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December 29, 2003

Toxicology

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Impact of Environmental Exposures on the Mutagenicity/Carcinogenicity of Heterocyclic Amines

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Abstract

Carcinogenic heterocyclic amines are produced from overcooked foods and are highly mutagenic in most short-term test systems. One of the most abundant of these amines, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), induces breast, colon and prostate tumors in rats. Human dietary epidemiology studies suggest a strong correlation between either meat consumption or well-done muscle meat consumption and cancers of the colon, breast, stomach, lung and esophagus. For over 20 years our laboratory has helped define the human exposure to these dietary carcinogens. In this report we describe how various environmental exposures may modulate the risk from exposure to heterocyclic amines, especially PhIP. To assess the impact of food on PhIP metabolism in humans, we developed an LC/MS/MS method to analyze the four major PhIP urinary metabolites following the consumption of a single portion of grilled

chicken. Adding broccoli to the volunteers' diet altered the kinetics of PhIP metabolism. At the cellular level we have found that PhIP itself stimulates a significant estrogenic response in MCF-7 cells, but even more interestingly, co-incubation of the cells with herbal teas appeared to enhance the response. Numerous environmental chemicals found in food or the atmosphere can impact the exposure, metabolism, and cell proliferation response of heterocyclic amines.

Introduction

Well-done cooking of muscle meats results in the natural formation of heterocyclic aromatic amines that have been found to be potent mutagens in various assay systems. These same compounds produce tumors at multiple organ sites in both male and female mice and rats (Shira et al. 1997; Sugimura 1997). Furthermore, 100 percent of non-human primates given one of these heterocyclic amines (2-amino-3-methylimidazo[4,5-f]quinoline; IQ) developed hepatocarcinomas after a very short latency (Adamson et al. 1994; Adamson et al. 1990). More than two thirds of human epidemiological studies (both case-control and cohort) correlating meat intake and cooking practices have shown an increased risk of cancer for individuals that prefer well-done meat. These epidemiology studies identify breast, colon, stomach and esophagus as the primary target organs. For human prostate cancer, more studies are needed to determine if meat intake is correlated with the disease. Several authors have described cooking methods such as low temperature frying (Knize et al. 1994; Sko et al. 1995), pre-microwaving (Felton et al. 1994), marinating (Salmon et al. 1997), and frequently

turning the meat during frying (Salmon et al. 2000) that markedly reduce the HA levels in foods.

Schwab et al. (Schwab et al. 2000) wrote a comprehensive review of compounds that may inhibit the genotoxic/carcinogenic effects of HAs. They compiled data from more than 150 reports that described more than 600 agents that attenuated the effects of HAs. Most of the agents were evaluated in *Salmonella* TA98 mutation assays, but aberrant crypt foci, liver foci and DNA adducts were also used to assess *in vivo* effects. Protection was presumed to be a result of a number of different mechanisms, including 1) HA inactivation by a number of different mechanisms by compounds like chlorophyllin, 2) inhibition of the activating cytochrome P450 1A family of enzymes, 3) induction of detoxifying enzymes, or 4) enhancement of DNA repair mechanisms. This report will not attempt to review these studies, but give an account of new literature studies and those primarily ongoing in our laboratory, including those which show an enhancement (increased risk) rather than an inhibition of HA effects.

We have known for many years that HAs are metabolized by cytochrome P450 1A1 and 1A2 to an N-OH intermediate (Holme et al. 1989; Kaderlik et al. 1995) and that further metabolism by a series of conjugating enzymes is either further activating or detoxifying, depending on the specific compound (see Fig 2). It is reasonable to suspect that any polycyclic aromatic hydrocarbon, flavonoid, or other environmental inducer that changes the activity of the cytochrome P450 enzymes would impact the kinetics of HA metabolism. What is less well understood is the effect of competition at the CYP450 or other enzymatic active site by these, and other, environmental compounds.

Gooderham et al. (Gooderham et al. 2002) described an increased estrogenic response in cells exposed to PhIP. We have found a similar response in MCF-7 human breast cancer cells. This important finding may help explain why PhIP behaves as a complete carcinogen in rat breast tumorigenesis. Clearly, estrogenic behavior should increase cell proliferation and make the mammary cells quite susceptible to PhIP genotoxicity.

