Inhibition of 2-amino-3-methylimidazo[4,5-f]quinoline–DNA adducts by indole-3-carbinol: Dose–response studies in the rat colon

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Indole-3-carbinol (I3C) inhibits the formation of colonic aberrant crypt foci and DNA adducts in rats given heterocyclic amine colon carcinogens, such as 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). Mechanism studies indicate that I3C induces cytochromes P4501A1 and 1A2 (CYP1A1 and CYP1A2), isozymes that respectively metabolize IQ via ring hydroxylation or activate the carcinogen by N-dealkylation. The present study examined the dose–response for induction of CYP1A1 versus CYP1A2 by I3C, and compared the profiles of induction with the dose–response for inhibition of IQ–DNA adducts in the colon of the F344 rat. Dietary equivalent doses of I3C in the range 100–1000 p.p.m. increased in a dose-related manner both ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD) activities in the liver and colonic mucosa, and Western blots showed a corresponding induction of CYP1A1 and CYP1A2 proteins. However, dietary equivalent doses of I3C in the range 10–25 p.p.m. (i) reduced hepatic EROD and MROD activities and CYP1A protein levels compared with controls, (ii) increased the ratio of CYP1A2 versus CYP1A1, and (iii) activated IQ to a more potent mutagen when liver microsomes from rats given I3C were used for metabolic activation in the Salmonella assay. Rats given a single oral dose of I3C shortly before administering IQ (5 mg/kg body wt, p.o.) exhibited dose-related inhibition of colonic IQ–DNA adducts in the range 25–100 p.p.m. I3C, reaching 95% inhibition at doses ≥100 p.p.m. I3C, but IQ–DNA adducts were elevated slightly at the lowest I3C dose as compared with the controls. The possible significance of the low versus high dose effects of I3C are discussed in the context of human dietary exposures to I3C and the reported chemopreventive mechanisms of I3C in vivo.

Introduction

Indole-3-carbinol (I3C*), a natural constituent of cabbage, broccoli, cauliflower and other cruciferous vegetables, has been reported to exhibit anticarcinogenic activity in rats, mice and rainbow trout exposed to various initiating agents and carcinogen treatment protocols (1–6). In recent studies (7,8), I3C was shown to prevent against the formation of colonic aberrant crypt foci (ACF) and DNA adducts in rats given the heterocyclic amines 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). Both PhIP and IQ are known to be activated by CYP1A2, but these compounds can be ring hydroxylated by a competing pathway involving CYP1A1 (7,8). Metabolism studies indicated that I3C shifted the initial activation step from the exocyclic amine group to the 4 and 5 ring positions of PhIP and IQ, respectively, thereby favoring subsequent detoxification by phase II enzymes. As a consequence, I3C reduced the urinary excretion of sulfamates and increased the elimination of 4-hydroxy-PhIP and 5-hydroxy-IQ metabolites and their corresponding O-glucuronides and O-sulfates. Results from Western blots and enzyme assays showed that cytochromes P4501A1 and 1A2 were strongly induced by I3C, but CYP1A1 was induced to a significantly greater extent than CYP1A2 at a dietary exposure of 0.1% I3C (7,8). Because CYP1A1 and CYP1A2 compete to some extent for the initial step in heterocyclic amine metabolism (mainly ring versus N-hydroxylation respectively), we decided to examine the time-course and dose–response for I3C induction of CYP1A1 versus CYP1A2 and the relationship of this induction to the inhibition of IQ–DNA adducts in the colon.

Materials and methods

Materials

IQ, I3C and all other materials and supplies used in the in vivo studies, Western blots, enzyme assays and mutagenicity tests were from sources described previously (7).

Animals and treatments

Weanling male F344 rats were purchased through the University of Hawaii Laboratory Animal Services and housed in a climate-controlled room with a 12 h light/12 h dark cycle. Animals typically were housed two per cage and given AIN-76A diet (ICN Biomedicals Inc., Aurora, OH) and water ad libitum. Experiment 1 examined the time-course for induction of hepatic CYP1A1 and CYP1A2 by I3C. Thirty-two rats were randomly divided into eight groups of four animals, and these were killed at 0, 1, 3, 6, 12, 24, 48 h or 7 days after a single oral administration of I3C. Previous studies provided information on the average consumption of diet in rats given 0.1% I3C for several weeks, and hence the average daily exposure of each animal to I3C (7). In experiment 1, this amount of I3C was administered once to each rat by oral gavage, and although the vehicle was dimethyl sulfoxide (DMSO), we defined this as the ‘1000 p.p.m. dietary equivalent dose’ (0.67 mmol/kg body wt; in 0.5 ml DMSO/kg body wt). At the times selected, each colon was removed, opened longitudinally, cleaned of its contents and the mucosa scraped with a spatula. The liver from each animal was briefly perfused of blood in situ using ice-cold saline before being removed and chopped into small pieces. Liver and colon microsomes were prepared from individual rats as described previously and stored at −80°C (7,9).

