AN ABSTRACT OF THE THESIS OF

Sirinmas Katchamart for the degree of Doctor of Philosophy in Toxicology presented on January 3, 2000. Title: Indole-3-Carbinol and 3,3'-Diindolylmethane: Relative Potency as Modulators of Drug Metabolism and Carcinogenesis.

Abstract approved: Redacted for Privacy

David E. Williams

Indole-3-carbinol (I3C), the most abundant metabolite of glucobrassicins, is found in cruciferous vegetables such as broccoli, Brussels sprouts, cabbage and cauliflower. I3C has been shown to have anticarcinogenic activity against many chemical carcinogens in multiple target organs in animal models. However, the anticarcinogenic activity of I3C is considered indirect since I3C is converted irreversibly to its dimer and other condensation products under the low pH conditions of the stomach. 3,3'-Diindolylmethane (DIM), a primary breakdown product of I3C, also shows anticarcinogenic activity in a manner similar to I3C. Our laboratory has shown that I3C dramatically induces CYP1A (cytochrome P4501A) and inhibits FMO1 (flavin-containing monooxygenase form 1) expression in rat liver and intestine. We also showed that I3C induced hepatic CYP1A1/1A2 in male guinea pig, mouse and rabbit. There was no significant difference in FMO1 protein between control and I3C treated groups in these three species. The

simultaneous induction of CYP and repression of FMO may alter the metabolism, disposition and toxicity of drugs and/or xenobiotics that are substrates of both monooxygenases such as N, N-dimethylaniline, nicotine and tamoxifen. In liver microsomes from rats fed I3C or DIM, the ratio of FMO to CYP metabolites of the three chosen compounds decreased, mostly due to a reduction of N-oxide FMO-dependent formation. Major concerns associated with human dietary I3C supplementation are its estrogenic effect as seen in trout and its ability to induce cytochrome P450s involved in bioactivation. Evidence presented here also demonstrated a dose response of vitellogenin induction after 2 weeks of dietary I3C or DIM in rainbow trout. In immature female rat, I3C and DIM significantly induced uterine peroxidase, hepatic CYP1A1/1A2 and CYP2B1/2B2. All our results suggested that I3C and/or DIM, in combination with other drugs that are substrates for both monooxygenases, could alter the therapeutic effects or toxicity of drugs. Also the estrogenic activity in rainbow trout and immature female rat should be considered when assessing risk for the use of I3C and DIM as chemopreventive agents or dietary supplements.

INDOLE-3-CARBINOL AND 3,3'-DIINDOLYLMETHANE: RELATIVE POTENCY AS MODULATORS OF DRUG METABOLISM AND CARCINOGENESIS

by

Sirinmas Katchamart

A THESIS

Submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Completed January 3, 2000

Commencement June, 2000

Doctor of Philosophy thesis of Sirinmas Katchamart presented on January 3, 2000

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Redacted for Privacy

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Sirinmas Katchamart, Author

ACKNOWLEDGMENTS

I owe a debt of gratitude to numerous individuals who were encouraging and helpful in many ways during the course of graduate studies. Special thanks goes to Dr. David E. Williams, my advisor. I very much appreciate his guidance, patience and financial support. Thanks to Drs. Donald Reed, George Bailey, Mark Christensen and Wilbert Gamble for being my graduate committee and the staffs of the Laboratory Animal Resources and Food Toxicology and Nutritional Laboratory for their assistance taking care of my animals. Thanks also to following people who encouraged me: Dr. Viravan Prapapanich and her mother, Nupan Kheaomaingam, Dr. Nantana Jiramongkul and Aree Wangmaneerath. Additionally, I appreciate Sandy Segna for her expert editorial assistance and Angsana Phoosuntisumpunth, Dr. Siriporn Pipatsatayanuwong, Dr. Mei-Fei Yueh, Marilyn Henderson, Jin-Young Choi, Dr. Gayle Orner, Dr. David Carlson, Adam Shilling, Dustin Leibelt, and Margaret Pratt for their friendship. Finally, I'm grateful for my family in Thailand including my sister and her husband in Colorado for unlimited support, Department of Medical Sciences; Public Health Ministry and Royal Thai Embassy for the opportunity to study by their financial support.

CONTRIBUTION OF AUTHORS

Dr. David Williams was involved in experimental design, the choice of analytical methods and in the preparation of all manuscripts. He also performed N, N dimethylaniline N-oxygention and nicotine metabolism in Chapter 3 and assisted with the tail flick assay in Chapter 4. Drs. David Kupfer, David Stresser and Shangara Dehal conducted the tamoxifen metabolism in chapter 3. Dr. Paul Franklin provided instrumentation and instruction for the tail flick assay in Chapter 4. Dr. Cliff Pereira was responsible in statistical analysis in Chapter 4. Dr. David Carlson and Adam Shilling assisted with taking blood in trout and the vitellogenin analysis in chapter 5. Dr. Mei-Fei Yueh assisted with animal care and necropsy.

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This thesis is dedicated to my family:

Paiboon, Chutharath, Sunisa, Wanratchada and Akarapong

INDOLE-3-CARBINOL AND 3,3'-DIINDOLYLMETHANE: RELATIVE POTENCY AS MODULATORS OF DRUG METABOLISM AND CARCINOGENESIS.

Chapter 1

INTRODUCTION

Most cancers are caused by the interaction between genetics and the environment (Doll, 1996; Perera, 1996). Epidemiological studies show that approximately 80% of human cancer can be attributed to tobacco smokes and diet (Harris, 1991; ACS, 1995). Other factors are intense sun exposure, consumption of alcohol, chronic infections (Helbock *et al.*, 1998) and life-style influences such as lack of exercise, obesity and reproductive history (Henderson *et al.*, 1991; Feigelson and Henderson, 1996; Andersson *et al.*, 1997). Genetic factors are thought to explain only about 5% of all cancers (Knudson, 1985; Venitt, 1994). Given these observations, the majority of cancers are theoretically preventable. The approaches to cancer prevention including the following three steps. 1) First, reduce human exposure to environmental carcinogens through careful monitoring of the workplace and through educational approaches to encourage changes in lifestyle, 2) Second, identify individuals at high risk for cancer development through predisposing genetic or biochemical factors, and 3) Third, provide chemoprevention by dietary or synthetic substances (Stoner *et al.*, 1997).

Consumption of adequate fruits and vegetables is associated with a lower risk of degenerative diseases including cancer, cardiovascular disease, cataracts and brain dysfunction (Helbock *et al.*, 1998). Epidemiological studies also show the association between lack of adequate consumption of fruits and vegetables and cancer incidence (Block *et al.*, 1992; Steinmetz and Potter, 1996). A low intake of fruits and vegetables is associated with twice the cancer rate for lung, larynx, oral cavity, esophagus, stomach, colon, rectum, bladder, pancreas, cervix and ovary (Block *et al.*, 1992). There are many potential anticarcinogenic substances in fruits and vegetables. Some of the substances are widespread, like dietary fiber, whereas others are limited to one type of fruit or vegetable such as indole-3-carbinol (I3C) from cruciferous vegetables.

OCCURRENCE OF INDOLE-3-CARBINOL (I3C) AND 3,3'-DIINDOLYLMETHANE (DIM)

Glucobrassicins (indole glucosinolates) are found in high concentrations in cruciferous vegetables such as broccoli, Brussels sprouts, cabbage and cauliflower (Fenwick *et al.*, 1983). At neutral pH, glucobrassicins are hydrolyzed, yielding glucose, sulfate, 3-indolylmethyl isothiocyanate, thiocyanate ion and I3C. At a more acidic pH (3-4), the hydrolysis products are indole-3-acetonitrile (ICA), hydrogen sulfide and elemental sulfur (McDanell *et al.*, 1988) (Figure 1.1). I3C and ICA are the most abundant metabolites of glucobrassicins. I3C may undergo a

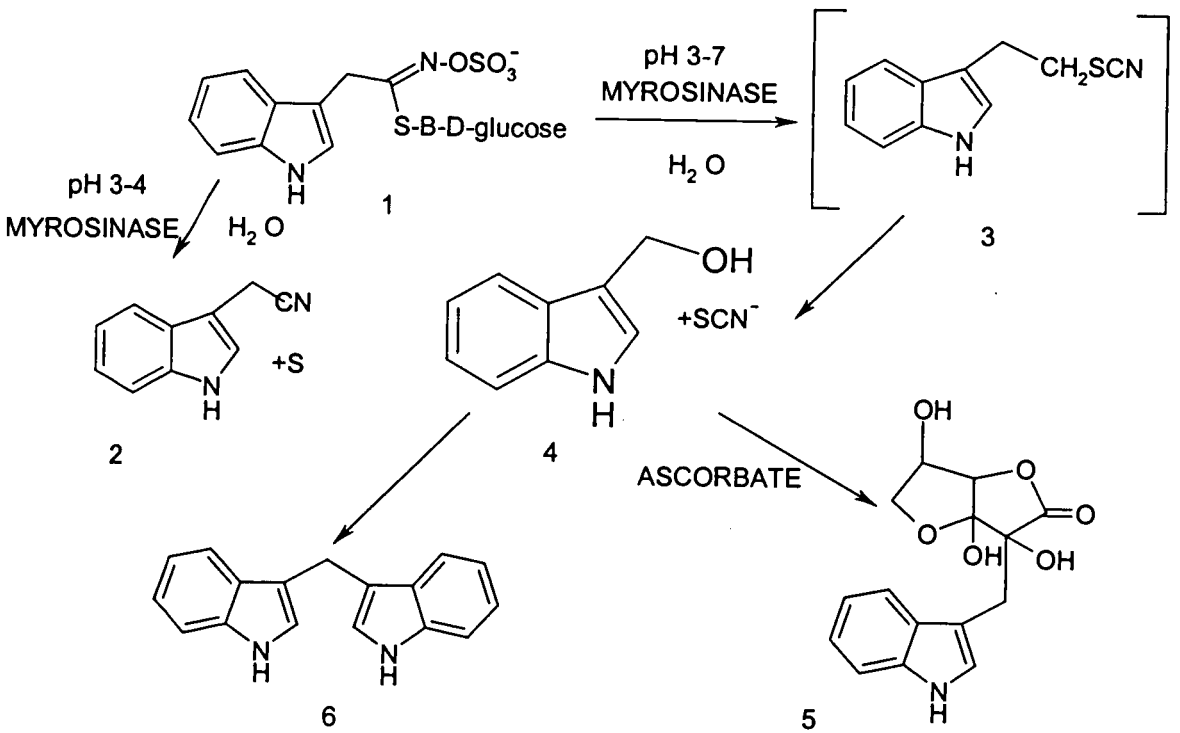

Figure 1.1 Enzymatic hydrolysis of glucobrassicin, found in cruciferous vegetables, and formation of I3C. 1 = Glucobrassicin; 2 = Indole-3-acetonitrile; 3 = 3-Indolylmethyl isothiocyanate; 4 = I3C; 5 = Ascorbigen; 6 = 3,3'-Diindolylmethane (DIM) (Taken from McDanell *et al.*, 1988).

condensation reaction to produce 3,3'-diindolylmethane (DIM) or react with ascorbic acid to form ascorbigen. Ascorbic acid is found at high levels in crucifers. The hydrolytic enzyme involved in glucobrassicin hydrolysis for both pH levels is myrosinase (thioglucoside glycohydrolase EC 3:2:3:1). This enzyme is found in the plant cell within a separate compartment from the glucobrassicin and in intestinal microflora. When the vegetables are cooked, myrosinase is inactivated by heat, and washed out, resulting in a 30-60% loss of intact glucobrassicin (de Vos and Bliijleven, 1988).

POSTULATED MECHANISM OF ANTICARCINOGENIC ACTIVITIES

Carcinogenesis is an accumulation of alterations in genes such as oncogenes, tumor suppressor genes, apoptosis regulating genes and DNA repair genes (Stanley, 1995) that regulate cellular proliferation and cell death. This process can be influenced by nutrition through hormonal, paracrine, autocrine, immune and metabolic mechanisms that modulate cellular proliferation, differentiation and apoptosis. Due to the slow and multistage nature of carcinogenesis, the strategies for the nutritional modulation and chemoprevention of cancer should focus on stopping carcinogenesis at the earliest possible point in the pathway.

The initiation stage is characterized by the mutation of DNA in response to exogenous or endogenous genotoxic agents Several chemical carcinogens are not demonstrably genotoxic, and are called epigenetic carcinogens (Tennant, 1991)

Generally, epigenetic carcinogens require high doses and prolonged exposure to be carcinogenic. Several mechanisms of epigenetic carcinogens have been proposed, including mutagenic processes such as oxidative stress (Guyton and Kensler, 1993), hormonal activity (Davis *et al.*, 1993) and increasing cell proliferation (Tennant, 1993). Genotoxic agents form reactive electrophiles, either spontaneously or from cellular metabolism, and react chemically with nucleophilic sites in the DNA. If specific genes regulating cellular growth, such as protooncogenes, tumor suppressor genes, apoptosis-regulating genes, or DNA repair, are damaged, the genetic changes such as point mutations, chromosomal translocations, deletions and inversions will be passed to the daughter cells during cell division, leading to a collection of cells expressing the mutant genes (Eng and Ponder, 1993). Alteration of procarcinogenic activation, enhancing carcinogen detoxification enzymes (Sumiyoshi and Wargovich, 1990), scavengers of electrophiles/reactive oxygen species (Perchellet *et al.*, 1995) and enhancing DNA repair (Weisburger *et al.*, 1995; Birt *et al.*, 1988) can modulate this early stage of carcinogenesis.

The promotion stage is characterized by transformation of initiated cells into a population of neoplastic cells, due to alteration in gene expression and cell proliferation. Promoting agents are not mutagenic, but rather they exert their effects on gene expression through specific receptors (Pitot, 1993) and perturbation of signal transduction pathways and cell cycle control (Fischer and DiGiovanni, 1995). The cell number homeostasis controlled by mitosis and apoptosis, is altered

during the promotion stage, resulting in increased proliferation and decreased apoptosis (Thompson *et al.*, 1992). Promoting agents can elicit the release and metabolism of arachidonic acid to a series of metabolites referred to as eicosanoids. Eicosanoids, including the prostaglandins and hydroperoxy forms of arachidonic acid, are involved in the inflammatory process, the immune response, tissue repair and cell proliferation (Fischer, 1995). Eicosanoids also affect several parameters associated with cell proliferation (Liu *et al.*, 1991). Modulation of tumor promotion can involve signal transduction pathways, cell cycle control, apoptosis and inflammatory processes.

The progression stage is characterized by the transformation of neoplastic cells to an invasive malignant cell mass, resulting from additional genetic alterations. Mutation in tumor suppressor gene p53 is frequently observed at this stage (Harris, 1993). The p53 gene product is a transcription factor that regulates the expression of a number of DNA-damage, cell-cycle and apoptosis-regulating genes. Genomic stability, which is regulated by p53, and cell homeostasis are lost during malignant progression (Livingstone *et al.*, 1992). DNA hypomethylation also contributes to this stage (Guinn and Mills, 1997). Thus, p53, other cell cycle and apoptotic regulators, as well as other genes regulating genomic instability and DNA methylation, are critical targets to prevent the progression stage of carcinogenesis.

Epidemiological and animal studies demonstrate the protective effect against carcinogenesis of greater fruit and vegetable consumption (Steinmetz and

Potter, 1996). Cruciferous vegetables are unique in their high contents of I3C. I3C has been shown to have anticarcinogenic activity against many chemical carcinogens such as aflatoxin B1 (AFB1) (Fong *et al.*, 1990), 2-amino-*N*-methyl-5-phenylimidazopyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (Guo *et al.*, 1995; Xu *et al.*, 1996), nitroazarene-1-oxide (Tanaka *et al.*, 1992), dimethylnitrosamine (Shertzer and Tabor, 1988), and 7, 12-dimethylbenz[a]anthracene (DMBA) (Hendricks *et al.*, 1994). Several studies have shown that I3C lowered cancer incidences in many target organs including endometrium (Kojima *et al.*, 1994), forestomach (Wattenberg and Loub, 1978), larynx (Newfield *et al.*, 1993), lung (Morse *et al.*, 1988; 1990), liver (Dashwood *et al.*, 1988; Morse *et al.*, 1988; Dashwood *et al.*, 1989; Bailey *et al.*, 1996; Oganesian *et al.*, 1997), mammary (Grubbs *et al.*, 1995), nasal mucosa (Morse *et al.*, 1990), stomach (Hendricks *et al.*, 1994), swim bladder (Hendrick *et al.*, 1994) and tongue (Tanaka *et al.*, 1992) in animal models.

The anticarcinogenic activity of I3C has been hypothesized to be the result of modulation of the metabolism of procarcinogens and prevention of formation of electrophilic intermediates, through alteration of biotransformation enzymes. Stresser *et al.* (1994a) found that after rats were given diets containing I3C, CYP1A and CYP3A were induced in liver, resulting in increased formation of AFM1 and AFQ1, detoxification products of AFB1. However, in rainbow trout, 1000 ppm I3C inhibits AFB1-DNA adduction without sustained CYP1A induction (Takahashi *et al.*, 1995).

Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (mercapturic acid synthesis) and conjugation with amino acids such as glycine, taurine and glutamic acid. Phase II enzymes increase the xenobiotic hydrophilicity by adding these endogenous groups to xenobiotics, thereby promoting excretion (with the exception of methylation and acetylation) (Timbrell, 1992). Therefore, induction of phase II enzymes is involved in detoxification of activated procarcinogens, preventing their binding to DNA and enhancing excretion. Studies have shown that I3C induces Phase II enzymes including UDP-glucuronosyl transferase (Jorgen *et al.*, 1989; Shertzer and Sainsbury, 1991a; b), glutathione transferase (Sparmin *et al.*, 1982; Shertzer and Sainsbury, 1991a; b; Danger *et al.*, 1992; Stresser *et al.*, 1994b), epoxide hydrolase (Cha *et al.*, 1985) and quinone reductase (Salbe and Bjeldanes, 1986; de Kruif *et al.*, 1991; Shertzer and Sainsbury, 1991a; b; Wortelboer *et al.*, 1992a; b). The efficiency with which phase II enzymes detoxify carcinogens is a critical factor in determining the carcinogenicity of particular xenobiotics.

Most carcinogens damage DNA through electrophilic intermediates (Miller and Miller, 1981). The electrophilic metabolites may themselves be reactive oxygen species (ROS) that can directly interact with DNA. Oxygen free radicals may also be involved in a step required for activation of a procarcinogen, thus the reactions involved in metabolic activation of carcinogens may release ROS that can in turn attack DNA (Perchellet *et al.*, 1995). Under oxidative stress conditions, ROS can interact with biomolecules including DNA (base modification and strand

breaks), proteins (carbonyl formation, methionine or cysteine oxidation, fragmentation) and lipids (peroxidation). Lipid peroxidation not only destroys lipids in cell membranes and membrane integrity, it also generates endogenous toxicants including free radicals such as alkoxyl, peroxyl radicals and electrophiles such as 4-hydroxynonenal. These endogenous toxicants are also reactive and can modify DNA and protein (Toyokuni *et al.*, 1994). The antioxidant and electrophilic scavenging properties of I3C (Shertzer, 1983; 1984; Shertzer *et al.*, 1987; 1988; Shertzer and Tabor, 1988; Sharma *et al.*, 1994; Arnao *et al.*, 1996) have been reported. Therefore, I3C is a plausible agent for modulating this early stage of carcinogenesis.

