AN ABSTRACT OF THE THESIS OF

Caitlin A. Crimp for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics and Honors Baccalaureate of Arts in Spanish presented on May 23, 2012.
Title: Gender-Dependent Mechanisms of Alpha-Tocopherol Protection from Benzo[a]pyrene Exposure in Rats.

Abstract approved: _____
Debbie Mustacich

Polycyclic aromatic hydrocarbons (PAHs), including benzo[a]pyrene (B[a]P), are environmental pollutants linked to increased disease susceptibilities. Alpha-Tocopherol (αT) supplementation decreases B[a]P-DNA adducts in smokers, particularly women; but the mechanism is unknown. To test the hypothesis that αT protection from B[a]P exposure is gender-dependent, male and female rats received 7 daily subcutaneous (SQ) injections of αT (100 mg αT/ kg body wt) or vehicle, followed by a single intraperitoneal injection of B[a]P (20 mg/kg, spiked with ^{3}H-B[a]P) on day 9. Urine and bile were collected pre- and post-B[a]P; plasma and tissues were collected 5 or 24 h post-B[a]P. αT supplementation increased αT levels to a greater extent in females than in males. Compared to vehicle, αT supplementation increased total urinary and biliary excretion of B[a]P and/or B[a]P metabolites more than 2.5-fold in females, but decreased total excretion in males ($p<0.05$). αT prevented B[a]P-induced increases in urine 8-isoprostanes (males) and decreased tissue malondialdehyde levels in a tissue- and gender-dependent manner. Thus, αT protection from B[a]P exposure is gender-dependent and occurs by both antioxidant and non-antioxidant mechanisms. Further elucidation of the mechanism(s) of αT protection against environmental toxins may lead to the development of protective strategies for occupational PAH exposures.

Key Words: Vitamin E, alpha-tocopherol, PAH, benzo[a]pyrene, gender
Corresponding e-mail address: crimpc@onid.orst.edu

Gender-Dependent Mechanisms of Alpha-Tocopherol Protection from Benzo[a]pyrene Exposure in Rats

by

Caitlin A. Crimp

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degrees of

Honors Baccalaureate of Science in Biochemistry and Biophysics (Honors Scholar) and
Honors Baccalaureate of Arts in Spanish (Honors Scholar)

Presented May 23, 2012
Commencement June 2012

Honors Baccalaureate of Science in Biochemistry and Biophysics and Honors Baccalaureate of Arts in Spanish project of Caitlin A. Crimp presented on May 23, 2012.

APPROVED:

_____

Mentor, representing Biomedical Sciences/ Linus Pauling Institute

_____

Committee Member, representing the School of Biological and Population Health Sciences/ Linus Pauling Institute

_____

Committee Member, representing Environmental and Molecular Toxicology/ Linus Pauling Institute

_____

Chair, Linus Pauling Institute

_____

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Caitlin A. Crimp, Author

ACKNOWLEDGEMENTS

Firstly, I would like to extend my sincere gratitude to Dr. Debbie Mustacich for serving as my mentor for the past four years, for the many hours that she has dedicated to instructing me in lab techniques, data presentation, and so much more. Her genuine commitment to training young investigators has allowed me many unique opportunities to grow as a scientist. This project would not have been possible without her.

Secondly, I would like to thank Dr. Maret Traber and Dr. Dave Williams for serving as committee members on my honors thesis project. My thanks for their time and for their evaluation of this work that has enriched my thesis experience.

Additionally, the work for this project could not have been completed without the guidance that I received from Scott Leonard and Alan Taylor, as they taught me several of the assays for this project and were always more than willing to help. Many thanks to Tammie McQuistan who worked beside me and helped in countless ways, including assistance with LSC data and animal experiments. I am very appreciative for the help of Lisa Poland and Liana Granum with animal experiments, and for that of the Hagen lab with the glutathione assay. The Baird lab was also very generous in sharing their space and equipment. I would also like to thank many members of the Traber lab for all of the great “lab meetings” at Brew Station and Bombs.

Two very special people have made a profound impact on my life here at OSU, and there are no words to describe my appreciation for Kevin Ahern and Indira Rajagopal; for their wisdom, support, and commitment to the quality education of their students.

Finally, I would like to thank my family, especially my parents, Greg and Shana Crimp, and my sisters, Lindsay and Carin, for their continuous support, love, and for always being there when I needed an ear to listen.

This work was supported by grants to Dr. Mustacich (NIEHS R21ES015872, NIEHS 3R21ES015872-02S1), an HHMI summer award, and a URISC summer grant. I would also like to thank Biomedical Sciences and the Linus Pauling Institute for the travel funds that they provided me so I could present my work at various conferences. I would also like to thank the Honors College for this experience and for the Honors Promise Finishing Scholarship that helped fund my last year.

TABLE OF CONTENTS

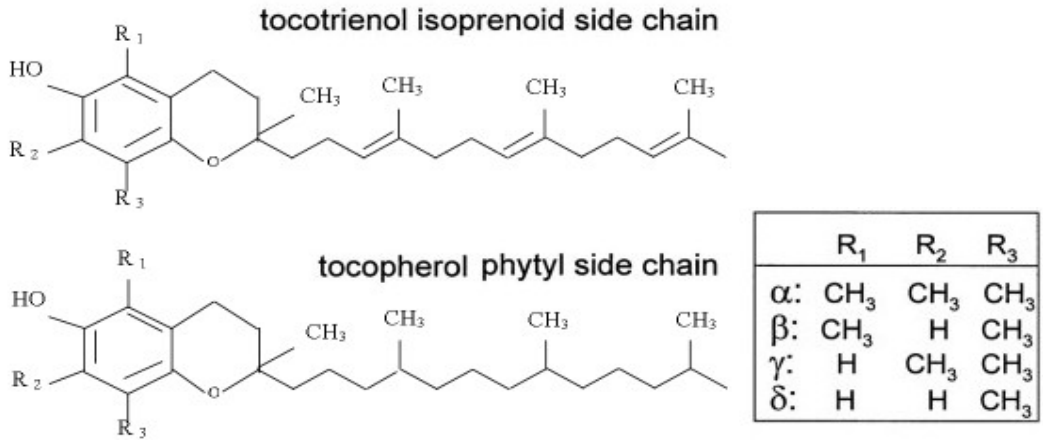
LIST OF FIGURES

Gender-Dependent Mechanisms of Alpha-Tocopherol Protection from Benzo[a]pyrene Exposure in Rats

INTRODUCTION

Vitamin E Structure

Vitamin E is the generic name for the family of lipid-soluble antioxidants that includes the α-, β-, γ-, and δ-tocopherols and the four corresponding tocotrienols. Both tocopherols and tocotrienols consist of a chromanol ring and a carbon side chain attached at the 2-position of the ring (Fig.1). The α-, β-, γ-, and δ- forms differ in the number and location of methyl substituents on the aromatic ring (Fig. 1). Tocotrienols differ from tocopherols in the degree of saturation of their side chain. Tocotrienols have an isoprenoid side chain containing three double bonds whereas tocopherols have a saturated side chain, also referred to as a phytyl tail (Fig. 1).



	R_1	R_2	R_3
α:	CH_3	CH_3	CH_3
β:	CH_3	H	CH_3
γ:	H	CH_3	CH_3
δ:	H	H	CH_3

Figure 1. Structure of vitamin E. Adapted from (Mustacich et al. 2007a).

The tocopherol forms contain chiral centers at position 2 in the chromanol ring and positions 4' and 8' in the phytyl tail. The naturally occurring forms of tocopherols possess the *R* configuration at all three chiral centers, i.e., *RRR*-α-tocopherol. However, synthetic α-tocopherol is an equimolar mixture of eight stereoisomers of both *R* and *S* configurations (*RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS*) called all-*rac*-α-tocopherol.

RRR-α-tocopherol is the most biologically active form of Vitamin E in part due to the selectivity of the α-tocopherol transfer protein (α-TTP) (Brigelius-Flohe and Traber 1999; Hosomi et al. 1997; Traber et al. 2004). Although all forms of natural and synthetic Vitamin E are absorbed in the intestine and secreted into the circulatory system via chylomicrons, once chylomicron remnants reach the liver, hepatic α-TTP preferentially salvages *RRR*-α-tocopherol and facilitates its secretion from the liver to circulating lipoproteins (Morley et al. 2008; Traber et al. 2004). In addition, non-α-tocopherol forms of Vitamin E are more rapidly metabolized and excreted than α-tocopherol (Birringer et al. 2002; Leonard et al. 2005; Traber 2007).

Antioxidant Activity of Alpha-Tocopherol

Free radical species are highly reactive molecules that contain at least one or more unpaired electron. Reactive Oxygen Species (ROS) is a term used to describe the partially reduced and reactive forms of oxygen capable of causing damage to cellular components including DNA, proteins, carbohydrates and lipids (Halliwell 1995). Antioxidants are molecules capable of preventing oxidation of substrates by ROS and free radical species in vivo (Halliwell 1995).

α-Tocopherol's most common and well-known function is that of a lipid-soluble antioxidant that scavenges lipid peroxyl radicals to terminate the propagation step in the oxidation of polyunsaturated fatty acids (PUFA) (Burton et al. 1983). Abstraction of a hydrogen ion from a polyunsaturated lipid (RH) leads to the formation of a carbon-centered radical (R•). R• spontaneously reacts with molecular oxygen leading to the formation of a lipid peroxyl radical (ROO•) (Fig. 2). In the absence of α-tocopherol the ROO• reacts with a neighboring PUFA to form a lipid hydroperoxide (ROOH) and a new R• (Fig. 2). The new R• rapidly reacts with oxygen to produce a new ROO• and thus leads to a chain reaction of continuing lipid peroxidation.

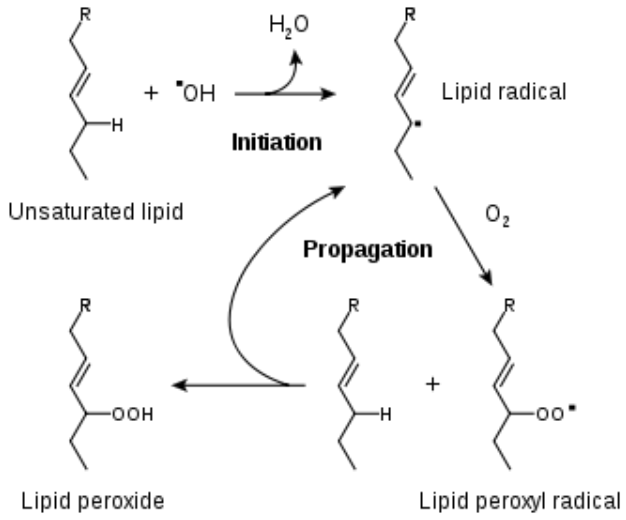


Figure 2. Lipid peroxidation reaction. Adapted from (Young and McEneny 2001).

