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We have hypothesize that the toxic manifestations of polyhalogenated cyclic hydrocarbons and heavy metals are at least in part induced through a series of events involving the production of reactive oxygen species, activation of the protein kinase C system and release of tumor necrosis factor-ä (TNFa). The sequence of these events is not known, and the relationship between these events and the induction of stress protein has not been studied. Both in vivo and in vitro effects on these parameters as well as acute and chronic studies will be determined. The above hypothesis will be tested by completing the following specific aims. Excellent progress has been made relative to the specific objectives of this research project. Based on the results which have been completed during the past three years, 21 manuscripts were published or are in press in refereed journals, and 14 presentations have been made at national and international scholarly meetings.

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MECHANISMS ASSOCIATED WITH THE TOXICITY OF POLYHALOGENATED CYCLIC HYDROCARBONS AND HEAVY METALS

by

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Submitted to

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Bolling AFB, DC 20332-6448

I. OBJECTIVES

We have hypothesized that the toxic manifestations of polyhalogenated cyclic hydrocarbons and heavy metals are at least in part induced through a series of events involving the production of reactive oxygen species, activation of the protein kinase C system and release of tumor necrosis factor-α (TNFα). The sequence of these events is not known, and the relationship between these events and the induction of stress protein has not been studied. Both in vivo and in vitro effects on these parameters as well as acute and chronic studies will be determined. The above hypothesis will be tested by completing the following specific aims.

Initial studies will be conducted in rats and mice using a single, oral, acutely toxic dose equivalent to one-half the LD_{50}. Polyhalogenated cyclic hydrocarbons (PCH) as lindane, TCDD, and endrin, and heavy metals as chromium and cadmium will be used. In addition, studies will be conducted with naphthalene. Since most occupational exposures involve chronic, low doses, low dose chronic studies will be conducted with dosing for up to 120 days using doses in the range of 1/10th to 1/100th the acute dose. Initial studies will focus on the identification and quantitation of biomarkers of toxicity including the urinary excretion of the lipid peroxidation products malondialdehyde, formaldehyde, acetaldehyde and acetone by HPLC, production of reactive oxygen species by spectroscopic methods, an increased incidence of DNA strand breaks by alkaline elution, enhanced production of TNFα, activation of the protein kinase C system, and induction of stress proteins by polyacrylamide gel elecrophoresis (PAGE). Selected antioxidants/free radical scavengers and membrane stabilizers will be used to evaluate the role of reactive oxygen species in the activation of the protein kinase C system, production of TNFα and induction of stress proteins.

II. STATUS OF EFFORT

Excellent progress has been made relative to the specific objectives of this research project. Based on the results which have been completed during the past three years, 21 manuscripts were published or are in press in refereed journals, and 14 presentations have been made at national and international scholarly meetings.

Studies have been conducted with chromium, cadmium and naphthalene as well as with structurally diverse pesticides and contaminants of pesticides including endrin, chlordane, lindane, DDT, TCDD, chlorpyrifos, fenthion and alachlor.

In summary, the results support the basic hypothesis that the mechanism of toxicity of structurally diverse xenobiotics and heavy metals is at least in part involves the production of a cascading series of events involving production of reactive oxygen species, glutathione depletion, altered calcium homeostasis, activation of the protein kinase C system, enhanced release of TNFα, induction of stress/heat shock protein (HSP) 90, oxidative tissue damage including lipid peroxidation, DNA strand breaks and decreased membrane fluidity, and apoptosis (programmed cell death). We have also hypothesized that stimulation of oncogene expression and inhibition of tumor suppressor genes are involved. A working hypothesis has

been developed and is presented in Appendix #1.

The HPLC determination of the urinary lipid metabolites malondialdehyde, formaldehyde, acetaldehyde and acetone has been developed in our laboratories as an effective biomarker system for assessing oxidative tissue damage. The methods for determining these lipid metabolites in blood and amniotic fluids have also been developed. The present studies have shown that excellent correlations exist between the abilities of structurally diverse xenobiotics to induce production of reactive oxygen species, and the production of these oxidative lipid metabolites.