Given these very interesting data, it is important to determine the extent to which these HAs show enhanced mutagenicity or carcinogenicity when environmental compounds are given prior to or at the same time as the heterocyclic amines are consumed.

Heterocyclic amine exposure

Extensive analysis of many different kinds of food has demonstrated the presence of heterocyclic amines in muscle meats cooked to a well-done state. Table 1 shows selected data for commonly consumed beef in North America. These data were obtained from meats cooked "well-done" in local restaurants. In these samples, MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), PhIP, and IFP (2-amino-(1,6-dimethylfuro[3,2-e]imidazol[4,5-b]pyridine) are found at levels that vary from below the level of detection (about 0.1 ng/g) to 19 ng/g. Studies of the amounts of HAs produced in foods as a result of regional cooking practices are reported for Great Britain (Murray et al. 1993), Sweden (Johansson et al. 1994; Skoget al. 1997), Switzerland (Zimmerli et al. 2001), Spain (Busquet et al. 2003) and Japan (Wakabayashi et al. 1993). In most

cases PhIP and MeIQx tend to be the most abundant HAs, but the levels are very dependent on the cut of the meat product and the cooking method.

Metabolism in rodents and humans

In mouse liver microsomes, PhIP is metabolized to two major metabolites, one of which is the direct α -acting mutagen, N⁷-OH-PhIP. The other major mouse metabolite has a hydroxylation at the 4' position of the phenyl ring and appears to be a detoxification product (Turteltaub et al. 1989). In rat hepatocytes PhIP is transformed to 4'-OH-PhIP as the primary product, followed by glucuronidation and sulfation of this metabolite (Langouet et al. 2002; Malfatti et al. 1994). Glucuronide and sulfate conjugates of 4'-OH-PhIP have been detected in human hepatocytes, but as much more minor products (Langouet et al. 2002). Thus, it is important to understand factors that favor formation of one or the other of these, as the ratio will affect the level of reactive intermediates available for DNA binding (adduct formation) and mutation. Other activation pathways besides P450 hydroxylation must be present in the mouse. Studies of transgenic CYP1A2 null mice demonstrated that PhIP was equally potent in causing lymphomas and tumors of the lung and liver in the mice lacking CYP1A2 as in wild type mice (Kimura et al. 2003). DNA adduct levels were reduced in the knockout mice, suggesting that the level of adducts is not directly correlated with the formation of blood, liver or lung tumors in this system (Snyderwine et al. 2002).

In humans, cytochrome P450 1A2 activation of the parent amine to the corresponding 2-hydroxyamino intermediate is the predominant step. As figure 1 shows, conjugating reactions can occur impacting the overall metabolism of the HA (Buonarati et al. 1990; Turteltaub et al. 1989). For PhIP, the N⁷-hydroxy intermediate can be

esterified by sulfotransferase and/or acetyltransferase to generate the highly electrophilic O-sulfonyl and O-acetyl esters (Buonarati et al. 1990). But unlike the rodent, human PhIP metabolism is dominated by glucuronidation. Both the N^2 and the N^3 positions of PhIP are glucuronidated directly (most likely these are non-reactive intermediates) and glucuronidation of N-hydroxy PhIP intermediates can be envisioned as a direct detoxification pathway (Malfatti et al. 2001).

Foods affecting HA damage

Table 2 reviews studies demonstrating that many foods have the potential to affect the mutagenic/carcinogenic effects of the heterocyclic amines, by a variety of mechanisms. This data represents studies published since the review by Schwab et al. (Schwab et al. 2000). With complex mixtures the exact mechanism and chemical or chemicals responsible for the effect are frequently unknown. But in some cases, specific food components are known to decrease the level of DNA adducts, suggesting a possible protective effect for cancer initiation. DNA adducts can be relevant biomarkers of cancer risk, but as stated above, at least in the mouse, they may not always be for these tumor sites. Table 3 shows that a variety of chemicals in food reduce PhIP-DNA adducts in rodents and in many cases at the site of tumor genesis in that species. In contrast, caffeine increased colon adducts in the rat after PhIP exposure (Takeshita et al. 2003). Much more needs to be done to understand the impact of dietary interactions, competing substrates and adduct levels on tumorigenesis.