α-Tocopherol acts to terminate the propagation step and prevents further oxidation of PUFAs by reacting with ROO• (Burton et al. 1985). The reaction kinetics of α-tocopherol with ROO• is much faster than the reaction of ROO• with biological targets such as PUFAs (Burton et al. 1985). The tocopheroxyl radical that is produced can be

reduced by Vitamin C or other hydrogen donors to reform α-tocopherol (Buettner 1993). If not terminated, lipid peroxidation in biological tissues leads to cell membrane damage and production of mutagenic compounds such as malondialdehyde (MDA). MDA is a lipid peroxidation product that can react with deoxyadenosine and deoxyguanosine in DNA to introduce mutagenic lesions (Shuker et al. 2002).

Xenobiotic Metabolism

Xenobiotics are any substances considered foreign to the body, including drugs, toxins, vitamins, and herbs. The enzymes and transporters involved in the metabolism of xenobiotics are divided into three classes, Phase I, II, and III. Phase I enzymes modify the xenobiotic by addition of a functional group to make the compound more reactive. Phase I modifications include oxidation, reduction, and hydrolysis reactions, among others. The cytochrome P450 enzymes (CYPs) are a superfamily of enzymes that catalyze the oxidation of various exogenous and endogenous compounds. The Phase II enzymes catalyze conjugation reactions and include the sulfotransferase (SULT), uridine diphosphate (UDP) glucuronosyl transferase (UGT), and glutathione S-transferase (GST) superfamilies.. These conjugation reactions make the compound more water-soluble. Phase III transporters are responsible for the import and export of xenobiotic compounds and metabolites across cellular membranes. Members of the ATP-binding cassette (ABC) superfamily, including the multidrug resistance (MDR) proteins, the multidrug resistance related proteins (MRP), and the breast cancer resistance protein (BCRP), are phase III transporters that are responsible for the excretion of xenobiotic metabolites from the liver and kidney into the bile and urine, respectively.

Alpha-Tocopherol Metabolism

Unlike other fat-soluble vitamins, α-tocopherol does not accumulate to toxic levels and studies suggest that mechanisms regulating metabolism and excretion are in place to prevent excess accumulation of α-tocopherol (Mustacich et al. 2006; Mustacich et al. 2007b; Schultz et al. 1995).

Both in vivo and in vitro data suggests that tocopherols are metabolized via xenobiotic metabolism and excretion pathways. α-Tocopherol initially undergoes ω-oxidation catalyzed by CYPs to form 13´-OH-α-tocopherol, followed by five rounds of β-oxidation within the peroxisomes and mitochondria leading to the formation of the final α-tocopherol metabolite 2,5,7,8-tetramethyl-2-(2´-carboxyethyl)-6-hydroxychroman (α-CEHC) (Fig. 3) (Abe et al. 2007; Birringer et al. 2001; Mustacich et al. 2006; Mustacich et al. 2010; Sontag and Parker 2002). Prior to excretion in either urine or bile, the majority of the α-CEHC is conjugated by either SULTs or UGTs that produce sulfate or glucuronide conjugates, respectively (Lodge et al. 2000; Pope et al. 2002; Schultz et al. 1995; Stahl et al. 1999). Finally, the ATP-binding cassette (ABC) transporter, Mdr2 (multidrug resistance protein 2) is required for biliary excretion of α-tocopherol in mice (Mustacich et al. 1998). (Note: In rats and humans ABC transport protein nomenclature is capitalized while in mice only the first letter is capitalized. Additionally the mouse ABC transport protein responsible for the efflux of α-tocopherol into the bile is designated Mdr2 in mice, while the equivalent protein in humans and rats is designated MDR3.) It has yet to be determined if additional Phase III transporters play a role in the excretion of α-CEHCs from the liver and kidney.

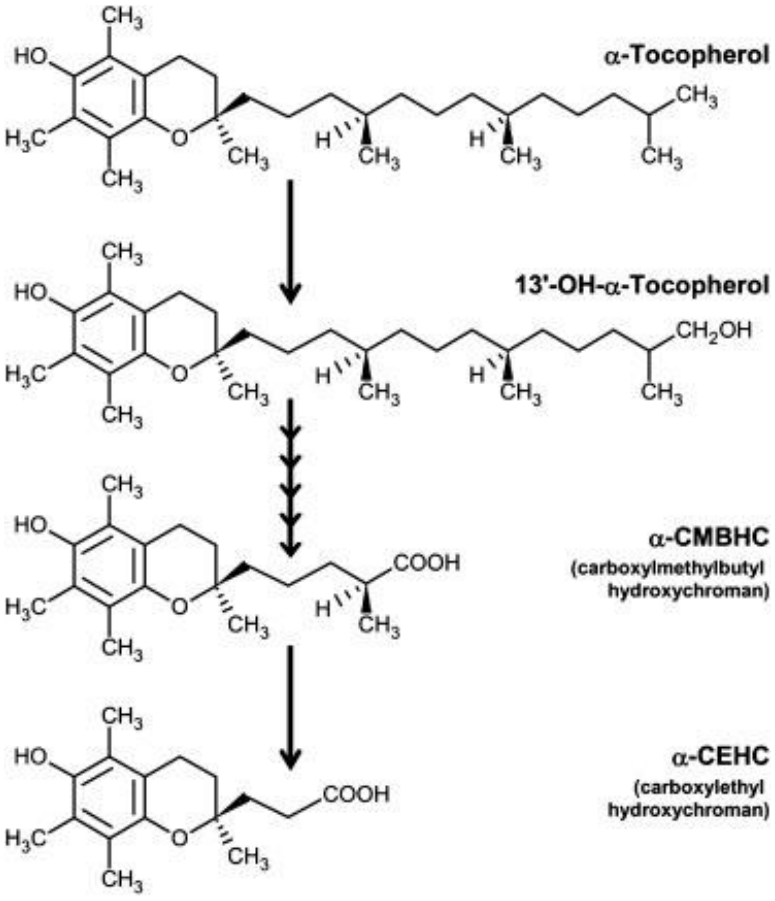


Figure 3. α-Tocopherol metabolism scheme. Adapted from (Traber 2010).

Alpha-Tocopherol Regulation of Xenobiotic Metabolism

In addition to its traditional role as an antioxidant, α-tocopherol is suggested to regulate xenobiotic metabolism, as reviewed in (Traber 2010). In a study in which rats were given 18 daily subcutaneous (SQ) injections of α-tocopherol (100 mg/kg body weight) hepatic levels of α-tocopherol and its metabolites increased, indicating that excess α-tocopherol leads to increases in its own metabolism (Mustacich et al. 2006). In this same study, hepatic protein levels of CYP3A, CYP2B, CYP2C, but not CYP4F, increased more than 2-fold and remained elevated over the course of the 18-day study

(Mustacich et al. 2006). These results were in agreement with a previous study that observed a correlation between hepatic α-tocopherol concentrations and Cyp3a, but not Cyp4f protein concentrations in mice (Traber et al. 2005). In addition, dietary α-tocopherol supplementation increased CYP2C levels in rats (Murray 1991) and measures of increased activity for hepatic CYPs 3A, 2B, and 2C were demonstrated in α-tocopherol supplemented Cebus monkeys (Meydani and Greenblatt 1990). Dietary α-tocopherol supplementation also increased the gene expression of P450 oxidoreductase and Cyp3a11, as well as the phase II enzymes, SULT2a and GSTm3 in female mice (Mustacich et al. 2009). Finally, Tampo and Yonaha determined that Vitamin E was important for GST activity in microsomes (1990). Importantly, CYP1A and CYP1B protein and mRNA levels were not altered by either dietary α-tocopherol supplementation or SQ α-tocopherol injections (Mustacich et al. 2009; Mustacich et al. 2006).

Hepatic MDR1 protein levels increased following 7 daily SQ injections of α-tocopherol, the same time point at which hepatic α-tocopherol and α-CEHC levels began to decrease (Mustacich et al. 2006). In another study, dietary α-tocopherol supplementation (1000 IU all-*rac*-α-tocopherol/kg diet) in female mice increased Mdr1a mRNA expression more than 2-fold compared to non-supplemented control mice (35 IU all-*rac*-α-tocopherol/kg diet) (Mustacich et al. 2009). However, biliary α-tocopherol levels in Mdr1 knock-out mice did not differ from wildtype mice when both were fed a chow diet containing 60 ppm α-tocopherol (Mustacich et al. 1998). Together these data suggest that excess α-tocopherol regulates specific xenobiotic enzymes and transporters by an as yet undefined mechanism. In addition, it is possible that Mdr1 plays a role in the

biliary excretion of α-tocopherol and/or α-CEHC only under conditions of excess hepatic α-tocopherol.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a large class of environmental toxins that are produced as a result of incomplete combustion of organic materials and are characterized by the presence of two or more fused aromatic rings. Exposure to PAHs may occur by inhalation, ingestion, or skin contact and from a variety of sources, including food, car exhaust, cooking smoke, tobacco smoke, residential heating (coal, wood, oil) and volcanic eruptions. Additionally, some individuals are occupationally exposed to high levels of PAHs, such as road pavers, roofers, coke oven workers, forest fire fighters, smoke house workers and those that work in aluminum production and iron and steel foundries (Boffetta et al. 1997). Although PAHs usually exist as complex mixtures, virtually all mixtures contain benzo[a]pyrene (B[a]P). B[a]P is the most studied PAH and is often used as a model compound in PAH exposure experiments. Additionally, the International Agency for Research on Cancer recently updated the classification of B[a]P to Group 1, thus classifying it as a known human carcinogen.

The ability of PAHs to induce human cancer has been known since 1775, when Sir Percival Pott described the first occupational cancer when he observed a higher incidence of scrotal cancer in chimney sweeps (Pott 1974). The B[a]P present in soot would later be determined as the causative factor for this cancer (Cook et al. 1933). In addition to the increased incidence of scrotal and skin cancer in chimney sweeps and tar workers, inhaled PAHs have been shown to induce lung cancer in coke-oven workers,

coal-gas workers, and employees of aluminum production plants (Boffetta et al. 1997). Results of epidemiologic studies show increased mortality due to cancer for both smokers and workers exposed occupationally to PAHs, e.g., roofers, coke oven workers, asphalt pavers and employees of aluminum production plants (Costantino et al. 1995; Hammond et al. 1976; McClean et al. 2004; Pavanello et al. 1999; Sasco et al. 2004).