In general, the present studies have also shown that excellent correlations exist between the ability to produce reactive oxygen species by structurally diverse xenobiotics, and other indices of oxidative tissue damage including DNA damage, altered membrane fluidity, and leakage of lactate dehydrogenase (LDH) from cells.

The authors have also used a wide range of techniques to assess the hypothesis which has been set forth. Production of superoxide anion has been determined by chemiluminescence and spectrophotometric methods as well as the use of the enzyme superoxide dismutase. Hydroxyl radical has been determined using chemiluminescence and a HPLC technique. Intracellular oxidized states of cells have been determined using laser scanning confocal microscopy, while apoptosis (programmed cell death) has been assessed based on DNA laddering and flow cytometry. Induction of genes associated with heat shock (stress) protein (HSP) 90 has been determined by Western and Northern blot analyses. The role of tumor necrosis factor α has been determined using TNFα antibody. The activity of protein kinase C was measured using a nonradioactive microwell spectrophotometric assay kit. Cell viability has been determined using Trypan blue exclusion. Molecular and cellular biological techniques are currently being used to assess the role of p53, bcl-2, and NF$\kappa\beta$ in the cascade of events associated with oxidative stress and the induction of oxidative tissue damage by structurally diverse xenobiotics.

III. SUMMARY OF ACCOMPLISHMENTS/NEW FINDINGS

Initial studies were conducted comparing the effects of a single acute oral (0.50 LD_{50}) dose of chromium (III) (895 mg/kg) and chromium (VI) (25 mg/kg) to produce reactive oxygen species including nitric oxide production, enhanced urinary excretion of lipid metabolites, and produce DNA damage in Sprague-Dawley rats. The results indicate that both chromium (VI) and chromium (III) induce an oxidative stress at equitoxic (0.50 LD_{50}) doses, while chromium (VI) induced greater oxidative stress as compared to chromium (III) in treated animals.

Since most exposure to chromium is obtained on a chronic basis as opposed to an acutely toxic exposure, rats were treated daily with an oral (0.20 LD_{50}) dose of 10 mg sodium dichromate dihydrate [Cr(VI)]/kg/day for a period of 90 days. Previous studies have shown that a single dose of 10 mg sodium dichromate/kg exhibits little or no toxicity. Increases in hepatic lipid peroxidation, the urinary excretion of lipid metabolites, and enhanced hepatic DNA damage

were observed after 15 days of treatment, while maximum increases in these tissue damaging effects were observed at approximately 45 days of treatment.

The effects of cadmium (II) chloride on reactive oxygen species production were examined following a single oral exposure (0.50 LD_{50}; 44 mg/kg) by assessing hepatic mitochondrial and microsomal lipid peroxidation, glutathione content in the liver, excretion of urinary lipid metabolites, and the incidence of hepatic nuclear DNA damage. Increases in lipid peroxidation, the urinary excretion of lipid metabolites, an increase in hepatic DNA single strand breaks, and a decrease in glutathione content support the hypothesis that cadmium induces production of reactive oxygen species which may contribute to the tissue-damaging effects of this metal ion.

The effects of oral, low (0.05 LD_{50}) doses of sodium dichromate [Cr(VI); 2.5 mg/kg/day] and cadmium chloride [Cd(II); 4.4 mg/kg/day] in water on hepatic and brain mitochondrial and microsomal lipid peroxidation, excretion of urinary lipid metabolites, and hepatic nuclear DNA single strand breaks were examined in Sprague-Dawley rats over a period of 120 days. The animals were treated daily using an intergastric feeding needle. Small but significant increases were observed in all parameters which were measured as early as the 15th day of treatment. However, maximum increases were observed in all parameters between 60-75 days of treatment. The results clearly indicate that low daily (0.05 LD_{50}) dose chronic administration of sodium dichromate and cadmium chloride induces an oxidative stress resulting in tissue damaging effects which may contribute to the toxicity and carcinogenicity of these two cations. Furthermore, these results, in conjunction with studies presented above, clearly indicate that an acute (0.50 LD_{50}) dose induces an oxidative stress and oxidative tissue damaging effects within 24-48 hours, while a dose of 0.20 LD_{50} on a daily basis results in maximal effects at approximately 45 days of treatment, and a dose of 0.05 LD_{50} requires 60-75 days of daily treatment in order to produce maximal oxidative effects.

