Conjugated linoleic acid inhibits mutagenesis by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in the prostate of Big Blue® rats

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Introduction

Prostate cancer is the second leading cause of cancer deaths for men in the USA, with African-Americans having the highest rate in the world, immediately followed by white Americans (Pienta and Esper, 1993). The etiology of human prostate cancer is essentially unknown, although certain dietary factors, such as the consumption of red meat and saturated fat, have been implicated in this disease (Kolonel, 1996). The identification of mutagenic and carcinogenic heterocyclic amines (HCAs) in cooked meat has raised the possibility that these dietary HCAs may play a role in the etiology of human prostate cancer.

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a pyrolysis product in meat and fish and is currently recognized as the principal mutagenic HCA in the human diet (Layton et al., 1995). The finding of PhIP in the urine of healthy volunteers eating a normal diet and that of PhIP-related DNA adducts in the urine of smokers of black tobacco suggests that humans are continuously exposed to PhIP (Peluso et al., 1991; Lynch et al., 1992; Felton et al., 1997). The possible association of PhIP with several dietary-related cancers, including those of mammary gland, colon and prostate, has attracted increasing interest, especially after the carcinogenicity of PhIP was demonstrated in rodent models, in which colon and prostate tumors were predominately induced in male rats (Ito et al., 1991; Shirai et al., 1999) and mammary gland tumors in female rats (Ito et al., 1991).

Similar to many chemical carcinogens, PhIP requires metabolic activation for DNA adduct formation and genotoxicity. PhIP is first metabolized to N-hydroxy-PhIP by cytochrome P4501A2 in the liver and is then further activated to reactive ester derivatives by phase II esterification enzymes in the liver or the target tissues (Wallin et al., 1990; Alexander et al., 1995). The major PhIP–DNA adduct has been identified as N-(deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP) (Lin et al., 1992), while others remain uncharacterized. Mutational events generated by PhIP have been observed primarily at G:C base pairs, consistent with the predominant formation of PhIP–DNA adducts at guanines (Carothers et al., 1994; Morgenthaler and Holzhauser, 1995). In the lacI gene of Big Blue® transgenic rats, G:C→T:A transversions and –1 frameshifts of G:C base pairs are the primary mutational classes induced by PhIP, followed by G:C→C:G and G:C→A:T transitions (Okonogi et al., 1997).

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid. The major dietary source of CLA is animal food, especially foods derived from ruminant animals (e.g. dairy products and beef) (Ip et al., 1994a). CLA has shown remarkable cancer prevention properties, including inhibiting chemically induced carcinogenesis (Ip et al., 1994b), inhibiting the growth of transplanted tumors (Visonneau et al., 1997) and suppressing the growth of cancer cells (Durgam and Fernandes, 1997). In a recent review paper, Pariza et al. (1999) proposed that CLA may influence the development and progression of cancer in three ways: (i) by directly affecting the process of carcinogenesis, including inhibition of the activation of carcinogens (Liew et al., 1995; Josyula and Schut, 1998), inhibition of cell proliferation (Shultz et al., 1992) and decreasing the formation of arachidonic acid by competitive inhibition of linoleic acid (LA) metabolism (Banni et al., 1999); (ii) by reducing excessive body fat accumulation, which indirectly influences cancer risk; (iii) by reducing cachexia, which is associated with advanced cancer and with certain cancer treatment strategies. The effective concentration of CLA is 0.1–1% of the diet (Ip et al., 1991, 1994b), representing concentrations close to human
consumption levels. This may imply that CLA has direct implications for human health.

In a previous study we showed that CLA inhibited PhIP-induced mutagenesis in the distal colon of Big Blue® rats (Yang et al., 2001). In this paper we have examined the modification of PhIP-induced mutagenesis by CLA in the prostate.

Materials and methods

Chemicals

PhIP (>98% pure) was obtained from Toronto Research Chemicals (Toronto, Canada). CLA (>94% pure, composition = 41% cis-9, trans-11 CLA, = 44% trans-11, cis-9 CLA, = 14% cis-10,12 CLA, = 5% cis-9,12 linoleate) was purchased from Nu-Chek-Prep (Elyria, MN). Tocopherol-stripped corn oil was from ICN (Costa Mesa, CA).

