Dietary Copper Affects Azoxymethane-Induced Intestinal Tumor Formation and Protein Kinase C Isozyme Protein and mRNA Expression in Colon of Rats\textsuperscript{1,2}

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ABSTRACT Previous studies have shown that changes in protein kinase C (PKC) isozyme expression may be related to increased susceptibility of copper-deficient rats to aberrant crypt formation. The purpose of this study was to determine whether dietary copper would affect azoxymethane-induced intestinal tumor formation and PKC isozyme expression in normal colonic mucosa and tumor samples. Eighty weaning Fischer-344 rats were randomly assigned to diets that contained either 0.8 or 5.3 \( \mu \text{g Cu/g diet} \). After 24 and 31 d of diet consumption, 30 rats/diet were administered azoxymethane (15 mg/kg i.p.) and 10 rats/diet were administered saline. Rats continued to consume their respective diets for an additional 38 wk. Rats injected with azoxymethane and fed the low copper diet had a significantly (\( P < 0.0001 \)) greater small intestinal and total tumor incidence compared with rats fed adequate dietary copper. However, dietary copper did not affect colon tumor incidence. Low dietary copper significantly (\( P < 0.004 \)) decreased PKC \( \alpha \) protein expression in normal but not in tumor tissue. In contrast, low dietary copper did not affect PKC \( \delta \) or \( \zeta \) protein expression in either the normal or tumor tissue. PKC \( \alpha \) and \( \delta \) protein and mRNA expression were lower in tumor tissue than in normal tissue. These results along with previous observations suggest that dietary copper-mediated changes in PKC \( \alpha \), \( \delta \) and \( \zeta \) protein expression are not as important for colon tumor promotion/progression as they are for tumor initiation. J. Nutr. 132: 1018-1025, 2002.

KEY WORDS: • colon cancer • protein kinase C • copper • rats

Colon cancer is the third most common newly diagnosed cancer in the United States and the third most common cause of cancer-related deaths (1). Approximately 130,000 people in the United States were diagnosed with colon cancer in 1999 (1). Diet is the single greatest contributor to human cancer, including colon cancer, and may be associated with 35–70\% of the incidence of the disease (2). Although various carcinogens are present in foods, their effects are minor compared with dietary components that inhibit the cancer process. One possible dietary factor that may increase the susceptibility to colon cancer is inadequate dietary copper. Recent studies (3,4) have shown that ingestion of a diet low in copper significantly increased the formation of 3,2'-dimethyl-4-aminobiphenyl and dimethylyhydrazine-induced aberrant crypt foci in rats. Aberrant crypt foci are preneoplastic lesions that have been detected in human colon resections and in experimental rats treated with chemical carcinogens (5,6). Two studies have shown that copper deficiency increased the incidence of chemically induced colon cancer in rats (7,8). Furthermore, a recent study has shown that copper deficiency significantly increased the small intestine tumor incidence in Min mice, a genetic model for human colon cancer susceptibility (9). Thus, low dietary copper may be a potential risk factor for colon cancer in humans.

We have recently shown that changes in protein kinase C (PKC)\textsuperscript{4} isozyme protein concentration may be related to increased susceptibility of copper-deficient rats to colon cancer (4). PKC constitutes a family of serine/threonine protein kinases that play central roles in transmembrane-signaling events and are involved in diverse biological processes, including cellular proliferation and differentiation. We observed that 1 wk after the second dose of dimethylyhydrazine (DMH), PKC \( \alpha \), \( \delta \) and \( \zeta \) protein content was significantly reduced in rats fed low dietary copper, compared with rats fed adequate dietary copper. There is extensive evidence that signals mediated via PKC serve to regulate colonic tumor development, possibly by influencing the actions of various growth factors and onco-genes (10–12). Several laboratories, utilizing DMH or its more proximate metabolite, azoxymethane (AOM), have demonstrated multiple biochemical changes in cellular signaling in both premalignant and malignant colonocytes (13,14). Studies in humans as well as in experimental animals have shown that

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\textsuperscript{4} Abbreviations used: AOM, azoxymethane; DMH, dimethlyhydrazine; PKC, protein kinase C.
decreased total PKC activity is observed during colonic carcinogenesis (10,15–17). These results suggest that alterations in PKC isofrom protein concentration may be related to increased susceptibility of copper-deficient rats to aberrant crypt formation.