The effects of chromium (VI) and chromium (III) on the production of superoxide anion, nitric oxide and DNA single strand breaks in J774A.1 macrophage cells in culture as well as the effects on LDH leakage and cell viability have been determined. Following a 48 hr incubation, over 2-fold increases in superoxide anion and nitric oxide production were observed at concentrations of approximately 0.30 μM Cr(VI) and 50 μM Cr(III). A 50% decrease in viability was observed at these concentrations. Excellent concentration-dependent correlations existed between the production of reactive oxygen species and LDH leakage, while an inverse relationship existed with respect to cell viability.

The toxic and apoptotic potentials of sodium dichromate [Cr(VI)] and cadmium chloride [Cd(II)] have been assessed in cultured J774A.1 macrophage cells. The overall intracellular oxidized states of the cells were measured by laser scanning confocal microscopy using 2,7-dichlorofluorescein diacetate as the fluorescent probe. Concentration- and time-dependent increases in fluorescence intensity, and DNA damage and apoptosis were observed.

The toxic and apoptotic potentials of Cr(VI) and Cd(II) in cultured chronic myelogenous leukemic (CML) K562, promyelocytic leukemic HL-60 and normal human peripheral blood mononuclear (HPBM) cells were determined at 0-100 μM concentrations of these cations for 0-48 hrs. Cell cycle modulation and apoptosis were determined by flow cytometry, while

changes in intracellular oxidized states were determined using laser scanning confocal microscopy. Both cations produced large increases in apoptosis 24 hrs after initiation of incubation with the K562 cells, but the level of apoptosis decreased markedly by 48 hrs. In HL-60 cells, no apoptosis was observed at 24 hrs, but significant apoptosis occurred after 48 hrs of incubation with both cations at 12.5 μM. Of particular interest was the fact that no apoptosis was observed at any of the time points with 25 μM concentrations or less of both cations with respect to the HPBM cells. The results clearly indicated that both Cr(VI) and Cd(II) cations induce cytotoxicity and tissue damaging effects through enhanced production of reactive oxygen species, oxidative DNA damage and apoptotic cell death in leukemic and premyelocytic leukemic cells, but had little effect on normal human cells at similar concentrations. Thus, the results demonstrate the roles of reactive oxygen species in the toxicity of these cations, and the differing susceptibilities of various cell types.

Quinone metabolites of naphthalene (NAP) are known to produce lipid peroxidation. However, the ability of naphthalene to induce oxidative stress in experimental animals has not been previously investigated. Female Sprague-Dawley rats were treated with a single oral dose of 1100 mg NAP/kg (0.50 LD_{50}). Tissue samples were collected up to 72 hrs after NAP treatment. NAP treatment resulted in significant increases in lipid peroxidation in liver and brain as well as significant increases in hepatic DNA single strand breaks. Furthermore, glutathione (GSH) decreased in hepatic and brain tissues, while membrane fluidity increased. Maximal excretion of urinary lipid metabolites occurred at 12-24 hrs after NAP administration. The results support the hypothesis that NAP induces oxidative stress and tissue damage. Furthermore, the antioxidant vitamin E succinate was capable of providing significant protection.

Primary exposure to naphthalene (NAP) normally involves low dose, chronic exposure. Therefore, the effects of an oral, low (0.05 LD_{50}) daily dose of NAP in corn oil (110 mg/kg/day) was determined in rats. Significant increases in hepatic and brain DNA fragmentation and lipid peroxidation were observed after 60-75 days of treatment while maximal increases occurred at 75-90 days of treatment. Greatest increases in the urinary excretion of lipid metabolites also occurred at 75-90 days of treatment. The results clearly demonstrate that low dose chronic exposure to naphthalene results in oxidative tissue damaging effects.

In order to determine whether naphthalene (NAP) can directly induce an oxidative stress, the concentration-dependent effects of NAP on lipid peroxidation, superoxide anion production, hydroxyl radical production, and DNA fragmentation were determined in cultured J774A.1 macrophage cells. Following incubation for 24 hrs, concentrations of NAP of 100 μM or higher resulted in significant increases in superoxide anion and hydroxyl radical production as well as significant increases in lipid peroxidation and DNA fragmentation. The results demonstrate that NAP may induce toxic manifestations by enhanced production of reactive oxygen species resulting in lipid peroxidation and DNA damage. Further studies will be required to determine the role of NAP metabolism in the production of reactive oxygen species.

