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Chemopreventive Properties of Indole-3-Carbinol (I3C): Inhibition of DNA Adduct Formation of the Dietary Carcinogen, 2-Amino-1-Methyl-6-Phenylimidazo [4,5-*b*]Pyridine (PhIP), in Female F344 Rats

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Abstract Indole-3-carbinol (I3C), a naturally occurring inhibitor of experimental carcinogenesis, was evaluated for its possible inhibitory effect on DNA-adduct formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a dietary mutagen, in female F344 rats. PhIP is a mammary carcinogen in female F344 rats and a colon carcinogen in male F344 rats. Four-week-old animals (4/group) were maintained on powdered AIN-76A diet with or without I3C (0.02% or 0.1%, w/w) for 58 days. PhIP (0.04%, w/w) was added to the diet from days 15 through 42. Animals were killed on days 43 and 58. DNA isolated from mammary epithelial cells (MECs), colon, liver, and white blood cells (WBCs) was analyzed for PhIP-DNA adducts by ^{32}P-postlabeling assays. On day 43, adduct levels of the group receiving 0.1% dietary I3C decreased in MECs (91.9%), colon (67.2%), liver (69.2%), and WBCs (82.3%). On day 58, DNA adduct formation was inhibited in the colon (81.3–82.2%) at both dietary I3C concentrations, and in liver (46.8%) only in the animals fed 0.1% I3C. When incorporated in the diet after exposure to dietary PhIP (0.04% for 2 weeks), I3C (0.1%) had no effect on the rate of removal of PhIP-DNA adducts over the next 28 days. It is concluded that dietary I3C inhibits PhIP-DNA adduct formation in the female F344 rat but does not affect adduct removal. I3C may be a promising chemopreventive agent in PhIP-induced carcinogenesis in rats. J. Cell. Biochem. Suppl. 27:42–51.

Key words: food mutagens; indole-3-carbinol; chemoprevention; DNA adducts; PhIP; heterocyclic amines

The heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), is quantitatively the most important bacterial mutagen that can be isolated from well-done ground beef [1]. PhIP has also been isolated from other cooked proteinaceous foods [2,3] and has been found in cigarette smoke [4] and in beer and wine [5].

In the F344 rat, PhIP has been shown to exert carcinogenicity in the mammary glands of females and in the colon and prostate of males [6,7]. The mammary carcinogenicity of PhIP in female rats has also been confirmed in other strains [8,9]. In the CDF_1 mouse, PhIP induces lymphomas [10], while in the newborn $B6C3F_1$ mouse model it induces adenocarcinomas of the liver [11].

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Most carcinogens, including PhIP, require metabolic activation to oxygenated derivatives before their carcinogenicity is expressed. These derivatives may then be converted to more electrophilic intermediates that can bind to DNA to form DNA adducts. PhIP is activated by *N*-hydroxylation of its exocyclic amino group, a reaction catalyzed by either cytochrome P450 1A1 or cytochrome P450 1A2 [12–16]. Further esterification by either cytosolic sulfotransferase or *O*-acetyltransferase yields a DNA-reactive electrophile [17].

PhIP-DNA adduct formation has been demonstrated in vitro [17,18] as well as in vivo [19–21]. Using ^{32}P-postlabeling methodology, we were able to detect three PhIP-DNA adducts in various tissues of the F344 rat [21] and cynomolgus monkey [19], the major one of which [21] has been identified as N^2-(deoxyguanosin-8-yl)-PhIP [22]. Regarding PhIP-DNA adducts in the rat mammary gland, we have shown that PhIP is first activated in the liver to *N*-hydroxy-PhIP, which, after transport to the mammary gland,

is further activated by mammary epithelial cell *O*-acetyltransferase to form DNA adducts [23].

Indole-3-carbinol (I3C) is a naturally occurring constituent of cruciferous vegetables such as broccoli, Brussels sprouts, cabbage, and cauliflower, where it is present as a glucosinolate [24]. Twenty years ago, I3C was reported to inhibit 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumors in rats and benzo[*a*]pyrene-induced forestomach tumors in mice [25]. The chemopreventive properties of I3C have been confirmed in several more recent studies; these include inhibition of diethylnitrosamine-induced hepatocarcinogenesis in ACI/N rats [26], inhibition of 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone-induced lung neoplasia in A/J mice [27], and inhibition of DMBA-induced mammary tumors in Sprague-Dawley rats [28].