Although copper deficiency increases the formation of aberrant crypt foci in rat colon, it is not known whether the increase in preneoplastic aberrant crypts leads to an increase in tumor formation. Accordingly, an objective of the current study was to determine whether dietary copper deficiency would increase AOM-induced intestinal tumor formation. In the previous study (4), the protein and message expression of various PKC isofroms were investigated in the cytosol of normal colonic tissue under short-term (2 and 8 wk after carcinogen administration) intakes of low dietary copper as a potential indicator of cancer susceptibility rather than actual tumor formation. Thus, a second objective of the current study was to determine the effect of long-term (38 wk after carcinogen administration) intakes of low dietary copper on PKC protein and mRNA expression in normal colonic tissue and colon tumors.

MATERIALS AND METHODS

Chemicals. AOM was purchased from Sigma Chemical (St. Louis, MO). Xylazine was purchased from Rompan Mobay (Shawnee, KS) and ketamine from Ketaset Aveco (Fort Dodge, IA). Rabbit anti-PKC α, δ and η antibodies and SuperScript II reverse transcriptase were purchased from Life Technologies (Rockville, MD). Streptavidin conjugated to horseradish peroxidase and vistra green were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Taq DNA polymerase and polynucleotides were purchased from Promega Life Science (Madison, WI).

Animals and diets. Eighty weanling, male Fischer-344 rats were purchased from Sasco (Ornall, NE). All animals were housed individually in stainless steel wire-bottomed cages in a room with controlled temperature and light. Rats had free access to demineralized water and food. Rats were anesthetized with xylazine and ketamine and killed by exsanguination. Blood was collected by cardiac puncture into syringes containing EDTA (2 mg/mL). Heart and liver were removed and weighed and homogenized in nine volumes of cold (4°C) homogenization buffer with six strokes of a Teflon pestle in a Potter-Elvehjem homogenizer. Homogenization buffer consisted of 20 mmol/L HEPES, pH 7.5, 0.25 mol/L sucrose, 1 mmol/L EGTA, 2 mmol/L EDTA, 2 mmol/L diithiothreitol, and 50 TIU/mL of aprotinin, soybean trypsin inhibitor, antipain, leupeptin, and pepstatin (19). A portion of the homogenate was saved for analysis of PKC isoforms. The remaining homogenate was centrifuged at 100,000 × g for 1 h and the supernatant was saved for analysis of PKC isoforms.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Homogenate and supernatant fractions from colon mucosa were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20) on 10% polyacrylamide gels. Supernatant samples were loaded (40 μg protein/lane) on 7 × 8 × 0.125-cm gels. Rat brain cytosol (15 μg protein/lane) was used as a control on each immunoblot. Proteins were transferred to polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA) by semidry electrophoretic transfer (Blot-Trans SD semi-dry transfer cell; Bio-Rad Laboratories, Hercules, CA) and their transfer to the membrane was verified by staining the polyacrylamide gels with Coomassie Blue R250. After the transfer of proteins, the membrane was processed by a method that takes advantage of the hydrophobicity of polyvinylidene fluoride to eliminate the blocking step and to reduce the number of wash steps (Millipore Technical Note RP562; Millipore Corp.). In brief, the dry blots were incubated for 2 h at room temperature with antibodies specific to individual PKC isoforms. The anti-PKC antibodies were diluted 1:1000 in buffer containing 50 g/L nonfat dry milk, 0.5 mol/L Tween-20, 0.081 mol/L Na3HPO4, 0.019 mol/L NaH2PO4, 0.145 mol/L NaCl, pH 7.4. The blots were then incubated for 1 h with horseradish peroxidase-coupled antirabbit IgG (Amersham, Arlington Heights, IL) diluted 1:8000 in the buffer described above. Visualization of the PKC isoforms was accomplished by chemiluminescence and exposure of the blots to luminence detection film (ECL Western Blotting detection reagents and Hyperfilm-ECL; Amersham). Specificity of the antibodies for PKC isoforms was determined by incubating the specific isoezyme peptides with antibody and demonstrating the elimination of the specific PKC band on the immunoblot. Immunoreactive bands were analyzed by imaging densitometry (GS-700 Imaging Densitometer; Bio-Rad Laboratories). PKC isofrom content was represented as a relative density calculated by dividing the scanned density of the isofrom peak in the mucosal samples on a blot by the density of the isoform peak in the brain cytosol standard on the blot. Densities for the peaks representing mucosal PKC were below the film saturation levels and were within the linear range of the densities for the peaks representing PKC in the brain standard.