Because reactive oxygen species may be involved in the toxicity of structurally diverse xenobiotics, we have examined the in vitro and in vivo effects of structurally dissimilar polyhalogenated cyclic hydrocarbons as endrin and chlordane, chlorinated acetamide herbicides as alachlor, and organophosphorous pesticides as chlorpyrifos and fenthion on hepatic and

brain lipid peroxidation and DNA strand breaks. These five xenobiotics all resulted in significant increases in lipid peroxidation and DNA single strand breaks in rat liver and brain. When cultured neuroactive PC-12 cells were incubated with the pesticides, a concentration-dependent increase in the release of LDH from the cultured cells was observed. Furthermore, concentration-dependent increases in the incidence of DNA single strand breaks was observed in these cells. The results suggest that reactive oxygen species may serve as common mediators of programmed cell death (apoptosis) in response to many toxicants and pathological conditions.

The possible role of heat shock (stress) protein (HSP) 90 in the oxidative stress produced by various pesticides has been examined in Sprague-Dawley rats and cultured PC-12 cells. Induction of HSP 90 was examined by Western and Northern blot analysis. Alachlor, endrin, chlorpyrifos and fenthion induce HSP 89α and HSP 89β in hepatic and brain tissues as well as in cultured PC-12 cells as determined by Northern blot analysis. These findings were substantiated by Western blot analysis using HSP 90 antibody. The results support the hypothesis that these genes may be mechanistically involved in protecting tissues against oxidative stress induced by the pesticides which were used.

We have hypothesized that protein kinase C may mediate the toxic effects of structurally diverse xenobiotics since it plays a key role in several cellular functions including transmembrane transduction of many signals during metabolism, growth and cell differentiation. Therefore, the effects of TCDD, endrin, chlordane, lindane, DDT, chlorpyrifos, fenthion, alachlor, cadmium chloride and sodium dichromate [Cr(VI)] on protein kinase C activity were determined in brain and liver tissues of female Sprague-Dawley rats and cultured PC-12 cells. Under the conditions which were employed, in hepatic tissues the greatest increases in protein kinase C activities were observed with TCDD, chlorpyrifos, endrin and cadmium chloride, while chlorpyrifos and fenthion exerted the greatest increases in brain tissues. In cultured PC-12 cells, greatest effects were induced by chlorpyrifos, fenthion, cadmium and chromium. The results suggest that protein kinase C is involved in the cascade of events associated with the toxicity of these xenobiotics.

TNFα sensitizes and activates phagocytic cells to agents that induce them to release reactive oxygen species. Therefore, TNFα may act as an amplifying loop in the induction of reactive oxygen species by structurally diverse xenobiotics. We have therefore examined the possible role of TNFα in mice exposed to TCDD. The results clearly demonstrated that TNFα is involved in TCDD-induced production of DNA single strand breaks in hepatic nuclei as well as in hepatic lipid peroxidation and activation of peritoneal lavage (primarily macrophage) cells. The results suggest that TNFα release may play a role in sensitizing and activating phagocytic cells following treatment with TCDD, contributing to the overall oxidative stress of animals following exposure to this xenobiotic.

IV. PERSONNEL ASSOCIATED WITH THE RESEARCH EFFORT

Dr. Sidney J. Stohs - Principal Investigator
Dr. Debasis Bagchi - Co-investigator
Dr. Ezdihar Hassoun - Co-investigator
Dr. Manashi Bagchi - Co-investigator
Dr. Terry Lawson - Collaborating Investigator
Dr. William Schlueter - Collaborating Investigator
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Dr. S.D. Ray - Collaborating Investigator
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Ms. Julia Kelly - Student
Mr. Phillip Vuchetich - Student
Mr. Jeff Moser - Student
Mr. Minh Tran - Student
Mr. Roger Krohn - Student
Mr. Amit Garg - Student
Mr. Lawrence Chinn - Student
Ms. Dipa Bagchi - Student
Ms. Bridget Buchanan - Student
Ms. Sheryl Sato - Student

V. PUBLICATIONS

1. Bagchi, D., Shara, M.A., Bagchi, M., Hassoun, E.A. and Stohs, S.J. Time-dependent effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on serum and urine levels of malondialdehyde, formaldehyde, acetaldehyde and acetone in rats. Toxicol. Appl. Pharmacol. 123, 83-88 (1993).