Experiment 2 determined the dose–response for induction of CYP1A1 and CYP1A2 by I3C. Forty rats were randomly divided into eight groups of five animals and each was given a single oral injection of I3C at the following dietary equivalent doses: 0, 10, 25, 50, 100, 200, 500 or 1000 p.p.m. All of the rats were killed 12 h thereafter, and liver and colon microsomes were prepared and stored at −80°C.

Experiment 3 examined the I3C dose–response for inhibition of IQ–DNA adducts...
adducts in the colon. Rats were randomly divided into groups of four animals. One group of rats was given the 1000 p.p.m. dietary equivalent dose of I3C (in 0.1 ml DMSO) for 7 consecutive days before treatment with IQ. The other groups were given DMSO alone for 6 days and then one injection of IQ on day 7 at dietary equivalent doses <1000 p.p.m. At 6 h later, each rat received a single oral injection of 5 mg IQ/kg body wt. At 8 h after carcinogen dosing, at the peak of adduct formation (7), the rats were killed by CO2 inhalation.

Quantification of IQ–DNA adducts

Procedures used for the isolation of DNA from colonic mucosa and for the quantification of adducts by 32P-postlabeling under intensification conditions have been described elsewhere (7,10).

Western blotting

Microsomal protein was run on a 7% polyacrylamide mini-gel and probed with an antibody to CYP1A1 and CYP1A2 according to a recently published methodology (7).

Mutagenicity assays

Liver microsomes from experiment 2 were used as a source of metabolic activation in the Salmonella mutagenicity assay, using Salmonella typhimurium strain TA98 kindly provided by Dr Bruce Ames. The microsomes were incorporated at a level of 10% into the cofactor solution (3.4 mg protein/ml) and sterilized by passage through a 0.45-µm filter. The order of addition to 2 ml soft agar was as follows: mutagen (10 µl), overnight culture of TA98 (0.2 ml) and activation system (0.4 ml). After pouring onto minimal glucose plates and incubating at 37°C for 2 days, hisr revertant colonies were counted on each plate. The number of spontaneous revertant colonies was typically on the order of 22 ± 3 per plate (mean ± SD, n = 3).

Results

Rats given a single p.o. injection of the 1000 p.p.m. dietary equivalent dose of I3C showed marked induction of EROD and MROD activities in hepatic microsomes, but little induction in the microsomes isolated from the colonic mucosa (Figure 1). The peak of induction occurred 12 h after I3C treatment, representing a 24-fold induction of EROD and a 7.6-fold induction of MROD versus controls (Figure 1a and b respectively).

The 12-h time-point was used in subsequent studies of the dose–response for induction by I3C. As shown in Figure 2, hepatic EROD and MROD activities were induced in a dose-related fashion by dietary equivalent doses of I3C in the range 100–1000 p.p.m. No induction occurred in the range 10–50 p.p.m. I3C; in fact, the EROD and MROD activities were slightly lower in these groups than in the controls given 0 p.p.m. I3C (Figure 2, inset). In contrast to the results in liver, EROD activities in the colonic mucosa increased with I3C dose over the entire range 10–1000 p.p.m., whereas only marginal induction of MROD was detected in the colon (Figure 3). The absolute levels of EROD and MROD were significantly lower in the colon than in the liver at corresponding doses of I3C (compare ordinates in Figures 2 and 3).

Western analysis gave results that were largely consistent with the data from the hepatic EROD and MROD assays (Figure 4). Compared with controls, dietary equivalent doses >50 p.p.m. I3C markedly induced both CYP1A1 and CYP1A2 (Figure 4), and scanning densitometry showed the induction of both isozymes to be dose-related in the range of 50–1000 p.p.m. I3C (Figure 4). Whereas the highest dose of I3C (1000 p.p.m.) induced CYP1A1 to a greater extent than CYP1A2, slightly higher CYP1A2 versus CYP1A1 levels were detected at dietary equivalent doses of I3C <500 p.p.m. At I3C doses of 10 p.p.m. and 25 p.p.m., CYP1A1 protein levels were below the limit of detection (Figure 4).