Benzo[a]pyrene Detoxification and Bioactivation

PAHs, including B[a]P, exert their mutagenic and carcinogenic activity through metabolic conversion to chemically reactive metabolites, particularly the benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE) metabolite that readily forms DNA adducts (Baird et al. 2005) (Fig. 4). The production of B[a]PDE in the liver and lung requires bioactivation by CYP1A1/1A2 and CYP1B1 as reviewed in (Baird et al. 2005). Bioactivation by CYP enzymes initially results in the production of B[a]P-oxides that are either conjugated by GST enzymes or converted to their respective phenols or diols. Most of the B[a]P phenols and diols are conjugated by phase II enzymes (GSTs, SULTs and UGTs) and excreted (Fig. 4). However, if the B[a]P-7,8-oxide is not conjugated it will undergo further bioactivation by epoxide hydrolase (EH) to form B[a]P-7,8-dihydrodiol. B[a]P-7,8-dihydrodiol is further metabolized to DNA reactive metabolites by one of two pathways. In the first, B[a]P-7,8-dihydrodiol undergoes conversion to B[a]PDE. In the second pathway B[a]P-7,8-dihydrodiol undergoes conversion to B[a]P-7,8-catechol via aldo-keto reductase (AKR) (Fig.4). B[a]P-7,8-catechol undergoes autoxidation to B[a]P-7,8-dione via the semiquinone intermediate, producing ROS in a redox cycling process

(Fig.4). B[a]P-7,8-dione and B[a]PDE are the ultimate carcinogens as they form covalent adducts with DNA (Fig.4)

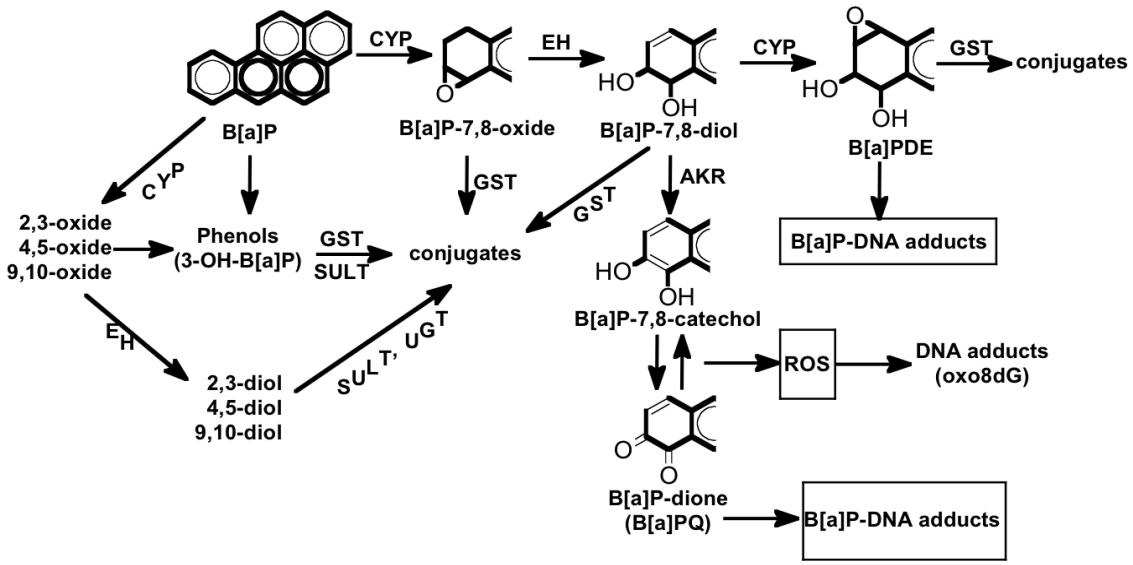


Figure 4. Proposed pathways for metabolism of B[a]P by xenobiotic enzymes. Adapted from (Baird et al. 2005).

Alpha-Tocopherol and Benzo[a]pyrene Interactions

CYP1A1/CYP1A2 and CYP1B1 initiate metabolic activation of B[a]P leading to production of both DNA-damaging and non-damaging metabolites, while CYP3A4 and CYP2C participate only in the metabolic detoxification of B[a]P to non-DNA-damaging metabolites, i.e., 3-OH-B[a]P (reviewed in (Alexandrov et al. 2010). Furthermore, inhibition of pulmonary CYP2B decreased the production of 3-OH-B[a]P, thus suggesting CYP2B also participates in the metabolic detoxification of B[a]P (Furman et al. 1991). Importantly, Mooney et al. (2005) found that α-tocopherol supplementation decreased white blood cell B[a]PDE-DNA adduct levels in female smokers to a greater

extent than male smokers and this was particularly true for females with the GSTM1 null genotype. The mechanism by which α-tocopherol decreases B[a]PDE-DNA adducts in either male or female smokers has not been determined. However, as discussed above, α-tocopherol supplementation selectively increases hepatic protein expression of CYPs 3A, 2B, and 2C (Mustacich et al. 2006; Mustacich et al. 2007b). Thus, α-tocopherol supplementation may protect tissues from B[a]P exposure by modulating B[a]P metabolism and decreasing the production of DNA reactive B[a]P metabolites.

As described above, the phase II enzymes (GSTs, SULTs and UGTs) are involved in the detoxification of B[a]P to polar products that are excreted via either renal or biliary routes. However, regardless of route of administration, biliary excretion and elimination through the feces is the major route by which B[a]P metabolites are removed from the body (Vandewiel et al. 1993). Exposure to B[a]P increases in vitro expression of the Mdr1 biliary transporter in mouse hepatocytes (Mathieu et al. 2001) and rat liver cells (Fardel et al. 1996). In addition, B[a]P pretreatment increases biliary excretion of its own metabolites (Schlede et al. 1970). As discussed above, α-tocopherol has been shown to increase mRNA and protein levels of MDR1 (Mustacich et al. 2006; Mustacich et al. 2007b). Taken together these data suggest that (1) MDR1 plays a role biliary excretion of B[a]P and (2) up-regulation of MDR1 by α-tocopherol would increase biliary excretion of B[a]P and thereby decrease B[a]P exposure and subsequent B[a]P-induced tissue damage.

The ability of α-tocopherol supplementation to increase the expression of CYPs involved in B[a]P detoxification, as well as increase hepatic MDR1, combined with the decreased B[a]PDE-DNA adducts in α-tocopherol supplemented smokers, suggests that

non-antioxidant mechanisms are involved in α-tocopherol protection from B[a]P-induced tissue damage.

Hypothesis and Experimental Design

We hypothesized that α-tocopherol would prevent B[a]P-induced damage in rats by two synergistic mechanisms, (1) antioxidant protection from B[a]P-induced oxidative damage and (2) upregulation of B[a]P metabolism and/ excretion of B[a]P and B[a]P metabolites. Additionally, based on the results of studies in male and female smokers, we hypothesized that this protection would be greater in female rats compared to male rats. To test this hypothesis, using a subcutaneous α-tocopherol dosing regimen in male and female rats, we measured α-tocopherol levels in tissues, plasma and bile, reduced and oxidized glutathione in tissues, as well as 2 biomarkers of oxidative stress (urine 8-isoprostanes and tissue malondialdehyde). In addition, we measured total biliary and urinary excretion of B[a]P and B[a]P metabolites.

MATERIALS AND METHODS

Materials

The Vitamin E solution used for injections was Vital E-500 (1 mL contains 500 IU *RRR*-α-tocopherol compounded with 18% ethanol and 1% benzyl alcohol in an emulsifiable base). Vital E-500 is supplied as a sterile multi-dose solution for subcutaneous injection (Stuart Products, Inc Bedford, TX). HPLC-grade methanol, hexane, ethanol, butanol, and glacial acetic acid were obtained from Fisher (Fair Lawn, NJ). All other chemicals were obtained at reagent grade quality from suppliers.

Animal Experiments

5 Hour Animal Experiments

Male and female bile-cannulated Sprague-Dawley rats (Charles-River, Wilmington, MA), 300-350 g and 250-300 g, respectively, were acclimated to the Oregon State University animal facility for one week prior to the beginning of the injection regimen. Rats were kept on a 12 h light/dark schedule and fed the Constant Nutrition Rat Diet 5012 (35 IU *dl*-α-tocopheryl acetate/kg diet, LabDiet, PMI Nutrition International, Saint Louis, MO) and water *ad libitum*. Bile cannulas were flushed daily with saline. Rats were given seven daily subcutaneous (SQ) injections of either 10 mg *RRR*-α-tocopherol /100 g body weight as Vital E-500 (Stuart Products) diluted in 2 mL saline or an equivalent volume of saline (vehicle) (n=5-8/gender/treatment). Dilution of Vital E-500

in saline just prior to injection does not alter the efficacy or stability of the α-tocopherol emulsion (personal communication, Rob Stuart, Stuart Products). Injections were administered between 4 p.m. and 5 p.m. daily.

On the morning of the eighth day of injections rats were transferred to metabolic cages for 24 h urine and feces collection (Pre-B[a]P samples). On the morning of the ninth day, following a 4 h fast, pre-B[a]P (zero hour) bile was collected into cryogenic vials containing liquid nitrogen. The liquid nitrogen filled cryogenic vials were kept cold by placing them in a shallow-form dewer containing a bed of "angel hair" (spun glass) and liquid nitrogen. After an hour of bile collection, rats received an intraperitoneal (ip) injection of unlabeled B[a]P (20 mg B[a]P/kg body weight) spiked with 6.35 µCi ^{3}H-B[a]P and dissolved in tocopherol-stripped corn oil. ^{3}H-B[a]P was generally labeled with ^{3}H. Following the B[a]P injection, bile was collected into a new cryogenic vial placed in the same shallow-form dewer filled with liquid nitrogen. Post-B[a]P bile was collected for five hours. Post-B[a]P urine was collected during the five hours following the ip B[a]P injection. Urine was centrifuged (3000 x g, 10 min) to separate urine from food particles that fall down the collection spout. Urine aliquots were frozen in liquid nitrogen prior to storage at -80 ˚C.

At the end of the 5 h bile and urine collection, rats were euthanized with an ip injection of sodium pentobarbital (80 mg/100 g body weight). Blood was collected from the heart with a 6 mL syringe and 21-gauge needle, transferred into a 6-mL Vacutainer tube containing 1 mg/mL EDTA, and placed on ice. Plasma was obtained by centrifugation (1500 X g, 15 min) and stored at -80˚C. Following blood collection; liver, lung, and kidneys were perfused with 0.9% saline containing 2 U/mL heparin by

inserting a 21-gauge needle into the left ventricle of the heart and making a small incision in the right auricle. Immediately following perfusion, liver, lung and kidneys were removed, placed in cryogenic vials, and frozen in liquid nitrogen prior to storage at -80˚C.

24 Hour Animal Experiments

The experimental design for the 24 h B[a]P exposure study is similar to that described above with a few modifications. The male and female Sprague-Dawley rats (Charles-River), 300-350 g and 250-300 g, respectively, were not bile duct cannulated. Rats received seven daily SQ injections of either 10 mg *RRR*-α-tocopherol/100 g body weight as Vital E-500 (Stuart Products) diluted in 2 mL saline or an equivalent volume of saline (vehicle) (n=6/gender/treatment). On the morning of the eighth day following the start of α-tocopherol injections rats were transferred to metabolic cages and urine and feces were collected for 24 hours. On day nine, rats received an ip injection of unlabeled B[a]P (20 mg B[a]P/kg body weight) spiked with 6.35 µCi generally labeled ^{3}H-B[a]P dissolved in tocopherol-stripped corn oil. Urine was collected for 24 h post-B[a]P exposure. Following a 4 h fast rats were euthanized with sodium pentobarbital (80 mg/100 g body weight). Exsanguination, perfusion, and excision of tissues were performed as described above for the 5 h experiments. Plasma was obtained by centrifugation and all samples were stored at -80˚C until analysis.