Treatment of rats

Male and female F344 Big Blue® rats (10 weeks old) obtained from Stratagene (La Jolla, CA) were used for in-house breeding. The offspring male rats were weaned at 21 days old and were given the powdered basal diet AIN-93G without the antioxidant t-butylhydroquinone (Dyets Inc., Bethlehem, PA). Food and water were provided ad libitum. The rats were weighed weekly and the food consumed was measured twice weekly. Food intake and animal weight were described in Yang et al. (2002). The rats were divided into five groups consisting of 4–6 rats each, with the following supplementation to the basal diet: (1) none; (2) corn oil; (3) corn oil + PhIP; (4) corn oil + CLA; (5) corn oil + PhIP + CLA. The number of animals per group was chosen as recommended by a statistical analysis of mutation studies in transgenic mice by Carr and Gorelick (1995). When the rats were 50 days old, PhIP (100 p.p.m.) was incorporated into the diet. CLA (1% w/w) was added 1 week prior to supplementation with PhIP and continued until the end of PhIP treatment. Corn oil (2% w/w) was incorporated into the powdered diet of groups 2–5 during the PhIP exposure period to reduce the formation of dust. After 47 days PhIP treatment the rats were returned to the basal diet and killed 1 week later. Prostate tissues, including the ventral and dorsolateral lobes, were removed immediately, frozen in liquid nitrogen and stored at −80°C until DNA isolation.

lacI mutational assay and statistical analyses

High molecular weight genomic DNA was isolated as previously described (Yang et al., 2001). The genomic DNA was added to a λ phage packaging extract (Transpack; Stratagene) and the resulting phages were plated on Escherichia coli SCS-8 bacteria on trays of NZY medium containing 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside. The mutant frequency was determined by the ratio of the number of blue mutant plaques to the total number of plaques. The lacI gene from mutants was amplified by PCR (Erle et al., 1996), purified using a Qiagen column and sequenced on a LICOR automated fluorescence sequencer, thus establishing a mutational spectrum (MS) for each treatment.

Mutation frequencies (MFs) were compared using the computer program COCHARM (Procter and Gamble, Cincinnati, OH) that executes the Generalized Cochran–Armitage test (Carr and Gorelick, 1995). Statistical comparisons of MS were made using the Monte Carlo method of Adams and Skopek (1987). The comparison of individual mutational classes was performed by a statistical analysis of mutation studies in transgenic mice by Carr and Gorelick (1995). The comparison of individual mutation classes was performed by a statistical analysis of mutation studies in transgenic mice by Carr and Gorelick (1995).

Results

Mutation frequencies

We recovered mutants from the entire prostate as the tissues are too small to isolate sufficient DNA from the ventral and dorsolateral lobes individually. The MFs of the lacI gene in the prostate tissue from various treatment groups are summarized in Table I. The spontaneous MF in the prostate was not significantly different from the MF of the corn oil- or CLA-supplemented control group (P > 0.6). Supplementation with 100 p.p.m. PhIP for 47 days induced a lacI MF of 14.6 ± 3.2 × 10⁻³, a 5.1-fold increase over the control group (2.4 × 10⁻⁵). Addition of CLA to the PhIP-containing diet resulted in a MF of 9.1 ± 1.2 × 10⁻³, significantly decreasing the PhIP-induced MF by 38% (P = 0.03).

Mutation spectra

Randomly selected lacI mutants recovered from each prostate sample were sequenced to establish the individual MS for each treatment (Table II). A total of 65, 32, 41 and 97 independent mutants (identical mutations occurring at a single nucleotide position in the same organ of a single rat were scored only once) were identified from the spontaneous, control (corn oil), CLA, PhIP and PhIP + CLA groups, respectively. The predominant spontaneous mutation was G:C→A:T transition, followed by G:C→T:A transversion and by deletion in descending order. A significantly different MS (P < 0.01) was obtained when the rats were supplemented with corn oil, in which −1 frameshifts were recovered as a surprisingly high proportion (20.9%), similar to those of G:C→T:A transversions and G:C→A:T transitions. In the CLA treatment group similar proportions of G:C→A:T transitions, G:C→T:A transversions and −1 frameshifts were seen. The MS after CLA exposure was not significantly different from the control nor from the spontaneous MS. The PhIP-induced MS was significantly different from the control MS and from the spontaneous MS. The leading class of mutation induced by PhIP was −1 frameshifts (51.4%), followed by G:C→T:A transversions (17.6%) and G:C→A:T transitions (12%). CLA supplementation significantly changed the PhIP-induced MS (P = 0.016), with −1 frameshifts (38.1%) and G:C→T:A transversions (29.9%) as the major mutational types, followed by deletions (13.4%) and G:C→A:T transitions (10.3%).

The frequencies of the major classes of mutations were calculated and are presented in Figure 1. The addition of CLA resulted in a 50% decrease in the frequency of G:C→A:T transitions in the control group as well as the frequencies of G:C→A:T transitions and −1 frameshifts in the PhIP-treated group.