Total RNA extraction and reverse transcriptase polymerase chain-reaction (RT-PCR) analysis. Total RNA was isolated by guanidine isothiocyanate lysis and acid phenol extraction (21). The relative mRNA levels of PKC isoforms were quantified by using rapid competitive PCR as previously described (4,21,22). First strand cDNA was synthesized in 20 μL total volume by using 0.02 μmol/L 3’ oligonucleotide (see below) and SuperScript II reverse transcriptase. Briefly, rat colon total RNA (1 μg) and 0.02 μmol/L 3’ oligonucleotide were denatured by heating to 70°C for 10 min, quickly chilled on ice, and subsequently reverse-transcribed by incubation with 0.01 mol/L diithiothreitol, 1 mmol/L dNTP and 200 U SuperScript II reverse transcriptase for 50 min at 42°C, followed by enzyme denaturation at 70°C for 15 min. Incubations containing no reverse transcriptase were used as negative controls.

Primer pairs for construction of internal standards and amplification of PKC isozyme mRNA were: PKC α competitor (293 bp) forward 5’-TGAACCCTCTGAGATAATGCT-3’; reverse 5’-GGCTGTCCCTGTCTTCTGAATTGTTTCTCGAAC-3’; PKC α mRNA (325 bp) forward 5’-TGAACCCTCTGAGATGAGT-3’; reverse 5’-GGCTGTCCCTGTCTTCTGA-3’; reverse 5’-GGCTGTCCCTGTCTTCTGA-3’; PKC δ competitor (584 bp) forward 5’-GAGTGGGGTGAATGGAACTGGCTGCTTCCTGTCTTCTGAACTTGGCTTTCTCGAAC-3’; PKC δ mRNA (299 bp) forward 5’-GAGTGGGGTGAATGGAACTGGCTGCTTCCTGTCTTCTGAACTTGGCTTTCTCGAAC-3’; reverse 5’-GGCTGTCCCTGTCTTCTGA-3’; reverse 5’-GGCTGTCCCTGTCTTCTGA-3’. Primer pairs for construction of internal standards and amplification of PKC isozyme mRNA were: PKC α competitor (293 bp) forward 5’-TGAACCCTCTGAGATAATGCT-3’; reverse 5’-GGCTGTCCCTGTCTTCTGAATTGTTTCTCGAAC-3’; reverse 5’-GGCTGTCCCTGTCTTCTGA-3’; reverse 5’-GGCTGTCCCTGTCTGTTTGC-3’; PKC γ competitor (584 bp) forward 5’-GAGTGGGGTGAATGGAACTGGCTGCTTCCTGTCTTCTGAACTTGGCTTTCTCGAAC-3’; PKC γ mRNA (680 bp) forward 5’-GAGTGGGGTGAATGGAACTGGCTGCTTCCTGTCTTCTGAACTTGGCTTTCTCGAAC-3’; reverse 5’-GGCTGTCCCTGTCTTCTGA-3’; reverse 5’-GGCTGTCCCTGTCTTCTGA-3’.
competition experiments were carried out for each message analyzed; the internal standard contains the same sequence as the message and utilizes the same primers as the message of interest but is smaller in length. Internal standards were synthesized using cDNA of the PKC isozyme of interest and the primers listed above. Thus, the internal standard contains the same sequence as the message and utilizes the same primers as the message of interest but is smaller in length. A 50-µL PCR reaction contained the following: 0.1 mmol/L dNTP, 1.5 mmol/L MgCl₂, 1× Taq DNA polymerase buffer, 20 mM/L DMSO, 0.2 µmol/L of each forward and reverse primer, internal standard generated for the message of interest, 1.25 U of Taq DNA polymerase, and 10 µL of the RT reaction. Quantitative competition experiments were carried out for each message analyzed; a fixed amount of sample RNA (1 µg) was reverse transcribed and amplified with increasing amounts of synthetic internal standard cDNA, resulting in a product from the endogenous message and a product from the internal standard. Equal amplification efficiency (similar band intensity) of the target sequence and internal standard were obtained when 5, 2.5 and 12.5 pg of PKC α, δ and ζ internal standard cDNA, respectively, were used. PCR was performed on a PTC-100 Programmable Thermal Controller (MJ Research, Incline Village, NV). The reaction was performed for 35 cycles as follows: denaturation, 93°C for 30 s; annealing, 60°C for 45 s; and extension 74°C for 45 s. The final cycle included an additional 10 min at 74°C for complete strand extension. PCR products were incubated 1:10,000 with Vistra Green for 15 min before electrophoresis on 4% agarose gels. The fluorescence intensity of the endogenous target and the internal standards were quantified with a Storm 860 (Molecular Dynamics, Sunnyvale, CA). Results are expressed as the ratio of sample:internal standard for each sample.