In using dietary additives such as selenite, garlic powder, or rosemary extract as chemopreventive agents, the amount added to the diet was found to be related to both the extent of inhibition of DMBA-DNA adducts in mammary epithelial cells (MECs) and the extent of inhibition of DMBA-induced mammary tumor formation in rats [29–31]. Thus, DNA adducts may be an excellent predictive tool for gauging the effectiveness of dietary agents in preventing carcinogen-induced tumors.

In the present experiments, we sought to assess the potential chemopreventive properties of I3C in PhIP-induced carcinogenesis by evaluating its inhibitory effect on PhIP-DNA adduct formation in the female F344 rat. To approximate human intake, both PhIP and I3C were given in the diet at relatively low concentrations.

MATERIALS AND METHODS

Materials

Four-week-old female F-344 rats (35–50 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). They were housed, four per cage, in a temperature (22 ± 2°C) and light-controlled (12 h light/12 h dark) room. Food and water were provided ad libitum. PhIP was purchased from Toronto Research Chemicals (North York, ON, Canada). I3C was purchased from Sigma Chemical Company (St. Louis, MO). Materials for the ^{32}P-postlabeling assay were obtained from the same sources as before [32]. Powdered AIN-76A diet was prepared in the laboratory as described before [33], except that the antioxidants were omitted. Food was prepared in batches of 1 kg and stored in airtight containers at −20°C. Animals received fresh food every third day.

Animal Treatment

In experiment 1 (Fig. 1), after 1 week of acclimation on AIN-76A diet, rats were maintained on the diet containing different amounts of I3C (0.02 or 0.1%, w/w) throughout the experimental period (1–58 days). From days 15 to 42 (4 weeks), all animals received 0.04% (w/w) PhIP in their diet. All animals were weighed weekly. On the first day and sixteenth day after cessation of PhIP feeding (days 43 and 58, respectively), four rats from each group were killed. A heparin solution (~2,500 U/kg, i.p.) was administered, followed 15 minutes later by a sodium pentobarbital solution (~175 mg/kg, i.p.). After complete anesthesia (~3–5 min.), the abdominal cavity was opened and whole blood was collected from abdominal veins (4–6 ml) and placed in lithium heparin collection tubes. The liver and colon were removed and placed in 5 ml of phosphate-buffered saline and then stored at −70°C until isolation of DNA. Blood and mammary glands were removed and processed immediately. In experiment 2 (Fig. 2), 32 rats were allowed to acclimate for 8 days on the AIN-76A diet, after which 0.04% (w/w) PhIP was added to the diet of all animals. After 14 days of PhIP feeding, 8 rats were killed (day 0). Twelve rats were put on 0.1% (w/w) I3C-containing diet while the remaining 12 were put back on AIN-76A control diet. At 7, 14, and 28 days after the change of diet, animals (4 per group) were killed. Blood, mammary glands, colon, and liver were collected as for experiment 1.

Tissue Processing and DNA Isolation

Whole blood (4–6 ml) was mixed with 45 ml lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, 0.04% tetrasodium EDTA, pH 7.3) for ~8 min. White blood cells (WBCs) were pelleted by centrifugation (3,000*g* for 5 min.). The supernatant was removed, the WBC pellet was resuspended in 2 ml nuclei lysis buffer (10 mM Tris-HCl, 400 mM sodium chloride, 2 mM tetrasodium EDTA, pH 8.2), and stored at −70°C until isolation of DNA.

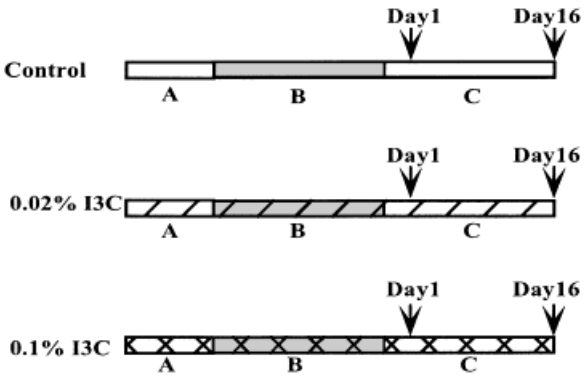

Fig. 1. Protocol of experiment 1. Animals were given control AIN-76A diet without I3C (▭), with 0.02% (w/w) I3C (▨), or with 0.1% (w/w) I3C (▩) for the entire duration of the experiment. An initial 2-week feeding period (period A) was followed by a 4-week period of 0.04% (w/w) PhIP in the diet (▬, period B), and a 16-day period after removal of PhIP from the diet (period C). Animals received fresh food every third day. Groups of 4 rats were killed on days 1 and 16 of period C.

