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Free Radical Mechanisms of Xenobiotic Mammalian Cytotoxicities

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Our initial goal was to identify if free radical mechanisms are involved in the cytotoxicity of a number of IRP volume I and II chemicals. We found that a number of these agents act to enhance membrane lipid peroxidation in response to a standard dose of exogenous free radicals. Using chlorinated hydrocarbons (carbon tetrachloride, trichloroethylene, dichloroethylene, trichloroethane, dichloroethane) as a model for other IRP chemicals, we established conditions to measure lipid peroxidation in cultured smooth muscle and endothelial cells. These agents induced lipid peroxidation in the presences of physiological levels of iron in these vascular cells by a mechanism that doesn't require cytochrome P-450. Antiradical treatement with deferoxamine and Probucol (but not SOD, catalase, or mannitol) appear to reduce the toxicity of these agents. We have also detected the presences of free radicals in the cultured cells by ESR spin trapping following exposure to iron and chlorinated hydrocarbons. Although this free radical production does not appear to require biotransformation by cytochrome P-450, it is also not a result of spontanous oxidation of the IRP chemicals. Instead, it appears that

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First Year Progress Report for AFOSR-88-0016

"Free Radical Mechanisms of Xenobiotic Mammalian Cytotoxicities"

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Projected Duration
November 1, 1987 to October 31, 1990

Submitted, November 30, 1988

(A) Personal

During the first year of this project, Dr.'s Dickens, Weglicki, Phillips, Arroyo, and Tse have all contributed to the progress of this project. However, three significant changes in our staff will occur during year two. First, our post-doctoral fellow, Dr. S. Tse, recently left our group to accept a permenant position at Georgetown University. Second, the spin trapping experiments (specific aim #1), have been slowed by the delay in the arrival of the interface for the computer signal averager from Bruker. With this, Dr. Arroyo, who is involved in spin trapping experiments, is reducing her effort from 75% to 25%. The chronic exposure of cells to xenobiotics and the effect on intracellular macromolecules, such as phospholipids, glutathione, vitamin E, etc. will require more analytical quantification than has been performed in year 01. Therefore, Dr. Dickens percent effort will be increased from 20 to 50% so that he will have enough time committed to this project to perform these analytical studies.

In the 6-month report, we stated that the technician working on this project had quit and that we were seeking a replacement. Since then, we have hired Ellyn Knigin. She is a recent graduate of GWU and has proven a quite efficient lab technician. Also, a new ESR technician, Jin-Hu Ling, has been moved from 100% on another project to 50% effort on this project to help offset the loss of Dr. Tse and the reduction in effort by Dr. Arroyo. The following table gives the make up and percent effort of the staff working on this proposal during year two.

Dr. Dickens, PI	50%
Dr. Weglicki	5%
Dr. Phillips	10%
Dr. Arroyo	25%
Ellyn Knigin	100%
Jin-Hu Liang	50%

(B) Facilities

Computer interface for the ESR: In the six month progress report, we mentioned that we have had to put together a package from 4 different vendors for the computer ESR- computer signal averager since IBM instruments had gone out of business. The computer, interface boards, cables, and software have all arrived. However, we are still waiting for Bruker Instruments to ship to us from Germany the $1575.00 ESR interface to connect the ESR to the computer.

Also mentioned in the six month progress report, the tissue culture incubator funded by this project has been installed. The primary use of this incubator is for studies involving chronic exposure to these xenobiotics.

In addition, the second year of this proposal will make use of a new multi- user facility that has been set-up within the Division of Experimental Medicine under Dr. Dickens' direction. Specifically, the two units multi- user units to be utilized are:1) HPLC with radioactive flow-through detector. This unit will allow the cultured cells to be grown in the presences of

exogenous radioactive substrates and the effect of IRP chemical exposure on cellular macromolecules containing these radioactive compounds investigated. 2) GCMS. This instrument will be used to follow changes in membrane conjugated dienes more precisely that that using MDA to measure lipid peroxidation.

Bioquant densitometry. Another useful addition to our facility was the recent purchase by Dr. T. Phillips, W. Weglicki and M. Cassidy of a Bioquant system consisting of a fluorescent/phase contrast microscope, a video camera connected to computer aided software. Using this system, we are able to save fields of cells for trypan blue counting at a later date. We can also quantify such events as lipid droplet accumulation and membrane blebing by densitometry.

(C) Scientific Results

The data we have collected in the first year of this proposal provides strong support for the central hypothesis of this project (which was that **Free radical-induced injury participates in the pathogenic mechanisms of many lipophilic xenobiotic compounds**). The most dramatic finding is our demonstration that five chlorinated hydrocarbons (carbon tetrachloride, trichloroethylene [TCE], 1,1,1-trichloroethane [TCA], trans-1,2- dichloroethylene [DCE], and 1,2-dichlorethane [DCA]), are all capable of inducing lipid peroxidation in cultured mammalian cells apparently by cytochrome P-450 independent mechanisms. This is unlike the condition in hepatic cells, which are rich in cytochrome P-450, where this microsomal system plays a *key* role in the lipid peroxidation induced by CCl_4 and other chlorinated hydrocarbons. An important question for year two is to what extent other IRP chemicals also display the ability to produce non-cytochrome P-450 lipid peroxidation in vascular cells. This proposal consisted of four separate hypotheses, each with its own specific aim. The majority of the highlights during months 7 through 12 has been made in the area of specific aim #2. The highlights by specific aim are:

> Specific Aim #1 was to classify the xenobiotic compounds on the IRP list (volume 1 and 2) which, when introduced to a typical mammalian cell, leads to free radical production.

In the six month progress report, we showed that CCl_4, paraquat, and TCE all resulted in endogenous free radical production in cultured endothelial cells. At that time, we anticipated the arrival of the signal averager during the second six months, at which time we would extend these studies to other IRP chemicals. Unfortunately, we have been limited in our ability to investigate the free radical production induced *within* cultured cells by the delay in interfacing the computer to our ESR instrument. Bruker Instruments suggest delivery of the ER 133 ESR Computer interface in late November or Early December. With the arrival of this interface, the computer signal averager will be completed. We had predicated that we would need the signal averager to reduce the number of cells we had to use in order to obtain a detectable ESR signal. However, we have discovered an even more pressing need for it: We are currently using from 17 to 100 mM spin trap to detect endogenously-produced free radicals.

Unfortunately, control experiments demonstrate that these nitroxide-containing compounds are highly toxic to vascular cells and, at these levels, up to 50% of the cells are killed by the spin trap prior to exposure to the IRP chemicals being tested. This observation has raised serious concerns about the significance of the radicals trapped in the presences of IRP chemicals. Thus, we now realize that in addition to decreasing the number of cells per spin trapping experiment, the ESR signal averager also will provide us the opportunity to reduce the level of toxic spin traps to a non-lethal level.

While awaiting the computer averager, we have not turned away from spin trapping studies. The possible free radical mechanisms proposed to account for IRP chemical toxicity can be divided into six fairly specific areas (figure 1). A xenobiotic free radical would be ex-

POSSIBLE MECHANISM OF IRP CHEMICALS WHICH MAY PROMOTE CELLULAR FREE RADICAL DAMAGE.

DIRECT IRP RADICAL PRODUCTION:

1) DIRECT SPONTANOUS PRODUCTION OF FREE RADICALS (AS IS THE CASE FOR DIHYDROXYFUMERATE),

2) BIOTRANSFORMATION BY MICROSOMAL CYTOCHROME P-450

3) DIRECT INTERACTION WITH OTHER CELLULAR RADICAL (LIPID RADICALS) OR MEMBRANE LIPID HYDROPEROXIDES AND IRON TO PRODUCE AN IRP-RADICAL WHICH PROMOTES LIPID PEROXIDATION BETTER THAN THE INITIAL RADICAL OR HYDROPEROXIDE.