Discussion

The effect of CLA on DNA adduct formation and on carcinogenesis in the mammary gland, colon and liver in rodents (especially in female rats) has been extensively studied (Josyula and Schut, 1998; Josyula et al., 1998; Snyderwine et al., 1998) while research on the prostate is relatively rare. The first in vivo study on the inhibitory effect of CLA on prostate tumors was performed by Cesano et al. (1998), who demonstrated opposite effects of CLA and linoleic acid on inoculated human prostatic cancer cells in severe combined immunodeficient mice. Addition of CLA to the diet reduced local prostatic tumor growth and systemic spread of the prostatic tumor. Although there is no direct evidence showing that CLA is protective against human prostate cancer, epidemiological studies do suggest that the non-fat portion of milk is associated with an increased risk of prostate cancer (Grant, 1999). Since CLA is a fatty acid in the fat of milk, the promotional effect of non-fat milk may be related to the absence of CLA. The effect of CLA on chemically induced mutagenesis/carcinogenesis in the prostate has not been reported hitherto. Results from the present investigation show for the first time that CLA protects against mutagenesis induced by a dietary HCA in the prostate.

The daily food consumption and growth of experimental animals were dependent on the dietary supplementation and were previously described in Yang et al. (2002). PhIP appears to be toxic, as was evident from the lower food intake and slower gain in body weight. CLA has no obvious effect on animal growth and food intake. Thus we may conclude that...
Linoleic acid inhibits mutagenesis by PhIP

Table I. Mutation frequencies in the prostates of Big Blue® rats with different dietary supplementation

<table>
<thead>
<tr>
<th>Group</th>
<th>Supplementation</th>
<th>No. of rats</th>
<th>Total p.f.u.</th>
<th>MF (mean ± SD) (×10⁻⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous</td>
<td>None</td>
<td>6</td>
<td>3 049 437</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Control</td>
<td>Corn oil</td>
<td>4</td>
<td>1 434 840</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>CLA</td>
<td>CLA and corn oil</td>
<td>5</td>
<td>2 680 211</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>PhIP</td>
<td>PhIP and corn oil</td>
<td>5</td>
<td>2 390 060</td>
<td>14.6 ± 3.2²</td>
</tr>
<tr>
<td>PhIP + CLA</td>
<td>PhIP, CLA and corn oil</td>
<td>5</td>
<td>1 878 600</td>
<td>9.1 ± 1.2²</td>
</tr>
</tbody>
</table>

Big Blue® rats were exposed to different supplements as indicated in the table. PhIP (100 p.p.m.) was incorporated in the diet for 47 days. Corn oil was mixed into the diet during the period of PhIP exposure. CLA (1% w/w) was added 1 week prior to supplementation with PhIP and continued until the end of PhIP treatment. The lacI MF of each group was determined by the standard Big Blue® transgenic assay.

Table II. Mutation spectra recovered from the prostate of Big Blue® transgenenic rats after various treatments

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Spontaneous (65)a</th>
<th>Controlb (43)</th>
<th>CLA (41)</th>
<th>PhIP,c,d (142)</th>
<th>PhIP + CLA c,d (97)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G:C→A:T</td>
<td>35.4</td>
<td>32.6</td>
<td>22.2</td>
<td>12.0</td>
<td>10.3</td>
</tr>
<tr>
<td>A:T→G:C</td>
<td>4.6</td>
<td>2.3</td>
<td>9.8</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>G:C→T:A</td>
<td>20.0</td>
<td>25.6</td>
<td>19.5</td>
<td>17.6</td>
<td>29.9</td>
</tr>
<tr>
<td>G:C→C:G</td>
<td>4.6</td>
<td>0.0</td>
<td>0.0</td>
<td>5.6</td>
<td>1.0</td>
</tr>
<tr>
<td>A:T→T:A</td>
<td>1.5</td>
<td>4.7</td>
<td>4.9</td>
<td>2.1</td>
<td>0.0</td>
</tr>
<tr>
<td>A:T→C:G</td>
<td>3.1</td>
<td>4.7</td>
<td>7.3</td>
<td>0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>+1 frameshifts</td>
<td>7.7</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>–1 frameshifts</td>
<td>9.2</td>
<td>20.9</td>
<td>19.5</td>
<td>51.4</td>
<td>38.1</td>
</tr>
<tr>
<td>A:T deletions</td>
<td>4.6</td>
<td>7.0</td>
<td>9.8</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>G:C deletions</td>
<td>4.6</td>
<td>14.9</td>
<td>9.8</td>
<td>50.0</td>
<td>37.1</td>
</tr>
<tr>
<td>Deletions</td>
<td>12.3</td>
<td>2.3</td>
<td>7.3</td>
<td>5.6</td>
<td>13.4</td>
</tr>
<tr>
<td>Insertions</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Complex changes</td>
<td>1.5</td>
<td>0.0</td>
<td>2.4</td>
<td>2.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Double mutants</td>
<td>0.0</td>
<td>7.0</td>
<td>4.9</td>
<td>0.7</td>
<td>2.1</td>
</tr>
</tbody>
</table>

