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

Freda L. Arlow, Steven M. Walczak, Gordon D. Luk, and Adhip P. N. Majumdar

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 as well as tyrosine specific phosphorylation of membrane proteins were determined. 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 two distinct colonic mucosal membrane proteins with $M_r$ of 57,000 and 59,000. Again, dietary calcium suppressed the stimulation.

In the third set of experiments, organ culture was utilized. Methylazoxymethanol, the active metabolite of AOM, was used instead of AOM in this part of study. Four hour exposure of mucosal explants to methylazoxymethanol (1 $\mu$g/ml) resulted in a significant (20–30%) increase in ODC and Tyr-k activity when compared to controls. Addition of either $CaCl_2$ (2 $\mu$mol/ml) or difluoromethylornithine (2 $\mu$mol/ml) the irreversible inhibitor of ODC, significantly suppressed the methylazoxymethanol-induced activity of both ODC and Tyr-k.

We conclude that calcium may have a chemoprotective role and tyrosine kinases may have a regulatory role in the early stages of AOM induction of colon cancer.

INTRODUCTION

AOM, an established rodent colonic carcinogen, produces tumors in up to 100% of treated animals (1) and is associated with the multistep induction of ODC (2). Similar increases in colonic mucosal ODC (3) and DNA synthesis (4), indicative of a hyperproliferative mucosa, have been reported in patients at risk for colon cancer. Lipkin and Newmark (5) observed that calcium might bind suppressed by calcium. AOM also produced an increase in the rate of tyrosine specific phosphorylation of two distinct colonic mucosal membrane proteins with $M_r$ of 57,000 and 59,000. Again, dietary calcium suppressed the stimulation.

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Tyrosine Kinases and Tyrosine-specific Phosphorylation of Proteins

prepared from colonie mucosa homogenized in Tyr-k buffer containing albumin as the standard. was determined by the method of Bradford (14), utilizing bovine serum

At the end of the second incubation, the Eppendorf vials were removed, scintillation vials were quickly recapped and reincubated for 60 min.

hydroxide in methanol were placed on the bottom of the scintillation vial. The scintillation vials were tightly capped and incubated at 37°C ('.

period, expiants were removed and homogenized in ODH buffer or 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, DFM0, 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 and colonie tissue was obtained from the abdominal cavity. After careful cleansing in ice-cold saline, four 2 x 2-mm mucosal biopsies, one from each quadrant, were placed into each 60-mm Petri dish. Following a 4-h equilibration period in a 37°C CO2/oxygen (5/95%) incubator, the media were replaced, saline, four 2 x 2-mm mucosa! biopsies, one from each quadrant, were obtained 50 mM HEPES (pH 7.4), 1.05 mM EDTA, 0.25 mM pyridoxol-5-phosphate, and 1.0 mM dithiothre.

ODC Activity.

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, DFM0, the specific, irreversible inhibitor of ODC was used to show that ODC rather than nonspecific decarboxylases was being measured in vitro (11).

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Biochemical Assays

ODC Activity.

ODC activity in colonie 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 mM EDTA, 0.025 mM pyridoxol-5-phosphate, and 1.0 mM diithiohretol. The enzyme activity in colonie 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 mM EDTA, 0.25 mM pyridoxol-5-phosphate, 1.0 mM diithiohretol, 0.144 µCi of L-[1-14C]ornithine (40–60 mCi mmol; NEN, Boston, MA) in 0.01 N HCl. A 10-µl sample of the supernatant extract of the tissue homogenate was mixed with 10 µl of assay reagent in a 1.5-ml Eppendorf vial. This was placed inside a test, and Kruskal-Wallis one-way analysis of variance taking P < 0.05 as the level of significance.

RESULTS

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) colonie 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, colonie 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 colonie mucosal ODC activity (P = 0.005) compared to AOM treatment alone. Oral calcium carbonate alone had no significant effect on colonie mucosal ODC activity (Fig. 2A). A similar decrease of 37% (P < 0.025) in Tyr-k activity was observed with oral
CALCIUM ATTENUATION OF AOM-INDUCED ODC AND Tyr-k

**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). Colonic 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 CO$_2$ released/mg protein/hr. Tyr-k activity is expressed in pmol $^{32}$P incorporated/mg protein. Points, mean from three animals; bars, SEM.

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%.

**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 CO$_2$ released/mg protein/hr while Tyr-k results are expressed as pmol $^{32}$P incorporated/mg protein. Points, mean from four to five animals; bars, SEM.

**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.)
Calcium attenuates AOM-induced ODC and Tyr-k activity in colon mucosa explants. 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. Point, 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 suppressant 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 multisite 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...
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 tyrone 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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