INDIRECT IRP EFFECTS ON FREE RADICAL MEMBRANE INJURY:

4) POISONING OF THE ELECTRON TRANSPORT CHAIN AND THE RESULTING RISE IN MITOCHONDRIAL REDUCING EQUIVALENTS

5) REDUCING INTRACELLULAR ANTI-RADICAL DEFENSES BY REACTING WITH GLUTATHIONE OR REDUCING INTRACELLULAR VITAMIN E LEVELS

6) CHANGING MEMBRANE PHYSICAL STATE SUCH THAT THE AVERAGE NUMBER OF LIPID MOLECULES INVOLVED IN THE AUTO-CATALYZED CHAIN OF LIPID PEROXIDATION IS INCREASED PRIOR TO THE OCCURANCE OF A CHAIN BREAKING EVENT.

Figure 1

pected in the first three of these mechanisms while, in the latter three mechanisms, no such free radical would be required. Using spin trapping, it is obvious that we can directly test for xenobiotic free radicals in the first three of these areas. In cases where the first mechanism occurs, IRP chemicals would be able to spontaneously produced free radicals in solution. An example of a compound with this ability is dihydroxyfumerate, which spontaneously produces superoxide when dissolved in an oxygenated buffer. One advantage in looking for the spontanous production of free radicals in solution is that vascular cells are not required. In these studies we can use extremely high levels of spin trapping agents and IRP chemicals. So far, we have tested most of the IRP volume I and II chemicals which we originally proposed to study. To date, we have found no evidence to suggest that any of thes agents are capable of spontaneously producing free radicals in solution. In our studies on lipid peroxidation within cultured cells (see specific aim #2), we found that iron plays a key role not only in the free radical-mediated lipid peroxidation, but also in the toxicity of these compounds. Therefore, we have begun testing the ability of these compounds to produce free radicals in solutions with

various levels of iron. Once again, we have yet to find a positive result. This negative data does not entirely rule out the possibility of spontaneous free radical formation since we may simply not be trapping the produced radicals. However, as observed in the six month progress report, we were able to see free radicals in some experiments using cultured cells and IRP chemicals. This seems to suggest that if spontanous radical formation occurred, we would have been able to detect it.

The second mechanism, which involves the enzymatic formation of free radicals though cytochrome P-450 mediated events, was the most likely candidate for producing IRP chemical-related free radical injury at the beginning of this project. The observed increase in vascular cell lipid peroxidation following exposure to chlorinated hydrocarbons coupled with the negative data for spontanous free radical production from these chemicals initially supported a role for cytochrome P-450. To our surprize, however, when we used heat (5 minute exposure to boiling water) to inactivate the cellular enzymes, the IRP chemicals completely retained their ability to induce lipid peroxidation. These results suggested that a mechanism *independent* of enzymatic processes (ie cytochrome P-450) was involved in the lipid peroxidation. Combining these two sets of information with the spin trapping experiments reported in the first progress report seems to suggest that the third mechanism in figure one is playing a significant role in the toxicity of at least the chlorinated hydrocarbons. The fact that iron plays a major role in the both the cellular toxicity (figure 2) and lipid peroxidation (figures 3-6) of the chlorinated hydrocarbons supports this mechanism. It should be pointed out, however, that iron plays a major role in membrane lipid peroxidation events: it is only the combination of our experimental observations that point strongly towards mechanism #3.

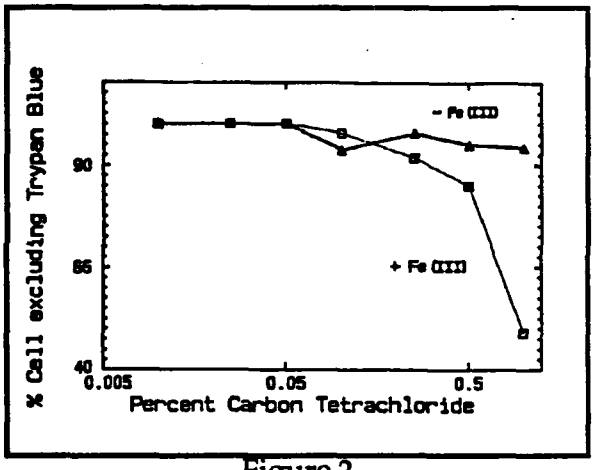

Figure 2

Effect of CCl4 ± 6 μM Fe(III) on endothelial cell viability as measured by trypan blue exclusion.

Specific Aim #2 was to follow free radical participation in cellular injury as indicated by lipid peroxidation and membrane structural alterations.

In the previous report, we showed that none of thirty different IRP chemicals could initiate measurable lipid peroxidation in isolated membranes alone, but that about 1/3 of those chemicals were able to enhance lipid peroxidation in the presences of an exogenous free radical generating system. We have screened this same list of compounds against cultured cells (in the absences of added iron) and found the same result. That is, none of the tested compounds are able to induce lipid peroxidation in cultured cells when the cells are exposed to the agent alone.

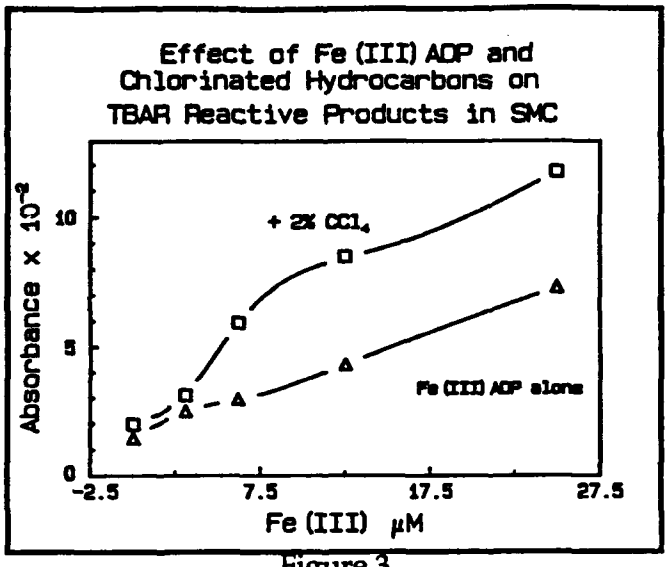

Figure 3

To investigate the effectiveness of IRP chemicals to induce membrane damage and promote lipid peroxidation, we chose to first study the primary chlorinated hydrocarbons. This choice was based upon our preliminary finding that was reported in the six month progress report (table two of that report) that in the presences of low levels of added iron, these compounds seemed able to promote lipid peroxidation. These studies, which are the bases of the paper submitted to Free Radical, Biology & Medicine (Appendix I), indicate that chlorinated hydrocarbons can induce lipid peroxidation in the presence of physiological iron concentrations. It is interesting that the need for iron is not only present in lipid peroxidation, but iron also plays a significant role in the toxicity of these agents towards the cultured cells (Figure 2). In this figure, cultured smooth muscle cells were exposed to various concentrations of carbon tetrachloride plus and minus 6 μM Fe(III) (chelated with ADP). The effect of carbon tetrachloride on lipid peroxidation in smooth muscle cells is shown in figure 3. In this figure, either iron alone or iron in the presences of 2% CCl_4 was used. These two figures demonstrate the synergistic toxic effect between iron and CCl_4. These results are also typical for the class of chlorinated hydrocarbons, except that, for reasons that we do not fully understand, the LD_{50} concentration seems to vary from day to day. One possible explanation is in difference in handling (ie degree of trypsinization) the cells prior to exposure to the IRP chemicals. To overcome this problem, we have initiated similar experiments using cells cultured in multi-well dishes. It is interesting to note that of the five tested chlorinated hydrocarbons (CCl_4, TCE, TCA, DCE, DCA), iron clearly adds to the toxicity of all except DCA. This is in agreement with the effect iron has on lipid peroxidation for these five compounds in both smooth muscle and endothelial cells, where only DCA fails to cause a stastically significant production of lipid peroxidation (Figure 6 and appendix I).