The eukaryotic cell cycle can be divided into four phases: G_1, S, G_2 and M phases. The G_1 phase is the interval between the completion of the M phase and the beginning of the S phase. The G_2 phase is the interval between the end of S phase and the beginning of M phase. The M phase consists of mitosis (the nuclear division) and cell division. DNA replication occurs during the S phase. Cells in G_1 can also pause in their progress for extended periods and enter a specialized resting state called G_0. There is a regulatory network of growth-inhibiting and growth-stimulating signals transduced from the extracellular environment that converge on G_1-acting components (Sherr, 1996; Stillman, 1996). The final targets of these growth signaling pathways are specific sets of cyclin-dependent kinase (CDK) protein complexes. In the G_1 phase, cyclins C, D1, D2, D3 and E are necessary for activating the G1 CDKs (CDK2, CDK4 and CDK6), while several of the small

proteins associated with cyclin-CDK complexes (p15, p16/Ink4a, p21/Waf1/Cip1, p27/Kip1 and p57/Kip2) act as specific inhibitors of cyclin-dependent-kinase activity (Elledge and Harper, 1994; Sherr and Robert, 1995; Gartel *et al.*, 1996; Alessandrini *et al.*, 1997). There is a loss of normal cell cycle control in G_1 in mammary tumor development and differentiation (Buckley *et al.*, 1993; Keyomarsi and Pardee, 1993). I3C can alter the cell cycle by decreasing the expression and activity of cyclin-dependent kinase-6 (CDK6), resulting in the G_1 arrest of breast cancer cells by an estrogen receptor (ER)-independent pathway (Cover *et al.*, 1998).

Cells in tissues are interconnected by gap junctions, with clusters of well-insulated cell-to-cell channels contained in specialized plasma membrane regions. It has been proposed that the loss or alteration of gap junctional intercellular communication (GJIC) plays an important role in the process of carcinogenesis (Holder *et al.*, 1993). Adhesion of tumor cells to vascular endothelium is a primary step in the colonization of select target organs by blood-borne cancer cells (El Sabban and Paule, 1994). Rijinkels *et al.* (1998) reported that I3C prevented a decrease in intercellular communication in human colon carcinoma cell lines caused by stearic acid. Due to the importance of GJIC in cell proliferation and differentiation, the inhibition of GJIC may be related to the tumor promotion stage.

The anticarcinogenic activity of I3C is indirect because I3C is converted irreversibly via acid condensation reactions to dimers, trimers, tetramers and other condensation products (Figure 1.2). Intravenous administration of I3C, which

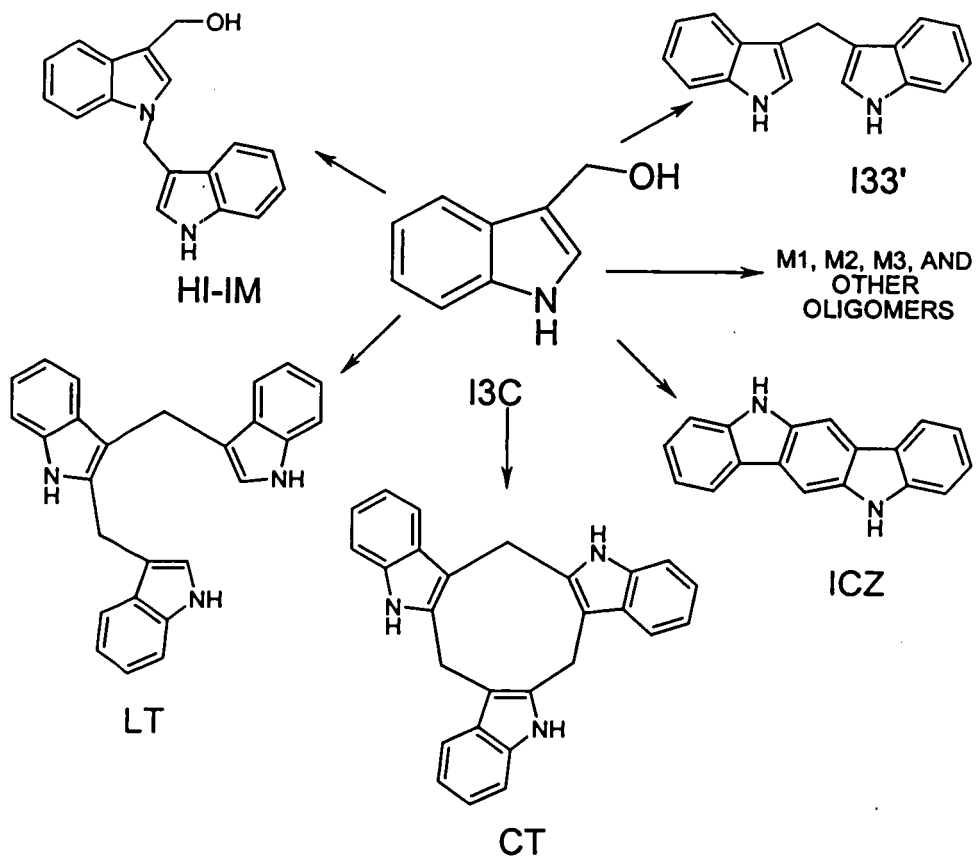

Figure 1.2 Structure of I3C acid condensation products found in liver extracts of rats given I3C orally. I3C itself was not detected in liver extracts. I33', 3,3'-diindolylmethane; LT, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane; HI-IM, (hydroxymethyl)indolyl-3-indolylmethane; CT, 5,6,11,12,17,18-hexahydroclonal[1,2-b:4,5-b':7,8-b]:triindole; ICZ, 3,2-b-indolocarbazole (Taken from Stresser *et al.*, 1995)

bypasses the acid environment of the stomach, does not result in biological efficiency. 3,3'-Diindolylmethane (DIM) is a primary breakdown product *in vivo* after oral administration of I3C (Stresser *et al.*, 1995) and *in vitro* (Spande, 1979). DIM inhibits AFB1-DNA binding in trout (Dashwood *et al.*, 1994) and mammary tumor formation in the rat (Wattenberg and Loub, 1978; Chen *et al.*, 1998). DIM also induces phase I and phase II enzymes such as CYP1A1, CYP1A2, and CYP2B2 (Jellinck *et al*, 1993) and glutathione S-transferase (Wortelboer *et al.*, 1992a; b). An enhancement of apoptosis is observed in human cancer cells treated with DIM, independent of the p53 mediated apoptosis pathway (Ge *et al.*, 1996).

ENDOCRINE EFFECTS

Estradiol and estrone, endogenous estrogens, that are distributed widely in mammalian tissues, have been shown to be oxidized by cytochrome P450 enzymes. 2-and 4-Hydroxylations of estradiol and estrone are mainly catalyzed by CYP1A2 in human liver (Yamazaki *et al.*, 1998). In human, CYP1B1 is an extrahepatic 17β-estradiol 4-hydroxylase under the regulatory control of the AhR (Spink *et al.*, 1997). CYP3A4 and CYP2C9 also catalyze these reactions to lesser extents than do CYP1A2 and CYP1B1. Estradiol 16α-hydroxylation is catalyzed primarily by CYP1A2 and CYP3A4, whereas estrone 16α-hydroxylation is catalyzed solely by CYP3A4 in human liver microsomes (Yamazaki *et al.*, 1998). Human CYP1A2 is expressed only in liver, but CYP3A4 is also expressed at lower concentrations in a

number of extrahepatic tissues (Guengerich, 1995) and is the major CYP in the intestine (Krishna and Klotz, 1994).

2-Hydroxyestradiol has little or no carcinogenic activity (Liehr *et al.*, 1986a; Li and Li, 1987) and exhibits antiestrogenic activity based on its inhibition of estrogen-stimulated MCF-7 cell growth (Liehr *et al.*, 1986b). 2-Hydroxyestradiol and estrone can bind to the estrogen receptor but with a markedly reduced binding affinity (Ball and Knuppen, 1980; MacLusky *et al.*, 1983; van Aswegen *et al.*, 1989; Feigelson and Henderson, 1996), resulting in weaker hormonal potency as compared with estradiol (Martucci and Fishman, 1979; Ball and Knuppen, 1980; Fishman 1981; Schutze *et al.*, 1993; 1994). 4-Hydroxyestradiol is a strong carcinogen in the hamster kidney (Liehr *et al.*, 1986a; Li and Li, 1987). 16α-Hydroxyestrone is genotoxic based on the increase in unscheduled DNA synthesis in mouse mammary epithelial cells (Telang *et al.*, 1992; 1993). There is a correlation between high levels of 16α-hydroxylation and mammary tumor incidence in humans and in several strains of mice (Schneider *et al.*, 1982; Bradlow *et al.*, 1985). In contrast to 2-hydroxylation of estradiol and estrone, 4-and 16α-hydroxylation estrogens can bind to the estrogen receptor with the same affinity as estradiol, and then activate the estrogen receptor (Martucci and Fishman, 1979; Ball and Knuppen, 1980; Fishman and Martucci, 1980; MacLusky *et al.*, 1983; van Aswegan *et al.*, 1989).

2- and 4-Hydroxylation metabolites can undergo metabolic redox cycling (Liehr *et al.*, 1986a; Liehr, 1990; Liehr and Roy, 1990) and generate free radicals

and the chemically-reactive estrogen semiquinone/quinone intermediates, resulting in damage to DNA and other cellular constituents (Nutter *et al.*, 1991; Han and Liehr, 1994a; b; Cavalieri *et al.*, 1997). 2-Hydroxylation of estradiol and estrone to a catechol is a major metabolic pathway in the liver (Dannan *et al.*, 1986; Kerlan *et al.*, 1992; Zhu *et al.*, 1993; Suchar *et al.*, 1995) whereas 4-hydroxylation of estrogens to a catechol represents a major pathway in several extrahepatic target tissues (Bui and Weisz, 1988).

2- and 4-Hydroxylated metabolites can be detoxified to *O*-methylated products by catechol *O*-methyltransferase (COMT). However, the metabolic clearance of 4-OH estradiol is slower than that of 2-OH estradiol. The *O*-methylation of 4-OH estradiol is inhibited by 2-OH estradiol (Roy *et al.*, 1990). It is possible that 4-OH estradiol persists in the body and undergoes redox cycling, resulting in increased oxidative DNA damage. Male Syrian hamster kidney, CD-1 mouse uterus and rat pituitary, the estradiol-induced tumor sites, have very high levels of endogenous catecholamines (up to 50-fold higher than in several non-target tissues in the same animal or in other strains or species). High concentration of catecholamines in target tissues may inhibit COMT, resulting in increased tissue concentration of 2 and 4-hydroxy metabolites and decreased 2 and 4-methoxy metabolites (Zhu and Liehr, 1993). Earlier studies also found that stressed mice had an increased spontaneous breast cancer incidence (Riley, 1975). Epidemiological studies have suggested that chronic stress associated with an

increase in endogenous catecholamine levels increases the risk for breast cancer (Cooper *et al.*, 1989; Faragher and Cooper, 1990; Forsen, 1991).

The formation of 16α-hydroxylation estrone is elevated in women with breast cancer, in women at high risk of breast cancer, and in strains of mice with a high incidence of spontaneous mammary tumors (Schneider *et al.*, 1982; Bradlow *et al.*, 1985; Osborne *et al*, 1988). Increased 2-hydroxylation is linked to a reduced risk of estrogen-dependent tumors, as observed in cigarette smokers (Michnovicz *et al.*, 1986), reduced body weight (Fishman *et al.*, 1975), aerobic exercise (Snow *et al.*, 1989), thyroid supplementation (Fishman *et al.*, 1965) and dioxin exposure (Bertazzi *et al.*, 1989). Several factors are known to decrease estradiol 2-hydroxylation, including obesity (Schneider *et al.*, 1983), high fat diets (Musey *et al.*, 1987), hypothyroidism (Fishman *et al.*, 1965) and cimetidine therapy (Galbraith and Michnovicz, 1989). To date, there are no known modulators of estradiol 16α-hydroxylation.

Kojima *et al.* (1994) showed that the spontaneous occurrences of endometrial adenocarcinoma and preneoplastic lesions were reduced in Donryu rats treated with 200, 500 and 1000 ppm I3C for 660 days, compared to controls. In an 8-month feeding study, I3C at doses of 500 and 2000 ppm, significantly lowered the incidence and multiplicity of spontaneous mammary tumors in C3H/OuJ mice (Bradlow *et al.*, 1991). The alteration of estrone 2-hydroxylation associated with CYP1A2 in MCF-7 cells (Tiwari *et al.*, 1994) and in humans (Michnovicz and Bradlow, 1991; Bradlow *et al.*, 1994; Wong *et al.*, 1997) is possibly the mechanism

involved in lowering the incidence of hormone-dependent cancer mediated by I3C. I3C also inhibited the growth of an estrogen-dependent human breast cancer cell line in a manner independent of the estrogen receptor (Cover *et al.*, 1999).

Estrogens are classified as epigenetic carcinogens and promoters based on their hormone receptor-mediated effect on cell proliferation (Yager *et al.*, 1991; Yager and Zurlo, 1995). Few hepatic tumors are found in most experimental animals after prolonged treatment with estrogens alone. However, when used in conjunction with a known carcinogen or initiator agent such as diethylnitrosamine, estrogens can act as a promoting agents by dramatically enhancing the effects of a carcinogen initiator (Diwan *et al.*, 1991).

In addition to antiestrogenic effects, I3C exhibits estrogenic activity as shown by induction of plasma vitellogenin during I3C promotion of aflatoxin B1-initiated liver tumors in trout (Oganesian *et al.*, 1999). Nunez *et al.* (1989) reported that immature rainbow trout, initiated with subcarcinogenic doses of aflatoxin B1 and fed 17β-estradiol, showed reduced growth and increased DNA synthesis, mortality, and liver tumor incidence. In the C57BL/6J infant mouse model, long-term dietary I3C inhibited diethylnitrosamine-induced tumor multiplicity. This result is consistent with a hypothesis supporting an estrogenic mechanism of I3C, as it is known that estrogen inhibits chemically induced liver tumors in mice (Poole and Drinkwater, 1996).

OTHER BIOLOGICAL PROPERTIES

Dunn and LeBlance (1994) showed that I3C and its acid condensation products lowered serum LDL/VLDL cholesterol levels in mice, resulting from the inhibition of acyl-CoA:cholesterol acytransferase (ACAT). This enzyme is responsible for esterification of free cholesterol to be stored in the liver as lipid droplets or incorporated into lipoproteins for systemic distribution.

Three I3C acid condensation products DIM, CTI (5,6,11,12,17,18-hexahydrocyclononal (1,2-b:4,5-b':7,8-b'') triindole) and BII (2,3-bis(3-indolylmethyl)indole) competed with vinblastine and doxorubicin, for binding to P-glycoprotein, resulting in increased cellular accumulation of these chemotherapeutic agents and increased efficacy of these agents *in vitro*. In nude mice, I3C can reverse multidrug resistance in chemotherapeutic drugs to tumors without toxicity (Christensen and LeBlanc, 1996).

TUMOR PROMOTION AND ENHANCEMENT

Although I3C has received particular interest as a possible chemopreventive agent, several studies provide clear evidence for promotion or enhancement of carcinogenesis by I3C. Administration of I3C during the promotion stage or post initiation enhanced liver and thyroid gland tumors induced by diethylnitrosamine in the rat (Kim *et al.*, 1994; 1997), colon cancer induced by 1,2-dimethylhydrazine (DMH) in the rat (Pence *et al.*, 1986), liver tumors induced by AFB1 in trout

(Nixon *et al.*, 1984; Bailey *et al.*, 1987; Dashwood *et al.*, 1991) and by 7,12-dimethylbenz[a]anthracene (DMBA) in trout (Hendricks *et al.*, 1994), and ornithine decarboxylase (ODC) activity induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse skin (Birt *et al.*, 1986). At lower dietary I3C levels, promotion of aflatoxin B1-initiated hepatocarcinogenesis in trout may be associated with estrogenic activity, whereas at high I3C levels CYP1A induction may also play a role in an additive promotional phenomenon (Oganesian *et al.*, 1999). The time of I3C exposure is important since the previous studies showed that inhibition could occur only when I3C is given before and/or during carcinogenic exposure. Administration of I3C after carcinogenic exposure can promote tumor formation. However, recently a study showed that infant mice exposed to I3C through lactation exhibited enhanced liver tumorigenesis after initiation with diethylnitrosamine (Oganesian, 1998).

OTHER TOXICITIES

I3C was not teratogenic in rats treated subcutaneously on gestation days eight and nine at doses of 200 or 300 mg/kg body weight. A significant decrease in fetal weight was observed at a dose of 200 mg/kg body weight, but not at a dose of 300 mg/kg. I3C has shown no effect on kidney, liver and thyroid weight of pregnant rats (Nishie and Daxenbichler, 1980).

I3C alone was not mutagenic with or without metabolic activation in the Ames test using *Salmonella typhimurium* strains TA98 and TA100 or in CHO cells

(Reddy *et al.*, 1983, Birt *et al.*, 1986; Kuo *et al.*, 1992). Studies showed that I3C was mutagenic when cotreated with nitrites at pH 3 (Tiedink *et al.*, 1989; Sasagawa and Matsushima, 1991), suggesting that under the low pH conditions in the stomach, I3C might be indirectly involved in initiation events.

The chemoprevention Branch of the National Cancer Institute sponsored several I3C toxicity studies, including an acute oral toxicity study in rats, and 28-day and 90-day chronic studies in rats and dogs. The results from the acute oral toxicity study showed an LD_{50} estimated to be more than 2,250 mg/kg body weight. The 28-day and 90-day studies in rats documented toxic effects to the hematopoietic system, liver, hair coat and testes at doses of 20, 60, 200, 600 and 2000 mg/kg body weight, and increases in liver weight associated with induction of smooth endoplasmic reticulum at doses of 20 and 100 mg/kg body weight, respectively. Diarrhea was observed in the 28-day and 90- day studies in both sexes of dogs administered I3C at doses of 15, 50 and 150 mg/kg body weight. I3C at a dose of 150 mg/kg body weight lowered body weight and caused anemia due to gastrointestinal disturbances, and caused thymic atrophy in both sexes and testicular degeneration (Kelloff *et al.*, 1996).

In some cases, conjugation with glutathione enhances the toxicity of a xenobiotic including dihaloalkanes (Monks *et al.*, 1990; Dekant and Vamvakas, 1993). Two dihaloalkanes that have received particular interest are dichloromethane (DCM) and dibromoethane (DBE). DCM is widely used in paint, varnish strippers, the synthesis of plastics, the manufacture of film, and the

synthesis of pharmaceutical drugs. DBE is primarily used as a lead-scavenging agent in anti-knock mixtures added to gasoline (Andersen *et al.*, 1987). DCM increases pulmonary and hepatic neoplasm incidences in female and male mice. DBE gave positive results in carcinogenicity tests sponsored by the National Toxicology Program (NTP, 1982; 1986). There are two major biotransformation of dihalolkane: detoxification and toxification pathways. The oxidation of dihaloalkane by CYP2E1 is the detoxification pathway (NTP, 1982). The toxification pathway of both compounds is a nucleophilic substitution of thiolate for halide that occurs in a reaction catalyzed by glutathione S-transferase, yielding S-haloalkylglutathione conjugates which are more reactive than the parent compound (Ahmed and Anders, 1976). S-Chloromethyl-glutathione, the DCM intermediate, undergoes a further nucleophilic substitution with cellular macromolecules including DNA. S-2-Bromoethylglutathione, the DBE intermediate, rearranges to eliminate the remaining halogen atom, resulting in an episulfonium, a strong electrophile. The class theta glutathione S-transferase T1-1 is almost exclusively responsible for the activation of DCM, whereas other classes of glutathione S-transferase (alpha, mu and theta T1-1) play a role in DBE activation (van Bladeren *et al.*, 1980; Meyer *et al.*, 1991; Thier *et al.*, 1993; Hayes and Pulford, 1995; Ploeman *et al.*, 1997). After administration of 0.5% I3C in control diet for 2 weeks to male rats, the hepatic GSTT1 protein was induced 6.2 fold, associated with an increase in the steady-state level of mRNA, compared with the control rats. Hepatic CYP2E1 was unchanged by I3C (Sherratt *et al.*, 1998).

