production of cytokines stimulated by the soluble and insoluble components of FSD warrants future studies of the pro-inflammatory effects of the dust.

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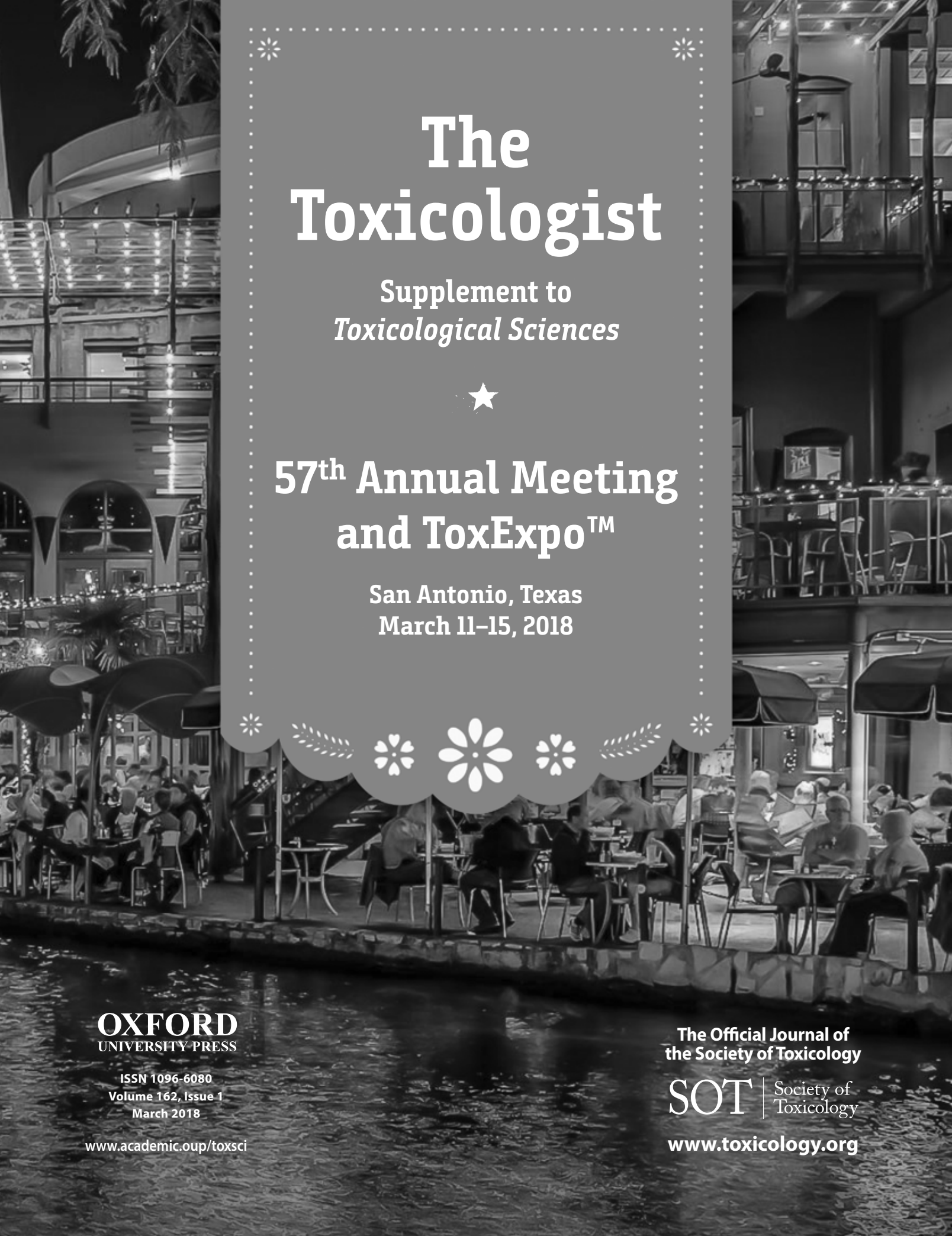

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