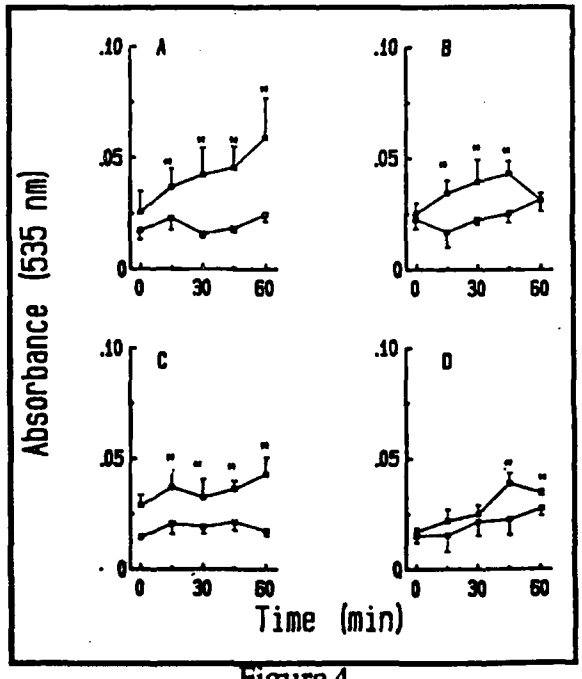

Figure 4

Time course of lipid peroxidation induced by 2% chlorinated hydrocarbons ±12.5 mM Fe(III). The upper curve in each panels contains the iron. The panels are: A) CCl_4; B) TCE; C) TCA; and D) DCE.

Specific Aim #3 was to investigate lipophilic IRP compounds to determine which ones reduce cellular conditioning against (resistance to) oxidative stress.

The primary result in this specific aim was reported in the six month progress report. That is, several general groups of experiments were performed to investigate whether or not IRP chemicals condition cells and/or membranes to make them more sensitive to oxidative stress (table one of the six month progress report). The first group of these experiments dealt with the effectiveness of these agents to enhance injury of isolated microsomal membranes when exposed to a standard dose of free radical generating system. Those results can be summarized by saying that the chlorinated hydrocarbons and the aromatic hydrocarbons proved effective in enhancing free radical injury with an exogenous free radical generating system. It was these experiments that helped us identify the chlorinated hydrocarbons as the first group of chemicals to test further. This mechanism of enhanced free radical-mediated lipid peroxidation provides support for several of the proposed mechanism in figure 1. First off, it supports mechanism #3, in that the exogenously-added free radical generating system produces lipid peroxidation which was then further promoted in the presences of the appropriate IRP chemicals. This interpretation is not the only one possible, however, since it is obvious that at least some of the xenobiotic can interact directly with the superoxide and hydroxyl radicals being produced in this artificial system to give rise directly to xenobiotic radicals (for example the trichloromethyl radical from CCl_4). However, in this case, where the xenobiotic competes with the membrane lipids for reactive oxygen radicals, the resulting xenobiotic radical must be more toxic to the cell than the initiating oxygen radical in that lipid peroxidation is promoted in spite of an *a priori* reduction in oxygen free radicals by interaction with these agents. The observation that at least some of the IRP chemicals "condition" membranes for lipid peroxidation in the presences of exogenously provided superoxide anion and hydroxyl radicals is also consistant with mechanisms #6 (Figure 1).

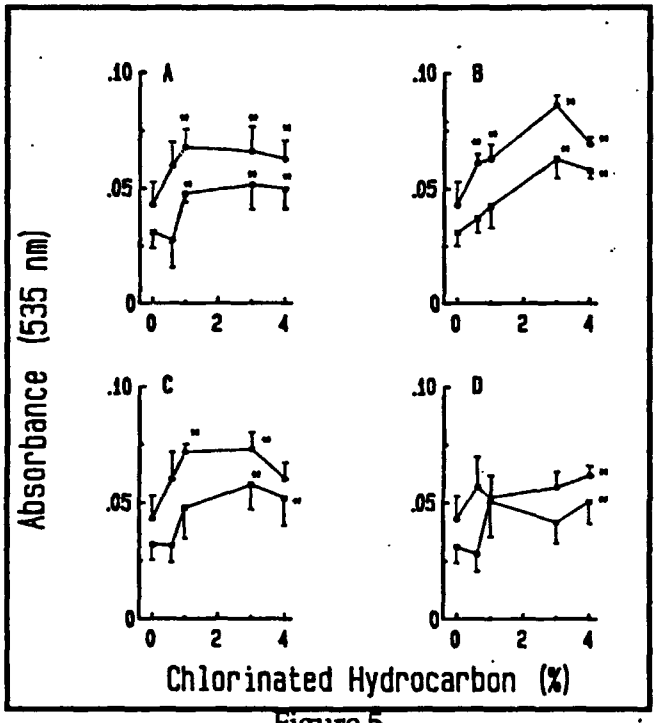

Figure 5

Effect of xenobiotics on smooth muscle cells in the precences of 6 (lower curves) and 12 μM (upper curves) or iron. Panels are: A) CCl_4, B) TCE, C) TCA, D) DCA

Besides simply making an exogenous generating system more toxic, there are other ways in which IRP chemicals may condition cells for free radical damage. One of these is to form

"inactive" conjugates of glutathione, thereby reducing a key intracellular anti-radical defensive mechanism. Another is cause long-term reductions in vitamin E levels. It would seem that the these indirect radical injury promoting mechanisms (figure 1, #'s 4 and 5) would require longer term exposures to detect. In fact, the many IRP chemicals which tested negative for promoting lipid peroxidation in isolated membranes may very well test positive in cells chronically exposed to these agents. Such conditioning mechanisms will be investigated during the next twelve months as part of the experiments involving chronic exposure of vascular cells to the our selected list of IRP chemicals.

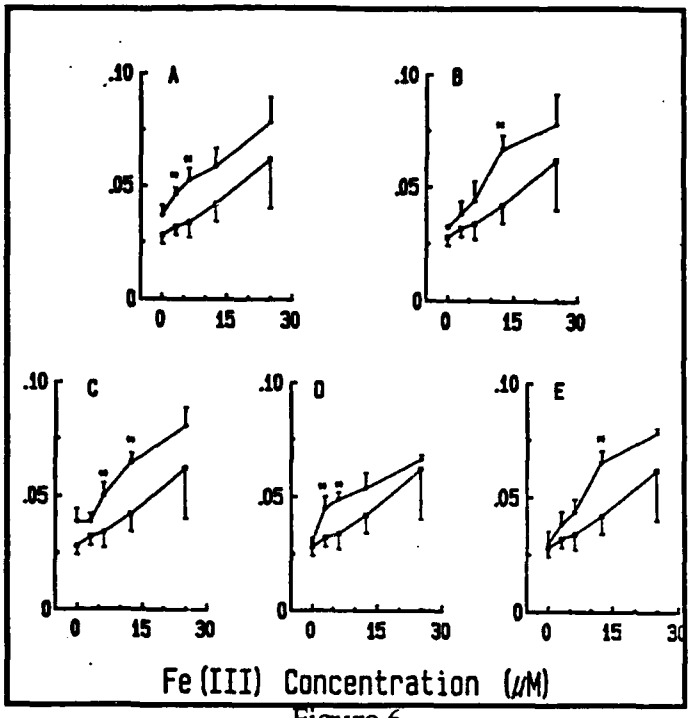

Figure 6

Effect of IRP chemicals on cultured endothelial cells. Conditons same as in figure 5, except that condition E contains DCA. In this case, 2% of the IRP chemicals was added ±Fe(III) as indicated in the figure.

Specific Aim #4 was to investigate the protective effects of a number of antiradical treatments against IRP chemical-related cytotoxicity.

