Attenuation of Azoxymethane-induced Colonic Mucosal Ornithine Decarboxylase and Tyrosine Kinase Activity by Calcium in Rats

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ABSTRACT
Two in vivo and one in vitro studies were performed to evaluate the chemoprotective role of calcium during the early period of azoxymethane (AOM) induction. In the first set of experiments, groups of male Fischer 344 rats were s.c. injected with either AOM (20 mg/kg) or water (controls) and sacrificed immediately (0 time), and 1, 3, 5, and 7 days postinjection. In the second set of experiments, animals were injected with the same dose of AOM and subsequently pair-fed with rat chow containing either calcium carbonate or diet devoid of added calcium. The amount of calcium consumed was calculated to be 250 mg/kg b.w. In both experiments, colonic mucosa was assayed for ornithine decarboxylase (ODC). In addition, tyrosine kinase (Tyr-k) activity was evaluated in the same colon sections. Results revealed that maximal stimulation by AOM of ODC and Tyr-k activity occurred 5 days postinjection. This stimulation was significantly suppressed by calcium. AOM also produced an increase in the rate of tyrosine-specific phosphorylation of membrane proteins. These results indicate that calcium may have a chemoprotective role in the early stages of AOM treatment in rats and report on calcium's antiproliferative properties in this model. Furthermore, the development of chemoprevention strategies for the early stages of colon cancer becomes more attractive when one considers the high incidence of colon cancer in populations that consume high fat "western" diets (6), and that calcium might bind increased fecal bile acids (known tumor promoters), present in populations that consume high fat "western" diets (6). We conclude that calcium may have a chemoprotective role and colon cancer, suggesting a chemoprotective role for calcium. In the present study we have evaluated the role of calcium during the early stages of AOM treatment in rats and report on calcium's antiproliferative properties in this model. Furthermore, in order to begin to elucidate the mechanism for the changes in proliferative activity induced by AOM and calcium, we have evaluated Tyr-k activity. Tyrosine kinases have recently been shown to be important in cellular proliferation, differentiation, and transformation (9).

MATERIALS AND METHODS

Animal and Diets
Pathogen-free male Fischer 344 rats were obtained at 8 weeks of age from Harlan Breeding Labs, Walkersville, MD. Animals were housed in our facilities for 2 weeks prior to experimentation, at which time their weight was 150–200 g. Animals were housed in metal cages in a light (12 h/day)- and temperature (20°C)-controlled rodent colony with daily care. Animals were given water and Ralston Purina 5002 powdered rat chow diet ad libitum. After carefully measuring the amount of food ingested by the rats per day over several days, animals were pair-fed and consumed 10 g/day. The amount of calcium carbonate required to provide 130 mg/day of calcium (3.8 mg calcium/kcal) was mixed with the chow and dispensed to animals during the 5 days after injection with AOM. This increased the basal calcium intake by 63%. The experiment was also repeated with rats fed 180 mg/day of calcium, doubling the supplemental calcium, in order to determine whether this would result in further suppression of ODC by calcium. Age, weight-matched control animals received the basal powdered rat chow (80 mg/day calcium or 2.4 mg calcium/kcal) without additional calcium. All were given water ad libitum.

Carcinogen Procedures
All procedures involving carcinogen were reviewed and approved by the Wayne State University Animal Use Committee. AOM and MAOM acetate, were purchased from Ash Stevens Co., Detroit, MI. AOM was diluted to a concentration of 50 mg/ml with pyrogen- and preservative-free sterile water. AOM was stored at 4°C in the dark until use. Animals were given one s.c. injection of AOM at a dose of 20 mg/kg b.w. at 8 a.m. following an overnight fast. This dose was chosen for the short-term experiment because of its lack of toxicity and to accentuate the induced changes (1). Also, high doses of AOM produce left-sided tumors, similar to the clinical finding in humans (1). Injection procedures were carried out in a chemical fume hood with an air flow of 150 linear ft/min. MAOM was diluted in sterile water to a concentration of 50 mg/ml for use in organ culture studies, and also stored at 4°C in the dark until used.