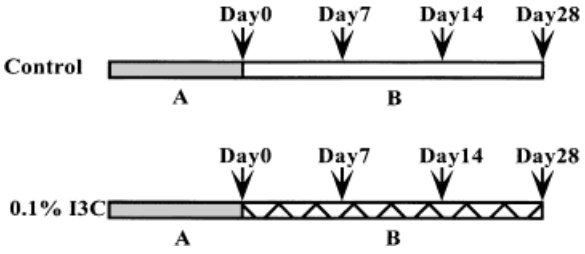

Fig. 2. Protocol of experiment 2. After an acclimation period of 8 days on control AIN-76A diet, all animals were given 0.04% (w/w) PhIP in the diet for 2 weeks (▬), (period A), followed by a 28-day period (period B) in which animals were separated into a control group without I3C in the diet (▭), and an experimental group with 0.1% I3C (w/w) in the diet (▩). Animals were given fresh food every third day. Groups of 4 rats were killed on the last day of period A (day 0), and on days 7, 14, and 28 of period B.

The inguinal mammary glands from two rats were pooled to form one sample. The tissue was minced into small pieces (<2 mm^2) in 10 ml of phosphate-buffered saline and then digested with collagenase I and collagenase IV as described before [23]. After filtration and centrifugation [23], MECs were resuspended in 2 ml nuclei lysis buffer and stored at -70°C until isolation of DNA. Liver was minced into small pieces (<2 mm^2) and a random sample of $\sim$0.3 g was taken and homogenized in 5 ml nuclei lysis buffer. The colon was cut open longitudinally, rinsed free of its content, and the epithelial cells were scraped off and placed in 5 ml nuclei lysis buffer. DNA was isolated from WBCs, MECs, liver, and colon as described previously [33].

^{32}P-Postlabeling Assay for PhIP-DNA Adducts

Plastic-backed polyethyleneimine (PEI)-cellulose thin layer chromatography sheets were prepared in the laboratory [34] and [γ-^{32}P]ATP was prepared fresh for each assay [34]. As before [21,35], the intensification version (ATP-deficient) of the ^{32}P-postlabeling assay was used to isolate and quantitate PhIP-DNA adducts. In brief, DNA was first digested by micrococcal nuclease and spleen phosphodiesterase. T4 polynucleotide kinase was then used to catalyze the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP to the 5'-hydroxy of 3'-mononucleotides. To separate PhIP-adducted bisphosphates from normal nucleotide bisphosphates and to resolve individual adducts, ion-exchange chromatograph on PEI-cellulose thin layers was employed as described previously [21,35]. The solvents used in chromatography were: D1, 2.3 M sodium phosphate, pH 5.8; D3, 2.81 M lithium formate, 6.63 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris-HCl, 7.4 M urea, pH 8.0; D5, 1.0 M magnesium chloride.

^{32}P-Postlabeled PhIP-DNA adducts on the PEI-cellulose plates were located as radioactive spots by autoradiography at -70°C for varying time periods. They were cut out and then counted by Cerenkov counting [34]. Adduct levels were expressed as relative adduct labeling (RAL) values, calculated by dividing the c.p.m. in each adduct by the c.p.m. in normal nucleotides, after adjusting for dilution factors [21,35]. values obtained under intensification conditions were converted to RAL values (true RALs under ATP-saturating conditions) using the intensification factors for the individual PhIP-DNA adducts [21].

Statistical Analysis

The effect of diet on weight gain and PhIP-DNA adduct levels in organs/cells in experiment 1 was analyzed by one-way analysis of variance (ANOVA), while Fisher's Least Significant Difference test was used to test for differences between the individual diets. Student's *t*-test was used to examine the effect of time on adduct levels in experiment 1 and the effect of I3C on adduct levels and weight gain in experiment 2.