This study suggested that taking I3C might increase susceptibility to the neoplastic effects of DCM and DBE.

As with the Ah receptor agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), *in utero* exposure to a single dose of I3C (1.0 or 100 mg/kg) resulted in abnormalities in the male rat offspring including decreased sperm production and decreased transit rate of sperm (Wilker *et al.*, 1996). Our laboratory has preliminary evidence that the linear trimer is the only metabolite found in the liver of neonates and fetuses from maternal mice or rats fed I3C (Oganesian, 1998; Larsen-Su, 1998).

SUMMARY

The anticarcinogenic activity of I3C has been investigated for many years in an attempt to understand its anticarcinogenic mechanisms before I3C is used as a supplement for chemoprevention. Numerous studies have provided evidence for mechanisms of anticarcinogenic activity including inhibition of CYP bioactivation, induction of CYP detoxification, and induction of phase II enzymes. Electrophilic or free radical scavenging, alteration of cell cycle via decreasing the expression and activity of cyclin-dependent kinase-6 (CDK6), and preventing a decrease in intercellular communication also may be important. However, the anticarcinogenic activity of I3C is indirect since I3C is converted irreversibly to its dimer and other condensation products under the low pH condition of the stomach. 3,3'-

Diindolylmethane (DIM), the primary breakdown product, also shows anticarcinogenic activity in a manner similar to I3C.

Our laboratory has shown that I3C dramatically inhibits FMO1 and induces CYP1A1 expression in rat liver and intestine. 2000 ppm I3C administered to male guinea pig, mouse and rabbit induced hepatic CYP1A1/1A2. There was no significant difference in FMO1 between control and I3C-treated groups of guinea pig, mouse and rabbit (Chapter 2). The alteration of FMO and CYP-mediated drug metabolism *in vitro* by I3C and DIM suggested a potential enhance therapeutic efficacy and/or toxicity of nicotine, tamoxifen and codeine *in vivo* (Chapter 3-4).

In addition to antiestrogenic effects, presumably mediated by induction of estrogen 2-hydroxylation, I3C exhibits estrogenic activity by inducing plasma vitellogenin and uterine peroxidase activity in trout and rat, respectively (Chapter 5). These and other considerations are important when assessing risk for the use of I3C and/or DIM as a chemopreventive agent or dietary supplement.

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Chapter 2

SPECIES-SPECIFIC VARIATIONS IN DIETARY INDOLE-3-CARBINOL INHIBITION OF FLAVIN-CONTAINING MONOOXYGENASE FORM 1 EXPRESSION IN GUINEA PIG, MOUSE AND RABBIT LIVER

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ABSTRACT

Indole-3-carbinol (I3C) administered in the diet to male Fischer 344 rats has been shown to induce hepatic levels of CYP1A1 and inhibit both the expression and activity of FMO1. However, little is known about the effects of I3C administered in the diet to other common laboratory species. The purpose of this study was to determine the effect of I3C on hepatic expression of CYP1A1/1A2 and FMO1 in guinea pig, mouse and rabbit. I3C at a dose of 2000 ppm induced hepatic CYP1A1/1A2 in male guinea pigs, mice and rabbits, however there was no significant difference in hepatic FMO1 protein level between control and I3C treated groups

INTRODUCTION

Microsomal flavin-containing monooxygenase (FMO, EC1.14.13.8) catalyzes oxidative NADPH metabolism of a wide variety of soft nucleophilic substrates containing nitrogen, sulfur, phosphorus, boron, iodine or selenium heteroatoms (Ziegler, 1993; Cashman, 1995), resulting in detoxification and toxification (Ziegler, 1988; 1990). Based on the relationship between DNA sequences, not on catalytic activity, FMO genes are divided into families (FMO1 through FMO5) which share 50-60% identity; orthologs have greater than 80% identity (Hines *et al.*, 1994; Lawton *et al.*, 1994).

The expression of different FMO isoforms in animals may vary in relation to various biological or physiological factors such as tissue, sex, species, developmental stage and nutrition. The major FMO isoform found in the liver of all mammalian species is FMO1, except adult primates and female mice in which FMO3 is the major isoform (Phillips *et al.*, 1995; Falls *et al.*, 1995). FMO1 is also present in high amount in rabbit nasal microsomes (Shehin-Johnson *et al.*, 1995). The expression of FMO1 in liver is also sex-specific in rats and mice, being higher in the male in rat, and the female in mouse (Cherrington *et al.*, 1998). Previous studies showed that in rat testosterone and estrogen were stimulatory and inhibitory, respectively for FMO expression (Dunnan *et al.*, 1986), but in mice the opposite pattern is observed (Fall *et al.*, 1997). Interestingly, the developmental pattern observed for mouse FMO3 was similar to human FMO3, which is found at low levels in fetal liver and then becomes the predominant isoform in adult liver. In

contrast, hepatic FMO1 is expressed at high levels in fetus and disappears in the adult mouse and human (Cherrington *et al.*, 1998). In rabbit, high levels of progesterone and cortisol produced during pregnancy, increase the expression of FMO1 and FMO2 in liver and lung (Lee *et al.*, 1995). Nutritional factors also play a role. FMO expression and activity were inhibited by diet restriction in mice and guinea pig, ascorbic acid deficiency in guinea pig (Dixit and Roche, 1984; Brodfuehrer and Zonnoni, 1986) and by changing from a laboratory chow to a semi-synthetic diet in rat (Kaderlik *et al.*, 1991).

Indole-3-carbinol (I3C), a major component of cruciferous vegetables, and its acid condensation products bind to the aryl hydrocarbon receptor (AhR) and strongly induce many Phase I and Phase II enzymes, both *in vivo* and *in vitro* (Bjeldenas *et al.*, 1991; Jellinck *et al.*, 1993; Loub *et al.*, 1975; Stresser *et al.*, 1995). Our laboratory has shown that indole-3-carbinol (I3C) administered in the diet to male Fischer 344 rats inhibits both the level and catalytic activity of FMO1 protein and induces CYP1A (Larsen-Su and Williams, 1996). After consumption 300 g of cooked Brussel sprouts per day for 3 weeks, the ratio of human urinary trimethylamine and trimethylamine N-oxide was increased 2.6-3.2 fold, suggesting that FMO activity was decreased (Cashman *et al.*, 1999). However, little is known about the effects on FMO1 of I3C administered in the diet in other species. The purpose of this study was to address the species-specificity of dietary modulation of FMO1 by I3C from guinea pig, mouse and rabbit.

MATERIALS AND METHODS

Chemicals and Diet

I3C was purchased from Aldrich Chemical Co. (Milwaukee, WI). I3C was incorporated into diet prepared without preservatives and just before initiation of the experiment and stored frozen until the day before feeding. Goat anti-rabbit CYP1A1/1A2 was from Gentest (Woburn, MA). Rabbit anti-hog liver FMO1 was a generous gift of Dr. Daniel Ziegler, University of Texas at Austin. Rabbit anti-goat and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase were from Bio-Rad (Richmond, CA). The chemiluminescence kit was obtained from Amersham Corp. (Arlington Heights, IL).

Animals

Three-month-old male Hartley guinea pigs, three-month-old male CD-1 mice and four-month-old male NZW rabbits were acclimated for 7 days before being switched to diet containing 2000 ppm I3C and fed ad libitum for 4 weeks. The animals were euthanized and livers were removed, frozen in liquid N_2 and stored at -80°C until analysis.

Microsome Preparation and Immunodetection of CYP1A1/1A2 and FMO1

Liver microsomes were prepared by ultracentrifugation according to Guengerich (1989). Protein was measured by the method of Lowry *et al.* (1951). The liver microsomal proteins were separated by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membrane (Towbin *et al.*, 1979). The blots were incubated in 2% BSA in phosphate buffer saline (PBS), followed with goat anti-rat CYP1A1/1A2 or rabbit anti-hog liver FMO1. After washing in PBS-Tween 20, the blots were probed with rabbit anti-goat or goat anti-rabbit secondary antibody conjugated to horseradish peroxidase and then visualized using a chemiluminescense kit. Quantitation was performed by densitometry, using an HP ScanJet IIcx flatbed scanner employing NIH Image version 1.54 software (public domain, Wayne Rasband, National Institutes of Health).

Statistical Analysis

Statistical analyses of the data were performed using Student's t-test. All data points are the mean ± SD for four animals per group. The p values less than 0.05 were considered significant.

RESULTS

Dietary administration for four weeks of 2000 ppm I3C to male guinea pig, mouse and rabbit increased liver CYP1A1/1A2 protein levels as previously report by our laboratory (Fig. 2.1) (Larsen-Su and Williams, 1996). There was no significant difference in hepatic FMO1 protein between control and I3C treated groups of guinea pig (25.5 ± 4.9 versus 39.5 ± 11.9 pmol/mg, in control and I3C treated guinea pigs, respectively), mouse (6.2 ± 1.4 versus 5.1 ± 1.0 pmol/mg in control and I3C treated mice, respectively) and rabbit (35.8 ± 16.5 versus 20.9 ± 2.1 pmol/mg in control and I3C treated rabbits, respectively) (Fig. 2.2).

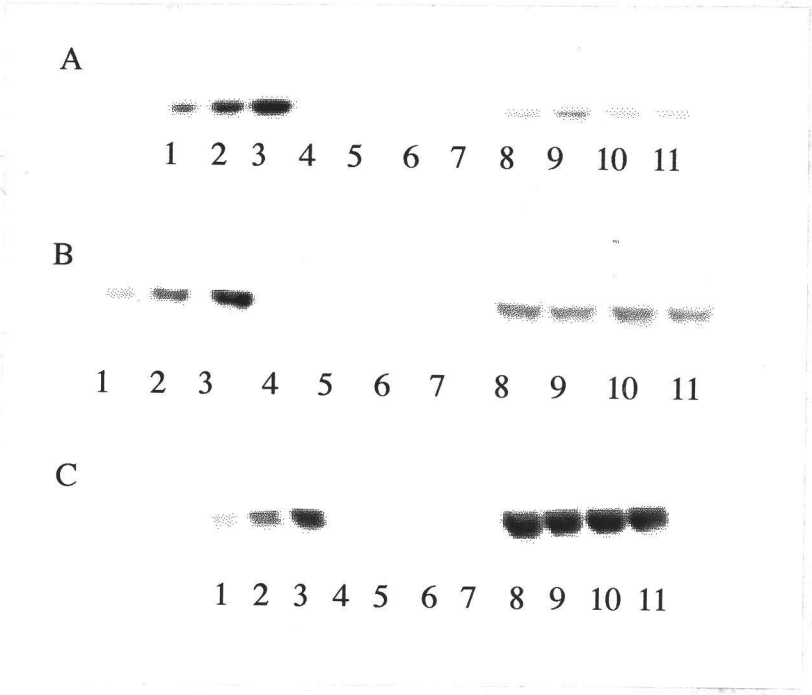

Figure 2.1. Western blots of hepatic microsomal protein probed with antibody to CYP1A1/1A2. Lanes 1, 2 and 3 represent 0.179, 0.452 and 1.074 pmole purified CYP1A1/1A2. Lanes 4-7 and 8-11 represent 20 μg microsomal protein of control and 2000 ppm I3C treated groups, respectively. (A) guinea pig. (B) mouse. (C) rabbit.

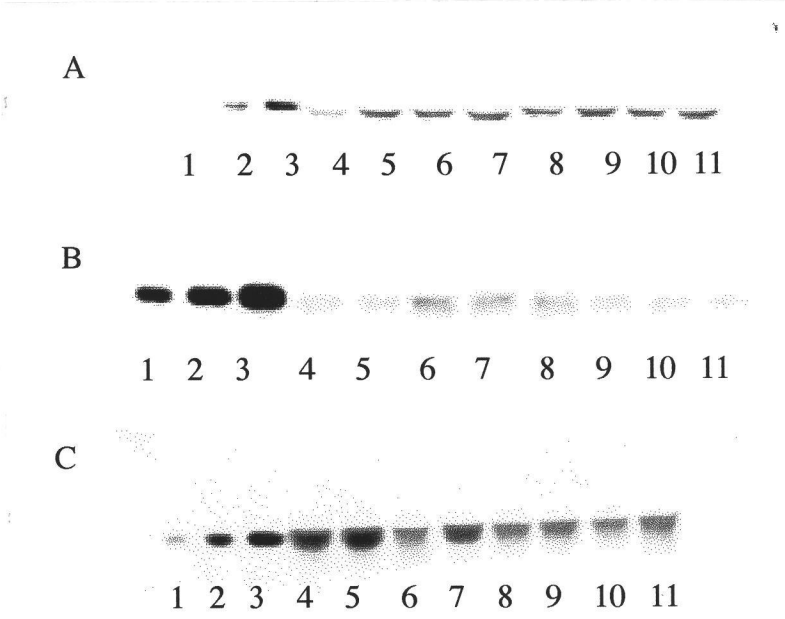

Figure 2.2. Western blots of hepatic microsomal protein probed with antibody to FMO1. Lane 1,2 and 3 represent 0.5, 0.9 and 1.8 pmole purified FMO1. Lanes 4-7 and 8-11 represent 40 μg (guinea pig and rabbit) and 48 μg (mouse) microsomal protein of control and 2000 ppm I3C treated group, respectively. (A) guinea pig. (B) mouse. (C) rabbit.

DISCUSSION

In this study we have demonstrated that I3C administered in the diet to male guinea pigs, mice and rabbits for 4 weeks significantly induced hepatic CYP1A1/1A2. No CYP1A1/1A2 was detected in liver microsomes from any control animals. There was no statistically significant difference in FMO1 protein levels between control and I3C-treated guinea pig, mouse and rabbit. In contrast to our previous laboratory result (Larsen-Su and Williams, 1996), I3C inhibited FMO1 and induced CYP1A protein levels in rat. This study suggests that there is no correlation or common mechanism between FMO1 inhibition and CYP1A1/1A2 induction as mediated by I3C.

FMO activity, using N, N-dimethyaniline (DMA) as the substrate, was reduced significantly in ascorbic deficient and/or diet restricted guinea pigs (Brodfuehrer and Zannoni, 1986). Concurrently, the physiological and nutritional factors which determine expression of FMO in guinea pig are not known. Thus, it will be important to investigate the mechanism by which FMO1 is regulated in guinea pig.

Due to negative regulation by testosterone, male mice have FMO1 levels 2-3 times lower than the female (Duffel *et al.*, 1981). Castration or 17-β-estradiol administration (24 μg/day for 3 weeks) can increase FMO1 mRNA expression in male mice (Falls *et al.*, 1997). Bradlow *et al.* (1991) showed that I3C lowered the level of endogenous estrogens in female mice associated with an increase in estradiol 2-hydroxylase, resulting in a decreased spontaneous mammary tumor

incidence. Serum testosterone levels in male mice fed diet containing 750 ppm for 7 days were decreased to less than 25%, compared to control (Wilson *et al.*, 1999). However, there was no induction of FMO1 protein expression in our study in contrast to the castration effect.

Hormones other than sex steroid hormones have been shown to modulate liver FMO1. Rabbit hepatic FMO1 protein levels increased during gestation on days 10, 15, 20 and 31. Subcutaneous administration of progesterone or dexamethasone to male rabbits increased liver FMO1 mRNA levels. However, estradiol and aldosterone had no significant effect on FMO1 (Lee *et al.*, 1995). Here, we have demonstrated that I3C has no effect on rabbit hepatic FMO1 protein. Further studies on the regulation of FMO1 in rabbit will be required to verify this result.

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Chapter 3

INHIBITION OF FMO PROTEIN EXPRESSION AND FMO-MEDIATED TAMOXIFEN METABOLISM BY DIETARY INDOLE-3-CARBINOL AND 3,3'-DIINDOLYLMETHANE IN THE RAT

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ABSTRACT

Our laboratory has previously shown that dietary administration of indole-3-carbinol (I3C) to male Fischer 344 rats has the very unusual property of inducing hepatic levels of a number of cytochrome P450s (CYPs), especially CYP1A1, while markedly inhibiting the levels of FMO1 protein and its catalytic activity. We hypothesized that rats fed I3C or 3,3'-diindolylmethane (DIM), one of its major acid condensation products formed *in vivo*, should exhibit a marked shift in the metabolic profiles of drugs or xenobiotics that are substrates for both monooxygenase systems. Male rats were fed AIN-76A powdered diets containing 0, 1000 or 2500 ppm I3C or DIM for 4 weeks. Dietary I3C and DIM reduced FMO1 protein levels (8% reduction with I3C and 84% with DIM at 1000 ppm, and 90% reduction with I3C and 97% with DIM at 2500 ppm) in hepatic microsomes. The ratio of FMO-(N-oxygenation) to CYP-(N-demethylation) mediated metabolism of N,N-dimethylaniline (DMA) decreased in liver microsomes from I3C or DIM fed rats from near unity to 0.02 at the highest dietary doses. The ratio of FMO to CYP metabolites of nicotine decreased, due to a reduction in N-oxygenation. Similarly, FMO-mediated N-oxygenation (tamoxifen N-oxide) was decreased, whereas CYP-mediated (N-desmethyl tamoxifen and 4-OH tamoxifen) metabolism of tamoxifen was unchanged in liver microsomes from rats fed I3C or DIM. This study demonstrates alteration of FMO and CYP-mediated drug metabolism *in vitro* by dietary I3C or DIM and suggests the potential for altered toxicity of nicotine and tamoxifen *in vivo*.

INTRODUCTION

Indole glucosinolate (glucobrassicin) is the most abundant of all glucosinolates and is found in high concentrations in cruciferous vegetables such as broccoli, cabbage, cauliflower and Brussels. The glucosinolate hydrolysis products from myrosinase (thioglucoside glycohydrolase EC 3:2:3:1) at neutral pH are glucose, sulfate, 3-indolylmethyl isothiocyanate, indole-3-carbinol (I3C) and thiocyanate ion. I3C may condense to 3,3'-diindolylmethane (DIM) and higher MW oligomers or react with ascorbic acid to form ascorbigen (McDanell *et al.*, 1988). A number of studies have shown I3C to be chemoprotective against cancer in multiple target organs such as mammary tissue (Grubbs *et al.*, 1995), liver (Bailey *et al.*, 1991), endometrium (Kojima *et al.*, 1994), lung (Morse *et al.*, 1990), and colon (Guo *et al.*, 1995) in animal models. I3C has been proposed for chemoprevention of breast cancer in healthy women (Wong *et al.*, 1997). Both I3C and DIM are marketed to the public as dietary supplements.

There are many proposed mechanisms involved in the anticarcinogenic activity of I3C including alteration of phase I and phase II enzymes (Stresser *et al.*, 1994a; b), free radical scavenging (Arnao *et al.*, 1996) and alteration of the cell cycle, resulting in the G_1 arrest of breast cancer cells (Cover *et al.*, 1998). However, the anticarcinogenic activity of I3C depends on the timing of I3C treatment. Chemoprotection is observed when I3C is given before and/or during carcinogenic exposure (Wattenberg, 1977). Long-term post-initiation exposure can result in tumor promotion (Bailey *et al.*, 1987).

