stress sensitizes IκB superrepressor-expressing hepatocytes to the TNFα-induced MPT and apoptosis. To induce mild oxidative stress, IκB-expressing hepatocytes were exposed to 2 μM t-BuOOH for 8 h. Hepatocytes were then treated with TNFα (30 ng/ml), and cell viability was determined 12 h after TNFα by propidium iodide fluorometry. t-BuOOH pre-exposure increased TNFα-induced cell killing to 43 ± 5% (mean ± SE) from less than 15% after either TNFα alone or t-BuOOH alone ($p<0.05$, n=3). Cyclosporin A (CsA, 2 μM), a specific inhibitor of the MPT, decreased sensitized cell killing to 16 ± 4.2% ($p<0.05$, n=3), suggesting the specific involvement of the MPT. In CsA inhibitor chase experiments, cytoprotection by CsA was lost when CsA was added more than 4 h after TNFα, suggesting the MPT occurred within 4 h. Confocal microscopy of calcein- and TMRM-labeled hepatocytes showed directly mitochondrial depolarization and inner membrane permeabilization beginning 3 h after exposure to TNFα in hepatocytes pre-exposed to t-BuOOH. In the absence of t-BuOOH, the MPT began after 8 h. Pre-exposure to t-BuOOH also increased mitochondrial ROS formation immediately after TNFα addition, as indicated by dichlorofluorescin fluorometry. In conclusion, mild non-toxic oxidative stress sensitizes cultured rat hepatocytes to TNFα-induced apoptosis. This sensitization is manifested by accelerated mitochondrial ROS formation and earlier onset of the MPT, which suggests that mitochondrial ROS generation is a key factor modulating TNFα apoptotic signaling in hepatocytes.

379 EFFECTS OF ALCOHOL CONSUMPTION ON FAS-MEDIATED APOPTOSIS.

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It has been suggested that fulminant hepatitis in humans may be mediated by activated cytotoxic T-lymphocytes (CTLs). CTL engagement of Fas on infected hepatocytes may normally occur to remove virus infected cells, but, if exaggerated, may lead to hepatitis. It has been shown that administration of anti-Fas antibody to mice results in death of the mouse due to severe damage to the liver by apoptosis. We are interested in the role of chronic alcohol consumption on Fas-mediated apoptosis in the liver. In order to assay this phenomenon in a mouse model, we are using biochemical, molecular, and histochemical approaches. We have found that mice who consume alcohol undergo apoptosis caused by Fas at time points earlier then pair and chow-fed control mice. Two hours after 15 micrograms of anti-Fas antibody was delivered intra-peritoneally, 6% of the hepatocytes in the alcohol-fed mice are apoptotic as compared to 2% of the hepatocytes in the control mice. Understanding of the molecular mechanisms of Fas-mediated liver injury will allow for identification of potentially selective therapeutic intervention strategies that can be used to prevent hepatitis without affecting vital host-defense functions. Supported by NIH grant AA12450.