RESULTS

Starting with an average body weight of 76.3 g, animals on control diet, 0.02% I3C diet and

0.1% I3C diet in experiment 1 gained an average of 24.0, 22.1, and 20.5 g, respectively, during week 1. During week 2, these gains were 12.6, 15.1, and 15.6, respectively, while during weeks 3–6 (the 4 weeks of PhIP feeding, Fig. 1), weight gains averaged only 3.8–6.4 g, 6.4–9.0 g, and 7.0–11.6 g for animals on the control diet, 0.02% I3C diet, and 0.1% I3C diet, respectively. Weekly weight gains were not different ($P > 0.05$) between the three diets. In experiment 2, the average body weight gain during the 2-week PhIP-feeding period (Fig. 2) was 5.3 g/week.

PhIP-DNA adduct patterns were identical in all four tissue/cell types (Fig. 3). After correction for intensification factors, the distribution of adducts (% of total) was as follows: adduct 1, 48.6% (range 24.5–72.7%); adduct 2, 39.9% (range 27.3–57.4%); and adduct 3, 12.8% (range 0–27.3%). Minor adduct 4, which was not well separated from adduct 2 (Fig. 3), could not be detected under standard labeling conditions; it constituted 5.9% (range 0–9.4%) of the total of the intensified adducts. These distributions were independent of the type of diet (data not shown).

Adduct levels were highest in WBCs, followed by colon, MECs, and liver (Fig. 4). With the exception of those in MECs, adduct levels in WBCs, colon, and liver decreased significantly ($P < 0.05$) during the 16-day observation period. In animals on control diet, day 16 levels in WBCs, colon, and liver were 7.8, 47.0, and 26.3% of those on day 1; in animals on 0.02% I3C diet, the day 16 levels decreased to 23.4, 11.6, and 52.4%, respectively, while in animals on 0.1% I3C diet, day 16 adduct levels in WBCs, colon, and liver were 43.8, 25.5, and 45.4%, respectively, of those on day 1 (Fig. 4).

In many experimental systems testing the effectiveness of chemopreventive agents, the carcinogen is administered, often i.p., as a bolus dose. In the present experiment, we chose to provide both the carcinogen, PhIP, and the chemopreventive agent, I3C, at low levels in the diet in order to approximate more closely the manner and rate of human intake of these chemicals. I3C was provided not only simultaneously with PhIP, but also during a period of 2 weeks prior to exposure to PhIP (Fig. 1), so that any stimulatory or inhibitory effects of this agent on cellular processes would be stabilized before exposure to the carcinogen. Under these experimental conditions, 0.1% I3C in the diet strongly reduced PhIP-DNA adducts on day 1 in MECs (91.9% inhibition), WBCs (82.3% inhibition), colon (67.2% inhibition), and liver (69.2% inhibition) (Fig. 4). On day 16, this concentration of I3C was inhibitory only in the colon (82.2% inhibition, Fig. 4C) and liver (46.8% inhibition, Fig. 4D). Lowering the dietary I3C concentration to 0.02% resulted in significant inhibition only on day 16 in the colon (81.3% inhibition, Fig. 4C).

Susceptibility to experimental carcinogenesis may be related to the persistence of carcinogen-DNA adducts in the target organ [36]. Thus, the observed inhibition of PhIP-DNA adducts (Fig. 4) may have resulted from inhibition of adduct formation and/or acceleration of adduct removal. To examine the latter possibility, in experiment 2, 0.1% I3C was added to the diet after exposure to PhIP (Fig. 2), and PhIP-DNA adduct levels were followed over a period of 4 weeks. Analysis of PhIP-DNA adducts in MECs, WBCs, colon, and liver showed no significant acceleration of adduct removal due to I3C during the 28-day period following the cessation of exposure to dietary PhIP (Fig. 5).

DISCUSSION

PhIP-DNA adduct patterns were identical in all tissues and cells (Fig. 3), confirming our previous findings in the F344 rat [21,35]. We have previously identified adduct 1 (Fig. 3), which comprises approximately 50% of the total on average, as *N*-(deoxyguanosin-8-yl)-PhIP [22]. The identity of adducts 2, 3, and 4 (Fig. 3) is not known, but they may comprise dimers or other undigested oligomers containing a guanine-PhIP moiety [22,37,38].