Plasma samples were analyzed for enzymatic ceruloplasmin activity with a Cobas Fara II Centrifugal Analyzer (Roche Diagnostics Systems, Montclair, NJ) by the method of Sunderland and Nomata (24), which measures its p-phenylenediamine oxidase activity. Plasma samples were precipitated with 30 g/L trichloroacetic acid and 6 mol/L HCl for mineral analysis. The supernatant was analyzed by inductively coupled argon atomic emission spectrometer (Liberty Series II; Varian Associates, Sugarland, TX). Control samples containing demineralized water that had been collected through the syringes containing 1 g EDTA/L water and processed similarly to the plasma samples were not found to contain any copper, iron or zinc contamination.

Samples of liver were analyzed for copper, iron and zinc by inductively coupled argon atomic emission spectrometry (Liberty Series II; Varian Associates) as previously described (4). Liver standard reference material (1577b; National Institute of Standards and Technology, Gaithersburg, MD) was analyzed with each batch of tissue samples for quality control. Liver samples (n = 4) were determined to contain 100%, 103% and 108% of the certified values for copper, iron and zinc, respectively.

Statistical analyses. The data were analyzed by a two-way ANOVA to determine the significance of the main effects of dietary copper and AOM treatment and the interaction of dietary copper and AOM administration using a SAS general linear model program (SAS Version 6.12; SAS Institute, Cary, NC). Because the data for hematocrit and hemoglobin concentrations did not follow a normal distribution, data were transformed by using the natural log before statistical analysis. Because the variances were proportional to the means, a square root transformation was performed on PKC α, δ and ζ mRNA expression and on PKC ζ protein expression before the analysis. If the interaction was significant, Tukey’s contrasts were used to differentiate among means. Tumor incidence was analyzed by a generalized linear model. Values are reported as means ± SEM except where indicated otherwise.

RESULTS

Dietary copper did not affect the weight gain of rats (Fig. 1). However, beginning at 30 d of age, rats injected with AOM had a significantly (P < 0.05) lower body weight than rats injected with saline.

Rats fed the low copper diets and injected with azoxymethane had a (P < 0.0001) significantly greater total tumor incidence than rats fed adequate dietary copper and injected with azoxymethane (Fig. 2). However, this difference reflected a higher incidence of tumors in the small intestine and no difference in colon tumor incidence. There were no differences in tumor mass or total tumor burden between rats fed low and adequate dietary copper and injected with azoxymethane (data not shown). No tumors were observed in the rats injected with saline.

Dietary copper had no effects on hematocrit or hemoglobin concentrations; however, rats fed low dietary copper had lower (P < 0.0001, ANOVA) ceruloplasmin activity and plasma and liver copper concentrations than rats fed adequate dietary copper (Table 1). There was a significant (P < 0.05) diet by treatment interaction in both ceruloplasmin activity and plasma copper concentrations such that carcinogen treatment increased ceruloplasmin activity and plasma copper concent-

![FIGURE 1](image1)

![FIGURE 2](image2)
PKC isozymes present in the cytosolic versus the homogenate fraction. The homogenate includes PKC in both compartments (Fig. 3). Dietary copper reduced (P < 0.004) PKC α protein expression in the homogenate samples in the normal mucosa (Fig. 3B). The same trend for decreased PKC α protein expression in the cytosolic fraction (Fig. 3A) and decreased PKC α mRNA expression (Table 2) when rats were fed low dietary copper was observed; however, these effects were not significant (P < 0.05) because of the variability between samples. Tumor tissue had lower PKC α protein and mRNA expression than normal mucosa (compare Fig. 3C with Fig. 3A and 3B and Table 3 with Table 2). Dietary copper did not affect PKC α protein and mRNA expression in tumor tissue.