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18. Bagchi, D., Vuchetich, P.J., Bagchi, M., Hassoun, E.A., Tran, M.X., Tang, L. and Stohs, S.J. Induction of oxidative stress by chronic administration of sodium dichromate [Cr(VI)] and cadmium chloride [Cd(II)] to rats. Free Rad. Biol. Med. (in press).

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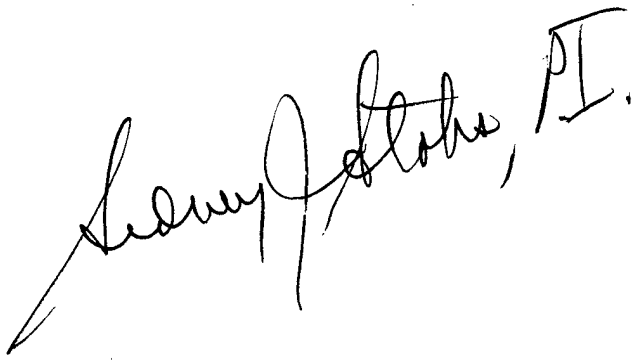
21. Bagchi, D., Wetscher, G.J., Bagchi, M., Hinder, P.R., Perdikis, G., Stohs, S.J., Hinder, R.A. and Das, D.K. Interrelationship between cellular calcium homeostasis and free radical generation in myocardial reperfusion injury. Free Rad. Biol. Med. (in press).

VI. INTERACTIONS/TRANSITIONS

Our laboratory has developed a HPLC system for assessing the urinary excretion of the lipid metabolites malondialdehyde, formaldehyde, acetaldehyde and acetone as biomarkers of lipid peroxidation. These techniques were developed in rodents and the technology has been applied to human subjects. These techniques have been used by Professor D.K. Das, Department of Surgery, University of Connecticut School of Medicine and at least three papers have been published using these techniques. Professor T.E. Adrian of the Creighton University School of Medicine Cancer Center has utilized these techniques as has Professor P.M. Pour at the Eppley Cancer Institute, University of Nebraska Medical Center. These techniques have been used to analyze serum and urine samples of trichloroethylene-exposed human subjects through the ad hoc Trichloroethylene Stakeholder Committee of the Wright-Patterson Air Force Base.