Human studies with broccoli

Identification of the four major metabolites of PhIP in humans allowed our laboratory to develop LC/MS/MS detection and quantification methods for these metabolites in urine after a single meal of cooked well-done chicken (Kulpetal.2003; Kulpetal.2000). Measuring changes in the amount of total metabolites excreted or the rate of excretion of specific metabolites with time can be used to understand individual differences in metabolism, as well as whether environmental agents can impact the metabolism of PhIP.

Using this assay, we are now doing intervention studies to determine whether environmental chemicals such as isothiocyanates and/or indole-3-carbinols in broccoli and other cruciferous vegetables can impact PhIP metabolism. In a preliminary study, eight healthy men were fed a single meal of well-done chicken after abstaining from broccoli or related cruciferous vegetables for 3 days. Metabolites were determined in urine collected in 6h increments. After eating cooked broccoli for 3 days, the protocol with well-done chicken was repeated. We found that the percent of the total metabolites excreted in the first 6h after chicken consumption was increased in all but one individual after the broccoli intervention (Figure 2). This suggests chemicals in the broccoli increase the metabolic rate for PhIP metabolism, possibly by increasing the oxidation by P4501A2.

Murray et al. fed a group of 20 volunteers broccoli and Brussels sprouts and showed induction of CYP1A2 activity and a decrease of the parent MeIQx and PhIP in the urine (Murray et al. 2001). They did not measure the metabolites, but the finding is consistent with that from our laboratory.

Computational simulation of environmental chemical effects

To understand the role of compounds that either induce enzyme activity or compete at the P450 active site we have begun to model the active site of human cytochrome P450 1A2. This is possible by homology modeling of the structure of other P450s from rabbit and bacteria. By calculating the molecular dynamics of the docking of the small molecules into the active site we can correlate mutagenic activity of various HAs and at the same time get comparative active site-binding parameters (Colvin et al. 1998; Sasaki et al. 2002).

Inhibition of mutagenic activity in bacteria by flavonoids

We were able to compare the inhibition of PhIP and MeIQx mutagenicity by co-incubating the HAs with 2 flavonoids, naringenin and apigenin, in an Ames *Salmonella typhimurium* TA98 assay (Figure 3). Interestingly, although they are structurally very similar, they produced a different inhibitory response. Apigenin was able to inhibit the mutagenicity of PhIP by 90% and MeIQx by 69%. Naringenin, which differs by a single double bond, was only able to inhibit PhIP mutagenicity by 16%, although the compound was more potent in the presence of MeIQx, inhibiting mutagenicity by 44%.

Impact of dietary supplement on PhIP induced mutagenicity, cell proliferation and estrogen response

Fifty percent of North Americans use some form of dietary supplement, natural product, or alternative therapy (Ernst et al. 1999; Kaegi 1998; Smith et al. 1999). People

diagnosed with cancer are motivated to try alternative products because they do not want to leave unexplored any option that could treat their cancer, prevent recurrence, or improve their quality of life (Richardson et al. 2000). The literature on dietary supplements is widely disseminated in the lay press (Percival 1994; Stainsby 1992; Whitaker 1995) and on websites where information, presented as factual, is rarely backed by scientific investigation (Stainsby 1992). The consumers choosing to use these products are not only emotionally vulnerable to spending money on expensive products that may or may not have value, but as cancer survivors with increased risk of recurrence, may be physically vulnerable to further exposure. We are interested in understanding how breast cancer survivors who take these products while continuing to eat well-done meat may be impacting their health.

Flor-Essence® Herbal Tea is a widely consumed herbal tonic, available at health food stores and on the internet in North America. The product is a mixture of eight herbs, including burdock root (*Arctium lappa*), sheep sorrel (*Rumex acetosella*), slippery elm (*Ulmus rubra*), Turkish rhubarb (*Rheum palmatum*), watercress (*Nasturtium officinale*), blessed thistle (*Carduus benedictus*), red clover (*Trifolium pratense*) and kelp (*Laminaria digitata*). Individually, these herbs have been shown to have anti-carcinogenic, estrogenic, anti-estrogenic, and anti-oxidant properties, among others.