380 SIGNALING FOR APOPTOSIS AND REPAIR *IN VITRO*.

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We demonstrated the role of cytokines in ethanol (EtOH)-induced liver cell apoptosis and repair. (Gastroenterology, 114(7):157-169, 1998). Polyenylphosphatidylcholine (PPC) was postulated to protect against cirrhosis in baboons. The present study evaluated whether PPC affects apoptosis, *in vitro* in a co-culture of normal human stellate cells with HepG2. Cells were treated with 0-80 mM EtOH for 24 hrs and with 2 doses of 80 mM EtOH (1/24 hrs) in the presence or absence of 20 mmol/L of PPC. After 24 hours in culture, the media were collected for tumor necrosis alpha (TNFα) measurements by enzyme-linked immunosorbent assays (ELISA). By transmission electron microscopy (TEM) 6000 cells were analyzed for each condition. In cells treated with 60-80 mM EtOH the cytoplasm density is higher and nuclear chromatin condenses into compact masses. The integrity of organelles and plasma membrane remained intact, but the cell size decreased (70% *vs.* control), the shape become elongated and fragmented into apoptotic bodies. At the molecular level we assayed in twelve cells/treatment: 1- the DNA fragmentation by ELISA (which quantitates histones associated with DNA fragments formed by cleavage at the internucleosomal linker region) and 2- measurement of 3 hydroxyl ends of doubled strand breaks (TUNEL). Exposure to 40 mM EtOH resulted in 12.5 % apoptosis (*vs.* control). The process of apoptosis is dose dependent: 60 mM EtOH resulted in 30% apoptotic cells ($p<0.05$), 80 mM dose of EtOH resulted in 42% ($p<0.001$) apoptosis (*vs.* control). Two consecutive doses of 80 mM EtOH for 24 hours each caused 66% ($p<0.001$ *vs.* control and $p<0.05$ *vs.* previous dose). PPC significantly reduced apoptosis (*vs.* non PPC exposed): 60 mM-15% ($p<0.05$) 80 mM- 25% ($p<0.05$) 2x80 mM- 30% ($p<0.05$). Anti-TNF also inhibits apoptosis. We conclude that PPC downregulates apoptotic process. High doses and repetitive application of EtOH signal for apoptosis in liver cells, *in vitro*.

381 PPARα-DEPENDENT ALTERATION OF GRP94 EXPRESSION IN MOUSE HEPATOCYTES.

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The adverse effects of the peroxisome proliferators (PPs), a class of rodent nongenotoxic hepatocarcinogens, include suppression of apoptosis, induction of hepatocyte proliferation and liver enlargement which eventually leads to tumors. The response to PPs is mediated by the peroxisome proliferator activated receptor α(PPARα). We carried out proteomic analyses of PP treated hepatocytes from wild type and PPARα null mice to identify the molecular pathways underlying the adverse effects of PPs. We have identified eighteen protein spots exhibiting differential expression in PP treated wild type mouse hepatocytes. Several proteins involved in lipid metabolism pathways, but also ATP synthase b subunit, which are regulated by PPs were identified. In addition, both 2D silver stained gels and western blotting analysis indicated that the anti-apoptotic glucose regulated protein 94 (GRP94) is consistently over-expressed upon stimulation with PPs, providing us with novel insights into the anti-apoptotic mechanism activated by PPs.

382 HISTOCHEMICAL ASSESSMENT OF HEPATIC APOPTOSIS UPON SUBCHRONIC TREATMENT OF MALE MICE WITH THIAMETHOXAM.

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Hepatic apoptosis was histochemically identified and quantitatively assessed in mice treated with dietary concentrations of 0, 100, 500, and 2500 ppm thiamethoxam for 3, 7, 13, 27, and 59 days, and for 9 months (0 and 2500 ppm only). Paraffin liver sections from a subchronic cell proliferation study and from the interim sacrifice of an 18-month long-term study were submitted to a TUNEL (terminal deoxy-nucleotidyl transferase mediated dUTP nick end labeling) histochemical procedure, followed by morphometry in purpose to quantify the extent of apoptotic activity. In small intestine sections, serving as positive controls, TUNEL staining was found to label dying enterocytes at the villus tips, indicating the method's ability to detect apoptotic cells. The negative control sections (without the DNA label-linking enzyme "terminal deoxynucleotidyl transferase") revealed no TUNEL-specific staining at all. Significantly increased numbers of apoptotic figures per unit area were seen at 500 and 2500 ppm after 59 days and at 2500 ppm after 9 months of treatment. No significant increase was seen at 100 ppm at any time. These results confirm previously reported late appearance of apoptosis with thiamethoxam and is consistent with the overall concept of persistent and exacerbating cell loss in the liver of mice after prolonged treatment with high doses of thiamethoxam.