Under the low pH conditions of the stomach, I3C undergoes a series of condensation reactions resulting in the production of various dimers, linear and cyclic trimers, and tetramers (Bjeldanes *et al.*, 1991). A major product *in vivo* after oral administration of I3C (Stresser *et al.*, 1995a) and *in vitro* (Spande, 1979) is DIM. When DIM is coinjected with aflatoxin B1 (AFB1), it reduces hepatic AFB1-DNA binding and tumor incidence in rainbow trout embryos (Dashwood *et al.*, 1994). DIM is a potent non-specific inhibitor of rat and human CYP1A1, human CYP1A2, and rat CYP2B1 (Stresser *et al.*, 1995b). Chen *et al.* (1998) showed that DIM was an aryl hydrocarbon (Ah) receptor ligand and induced CYP1A1 in MCF-7 cells at a concentration of 100 μM. DIM also inhibited E2-induced proliferation of MCF-7 cells and down-regulated the nuclear estrogen receptor. Growth of 7, 12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors in Sprague-Dawley rats was inhibited by DIM at a dose of 5 mg/kg given every other day under conditions in which no induction of hepatic CYP1A1 was observed (Chen *et al.*, 1998).

Our laboratory has previously shown that I3C administered in the diet to male Fischer 344 rats has the very unusual property of inducing hepatic levels of a number of cytochromes P450 (CYPs), especially CYP1A, while markedly inhibiting FMO1 in both a dose- and time-dependent manner (Larsen-Su and Williams, 1996). In our current study, we report that I3C and DIM each induced CYP1A1/1A2 and inhibited the expression and activity of FMO1 in liver of male rats. Simultaneously, they exhibited a marked shift in the metabolic profiles of

xenobiotics such as N,N-dimethylaniline and drugs such as nicotine and tamoxifen, which are substrates for both monooxygenases. Alteration of the FMO/CYP ratio may have marked effects on toxicological and/or therapeutic properties, depending upon the drug or xenobiotic.

MATERIALS AND METHODS

Chemicals and Diet

I3C was purchased from Aldrich Chemical Co. (Milwaukee, WI). DIM was the kind gift of Dr. Michael Zeligs of BioResponse L.L.C. (Boulder, CO). I3C and DIM were incorporated into powdered semi-synthetic AIN-76A diet prepared without preservatives. The diet was prepared just before initiation of the experiment and stored frozen until the day before feeding. N,N-Dimethylaniline (15.5 mCi/mmol, UL-ring) was purchased from Sigma Chemical Co. (St.Louis, MO). (*S*)-5-^{3}H-Nicotine (32 Ci/mmol), prepared by the catalytic tritiation of (*S*)-5-bromonicotine (Shigenaga *et al.*, 1987) was a gift of Dr. Mark Shigenaga. [^{3}H-N-methyl] Tamoxifen (85.6 Ci/mmol) was obtained from DuPont -NEN (Boston, MA).

Animals

Four-week-old male Fischer 344 rats were acclimated to AIN-76A diet for 7 days before being switched to AIN-76A diet containing I3C or DIM at levels of 0,

1000 or 2500 ppm and fed ad libitum for 4 weeks. The rats were killed by CO_2 asphyxiation, and livers were removed, frozen in liquid N_2 and stored at -80°C until analysis. The protocols used were approved by the Oregon State University IACUC.

Microsome Preparation and Immunodetection of FMO1

Liver microsomes were prepared by ultracentrifugation according to Guengerich (1989). Protein was measured by the method of Lowry *et al.* (1951). The liver microsomal proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979). The blots were probed with a polyclonal antibody specific to pig liver FMO1 (a generous gift of Dr. Daniel Ziegler, University of Texas at Austin), followed with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Bio-Rad, Richmond, CA) and then visualized using a chemiluminescense kit (Amersham Corp., Arlington Heights, IL). Quantitation was performed by densitometry, using an HP ScanJet IIcx flatbed scanner and NIH Image software version 1.54 (public domain, Wayne Rasband, National Institutes of Health).

N,N Dimethylaniline (DMA) N-oxygenation

FMO and CYP activity toward [^{14}C]-DMA was determined utilizing a high performance liquid chromatography assay with a reverse-phase ACT-1 column and

radiochemical detection (Williams, 1991; Shehin-Johnson *et al.*, 1995). FMO-mediated N-oxygenation and CYP-mediated N-demethylation can be determined simultaneously by this method.

Nicotine Metabolism

Nicotine metabolism was assayed according to Williams *et al.* (1990a). Nicotine, nicotine N-1'-oxide, nornicotine and nicotine $\Delta^{1,5}$-iminium metabolites are readily resolved utilizing a Beckman Ultrasphere C18 ODS (5 μm, 4.6 mm x 25 cm) column with quantification via on-line radiochemical detection.

Tamoxifen Metabolism

The incubations, containing rat liver microsomes, radiolabeled tamoxifen and an NADPH-regenerating system in phosphate buffer (pH 7.4), were carried out as previously described (Dehal and Kupfer, 1997). After a 1 hour incubation, the reaction was terminated, and metabolites resolved on silica gel TLC plates (Whatman, Inc., Clifton, NJ) with $CHCl_3$: CH_3OH: NH_4OH (80:20: 0.5% v/v/v). Radiolabeled metabolites on TLC were analyzed and quantified by radioscanning using the System 2000 imaging scanner (Bioscan, Inc., Washington, DC).

Statistical Analysis

Statistical analyses of the data were performed using Student's t-test. All data points are the mean ± SD for six rats per group. p values less than 0.05 were considered significant.

RESULTS

Dietary administration for four weeks of I3C or DIM to male Fischer 344 rats resulted in a dose-dependent reduction in liver microsomal FMO1 protein levels (Fig. 3.1) as previously reported by our laboratory for I3C (Larsen-Su and Williams, 1996). The higher dose of I3C, 2500 ppm, reduced FMO1 protein levels to 10% that of controls. DIM was markedly more potent than I3C, reducing FMO1 levels to 16% and 3% of controls at 1000 and 2500 ppm, respectively.

N,N-Dimethylaniline metabolism documents clearly the effects of I3C and DIM on FMO- and CYP-monooxygenation in liver microsomes of rats following dietary administration (Fig. 3.2). FMO-dependent formation of the N-oxide is inhibited in a dose-dependent manner; concurrently CYP-dependent N-demethylation is induced. Consistent with the western blotting results, DIM proved to be more potent than I3C; the higher dose of I3C reduced N-oxygenation of N,N-dimethylaniline to 28% of control levels, whereas the inhibition with DIM was to 7% of control levels. CYP-dependent N-demethylation was induced 3-5-fold, but the effect was not dose-dependent. The ratio of FMO/CYP metabolism of N,N-dimethylaniline decreased by 50-fold at the higher dose of DIM.

Although the major CYP-mediated pathways of (S)-nicotine metabolism, N-demethylation to nornicotine and formation of the $\Delta^{1,5}$-iminium ion, were unchanged by dietary I3C or DIM, FMO-catalyzed N-oxygenation of nicotine was markedly reduced (Fig. 3.3).

As was the case with nicotine, dietary exposure of rats to I3C and DIM markedly reduced the N-oxygenation of tamoxifen by liver microsomes without a marked increase in CYP-dependent N-demethylation and 4-hydroxylation (Fig. 3.4).

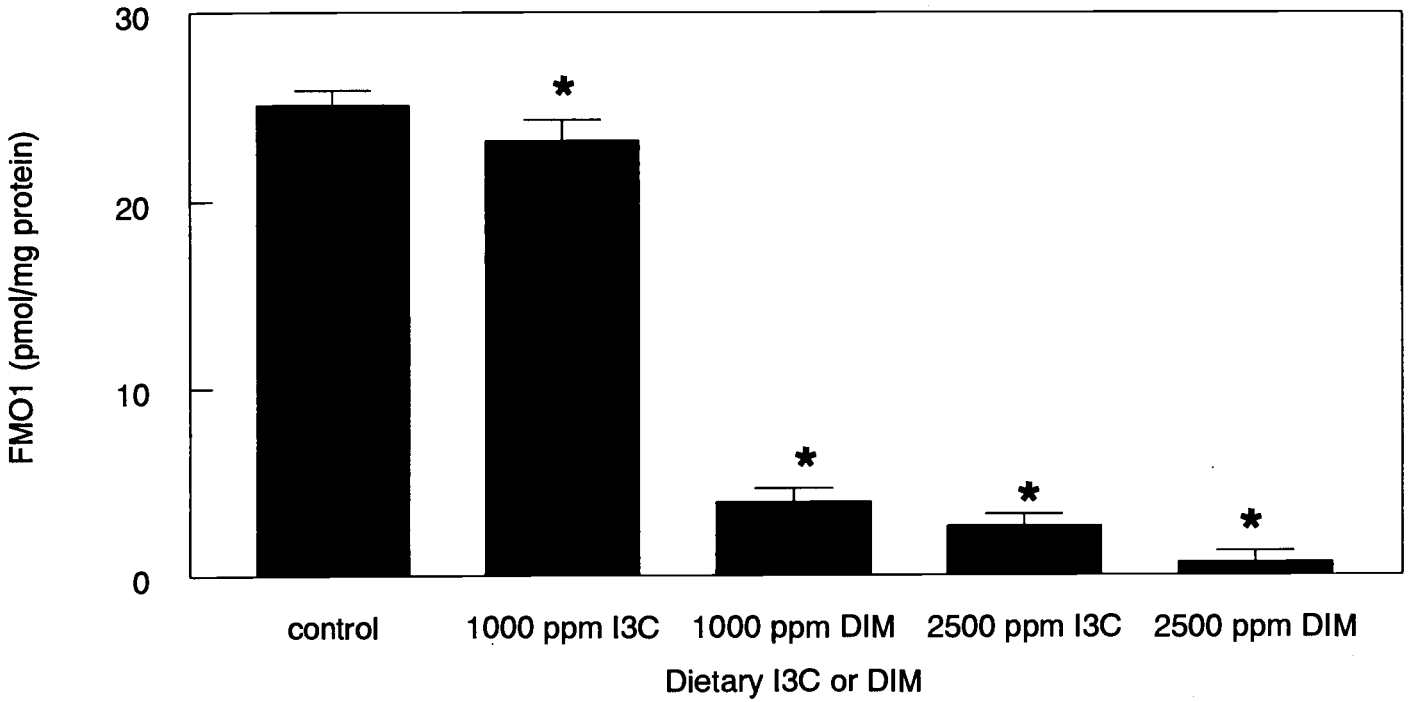

Figure 3.1. Western blotting analysis of FMO1 protein levels in liver microsomes from rats treated with I3C or DIM at doses of 1000 and 2500 ppm for 4 weeks. * Indicates significantly lower than controls at $p < 0.05$.

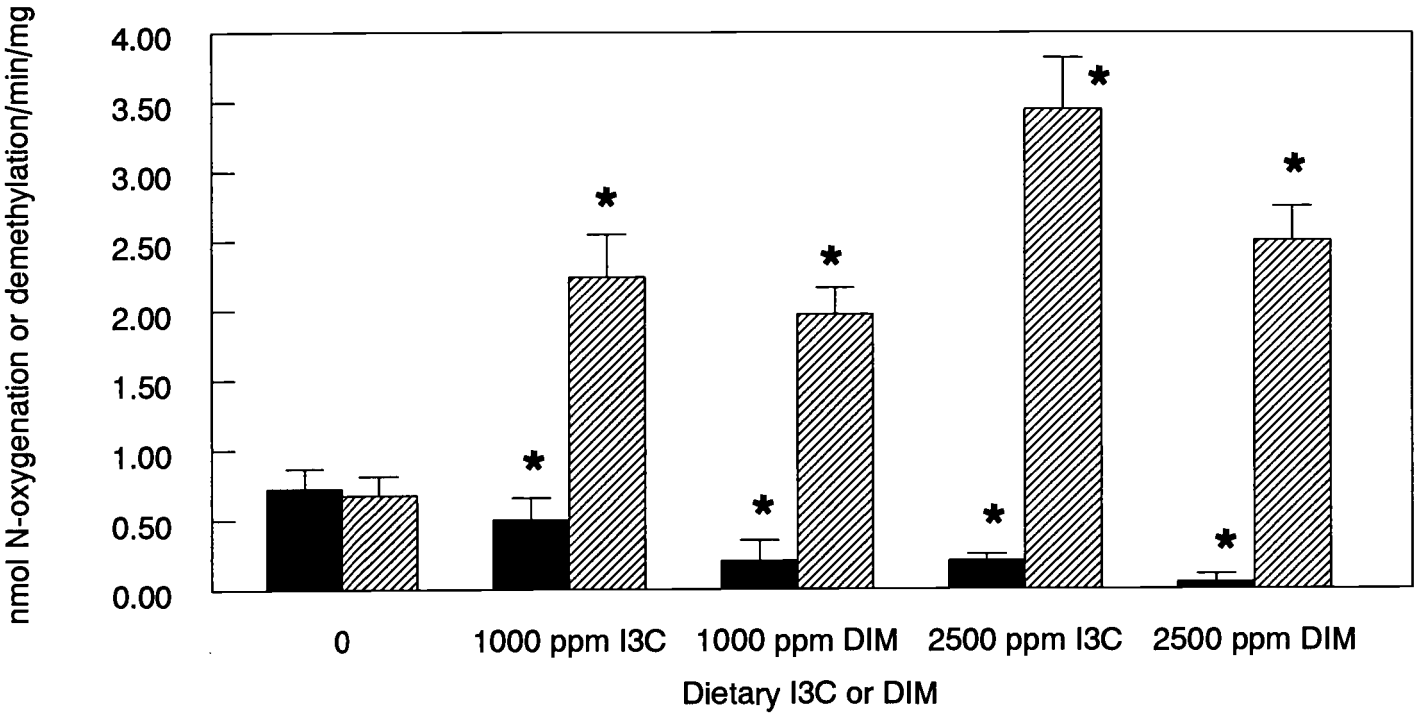

Figure 3.2. FMO-dependent N-oxygenation (solid bars) and CYP-dependent N-demethylation (striped bars) of N, N-dimethylaniline (DMA) in liver microsomes from rats treated with I3C or DIM at doses of 1000 and 2500 ppm for 4 weeks. N-oxygenation was significantly ($p<0.05$) decreased and N-demethylation significantly increased at all dietary levels of I3C and DIM.

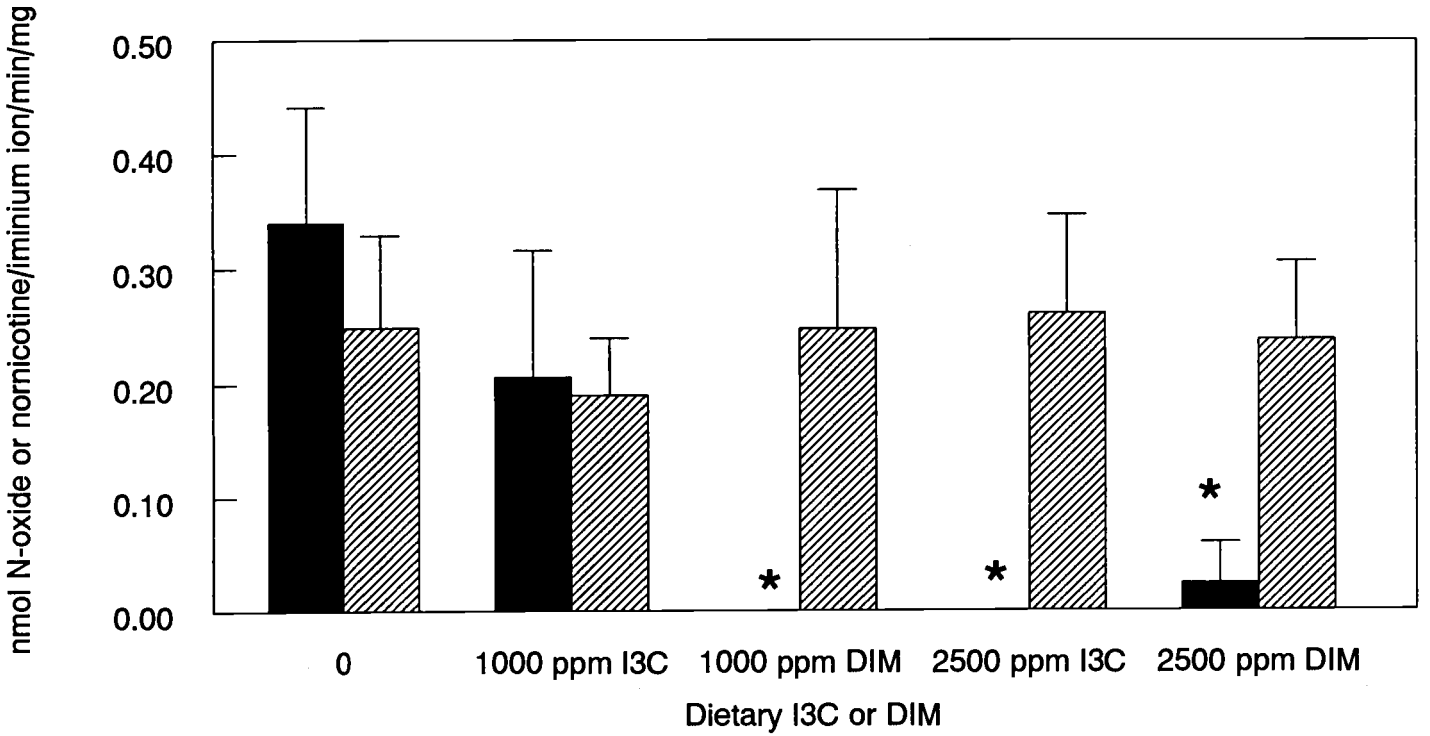

Figure 3.3. CYP-and FMO-mediated metabolism of ^{3}H-(S)-nicotine. Liver microsomes from rats fed 0, 1000 or 2500 ppm I3C or DIM for 4 weeks were incubated with ^{3}H-(S)-nicotine and the major metabolites nornicotine, nicotine $\Delta^{1,5}$-iminium ion (CYP, hatched bars) and nicotine N-1'oxide (FMO, solid bars) resolved and quantified by HPLC as described in materials and methods. * Indicates significantly lower than controls at $p<0.01$.