We evaluated Flor-Essence® in the Ames test to determine if the anti-mutagenic activity described by others for one of its components, Turkish rhubarb (Horikawa et al. 1994; Lee et al. 1991), was detectable in this complex mixture. Using the *S. typhimurium* strain TA98, we determined that Flor-Essence® tea inhibits the mutagenic activity of both PhIP ($p = 0.04$) and IQ ($p = 0.002$) in a dose-dependent manner (Figure 4). In

contrast, Flor -Essence® did not inhibit the mutagenicity of DMBA and benzo[a]pyrene using the TA100 *S.typhimurium* strain (data not shown). TA100 is a bacterial strain that is sensitive to base substitutions, and is commonly used to quantify DMBA and benzo[a]pyrene mutagenicity. These results suggest that Flor -Essence® may be inhibiting pathways that are important for PhIP activation, while having no effect on DMBA activation.

N-OH-PhIP causes cytotoxicity in LNCaP and PC3 prostate cells at concentrations above 1 µg/ml. Adding Flor -Essence® herbal tonic with the N -OH-PhIP protects prostate cancer cells from cytotoxicity (Figure 5). Cell toxicity was measured using the CellTiter 96 Nonradioactive Cell Proliferation Kit. The results from these experiments suggest that Flor-Essence® herbal tonic may prevent the formation of DNA adducts known to be related to N -OH-PhIP-induced cytotoxicity. Taken together, these experiments suggest that Flor -Essence® can influence PhIP -induced DNA damage in both bacterial and mammalian cells.

We have been investigating the estrogenic response in MCF -7 human breast cancer cells after PhIP exposure using computational, analytical and biochemical tools. Early data show that PhIP induces a weak, but significant, dose -dependent activation of this response in these cells. Interestingly, Flor -Essence® herbal tonic, which is highly estrogenic in this assay system, is able to enhance the estrogenic response caused by PhIP in these cells. Figure 6 shows the effect of Flor -Essence® and PhIP on estrogen receptor activation in MCF -7 cells. Estrogen receptor activation is measured using a standard estrogen responsive reporter plasmid containing three estrogen responsive elements (EREs) upstream of the luciferase reporter gene. Flor -Essence® at 2%

concentration gave a 110% response that was not significantly different than physiological concentrations of estrogen. When the tea and PhIP were co-administered the estrogen activation was 150%. This was additive for the two compounds and clearly higher than estrogen alone. More work will need to be done to understand the ramifications of strong genotoxic agents like PhIP that may also have an effect on cell growth. Our experiments investigating the effects of an herbal supplement on PhIP exposure are an excellent example of how being exposed to complex mixtures of chemicals may have a competing effect on the ultimate risk of cancer development; in this case we see both protection and enhancement of tumorigenesis. The ultimate understanding of the effects of exposure to complex mixtures will come after we learn much more about the details and kinetics of the competing pathways.

Conclusions

The impact of environmental exposures on the mutagenicity and carcinogenicity of heterocyclic amines has been shown to be significant and real. Upregulation of the cytochrome P450 1A2 level is not unexpected, as we see enzyme inducers like polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and flavonoids induce rodent hepatic enzyme levels in our experimental studies. From these animals we make S9 or microsomes to activate HAs for mutagenicity testing. Now, because of the sensitivity of LC/MS/MS techniques, we can study various food ingredients, such as those found in broccoli, for their ability to change the human pharmacokinetics of the HAs in different individuals. Possibly, eating cruciferous vegetables with meat could lower risk, but more needs to be done to understand these interactions fully. Finally, the

cell proliferation activity of HAssee is enhanced with other dietary exposures, such as specific herbal teas. Specifically, these combinations might be avoided in women susceptible to breast cancer or those trying to prevent re-occurrence of this disease. Understanding complex dietary exposures and competing risks should be studied in cell culture and rodents, but the ultimate understanding of these risks will only happen when we study humans individually and in large populations.

Acknowledgements

Work performed under the auspices of the USD DOE by UC, LLNL under contract W-7405-Eng-48. This work was supported by: NCI grants CA55861 and CA94709; DOD Prostate Cancer Research Program DAMD17-00-1-0011; California Breast Cancer Research Program 7IB-003 and LDRD02-FS-006.

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Table 1. Heterocyclic amines are found in various quantities in restaurant -cooked meat
(ng heterocyclic amine per g cooked meat).

