CONCURRENT FLAVIN-CONTAINING MONOOXYGENASE DOWN-REGULATION AND CYTOCHROME P-450 INDUCTION BY DIETARY INDOLES IN RAT: IMPLICATIONS FOR DRUG-DRUG INTERACTION

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(Received January 28, 2000; accepted April 14, 2000)

This paper is available online at http://www.dmd.org

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 flavin-containing monooxygenase (FMO) 1 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 decreased in liver microsomes from I3C- or DIM-fed rats from near unity to 0.02 at the highest dietary doses. FMO-mediated N-oxygenation (nicotine N-1-oxide) was decreased, whereas CYP-mediated (nicotinamide and nicotine Δ1'-iminium ion) metabolism of nicotine was unchanged in liver microsomes from rats fed I3C or DIM. Similarly, the ratio of FMO to CYP metabolites of tamoxifen decreased due to a reduction in N-oxygenation. 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 tamoxifen and nicotine in vivo.

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 sprouts. The glucosinolate hydrolysis products from myrosinase (thioglucoside hydrolysis EC 3.2.2.1) at neutral pH are glucosinate, 3-indolylmethyl isothiocyanate, indole-3-carbinol (I3C), and thiocyanate ion. I3C may condense to 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 decreased in liver microsomes from I3C- or DIM-fed rats from near unity to 0.02 at the highest dietary doses. FMO-mediated N-oxygenation (nicotine N-1-oxide) was decreased, whereas CYP-mediated (nicotinamide and nicotine Δ1'-iminium ion) metabolism of nicotine was unchanged in liver microsomes from rats fed I3C or DIM. Similarly, the ratio of FMO to CYP metabolites of tamoxifen decreased due to a reduction in N-oxygenation. 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 tamoxifen and nicotine in vivo.

This was supported by U.S. Public Health Service Grants HL38650 (D.E.W.) and ES00834 (D.K.).

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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 G1 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 postinitiation 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., 1995b) and in vitro (Spande, 1979) is DIM. When DIM is coxjugated with aflatoxin B1, it reduces hepatic aflatoxin B1-DNA binding and tumor incidence in rainbow trout embryos (Dashwood et al., 1994). DIM is a potent nonspecific inhibitor of rat and human cytochrome P450 (CYP) 1A1, human CYP1A2, and rat CYP2B1 (Stresser et al., 1995a). Chen et al. (1998) showed that DIM was an aryl hydrocarbon 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. 7,12-Dimethylbenz[a]anthracene-induced mammary tumor growth 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.

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 CYPs, especially CYP1A, while markedly inhibiting flavin-containing monooxygenase (FMO) 1 in both a dose- and time-dependent manner (Larsen-Su and Williams, 1996). In this 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 (DMA) and drugs such as tamoxifen and nicotine, which are substrates for both mono-oxygenases. Alteration of the FMO/CYP ratio may have marked effects on toxicological and/or therapeutic properties, depending on the drug or xenobiotic.

Materials and Methods

Chemicals and Diet. I3C was purchased from Aldrich Chemical Co. (Milwaukee, WI). DIM was the gift of BioResponse L.L.C. (Boulder, CO). I3C and DIM were incorporated into powdered semisynthetic AIN-76A diet prepared without preservatives. The diet was prepared just before initiation of the experiment and stored frozen until the day before feeding. DMA (15.5 mCi/mmol, UL-ring) was purchased from Sigma Chemical Co. (St. Louis, MO). [1H-N-methyl]Tamoxifen (85.6 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). [S]-5-3H-Nicotine (32 Ci/mmol), prepared by the catalytic tritiation of (S)-5-bromonicotine (Shigenaga et al., 1987) was a gift from Dr. Mark Shigenaga.

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 sacrificed by CO2 asphyxiation, and livers were removed, frozen in liquid N2, and stored at −80°C until analysis. The protocols used were approved by the Oregon State University Institutional Animal Care and Use Committee.

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 SDS-polyacrylamide gel electrophoresis 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 from 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 chemiluminescence kit (Amersham Corp., Arlington Heights, IL). Quantitation of the blotting results, DIM proved to be more potent than I3C; 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 (DMA) and drugs such as tamoxifen and nicotine, which are substrates for both mono-oxygenases, were sacrificed by CO2 asphyxiation, and livers were removed, frozen in liquid N2, and stored at −80°C until analysis. The protocols used were approved by the Oregon State University Institutional Animal Care and Use Committee.

Results

Dietary administration for 4 weeks of I3C or DIM (Fig. 1) to male Fischer 344 rats resulted in a dose-dependent reduction in liver microsomal FMO1 protein levels (Fig. 2) 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.