In contrast to PKC α, most of PKC δ protein was in the homogenate fraction of the normal mucosa samples (Fig. 4A and B) but in the cytosolic fraction of the tumor samples (Fig. 4C). Dietary copper did not affect PKC δ protein expression in

![FIGURE 3](image)

PKC α protein was predominantly located in the cytosolic compartment (Fig. 3). Dietary copper reduced (P < 0.004) PKC α protein expression in the homogenate samples in the normal mucosa (Fig. 3B). The same trend for decreased PKC α protein expression in the cytosolic fraction (Fig. 3A) and decreased PKC α mRNA expression (Table 2) when rats were fed low dietary copper was observed; however, these effects were not significant (P < 0.05) because of the variability between samples. Tumor tissue had lower PKC α protein and mRNA expression than normal mucosa (compare Fig. 3C with Fig. 3A and 3B and Table 3 with Table 2). Dietary copper did not affect PKC α protein and mRNA expression in tumor tissue.

In contrast to PKC α, most of PKC δ protein was in the homogenate fraction of the normal mucosa samples (Fig. 4A and B) but in the cytosolic fraction of the tumor samples (Fig. 4C). Dietary copper did not affect PKC δ protein expression in
either the cytosolic or the homogenate fractions of normal or tumor samples (Fig. 4, A and B). Both dietary copper and carcinogen treatment increased ($P < 0.03$) PKC $\delta$ mRNA levels (Table 2). There was a diet $\times$ carcinogen interaction ($P < 0.05$) such that the effect of dietary copper was more pronounced in rats injected with AOM. Dietary copper did not affect PKC $\delta$ mRNA expression in the tumor samples (Table 3).

Although two bands are observed in the representative blots of PKC $\zeta$ (Fig. 5), only the lower band represents PKC $\zeta$ (as demonstrated by its disappearance when specific PKC $\zeta$ peptides were incubated with the antibody). Similar to PKC $\delta$, a large portion of PKC $\zeta$ protein was in the homogenate of the normal mucosa samples (Fig. 5B) but in the cytosolic fraction of the tumor samples (Fig. 5C). Dietary copper did not affect PKC $\zeta$ protein expression or mRNA levels in the normal
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Effect of dietary copper on PKC mRNA expression in intestinal tumor tissue of rats treated with AOM

<table>
<thead>
<tr>
<th>Copper</th>
<th>PKC α</th>
<th>PKC δ</th>
<th>PKC ζ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>0.8</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinogen</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tissue</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

FIGURE 6  Representative PCR bands obtained from the RT-PCR analysis of PKC α, δ, and ζ from rats fed low (1) or adequate (6) concentrations of dietary copper (by analysis, 0.8 and 5.3 µg Cu/g diet) and injected with either azoxymethane (+carcinogen) or saline (−carcinogen), and their synthetic internal standard cDNA. Samples were obtained from either normal mucosa (N) or tumor (T) samples.

mucosa or in the tumor samples (Fig. 5; Table 2; Table 3). However, low dietary copper increased (P < 0.01) the ratio of PKC ζ protein expression in the homogenate to PKC ζ protein expression in the cytosol (3.80 vs 2.53, respectively) compared with rats fed adequate dietary copper. This suggests that low dietary copper caused a translocation of PKC ζ to the membrane fractions.

Carcinogen treatment did not affect PKC α, δ, or ζ protein expression in either the cytosolic or homogenate fractions (Fig. 3, A and B; Fig. 4, A and B; Fig. 5, A and B). Carcinogen treatment increased (P < 0.05) PKC α and PKC δ but not PKC ζ mRNA expression in the normal mucosa (Table 2).