In the proposal, this specific aim was identified as being one that would be investigated late, perhaps in year three. However, we have begun studies of the effectiveness of antiradical treatments against these agents. For the chlorinated hydrocarbons that were extensively studied in the last six months, SOD, catalase, SOD plus catalase, and mannitol were equally ineffective in preventing lipid peroxidation and cell death (by trypan blue exclusion). Deferoxamine, an iron chelator and frequently used antiradical agent, provide protection against chlorinated hydrocarbon lipid peroxidation. This, however, was not surprising since we had to add low levels of iron to induce this xenobiotic-associated lipid peroxidation. It will be worthwhile to closely investigate the cytotoxicity of IRP agents in the presences of deferoxamine to determine if intracellular iron levels may be playing a role in the cytotoxicity of these IRP chemicals in the absences of added extracellular iron. In this case, the LD_{50} dose of each IRP chemical may be increased in the presences of deferoxamine. In the six month progress report, we suggested also looking at vitamin E, BHA, and BHT as antiradical treatments. Vitamin E supplementation experiments are being initiated simultanously with the chronic IRP chemical exposure experiments (some cells being exposed to IRP chemicals, some to the chemicals plus exogenous vitamin E). However, instead of BHA or BHT, we have chosen

to use a drug, probucol, which is used clinically. Probucol is little more than two BHT molecules containing two sulfur groups that are interconnected through a methyl-like bridge (Figure 7). In studies in our laboratories, probucol is a tremendously effective antiradical agent, perhaps

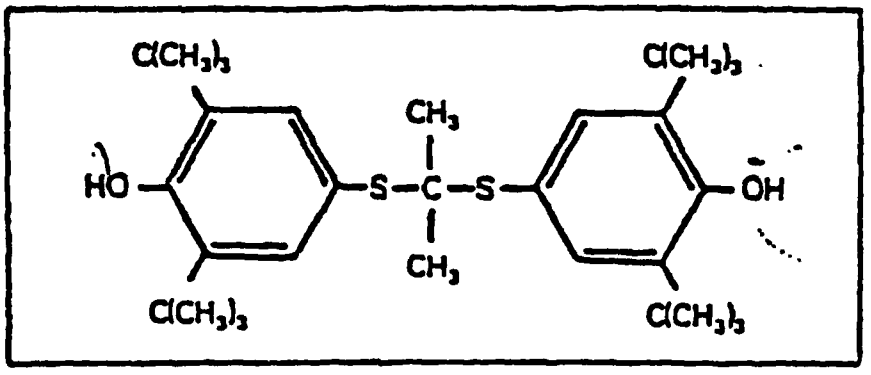

Figure 7 Structure of Probucol

as good as vitamin E. In response to exogenously provided free radicals (DHF + Fe(III)ADP), very low levels of probucol (0.2 and 2 uM) were equally effect as vitamin E in significantly reducing lipid peroxidation in cultured endothelial cells. In these experiments, 2 uM probucol reduced lipid peroxidation by 42% while the same concentation of vitamin E reduced it by 49%. We are hopeful that either probocol, deferoxamine, or a combination of these agents will prove effective in reducing the toxicity of many of these IRP chemicals.

SUMMARY: Our initial goals were to identify if free radical mechanisms are involved in the toxicity of a number of hydrophobic IRP volume I and II chemicals. In preliminary experiments we found that a number of these agents acted to enhance membrane lipid peroxidation in response to a standard exogenous dose of free radicals. Using chlorinated hydrocarbons, a class of IRP chemicals which tested positive for enhancing lipid peroxidation, we found that these agents were able to induce vascular cell lipid peroxidation in the presences of physiological levels of iron by a non-cytochrome P-450 mechanism. In addition, we have preliminary evidence that antiradical treatment with probucol or deferoxamine (but not SOD, catalase, or mannitol) reduce the toxicity of chlorinated hydrocarbons. We have also detected the presences of free radicals in cultured endothelial cells upon exposure to iron and these chlorinated hydrocarbons. The data on lipid peroxidation, when combined with our spin trapping studies has lead us to the following current working hypothesis to explain the free radical-mediated component of chlorinated hydrocarbon toxicity in cultured vascular cells. (Figure 8).

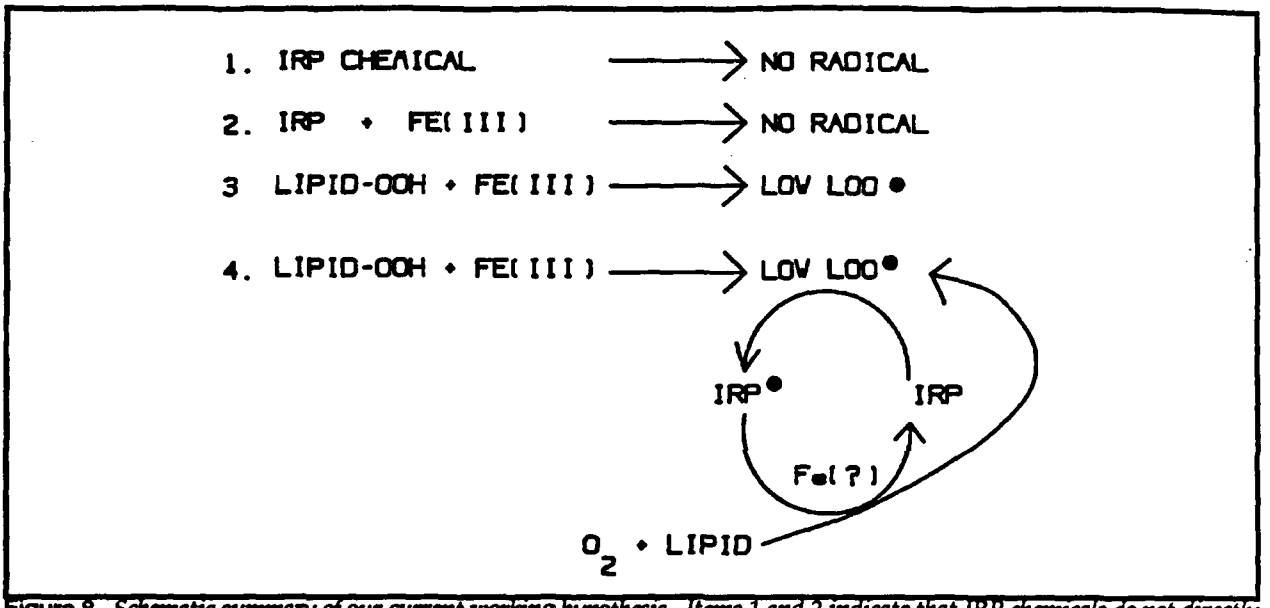

Figure 8. *Schematic summary of our current working hypothesis. Items 1 and 2 indicate that IRP chemicals do not directly induce free radical production (or lipid peroxidation). However, IRP agents interact with naturally occuring, low levels of endogenous lipid hydroperoxides (or initiating lipid radicals promoted by the presences of iron) giving rise to a hydrophobic IRP radical. In this model, the hydrophobic IRP radical is more mobile in the membrane bilayer than a normal lipid radical and is thus a better promotor of continued membrane lipid peroxidation than the initial lipid radical. Iron, and perhaps oxygen appears to play a role in this cycle.*

(D) Goals for Month's 13-24

Specific Aim #1: With the addition of computer-aided-ESR-signal collection, we anticipate that the problems of poor ESR spectral problems, as discussed in the six month progress report, will be overcome. It is further hoped that we will be able to reduce the spin trap levels to non-lethal levels. We also anticipate expanding our studies to include more of the IRP chemicals and to complete our investigations as to whether the free radicals produced are a consequence of spontaneous oxidation of the xenobiotic, or of oxidase-assisted biotransformation, or, as currently seems likely, due to an interaction with naturally occuring endogenous lipid hydroperoxides (or lipid radicals). Also, a more systematic study of the nature of the intracellularly spin trapped radicals will be initiated as soon as conditions and instrumentation are established to maximize adduct signals.