Tissue Preparation
In the first set of experiments, groups of three to five animals were killed immediately (0 h), after one s.c. injection of AOM or water

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³ The abbreviations used are: AOM, azoxymethane; ODC, ornithine decarboxylase; Tyr-k, tyrosine kinase; MAOM, methylazoxymethanol; DFMO, difluoromethylornithine; HEPES, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
⁴ Ralston Purina 5002 chow contains a minimum of 20.0% protein; 4.5% fat; a maximum of 5.5% fiber; 7.0% ash; 0.8% calcium; 0.6% phosphorus; 0.21% magnesium; 0.86% potassium; 0.3% sodium; 0.41% chlorine. In ppm: 168.0 iron, 76.1 zinc, 74.5 manganese, 16.9 copper, 0.6 cobalt, 0.53 iodine, 2.0 chromium, 0.2 selenium, 5.6 carotene, 13.3 thiamine, 8.0 riboflavin, 60.0 niacin, 17.0 panthothenic acid, 18.0 choline × 100, 4.0 folic acid, 6.0 pyridoxine, 0.13 biotin, 19.8 kCal/kg, vitamin A 17.6 IU/g, vitamin D 2.2 IU/g, vitamin E 66.1 IU/kg.
Tyrosine Kinases and Tyrosine-specific Phosphorylation of Proteins

Prepared from colonic mucosa homogenized in Tyr-k buffer containing albumin as the standard. The protein content was determined by the method of Bradford (14), utilizing bovine serum scintillation cocktail were added for assay of 14CO2. Protein content rinsed with 2 ml of 95% ethanol and discarded, and 10 ml of liquid hydroxide in methanol were placed on the bottom of the scintillation vial. The scintillation vials were tightly capped and incubated at 37°C (11). The reaction mixture in a 1.5 ml Eppendorf vial. This was placed inside a phosphate, 1.0 mM dithiothreitol, 0.144 µCi of L-[l-14C]ornithine (40-60 mCi mmol; NEN, Boston, MA) or 50 µg of Glu-Tyr polymer. The reaction was initiated with membrane preparations (10-20 µg protein). Orthovanadate was added to inhibit degradation of ATP and dephosphorylation of the phosphoprotein. The reaction was terminated by applying 20 µl of the reaction mixture onto 3-cm2 Whatman No. 2 MM filter paper. The filters were washed three times in 10 ml of 10% trichloroacetic acid containing 10 mm sodium pyrophosphate, rinsed with ethanol, dried, and radioactivity quantitated in 5-ml scintillation cocktail. Results are expressed as pmoI 32P-incorporated per milligram protein.

The autophosphorylation of colonic mucosal membrane proteins was carried out essentially according to our previously described method (16). Briefly, the reaction mixture contained a final volume of 0.05 ml: 2.5 µmol of HEPES (pH 7.8), 2.5 µmol of MgCl2, 0.5 mmol of orthovanadate, 0.5 mmol of [γ-32P]ATP (4 x 106 dpm) and 0.02% Triton X-100. The reaction at 0-2°C for 30 min was initiated with 0.02 µmol of membrane preparation (0.05 mg protein) from the colonic mucosa and terminated by adding 0.05-ml hot (100°C) incubation buffer [62.5 mM Tris-HCl (pH 6.8)/5% SDS/20% glycerol/10%-mercaptoethanol]. A small aliquot (0.01 ml) was spotted onto 3-cm2 Whatman No. 3 MM filter paper, and then treated the same way as described above for Tyr-k activity. The remaining samples were heated at 100°C for 2 min and subsequently electrophoresed on a 7.5% polyacrylamide slab gel (1.5 mm) containing 0.1% SDS (16). Following electrophoresis, the gels were fixed overnight in fixing buffer (500 ml methanol/100 ml acetic acid/400 ml water), washed thoroughly in water and then incubated with 1 M KOH at 56°C for 2 h. Phosphorylase b bonds have been shown to be resistant to this alkali treatment (15, 16, 18). After KOH treatment the gel was washed with water, fixed overnight in 10% acetic acid/10% isopropanol, dried, and finally exposed to Kodak X-Omat AR film for 3 days at ~70°C. Molecular weights of the 32P-labeled protein bands were calculated from standard protein markers run concurrently. Protein was measured by the method of Bradford (14).

Statistical Analysis

Where applicable data were analyzed using the unpaired Student’s t test, and Kruskal-Wallis one-way analysis of variance taking P < 0.05 as the level of significance.

RESULTS

In vivo studies of age-weight matched, pair-fed animals given supplemental calcium carbonate and sacrificed on the 5th day after s.c. injection of AOM showed a 39% reduction in colonic mucosal ODC activity (P < 0.005) compared to AOM treatment alone. Oral calcium carbonate alone had no significant effect on colonic mucosal ODC activity (Fig. 2A). The last experiment was performed in vitro to determine whether calcium exerts a direct effect on MAOM-induced ODC and Tyr-k activity. For this purpose, an organ culture system was used. MAOM was used instead of AOM because MAOM no longer required metabolism to be an active carcinogen (10). In addition, DFMO, the specific, irreversible inhibitor of ODC was used to show that ODC rather than nonspecific decarboxylases was being measured in vitro (11).