The relatively high PhIP-DNA adduct levels in the colon and WBCs (Figs. 4 and 5) confirm our previous results obtained in the male F344 rat [21,35]. Since the colon of the male is much more sensitive to the carcinogenic action of PhIP than the female F344 rat [6], our present results show that PhIP-DNA adducts in target organs may not be influenced by sex, even though the target organ's sensitivity to PhIP is vastly different between males and females. It is likely that the relatively high level of PhIP-DNA adducts in WBCs is a reflection of relatively high concentrations of circulating *N*-hydroxy-PhIP. This is supported by our finding that, in the female rat, PhIP-DNA adducts in MECs probably arise from circulating *N*-hydroxy-PhIP of hepatic origin. MECs possess

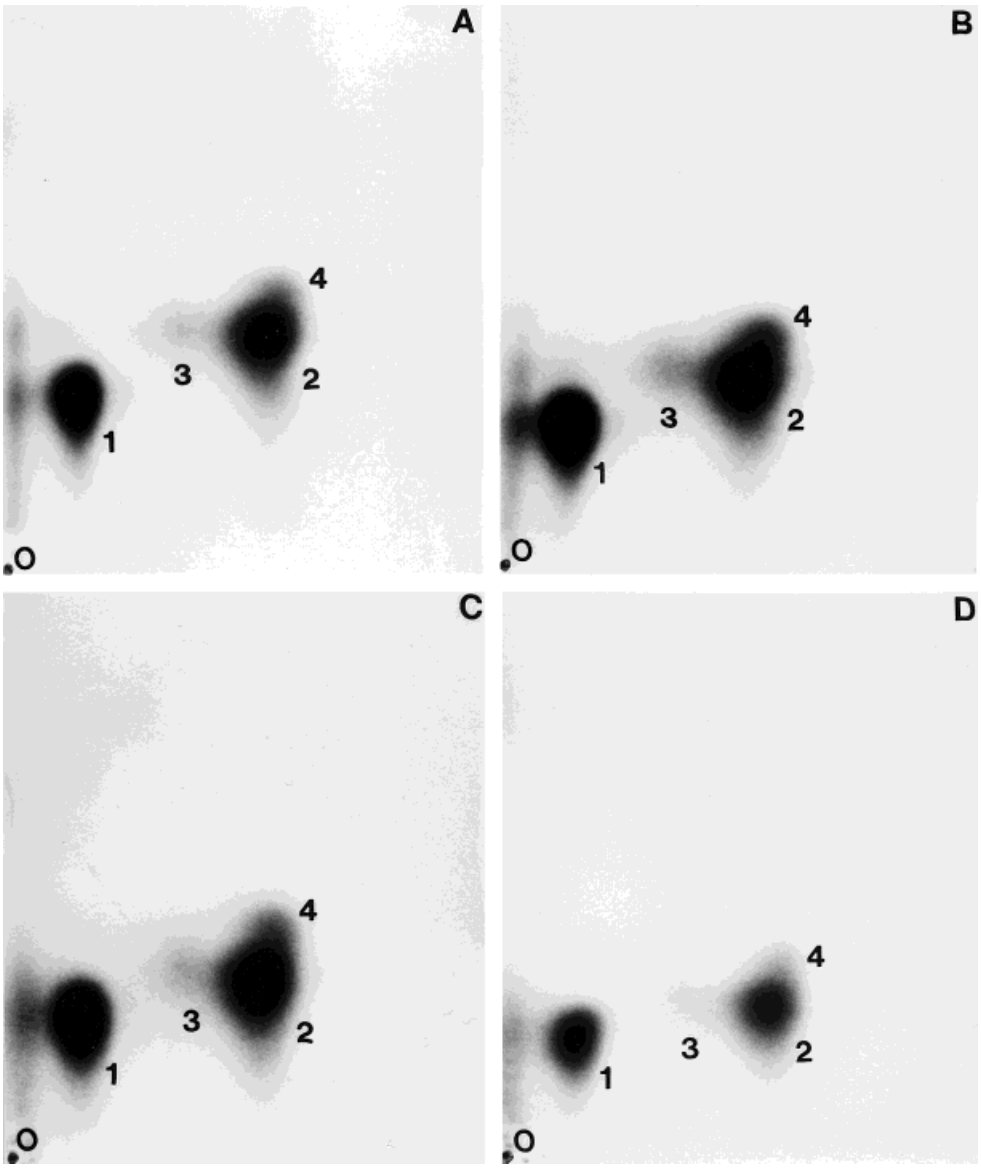

Fig. 3. PhIP-DNA adduct patterns, determined by ^{32}P-postlabeling, in mammary epithelial cells (**A**), white blood cells (**B**), colon (**C**), and liver (**D**) of female F344 rats after a 4-week period of feeding 0.04% (w/w) PhIP in the diet. ^{32}P-Postlabeling assays were run under intensification conditions as before [21,35]. Adduct 1 has been identified as *N*-(deoxyguanosin-8-yl)-PhIP [21,22]. O, origin.

