Supplemental Calcium Suppresses Colonic Mucosal Ornithine Decarboxylase Activity in Elderly Patients with Adenomatous Polyps

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ABSTRACT

Epidemiological and animal studies suggest a role for calcium in the chemoprevention of colorectal neoplasia. This study was designed to investigate whether supplemental oral calcium has a suppressant effect on colonic mucosal ornithine decarboxylase (ODC) and tyrosine kinase activities in patients with adenomatous polyps or a history of adenomatous polyps and whether this is affected by age. ODC and tyrosine kinase activities were measured in rectal mucosal biopsies of 19 male patients (age, years 46–85 years; mean, 66 years) with adenomatous polyps or a history of adenomatous polyps before and after 1 week of calcium supplementation p.o. (Caco2; 2500 mg/day) and 2 weeks after cessation of calcium treatment. The basal rectal mucosal ODC activity of patients ≥64 years old was nearly 4-fold higher than that of patients <64 years old (P < 0.005). In patients ≥64 years old, there was a significant decrease in rectal mucosal ODC activity following 1 week of calcium p.o. compared to those age <64 years (P < 0.05). Overall tyrosine kinase activity did not differ significantly in either patient group before or after calcium supplementation p.o. However, the concentration of phosphotyrosine membrane proteins with molecular weights between 40,000 and 60,000, and between 80,000 and 100,000 were suppressed in patients age ≥64 years after 1 week of calcium treatment p.o. These patients also had a corresponding decrease in their rectal mucosal ODC activity. Alternatively, patients whose ODC was not affected by calcium showed no apparent change in the relative concentration of rectal mucosal phosphotyrosine membrane proteins. Our data indicate that there is an age-related increase in basal rectal mucosal ODC activity in patients with adenomatous polyps which can be suppressed with calcium supplementation p.o., suggesting a role for dietary calcium in the chemoprevention of colorectal neoplasia.

INTRODUCTION

Colorectal cancer is one of the most common malignancies in the United States and is thought to originate within previously benign adenomas (1, 2). Numerous studies have demonstrated that the activity of ODC, the rate-limiting enzyme in polyamine biosynthesis, is greatly elevated in colon carcinomas (3–6). Polyamines are considered to be intracellular mediators of cell proliferation (7). Furthermore, increased ODC activity has been demonstrated in benign colonic adenomas (3–6, 8) as well as the normal appearing mucosa adjacent to adenomas (8, 9) reflecting the underlying hyperproliferative state of the colonic mucosa.

Epidemiological studies strongly suggest a relationship between increased dietary intake of fat and risk of developing colon cancer (10–12). In murine studies with established colorectal carcinogens, dietary fat seems to function as a promoter of colon carcinogenesis (13, 14). This effect may be mediated by the colonic mucosal toxicity of bile acids (15) or fatty acids (16) resulting in hyperproliferation of colonic epithelial cells. It has been suggested that oral calcium may combine with fecal bile and fatty acids to render them nontoxic to colonic mucosa, thus reducing their tumor-promoting properties. Recent studies examining the effects of calcium supplementation in animal models of carcinogenesis (15–17) as well as patients at high risk for colorectal cancer (18, 19) further support this hypothesis.

The present study was undertaken to determine whether supplemental oral calcium has a suppressant effect on colonic mucosal proliferation as reflected by ODC activity in patients with adenomatous polyps. In addition, we have measured Tyr-k activity and the relative concentration of phosphotyrosine membrane proteins, since Tyr-k is thought to play a role in cell proliferation and differentiation (20–23).

MATERIALS AND METHODS

Patients

Patients undergoing colonoscopy who were found to have colonic polyps (later confirmed histologically to be adenomas) or were having a follow-up examination for previous adenomatous polyps were invited to participate. Patients consumed a standard oralavage preparation of Colyte (4–6 liters) which has been shown to have no effect on colonic mucosal ornithine decarboxylase activity (24). All patients had 6–8 forceps mucosal biopsies obtained from macroscopically normal appearing rectum about 10 cm from the anus. Biopsies were immediately frozen in liquid N2 and stored at −70°C to be simultaneously assayed for ODC and Tyr-k activities at a later date. A complete dietary history was obtained from each patient by a registered dietitian.