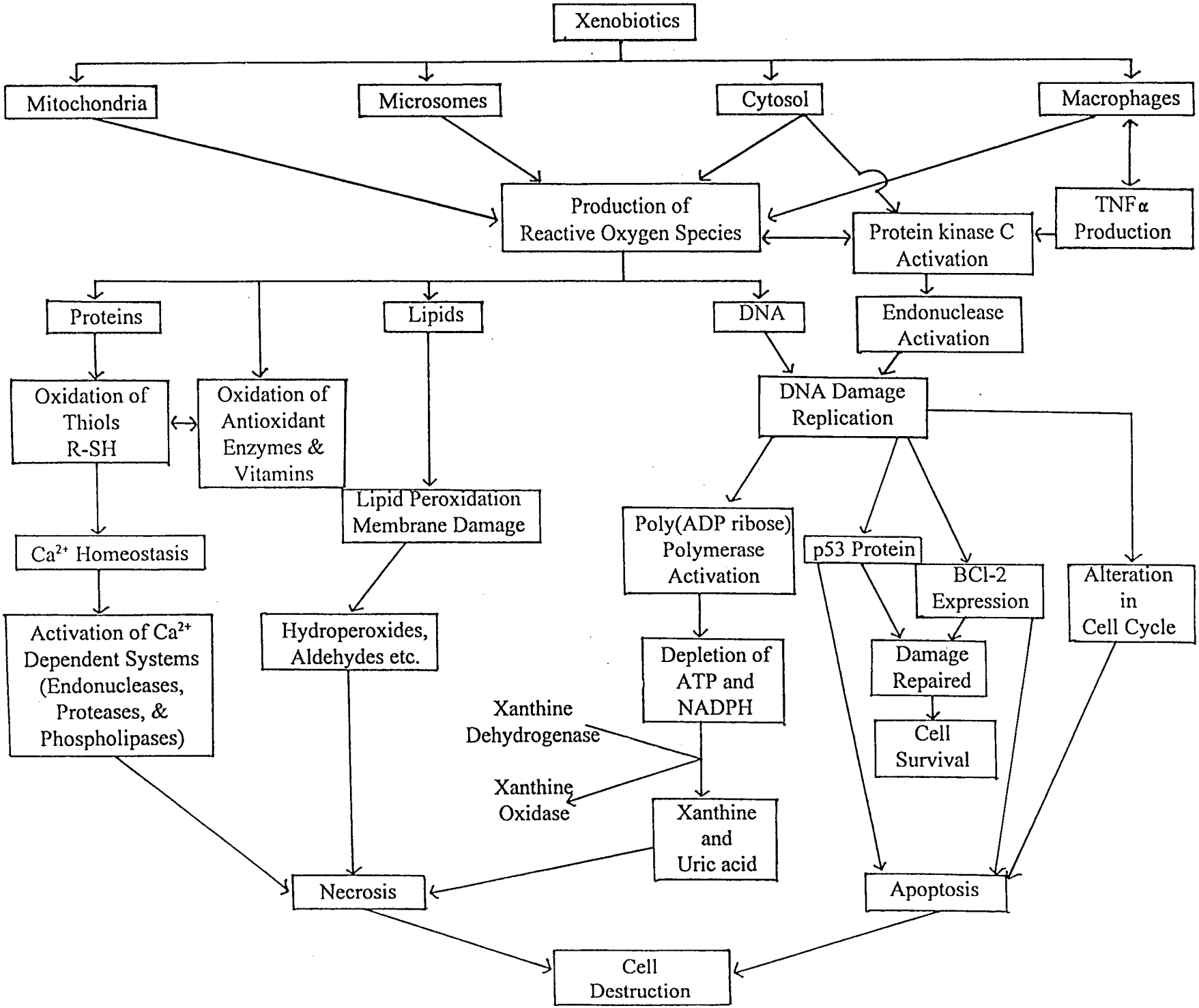
The investigators have presented numerous papers at national and international meetings.

The principal investigator has been appointed to the EPA Science Advisory Board on Dioxin Review Panel, and he has been appointed to the USP Division of Standards Development Advisory Panel on Identification and Standardization of Natural Products. Furthermore, he has participated in 1995 and 1996 in the Air Force Office of Scientific Research Predictive Toxicology Program Review, Fairborn, Ohio. Dr. Stohs also was an invited participant in an international symposium where he discussed the role of metal ions in the toxicity of tobacco smoke (1997).



APPENDIX #1

WORKING HYPOTHESIS REGARDING THE CASCADE OF EVENTS ASSOCIATED WITH THE MECHANISMS OF TOXICITY OF STRUCTURALLY DIVERSE ENVIRONMENTAL TOXICANTS


Xenobiotics
Mitochondria
Microsomes
Cytosol
Macrophages
Production of Reactive Oxygen Species
Protein kinase C Activation
TNFα Production
Proteins
Lipids
DNA
Endonuclease Activation
Oxidation of Thiols R-SH
Oxidation of Antioxidant Enzymes & Vitamins
DNA Damage Replication
Lipid Peroxidation Membrane Damage
Ca2+ Homeostasis
Poly(ADP ribose) Polymerase Activation
p53 Protein
BCl-2 Expression
Alteration in Cell Cycle
Activation of Ca2+ Dependent Systems (Endonucleases, Proteases, & Phospholipases)
Hydroperoxides, Aldehydes etc.
Depletion of ATP and NADPH
Damage Repaired
Xanthine Dehydrogenase
Cell Survival
Xanthine Oxidase
Xanthine and Uric acid
Necrosis
Apoptosis
Cell Destruction