383 CAFFEIC ACID ENHANCEMENT OF COCAINE-INDUCED HEPATOTOXICITY.

D. J. Price[1], C. A. Muro-Cacho[2], A. P. Kulkarni[1] and R. D. Harbison[1,2]. [1]*College of Public Health, University of South Florida, Department of Environmental and Occupational Health, Tampa, FL and* [2]*College of Medicine, University of South Florida, Department of Pathology, Tampa, FL.*

Cocaine has been reported to produce hepatic necrosis and apoptosis. Caffeic acid is a ubiquitous ingredient in beverages, tobacco, fruits and vegetables. The objective of this study was to determine if concomitant treatment with cocaine and caffeic acid would enhance cocaine-induced hepatotoxicity. Male ICR mice were administered caffeic acid (125 mg/kg, i.p.) and 20-min later cocaine (60 mg/kg, i.p.), with sacrifice 24 hr after administration of cocaine. A 4-fold increase in serum alanine aminotransferase activity (ALT) occurred in animals treated with caffeic acid and cocaine (12,000 SF Units/mL) compared to animals treated with cocaine alone (3,000 SF Units/mL). Caffeic acid pretreatment did not significantly reduce hepatic glutathione or esterase activity. The pretreatment did, however, increase cocaine-induced hepatic necrosis and cause a 2 fold increase in apoptosis at 4 hr, a 6 fold increase at 24 hr, and a 12 fold increase at 7 days. Apoptosis was measured using the TUNEL method. Histopathological evaluation indicated that with caffeic

acid pretreatment, necrosis was in the same compartments as apoptosis, and occurred at the same time periods. The profound hepatic necrosis was observed in the midzonal and centrilobular areas. The results of this study indicate that caffeic acid can significantly increase cocaine-induced hepatotoxicity, and this enhancement does not appear to be a glutathione or esterase dependent mechanism. (Supported by NIOSH Grant #412874-03)

384 ALTERATIONS OF CELL PROLIFERATION, APOPTOSIS AND P53 PROTEIN IN THE EXOCRINE PANCREAS OF RATS TREATED WITH 4-HYDROXYAMINOQUINOLINE 1-OXIDE.

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Cell proliferation, apoptosis and p53 protein expression in the pancreas induced by 4-hydroxyaminoquinoline 1-oxide (4HAQO) was investigated in male 6-week-old Sprague-Dawley rats to clarify early changes associated with carcinogenesis by genotoxic carcinogens. Rats were given a single iv injection of 4HAQO at a dose of 20 mg/kg bw, and sequentially killed after 2-72 hrs. Control rats were killed after 4, 24 and 48 hrs. Part of pancreas and non-target livers were immediately fixed rutine HE staining, immunohistochemistry for p53 protein, PCNA, and apoptosis (TUNEL method). A part of the tissues were fixed for electron microscopic observation. Apoptosis and p53 expression were preferentially observed in the pancreas of the 4HAQO-treated animals but scarcely in the livers. The incidences of these biomarkers were greatest at 24 hrs after the treatment, being consistent with the peak for PCNA-labeling index. Nucleolar alteration, demonstrable by electron microscopy as segregation of nucleolar components into granular and fibrillar compartments, was evident in cells of the target organ, exocrine pancreas, but not of the non-target liver parechyma. Sequential observation clarified that such alteration was highest in frequency 6 hrs after 4HAQO administration in pancreatic acinar cells. These the results of the present study suggest that apoptosis, p53 and enhanced cell replication are closely related phenomena involved in the carcinogenesis of 4HAQO following DNA adduct formation and nucleolar segregation.