relatively high *O*-acetyltransferase activity, which, after esterifying *N*-hydroxy-PhIP, produces an electrophile, probably the nitrenium ion, that reacts with MEC DNA [23]. Similarly, indirect evidence indicates that PhIP-DNA adducts in the colon are also formed after local esterification of *N*-hydroxy-PhIP of hepatic origin [39]. The relatively low levels of PhIP-DNA adducts in the liver (Figs. 4D and 5D) confirm our previous findings [35,40], and probably reflect efficient detoxification and/or rapid clearance of locally-formed *N*-hydroxy-PhIP.

The potent inhibition by I3C of PhIP-DNA adduct formation in MECs (Fig. 4A) confirms our previous results [41] and is in agreement with its inhibitory effect on the chemical induction of rat mammary carcinogenesis [25,28]. In inhibiting rat mammary tumors induced by DMBA, I3C probably acts on the initiation phase by inhibiting the activation of DMBA and/or enhancing its detoxification. The modulating effects of I3C on cytochromes P450 and phase II enzymes acting on a variety of carcinogenic substrates are well known [28,42–47]. Our pre-

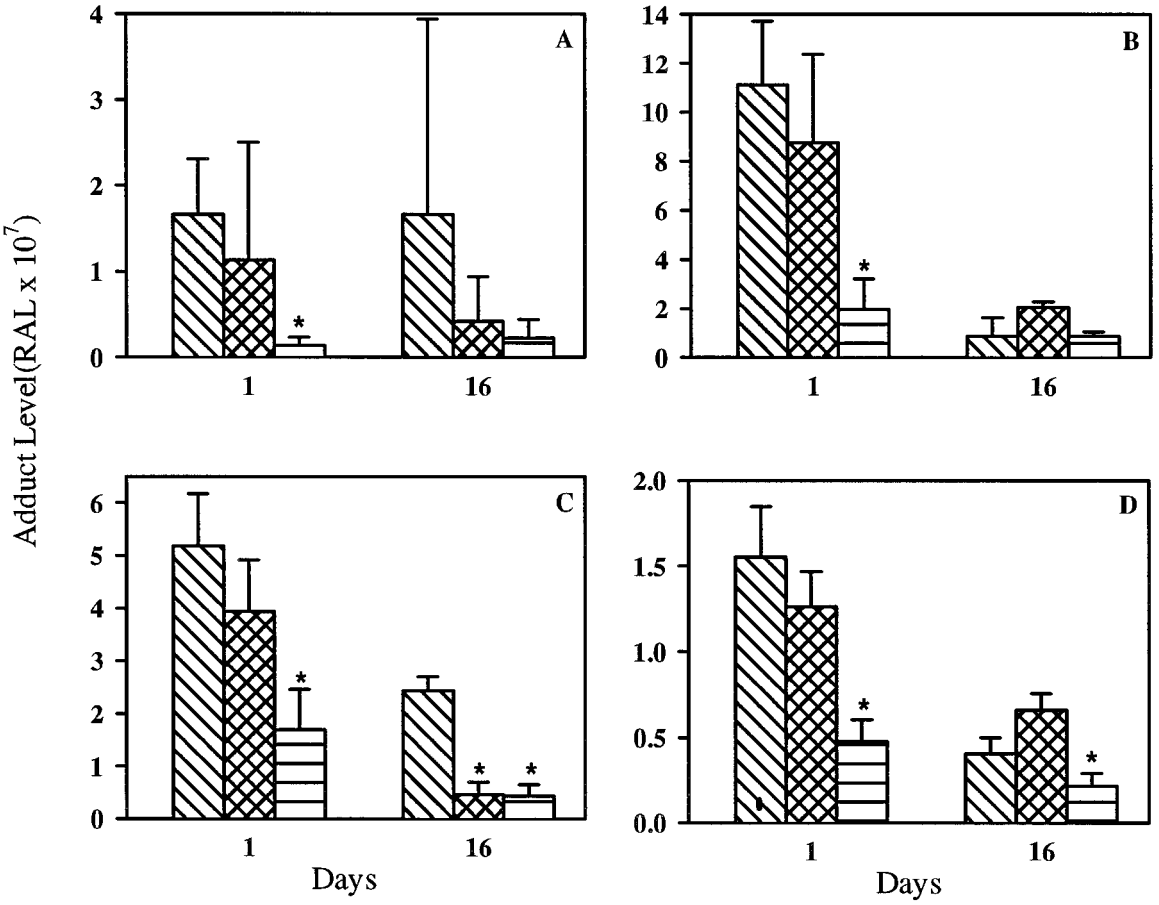

Fig. 4. Inhibition of PhIP-DNA adduct formation in mammary epithelial cells (**A**), white blood cells (**B**), colon (**C**), and liver (**D**) of female F344 rats, 1 or 16 days after a 4-week period of feeding 0.04% (w/w) PhIP in the diet. (▨), animals on control AIN-76A diet; (▩), animals on 0.02% (w/w) I3C diet; (▤), animals on 0.1% (w/w) I3C diet. RAL values are means ± SD; N = 4, except for A, where N = 2. Day 16 adduct levels were, with the exception of mammary epithelial cells (A), significantly lower ($P < 0.05$) than the corresponding day 1 adduct levels. The 