Specific Aim #2: The basic experimental conditions that give rise to vascular cell lipid peroxidation in response to acute exposure of IRP chemicals have now been well established using chlorinated hydrocarbons. In the next few months, we plan to complete the testing of all of the IRP chemicals on our list for their effectiveness under similar conditions. There appears to be a marked correlation between the interaction of iron and chlorinated hydrocarbons on vascular cell lipid peroxidation and on the toxicity of these agents on these cells (figures 2 & 3). This interaction will be investigated more closely to determine the exent of such a correlation (see also aim #4, page 10). In order to rapaidly screen a large number of conditions, the trypan blue studies of cellular viability will be augmented by Chromium-51 release studies. A minor drawback to our lipid peroxidation data is the relatively low levels of malondialdehyde

(MDA) detected in these experiments. Since MDA binds cellular macromolecules and can also be metabolized by cells, we are developing GCMS methods for directly detecting and quantifying lipid peroxidation products to further validate our findings on lipid peroxidation.

Specific Aim #3: As mentioned in the six month progress report, year two will see the initiation of experiments involving chronic, long term exposure of cultured cells to the IRP chemicals. Sub-lethal doses of the xenobiotics will be given to the cells and these cells cultured for several generations. Internal level of lipid peroxides (both as MDA and by the developing GCMS methodologies) following this long-term exposure will be evaluated. Furthermore, the ability of the cells treated in such a manner to resist further insult by a standard dose of exogenously supplied free radical generating systems will be determined. In those cells that show a positive result for free radical associated injury by either of the above manipulations (either direct evidence of free radical injury by elevated lipid peroxides after culture, or enhanced free radical injury following this chronic exposure) will be further investigated to determine if endogenous anti-radical defense systems were effected by the chronic treatment.

Specific Aim #4: Modulation by antiradical treatments of the correlation between iron-mediated IRP chemical lipid peroxidation and cell viability may also help to determine the extent to which free radical mechanisms contribute to the cytotoxicity of these agents. Therefore, more emphasis will be placed on the preventing of toxicity through the use of anti-radical agents. The results to date suggest that oxygen radicals are not play a role in the toxicity of these agents. However, clearly iron has a key role in this toxicity so that iron chelators, like deferoxamine, will need to be investigated. Also, cells will be enriched in vitamin E, in an attempt to provide protection against IRP chemicals. If, as it seems, the toxicity of these agents is at least partly mediated through free radical mechanisms, it should be possible to show a relationship between endogenous vitamin E levels in supplemented cells and IRP chemical toxicity. Also, as mentioned earlier, we have found that the drug probucol is an effective antiradical treatment. In one preliminary study, probucol was added to one tube during an experiment investigating the toxicity of CCl_4 on cultured smooth muscle cells. In this experiment, probucol appeared to provide a significant degree of protection against CCl_4 + Fe(III)ADP toxicity as measured by trypan blue exlusion. This finding suggest that we may discover treatment, perhaps with combinations of iron chelation and probocol or vitamin E treatment, which will prove useful in attenuating both acute and chronic exosures to these agents.

(E) Appendix including publications and abstracts

The following material has been presented or submitted for publication.

Appendix I. Tse, S.Y.H., Mak, I.T., Weglicki, W.B., and Dickens, B.F. Lipid peroxidation in vascular smooth muscle and endothelial cells in response to chlorinated hydrocarbons. Free Radical Biology & Medicine, submitted November 1988.

Appendix II. Tse, S.Y.H., Mak, I.T., Weglicki, W.B., and Dickens, B.F. Chlorinated hydrocarbons enhance lipid peroxidation in cultured endothelial cells and smooth muscle cells. J. Mol. Cell. Cardiol. 20 (Suppl. 3):S-36, 1988.

Appendix III. Dickens, B.F., Tse, S.Y.H., Mak, I.T., and Weflicki, W.B. Pro-oxidant effect of chlorinated hydrocarbons on cultured vascular cells. Abstract submitted to the 1989 FASEB meeting to be held in New Orleans, LA.

Lipid Peroxidation in Vascular Cells: Synergistic Interaction between Iron and Chlorinated Aliphatic Hydrocarbons

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Running title: cardiotoxicities of halocarbons

Lipid Peroxidation in Vascular Cells: Synergistic Interaction between Iron and Chlorinated Aliphatic Hydrocarbons

Abstract

Carbon tetrachloride and chloroform are potent hepatotoxins, acting by production of reactive free radicals. These and related chlorinated aliphatic hydrocarbons, eg. trichloroethylene and trichloroethane, also cause acute and chronic cardiotoxicities, via mechanisms that are still obscure. Some of these toxic effects may result from free radical associated membrane damage to the vascular tissues. The pro-oxidant effects of carbon tetrachloride, trichloroethylene, 1,1,1-trichloroethane, 1,2 - dichloroethane and *trans* -1,2 - dichloroethylene were assessed in cultured arterial endothelial and aortic smooth muscle cells. Treating the cells with the above chemicals at final concentrations of 0.6 to 4% did not increase the formation of thiobarbiturate reactive products above background levels. Lipid peroxidation was significantly enhanced (up to 2-fold) in the presence of extracellular iron (3.2 to 25 μM $FeCl_3$ chelated by 32 to 250 μM ADP). The synergistic interaction between iron and chlorinated hydrocarbons in promoting lipid peroxidation may contribute to the cardiotoxicity of these agents.

Key words: halogenated hydrocarbons, lipid peroxidation, iron, endothelium, vascular smooth muscles.

Introduction

Exposures to chlorinated hydrocarbons can cause acute and chronic cardiotoxicities[1]. For example, the primary manifestations of acute trichloroethane (TCA)[2] and trichloroethylene (TCE)[3] poisoning include cardiac and pulmonary depression, ventricular fibrillation and sudden deaths. In the cases of chronic exposures to these volatile chlorinated solvents, such as in both industrial settings and in solvent abuse, manifestations such as myocarditis - like syndromes[4], ventricular dilation and hypertrophies[5], hemolytic anemia[6], peripheral vasculitis and vasodilatation have been reported. Therefore, apart from being toxic to the liver and the kidney, chlorinated hydrocarbons also induce toxic effects to the myocardium, the red blood cells and the peripheral vasculatures. It has been postulated that acute cardiac toxicities of chlorinated solvents are due to increased myocardial sensitivity to catecholamines, resulting in decreases in ventricular fibrillation threshold[1]. However, mechanisms mediating the chronic toxic effects are still obscure.

In the liver, chlorinated hydrocarbons such as carbon tetrachloride (CCl_4) and chloroform are metabolized by the cytochrome P450 system to toxic free radicals, which are responsible for the hepatotoxicities[7,8]. Production of free radicals from carbon tetrachloride[9-12], chloroform[13], halothane[14], and bromotrichloromethane[9] in hepatic tissues have been demonstrated by ESR- spin trapping techniques. Using similar techniques, but under anaerobic conditions, free radicals are also formed from dichloroethanes and trichloroethanes[15], although at a much lower rates than from carbon tetrachloride and chloroform. These reactive free radicals can induce lipid peroxidation[16-18], protein cross-linking, and denaturation[19] and DNA damage[20].

Cytochrome P450 systems are also found in the myocardium and in the endothelial cells[21]. Although the concentration of cytochrome P450 is small compared to that of the liver (about 0.1%), cardiovascular tissues may be more susceptible to oxidative injury because of the deficiency in antioxidative mechanisms, such as glutathione peroxidase, epoxide hydratase and conjugating enzymes[22]. In fact, many similarities exist between the cardiotoxicities induced by halogenated hydrocarbons and those induced by free radical mechanisms. For example, superoxide anion (O_2^-) and hydroxyl radical (• OH) have been reported to be potent vasodilators[23-25]. Hydroxyl radicals also increases vascular permeability[26,27] and produces vasculitis like syndromes[28]. It is known that iron plays an important role in the generation of hydroxyl radical and in lipid peroxidation. Iron has also been shown to play a key role in in vivo lipid peroxidation during exposure to chlorinated hydrocarbons. Ethane exhalation in rats exposed to CCl_4 was greatly decreased in the presence of the iron chelator desferrioxamine[29]. Desferrioxamine also inhibited hepatic necrosis in the same model. In this study, we are investigating whether chlorinated hydrocarbons induce lipid peroxidation in cultured vascular cells, and whether iron and the generation of free radicals play any role in the cardiotoxicities of these chlorinated solvents.