Sample	Restaurant- doneness	IFP	MeIQx	PhIP
Toploinsteak	A-welldone	7.0	1.3	7.7
Toploinsteak	B-welldone	nd	1.3	0.86
Flanksteak	C-welldone	8.2	1.9	19
Primerib	C-welldone	nd	nd	nd
Beef(fajitas)	D-unspecified	1.4	0.93	1.7

nd=notdetected.

Table 2. Recent reports of foods or contaminants shown to affect HA effects *in vitro* or *in vivo*.

Food/exposure	Effect	Reference
Green, black, white tea	Inhibit various enzymes, Prevent DNA binding	(Santana-Rios et al. 2001), (Kru et al. 2001), (Muto et al. 2001), (Lin et al. 2003), (Lin et al. 2003)
Cruciferous vegetables	Induce GST, P4501A2	(Steinkellner et al. 2001), (Murray et al. 2001)
Wheat bran fiber	Lignin binding	(Yu et al. 2001)
Coffee	Induce glucuronosyl and glutathione transferases	(Huber et al. 2002)
Milk products	Binding to bacteria	(Knasmüller et al. 2001)
Cigarette smoke	Induce CYP1A2	(Mori et al. 2003)
Fat	Increase intestinal tumors in rats	(Ubagai et al. 2002)
Organophosphate insecticides	Enhances mutagenic response	(Wagner et al. 2003)

Table 3. Food components reducing PhIP-DNA adducts in rodents.

Food component	Target tissue	Reference
Chlorophyllin	Colon	(Guo et al. 1995)
Indole-3-carbinol	Colon	(Guo et al. 1995)
	Mammary, colon, liver	(Hee et al. 1997)
	11 of 12 tissues reduced	(Hee et al. 2000)
Conjugated linoleic acid	Liver and mammary gland	(Schut et al. 1997) ,
		(Futakuchi et al. 2002)
Resveratrol	Mammary gland	(Dubuisson et al. 2002)
Omega-3 fatty acids	Liver, spleen, small intestine in	(Josyula et al. 1998)
	mouse, not rat	
Quercetin, genestein, tangeretinor β-Naphthoflavone	Colon	(Breinholt et al. 1999)
Docosahexaenoic acid	Colon	(Takahashi et al. 1997)

Figure Legends.

Figure 1. Pathways for the activation and detoxification of PhIP.

Figure 2. Effect of broccoli on the percent of PhIP metabolites excreted in the urine 0-6 hours after consuming well-cooked chicken. Black bar represents percent of total PhIP metabolites excreted during the 0-6 hours collection time before the broccoli intervention. Grey bars represent excretion during the same time period after the intervention.

Figure 3. Inhibition of mutagenic activity of MeIQx (open bar) and PhIP (filled bar) activity in *Salmonella typhimurium* TA98. Apigenin and naringenin differ in only a single double bond, indicated by an arrow, yet apigenin is a more potent inhibitor of both MeIQx and PhIP. Both flavonoids were used at 15 nmoles/plate.

Figure 4. Effect of Flor-Essence® on PhIP (triangle) and IQ (circle) induced mutagenicity using *Salmonella typhimurium* strain TA98 to test Flor-Essence® for anti-mutagenic activity. Flor-Essence® herbal tonic inhibits the mutagenic activity of PhIP and IQ in a dose-dependent manner. The slope of each line is significant for IQ ($p = 0.002$) and PhIP ($p = 0.04$).

Figure 5. PhIP-induced toxicity in LNCaP and PC3 prostate cell lines is inhibited by 2% and 4% Flor-Essence® administration. Cells were pretreated with 1 µg/ml NOH-PhIP, 2% or 4% Flor-Essence®, or 1 µg/ml NOH-PhIP with 2% or 4% Flor-Essence® added. In both LNCaP and PC3 cells 4% Flor-Essence® protects from the cytotoxic effects of NOH-PhIP. a: significantly less than control, $p < 0.05$; b: significantly less than control, $p < 0.01$; c: significantly greater than NOH-PhIP alone, $p < 0.05$; d: significantly greater than NOH-PhIP alone, $p < 0.01$.

Figure 6. Effect of PhIP and Florsalicylic acid on estrogen receptor activation in MCF-7 human breast cancer cells. Data are represented as percentage of the response of estrogen. Error bars represent standard error of the mean. a: significantly greater than control, $p < 0.01$; b: significantly different than estradiol, $p < 0.01$.