Measurement of Total Radioactivity

Total tritium (^3H) radioactivity was measured by liquid scintillation counting (LSC) on a multi-purpose liquid scintillation counter (Beckman LS 6500). Aliquots of plasma, urine, and bile (100 µL each) were added directly to 20 mL glass scintillation vials containing either 10 mL Ultima Gold LSC cocktail (plasma and urine) or 8 mL Ultima Gold (bile) (PerkinElmer, Waltham, MA). Samples were mixed and allowed to adjust to room temperature and light for one hour prior to counting. As appropriate, 10 or 8 mL Ultima Gold aliquots were used as counting blanks. Results in counts per minute (CPM) were converted to disintegrations per minute (DPM) based on the counting efficiency of the Beckman LS 6500 for ^3H.

Measurement of Alpha-Tocopherol

Plasma, bile, and tissue α-tocopherol was measured by a modification of the method described by Podda et al. (1996). Briefly, samples were aliquoted into a 10 mL glass screw top tube containing 1 mL distilled H_2O plus 2 mL 1% ascorbic acid/ethanol solution to prevent oxidation of the α-tocopherol. Samples were saponified with 0.3 mL saturated potassium hydroxide, then 1 mL ascorbic acid (1% in distilled water) and 25 µL butylhydroxytoluene (1 mg/mL EtOH) were added to further prevent oxidation. The sample was extracted with hexane, dried under nitrogen, and then suspended in 1:1 ethanol:methanol. Samples were analyzed by HPLC (Waters system with e2695 Separations Module and multi λ fluorescence detector, Waters 2475) using an Ascentis C18 column, (100 x 4.6 mm, 3 µm particle size, SigmaAldrich), an isocratic mobile

phase delivery system of 97% methanol and 3% water and a excitation/emission set at 284 nm/326 nm. Quantification was performed using Empower software (Waters) and comparison to standard curves generated by authentic α-tocopherol.

Measurement of 8-Isoprostanes

Urine F2-isoprostanes were extracted by the SPE method described by Taylor et al. (2006). Briefly, 300 µL urine was added to a tube containing 500 µL methanol and 2 mL of 0.02 M bis-tris-HCl buffer and vortexed briefly. The pH of the sample was adjusted to 6.0 ± 0.1 using H_3PO_4 or KOH. Strata X-AW cartridges (100 mg/3 mL, Phenomenex, Torrance, CA) were conditioned with 2 mL methanol containing 2% (v/v) formic acid, followed by 2 mL distilled water. pH adjusted urine samples were loaded onto the cartridge, cartridges were sequentially washed with 2 mL water, 4 mL 25% methanol in water (v/v), 2 mL acetonitrile, and then cartridges were dried with mild suction (~5 Hg) for 30 seconds. Cartridges were then eluted with three aliquots of 1 mL methanol with mild suction between each aliquot. Methanol samples were dried under nitrogen gas, reconstituted in 200 µL methanol containing 0.1% formic acid (v/v), and stored at -80˚C until analysis.

On the day of analysis, stored samples were dried under nitrogen gas and reconstituted in 300 µL EIA buffer. An ELISA method was used to measure the 8-iso Prostaglandin $F_{2\alpha}$(8-iso $PGF_{2\alpha}$) as described by the manufacturer (8-Isoprostane EIA kit, Cayman Chemical). In this assay the 8-iso $PGF_{2\alpha}$ in the standard or sample competes with an 8-isoprostane-acetylcholinesterase conjugate (tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites (Cayman Chemical). The antiserum

complex, containing either free 8-isoprostane or tracer bound 8-isoprostane binds to the rabbit IgG mouse monoclonal antibody that is attached to the bottom of the wells in the manufacturer supplied plates. After addition of standard or sample, tracer, and antiserum to the wells, the plate was washed to remove unbound reagents. Ellman's reagent was then added to the wells. The product of the enzymatic reaction between acetylcholinesterase and Ellman's Reagent absorbs strongly at 412 nm. Absorbance was read at 412 nm (Spectra Max 190 Spectrophotometer, Molecular Devices, Sunnyvale, CA). The absorbance is proportional to the amount of tracer bound to the well, which is inversely proportional to the amount free 8-isoprostane in the well. Sample concentrations were determined by comparison to standard wells using SoftMax Pro 5.2 software (as described by manufacturer).

Urine 8-iso $PGF_{2\alpha}$ concentrations were normalized to creatinine concentrations. Urine creatinine concentrations were determined with a colorimetric detection kit using the Jaffe reaction (Creatinine Colorimetric Detection Kit, Assay Designs). Creatinine concentrations were determined by comparison to standard values using SoftMax Pro 5.2 software package.

Measurement of Malondialdehyde

Tissue malondialdehyde (MDA) was determined by a modification of the method described by Lykkesfeldt (2001). Tissue was homogenized in 2 mL (liver) or 1.5 mL (lung and kidney) 1.15% potassium chloride and 500 µL of the homogenate was combined with 500 µL distilled water, 100 µL 0.2% butylhydroxytoluene (BHT), 200 µL 8% sodium dodecyl sulfate, and mixed by inversion. Addition of BHT minimizes

formation of artifacts. 2-Thiobarbituric acid (TBA) reagent (2 mL 0.6% TBA) and 1 mL 8.5% H_3PO_4were then added and the sample was again mixed by inversion. Samples were incubated at 95˚C for 30 minutes. The heating of the samples with acid leads to the production of MDA from lipid hydroperoxides. Addition of TBA reagent produces MDA-TBA adducts. The MDA-TBA adducts were then extracted with 3 mL 1-butanol to increase sensitivity. Samples were analyzed by HPLC (Waters e2695 Separations Module with multi λ fluorescence detector (Waters 2475)) using a Luna C18 column, (250 x 4.6 mm, 5 µm particle size (Phenomenex)), an isocratic mobile phase of 50% methanol: 50% potassium phosphate buffer (25 mM, pH 6.5) with 532 nm excitation and 553 nm emission. Quantification was determined by comparison to standard curves using MDA-TBA standards made from 1,1,3,3-tetramethoxypropane and Empower software (Waters).

Measurement of Reduced and Oxidized Glutathione

Hepatic reduced (GSH) and oxidized (GSSG) glutathione was measured as described by Farris and Reed (1987). Tissue samples were frozen in liquid nitrogen and pulverized to powder. Powdered tissue (50-100 mg) was added to a microcentrifuge tube containing 990 µL of 10% PCA with 1 mM bathophenanthrolinedisulfonic acid and 110 µL of the internal standard, γ-Glutamyl-glutamate (γ-Glu-Glu) (800 µM). The extract was sonicated, frozen and thawed, and centrifuged at 15,000 X g for 3 min. PCA extract (250 µL) was then transferred to a new microcentrifuge tube for derivitization with 50 µL of 100 mM iodoacetic acid in 0.2 mM m-cresol purple to form S-carboxymethyl derivatives. The solution was then instantly brought to pH 8-9 by the addition of 250 µL of the working solution (2 M KOH and 2.4 M $KHCO_3$) and allowed to incubate in the dark at

room temperature for 1 h. Then, 500 µL of 1% 1-fluoro-2,4-dinitrobenzene was added and samples were incubated overnight at 4˚C to allow DNP chromophore derivatization of primary amines. Samples were then centrifuged at 13,000 X g for 1 min. and 250 µL of the supernatant was transferred into an HPLC vial for analysis.

DNP derivatives were separated and measured using a 3-aminopropyl column (20 cm x 4.6 mm, 5 µm) and a UV detector set at 365 nm. The gradient was as described (Farris and Reed 1987) using 80% methanol (mobile phase A) and 0.5 M sodium acetate in 64% methanol (mobile phase B). GSH and GSSG concentrations were determined by comparison to standard curves generated from GSH and GSSG standard solutions processed exactly as the samples and including the same internal standard (γ-Glu-Glu).

Statistical Analysis

Statistical analysis was performed using Prism version 5.0 (Graphpad Software, San Diego, CA). Where necessary, data were log-transformed to account for unequal variances between groups prior to statistical analysis. The main effects of gender and α-tocopherol treatment and their interaction were analyzed using 2-way ANOVA, followed by Bonferroni post hoc tests when the interaction was significant. Non-transformed data are shown in figures and data are expressed as the mean ± standard error (SE). A p value < 0.05 was considered statistically significant.

RESULTS

Tissue, Plasma and Bile Alpha-Tocopherol Concentrations

Male and female rats were given daily SQ injections of α-tocopherol (10 mg/100 g body weight), or saline (vehicle, non-supplemented), for 7 days prior to exposure to B[a]P on day 9. Rats were sacrificed 5 or 24 h after B[a]P exposure. To assess the ability of the supplementation regimen to increase hepatic and extrahepatic tissue α-tocopherol concentrations, α-tocopherol was measured in liver, kidney, lung, plasma, and bile.

In agreement with previous data in which male rats were given 9 daily SQ α-tocopherol injections (Mustacich et al. 2007), α-tocopherol levels in liver, plasma and extrahepatic tissues increased with α-tocopherol supplementation.

At 5 h post-B[a]P exposure, but not 24 h post-B[a]P, the interaction of gender and treatment significantly affected hepatic α-tocopherol concentrations (p-interaction<0.001) (Fig. 5A). Hepatic α-tocopherol concentrations in vehicle treated females were double those of vehicle treated male rats at both 5 and 24 h post-B[a]P ($p<0.001$). Thus, although α-tocopherol supplementation increased hepatic α-tocopherol levels to approximately 500 nmol/g in both genders 5 h post-B[a]P ($p<0.001$), the fold-increase following supplementation was greater in males. α-Tocopherol supplementation increased hepatic α-tocopherol concentrations approximately 7-fold in both males and females 24 h post-B[a]P compared to their respective 24 h vehicle controls (Fig. 5B).

Similar to liver, kidney and lung α-tocopherol levels were higher in female vehicle treated rats compared with those in male vehicle treated rats ($p<0.001$, Fig. C-F). α-Tocopherol supplementation increased kidney α-tocopherol levels nearly 2-fold in both genders at 5 and 24 h post-B[a]P, thus there was no interaction between gender and treatment ($p<0.001$, Fig. 5C and D).

Compared to non-supplemented rats, α-tocopherol supplementation increased lung α-tocopherol levels approximately 3-fold in both genders 5 h post-B[a]P ($p<0.001$, Fig. 5E). However, 24 h post-B[a]P, α-tocopherol supplementation increased lung α-tocopherol levels nearly 3-fold in males ($p<0.001$), but only 2-fold in females ($p<0.001$), compared with non-supplemented gender matched controls. Therefore an interaction occurred between gender and treatment at the 24 h time point (p-interaction=0.007, Fig. 5F).