Methods:

Cell Culture: Bovine pulmonary arterial endothelial cells (ATCC#CCL207) were cultured in Media 199 (Media Tech) supplemented with 5% penicillin- streptomycin, HEPES, and 20% fetal calf serum (GIBCO). The fetal calf serum was inactivated at 57°C for 30 minutes prior to use. Rabbit aortic smooth muscle cells were cultured in the same media, except the serum concentration was reduced to 10%. Confluent plates were

trypsinized with 0.5% trypsin - 0.2% EDTA solution (Sigma). As soon as the cells are detached from the plates, the digestion was stopped by the addition of 5 ml of growth media with serum. The cells were pelleted and washed twice with potassium sucrose buffer (120 mM KCl, 50 mM sucrose, and 10 mM K_2HPO_4/KH_2PO_4, pH 7.2) at room temperature and then suspended in the same buffer at 3 to 5 million cells per ml.

Incubations: Briefly, 200 μl of cell suspension prepared as described above was diluted to a final volume of 500 μl in the same buffer. Test compounds were added to the buffer, either directly or as ethanolic solutions, to a final concentration of 0.6 to 4 % (v/v) with or without Fe(III)ADP. Compounds tested were: carbon tetrachloride, trichloroethylene, 1,1,1 trichloroethane, trans-1,2- dichloroethylene and 1,2-dichloroethane. The structures of these compounds are shown in Figure 1. Stock solution of Fe(III)ADP was made up with 100 μM of $FeCl_3$, chelated by 1 mM of ADP. Dilutions were made from the stock solution to achieve nominal concentrations of 3.2 to 25 μM $FeCl_3$. Except when noted, the cell suspensions were incubated at 37°C. Lipid peroxidation was assessed by the thiobarbiturate method as previously described[19]. The incubation was stopped by the addition of 50 μl of 5% trichloroacetic acid and 500 μl of 0.5% thiobarbituric acid (TBA). The mixture was then heated at 80°C for 20 minutes, then 500 μl of ice cold trichloroacetic acid (70%) was added to stop the color development. The mixture was centrifuged (10 minutes at 1000 x g) and the supernatant absorbance was measured at 535 nm.

Chemicals: Carbon tetrachloride, trichloroethylene, 1,1,1 trichloroethane, and 1,2 trichloroethane was obtained from Fisher Scientific. *Trans* 1,2 dichloroethylene was

purchased from Eastman Kodak. All other chemicals were obtained from Sigma Chemical Co.

Results

Incubation of cultured endothelial or smooth muscle cells with chlorinated hydrocarbons alone did not induce lipid peroxidation (Table 1). Also shown were the effects of increasing iron concentrations. At low concentrations of iron (3.1 to 6.2 μM), no notable increase in lipid peroxidation was observed compared to blank control. However, higher concentrations of iron (12.5 to 25 μM) stimulated lipid peroxidation. The effect of incubating endothelial cells in the presence of both iron (3.1 to 25 μM) and 2% chlorinated hydrocarbons is shown in Figure 2. Lipid peroxidation was potentiated at the lower doses of iron. At the higher iron concentrations, while lipid peroxidation was measurable in the absence of the chlorinated hydrocarbons, these compounds nonetheless enhanced lipid peroxidation significantly above that observed with the iron chelate alone. The effect of varying the dose of chlorinated hydrocarbons on lipid peroxidation is shown in Figure 3. As can be seen, increasing the dose of CCl_4, TCE, TCA, DCE or DCA from 0.6 to 2% (V/V) generally resulted in a dose related increase in the production of TBA-reactive materials.

Smooth muscle cells also displayed lipid peroxidation when incubated in the presence of the test compounds and Fe(III)ADP (Table 2). DCA did not appear to induce lipid peroxidation in smooth muscle cells. The synergistic effects between chlorinated hydrocarbons and iron was observed between iron concentrations of 3.2 to 25 μM, but not at 50 μM: presumably because this higher concentration of iron resulted in significantly elevated background TBA- reactive material. Lipid peroxidation also increased with

increasing dose of CCl_4, TCE, TCA, or DCE in the incubation medium (Figure 4), or with increasing incubation time (Figure 5). Similar dose response curves were obtained when the test compounds were added as ethanolic solutions (data not shown).

To investigate whether superoxide and hydroxyl radical production was involved in the observed effects, we added superoxide dismutase (bovine erythrocyte SOD, 30,000 U/mg, Sigma) and catalase (bovine liver, 11,000U/mg, Sigma) to the incubation mixture. SOD, catalase and a combination of both have little inhibitory effect on the formation of TBA reactive materials. Use of Fe(II)ADP instead of Fe(III)ADP exhibited similar interaction with chlorinated hydrocarbons with no difference in the magnitude of response between the two iron species.

Discussion

The amount of cytochrome P450's in the cardiovascular tissues is about 0.1% compared to that found in the liver[22]. Our results suggest that such low levels of cytochrome P450 were not sufficient to metabolize carbon tetrachloride and the other chlorinated hydrocarbons to an appreciable extent, and there was little observable peroxidation when the cells were incubated with these compounds alone. However, when iron is added, at micromolar concentrations to incubation mixtures containing cultured vascular cells and chlorinated hydrocarbons a significant enhancement of lipid peroxidation was observed. This synergistic interaction was similar between endothelial cells and smooth muscle cells, despite differences in their metabolic capabilities. In the hepatocytes, metabolism of carbon tetrachloride to the trichloromethyl radical is at least 1000 fold faster than the corresponding reactions of dichloro- and trichloroethanes[30]. Our results indicate

that metabolism by cytochrome P450 is probably not a major determinant in the peroxidative effects observed in vascular cells, as the formation of TBA-reactive materials was within the similar order of magnitude for the five compounds tested.

In the membrane, lipid peroxidation proceeds via three phases : a) initiation by free radicals; b) propagation and amplification by radical chain reactions in the membrane; and c) termination, when radicals are quenched by antioxidants or two radicals react to form a non radical. Trivalent iron induced lipid peroxidation by two possible mechanisms. First, ferric ion can be reduced by superoxide anion to ferrous ion and subsequently, ferrous ion catalyses the formation of hydroxyl radical via Fenton's reaction[31]. Alternatively, ferric and/or ferrous ion can catalyze the decomposition of lipid peroxide in the membrane. With sufficient propagative ability, the lipid and lipid peroxy radicals can go on to give observable peroxidative endpoints, such as increase formation of TBA- reactive materials, even in the absence of superoxide and hydroxyl free radicals. In vascular cells, the latter mechanism may be more probable in explaining the synergistic interactions between ferric ion and chlorinated hydrocarbons. In this case, these cells did not possess sufficient cytochrome P450 to metabolize these xenobiotics to the free radical intermediates. However, adding ferric ion chelates may catalyze the decomposition of lipid hydroperoxide in the membrane. The presence of the chlorinated hydrocarbons, which are all very lipophilic may promote the formation of chain-carrying lipid peroxy radicals or facilitate the propagation of the radical chain reaction. There are several lines of evidence in support of this notion. First, the chlorinated hydrocarbons that we have tested did not induce lipid peroxidation in the absence of iron, indicating that chloromethyl radicals were not formed in sufficient

quantities to initiate lipid peroxidation. Also, SOD and/or catalase did not inhibit the peroxidation, thus, the effects were not mediated by the generation of hydroxyl radicals via the Fenton's reaction. Second, higher doses of iron also induced lipid peroxidation, although the effects were invariably enhanced by chlorinated hydrocarbons. Third, Fe(II)ADP was as effective as Fe(III)ADP in the synergistic effects, as Fe(II) and Fe(III) are similarly active in catalyzing lipid peroxide decomposition[32,33].

