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Effects of D,L-2-Difluoromethylornithine and Indomethacin on Mammary Tumor Promotion in Rats Fed High n-3 and/or n-6 Fat Diets[1]

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ABSTRACT

Virgin female Sprague-Dawley rats (50 days of age) were administered a single intragastric 10-mg dose of 7,12-dimethylbenz(*a*)anthracene (DMBA). Twenty-one days later they were placed on diets containing either 20% corn oil (CO), 15% menhaden oil plus 5% corn oil (MO + CO), 20% CO plus 0.5% w/w of the irreversible ornithine decarboxylase inhibitor, D,L-2-difluoromethylornithine (CO + DFMO), 20% CO plus 0.004% w/w of the cyclooxygenase inhibitor indomethacin (CO + INDO), 20% CO + 0.004% INDO + 0.5% DFMO (CO + INDO + DFMO), or 15% MO + 5% CO + 0.5% DFMO (MO + CO + DFMO). The incidence of DMBA-induced mammary tumors was significantly reduced in rats fed diets containing DFMO but not in rats fed the diet containing indomethacin. Incidences of mammary tumors at 16 weeks post-DMBA were 86% in rats fed the CO diet, 83% in rats ingesting the diet containing CO + INDO, 28% in rats fed CO + DFMO, 32% in rats fed diet containing CO + INDO + DFMO, 59% in rats fed the MO + CO diet, and 24% in rats fed the MO + CO + DFMO diet. The average number of tumors and tumor burden per tumor-bearing rat were reduced and tumor latency was increased in all rats fed diets containing DFMO. Body weight gain, but not food intake, of rats fed the 20% fat + 0.5% DFMO diets was significantly less than in rats fed the 20% fat diets. Prostaglandin E and leukotriene (LTB_4) syntheses, ODC activity and mammary tumorigenesis were significantly inhibited by feeding the diet containing menhaden oil or by adding 0.5% DFMO to any of the high fat diets. Feeding a 20% CO diet containing 0.004% INDO significantly reduced prostaglandin synthesis and ODC activity and increased LTB_4 synthesis of mammary tumors but did not inhibit mammary tumorigenesis. This study suggests that the 5-lipoxygenase product LTB_4 may be involved in mammary tumor production. Whereas a decrease in LTB_4 appears to be associated with a decrease in tumorigenesis, an increase (as seen in the indomethacin group) was not associated with any change in the tumorigenic response.

INTRODUCTION

The promotion of carcinogen-induced, spontaneous, and transplantable mammary tumors is enhanced in rats fed increasing levels of the n-6 fatty acid linoleate (1). This fatty acid may, in part, promote tumor growth and development by increasing synthesis of eicosanoids, particularly arachidonic acid products that have been shown to enhance cell division, depress immune responses and promote tumor growth (2). Alternatively, diets containing high levels of n-3 fatty acids have been shown to inhibit development of several carcinogen-induced cancers, action which appears to be mediated through its ability to inhibit arachidonic acid metabolism by both cyclooxygenase and lipoxygenase (2, 3).

Indomethacin, an inhibitor of cyclooxygenase, has been shown to inhibit DMBA[3]-induced mammary carcinogenesis in both the early and late stages. In the early stage, indomethacin appears to modulate carcinogen metabolism through the inhibition of prostaglandin H synthase (4–6). The possible late-stage effects of indomethacin may be explained by the fact that it inhibits cell proliferation in a variety of normal and neoplastic mammalian cells *in vitro* (7–9). Furthermore, it has been demonstrated that indomethacin can inhibit the growth of DMBA-induced mammary tumors *in vivo* (6) as well as the stimulatory effect of fat on DMBA-induced mammary tumor development (10–12). In contrast to these studies, others have reported that indomethacin did not inhibit tumor growth (13–16) although PGE levels were significantly reduced (13, 14, 16).

In the multistage carcinogenesis model for mouse skin, phorbol esters have been shown to inhibit intercellular communication (17, 18) through alterations in biochemical responses involving phospholipid metabolism, fatty acid and eicosanoid synthesis, and activation of ODC (19–23). Furthermore, the activity of ODC correlates with eicosanoid synthesis (23, 24) and is elevated in various proliferative cell systems including promotional stages I and II of neoplastic growth (25–27). The biochemical mechanism of ODC induction is not fully understood. However, it appears that phospholipase A_2 stimulation (19, 28) and resultant production of arachidonate metabolites (*i.e.*, cyclooxygenase and lipoxygenase products) are involved (24, 28, 29). Moreover, indomethacin has been shown to block the induction of ODC (23, 30).

ODC catalyzes the formation of putrescine from ornithine, the immediate precursor of polyamines spermidine and spermine (31). Polyamines are essential for cell growth and proliferation of several tissues of the body, including the breast (32, 33). Accumulation of these polycationic amines appears essential for rapid neoplastic growth (31, 34). Therefore, interference with polyamine accumulation can inhibit tumor development (31, 34, 35). In particular, inhibition of ODC activity with a specific enzyme-activated irreversible inhibitor, *e.g.*, D,L-2 difluoromethylornithine (DFMO), inhibits growth of chemically induced mammary tumor during promotion (36–39).