Liver microsomes from the I3C dose–response study (experiment 2) were used as source of metabolic activation in the Salmonella assay (Figure 5). Only the microsomes from rats given 10 p.p.m. or 25 p.p.m. I3C activated IQ to any significant extent in the Salmonella assay. As indicated above, these were the I3C groups in which EROD activities and CYP1A1 levels were essentially non-detectable, presumably leaving CYP1A2 levels sufficiently high to activate IQ in the absence of a competing pathway for ring hydroxylation.

Because several enzymes in addition to CYP1A1 and CYP1A2 are important for the metabolism of IQ in vivo (11–15), we next examined the dose–response for I3C inhibition of IQ–DNA adducts in the colon. As shown in Figure 6, IQ–DNA adducts were increased at the lowest I3C dose tested, but they were inhibited in a dose-related manner at dietary equivalent doses in the range 25–100 p.p.m. I3C. In all groups given >100 p.p.m. I3C, IQ–DNA adducts were inhibited by >95%, including the group receiving seven consecutive doses of the 1000 p.p.m. dietary equivalent dose (open circle, Figure 6). EROD and MROD
assays and Western blots showed the levels of CYP1A induction to be similar in rats given either a single or seven consecutive doses of the 1000 p.p.m. dietary equivalent dose of I3C (data not presented).

Dose–response inhibition of IQ–DNA adducts by I3C

Fig. 2. Dose–response for induction of EROD and MROD in rat liver microsomes by I3C. Rats were treated with a single oral dose of I3C equivalent to that received from a daily exposure to 0, 10, 25, 50, 100, 200, 500 or 1000 p.p.m. I3C in the diet. Animals were killed 12 h after I3C dosing and the liver microsomes were used for the detection of EROD (●) and MROD (■) activities. Results were obtained from five rats per dose point (mean ± SD). Insert: Enlarged view of the data for I3C doses in the range 0–100 p.p.m.

Fig. 3. Dose–response for induction by I3C of EROD and MROD activities in the colonic mucosa. Rats were treated as described in Figure 2, and colon microsomes were used for the detection of EROD (○) and MROD (□) activities. Results were obtained from five rats at each dose point (mean ± SD).

Fig. 4. Dose–response induction of CYP1A1 and CYP1A2 in rat liver microsomes following treatment with I3C. Immunoblot analysis of liver microsomal protein (20 µg) from control and I3C-treated rats after separation by electrophoresis on a 7% (w/v) SDS–polyacrylamide gel, transfer onto PVDF-plus membrane, and incubation with polyclonal anti-cytochrome P4501A1/1A2 (0.01 mg/ml). Immunoblots were visualized after incubation for 30 s with peroxidase-linked anti-rabbit IgG. Results obtained from the Western blots following scanning-densitometry (arbitrary units). The results shown are from a single experiment, but are representative of the findings from three separate in vivo studies.

Fig. 5. Activation of IQ in the Salmonella mutagenicity assay using liver microsomes from rats given various doses of I3C. Salmonella typhimurium strain TA98 (0.2 ml), IQ (20 ng), and liver microsomes (1.36 mg protein/plate) were added to 2 ml soft agar and the mixture was poured onto minimal glucose plates. His\(^{+}\) colonies were counted after 2 days incubation at 37°C. Each data point and bar represents the mean ± SD (n = 3); background counts for spontaneous revertants in the assay shown were 22 ± 3 colonies/plate.
than those of CYP1A1 at all I3C doses to the F344 rat, and indicates that CYP1A2 levels are higher whereas 200 p.p.m. I3C induced CYP1A2 to a greater extent I3C dose induced CYP1A1 more effectively than CYP1A2 treatment with 200 p.p.m. and 500 p.p.m. I3C, but the highest were induced in a dose-related manner following dietary dose tested (20). In the Wistar rat, CYP1A1 and CYP1A2 some evidence of toxicity in the intestine at the highest I3C activity was clearly dose-related in the liver and intestine, with for the controls given 0 p.p.m. I3C, but induction of EROD (but not MROD) induction was examined Wistar and Sprague–Dawley rats (20,26). In the Sprague–investigated in the F344 rat, particularly at doses in the lower isozymes to induction has not been thoroughly animes (20–26). Concentrations of I3C p.p.m. induce both CYP1A1 and CYP1A2, but the relative sensitivities of these specific isozymes to induction has not been thoroughly investigated in the F344 rat, particularly at doses in the lower range of possible human dietary intake of I3C.