There are several possible mechanisms by which chlorinated hydrocarbons may promote or propagate lipid peroxidation. First, by a general solvent effect, partitioning of lipid soluble chlorinated hydrocarbons into the membrane may cause changes in membrane fluidity, in turn facilitating the reaction between lipid peroxide and other lipid molecules. Alternatively, these compound may react with lipid radicals or lipid peroxides, forming secondary radicals within the membrane, and carry on the peroxidative chain. Our results indicated that there was a gradation of potencies among the five compounds tested: carbon tetrachloride and trichloroethylene were similar in potencies, and trichloroethane, dichloroethylene and dichloroethane displayed a descending order of potency. This suggests that the ability of the compound to promote lipid peroxidation in the presence of iron may be related to the number of chlorine substitutions (for example between trichloroethylene and dichloroethylene) and the degree of saturation (such as between trichloroethylene and trichloroethane, and between dichloroethylene and dichloroethane). Furthermore, dichloroethane had virtually little or no effect in the smooth muscle cells, arguing against a general solvation mechanism.

Toxic injuries and lipid peroxidation of the endothelial lining and the vascular smooth muscle cells may be important in a variety of pathological conditions[34-36]. For example, necrosis of vascular cells may result in the release of chemotactic factors and platelet adhesion factors[37] which may be important in the initiation of atherosclerotic plaques. Besides, lipid peroxidation of the membrane may release toxic secondary metabolites, such as malondialdehyde and lipid peroxides, which may bind to proteins and DNA[18,19]. Our studies have demonstrated that commonly encountered chlorinated solvents can interact with iron to induce lipid peroxidation. The concentrations of iron required are in the micromolar range. It is probable that acute toxic injury of vascular cells and blood cells may cause release of iron into the micro-environment of the endothelial lining. Lipid peroxidation in the presence of both iron and chlorinated hydrocarbons may cause further necrosis of the surrounding cells and may contribute to the chronic cardiotoxic effects of these agents.

Acknowledgments

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Abbreviations

CCl_4: carbon tetrachloride
TCE: trichloroethylene
TCA: Trichloroethane
DCE: trans 1,2 dichloroethylene
DCA: 1,2 dichloroethane
SOD: superoxide dismutase
TBA: thiobarbituric acid

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Table 1

Formation of thiobarbituric acid reactive materials in vascular cells incubated with either 2% of chlorinated hydrocarbons or with Fe(III)ADP, 3.1 to 25 μM.

Conditions		TBA-Reactive Material (535 nm) Endothelial Cells	Smooth Muscle Cells
Control		0.028 ±0.004	0.014 ± 0.005
CCl_4		0.033 ±0.006	0.020 ± 0.006
TCE		0.031 ±0.002	0.024 ± 0.007
TCA		0.033 ±0.008	0.019 ± 0.007
DCE		0.028 ±0.003	0.018 ± 0.018
DCA		0.026 ±0.007	0.020 ± 0.006
Fe(III)-	3.1 μM	0.032 ±0.004	0.025 ±0.006
ADP	6.2 μM	0.034 ±0.007	0.031 ±0.007
	12.5 μM	0.042 ±0.008*	0.043 ±0.010*
	25.0 μM	0.062 ±0.022*	0.073 ±0.016*

Values are means of n = 4-6, ± S.E., * $p < 0.05$

Table 2

Lipid peroxidation in vascular smooth muscle cells incubated with 2% chlorinated hydrocarbons and Fe(III)ADP, 3.1 to 25 μM.

	Fe(III)ADP (μM)				
	0	3.1	6.2	12.5	25
Control	0.0143 ±.005	0.0248 ±.006	0.0294 ±.007	0.0430 ±.010	0.0730 ±.0016
CCl_4	0.0197 ±.006	0.0310 ±.004	0.0592 ±.011	0.0848 ±.012	0.1172 ±.011
TCE	0.0244 ±.006	0.0350 ±.009	0.0519 ±.010	0.0766 ±.008	0.1032 ±.011
TCA	0.0188 ±.007	0.0374 ±.004	0.0480 ±.008	0.0865 ±.011	0.0978 ±.022
DCE	0.0177 ±.005	0.0338 ±.003	0.0407 ±.004	0.0752 ±.006	0.1234 ±.027
DCA	0.0199 ±.006	0.0222 ±.005	0.0404 ±.015	0.0797 ±.008	0.0764 ±.010

Values are absorbance at 535 nM, n = 5 ± S.E.

Table 3

Effects of SOD and / or catalase on MDA formation in vascular smooth muscle cells.

	TBA-Reactive Material (Abs. 535nm)					
	Control	CAT	Control	SOD	Control	SOD/CAT
CCl_4	0.077 ±.012	0.083 ±.009	0.077 ±.003	0.063 ±.003	0.053 ±.002	0.078 ±.006
TCE	0.072 ±.009	0.073 ±.004	0.073 ±.005	0.059 ±.004	0.050 ±.007	0.059 ±.004
TCA	0.077 ±.006	0.114 ±.003	0.073 ±.007	0.069 ±.006	0.061 ±.003	0.072 ±.007
DCE	0.066 ±.012	0.101 ±.004	0.059 ±.003	0.052 ±.003	0.068 ±.008	0.071 ±.011

Concentration of test chemicals was 2% in each case. SOD: 3000 U/ml; catalase 1100 U/ml. Data represents mean ± s.e.m. of 4 experiments.

Figure Legends

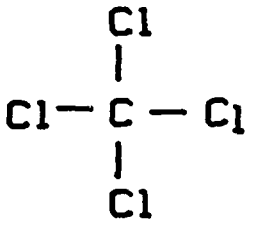
Figure 1. Chemical structure of chlorinated hydrocarbons tested.

Figure 2. Increased production of lipid peroxidation (measured as thiobarbiturate reactive products, absorbance at 535 nm) in cultured endothelial cells. A. CCl_4; B. TCE; C. TCA; D. DCE; and E. DCA. Closed symbol: cells incubated with 2% chlorinated hydrocarbon and 3.1 to 25 μM of Fe(III)-ADP chelate. Opened symbols: control, cells incubated with the Fe(III)ADP alone. Each data point represents average of 3 experiments. * $p < 0.05$ (compared to control values, Student's t-test, two tailed).

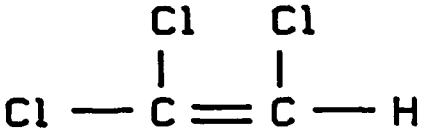
Figure 3. Production of thiobarbiturate reactive products by endothelial cells at increasing doses of halogenated hydrocarbons. All incubations were performed in the presences of 12.5 μM Fe(III). Chlorinated hydrocarbons were: A. CCl_4, B. TCE; C. TCA; D. DCE; and E. DCA

Figure 4. Production of thiobarbiturate reactive products in smooth muscle cells incubated with 0.6 to 4% chlorinated hydrocarbons. Incubation were in the presence of 6.25 μM (closed symbols) or 12.5 mM Fe(III)ADP (open symbols). Chlorinated hydrocarbons tested were: A. CCl_4, B. TCE; C. TCA; and D. DCE. Each data point represents average of 4 experiments. * $p < 0.05$ compared to controls (Student's t-test, 2 tailed).

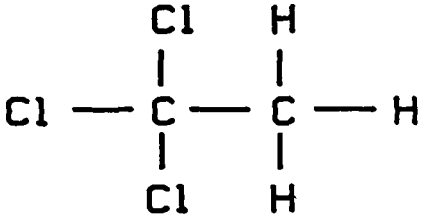
Figure 5. Time course of increase in thiobarbiturate reactive products in smooth muscle cells incubated with 2% of halogenated hydrocarbons in the presences (closed symbols) and absences (open symbols) of 6.25 μM of Fe(III)ADP. Each data points represents average of 4 experiments. * $p < 0.05$ (Student's t-test, two tailed).