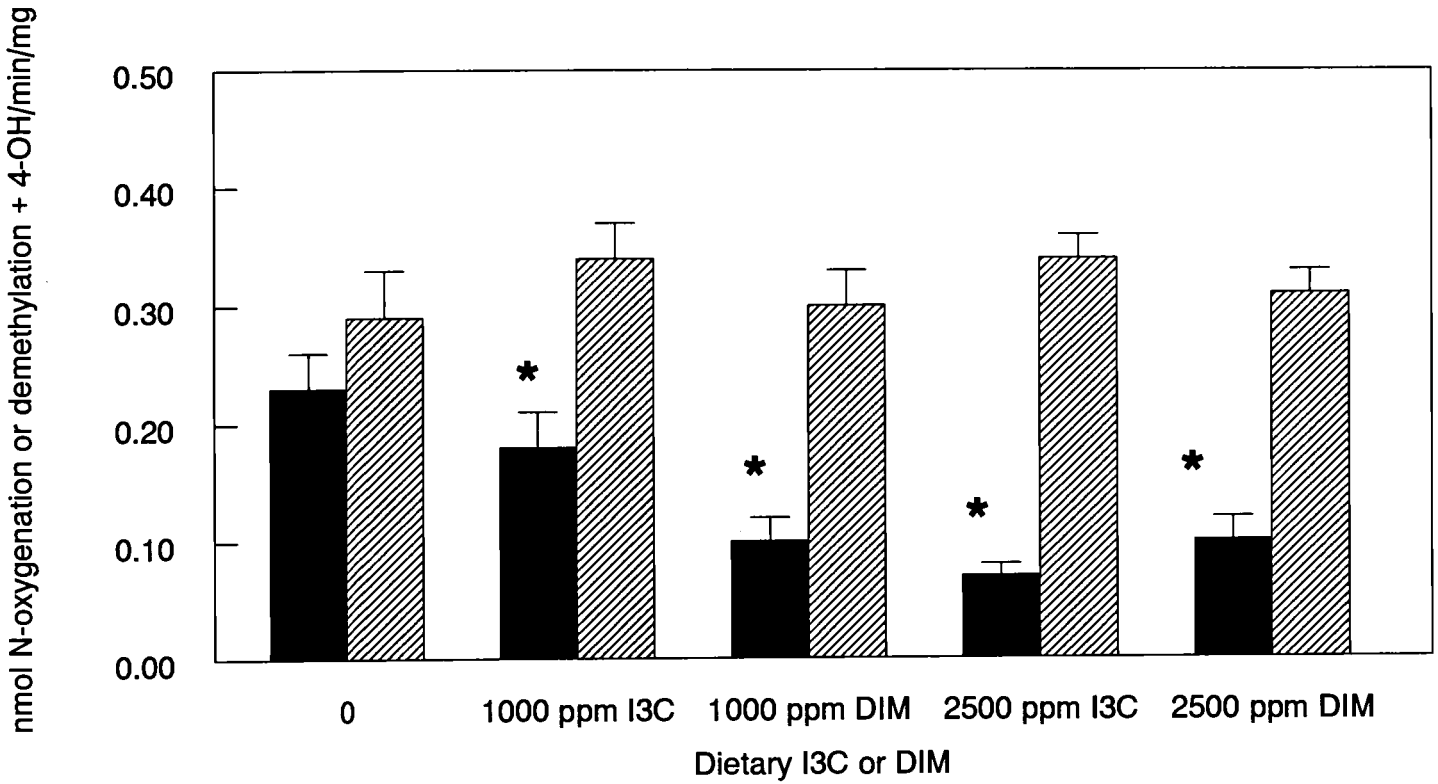

Figure 3.4. FMO-mediated TAM N-oxide (solid bars) and CYP-mediated 4-hydroxy TAM and N-desmethyl TAM (striped bars) formation by liver microsomes from rats treated with I3C or DIM at doses of 1000 and 2500 ppm for 4 weeks. * Indicates significantly lower than controls at $p<0.01$.

DISCUSSION

Our laboratory and others have documented that dietary exposure of I3C to rats induces a number of CYP isoforms. CYP1A1 is induced by greater than 20-fold, whereas more modest increases of 2-4-fold are observed for CYP1A2, CYP2B1/2 and CYP3A (Stresser *et al.*, 1994a; Bradfield and Bjeldanes, 1987; Bjeldanes *et al.*, 1991; Wortelboer *et al.* 1992a; b; Manson *et al.*, 1998). Concurrent with this up-regulation of CYP-dependent metabolic pathways in the rat, the levels of FMO1 protein and FMO-dependent catalytic activity is markedly inhibited by dietary I3C in a dose and time-dependent fashion (Larsen-Su and Williams, 1996).

Based upon these findings, we postulated that dietary I3C exposure could significantly alter the metabolic profile of drugs and xenobiotics that are substrates for both monooxygenase systems. The three compounds chosen in the present study to test this hypothesis, N,N-dimethylaniline, nicotine and tamoxifen, are all tertiary amines. Tertiary aliphatic amines are typically excellent substrates for FMO, yielding the water-soluble and usually non-toxic N-oxide metabolites (Ziegler, 1993). Tertiary aliphatic amines are preferentially N-demethylated by CYP; in only rare cases in which α-hydrogens are absent does CYP produce N-oxides in significant amounts (Guengerich and MacDonald, 1984; Williams et al., 1989).

In the case of N,N-dimethylaniline, although Hlavica and Kunzel-Mulas (1993) found that CYP2B4-dependent superoxide anion radical production lead to

N,N-dimethylaniline-N-oxide production, others studies have found that, relative to FMO, this pathway is insignificant (Pandey *et al*, 1989; Seto and Guengerich, 1993). In the present study, N,N-dimethylaniline-N-oxygenation was inhibited by dietary I3C in a dose-dependent manner. N-demethylation, presumably mediated predominantly by CYP2B1, was enhanced 3-5-fold, consistent with our previous documentation of CYP2B1/2 induction by dietary I3C in these rats (Stresser *et al.*, 1994a). The pattern of inhibition of N,N-dimethylaniline N-oxygenation (Fig. 3.2) closely resembled that of FMO1 protein repression (Fig. 3.1). At the higher dose of DIM, greater than 90% of N,N-dimethylaniline-N-oxygenation was inhibited. The relative contribution of CYP- and FMO- mediated metabolism of N,N-dimethylaniline is thus dramatically altered and serves as an example of how I3C and DIM could alter the therapeutic efficacy and/or toxicity of drugs or xenobiotics which are substrates for both monooxygenases.

Pretreatment with either I3C or DIM in the diet also altered the *in vitro* liver microsomal metabolic profile of (S)-nicotine. CYP-dependent N-demethylation to nornicotine and formation of the nicotine-$\Delta^{1',5'}$-iminium ion were unaffected, whereas, yield of the FMO-catalyzed nicotine N-1'-oxide was reduced to at or below the limits of detection.

Previous studies have demonstrated that the CYP2A and 2B subfamilies are active toward nicotine. In rabbit nasal tissue, CYP2A10/11 (P450 NMa) exhibited high activity toward nicotine (Williams *et al.*, 1990b). In rat liver, phenobarbital treatment markedly enhances nicotine C-oxidation, due to induction of CYP2B1

(Hammond *et al.*, 1991). The rabbit ortholog in lung, CYP2B4, is also the major nicotine oxidase in that organ (Williams *et al.*, 1990a). In human liver, CYP2A6 is the major isoform catalyzing C-oxidation of nicotine (Berkman *et al.*, 1995; Nakajima *et al.*, 1996; Messina *et al.*, 1997). Based on the modest induction of CYP 2B1/2 by I3C in the rat liver, it is somewhat surprising that we saw no induction of C-oxidation.

Nicotine is oxygenated at the N-1'-position by FMO1 and FMO3 (Damani *et al.*, 1988; Cashman *et al.*, 1992; Park *et al.*, 1993). The stereoselective production of (S)-nicotine trans N-1'-oxide has been proposed as a mechanism for phenotyping individuals for liver FMO3 (Park *et al.*, 1993). The virtual elimination of this pathway in liver microsomes from rats fed high concentrations of I3C and DIM, is consistent with the down-regulation of FMO1 protein and N,N-dimethylaniline N-oxygenation discussed above. It could be speculated that I3C or DIM inhibition of nicotine- N-1'-oxide production *in vivo* could alter the pharmacokinetics of nicotine and provide protection against nicotine addiction and reduce the number of cigarettes smoked in a manner analogous to that seen with the polymorphism which results in non-functional CYP2A6 (Pianezza *et al.*, 1998). It has been observed that trimethylaminuria (a genetic defect in FMO3) patients exhibit impaired nicotine N-1'-oxygenation (Ayesh *et al.*, 1988). One major caveat to this hypothesis involves the question of whether or not human liver FMO3 responds to dietary I3C and DIM as does rat liver FMO1.

Tamoxifen, an antiestrogen, is the therapeutic drug most often used in the treatment of breast cancer (Jordan, 1993) and, based on the results of a recent large clinical trial, is advocated as a chemopreventive agent for women at high risk of developing breast cancer (Fisher *et al.*, 1998). Of concern with the long-term use of tamoxifen is an enhanced incidence of endometrial cancers (Killackey *et al.*, 1985) and the observation that it is hepatocarcinogenic in the rat (Williams *et al.*, 1993). Tamoxifen is bioactivated by CYPs to yield 4-hydroxytamoxifen which is markedly more potent as an antiestrogen than the parent compound (Jordan *et al.*, 1977). Another major CYP metabolite is N-desmethyl tamoxifen. Further hydroxylation of 4-hydroxytamoxifen results in formation of tamoxifen catechol, a redox active metabolite that covalently binds to macromolecules (Dehal and Kupfer, 1999). The major CYPs active toward tamoxifen and 4-hydroxytamoxifen are CYP3A4, 2D6 and 2C9 (Dehal and Kupfer 1997; 1999; Crewe *et al.*, 1997). N-oxygenation of tamoxifen is mediated by FMO and the N-oxide is found in the serum of women taking the drug (Mani *et al.*, 1993; Poon et al., 1995).

In this study we document a significant reduction in the N-oxygenation of tamoxifen catalyzed by liver microsomes of rats fed I3C or DIM. In liver microsomes from control rats, the ratio of CYP-mediated N-demethylation and 4-hydroxylation to FMO-mediated N-oxygenated is approximately unity; at the higher dose of I3C and both doses of DIM, the ratio increases to 3-4. A reduction in N-oxygenation may actually decrease tamoxifen toxicity. Previous work has provided evidence that FMO activity could enhance tamoxifen-dependent covalent

binding (Mani and Kupfer, 1991). Recent studies document that tamoxifen N-oxide and metabolites covalently bind to DNA and the authors state that there is evidence for dG-N^2-tamoxifen N-oxide DNA adducts in human (Umemoto *et al.*, 1999). Based on these findings, we hypothesize that, if a similar alteration occurs in humans, women taking tamoxifen, in concert with diets high in cruciferous vegetables and/or taking I3C supplements, could enhance their risk of developing toxic side effects.

As mentioned above, whether or not these present studies with rat can be extrapolated to humans could depend, in large part, on whether or not down-regulation of human liver FMO3 is analogous to rat FMO1. Humans fed 300 g/day of Brussels sprouts, providing an estimated dose of 0.002-0.014 mmol I3C/Kg/day, exhibited a significant decrease in urinary trimethylamine N-oxide, presumably due to inhibition of liver FMO3 (Cashman *et al.*, 1999). This dose is markedly lower than the highest inhibitory dose of dietary I3C (0.46 mmol /Kg/day) given to rats in this study. It must be kept in mind, however, that Brussels sprouts contain numerous other phytochemicals including isothiocyanates and dithiolanes that may effect FMO.

It may not be necessary for FMO3 protein to be down-regulated by I3C (as is the case with rat liver and intestinal FMO1) to observe inhibition, as we have found that I3C acid condensation products can directly inhibit FMO catalytic activity. DIM and indole[3,2-*b*]carbazole (ICZ) inhibited the catalytic activity of rat FMO1 *in vitro* with K_is of 47 and 31 μM, respectively (Larsen-Su, 1998). ICZ,

DIM and I3C also directly inhibit the catalytic activity of the major FMO in human liver, FMO3, with K_is in the low μM range (Cashman *et al.*, 1999). Of these three compounds, only DIM would be expected to be present in liver following I3C oral administration at levels capable of eliciting this response. Studies following the pharmacokinetics of ^{3}H-I3C after oral administration to rats found no I3C in liver and ICZ levels were estimated to be 1.6 nM, however DIM levels were estimated to be 3-6 μM (Stresser *et al.*, 1995a). Interestedly, DIM also directly inhibits rat and human CYP1A1, human CYP1A2 and rat CYP2B1 with K_is again in the low μM range (Stresser *et al.*, 1995b). In this study DIM was demonstrably more effective than I3C. The 1000 and 2500 ppm diets correspond to 6.2 and 15.5 mmoles/Kg for I3C and 4.1 and 10.2 mmoles/Kg for DIM; therefore DIM was even more effective on a mole basis.

In summary, we have demonstrated that administration of the indoles I3C and DIM, present in cruciferous vegetables and sold as dietary supplements, markedly alter the metabolism in vitro of drugs that are substrates for both CYP and FMO monooxygenases. Dietary I3C and DIM inhibit the expression of FMO1 protein. Furthermore, DIM is capable of directly inhibiting the catalytic activity of FMO1 and FMO3 as well as a number of CYPs. The consequences of this "drug-drug" interaction could be manifest as alterations in therapeutic efficacy or toxicity.

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Chapter 4

INHIBITION OF FMO-MEDIATED CODEINE ANALGESIA BY DIETARY INDOLE-3-CARBINOL AND 3,3'-DIINDOLYLMETHANE IN THE RAT

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ABSTRACT

Dietary administration of indole-3-carbinol (I3C) to male Fischer 344 rats has been shown to markedly induce many Phase I and Phase II enzymes , while inhibiting the expression and activity of FMO1. To test the effect of I3C or 3,3'-diindolylmethane (DIM), the major breakdown product of I3C, on the metabolism of xenobiotics which are the substrates for both monooxygenases *in vivo*, rats fed I3C or DIM were gavaged with codeine and analgesic efficacy estimated by the tail flick assay. The time from onset of the heat stimulus to withdrawal of the tail was longer in rats treated with I3C or DIM than in controls. However, the results were highly variable between rats. Western blotting showed that both I3C and DIM significantly reduced FMO1 protein. CYP2D1 activity was significantly increased in liver microsomes pretreated with I3C or DIM. Further study is needed to confirm the difference in the tail flick assay between control and treated groups by increasing the sample size and finding a response that doesn't have to be censored.

INTRODUCTION

Indole-3-carbinol is the hydrolysis product of indole glucosinolate, which is found in high concentration in cruciferous vegetables such as broccoli, cabbage, cauliflower and Brussels sprouts (McDanell *et al.*, 1988). Numerous studies have shown I3C to be chemoprotective against cancer in multiple target organs such as mammary tissue (Grubbs *et al.*, 1995), liver (Bailey *et al.*, 1991), endometrium (Kojima *et al.*, 1994), lung (Morse *et al.*, 1990) and colon (Guo *et al.*, 1995) in animal models. Alteration of phase I and phase II enzymes (Stresser *et al.*, 1994a; b), free radical scavenging (Arnao *et al.*, 1996), modulation of intercellular communication (Rijinkels *et al.*, 1998), and alteration of cell cycle (Cover *et al.*, 1998) are proposed mechanisms involved in the anticarcinogenic activity of I3C.

I3C is not a stable compound, especially under the low pH conditions of the stomach. I3C undergoes a series of condensation reactions resulting in the production of various dimers, linear and cyclic trimers, and tetramers (Bjeldanes *et al.*, 1991). A major product *in vivo* after oral administration of I3C (Stresser *et al.*, 1995) and *in vitro* (Spande, 1979) is 3,3'-diindolylmethane (DIM). Like I3C, DIM decreases aflatoxin B1-induced liver tumors in rainbow trout (Dashwood *et al.*, 1994) and 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor growth in rat (Chen *et al.*, 1998).

Dietary administration of I3C to male rats markedly induced levels of hepatic CYP1A1, while dramatically down-regulated both the expression and activity of flavin-containing monooxygenase (FMO), form 1 (Larsen-Su and

Williams, 1996). Our previous study documented the alteration of FMO and CYP-mediated N, N-dimethylaniline, nicotine and tamoxifen *in vitro* by dietary I3C or DIM. The objective of this study was to determine the analgesic response of codeine mediated by FMO and CYP in rats treated with I3C or DIM.

MATERIALS AND METHODS

Chemicals and Diet

I3C was purchased from Aldrich Chemical Co. (Milwaukee, WI). DIM was the kind gift of Dr. Michael Zeligs, BioResponse L.L.C. (Boulder, CO). I3C and DIM were incorporated into powdered semi-synthetic AIN-76A diet prepared without preservatives. The diet was prepared just before initiation of the experiment and stored frozen until the day before feeding. Rabbit anti-hog liver FMO1 was a generous gift of Dr. Daniel Ziegler, University of Texas at Austin. Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase was from Bio-Rad (Richmond, CA). The chemiluminescence kit was obtained from Amersham Corp. (Arlington Heights, IL). Bufuralol and 1'-hydroxylbufuralol were from Gentest (Woburn, MA).

Animals

Four-week-old male Fischer 344 rats were acclimated to AIN-76A diet for 7 days before being switched to AIN-76A diet containing I3C or DIM at doses of 0

and 2500 ppm and fed ad libitum for 10 weeks. The rats were gavaged with codeine 25 mg in 0.9% NaCl, and the analgesic effect determined by the tail flick assay (measurements of codeine analgesia were made at 0, 15, 45 and 90 mins). The rats were killed by cervical dislocation, livers were removed, frozen in liquid N_2 and stored at -80°C until analysis.

Tail Flick Assay

The analgesic response was measured by exposure of the rat's tail to radiant heat. With the rat held in a restraining tube, heat was focused on a surface area of the rat's tail. For each rat, the time from onset of the stimulus to withdrawal of the tail was recorded and determined as time = 0. After gavage of codeine 25 mg, the time from onset of the stimulus to withdrawal of the tail was measured at 15, 45 and 90 min. The maximum time for the tail to be exposed to the heat was set at 15 sec to avoid tissue injury caused by long exposure to the heat stimulus (Beacher, 1957).

Microsome Preparation and Immunodetection of FMO1

Liver microsomes were prepared by ultracentrifugation according to Guengerich (1989). Protein was measured by the method of Lowry *et al.* (1951). The liver microsomal proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979). The blots were

incubated in 2% BSA in phosphate buffer saline (PBS), followed with a rabbit anti-hog liver FMO1. After washing in PBS-Tween 20, the blots were probed with a secondary antibody conjugated to horseradish peroxidase and then visualized using a chemiluminescense kit. Quantitation was performed by densitometry, using an HP ScanJet IIcx flatbed scanner employing NIH Image version 1.54 software (public domain, Wayne Rasband, National Institutes of Health).

Bufuralol 1'-Hydroxylase assay

CYP2D1 activity was determined in liver microsomes, as previously described (Kronbach *et al.*, 1987). The final reaction mixture contained 1 mM bufuralol in 0.1 M potassium phosphate pH 7.4, 20 mg/ml glucose 6-phosphate, 20 mg/ml NADP, 13.3 mg/ml $MgCl_2$-H_2O, 40 U/ml glucose 6-phosphate dehydrogenase in 5 mM sodium citrate, 0.1 M potassium phosphate pH 7.4 and 50 μg microsomal protein in a volume of 250 μl. Samples were preincubated at 37° C for 5 minutes before initiating the reaction with bufuralol. The reaction was terminated at 20 minutes with the addition of cold 60% perchloric acid 25 μl, cooled on ice 10 min and centrifuged at 10,000 g for 20 min at 4° C to precipitate protein. The 1'-hydroxybufuralol was quantified by HPLC with fluorescence detection: excitation at 252 nm and emission at 302 nm. The mobile phase was 37% acetonitrile, 63% water and 0.9 mM perchloric acid. The column used was a Beckman Ultrasphere C-18 analytical column, 4.6 mm × 25 cm, 5 μm pore size. The flow rate was constant at 1 ml/min.

Statistical Analysis

The tail flick assay data were analyzed by Kruskal-Wallis test. Statistical analyses of immunoquantitation of the FMO1 protein and CYP2D1 activity were performed using Student's t-test. All data points are the mean ± SD. p values less than 0.05 were considered significant.