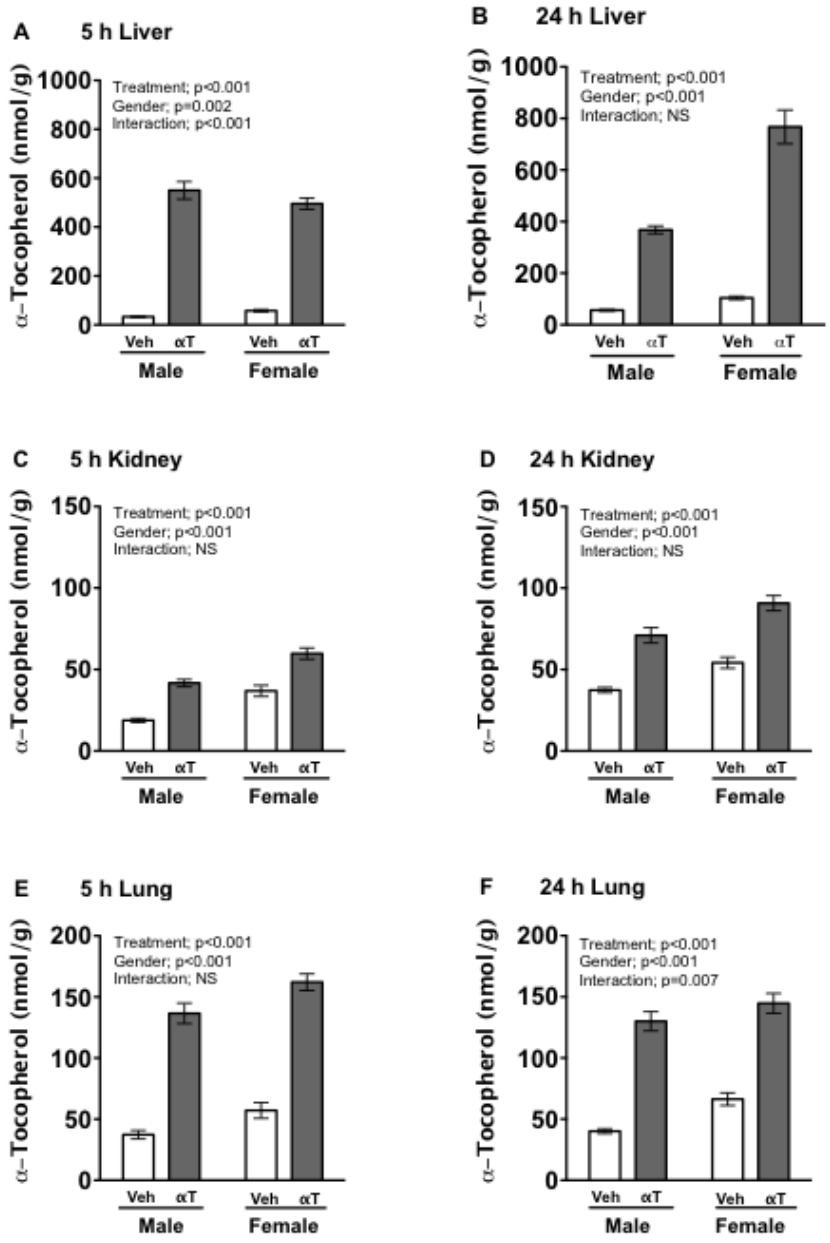


Figure 5. **Tissue α-tocopherol levels are altered in response to SQ vehicle or α-tocopherol injections**. Male and female rats received 7 daily SQ injections of either vehicle (saline) or α-tocopherol (10 mg/ 100 g body wt, dissolved in saline). On day 9, rats were given an ip injection of unlabeled B[a]P (20 mg/kg body wt) spiked with ^{3}H-B[a]P (6.35 µCi/rat). Plasma and tissues were collected at 5 or 24 h post-B[a]P and α-tocopherol levels were determined as described in Materials and Methods. α-Tocopherol levels 5 h post-B[a]P: (A) liver, (C) kidney, (E) lung, and 24 h post-B[a]P: (B) liver, (D) kidney, and (F) lung. Values are expressed as mean ± SE, n=5-8/group, open bar=vehicle, closed bar= α-tocopherol supplemented.

As observed in the tissues α-tocopherol plasma levels were higher in vehicle females compared to vehicle males ($p<0.001$). However, plasma α-tocopherol levels increased nearly 3-fold in α-tocopherol supplemented male rats compared with non-supplemented males 5 h post-B[a]P ($p<0.001$), while plasma α-tocopherol levels in supplemented female rats increased less than 2-fold compared with vehicles ($p<0.001$, Fig. 6A). Thus, a significant interaction of treatment and gender was observed (p-interaction=0.004). At 24 h post-B[a]P exposure, plasma α-tocopherol concentrations of supplemented male and female rats remained elevated compared to their respective vehicle controls ($p<0.01$) with supplemented females maintaining higher levels of plasma α-tocopherol than those of supplemented males ($p<0.001$). Thus, a significant interaction of treatment and gender was observed 24 h post-B[a]P (p-interaction=0.003, Fig. 6B).

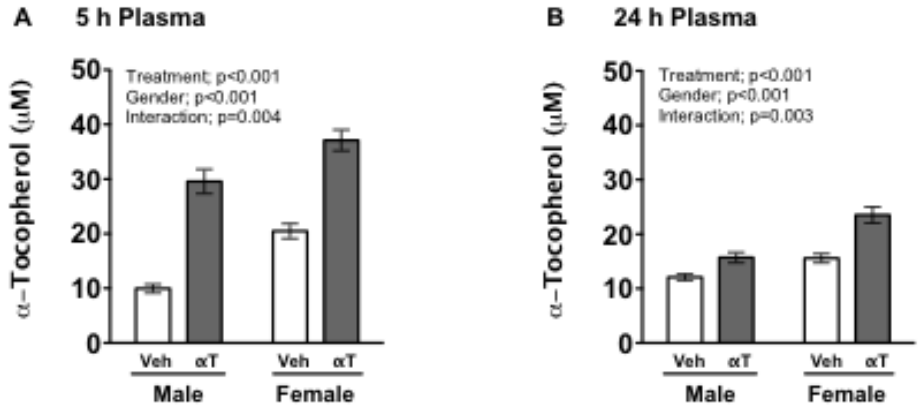


Figure 6. α-Tocopherol supplementation increases plasma α-tocopherol levels in male and female rats. α-Tocopherol levels in (A) 5 h post-B[a]P plasma and (B) 24 h post-B[a]P plasma were determined from SQ vehicle- and α-tocopherol-injected rats, as described in Fig. 5 and Materials and Methods.

α-Tocopherol levels were determined in bile collected for one hour prior to B[a]P injection and for 5 h after B[a]P injection. Post-B[a]P bile α-tocopherol levels did not differ from Pre-B[a]P bile α-tocopherol levels for any given treatment group (data not shown). Similar to tissues and plasma, α-tocopherol levels were higher in female rats compared with males

($p<0.001$) and α-tocopherol supplementation increased bile α-tocopherol levels in females and males ($p<0.001$). However, in contrast to the plasma data, there was no interaction between gender and treatment with respect to biliary α-tocopherol levels.

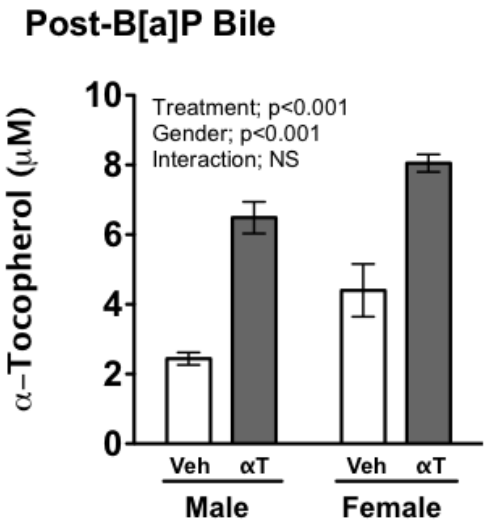


Figure 7. α-Tocopherol supplementation increases bile α-tocopherol levels 5 h post-B[a]P exposure. α-Tocopherol levels in bile of male and female vehicle- and α-tocopherol-injected rats were determined as described in Fig. 5 and Materials and Methods.

Tissue Malondialdeyhde Concentrations

Malondialdehyde (MDA) was measured in the tissues of vehicle- and α-tocopherol-injected rats 5 and 24 h post-B[a]P as a biomarker of lipid peroxidation within the tissues. Hepatic MDA levels in female rats were significantly higher than males at both time points, irrespective of treatment ($p\leq0.001$, Fig. 8A and B). Compared with vehicle controls, α-tocopherol supplementation decreased hepatic MDA levels in male and female rats 5 and 24 h post-B[a]P ($p=0.002$ and $p<0.001$, respectively, Fig. 8A-B).

Interestingly, α-tocopherol supplementation did not consistently decrease MDA levels in extrahepatic tissues. At 5 h post-B[a]P, but not 24 h post-B[a]P, there was a significant interaction

of treatment and gender on kidney and lung MDA levels (p-interaction=0.047 and <0.001, respectively). In contrast to liver, supplemented females had lower kidney MDA levels than supplemented males ($p<0.01$) at both time points (Fig. 8C-D). In addition, at 5 h post-B[a]P lung MDA levels decreased with α-tocopherol supplementation in males ($p<0.05$), but increased with α-tocopherol supplementation in females ($p<0.05$) (p-interaction<0.001, Fig. 8E). However, at 24 h post-B[a]P, α-tocopherol supplementation had no affect on lung MDA levels in males or females (Fig. 8F).