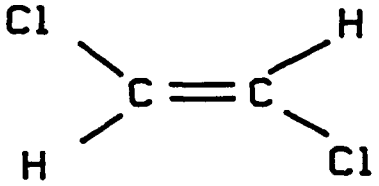
(A) CARBON TETRACHLORIDE



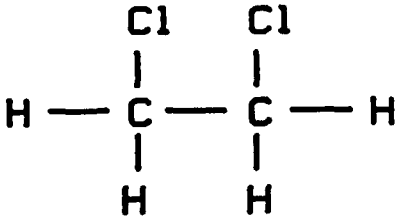
(B) TRICHLOROETHYLENE



(C) 1.1.1-TRICHLORO-ETHANE



(D) trans-1.2-DICHLOROETHYLENE



(E) 1.2-DICHLORO-ETHANE

Free Radical Mediated injury by Xenobiotics

1. Spontenous production of free radicals
   (DHF is an example)
2. Biotransformation of xenobiotic
   (Perequat. Carbon Tetrachloride)
3. Direct Poisoning of anti-radical Defenses
   (phenezine methosulfate)
4. Direct effect on the redox state of the cell
   (metabolic poisons)
5. Direct effect on auto-catalyzed chain of
   lipid peroxidation.

Appendix II

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106 LYSOLECITHINS AND PHOSPHOLIPASE A_2 AS A NEW GROUP OF ENDOTHELIUM-DERIVED RELAXING FACTORS. Wolf A, Saito T, Alves C, Saeed M, Bing RJ. Department of Experimental Cardiology & Scientific Development, Huntington Medical Research Institutes, Pasadena, California

Lysolecithins (monacylphosphatidylcholines) derived from lecithin through the action of Phospholipase A_2 have been found to be effective relaxants of rabbit aortic strips. Relaxation is slower but lasts longer than that of acetylcholine; it is inhibited by hemoglobin, methylene blue and nordihydroguiaretic acid, while indomethacin and superoxide dismutase have no effect. Lysolecithins derived from egg yolk (palmitoyl and stearoyl) are the most effective relaxants, followed by synthetic lysolecithin; their degree of relaxation depends on aliphatic chain length with longer chains exhibiting the greatest relaxation. Relaxation is endothelium dependent and is related to the activation of guanylate cyclase. Phospholipase A_2 when injected into the left atrium of rabbits in situ, causes prolonged decline in coronary vascular resistance. Lysolecithins differ in several respects from EDRF and NO. They may represent a new type of endothelium dependent muscle relaxant.

107 CHLORINATED HYDROCARBONS ENHANCE LIPID PEROXIDATION IN CULTURED ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS. S.Y.H. Tse, I.T. Mak, W.B. Weglicki and B.F. Dickens. Dept. of Medicine, George Washington University, Washington D.C. 20037.

Chlorinated aliphatic hydrocarbons (e.g. trichloroethylene, trichloroethane) are known to cause arrythmias in human subjects (Hamilton and Hardy's Industrial Toxicology, 4th ed., pg. 232, 235), the nature and mechanism of these arrhythmogenic effects are not well understood. Since some arrhythmogenic agents are known to be pro-oxidant in nature, we investigated the possible pro-oxidant effects of some aliphatic chlorinated hydrocarbons, including carbon tetrachloride, trichloroethylene, 1,1,1 trichloroethane, 1,2 dichloroethane and trans1,2dichloroethylene, in both cultured bovine pulmonary arterial endothelial cells (ATCC # CCL207) and rabbit aortic smooth muscle cells. None of the chemicals tested increased peroxide production above background, measured as thiobarbiturate reactive products. However, lipid peroxidation was significantly enhanced (20 to 200% increase compared to controls) in the presence of extracellular Fe(III)ADP (0.25 to 0.5 mM). The data suggest that chlorinated hydrocarbons may act by enhancing the production of free radicals, via synergistic interaction with iron. The promotion of lipid peroxidation may contribute to the cardiotoxicity of these compounds.

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108 HUMAN AND BOVINE VASCULAR ENDOTHELIAL CELLS PRODUCE METABOLITES WHICH INHIBIT BINDING OF [^{3}H]PYRILAMINE to HISTAMINE H_1 RECEPTORS. Patricia B. Williams, Miriam D. Rosenthal, and Julie Averitt, Departments of Pharmacology and Biochemistry, Eastern Virginia Medical School, Norfolk, VA 23501.

Vascular endothelial cells respond to histamine by mobilizing arachidonic acid and synthesizing prostacyclin. Although the histamine receptor is expressed on calf pulmonary artery endothelial (CPAE) cells, radioligand binding studies using [^{3}H]pyrilamine at 37°C are confounded by a cellular metabolite which interferes with binding of the ligand. Studies at 4°C minimize production of the metabolite and permit characterization of highly specific binding to H_1-type receptors with a K_d of 3.0×10^{-9}M and B_{max} of 4.2×10^{-8}M. The inhibitory metabolite can be removed from conditioned medium by dialysis, 3500 MW retention. Binding of [^{3}H]pyrilamine to human umbilical vein endothelial (HUVE) cells remains stable for >30 min. at 37°C. Medium conditioned by HUVE depresses binding to both CPAE and HUVE cell types. Thus HUVE cells, like CPAE, produce a metabolite which inhibits binding to the H_1 receptor; slower production by HUVE cells permits stable [^{3}H]pyrilamine binding at 37°C. These results may also explain why HUVE cells consistently mobilize arachidonic acid in response to histamine, while this response in CPAE cells is inconsistent. (Supported in part by NIH-RR05771, the A.H.A., and its VA Affiliate.)

APPENDIX III. ABSTRACT SUBMITTED TO 1989 FASEB MEETING IN NEW ORLEANS, LA.

Pro-oxidant effect of chlorinated hydrocarbons on cultured vascular cells.

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Acute and chronic cardiotoxicities can be induced by chlorinated aliphatic hydrocarbons. Free radical-mediated events, such as lipid peroxidation, is one proposed mechanism involved in this toxicity. We have investigated the possibility that chlorinated halocarbons (0.6-2.0% v/v) possess pro-oxidant effects in cardiovascular cells by exposing aortic smooth muscle and arterial endothelial cells to carbon tetrachloride (CCl_4), trichloroethylene (TCE), 1,1,1-trichloroethane (TCA), 1,2-dichloroethane (DCA), and trans-1,2- dichloroethylene (DCE), either alone or in the presences of extracellular Fe(III)(3.1 to 25 μM) chelated with ADP. Alone, none of these halocarbon induced membrane lipid peroxidation, as measured by thiobarbutaric acid reactive material (TBAR), in either cell type. Lipid peroxidation could be induced in these cells by the addition of the ADP chelate of Fe(III) at levels of 6.25 μM or higher. When chlorinated hydrocarbons were added to cells in the presences of the iron chelate, statistically significant increases in lipid peroxidation occurred. These elevations in lipid peroxidation were observed even at 3.1 μM Fe(III), a concentration at which lipid peroxidation was was not induced in the absence of added halocarbon suggesting a synergistic effect between iron and halocarbons on lipid peroxidation. The potency of these compounds for inducing a pro-oxidant effect in the presence of iron as measured by TBAR production is CCl_4 > TCE > TCA > DCE > DCA. Heating the cells to 80°C for 2 minutes prior to the addition of iron and halocarbon failed to block this pro-oxidant effect, suggesting that enzymatic biotransformation of the halocarbons by enzymes such as cytochrome P-450 oxidases is not a necessary step in the pro-oxidant effect of these compounds on lipid peroxidation in the presences of iron. (Supported by AFOSR F08671-88001600)