RESULTS

After feeding 2500 ppm of I3C or DIM for 10 weeks, the rats were gavaged with 25 mg codeine and the analgesic effect followed by the tail flick assay. The time from onset of the heat stimulus to withdrawal of the tail was longer in rats fed I3C or DIM than in controls. However, the result showed great variability in the response between rats. There was no response within 15 seconds in 3/4 rats fed I3C (Fig 4.1). There was no response within 15 seconds in 1/4 rats fed DIM (Fig 4.2). Other I3C- and DIM- treated rats responded similarly to controls. Western blotting showed that both I3C and DIM significantly reduced FMO1 protein levels (23% I3C and 31% DIM) (Fig. 4.3) as previously reported by our laboratory for I3C (Larsen-Su and Williams, 1996). In liver microsomes pretreated with I3C or DIM, the CYP2D1 activity, as determined by using bufuralol as the substrate, increased 52% and 85% in the I3C and DIM groups, respectively (Fig. 4.4).

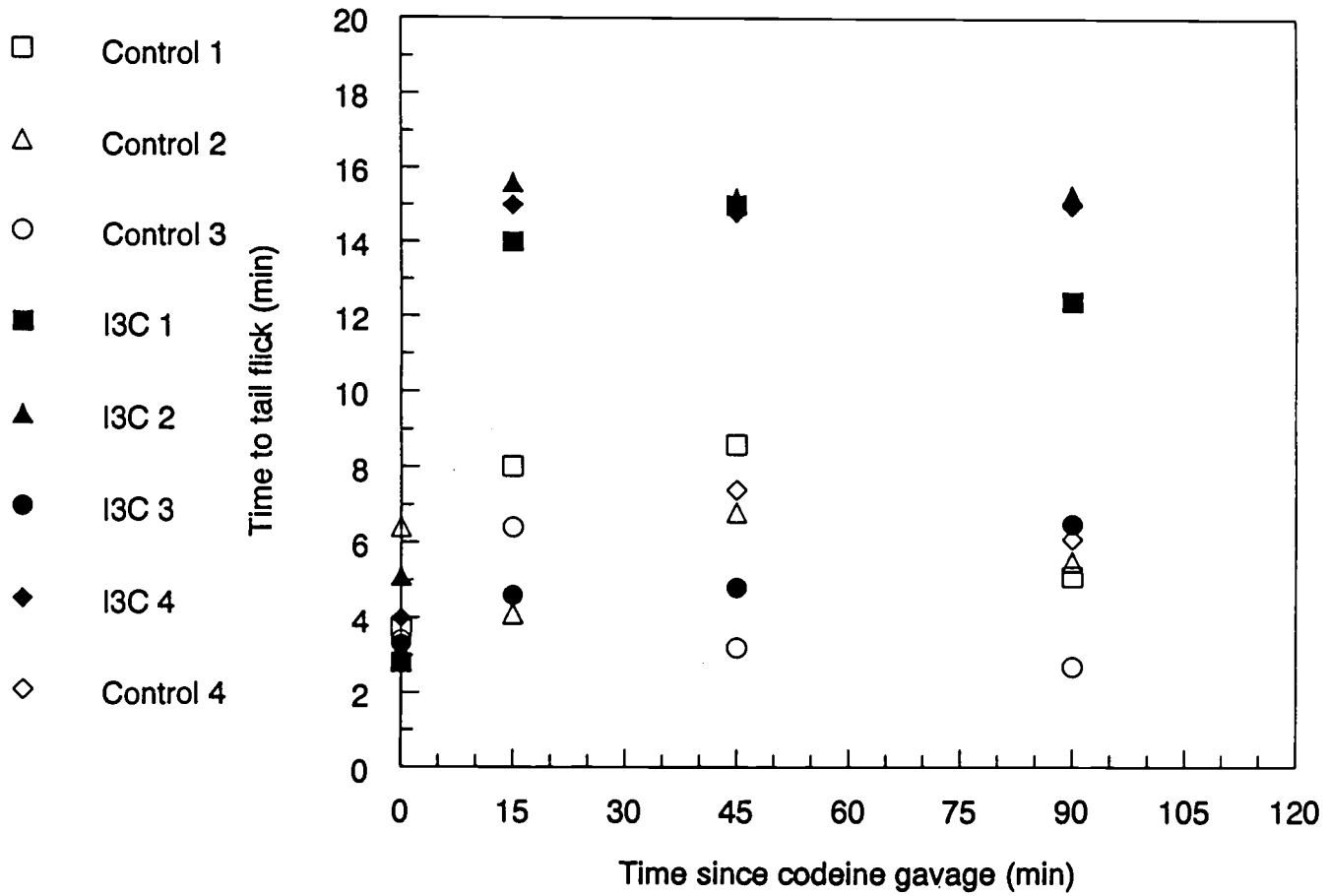

Figure 4.2 The analgesic response for codeine administration to I3C-pretreated rats.
Tail flick responses were measured at time 15, 45 and 90 min after codeine administration.

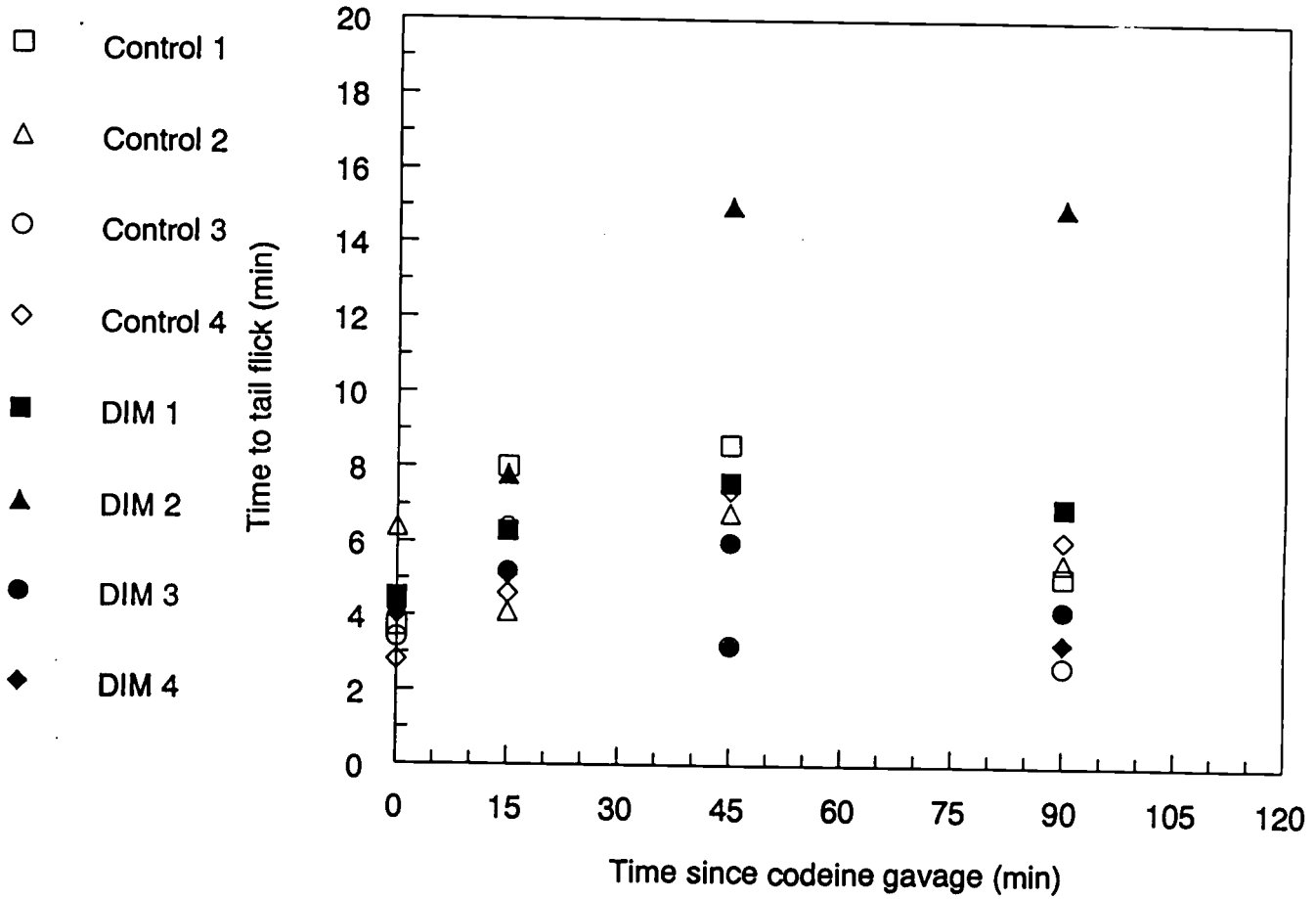

Figure 4.2 The analgesic response for codeine administration to DIM-pretreated rats. Tail flick responses were measured at time 15, 45 and 90 min after codeine administration.

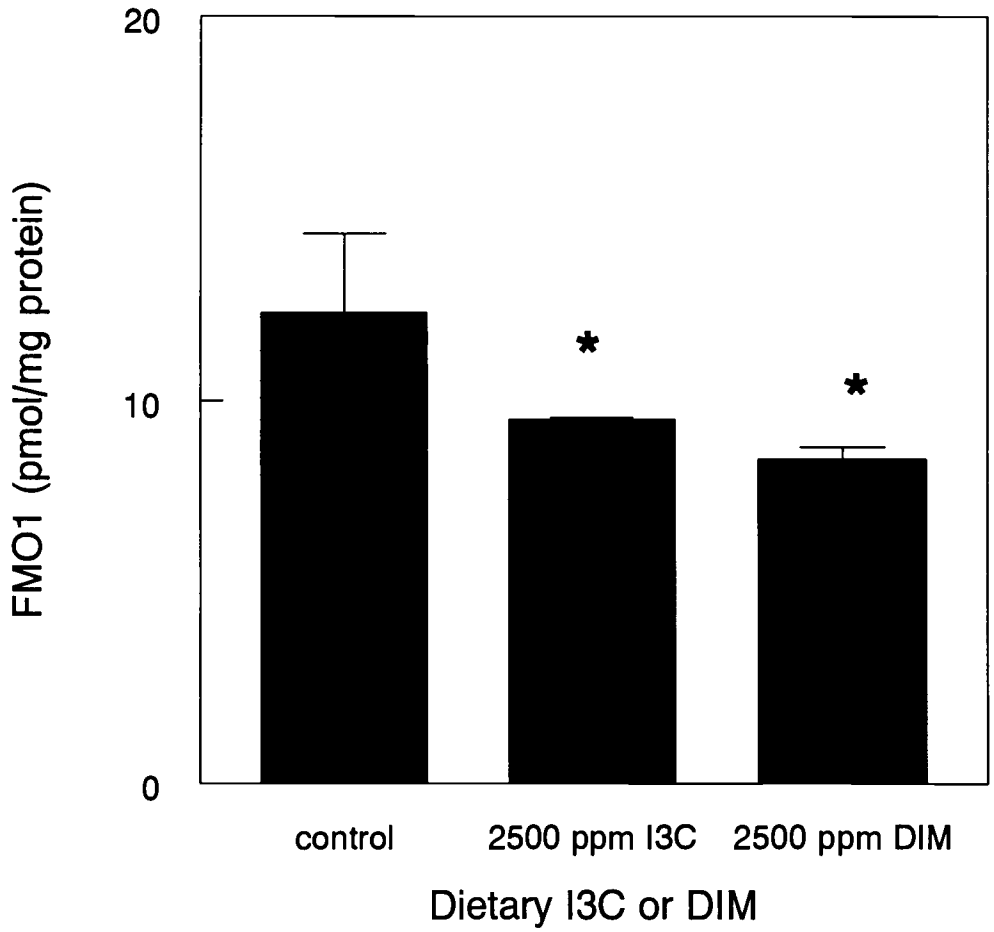

Figure 4.3. Western blotting analysis of FMO1 protein levels in liver microsomes from rats fed I3C or DIM at doses of 2500 ppm for 10 weeks. * Indicates significantly lower than controls at $p < 0.05$.

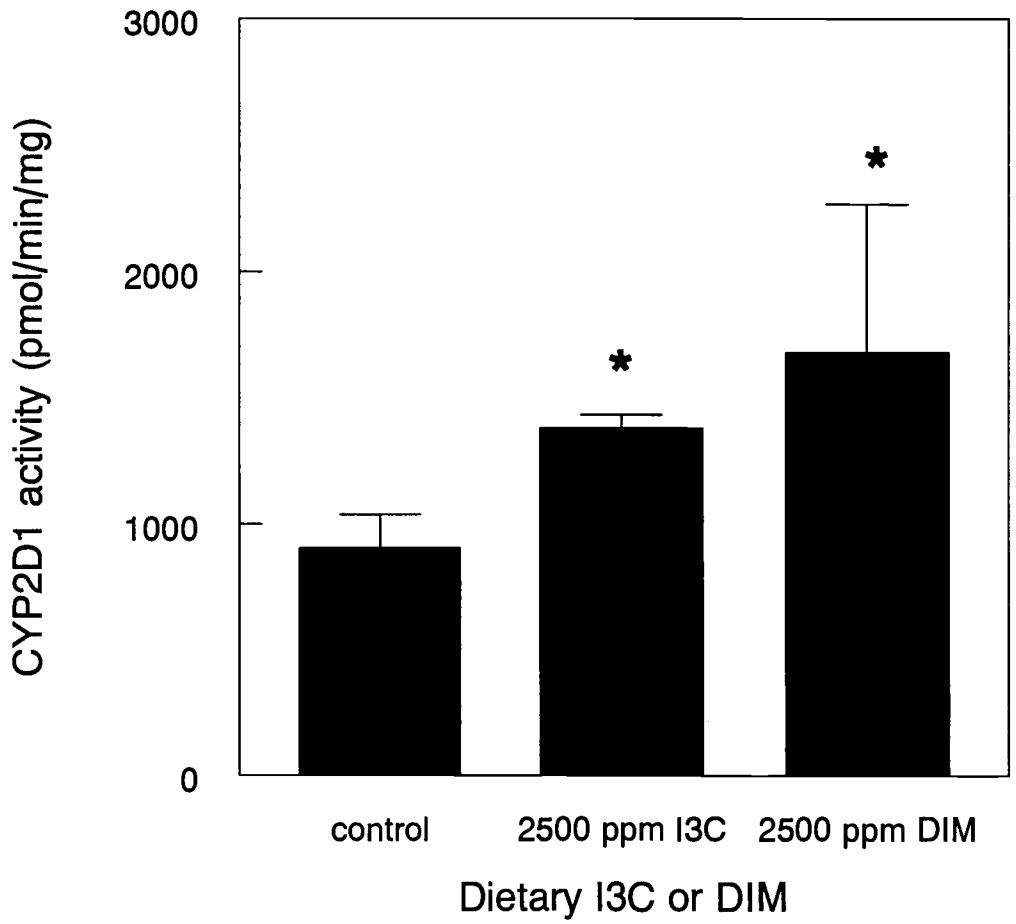

Figure 4.4. The CYP2D1 activity as the bufuralol 1'-hydroxylation in liver microsomes from rats fed I3C or DIM at doses of 2500 ppm for 10 weeks. * Indicates significantly higher than controls at $p < 0.05$.

DISCUSSION

Dietary administration of indole-3-carbinol (I3C) to male Fischer 344 rats has been shown to markedly induce many Phase I (Bjeldanes *et al.*, 1991; Wortelboer *et al.*, 1992; Jellinck *et al.*, 1993; Stresser *et al.*, 1994a) and Phase II (Sparnius *et al.*, 1982; Salbe and Bjeldanes, 1986; Danger *et al.*, 1992; Wortelboer *et al.*, 1992) enzymes, while inhibiting the expression and activity of FMO1 (Larsen-Su and Williams, 1996). Our previous study demonstrated that liver microsomes from rats fed I3C or DIM decreased the *in vitro* ratio of FMO to CYP metabolites of N, N-dimethylaniline, nicotine and tamoxifen, due primarily to reduction in FMO-N-oxygenation. In order to investigate the *in vivo* effect of alteration of the FMO/CYP ratio on the drugs or xenobiotics that are substrates for both monoxygenase systems, rats fed I3C or DIM were gavaged with 25 mg codeine followed determination of analgesic efficacy by the tail flick assay. The test compound in the present study was codeine, a tertiary amine substrate for FMO, (Ziegler, 1993). Codeine (methylmorphine) is an ancient analgesic, antitussive and anti-diarrheal drug whose therapeutic effects are principally produced by the O-desmethylated metabolite, morphine, catalyzed by CYP2D6 in human (Yue *et al.*, 1989) and CYP2D1 in the rat (Xu *et al.*, 1995). CYP3A4 appears responsible for norcodeine formation, the N-demethylation product (Caraco *et al.*, 1996). FMO catalyzes the N-oxygenation of both codeine and morphine, resulting in pharmacologically inactive metabolites (Yuno *et al.*, 1990).

The genetic polymorphism known as the debrisoquine polymorphism results in people being phenotyped as either poor or extensive metabolizers (PM and EM), based on CYP2D6 activity. Although CYP2D6 makes up only about 2-5% of the total CYP450 in the human liver, it is the major enzyme catalyzing metabolism of more than 30 clinically used drugs, including all of the tricyclic antidepressants, several neuroleptics, opiates, betablockers, and antiarrhythmics and, among the selective serotonin reuptake inhibitors (SSRIs), N-desmethylcitalopram, fluvoxamine and fluoxetine (Brsen, 1998). Eckhardt *et al.* (1998) showed that the percentage of the codeine dose (170 mg, p.o.) converted to morphine and its metabolites was 3.9% in EMs and 0.17% in PMs, resulting in the difference in analgesic response of codeine.

After dietary administration for ten weeks of I3C or DIM to male Fischer 344 rats, the animals were gavaged with 25 mg of codeine. The analgesic response was measured, using the tail flick assay. The tail flick response is a spinal reflex that rarely occurs spontaneously. This assay has been used for pharmacodynamics studies with many opioid analgesics such as morphine, oxycodone and oxymorphone (Plummer *et al.*, 1990).

Although there was no statistical difference between controls and treatment groups in the tail flick assay due to highly variable response between rats, FMO1 protein expression and CYP2D1 activity in liver microsomes showed a significant difference between control and treatment groups. The western blotting result showed that I3C and DIM reduced hepatic FMO1 protein levels. The CYP2D1

activity in liver microsomes pretreated with I3C or DIM was significantly induced, compared to control. Our results suggested that the enhanced analgesic effect (if real) was possibly due to higher morphine in I3C- or DIM- treated rats, resulting from a decrease of N-oxide metabolites of codeine and morphine by FMO1 and/or induction of morphine production by CYP2D1.

The analgesic action of codeine in rat was mediated by morphine formation both in the brain and in the liver (Chen *et al.*, 1990). Bergh and Strobel (1996) also showed the presence of P4502D mRNA in rat brain. Baum and Strobel (1996) showed that in ovariectomized rats, testosterone dramatically increases CYP2D expression, while estrogen increases a modest CYP2D expression in rat brain. Previous studies revealed the endocrine effects of I3C, for example, I3C induced plasma vitellogenin, the biomarker for estrogenic activity in rainbow trout (Oganesian *et al.*, 1999). *In utero* exposure to a single dose of I3C (1.0 or 100 mg/kg) causes abnormalities in the male rat offspring including decreased sperm production and decreased transit rate of sperm (Wilker *et al.*, 1996). The presence of FMO in brain of human (Bhamre *et al.*, 1995) and rat (Bhamre *et al.*, 1993) has been documented. The question to now address is whether or not I3C including its acid condensation products can alter the expression of FMO and CYP2D in the brain which would require passage through blood brain barrier and then whether they can exhibit estrogenic activity in the brain.