Limited I3C dose–response data have been reported in Wistar and Sprague–Dawley rats (20,26). In the Sprague–Dawley rat, EROD (but not MROD) induction was examined 20 h after p.o. injection of I3C, using dietary equivalent doses in the range 1 p.p.m. to 1500 p.p.m. No data were provided for the controls given 0 p.p.m. I3C, but induction of EROD activity was clearly dose-related in the liver and intestine, with some evidence of toxicity in the intestine at the highest I3C dose tested (20). In the Wistar rat, CYP1A1 and CYP1A2 were induced in a dose-related manner following dietary treatment with 200 p.p.m. and 500 p.p.m. I3C, but the highest I3C dose induced CYP1A1 more effectively than CYP1A2 whereas 200 p.p.m. I3C induced CYP1A2 to a greater extent than CYP1A1(26). The present study extends these findings to the F344 rat, and indicates that CYP1A2 levels are higher than those of CYP1A1 at all I3C doses <500 p.p.m. (i.e. greater induction of the isozyme which activates IQ). Interestingly, at the lowest I3C doses tested, the levels of CYP1A1 and CYP1A2 and their associated EROD and MROD activities were slightly lower than in the 0 p.p.m. I3C controls. In this I3C low dose ‘window’, IQ was activated more effectively in the Salmonella assay, and colonic IQ–DNA adduct levels were elevated in vivo compared with the controls. We also have noted this ‘low dose window’ behavior of I3C derivatives at the enzyme level in vitro. The predominant I3C derivative 3,3′-dindolylmethane inhibited dibenz[a,l]pyrene (DBP) metabolic activation by rainbow trout CYP1A1v3 expressed in yeast at concentrations above 150 µM, but enhanced the activation of DBP at lower concentrations (27).

To our knowledge, this is the first report to indicate that a low dose of I3C administered shortly before the carcinogen can increase IQ–DNA adducts. Previous studies showed that rats pretreated for 2 weeks with 1000 p.p.m. I3C in the diet had increased PhIP– and IQ–DNA adducts in the colon and other tissues 6–8 h after carcinogen exposure and decreased adducts at later time points (7,8). In another report, mice given four consecutive daily doses of I3C by gavage followed on the last day by a single i.p. injection of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK) had lower pulmonary but enhanced hepatic O6-methyl guanine adducts 2 h or 6 h after NNK dosing (3). In those studies, I3C pre-treatment increased the overall rate of carcinogen metabolism by the liver (3,7,8), leading to the more rapid formation and removal of DNA adducts.

One possible mechanism by which I3C might increase IQ–DNA adducts would be to inhibit the activity or constitutive expression of CYP1A1 to a greater extent than CYP1A2. Previous reports described the ability of I3C-derived oligomers (dimers and trimers) to act as reversible inhibitors of CYP1A isozymes (21,23,24), but the present study indicated a loss of CYP1A1 and a reduction of CYP1A2 at low I3C doses compared with controls (Figure 4). By what mechanism(s) might I3C induce CYP1A proteins at high doses but down-regulate their expression at low I3C exposure levels? Several oligomers formed from I3C at low pH are known to act as Ah receptor agonists (25,28–30), but the profile of these acid condensation products differs significantly at high versus low starting concentrations of I3C (31). Thus, whereas the profile of oligomers and their absolute levels at higher I3C concentrations probably favors Ah receptor binding and the induction of CYP1A proteins, at very low levels of I3C exposure certain oligomers or their metabolites might interfere with endogenous ligand(s) for the Ah receptor.

An important question arises as to how the doses used in this study compare with typical human exposures to I3C via the diet, and whether very low levels of I3C might facilitate DNA adduct formation by certain carcinogens. Fresh broccoli, which is a relatively rich source of I3C, contains in one 100 g portion ~50 mg of I3C, or 500 p.p.m. I3C (32). The ‘dilution’ of this amount of I3C by other foods in a meal might occur over a wide range, but concentrations as low as 25 p.p.m. I3C are predicted from the present study to offer significant protection against IQ. Concentrations of I3C on the order of 10 p.p.m. also are within the realm of possible human exposures, and we are now examining the chronic effects of very low dietary concentrations of I3C on heterocyclic amine metabolism in vivo.

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References

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