The patients were then given calcium carbonate 2500 mg/day p.o. in two divided doses for 1 week to supplement their conventional Western-style diet. Compliance was determined by performing a pill count and was uniformly greater than 90%. Flexible sigmoidoscopy and rectal mucosal biopsies were repeated as previously described after 1 week of calcium treatment and 2 weeks after cessation of calcium supplementation. All sigmoidoscopic procedures were preceded by a 24-h diet of clear liquids. No patients used enemas or rectal suppositories for bowel preparation. The following serum laboratory tests were obtained on all patients at the 3-day visit: triglycerides; cholesterol; ionized calcium; 1,25-dihydroxyvitamin D; COOH-terminal parathyroid hormone; and fasting gastrin.

Biochemical Assays

Determination of ODC Activity. Four mucosal biopsies were homogenized in 0.25 ml of homogenizing buffer containing 0.25 mM sucrose, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.4 mM pyridoxal 5'-phosphate, and 1 mM dithiothreitol in an Ultra-Turrax tissue homogenizer and then centrifuged at 20,000 × g for 50 min at 2°C. The supernatant was assayed for ODC activity by a slight modification (8) of the micromethod of Beaven et al. (25) in an Eppendorf microfuge in a final volume of 20 μl. The reaction mixture (final concentration) contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 1

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3 The abbreviations used are: ODC, ornithine decarboxylase; Tyr-k, tyrosine kinase; PBS, phosphate-buffered saline.
mm EDTA, 0.25 mm pyridoxal 5'-phosphate, 1 mm dithiothreitol, and 130 μM 1-[14C]ornithine (40.6 mCi/mmol; New England Nuclear, Boston, MA). The reaction at 37°C for 60 min was initiated with 10 μl of the 20,000 × g supernatant and terminated by adding 10 μl of 2 M citric acid. The liberated 14CO2 was collected for 50 min in 20 μl of 1 M sodium hydroxide in methanol in the scintillation vial. At the end of this period, the microfuge tubes were removed, rinsed with 2 ml of 95% ethanol, and discarded, and the contents of the scintillation vials were counted for radioactivity in a 10-ml scintillation cocktail. One enzyme unit was defined as 1 pmol of 14CO2 released/mg protein/h. Protein content was determined by the method of Bradford (26), utilizing bovine serum albumin as the standard.

Tyrosine Kinase Activity. For these experiments, membrane (30,000 × g pellet) fractions were prepared from colonic mucosa homogenized in Tyr-k buffer containing 10 mm 4-(2-hydroxyethyl)-piperazineethanesulfonic acid (pH 7.2), 150 mm NaCl, and 1 mm MgCl2 according to the method of Danog et al. (20) as described by Majumdar et al. (27). Tyr-k activity was measured using poly (l-glutamine-l-tyrosine) (4:1; Sigma Chemical Co., St. Louis, MO) as substrate (20, 26), which has been shown to be highly specific for Tyr-k (28). The reaction mixture in a final volume of 50 μl contained 2.5 μmol of Tris-HCl, 0.5 mmol of o-vanadate, 0.02% Triton X-100, 3 μmol of ATP, 0.4 μCi [γ-32P]ATP (11.7 Ci/mmol; New England Nuclear), and 50 μg of glutamine-tyrosine polymer. The reaction was initiated with membrane preparation (10–20 μg protein), o-vanadate was added to inhibit degradation of ATP and dephosphorylation of the phosphopeptide. The reaction was terminated by applying 20 μl of the reaction mixture onto 3-cm2 Whatman No. 2MM filter paper. The filters were extensively washed in 10% trichloroacetic acid containing 10 mm sodium pyrophosphate, rinsed with ethanol, and dried, and radioactivity was quantitated in a 5-ml scintillation cocktail. Results are expressed as pmol 32P incorporated/mg protein.