DISCUSSION

In this study, rats fed the low copper diet had significantly more small intestinal tumors than rats fed the adequate copper diet. Similarly, in a previous study, we observed that dietary copper deficiency increased the incidence of spontaneous small intestinal tumors that occur in the Min mouse (9). Those results are similar to those of other investigators who have observed that in Min mice, most of the tumors occur in the small intestine rather than in the large intestine (25). However, because of the genetic and histochemical similarities between small intestinal tumors in the Min mouse and colon cancer in humans, these mice are an accepted model for colon cancer in humans (25). Although azoxymethane is both a small intestine and a colon carcinogen, the relevance of the increased small intestinal tumor incidence observed in the current study is unknown. In humans, the small intestine contains 75% of the mucosal surface of the gastrointestinal tract, yet it is the site of only 2% as many malignancies as the colon (26). However, epidemiologic studies suggest that dietary correlates of adenocarcinoma of the small intestine are similar to those of colon cancer and at least of the same magnitude (27,28).

In this study, we did not observe an effect of dietary copper on colon tumor incidence. These results are surprising because in two previous studies (3,4) we observed increased chemically induced aberrant crypt formation in rats fed a low copper diet. Colon carcinogenesis is regarded as a multistage process. The target cells of colon carcinogens are colonic crypt epithelial cells. Studies in humans have suggested that colonic aberrant crypt foci are precursor lesions from which adenomas and adenocarcinomas will develop. During the last decade, numer-

TABLE 2

Effect of dietary copper and carcinogen administration on PKC mRNA expression in normal mucosa of rats

<table>
<thead>
<tr>
<th>Diet copper, µg/g</th>
<th>Treatment</th>
<th>PKC α</th>
<th>PKC δ</th>
<th>PKC ζ</th>
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<tbody>
<tr>
<td>0.8</td>
<td>AOM</td>
<td>1.08</td>
<td>0.87</td>
<td>0.73</td>
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<td>0.8</td>
<td>Saline</td>
<td>0.59</td>
<td>0.56</td>
<td>0.99</td>
</tr>
<tr>
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<td>AOM</td>
<td>1.52</td>
<td>3.43</td>
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<tr>
<td>5.3</td>
<td>Saline</td>
<td>0.82</td>
<td>0.63</td>
<td>0.95</td>
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Significant effects as determined by two-way ANOVA:

<table>
<thead>
<tr>
<th>Diet</th>
<th>Treatment</th>
<th>PKC α</th>
<th>PKC δ</th>
<th>PKC ζ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
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<td>0.03</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>NS</td>
<td>0.066</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NS indicates not significant.

1 Values are mean (±1 SEM; these numbers are not evenly distributed around the mean because of the square root transformation), n = 9–10 AOM-treated rats, n = 4–5 saline-treated rats. Values in a column without a common letter differ, P < 0.05.

2 Relative steady-state levels of mRNA were determined by semiquantitative RT-PCR. Values are the ratio of sample band intensity to internal standard band intensity.
rious studies including molecular analysis have focused on the importance of aberrant crypt foci as early events in colon carcinogenesis, and aberrant crypt foci are now regarded as putative premalignant lesions for colon cancers (4,29). Nevertheless, there is evidence that documents the lack of correlation between tumor development and the expression of aberrant crypt foci (30,31).

Furthermore, some compounds with the potency to prevent the occurrence of aberrant crypt foci, such as 2-(carboxyphenyl) retinamide or genistein have been found to enhance the development of colon cancers (32,33). A recent study (34) has suggested that β-catenin-accumulated crypts are independent lesions of aberrant crypt foci and are a more useful marker for premalignant lesions of azoxymethane-induced colon cancer in rats.