The procedure was the same as described previously in which the intestinal explants remained viable and metabolically active for up to 36 h (12). Briefly, adult male Fischer 344 rats 2-3 months old were sacrificed by CO2 narcotization and decapitation. Colonie tissue was obtained from the intestinal explants remained viable and metabolically active for up to 36 h (12). Briefly, adult male Fischer 344 rats 2-3 months old were sacrificed by CO2 narcotization and decapitation. Colonie tissue was immediately re

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Biochemical Assays

ODC Activity. ODC buffer contains 50 mM HEPES (pH 7.4), 1.05 mM EDTA, 0.025 mM pyridoxol-5-phosphate, and 1.0 mM diithiothreitol. The enzyme activity in colonic mucosa was determined by a modification of the micromethod of Beaven et al. (13), similar to that previously described from our laboratory (2). The assay reagent contained 50 mM HEPES (pH 7.4), 1.05 EDTA, 0.25 mM pyridoxol-5-phosphate, 1.0 mM diithiothreitol, 0.144 µCi of L-[1-14C]ornithine (40-60 mCi mmol; NEN, Boston, MA) as substrate (15, 16). Tyr-k activity was measured using poly(L-Glu-L-Tyr,4:1; Sigma Chemical Co., St. Louis, MO) as substrate (15, 16), which has been shown to be highly specific for Tyr-k (17). The reaction mixture in a final volume of 50 µl contained 2.5 µmol of Tris-HCl; 0.5 nmol of orthovanadate; 0.02% triton X-100; 3 µmol of ATP; 0.4 µCi [γ-32P]ATP (11.7 Ci/mmol; New England Nuclear, Boston, MA) and 50 µg of Glu-Tyr polymer. The reaction was initiated with membrane preparations (10-20 µg protein). Orthovanadate was added to inhibit degragation of ATP and dephosphorylation of the phosphoprotein. The reaction was terminated by applying 20 µl of the reaction mixture onto 3-cm2 Whatman No. 2 MM filter paper. The filters were washed three times in 10 ml of 10% trichloroacetic acid containing 10 mm sodium pyrophosphate, rinsed with ethanol, dried, and radioactivity quantitated in 5-ml scintillation cocktail. Results are expressed as pmoI 32P-incorporated per milligram protein.

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Statistical Analysis

Where applicable data were analyzed using the unpaired Student’s t test, and Kruskal-Wallis one-way analysis of variance taking P < 0.05 as the level of significance.

RESULTS

Kinetics of ODC and Tyr-k Activity after AOM. After one s.c. injection of AOM (20 mg/kg) colonic mucosal ODC activity began to drop to a nadir at 24 h that was 67% lower than at the beginning of the experiment. However, at 3 days after the initial injection ODC activity had increased 10-fold. It ultimately peaked on Day 5, 600% higher than at Day 1. By Day 7, colonic mucosal ODC activity had returned to basal levels (Fig. 1A).

Tyr-k activity followed a similar pattern dropping to a nadir 30% below basal levels on postinjection Day 1 and rising to a peak on Day 5, 75% higher than on Day 1. Tyr-k activity also returned to basal levels on Day 7 (Fig. 1B).

Effect of Oral Calcium on ODC and Tyr-k Activity. In vivo studies of age-weight matched, pair-fed animals given supplemental calcium carbonate and sacrificed on the 5th day after s.c. injection of AOM showed a 39% reduction in colonic mucosal ODC activity (P < 0.005) compared to AOM treatment alone. Oral calcium carbonate alone had no significant effect on colonic mucosal ODC activity (Fig. 2A). A similar decrease of 37% (P < 0.025) in Tyr-k activity was observed with oral...
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Fig. 1. Acute effect of administration of AOM on colonic mucosal ODC (A) and Tyr-k (B) activity. Groups of Fischer 344 rats were given one s.c. injection of AOM (20 mg/kg) and sacrificed at 0, 1, 3, 5, and 7 days after injection (3-day point not available for B). Colonie mucosal scrapings were homogenized for ODC measurement while membranes were prepared for Tyr-k measurement. Age-matched control animals showed no significant change in either ODC or Tyr-k activity from 0-h values. For ODC, results are expressed in pmol $^{14}\text{C}_2\text{O}_2$ released/mg protein/hr. Tyr-k activity is expressed in pmol $^{32}\text{P}$ incorporated/mg protein. Points, mean from three animals; bars, SEM.

calcium (Fig. 2B). In another set of experiments, doubling the added calcium from 50 to 100 mg/day resulted in no further suppression of ODC or Tyr-k over that obtained with 50 mg added calcium (results not shown). Although absolute values for ODC and Tyr-k in each experiment showed marked inter-assay variability, the magnitude of suppression induced by added calcium remained constant at approximately 35-45%.