0.1% I3C diet significantly decreased (*$P < 0.05$) day 1 adduct levels in all cases, but only in the colon (C) and liver (D) on day 16 (*$P < 0.05$). The 0.02% I3C diet did not significantly change adduct levels ($P > 0.05$) on either day 1 or day 16, except in the colon (C) on day 16 (*$P < 0.05$).

vious evidence suggests that I3C may act, in part, by enhancing the detoxification of PhIP in the male F344 rat [48].

I3C also inhibits rat mammary tumors induced by the direct-acting carcinogen *N*-methylnitrosourea (MNU) [28], which forms methylated DNA adducts. Our current results (Fig. 5) imply that in this case, I3C does not act by accelerating adduct removal, i.e., by induction of repair enzymes. Instead, it has been suggested [28] that, in this model, I3C causes an anti-estrogenic effect that comes about by increased cytochrome P450 1A2-mediated 2-hydroxylation of estradiol. I3C may also exert anti-estrogenic effects through other mechanisms [49], influencing the promotional/proliferative phase of chemically-induced rat mammary carcinogenesis.

Adducts in MECs of rats receiving 0.04% dietary PhIP for 4 weeks (Fig. 4A) were much lower than those in rats receiving this dietary regimen for 2 weeks (Fig. 5A). In WBCs, colon, and liver, such a difference was absent (Figs. 4 and 5). Because 0.04% dietary PhIP for 4 weeks was toxic, as judged by decreased weekly gains in body weight (see Results), it seems plausible that PhIP toxicity in MECs after this period results in lower adduct levels in these cells. Thus, MECs could be more sensitive in this respect than are WBCs, liver cells, or colon epithelial cells. Shortening the PhIP-feeding period to 2 weeks (Fig. 2) would result in lower