If our study with rat can be extrapolated to human, EM of both sexes taking codeine along with a diet rich in cruciferous vegetables and/or taking I3C

supplements could enhance the analgesic response to codeine and perhaps experience other side effects due to FMO1 protein repression, CYP2D1 activity induction and possible sex hormone effects. We cannot provide statistically significant data from this study because of the small sample size and the instrument set up that required termination of the stimulus at 15 seconds. Future studies should be done not only to find the effect of I3C or DIM on the analgesic response of codeine, but also to examine CYP2D1 activity and FMO expression in brain.

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Chapter 5

IN VIVO ESTROGENIC ACTIVITY OF INDOLE-3-CARBINOL AND 3,3'-DIINDOLYLMETHANE IN RAINBOW TROUT AND IMMATURE FEMALE RAT

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ABSTRACT

Indole-3-carbinol (I3C), the most abundant metabolite of glucobrassicin, is found in cruciferous vegetables. The primary breakdown product of I3C in aqueous solution, acidic solution, and *in vivo* after oral administration is 3,3'-diindolylmethane (DIM). I3C and DIM bind to the Ah receptor with a binding affinity of 2.6×10^{-7} and 7.8×10^{-5}, respectively, relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Both compounds also inhibit DMBA-induced mammary tumor growth in the rodent model. The purpose of this study was to investigate the estrogenic effect of both compounds in rainbow trout and immature female Sprague-Dawley rat. In trout fed 1000 ppm I3C, plasma vitellogenin, a biomarker of estrogenic exposure, was induced on days 5, 10, 15 and 20. Plasma vitellogenin was also induced in trout treated with 100-2000 ppm I3C or 10-250 ppm DIM for 2 weeks, in a dose-dependent manner. Rats were fed with a diet containing I3C or DIM for 5 days. I3C (25-2000 ppm) and DIM (10-250 ppm) significantly induced uterine peroxidase activity. I3C (25-2000 ppm) induced hepatic CYP1A1/1A2, CYP2B1/2B2 and CYP3A2 protein levels in a dose-dependent fashion. Hepatic CYP1A1/1A2, CYP2B1/2B2 and CYP3A2 proteins were induced by given DIM at doses of 250 ppm, 10-250 ppm, and 10-250 ppm, respectively. DIM-induced estrogenic response can be observed at doses of 10-100 ppm, at which dose CYP1A1/1A2 induction is undetectable, suggesting that the estrogenic activity at these doses may be Ah-R independent.

INTRODUCTION

Indole-3-carbinol (I3C), a naturally occurring component of cruciferous vegetables such as broccoli, cabbage, cauliflower and Brussels sprouts, has been shown to increase the activity of phase I and II enzymes including CYP1A1, CYP1A2, CYP2B1/2B2, CYP3A2 and glutathione S-transferase (Stresser *et al.*, 1994a, b). Alteration of biotransformation enzymes affects the toxicity, mutagenicity and tumorigenicity of specific compounds. For example, administration of I3C prior to aflatoxin B1 (AFB1) exposure in rainbow trout (Dashwood *et al.*, 1988) and rat (Stresser *et al.*, 1994b) decreases AFB1-DNA binding in liver and lowers the incidence of liver tumors. In mice, I3C given prior to CCl_4 (Shertzer *et al.*, 1988) or N-nitrosodimethylamine (NDMA) (Shertzer, 1984) protected against hepatotoxicity and decreased NDMA-DNA binding, respectively. However, the liver tumor rate was higher in rainbow trout treated with I3C after AFB1 exposure (Nixon *et al.*, 1984). The timing of I3C exposure is important in determining the activity of I3C as tumor inhibitor or promoter (Wattenberg, 1977; Dashwood *et al.*, 1991).

A significant reduction in the incidence of spontaneous mammary tumors in C3H/OuJ mice (Bradlow *et al.*, 1991) and spontaneous endometrial cancer in Donryu rats (Kojima *et al.*, 1994) was observed when I3C was incorporated in the diets. I3C also increased the estrogen metabolite ratio of 2-hydroxyestrone to 16-α -hydroxyestrone in MCF-7 cells (Tiwari *et al.*, 1994) and in urine of women taking a 300 mg oral dose of I3C for 4 weeks (Wong *et al.*, 1997). It was suggested that

I3C increased estradiol 2-hydroxylation, mainly catalyzed by CYP1A2, resulting in reduced estrogenic activity. Interestingly, estradiol-responsive MCF-7 cells were more sensitive to the growth inhibitory effects of I3C than the estradiol-nonresponsive MDA-MB-231 cells (Tiwari *et al.*, 1994).

A primary breakdown product of I3C in aqueous, acidic solution (Spande 1979), and *in vivo* after oral administration (Stresser *et al.*, 1995) is 3,3'-diindolylmethane (DIM). Hepatic CYP1A1/1A2, CYP2B1/2B2 and estradiol 2-hydroxylase were induced in rat after treatment with DIM (Jellinck *et al.*, 1993). DIM also induced quinone reductase and UDP-glucuronosyl transferase in rat hepatocytes (Wortelboer *et al.*, 1992). DIM and I3C bind to the aryl hydrocarbon (Ah) receptor with a binding affinity of 7.8×10^{-5} and 2.6×10^{-7}, respectively, relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Bjeldanes *et al.*, 1991). DIM inhibited estrogen-induced proliferation of MCF-7 cell and 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor growth in Sprague-Dawley rats (Chen *et al.*, 1998). DIM induced apotosis in human breast cancer cells and the induction was independent of the p53 pathway (Ge *et al.*, 1996).

I3C has shown chemopreventive activity in several different animal models, and is now in the clinical development for prevention of breast cancer, endometrial cancer and other estrogen-related cancers. Little is known, however, about endocrine effects of I3C. Therefore, more detailed information is needed to determine the risk of endocrine effects when taken as a supplement for

chemoprevention in humans. The purpose of this study was to investigate the estrogenic effects of I3C and DIM in rainbow trout and immature female rat.

MATERIALS AND METHODS

Chemicals

I3C was purchased from Aldrich Chemical Co. (Milwaukee, WI). DIM was the kind gift of Dr. Michael Zeligs, BioResponse L.L.C. (Boulder, CO). Tamoxifen was from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-chum salmon vitellogenin IgG and purified vitellogenin were provided by Dr. D.R. Buhler (antibody was originally isolated by Dr. A. Hara: Hara *et al.*, 1993). Goat anti-rat CYP1A1/1A2, CYP2B1/2B2 and CYP3A2 were obtained from Gentest (Woburn, MA). Rabbit anti-goat secondary antibody conjugated to horseradish peroxidase was from Bio-Rad (Richmond, CA). Biotinylated donkey anti-rabbit IgG, streptavidin linked horseradish peroxidase and the chemiluminescence kit were purchased from Amersham Corp. (Arlington Heights, IL). Other chemicals were all reagent grade and commercially available.

Animals

Rainbow trout (*Oncorhynchus mykiss*), Shasta strain, reared at the Marine/Freshwater Biomedical Sciences Center fish hatchery and histopathology facility at Oregon State University were used in all studies. The test compounds

were incorporated into Oregon Test Diet (OTD), a semi-purified trout diet, and expressed as parts per million (ppm) of dietary wet weight. In the time course study, three groups of 30 fry (weight approximately 10-20 g) were fed 1000 ppm I3C, 10 ppm tamoxifen or 1000 ppm I3C plus 10 ppm tamoxifen. On days 5, 10, 15 and 20, fry (seven fry/group) were anesthetized by an overdose of tricainemethanesulfonate (MS222) to collect blood. In another study, two-year-old rainbow trout were fed I3C (50, 100, 250, 500, 1000 and 2000 ppm) and DIM (2.5, 10, 25, 50, 100 and 250 ppm) for two weeks before collected blood.

Twenty-one-day-old female Sprague-Dawley rats were acclimated to an AIN-76A diet for 7 days before being switched to AIN-76A diet containing I3C (25, 100, 250, 500, 1000 and 2000 ppm), DIM (2.5, 10, 25, 100 and 250 ppm) or 17β-estradiol (1 ppm) and fed ad libitum for 5 days. The rats were killed by CO_2 asphyxiation, and livers and uterus were removed, frozen in liquid N_2 and stored at –80°C until analysis.

Vitellogenin Analysis

Plasma vitellogenin was determined by competitive enzyme linked immunosorbent assay (ELISA), according to Donohoe and Curtis (1996). High affinity 96-well plates were coated with 100 µl/well of purified rainbow trout vitellogenin standard in 0.05 M sodium carbonate buffer (pH 9.6) 250 ng/ml and incubated overnight at 4°C. Standard vitellogenin or diluted plasma sample containing 1% BSA and rabbit anti-chum salmon vitellogenin IgG were added in

low affinity 96-well plates and stored overnight at 4°C. After washing high affinity plates with phosphate buffer-saline-0.05% Tween 20 (PBST), non-specific binding was blocked with 1% BSA in PBST. The standard vitellogenin and samples were transferred to high affinity plates and stored overnight at 4°C. After washing, the plates were incubated with anti-rabbit IgG, followed with strepavidine-horseradish peroxidase at 37°C and then visualized by adding the 3,3',5,5'-tetramethylbenzidine dissolved in DMSO, 0.01% H_2O_2 (30%) and 0.01 M sodium acetate buffer, pH 6.0. The color development was stopped by adding 10% H_2SO_4 after 20 min incubation. Absorbance was measured at 450 nm on a Bio-Tek EL340 plate reader.

Microsome Preparation and Western Blot Analysis

Liver microsomes were prepared by ultracentrifugation (Guengerich, 1989). Protein was quantified by the method of Lowry *et al.* (1951). Microsomal proteins were separated on an 8% SDS-PAGE (Laemmli, 1970), and electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979). The blots were incubated in 2% BSA in PBS, followed with goat anti-rat CYP1A1/1A2 , CYP2B1/2B2 or CYP3A2. The membranes were probed with a rabbit anti-goat secondary conjugated to horseradish peroxidase and then visualized using a chemiluminescence kit. Quantitation was performed by densitometry, using an HP ScanJet IicX flatbed scanner and NIH Image software version 1.54 (public domain, Wayne Rasband, National Institutes of Health).

Uterine Peroxidase Assay

After each uterus was removed and trimmed free of fat, the uterine horn bisections from each group were pooled, homogenized in ice cold Tris-buffer (10 mM Tris-HCl, pH 7.2) and centrifuged for 45 min at 39,000 g at 4° C. The pellets were rehomogenized in TC buffer (10 mM Tris-HCl, 0.5 mM $CaCl_2$, pH 7.2) and centrifuged at 39,000 g for 45 min at 4°C. The reaction was started by adding 450 µl of assay mixture (13 mM guaiacol and 0.3 mM H_2O_2 in the TC buffer) and 150 µl supernatant (1 mg/ml protein) to a cuvette and guaiacol oxidation was recorded for 1 min on a spectrophotometer at 470 nm. Three determinations were performed from each pooled extract (Dickenson *et al.*, 1992).

Statistical Analysis

Statistical analyses of the data were performed using Student's t-test. All data points are the mean ± SD for four animals per group. The p values less than 0.05 were considered significant.

RESULTS

Results of the time course study showed that dietary administration of 1000 ppm I3C to trout produced a time-dependent induction in plasma vitellogenin (Fig. 5.1). Tamoxifen alone also induced vitellogenin production (10.2-307.8 µg/ml), compared to control (3.9-2.9 µg/ml: data not shown) from day 5-20. Cotreatment

of trout 1000 ppm I3C plus 10 ppm tamoxifen significantly reduced vitellogenin levels 23%, 25%, 15% and 44% on day 5, 10, 15 and 20, respectively, compared to 1000 ppm I3C alone. Plasma vitellogenin on day 5, 10, 15 and 20 was induced significantly in fish treated with I3C, tamoxifen and I3C plus tamoxifen.

Two week dietary exposure to 25-2000 ppm I3C or 2.5-250 ppm DIM resulted in the production of vitellogenin in a dose-dependent manner (Fig. 5.2-5.3). A statistically significant increase in plasma vitellogenin was observed in trout treated with 250-2000 ppm I3C and 50-250 ppm DIM.

I3C (25-2000 ppm) and DIM (10-250 ppm) significantly induced uterine peroxidase activity, related to control rat (0.001 unit/mg protein) (Fig. 5.4-5.5). However, there were no dose-dependent effects in uterine peroxidase activity for both compounds. 17β-Estradiol (1 ppm), with uterine peroxidase 5.4 units/mg protein, served as the positive control for this experiment. Densitometry results following western blotting showed the effects of five days of dietary feeding I3C (25-2000 ppm) or DIM (2.5-250 ppm) on liver CYP1A1/1A2, CYP2B1/2B2 and CYP3A2 in immature female rat (Fig. 5.6-5.7). No CYP1A1/1A2 or CYP2B1/2B2 was detected in liver microsomes from control rats. I3C (25-2000 ppm) significantly induced hepatic CYP1A1/1A2, CYP2B1/2B2 and CYP3A2 protein levels in a dose-dependent fashion. Hepatic CYP1A1/1A2 and CYP2B1/2B2 protein were induced by DIM at doses of 250 ppm, and 10-250 ppm, respectively.

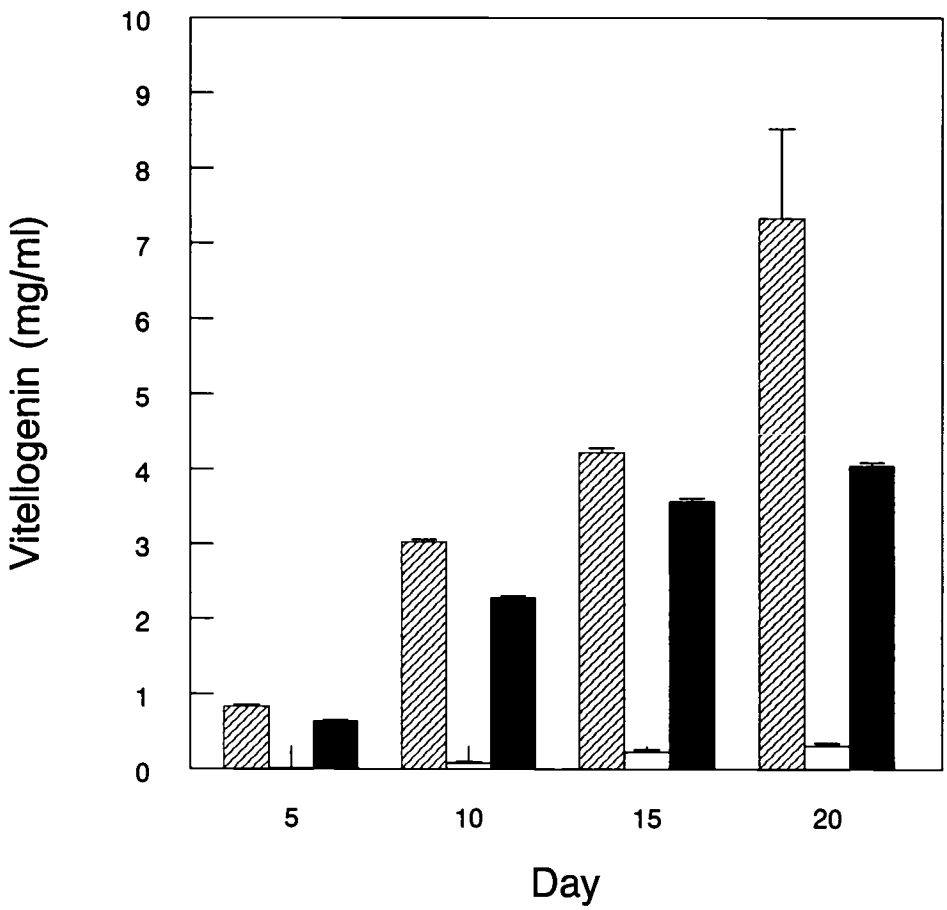

Figure 5.1. Time-course of plasma vitellogenin after 5-20 days dietary feeding 1000 ppm I3C (striped bars), 10 ppm tamoxifen (white bars) and 1000 ppm I3C plus 10 ppm tamoxifen 10 ppm (black bars) in rainbow trout.

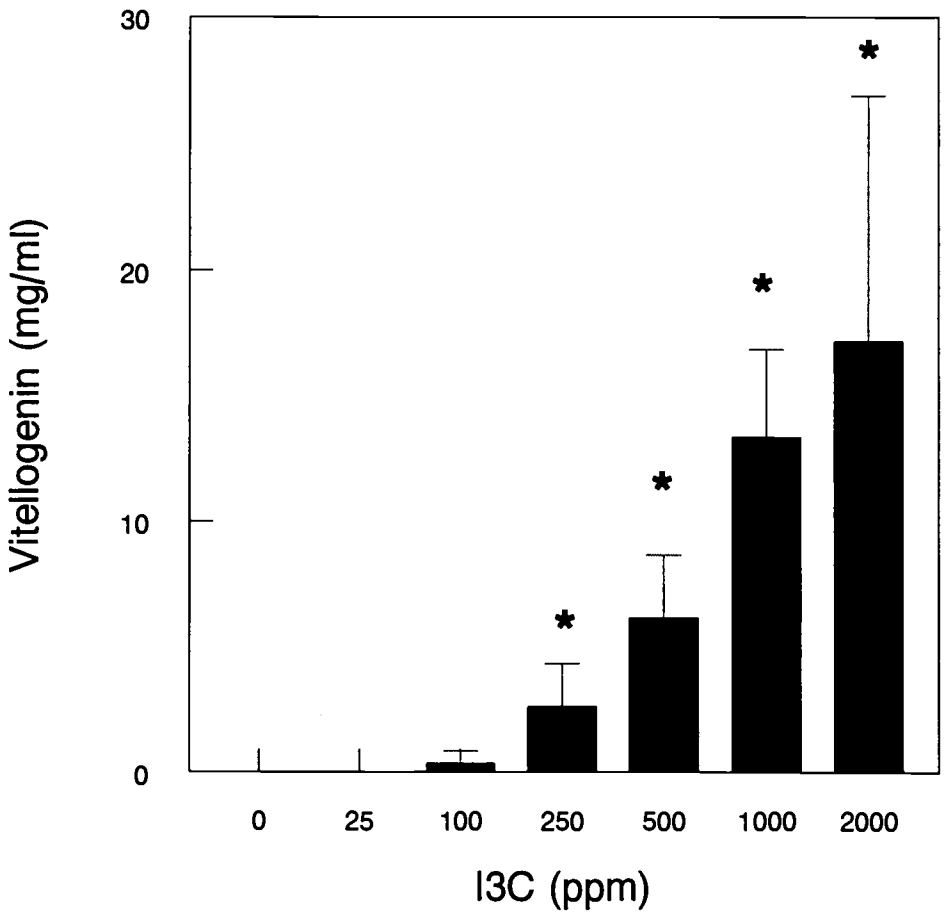

Figure 5.2. Dose-response of plasma vitellogenin after 2 weeks dietary feeding 0-2000 ppm I3C in rainbow trout. * Indicates significantly different from controls at $p < 0.05$.