Expression of Tyrosine-phosphorylated Membrane Proteins.

To further understand the mechanisms associated with the early induction of neoplasia with AOM, colonic mucosal membrane preparations from the animals in the first in vivo calcium experiment were subjected to SDS-polyacrylamide gel electrophoresis and KOH treatment to retain only tyrosine-specific proteins. Fig. 4 reveals that AOM increased the rate of expression of the tyrosine specific proteins with $M_r$ 57,000 and 59,000. However, the expression of these proteins was also attenuated by the additional dietary calcium, as evidenced by a 36% reduction in band intensity by densitometry. (Areas under curve: control, 3257; calcium, 2530; AOM, 8799; AOM + CaCO$_3$, 5617.)

Fig. 2. Attenuation of peak colonic mucosal ODC (A) and Tyr-k (B) by supplemental dietary calcium. Pair-fed Fischer 344 rats were sacrificed on the 5th day after a single injection of AOM (20 mg/kg). Test animals consumed rat chow containing 130 mg/day calcium, while control animals received sterile water injection and powdered rat chow containing 80 mg/day calcium. Scrapings were homogenized for ODC measurement while membranes were prepared for Tyr-k measurement. Animals receiving supplemental calcium showed a 39% reduction in colonic mucosal ODC activity ($P < 0.005$) and a 37% reduction in Tyr-k activity ($P < 0.025$) compared to AOM treatment alone. Animals receiving calcium carbonate and water injection showed no significant difference when compared to control animals receiving no added calcium. ODC results are expressed in pmol $^{14}\text{C}_2\text{O}_2$ released/mg protein/hr while Tyr-k results are expressed as pmol $^{32}\text{P}$ incorporated/mg protein. Points, mean from four to five animals; bars, SEM.

In vitro studies were accomplished to demonstrate that the effect of calcium on carcinogen induced ODC (Fig. 3A) and Tyr-k (Fig. 3B) activity was direct and not dependent on systemic factors. After the optimal concentrations for MAOM and CaCl$_2$ and DFMO were determined, explants incubated with these agents alone or in combination revealed that ODC activity induced by MAOM was suppressed 38% ($P < 0.025$) by CaCl$_2$ while the suppression by DFMO was only 15% (Fig. 3A). Tyr-k activity induced by MAOM was suppressed 45% ($P < 0.001$) by CaCl$_2$ and 60% by DFMO (Fig. 3B).

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Fig. 3. Inhibition of ODC (A) or Tyr-k (B) activity in MAOM treated colonic mucosal explants by CaCl2 and DFMO. Mucosal biopsies were incubated on paper rafts in Dulbecco's modified Eagle's medium plus 10% dialyzed fetal bovine serum with combinations of MAOM, CaCl2, DFMO, or water (controls). Significant inhibition was seen in ODC with CaCl2 (2 μmol/ml, P < 0.025) and in Tyr-k with CaCl2 (P < 0.001) and DFMO (2 nmol/ml, P < 0.001) when compared to MAOM alone. ODC values are expressed as pmol 14CO2 released/mg protein/h; Tyr-k values are expressed as pmol 32P incorporated/mg protein. Points, mean of four to six animals; bars, SEM.