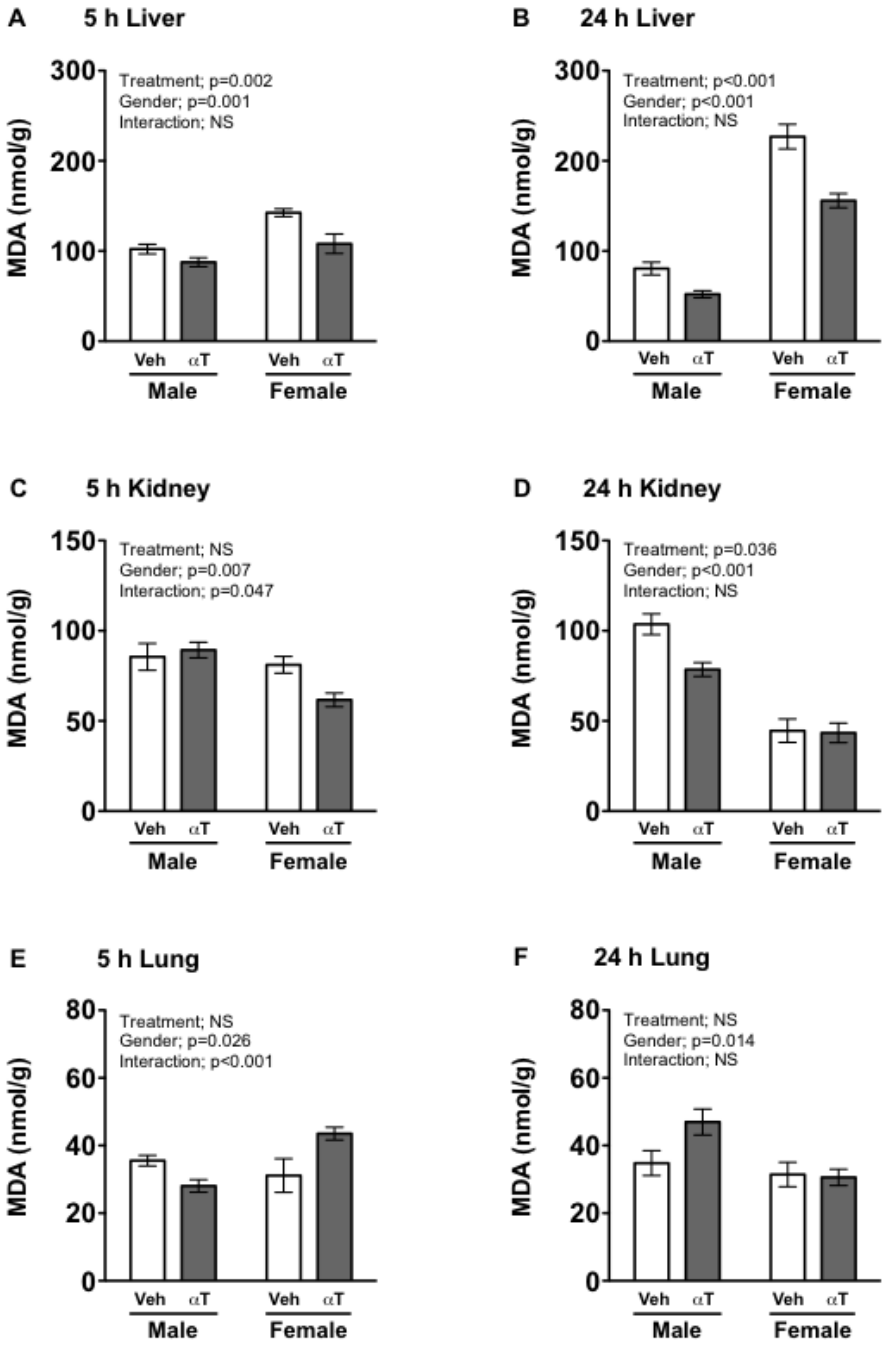


Figure 8. α-Tocopherol supplementation alters tissue malondialdehyde (MDA) levels. MDA concentrations were determined 5 h post-B[a]P: (A) liver, (C) kidney, (E) lung and 24 h post-B[a]P: (B) liver, (D) kidney, (F) lung as described in Materials and Methods. Values are expressed as mean ± SE, n=5-8/group, open bar=vehicle, closed bar=α-tocopherol supplemented.

Urine 8-Isoprostane Concentrations

8-isoprostanes (8-*iso* $PGF_{2\alpha}$) were measured in the urine of 24 h male and female rats pre- and post-B[a]P exposure as a biomarker of oxidative stress. Results were normalized to urine creatinine concentrations. Measurement of 8-*iso* $PGF_{2\alpha}$ before B[a]P exposure allowed rats to serve as their own controls. Results are presented as percent of Pre-B[a]P urine 8-*iso* $PGF_{2\alpha}$ levels (mean ± SE). B[a]P induced 8-*iso* $PGF_{2\alpha}$ levels were higher in females compared to males within the same treatment group (p=0.049, Fig. 9). α-Tocopherol supplementation decreased urine 8-*iso* $PGF_{2\alpha}$ levels to a greater degree in males compared to females (p=0.04).

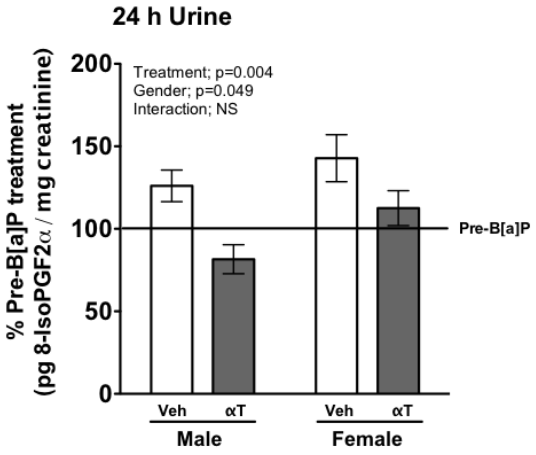


Figure 9. Urine 8-isoprostane concentrations in SQ vehicle- and α-tocopherol-injected rats 24 h post-B[a]P expressed as percent of pre-B[a]P levels. The horizontal line indicates pre-B[a]P levels designated as 100%. 24 h urine 8-isoprostane levels were determined pre-and post-B[a]P and normalized to urine creatinine, then post-B[a]P levels were expressed as percent of pre-B[a]P levels for individual rats. Values are expressed as mean ± SE, n=6/group, open bar=vehicle, closed bar=α-tocopherol supplemented.

Hepatic Reduced and Oxidized Glutathione Concentrations

Reduced (GSH) and oxidized (GSSG) glutathione were measured in the liver of male and female rats 24 h post-B[a]P exposure. Male and female hepatic GSH levels did not significantly differ within a treatment group and although there was a trend for α-tocopherol supplementation to decrease GSH in male rats, it did not reach significance (Fig. 10A). In contrast, hepatic GSSG levels in vehicle females were 2-fold greater than vehicle males. Additionally, GSSG levels in supplemented males decreased nearly 3-fold, while hepatic GSSG levels in supplemented females were unchanged. These opposing gender effects resulted in supplemented female GSSG levels that were 4-fold higher than GSSG levels in supplemented males (Fig. 10B).

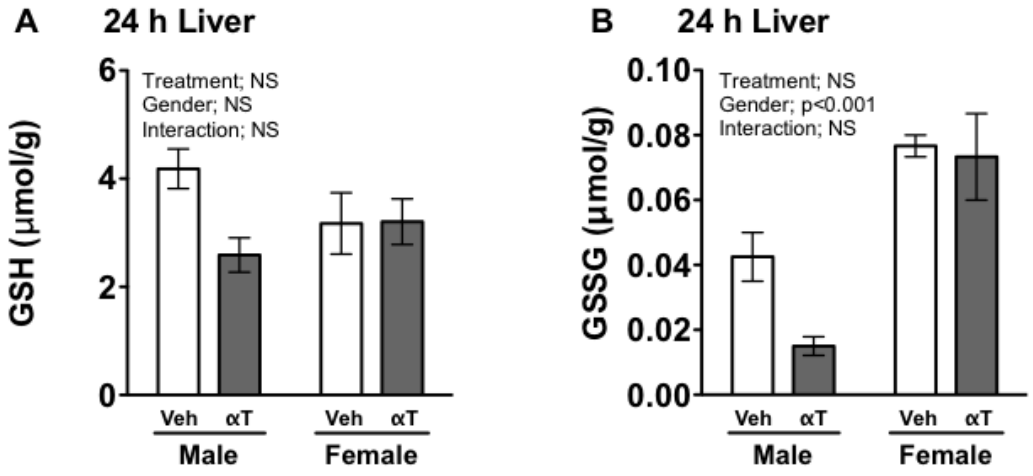


Figure 10. Hepatic reduced and oxidized glutathione levels in SQ vehicle- and α-tocopherol-injected rats 24 h post B[a]P. Hepatic GSH (A) and Hepatic GSSG (B). Values are expressed as mean ± SE, n=6/group, open bar=vehicle, closed bar=α-tocopherol supplemented.

Excretion of Tritium in Bile and Urine

To determine the ability of α-tocopherol to alter the excretion of B[a]P and/or B[a]P metabolites the total ^{3}H radioactivity present in bile 5 h post-^{3}H-B[a]P injection, as well as that present in urine 5 and 24 h post-^{3}H-B[a]P injection, was measured by LSC. At 5 h post-B[a]P, α-tocopherol supplementation in females increased biliary excretion of radioactivity 2.5-fold compared with non-supplemented females ($3.9 \times 10^{5} \pm 8.5 \times 10^{4}$ DPM/5h, $p<0.05$) (Fig. 11C). α-Tocopherol supplementation did not alter the biliary excretion of ^{3}H in male rats during the first 5h post-^{3}H-B[a]P. Similarly, in the urine collected for 5 h post-^{3}H-B[a]P, α-tocopherol supplementation increased ^{3}H excretion in females 3.2-fold compared to non-supplemented females ($p<0.014$), while male total urine radioactivity was unchanged (Fig. 11A). In urine collected for 24h post-^{3}H-B[a]P, α-tocopherol supplementation decreased ^{3}H (B[a]P/B[a]P metabolite) excretion in male rats 2.4-fold compared to non-supplemented males ($1.7 \times 10^{5} \pm 1.2 \times 10^{4}$ DPM/24h, $p<0.05$). Conversely, urine ^{3}H excretion increased 2-fold in 24 h urine from supplemented females compared to non-supplemented females ($1.3 \times 10^{5} \pm 2.9 \times 10^{4}$ DPM/24h, $p<0.01$) (Fig. 11B), resulting in an interaction of α-tocopherol treatment and gender (p-interaction<0.001) at 24 h post-B[a]P.