Phosphotyrosine Membrane Proteins. The relative concentrations of phosphotyrosine membrane proteins were determined by Western immunoblot. Briefly, the membrane fraction containing an equal amount of protein (25 μg) was electrophoresed on a 7.5% polyacrylamide gel as described previously (27).

Electrophoresed proteins were transferred onto nitrocellulose membranes (0.45 μm) using an LKB Trans-Blot apparatus according to the manufacturer's instructions. Membranes containing the proteins were incubated with the blocking buffer (PBS containing 3% bovine serum albumin, 0.25% gelatin, and 0.5% Tween 20) at 37°C for 3 h under constant agitation. After extensive washing with PBS containing 0.1% Tween 20, the membranes were incubated with antiphotophosphotyrosine antibody (monoclonal; ICN, Costa Mesa, CA) at 24°C for 2 h, subsequently washed with PBS/0.1% Tween, and then subjected to 1 h of incubation at 37°C with biotinylated anti-mouse IgG. The membranes were washed in PBS/0.1% Tween 20 and then incubated again at 37°C for 1 h with 125I-streptavidin (Amersham, Arlington Heights, IL). At the end of this incubation period, the membranes were washed extensively with PBS/0.1% Tween 20 and then exposed to Kodak X-Omat AR film. Molecular weights of the 32P-labeled protein bands were calculated from standard protein markers run concurrently. Protein was measured by the method of Bradford (26).

Statistics

Average data are expressed as the mean ± SEM unless otherwise stated. Where applicable data were compared using the unpaired Student's t test and analysis of variance for nonparametric data. P < 0.05 was considered significant.

RESULTS

Nineteen male patients completed the study. The age ranged from 46 to 85 years (mean, 66 years). No patients experienced any adverse effects during the study period, and none took additional vitamin or mineral supplements. The mean basal dietary intake of calcium was 681 ± 76 mg/day. ODC and Tyr-k Activities. The mean basal ODC activity in the rectal mucosa of patients ≥64 years (N = 14) was 357% higher than that of patients <64 years (N = 5) (P < 0.005) (Fig. 1). Following 1 week of calcium supplementation, the mean ODC activity decreased by 52 ± 8% in 11 of 14 patients in the older group while only 1 of 5 patients in the younger group showed diminished enzyme activity (P < 0.05). Two weeks following cessation of calcium, 7 of the 11 patients who responded to calcium still had decreased ODC activity, 2 patients returned to baseline, and 2 had an increase above baseline values.

In contrast to ODC, basal Tyr-k activity showed no correlation with age, nor were the basal values significantly affected by calcium supplementation (results not shown).

Phosphotyrosine Membrane Proteins. Calcium treatment resulted in a significant suppression of phosphotyrosine membrane proteins with molecular weights between 40,000 and 60,000 and between 80,000 and 100,000 in representative patients ≥64 years old (Fig. 2), while no suppression of these proteins was noted in patients <64 years old (Fig. 3).

Serum ionized calcium, parathyroid hormone, and vitamin D levels were not significantly altered by calcium supplementation.

DISCUSSION

In this study, we have examined the suppressant effect of calcium supplementation on rectal mucosal ODC and Tyr-k activities in patients with adenomatous polyps. Significant increases in ODC activity have been found in colonic adenocarcinoma as well as benign adenomas (3–6). Moreover, we have previously demonstrated increased levels of ODC in normal appearing rectal mucosa of patients with adenomatous polyps (9) and familial polyposis (8), suggesting a correlation between colonic mucosal proliferation and ODC. In the present study, we noted an age-related increase in ODC activity in the rectal mucosa of patients with adenomatous polyps. Although re-
Dietary Calcium and Colonic ODC

Fig. 1. Autoradiograph of phosphotyrosine membrane proteins in 3 representative patients >64 years before (A1, A2, and A3) and after (B1, B2, and B3) CaCO3 supplementation p.o. for 1 week. These patients had corresponding suppression of their rectal mucosal ODC activity after CaCO3 for 1 week. Phosphotyrosine proteins with molecular weights between 40,000 and 60,000 and between 80,000 and 100,000 kDa were suppressed after 1 week of CaCO3.

determine the duration of this effect and any subsequent benefit of continuous versus intermittent calcium supplementation.