aThe number of independent mutants.
bSignificantly different from the spontaneous MS (P < 0.05).
cSignificantly different from the control MS (P < 0.001).
dSignificantly different from the CLA-induced MS (P < 0.01).
eSignificantly different from the PhIP-induced MS (P < 0.007).

Fig. 1. The frequencies of several classes of mutations recovered from different treatments.

any differences in mutation between PhIP- and PhIP + CLA-treated animals are not the result of an effect of CLA on PhIP intake.

PhIP induced a 5.1-fold increase in MF in the prostate, confirming the finding by Stuart et al. (2000) that PhIP is mutagenic in the prostate. Two factors directly contribute to the induction of mutations by PhIP: formation of DNA adducts and elevation of DNA synthesis in the epithelial cells in the prostate (Shirai et al., 1997). Purewal et al. (2000) reported that supplementation with 100 p.p.m. PhIP for 1 week induced adduct levels of 3.39 ± 0.25×10⁻⁷, 1.98 ± 0.59×10⁻⁷ and 0.46 ± 0.10×10⁻⁷ in the prostate, colon, and liver, respectively. The higher level of PhIP–DNA adducts in the prostate may be related to its capacity to N-hydroxylate PhIP. Unlike the mammary gland and colon, which showed a low capacity to metabolically activate PhIP (Fan et al., 1995; Malfatti et al., 1996), the prostate was found to have a relatively high level of cytochrome P4501A2 and thus is able to convert PhIP to its hydroxy form. Although the formation of DNA adducts is considered an initiation event of mutagenesis and carcinogenesis, the level of PhIP–DNA adducts does not reflect the tissue-specific mutagenicity or carcinogenicity of PhIP. A lower MF and fewer tumors were found in the prostate than in the colon after exposure to the same PhIP doses (Hasegawa et al., 1993). Clearly, factors other than the number of PhIP adducts take part in determining the susceptibility to the mutagenicity/carcinogenicity of PhIP. One possibility is different rates of cell proliferation. Results from the proliferating cell nuclear antigen (PCNA) assay showed that in the colon the PCNA labeling index was ~20% while in the prostate it was only ~0.87–2.69% (Yao et al., 1998), suggesting a much higher cell division rate in the colon. Using immunohistochemistry, Takahashi et al. (1998) measured the kinetics of PhIP–DNA adducts in various tissues after a single dose of PhIP. PhIP–DNA adducts in the prostate appeared to be removed more slowly than those in the colon, which may be
indicative of faster cell division in the colon compared with the prostate after PhIP treatment.

The spontaneous MS in the prostate is similar to that in the liver (de Boer et al., 1996). However, the MS from the control animals (basal diet supplemented with corn oil) were significantly different from that recovered from animals on the basal diet alone. A surprisingly high proportion of –1 frameshifts (20.9%) was recovered from the prostate samples of these rats. In a previous study by Stuart et al. (2000), –1 frameshifts were only recovered at 9% of total mutations from the prostates of control rats. However, the sample size (11 mutants) in that study was very small. The high percentage of –1 frameshifts in the control samples may be due to the supplementation with corn oil, but the mechanism is unknown. The high prevalence of –1 frameshifts was not observed in the colon or cecum of these rats, in which only 8.6–11.6% (Yang et al., 2002) of the lacI mutants were identified as –1 frameshifts. It is therefore possible that the formation of –1 frameshifts is tissue specific.

A high percentage of –1 frameshifts was induced by PhIP, as expected. PhIP has been reported to induce –1 frameshifts, specifically G:C deletions, in the target tissues of rodents (Kakiuchi et al., 1995; Okonogi et al., 1997). In the current study 71 of the 73 –1 frameshifts are G:C deletions, significantly different from that observed in control samples, in which the frequency of A:T deletions is ~50% of the frequency of G:C deletions. This observation confirmed that guanine is the primary binding target of PhIP metabolites (Lin et al., 1992; Endo et al., 1995).