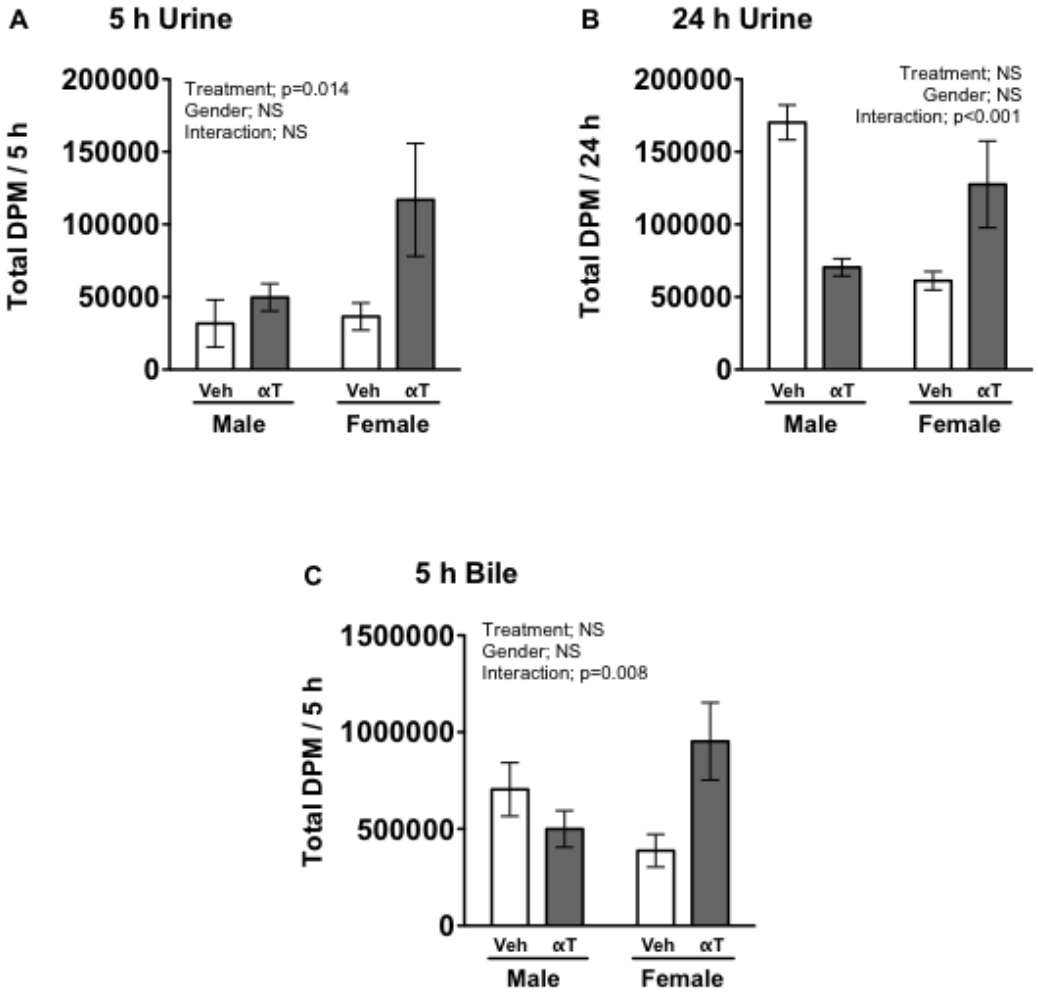


Figure 11. Total DPM excreted in urine at 5 and 24 h post-B[a]P and in bile at 5 h post-B[a]P. Total ^{3}H radioactivity was determined by LSC in (A) 5 h post-B[a]P urine, (B) 24 h post-B[a]P urine and (C) 5 h post-B[a]P bile as described in Materials and Methods. Values are expressed as mean ± SE, n=5-8/group, open bar=vehicle, closed bar=α-tocopherol supplemented.

DISCUSSION

In this study, male and female rats were SQ injected daily with either an injectable form of α-tocopherol (10 mg/100 g body wt, diluted in saline) or saline (vehicle, non-supplemented) for 7 days prior to a single ip injection of B[a]P (20 mg/kg body wt) spiked with ^{3}H-B[a]P administered on day 9. Rats were sacrificed 5 or 24 h after B[a]P-exposure and α-tocopherol, MDA, 8-isoPGF2α, glutathione, and excretion of B[a]P related radioactivity were determined. Importantly, our data show that α-tocopherol protection from B[a]P exposure is gender dependent and involves both antioxidant and non-antioxidant mechanisms.

We have previously demonstrated that the α-tocopherol dose used in the current study increases hepatic α-tocopherol to ~800 nmol/g tissue following 3 daily SQ injections in male rats, but female rats were not included in these earlier studies. Of note, despite continued daily SQ α-tocopherol injections, following 6-9 daily injections male hepatic α-tocopherol levels dropped to ~480 nmol/g (Mustacich et al. 2006). Importantly, cytochrome P450 (CYP) enzymes involved in the detoxification of B[a]P, i.e., CYPs 3A, 2B, and 2C, as well as the biliary transport protein, MDR1 (multidrug resistance protein 1, also ABCB1), were increased after 6 daily SQ injections (Mustacich et al. 2006). Our male data presented herein demonstrates similar increases in hepatic α-tocopherol levels following 7 daily injections. In the current study, α-tocopherol supplementation significantly increased kidney, lung, plasma, and bile α-tocopherol levels in both male and female rats. Of interest, female hepatic and extrahepatic α-tocopherol levels were significantly higher than male α-tocopherol levels in both supplemented and non-

supplemented rats. This data is in agreement with other studies that have demonstrated higher levels of tissue α-tocopherol in non-supplemented female rats compared with males. To date, the mechanism for this gender difference is undetermined, however, data from studies by (Feingold and Colby 1992; Feingold et al. 1993; Weglicki et al. 1969) suggest that gonadal hormones play a role in gender-dependent regulation of α-tocopherol levels. Furthermore, the ability of females to accumulate a higher level of tissue α-tocopherol supports human studies showing a greater degree of protection from B[a]P damage in female smokers compared to males (Mooney et al. 2005).

Tissue MDA was measured in liver, lung, and kidney of α-tocopherol supplemented and non-supplemented rats to determine the ability of α-tocopherol to prevent B[a]P-induced lipid peroxidation. α-Tocopherol supplementation protected male and female liver and kidney from B[a]P-induced lipid peroxidation, while lung tissue was not protected in either gender. Interestingly, although our data suggests that α-tocopherol supplementation protects female liver from B[a]P-induced lipid peroxidation, female hepatic MDA levels were more than 2-fold higher compared with MDA levels in male livers in both supplemented and non-supplemented rats. These data indicate that α-tocopherol protection from B[a]P-induced lipid peroxidation is both gender and tissue dependent.

B[a]P is a constituent of cigarette smoke and studies in smokers have shown decreased urinary 8-isoprostanes levels with α-tocopherol supplementation (Taylor et al. 2008). Therefore, to compare our rat data with the human smoker data, urine 8-isoprostanes were determined. In agreement with our liver and kidney MDA data, α-tocopherol supplementation decreased B[a]P-induced urine 8-isoprostane levels in both

genders; however the protection was greater in males. Again, a gender-dependent mechanism for α-tocopherol protection from B[a]P-induced lipid peroxidation is indicated.

α-Tocopherol supplementation increased excretion of ^{3}H (representing B[a]P and/or B[a]P metabolites) in both urine and bile of female rats, but not males. In fact, α-tocopherol supplementation decreased urinary excretion in male rats. Previously, α-tocopherol supplementation has been shown to increase protein levels of hepatic MDR1 in male rats and mRNA expression in female mice (Mustacich et al. 2009; Mustacich et al. 2006). Thus, increased excretion of B[a]P and/or B[a]P metabolites in the bile of female rats may be explained, in part, by the upregulation of the biliary transporter MDR1 by α-tocopherol supplementation and by B[a]P itself (Fardel et al. 1996; Mathieu et al. 2001). However, in light of the previous MDR1 data in male rats, and our data demonstrating decreased excretion in male bile and urine, the mechanism for B[a]P excretion requires further experimentation.

An increase in tissue GSSG/GSH ratio is indicative of increased oxidative stress, thus we determined the hepatic levels of reduced and oxidized glutathione in supplemented and non-supplemented rats. The combination of α-tocopherol supplementation and B[a]P did not alter hepatic GSH or GSSG levels, although female hepatic GSSG levels were significantly elevated compared males in both vehicle and α-tocopherol supplemented rats. Although not quite significant there was a trend for hepatic GSH and GSSG levels to decrease in male rats with supplementation. An increased consumption of GSH without a concurrent increase of GSSG may be due to increased production of B[a]P-glutathione conjugates. An increased consumption of glutathione

without a concurrent decrease in GSSG levels may be due to increased B[a]P metabolism, particularly increased production of B[a]P detoxification products requiring conjugation for subsequent excretion. Studies have shown that dietary α-tocopherol supplementation increases gene expression of GSTs (Mustacich et al. '09) in female mice. Paradoxical to this explanation of our glutathione data is our urine and bile tritium excretion data that does not support increased excretion in males. However, measurement of total ^{3}H, as used in these studies, does not distinguish one metabolite from another. It is possible that glutathione conjugated metabolites increased while total excretion of B[a]P or non-glutathione metabolites decreased. Thus, further in vivo studies are needed to determine the role, if any, that glutathione conjugation plays in α-tocopherol protection from B[a]P exposure.

In conclusion, our results indicate that α-tocopherol protection from B[a]P exposure occurs by both antioxidant (as evidenced by decreases in MDA and 8-isoprostanes) and non-antioxidant mechanisms (as evidenced by altered ^{3}H excretion). Furthermore, we have shown that the protection occurs by a gender-dependent mechanism.

Further studies are needed to elucidate the mechanisms of α-tocopherol protection from PAHs, such as B[a]P. We propose that α-tocopherol supplementation shunts metabolism away from DNA-reactive metabolites and towards non-DNA-reactive metabolites by upregulating B[a]P metabolic detoxification pathways. Identification of the specific B[a]P metabolites present in tissues, urine, and bile, as well as measurement of tissue B[a]P-DNA adduct levels is essential for the evidence based use of α-tocopherol

for protection from PAH exposure and prevention of B[a]P-induced damage, particularly in those occupationally exposed to high-levels of PAHs.

BIBLIOGRAPHY

Abe, C., Uchida, T., Ohta, M., Ichikawa, T., Yamashita, K. and Ikeda, S. (2007). "Cytochrome p450-dependent metabolism of vitamin e isoforms is a critical determinant of their tissue concentrations in rats." Lipids **42**(7): 637-45.

Alexandrov, K., Rojas, M. and Satarug, S. (2010). "The critical DNA damage by benzo(a)pyrene in lung tissues of smokers and approaches to preventing its formation." Toxicol Lett **198**(1): 63-8.

Baird, W. M., Hooven, L. A. and Mahadevan, B. (2005). "Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action." Environ Mol Mutagen **45**(2-3): 106-14.

Birringer, M., Drogan, D. and Brigelius-Flohe, R. (2001). "Tocopherols are metabolized in hepg2 cells by side chain omega-oxidation and consecutive beta-oxidation." Free Radic Biol Med **31**(2): 226-32.

Birringer, M., Pfluger, P., Kluth, D., Landes, N. and Brigelius-Flohe, R. (2002). "Identities and differences in the metabolism of tocotrienols and tocopherols in hepg2 cells." J Nutr **132**(10): 3113-8.

Boffetta, P., Jourenkova, N. and Gustavsson, P. (1997). "Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons." Cancer Causes Control **8**(3): 444-72.

Brigelius-Flohe, R. and Traber, M. G. (1999). "Vitamin e: Function and metabolism." FASEB J **13**(10): 1145-55.

Buettner, G. R. (1993). "The pecking order of free radicals and antioxidants: Lipid peroxidation, alpha-tocopherol, and ascorbate." Arch Biochem Biophys **300**(2): 535-43.