In contrast to ODC, rectal mucosal Tyr-k activity did not vary significantly with age and was unaffected by calcium supplementation in our study. Tyrosine kinases, which catalyze phosphorylation of tyrosine residues in proteins, are thought to play an important role in the regulation of cell growth (20–23). Tyr-k activity has been found to be associated with receptors for epidermal growth factor (39), platelet-derived growth factor (40), and several oncogene products (41). We have previously demonstrated increased Tyr-k activity in normal appearing mucosa of patients with adenomatous polyps (9). However, the present study suggests that increases in this enzyme in patients with adenomas are related to factors other than aging.

Although age-related changes in Tyr-k activity were not evident, the relative concentration of certain phosphotyrosine membrane proteins differed significantly between the two age groups following calcium supplementation. Since tyrosine kinases are composed of numerous enzymes, the synchronous stimulation of some and inhibition of others may explain the lack of correlation between Tyr-k activity and the content of phosphotyrosine membrane proteins. Calcium treatment markedly suppressed the phosphorylation of tyrosine membrane proteins in the Mr 40,000–60,000 and 80,000–100,000 range in elderly patients with adenomatous polyps. Although the identity of these proteins is unknown, we postulate that they may be related to the regulation of cell proliferation in these patients. In support of this hypothesis, we have previously demonstrated that azoxymethane-induced stimulation of colonic mucosal ODC activity and its suppression by calcium

regional variation in colonic mucosal ODC activity may exist, biopsies were obtained exclusively from the rectum to standardize our results and because of the aforementioned correlation of rectal mucosal ODC activity and established colonic hyperproliferative disorders (8, 9). The observed increase in ODC activity may explain, in part, the increased risk for colonic adenomas and carcinomas associated with aging in humans (29). Additionally, our findings are consistent with animal studies which have noted an increase in gastrointestinal mucosal proliferation associated with aging (30–33).

We have further demonstrated that calcium supplementation is effective in suppressing the increased colonic mucosal ODC activity in elderly patients with adenomas. This beneficial effect of calcium may be related to its interaction with free fatty acids in bile acids. The ionized forms of fatty acids and bile acids, which are not bound to calcium, may serve as a toxic irritant to the colonic epithelium (34–36). With injury to the colonic epithelial cells, repair and regeneration are initiated through an increase in cellular proliferation. Such increases in cellular proliferation can be attenuated by calcium p.o. in animals (15–17) as well as humans (18, 19, 37, 38) possibly by converting ionized fatty acids and bile acids to insoluble calcium compounds which are nontoxic to the colonic epithelium. However, our recent observation of the in vitro suppressant effect of calcium on azoxymethane-induced ODC activity in rat colonic mucosal explants (17) certainly suggests a direct effect for calcium on colonic epithelium. Interestingly, the majority of our elderly patients who responded to calcium treatment had a sustained decrease in ODC activity for 2 weeks after cessation of treatment. Further controlled studies are necessary, however, to
were associated with parallel alterations in the tyrosine phosphorylation of several mucosal proteins (17).

In summary, our study demonstrated that patients with adenomatous polyps have an age-related increase in rectal mucosal ODC activity which can be suppressed by calcium supplementation for 1 week. Additionally, tyrosine-specific phosphorylation of mucosal membrane proteins is also suppressed by calcium in these patients. Further studies are necessary to determine whether the mechanisms of action are related to a direct effect of calcium on colonic epithelial cells or an indirect effect through alterations in the intestinal milieu. Finally, the clinical significance of the antiproliferative effects of calcium in terms of cancer chemoprevention have yet to be elucidated.

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