385 RADIATION INDUCED GENE EXPRESSION, APOPTOSIS, AND PRE-B CELL CFU SURVIVAL DYSREGULATION BY N-ACETYL-L-CYSTEINE.

J. E. French, A. Quinn, H. Honeycutt, K. R. Martin, J. C. Barrett and F. W. Kari. *NIEHS, Research Triangle Park, NC.*

Radiation induced free radicals cause DNA damage and induce apoptosis in lymphohematopoietic cells. N-acetyl-L-cysteine (NAC) modulates free radical mediated signal transduction pathways, scavenges free radicals and provides substrate for glutathione synthesis, and, thus, may alter radiation induced DNA damage. We observed a greater incidence of lymphoid tumors in 4 Gyγ-irradiated FVB/N p53 deficient mice on diets with 3% NAC than with control diet. To investigate, we irradiated FVB/N mice with 4 Gyγ-irradiation after acclimation to a basal or basal plus 3% NAC diet. Three hours post-irradiation, mice were killed by CO_2 narcosis and femoral bone marrow cells were removed and cultured in a methylcellulose growth medium with IL-7 to enhance pre-B colony forming unit (CFU) growth or resuspended in a medium stabilizing RNA. Patterns of gene expression associated with apoptosis were determined using total RNA and cDNA expression arrays. Altered patterns of mRNA expression were observed 3 hrs post-irradiation in pathways that showed NAC suppression of Rb and NFκB while inducing the transcription factor E2F1, Trp53 dependent Gadd45, Bax and Mdm2, but not p53. Bad, Bcl-w, and caspases, but not Bax, Bcl-2, or Trail, expression was increased by NAC. Although the total number of cells/femur and total myeloid CFU/femur were not significantly reduced, pre-B CFU survival was greatly reduced, requiring 10X greater plating density than normal. However, NAC significantly increased the number of abnormal pre-B CFU observed (see below). We speculate that high levels of intracellular NAC alters signal transduction pathways in pre-B cells resulting in suppression of apoptosis in radiation induced DNA damaged pre-B CFU in the bone marrow permitting selection and clonal amplification of premalignant lymphoid cells. The mechanistic basis for this is under investigation.

Radiation Plus	Cells/femur (x10⁷)	Myeloid CFU/femur	Pre-B CFU/femur
Basal diet	2.6 ± 0.5	9100 ± 2600	71.0 ± 17.5
Basal diet + 3% NAC	2.0 ± 0.4	11000 ± 5400	314.0 ± 65.6 p≤0.01

386 ROLE OF P53 IN THE INDUCTION OF APOPTOSIS BY PSORALEN AND ULTRAVIOLET-A RADIATION (PUVA).

A. B. Santamaria[1], D. W. Davis[2], D. Nygiem[1], D. J. McConkey[2], S. E. Ullrich[1] and H. N. Ananthaswamy[1]. *[1]MD Anderson Cancer Center, Immunology, Houston, TX and [2]MD Anderson Cancer Center, Cancer Biology, Houston, TX.*

A combination of psoralen and ultraviolet-A (UVA, 320-400 nm) radiation, commonly referred to as "PUVA", is widely used in the treatment of psoriasis and other skin diseases. PUVA therapy is highly effective in killing hyperproliferative psoriatic skin cells but its mechanism of action has not been fully elucidated. In this study, we used immortalized JB6 mouse epidermal cells to investigate the molecular mechanism by which PUVA induces cell death *in vitro*. Treatment of JB6 cells with 10 μg/ml of 8-methoxypsoralen (8-MOP) followed by irradiation with 20 kJ/m² of UVA resulted in cell death by apoptosis. PUVA-treated JB6 cells exhibited the morphological and biochemical characteristics of apoptosis such as chromatin condensation, DNA ladder formation, and TUNEL-positivity. In addition, PUVA treatment caused stabilization of p53 in a time-dependent manner beginning at 6 hrs and peaking at 24 hrs post-treatment. Analysis of PUVA-treated cells by confocal microscopy revealed translocation of the p53 protein to the nucleus, suggesting that the p53 protein was transcriptionally active. This conclusion was supported by the fact that PUVA treatment also caused an induction of $p21^{Waf/Cip1}$ at 6-12 hr post-treatment. Interestingly, p53 was phosphorylated at serines 15 and 389 following PUVA treatment. In addition, suface fas expression was statistically increased at 48-72 hrs after PUVA treatment. *In vivo* studies were conducted to evaluate the role of p53 in PUVA-induced apoptosis. PUVA treatment induced apoptosis to an equal extent in $p53^{+/+}$ and $p53^{-/-}$ mouse skin. From these studies, we conclude that PUVA-induced apoptosis does not require p53 expression.