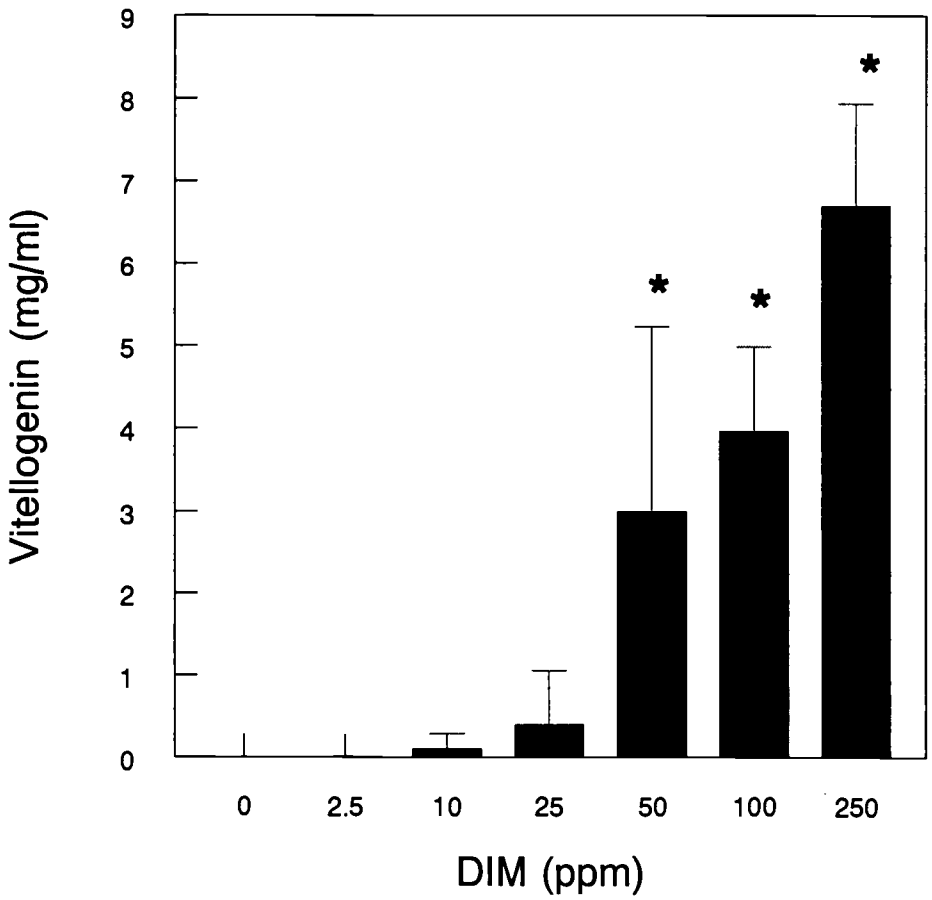

Figure 5.3. Dose-response of plasma vitellogenin after 2 weeks dietary feeding 0-250 ppm DIM in rainbow trout. * Indicates significantly different from controls at $p < 0.05$.

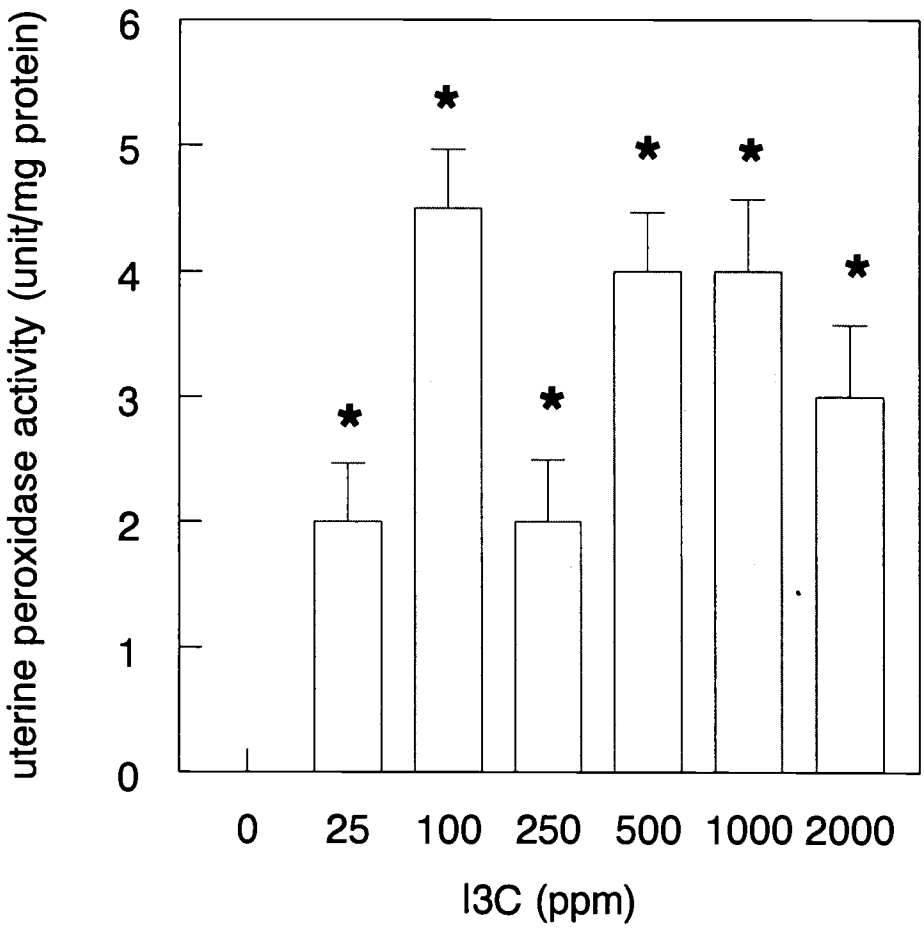

Figure 5.4. Dose-response of uterine peroxidase activity in the immature female Sprague-Dawley rat after 5 days dietary feeding 0-2000 ppm I3C. * Indicates significantly different from controls at $p < 0.05$.

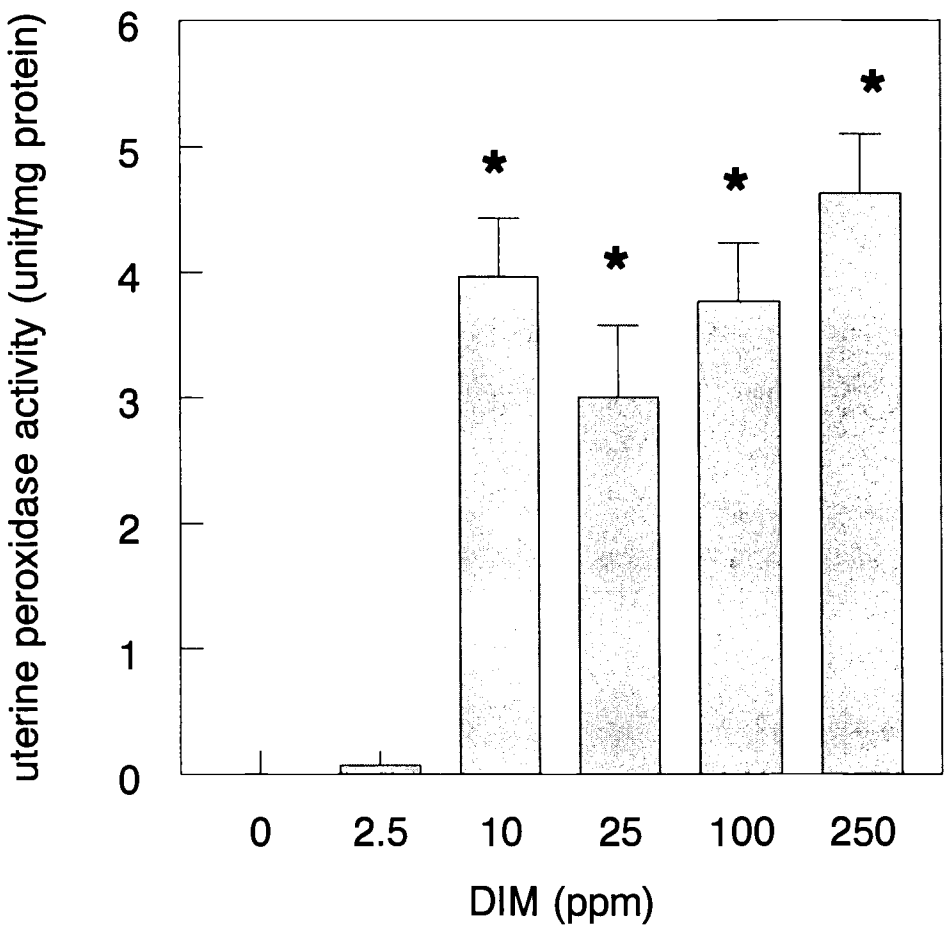

Figure 5.5. Dose-response of uterine peroxidase activity in the immature female Sprague-Dawley rat after 5 days dietary feeding 0-250 ppm DIM. * Indicates significantly different from controls at $p < 0.05$.

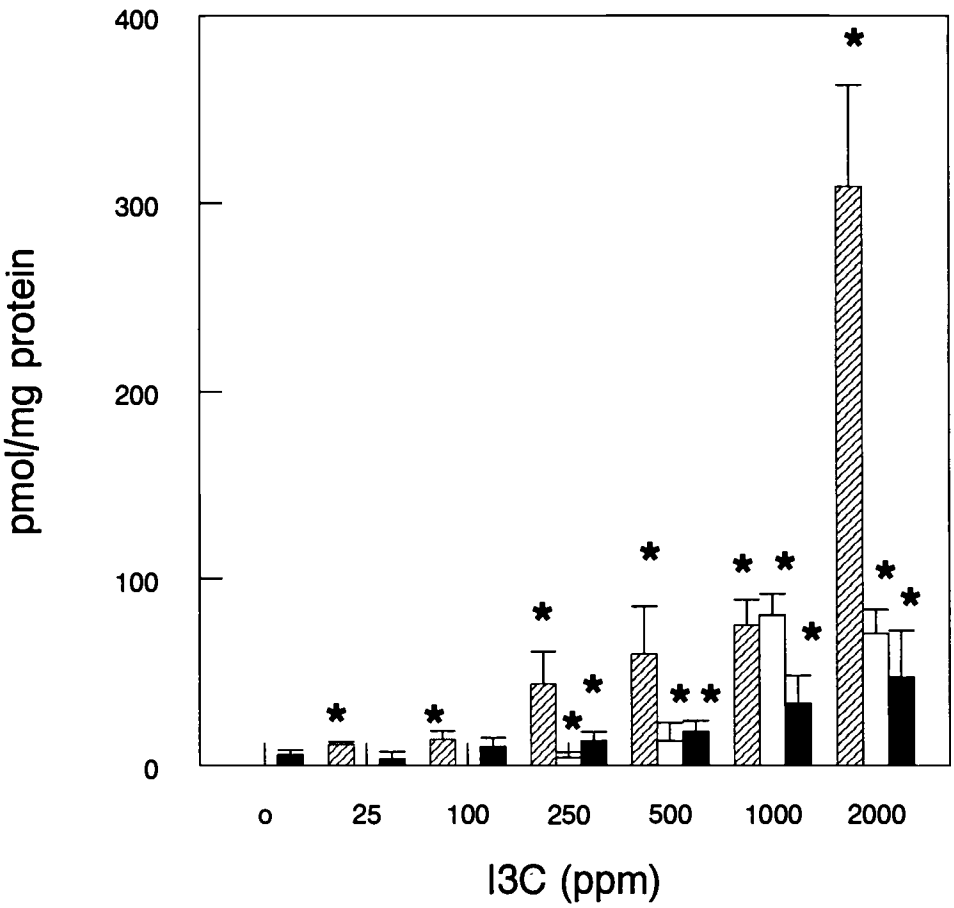

Figure 5.6. Dose-response of CYP1A1/1A2 (striped bars), CYP2B1/2B2 (white bars) and CYP3A2 (black bars) after 5 days dietary feeding 0-2000 ppm I3C in immature female rat. * Indicates significantly different from controls at $p < 0.05$.

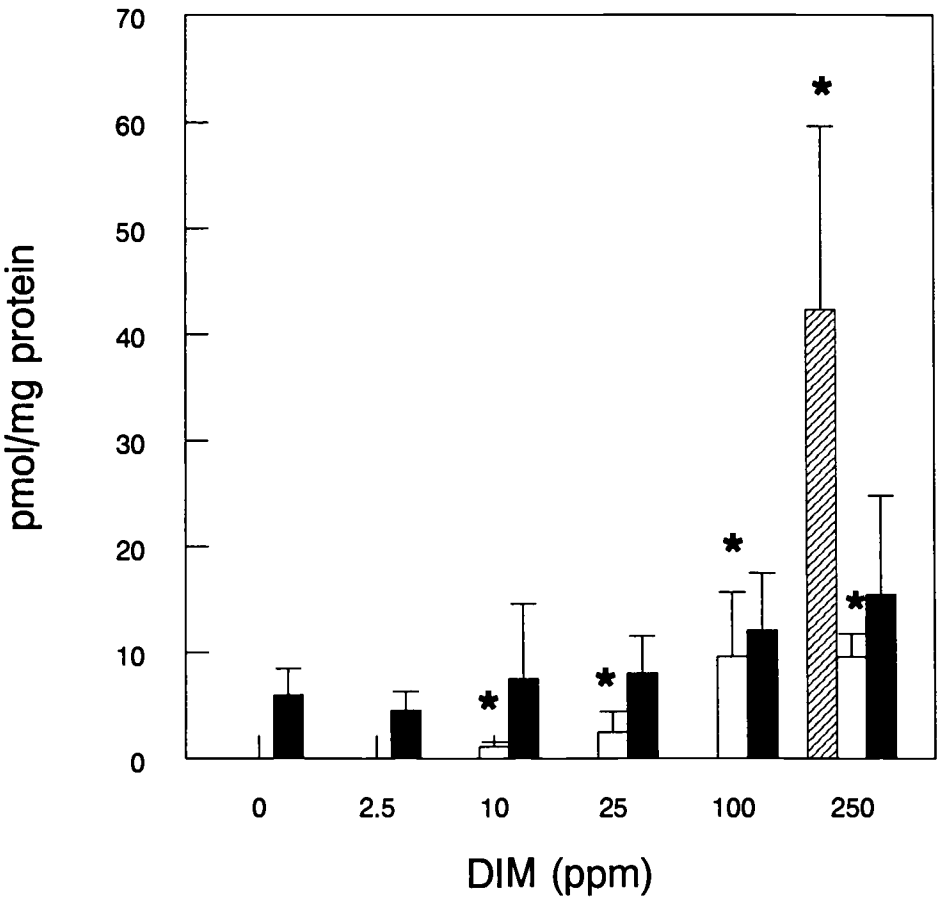

Figure 5.7. Dose-response of CYP1A1/1A2 (striped bars), CYP2B1/2B2 (white bars) and CYP3A2 (black bars) after 5 days dietary feeding 0-250 ppm DIM in immature female rat. * Indicates significantly different from controls at $p < 0.05$.

DISCUSSION

Mature female oviparous animals produce vitellogenin, an egg yolk protein precursor under the direct control of estrogen, but male and immature animals can also produce vitellogenin in response to estrogen. Vitellogenin has been used as a biomarker of estrogen and xenoestrogen exposure (Sumpter and Jobling, 1995; Donohoe and Curtis, 1996). Results from the time course study showed that in rainbow trout 10 ppm tamoxifen was weakly estrogenic compared to the effect seen with 1000 ppm I3C. The same dose of tamoxifen when given simultaneously with I3C only partially inhibit I3C-mediated vitellogenin production, suggesting that the trout estrogen receptor mRNA and vitellogenin mRNA transcription were saturated by 1000 ppm I3C (Flouriot *et al.*, 1996). Carlson *et al.* (1997) also showed that tamoxifen inhibited estrogen-mediated vitellogenin production only at sub-maximal estrogen doses.

DIM induced higher vitellogenin production than I3C at the same dose. Dashwood *et al.* (1989) showed that after given a single dose of I3C at 40 mg/kg body weight in the diet or by gavage for 48 hours, DIM comprised up to 40% of the total hepatic I3C condensation products in trout (1-1.5% of the I3C dose). If we assumed that I3C was metabolized to 1% DIM in trout liver as in Dashwood's study, then 1000 ppm I3C would be equivalent to 10 ppm DIM. However, vitellogenin induction by I3C at 1000 ppm was higher than that by DIM 10 ppm, 13 fold. One possible explanation was other I3C acid condensation products had estrogenic activity.

I3C (25-2000 ppm) and DIM (10-250 ppm) significantly induced uterine peroxidase activity, suggesting that the doses of both compounds were estrogenic in rat. Interestingly, the estrogenic activity of I3C and DIM was observed at doses of 25-2000 ppm, whereas CYP1A1/1A2 was induced at a dose of 250 ppm. It was possible that the estrogenic activity of DIM at doses of 10-100 ppm was Ah receptor independent.

I3C and DIM are estrogenic in a nonmammalian (trout) and mammalian (rat) models as determined by induction of plasma vitellogenin and uterine peroxidase, respectively. The results from this study suggested that I3C and DIM may act either as estrogens or antiestrogens depending on the target. However, the promotional potential of I3C and DIM in animal models for both hormone-independent and –dependent tumors, and the relation to human cancer risk, should be investigated before these compounds are used as the supplements for chemoprevention.

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Chapter 6

CONCLUSIONS

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SUMMARY

Our laboratory has previously shown that dietary administration of indole-3-carbinol (I3C) to male rats markedly induced hepatic levels of CYP1A, and dramatically down-regulated both the expression and activity of flavin-containing monooxygenase (FMO) form 1 (Larsen and Williams, 1996). In this thesis, we reported that there was no significant difference in hepatic FMO1 protein expression in male guinea pigs, mice and rabbits after feeding I3C 2000 ppm for 4 weeks. However, CYP1A1/1A2 was induced in all three species fed I3C.

We have also demonstrated that alteration of the FMO/CYP ratio mediated by I3C or DIM exhibited a marked shift in the *in vitro* metabolic profiles of drugs or xenobiotics that are substrates for both monooxygenase systems primarily due to the inhibition of FMO1 protein expression. The four compounds chosen in our study, N,N-dimethylaniline, nicotine, tamoxifen and codeine were all tertiary amines, typically excellent substrates for FMO, yielding the water soluble and usually non-toxic N-oxide metabolites (Ziegler, 1993). The metabolism of N, N-dimethylaniline, nicotine and tamoxifen was examined in liver microsomes of rats fed I3C or DIM. All N-oxide formation of three compounds was decreased, suggesting a potential for enhanced toxicity of three compounds *in vivo*. Additional studies are needed to examine whether human liver FMO3 responds to dietary I3C and DIM as does rat liver FMO1.

After dietary administration for ten weeks of I3C or DIM to male rats, the rats were gavaged with codeine, and analgesic response was determined using the

tail flick assay. There was no difference between control and treated rats in the tail flick assay due to highly variable response between rats. However, FMO1 protein expression was significantly decreased and CYP2D1 activity significantly increased in rat liver microsomes pretreated with I3C or DIM, compared to control. To investigate the effect of I3C or DIM on codeine metabolism, future studies should increase the sample size per group and set up the instrument to reduce experimental variation.

I3C exhibits estrogenic activity in trout by inducing plasma vitellogenin, the biomarker of estrogenic exposure. The estrogenic effects of I3C may be responsible for promotion of aflatoxin B1-initiated liver tumors in trout (Oganesian *et al.*, 1999). Our results also showed that I3C- and DIM- induced plasma vitellogenin in a dose-dependent manner. In immature female rats, both compounds induced uterine peroxidase activity at doses lower than required for CYP1A induction.

Overall, we have presented evidence that down-regulation of hepatic FMO1 expression mediated by I3C is species-specific in rat. *In vitro*, dietary I3C and DIM markedly shift the FMO/CYP ratio, suggesting that there could be an alteration of the metabolism, and perhaps therapeutic and toxicological effects of drugs and/or xenobiotics that are substrates for both monooxygenases *in vivo*. Both compounds exhibit estrogenic activity in rainbow trout and immature female rat. These results should be considered when assessing risk for the use of I3C and DIM as chemopreventive agents or dietary supplements.

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