DISCUSSION

In the present investigation we have utilized the rodent AOM model as a means for evaluating the potential chemoprotective role of calcium in colon cancer. We have demonstrated that modest amounts of supplemental calcium have a significant suppressive effect on the hyperproliferative state induced by AOM in vivo and MAOM in vitro during the latent period which precedes actual tumor formation. Shortly after injection, AOM produced a hyperproliferative mucosa as evidenced by increasing stimulation of ODC and Tyr-k activities. The maximal stimulation occurred on the 5th day after injection with activity returning to basal levels on the 7th day. In previous work using 2 mg/kg of AOM we have shown that ODC peaks at 7 days after initial injection (2). However, here we chose a much higher dose of AOM, 20 mg/kg, to accentuate the proliferative changes induced during the first week after injection. Although the precise effect of AOM is unknown, Zedeck et al. (19) have observed karyorrhexis in crypts of duodenum and colon 6 h after treatment with MAOM (a metabolite of AOM). Furthermore, DNA synthesis was observed to be rapidly inhibited, decreasing by 75% at 6 h and persisting for about 24 h, after which DNA synthesis began to recover. We have observed a similar decrease in Tyr-k and ODC activity during the first 24 h after AOM injection, which subsequently increased to a maximally stimulated level on the 5th day after injection. This may reflect an injury-recovery pattern. Calcium, on the other hand, does not appear to produce direct injury to cells. Our recent in vitro studies of colonic mucosal explants treated with high concentrations of calcium revealed no histological evidence of cell disruption (20). Thus, the decrease in ODC and Tyr-k activity observed with supplemental calcium is not likely to be due to a cytotoxic effect of calcium.

In addition, our present in vitro studies with colonic mucosal explants show that calcium exerts a direct effect and does not support a role for vitamin D metabolism or other systemic factors (21). Although a plausible explanation for the antiproliferative effect of calcium involves the binding of bile acids by calcium (8, 22, 23), our in vitro studies do not confirm this, since bile acids were not added. In fact, calcium has demonstrated antiproliferative properties for several other epithelial cells including mammary (24), esophageal (25), bronchial (26), and urothelial cells (27), in the absence of bile acids.

In the present study we have not addressed the question as to whether treatment with calcium after AOM will eventually result in fewer or no tumors. However, Appleton et al. (28) have recently demonstrated a 50% reduction in tumors in rats with small bowel resection treated with AOM and given calcium lactate in drinking water. An additional recent study reports the complete inhibition of skin tumors in mice treated with an epidermal carcinogen and calcium glucarate (29). Taken together, the results support the contention that dietary calcium may have a chemoprotective function.

The relationship between hyperproliferative colonic mucosa and neoplastic transformation is not fully understood, but is believed to involve the multistep induction of ODC (2). ODC, which is the rate-limiting enzyme in polyamine synthesis, plays an important role in normal and neoplastic cell proliferation (30) and is associated with the tumor-promoting ability of a variety of agents (2). We have observed the induction of ODC

Fig. 4. SDS-polyacrylamide gel electrophoresis autoradiographic analysis of 32P-labeled colonic mucosal proteins (tyrosine-specific phosphorylation) in rats 5 days after s.c. injection of AOM (20 mg/kg) while being pair-fed rat chow containing increased calcium (130 mg/day) or receiving the same with water injection (calcium-fed controls) or powdered chow minus added calcium (controls). Colonic mucosal homogenates containing the same amount of protein (50 μg) from each group was incubated with 1 γ32PATP at 0–2°C for 30 min. After termination of the reaction, the samples were electrophoresed on 7.5% polyacrylamide slab gel (1.5 mm) containing 0.1% SDS. The polyacrylamide gel containing the electrophoresed proteins was then incubated in 1 M KOH at 56°C for 2 h (to hydrolyze phosphothreonine and phosphoserine but not phosphotyrosine bonds), washed, dried, and finally exposed to a Kodak X-Omat AR film at −70°C for 2 days. Densitometric analysis of autoradiographs revealed a 36% reduction in intensity of the M, 57,000 and 59,000 bands.

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as well as Tyr-k in colonic mucosa of rats treated with AOM or its metabolite, MAOM, and have used this as a model to study the antiproliferative properties of calcium. Tyrosine kinases are also of interest because they have been implicated in cellular proliferation, differentiation, and neoplastic transformation (9). Recently, we have observed that highly proliferative tissues such as the gastric mucosa possess higher levels of Tyr-k than relatively stable organs like the liver and pancreas (16). Our current data show that AOM or MAOM induction of colonic mucosal proliferative activity and its suppression by calcium is associated with parallel alterations in Tyr-k activity. Furthermore, these changes are reflected in tyrosine-specific phosphorylation of two mucosal membrane proteins with apparent molecular size of M, 57,000 and 59,000. These observations suggest that tyrosine kinases in general and tyrosine-specific phosphorylation of certain proteins may play an important role in the AOM or MAOM regulation of colonic neoplasia. This is strengthened by our recent observation of increased ODC and Tyr-k activity in rectal mucosal of patients with known colonic polyps (3).

REFERENCES

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