387 CYTOTOXICITY OF ALKYLPHENOLS FROM GINKGO BILOBA - NECROSIS OR APOPTOSIS.

H. Hecker, R. Johanisson and C. P. Siegers. *Medical University Luebeck, Toxicology, Luebeck, Germany.*

Extracts from the leaves of Ginkgo biloba L. are among the most widely used phytopharmaceuticals. As hazardous constituents in crude extracts, ginkgolic acids (cardanols and cardols) have been identified. No contribution of these alkylphenols to the beneficial therapeutic effects of Ginkgo extracts has been reported, but allergenic, mutagenic and carcinogenic properties have been discussed for these substances. The cytotoxic potential of ginkgo alkylphenols, their effects on the human keratinocyte cell line HaCaT (Prof. Fusenig, DKFZ Heidelberg, Germany) and the rhesus monkey kidney epithelial cell line LLC-MK2 (ICN Biomedicals, Eschwege, Germany) were investigated. The effect of ginkgo extract EGb 761 as well as pure ginkgolic acids on cell growth and integrity was tested by uptake of neutral red as well as releases of enzymes, respectively. Following incubation of cells with the ginkgo extract EGb 761 a low toxic potential was found (IC50-values between 900 mg/l for HaCaT and 1480 mg/l for LLC-MK2). The corresponding IC50-values for the defined ginkgolic acids ranged between 21 mg/l (HaCaT) and 4.6 mg/l (LLC-MK2), respectively. In parallel to the inhibition of the neutral red uptake, we observed a concentration-dependent release of LDH into the medium. Toxic effects were also documented by electron microscopic analysis, which reveal mitochondrial damage indicative for uncoupling of oxidative phosphorylation. A possible induction of apoptosis by ginkgolic acids was evaluated by staining of HaCaT cells with the monoclonal antibody Apo2.7 (7A6 antigen). Following incubation of cells with ginkgolic acids for 18 h, the proportion of 7A6 positive cells increased concentration-dependently from a control level of about 10 % to more than 60 % at 30 mg/l and above. In conclusion, due to the toxic effects observed in the present investigation a removal of ginkgolic acids from Ginkgo biloba extracts to a technically possible level (e.g,. 2-5 ppm) is proposed to be appropriate.

388 DOWNSTREAM TARGETS OF p53 IN BLEOMYCIN-INDUCED LUNG INJURY.

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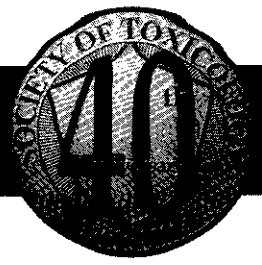
Chronic inflammation leading to pulmonary fibrosis develops in response to environmental pollutants, radiotherapy, or certain cancer chemotherapeutic agents. Our recent work demonstrated that intratracheal administration of bleomycin led to caspase-mediated DNA fragmentation characteristic of apoptosis. Using $p53^{-/-}$ and $iNOS^{-/-}$ mice, we demonstrated that nitric oxide activates p53 which



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Preface

This issue of *The Toxicologist* is devoted to the abstracts of the presentations for the symposium, platform, poster discussion, workshop, roundtable, and poster sessions of the 40th Annual Meeting of the Society of Toxicology, held at the Moscone Convention Center, San Francisco, California, March 25–29, 2001.

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