DMA metabolism documents clearly the effects of I3C and DIM on FMO- and CYP-monooxygenation in liver microsomes of rats after dietary administration (Fig. 3). 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 DMA to 28% of control levels, whereas the inhibition with DIM was to 7% of control levels. CYP-dependent N-demethylation was induced 3- to 5-fold, but the effect was not dose-dependent. The ratio of FMO/CYP metabolism of DMA 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 Δ1,5-iminium ion, were unchanged by dietary I3C or DIM, FMO-catalyzed N-oxygenation of nicotine was markedly reduced (Fig. 4). 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. 5).

**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- to 4-fold are observed for CYP1A2, CYP2B1/2, and CYP3A (Bradfield and Bjeldanes, 1987; Bjeldanes et al., 1991; Wortelboer et al., 1992a,b; Stresser et al., 1994a; 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 on 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 this study to test this hypothesis, DMA, nicotine, and tamoxifen, are all tertiary amines. Tertiary aliphatic amines are typically excellent substrates for FMO, yielding the water-soluble and usually nontoxic 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 DMA, although Hlavica and Kunzel-Mulas (1993) found that CYP2B4-dependent superoxide anion radical production led to DMA-N-oxide production, others studies have found that, relative to FMO, this pathway is insignificant (Pandey et al., 1989; Seto and Guengerich, 1993). In this study, DMA-N-oxygenation was inhibited by dietary I3C in a dose-dependent manner. N-demethylation, presumably mediated predominantly by CYP2B1, was enhanced 3- to 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 DMA-N-oxygenation (Fig. 3) closely resembled that of FMO1 protein repression (Fig. 2). At the higher dose of DIM, greater than 90% of DMA-N-oxygenation was inhibited. The relative contribution of CYP- and FMO-mediated metabolism of DMA 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 that 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-\(\Delta^{1,3}\)-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.

Fig. 3. FMO-dependent N-oxygenation (filled columns) and CYP-dependent N-demethylation (hatched columns) of 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 ($P < .05$) decreased and N-demethylation significantly increased at all dietary levels of I3C and DIM.

Fig. 4. 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 $\Delta^{1,3}$-iminium ion (CYP, hatched columns), and nicotine N-1'-oxide (FMO, filled columns), resolved, and quantified by HPLC as described under Materials and Methods.
(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 DMA 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 nonfunctional CYP2A6 (Pianezza et al., 1998). 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 the 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 (Crewe et al., 1997; Dehal and Kupfer, 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 demethylation and 4-hydroxylation to FMO-mediated N-oxygenation is approximately unity; at the higher dose of I3C and both doses of DIM, the ratio increases to 3 to 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-N2-tamoxifen N-oxide DNA adducts in humans (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 modulate their risk of developing toxic side effects.

As mentioned above, whether or not these 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 to 0.014 mmol of I3C/kg/day, exhibited a significant decrease in urinary trimethylamine N-oxide, presumably due to the 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 affect FMO.

It may not be necessary for FMO3 protein to be down-regulated by
I3C (as 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-[3,2-3C]carbazole (ICZ) inhibited the catalytic activity of rat FMO1 in vitro with K_i values 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_i values in the low micromolar range (Cashman et al., 1999). Of these three compounds, only DIM would be expected to be present in liver after I3C oral administration at levels capable of eliciting this response. Studies following the pharmacokinetics of 3H-I3C after oral administration to rats found no I3C in liver, and ICZ levels were estimated to be 1.6 mM; however, DIM levels were estimated to be 3 to 6 μM (Stresser et al., 1995b). Interestingly, DIM also directly inhibits rat and human CYP1A1, human CYP1A2, and rat CYP2B1 with K_i values again in the low micromolar range (Stresser et al., 1995a). In this study, DIM was demonstrably more effective than I3C. The 1000 and 2500 ppm diets correspond to 6.2 and 15.5 mmol/kg for I3C and 4.1 and 10.2 mmol/kg for DIM; therefore, DIM was even more effective on a per 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 of drugs that are substrates for both CYP and FMO monoxygenases. Dietary I3C and DIM down-regulate 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. These findings demonstrate the potential for previously unrecognized drug-drug interactions. The potential alterations in tamoxifen efficacy or nicotine toxicity in humans, as a result of a diet high in crucifers or through dietary supplementation with indoles, should be explored further.

Acknowledgments. We thank Dr. Daniel Ziegler for purified pig liver FMO1 and rabbit anti-hog liver FMO1, Dr. Mark Shigenaga for purified pig liver FMO1 and rabbit anti-hog liver FMO1, Dr. Mark Shigenaga for the potential for previously unrecognized drug-drug interactions. The potential alterations in tamoxifen efficacy or nicotine toxicity in humans, as a result of a diet high in crucifers or through dietary supplementation with indoles, should be explored further.

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