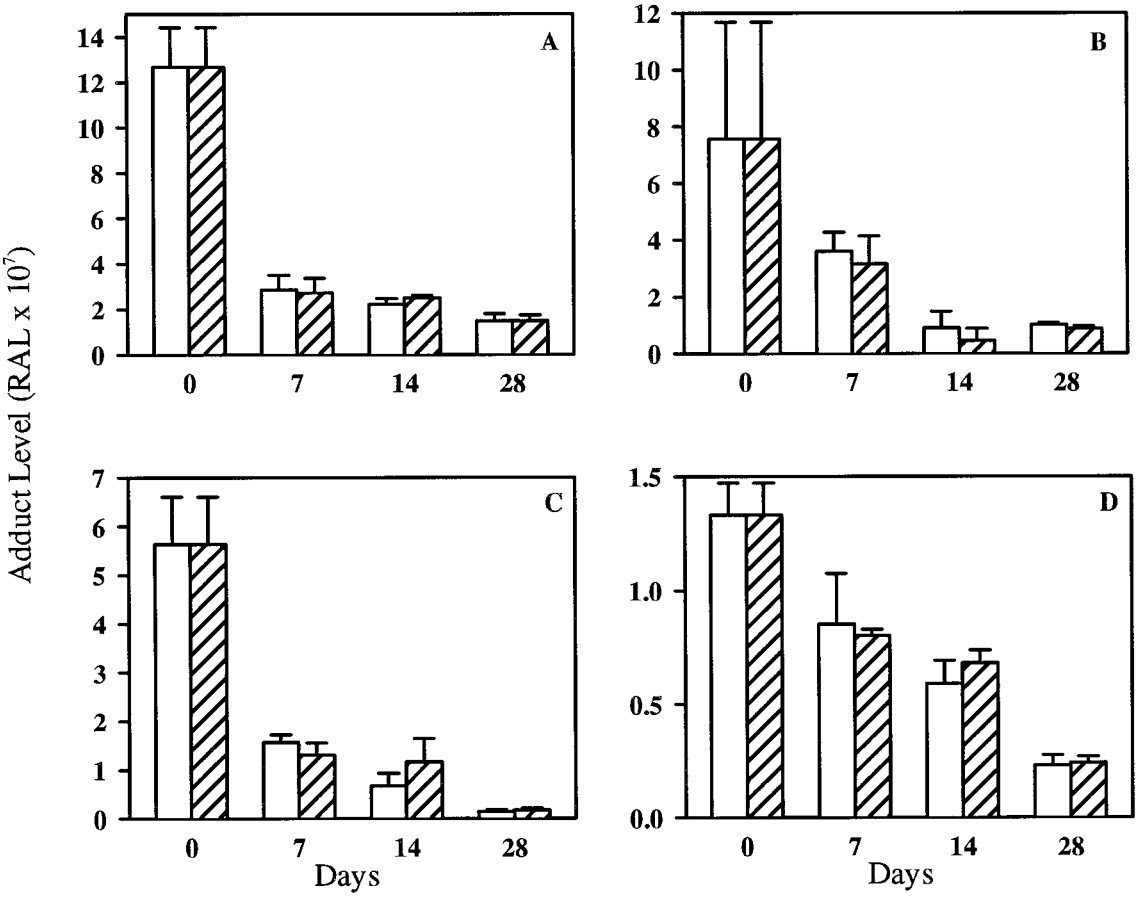

Fig. 5. PhIP-DNA adduct levels in mammary epithelial cells (**A**), white blood cells (**B**), colon (**C**), and liver (**D**) of female F344 rats 0–28 days after a 2-week period of feeding 0.04% (w/w) PhIP in the diet. (□), animals on control AIN-76A diet; (▨), animals on 0.1% (w/w) I3C diet. RAL values are means ± SD; N = 4, except for A, where N = 2. Adduct levels in animals on 0.1% I3C diet were not significantly different ($P > 0.05$) from those in animals on control diet at any of the 4 time points.

toxicity to MECs, as manifested by higher PhIP-DNA adduct levels than after 4 weeks of PhIP-containing diet. A toxic effect of PhIP in MECs is supported by the lack of repair of PhIP-DNA adducts in MECs after the 4-week PhIP-feeding period (Fig. 4A), as opposed to clear evidence of repair after the 2-week PhIP-feeding period (Fig. 5A). The rates of repair of PhIP-DNA adducts in liver and colon (Figs. 4C,D, 5C,D) are relatively slow when compared to those observed after a single oral bolus (50 mg/kg) of PhIP [35], and confirm similar results reported previously [50].

The inhibition of PhIP-DNA adduct formation in WBCs, liver, and colon, after giving 0.1% I3C in the diet before and during the feeding of PhIP (Fig. 4), confirms previous observations in the male F344 rat [48]. In the latter study [48], I3C almost completely prevented the PhIP-induced formation of aberrant crypt foci, which are putative premalignant lesions in the colon. Similarly, I3C also inhibited both DNA adduct formation and aberrant crypt foci induced by the related heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline [49].

I3C appears to have multiple positive attributes as a chemopreventive agent. It is active against both indirect- and direct-acting carcinogens [28] as well as a variety of experimental tumors, regardless of the carcinogenic agent used [25–31]; it modulates both phase I and phase II enzymes involved in carcinogen metabolism [42–47,49]; and it is active at low dietary concentrations [48–50]. When given only during the post-initiation phase of experimental carcinogenesis, however, I3C promotes tumor formation in certain systems [51,52]. These combined results show that for maximum ben-

eficiary effects, I3C should be given before, as well as during, the entire period of tumor induction.

The low toxicity of I3C in experimental systems, even after prolonged high oral doses [28], combined with its lack of toxic or untoward effects in humans [53] and its potential chemopreventive effect in smokers [54], make I3C an ideal candidate chemopreventive agent in humans. The recent demonstration of reversal of experimental multidrug resistance by I3C [55] indicates that I3C may also be beneficial in the area of cancer treatment.

ACKNOWLEDGMENTS

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