Burton, G. W., Cheeseman, K. H., Doba, T., Ingold, K. U. and Slater, T. F. (1983). "Vitamin e as an antioxidant in vitro and in vivo." Ciba Found Symp **101**: 4-18.

Burton, G. W., Doba, T., Gabe, E. J., Hughes, L., Lee, F. L., Prasad, L. and Ingold, K. U. (1985). "Autoxidation of biological molecules .4. Maximizing the antioxidant activity of phenols." Journal of the American Chemical Society **107**(24): 7053-7065.

Cook, J. W., Hewett, C. L. and Hieger, I. (1933). "The isolation of cancer-producing hydrocarbon from coal tar." J Chem Soc **395**: 1-3.

Costantino, J. P., Redmond, C. K. and Bearden, A. (1995). "Occupationally related cancer risk among coke oven workers: 30 years of follow-up." J Occup Environ Med **37**(5): 597-604.

Fardel, O., Lecureur, V., Corlu, A. and Guillouzo, A. (1996). "P-glycoprotein induction in rat liver epithelial cells in response to acute 3-methylcholanthrene treatment." Biochem Pharmacol **51**(11): 1427-36.

Farris, M. W. and Reed, D. J. (1987). "High-performance liquid chromatography of thiols and disulfides: Dinitrophenol derivatives." Methods Enzymol **143**: 101-109.

Feingold, I. B. and Colby, H. D. (1992). "Sex differences in adrenal and hepatic alpha-tocopherol concentrations in rats." Pharmacology **44**(2): 113-6.

Feingold, I. B., Longhurst, P. A. and Colby, H. D. (1993). "Regulation of adrenal and hepatic alpha-tocopherol content by androgens and estrogens." Biochim Biophys Acta **1176**(1-2): 192-6.

Furman, G. M., Silverman, D. M. and Schatz, R. A. (1991). "The effect of toluene on rat lung benzo[a]pyrene metabolism and microsomal membrane lipids." Toxicology **68**(1): 75-87.

Halliwell, B. (1995). "Antioxidant characterization. Methodology and mechanism." Biochem Pharmacol **49**(10): 1341-8.

Hammond, E. C., Selikoff, I. J., Lawther, P. L. and Seidman, H. (1976). "Inhalation of benzpyrene and cancer in man." Ann N Y Acad Sci **271**: 116-24.

Hosomi, A., Arita, M., Sato, Y., Kiyose, C., Ueda, T., Igarashi, O., Arai, H. and Inoue, K. (1997). "Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin e analogs." FEBS Lett **409**(1): 105-8.

Leonard, S. W., Paterson, E., Atkinson, J. K., Ramakrishnan, R., Cross, C. E. and Traber, M. G. (2005). "Studies in humans using deuterium-labeled alpha- and gamma-tocopherols demonstrate faster plasma gamma-tocopherol disappearance and greater gamma-metabolite production." Free Radic Biol Med **38**(7): 857-66.

Lodge, J. K., Traber, M. G., Elsner, A. and Brigelius-Flohe, R. (2000). "A rapid method for the extraction and determination of vitamin e metabolites in human urine." J Lipid Res **41**(1): 148-54.

Lykkesfeldt, J. (2001). "Determination of malondialdehyde as dithiobarbituric acid adduct in biological samples by hplc with fluorescence detection: Comparison with ultraviolet-visible spectrophotometry." Clin Chem **47**(9): 1725-7.

Mathieu, M. C., Lapierre, I., Brault, K. and Raymond, M. (2001). "Aromatic hydrocarbon receptor (ahr).Ahr nuclear translocator- and p53-mediated induction of the murine

multidrug resistance mdr1 gene by 3-methylcholanthrene and benzo(a)pyrene in hepatoma cells." J Biol Chem **276**(7): 4819-27.

McClean, M. D., Rinehart, R. D., Ngo, L., Eisen, E. A., Kelsey, K. T., Wiencke, J. K. and Herrick, R. F. (2004). "Urinary 1-hydroxypyrene and polycyclic aromatic hydrocarbon exposure among asphalt paving workers." Ann Occup Hyg **48**(6): 565-78.

Meydani, M. and Greenblatt, D. J. (1990). "Influence of vitamin-e supplementation on antipyrine clearance in the cebus monkey." Nutrition Research **10**(9): 1045-1051.

Mooney, L. A., Madsen, A. M., Tang, D., Orjuela, M. A., Tsai, W. Y., Garduno, E. R. and Perera, F. P. (2005). "Antioxidant vitamin supplementation reduces benzo(a)pyrene-DNA adducts and potential cancer risk in female smokers." Cancer Epidemiol Biomarkers Prev **14**(1): 237-42.

Morley, S., Cecchini, M., Zhang, W., Virgulti, A., Noy, N., Atkinson, J. and Manor, D. (2008). "Mechanisms of ligand transfer by the hepatic tocopherol transfer protein." J Biol Chem **283**(26): 17797-804.

Murray, M. (1991). "In vitro and in vivo studies of the effect of vitamin e on microsomal cytochrome p450 in rat liver." Biochem Pharmacol **42**(11): 2107-14.

Mustacich, D. J., Shields, J., Horton, R. A., Brown, M. K. and Reed, D. J. (1998). "Biliary secretion of alpha-tocopherol and the role of the mdr2 p-glycoprotein in rats and mice." Arch Biochem Biophys **350**(2): 183-92.

Mustacich, D. J., Leonard, S. W., Devereaux, M. W., Sokol, R. J. and Traber, M. G. (2006). "Alpha-tocopherol regulation of hepatic cytochrome p450s and abc transporters in rats." Free Radic Biol Med **41**(7): 1069-78.

Mustacich, D. J., Bruno, R. S. and Traber, M. G. (2007a). "Vitamin e." Vitam Horm **76**: 1-21.

Mustacich, D. J., Vo, A. T., Elias, V. D., Payne, K., Sullivan, L., Leonard, S. W. and Traber, M. G. (2007b). "Regulatory mechanisms to control tissue alpha-tocopherol." Free Radic Biol Med **43**(4): 610-8.

Mustacich, D. J., Gohil, K., Bruno, R. S., Yan, M., Leonard, S. W., Ho, E., Cross, C. E. and Traber, M. G. (2009). "Alpha-tocopherol modulates genes involved in hepatic xenobiotic pathways in mice." J Nutr Biochem **20**(6): 469-76.

Mustacich, D. J., Leonard, S. W., Patel, N. K. and Traber, M. G. (2010). "Alpha-tocopherol beta-oxidation localized to rat liver mitochondria." Free Radic Biol Med **48**(1): 73-81.

Pavanello, S., Favretto, D., Brugnone, F., Mastrangelo, G., Dal Pra, G. and Clonfero, E. (1999). "Hplc/fluorescence determination of anti-bpde-DNA adducts in

mononuclear white blood cells from pah-exposed humans." Carcinogenesis **20**(3): 431-5.

Podda, M., Weber, C., Traber, M. G. and Packer, L. (1996). "Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones." J Lipid Res **37**(4): 893-901.

Pope, S. A., Burtin, G. E., Clayton, P. T., Madge, D. J. and Muller, D. P. (2002). "Synthesis and analysis of conjugates of the major vitamin e metabolite, alpha-cehc." Free Radic Biol Med **33**(6): 807-17.

Pott, P. (1974). "Chirurgical observations relative to cancer of scrotum." Ca-a Cancer Journal for Clinicians **24**(2): 110-116.

Sasco, A. J., Secretan, M. B. and Straif, K. (2004). "Tobacco smoking and cancer: A brief review of recent epidemiological evidence." Lung Cancer **45 Suppl 2**: S3-9.

Schlede, E., Kuntzman, R. and Conney, A. H. (1970). "Stimulatory effect of benzo(alpha)pyrene and phenobarbital pretreatment on the biliary excretion of benzo(alpha)pyrene metabolites in the rat." Cancer Res **30**(12): 2898-904.

Schultz, M., Leist, M., Petrzika, M., Gassmann, B. and Brigelius-Flohe, R. (1995). "Novel urinary metabolite of alpha-tocopherol, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman, as an indicator of an adequate vitamin e supply?" Am J Clin Nutr **62**(6 Suppl): 1527S-1534S.

Shuker, D. E., Atkin, W., Bingham, S. A., Leuratti, C. and Singh, R. (2002). "Malondialdehyde-DNA adducts in relation to diet and disease risk--a brief overview of recent results." IARC Sci Publ **156**: 475-80.

Sontag, T. J. and Parker, R. S. (2002). "Cytochrome p450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin e status." J Biol Chem **277**(28): 25290-6.

Stahl, W., Graf, P., Brigelius-Flohe, R., Wechter, W. and Sies, H. (1999). "Quantification of the alpha- and gamma-tocopherol metabolites 2,5,7, 8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman and 2,7, 8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman in human serum." Anal Biochem **275**(2): 254-9.

Taylor, A. W., Bruno, R. S., Frei, B. and Traber, M. G. (2006). "Benefits of prolonged gradient separation for high-performance liquid chromatography-tandem mass spectrometry quantitation of plasma total 15-series f-isoprostanes." Anal Biochem **350**(1): 41-51.

Taylor, A. W., Bruno, R. S. and Traber, M. G. (2008). "Women and smokers have elevated urinary f(2)-isoprostane metabolites: A novel extraction and lc-ms methodology." Lipids **43**(10): 925-36.

Traber, M. G., Burton, G. W. and Hamilton, R. L. (2004). "Vitamin e trafficking." Ann N Y Acad Sci **1031**: 1-12.

Traber, M. G., Siddens, L. K., Leonard, S. W., Schock, B., Gohil, K., Krueger, S. K., Cross, C. E. and Williams, D. E. (2005). "Alpha-tocopherol modulates cyp3a expression, increases gamma-cehc production, and limits tissue gamma-tocopherol accumulation in mice fed high gamma-tocopherol diets." Free Radic Biol Med **38**(6): 773-85.

Traber, M. G. (2007). "Vitamin e regulatory mechanisms." Annu Rev Nutr **27**: 347-62.

Traber, M. G. (2010). "Regulation of xenobiotic metabolism, the only signaling function of alpha-tocopherol?" Mol Nutr Food Res **54**(5): 661-8.

Vandewiel, J. A. G., Fijneman, P. H. S., Duijf, C. M. P., Anzion, R. B. M., Theuws, J. L. G. and Bos, R. P. (1993). "Excretion of benzo[a]pyrene and metabolites in urine and feces of rats - influence of route of administration, sex and long-term ethanol treatment." Toxicology **80**(2-3): 103-115.

Weglicki, W. B., Luna, Z. and Nair, P. P. (1969). "Sex and tissue specific differences in concentrations of alpha-tocopherol in mature and senescent rats." Nature **221**(5176): 185-6.

Young, I. S. and McEneny, J. (2001). "Lipoprotein oxidation and atherosclerosis." Biochem Soc Trans **29**(Pt 2): 358-62.