The PhIP-induced spectrum in the current study was then compared with that reported by Stuart et al. (2000). Significant differences exist between the two spectra; notably, a higher proportion of –1 frameshifts and a lower proportion of G:C→T:A transversions were recovered in the current study. The differences may possibly be due to different PhIP doses. The frequencies of –1 frameshifts and G:C→T:A transversions almost doubled when the total PhIP intake increased from 0.071 to 0.183 g/rat (PhIP intake was calculated based on the observation that the average daily food intake of male rats is 15 g/rat), while insignificant increases in the MF of G:C→C:G and G:C→A:T transitions and deletions were observed. This observation is compatible with the preferential induction of –1 frameshifts and G:C→T:A transversions by PhIP and is also indicative of a dose-dependent effect of PhIP on the induction of MS.

In the current study CLA was found to inhibit prostate mutagenesis induced by PhIP. CLA has been reported to inhibit the formation of PhIP→DNA and IQ→DNA adducts in tumor target organs such as colon (Josyula and Schut, 1998). It is, therefore, possible that CLA inhibits the formation of PhIP→DNA adducts in the prostate. On the other hand, PhIP was also observed to induce oxidative DNA damage in the mammary gland (El Bayoumy et al., 2000). CLA is an antioxidant, inhibiting the oxidation of linoleic acid more efficiently than α-tocopherol (Ha et al., 1990). Thus, the inhibition of PhIP-induced mutagenesis by CLA may result from inhibition of the formation of PhIP→DNA adducts or of oxidative DNA damage. The inhibition of one or several specific DNA adducts by CLA would subsequently alter the MS induced by PhIP. Indeed, significant differences exist between the MS in the prostate recovered from PhIP- and PhIP + CLA-treated rats. Comparison of the MS from the PhIP- and PhIP + CLA-treated groups using Fisher’s exact test revealed that the PhIP + CLA treatment group had a significantly higher percentage of G:C→T:A transversions, as compared with the PhIP-treated group (P = 0.0068). However, when the MF is considered, the frequencies of G:C→A:T transitions and –1 frameshifts were selectively decreased by the addition of CLA (Figure 1). Interestingly, this reduction in the frequencies of these two classes of mutations by CLA is opposite to the effect of loss of function of the mismatch repair (MMR) gene MSH2 on spontaneous and PhIP-induced mutagenesis (Zhang et al., 2001), as determined in a MSH2 knockout lacI transgenic mouse model. The similar effects of CLA and MMR suggest that the anti-mutagenic effect of CLA in the prostate may be due to an enhancement of MMR. Some bulky aromatic amine adducts at the C8 position of guanine are bound by the mismatch recognition complex hMutSα (hMSH2/hMSH6) (Li et al., 1996), implicating MMR in the processing of such damage. The MMR gene hMSH2 was found to be mutated in the human prostate cancer cell line LNCaP (Leach et al., 2000), suggesting a possible association of impairment of the MMR pathway with prostate cancer occurrence and, vice versa, that an enhancement of MMR may reduce prostate carcinogenesis. The involvement of MMR in the prevention of prostate mutagenesis by PhIP should be further addressed.

Since CLA significantly changed the PhIP-induced MS, further analyses were performed to examine whether CLA has any effect on the induction of the fingerprint mutation of PhIP, a deletion of G:C base pairs at 5′-GGGA-3′ sequences. This deletion has been considered as the mutational fingerprint of PhIP and has been found to occur at a frequency of 3–10% of total mutations induced by PhIP (Nagao, 1999). This characteristic G:C deletion was identified in the tumor suppressor gene adenomatous polyposis coli in PhIP-induced tumors and in sporadic human colorectal cancer (Huang et al., 1996). In the prostate this fingerprint mutation constituted 9.9% (14/142) of the total mutations induced by PhIP. Of the 97 mutants from the PhIP + CLA treatment group, nine G:C deletions at 5′-GGGA-3′ were identified. The recovery of G:C deletions at 5′-GGGA-3′ sequences from PhIP- and PhIP + CLA-treated rats was similar, as judged by Fisher’s exact test. The G:C deletions at other sequences were also compared and no sequence-preferential inhibition was detected with CLA supplementation (P > 0.6).

In conclusion, we have shown that CLA inhibits PhIP-induced mutagenesis in the rat prostate, which suggests that CLA inhibits the initiation of PhIP-induced prostate carcinogenesis. The alteration in control and PhIP-induced MS by CLA may reflect an influence of CLA on the formation and removal of oxidative DNA damage and PhIP–DNA adducts. Two classes of mutations, G:C→A:T transitions and –1 frameshifts, were selectively inhibited by CLA, possibly via an enhancement of mismatch repair.

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References


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