Another explanation for the differential effect of dietary copper on aberrant crypt foci formation and tumor development is that dietary copper is having differential effects during different stages of tumorigenesis. Tumorigenesis is believed to be a multistage process. Dietary copper may be protective against aberrant crypt formation because of its effects on antioxidant enzymes. Two copper-containing enzymes, namely copper-zinc superoxide dismutase and ceruloplasmin, that may help protect against oxygen radical-mediated injury are significantly reduced in rats fed low copper diets (3,4). Copper-zinc superoxide dismutase functions to eliminate superoxide radicals and ceruloplasmin is hypothesized to inhibit iron-catalyzed radical formation (35,36). Substantial evidence suggests that free radicals, particularly oxygen radicals, are involved in both the initiation and promotion stages of carcinogenesis (37). Thus, low dietary copper may be a risk factor during the early stages of carcinogenesis. However, low dietary copper may be protective during the later stages of tumorigenesis. Both copper and the copper transport protein, ceruloplasmin, have been shown to be involved in angiogenesis and neovascularization (38–40). Ceruloplasmin mRNA has been shown to be at least threefold more abundant in tumor cells compared with normal tissue (40). Furthermore, the copper chelator, tetrathiomolybdate, is being used in the treatment of metastatic solid tumors because it causes mild copper deficiency and impairs neovascularization (40).

The differential effect of dietary copper on aberrant crypt formation and tumor formation may also be the result of different effects of dietary copper on PKC expression during different stages of tumorigenesis. Alterations in the expression and activation of specific PKC isoforms play a role in the malignant transformation process of the colon. Decreased levels of PKC activity have been observed in preneoplastic colonic mucosa and in colonic adenocarcinomas, indicating that alterations in PKC isozyme regulation occur early in the multistage process of colon carcinogenesis (11,19,41,42). In a previous study (4), we observed that low dietary copper significantly decreased the cytosolic protein concentration of PKC α, δ, and ζ at 2 wk but not at 8 wk after carcinogen administration. In this study, low dietary copper significantly reduced PKC α protein expression in the normal mucosa but did not affect PKC δ, β or ζ protein expression. Furthermore, dietary copper did not affect PKC α, δ, and ζ protein or mRNA expression in the tumors. This suggests that dietary copper-mediated changes in PKC α, δ, and ζ protein expression are not important for tumor development but may be important for aberrant crypt formation.

In this study, tumor tissue had a much lower PKC α protein expression than normal tissue (Fig. 3). Similarly, Gokmen-Polar et al. (43) observed that PKC α protein expression was slightly decreased in aberrant crypt foci and dramatically reduced in colon tumors. Consistent with these findings, a compelling body of evidence indicates that PKC α protein expression is associated with negative growth regulation and cell cycle arrest in various cell lines (44–48). In both our study and the study by Gokmen-Polar et al. (43) quantitative reverse transcription-PCR analysis revealed that PKC α mRNA levels do not directly correlate with PKC α protein levels. Similarly, in human colonic tumors, down-regulation of PKC α protein expression was not associated with decreased mRNA (41). These results indicate that PKC isozyme expression is likely regulated at the posttranscriptional/translational level.

In this study, the majority of PKC δ and PKC ζ in tumors was found in the cytosolic fraction, whereas these isoforms were found mostly in the homogenate of normal mucosa. The homogenate contains the totality of cellular membranes and organelles. When PKC is activated, there is net translocation of PKC from the cytosol to target membranes and organelles (12,13). Thus, the finding that almost all of the PKC δ and PKC ζ was in the cytosolic fraction of tumors but in the homogenate fraction of the normal mucosa suggests that PKC δ and PKC ζ activation is greatly reduced in tumor tissue compared with normal mucosal tissue. Consistently, we observed decreased PKC δ and ζ mRNA in the tumor samples compared with the normal samples (Tables 2 and 3). Similarly, previous studies have shown decreased PKC δ and ζ protein expression in azoxymethane-induced rats and sporadic human colon tumors and decreased PKC ζ mRNA levels in human colorectal tumors than in normal colonic mucosa (10,49–51). PKC δ is important in cell proliferation and apoptosis (52) and PKC ζ may play an important role in both inhibiting tumor initiation and promotion by regulating colonic epithelial cell ontogeny along the crypt axis (48,53,54).

Our results suggest that low dietary copper significantly increases small intestinal but not colon tumor development. These results have practical implications because many of the diets consumed in the United States do not contain the recommended amount of copper (55). Low dietary copper significantly decreased PKC α protein expression in normal but not in tumor tissue. However, long-term low dietary copper intake did not affect PKC δ or ζ protein expression in either the normal or tumor tissue. This result, along with previous observations, suggests that dietary copper-mediated changes in PKC α, δ and ζ protein expression are not as important for colon tumor promotion/progression as they are for tumor initiation.

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LITERATURE CITED


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