Since both ODC activity and eicosanoid levels are increased in cancerous tissues and blockade of their activation/synthesis is associated with reduced tumor incidence, these biochemical processes may be phenomena that are mechanistically related to mammary tumor promotion by high n-6 polyunsaturated fat diets in rats. Therefore, the objectives of this study were to block one or more events in the mammary tumor promotion and progression mediated by diets containing a high level of linoleate in the form of 20% corn oil, in the following manner: (*a*) by adding to the diet high levels of n-3 fatty acids, as found in menhaden oil, to competitively block cyclooxygenase and lipoxygenase; (*b*) by adding to the diet 0.004% indomethacin

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz(*a*)anthracene; DFMO, D,L-2-difluoromethylornithine; ODC, ornithine decarboxylase; CO, corn oil; MO, menhaden oil; INDO, indomethacin; PGE, prostaglandin E; LTB_4, leukotriene B_4; i.g., intragastric; EFA, essential fatty acids.

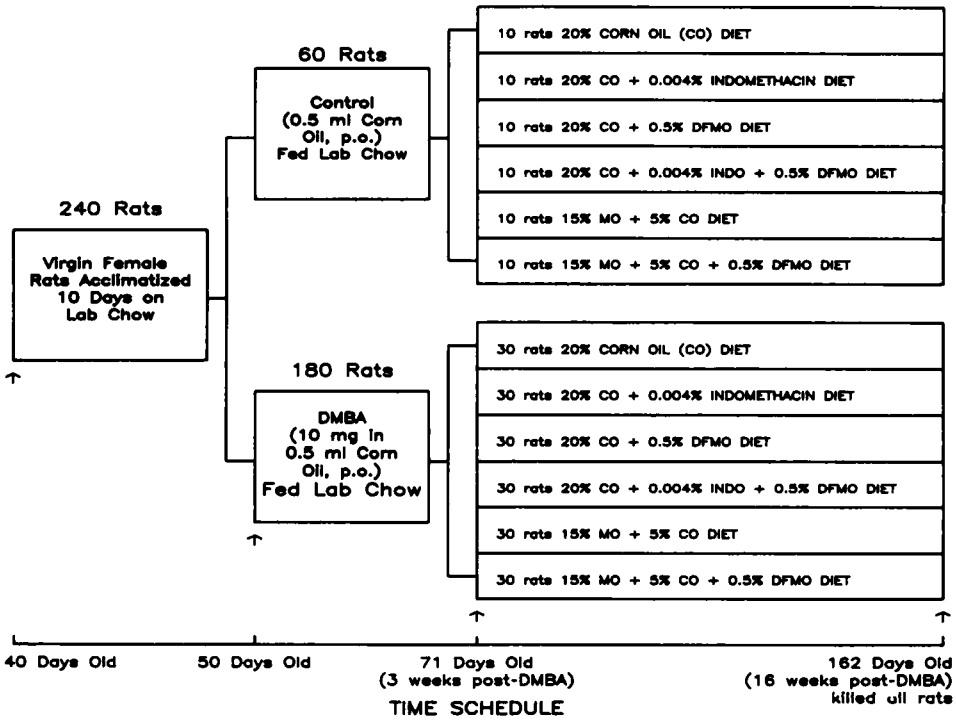

Fig. 1. Schedule of diets and treatments. Mammary tumors were initiated in 50-day-old virgin female Sprague-Dawley rats with 10 mg of DMBA administered i.g. At 3 weeks post-DMBA, promotion by high fat diets was begun. The high fat diets were 20% corn oil (CO), 20% corn oil plus 0.004% indomethacin (CO + INDO), 20% corn oil plus 0.5% difluoromethylornithine (CO + DFMO), 20% corn oil plus 0.004% INDO plus 0.5% DFMO (CO + INDO + DFMO), 15% menhaden oil plus 5% CO (MO + CO), 15% MO + 5% CO + 0.5% DFMO (MO + CO + DFMO). All rats were killed at 16 weeks post-DMBA.

to inhibit arachidonic acid metabolism by cyclooxygenase; (*c*) by adding to the diet containing n-3 and/or n-6 fatty acids, 0.5% DFMO to inhibit ODC activity; (*d*) by administering both indomethacin and DFMO in a 20% corn oil diet to establish whether additive or synergistic effect will result from inclusion of two inhibitors that act on tumor promotion by different but interrelated mechanisms.

MATERIALS AND METHODS

Diets, Feeding, and Tumor Induction. Two hundred forty female Sprague-Dawley rats, 40 days old, were purchased from Charles River Laboratories, Wilmington, MA. All animals were housed (four/cage) in suspended metal cages in a temperature-regulated (23 ± 0.5°C) and light controlled (12-h light/dark cycle) room and fed standard rat chow (Ralston Purina Co., St. Louis, MO). At 50 days of age, 180 rats were given a single dose of 10 mg of DMBA (Sigma Chemical Company, St. Louis, MO) via intragastric intubation in 0.5 ml corn oil. Sixty sham-treated rats each received 0.5 ml corn oil. After DMBA administration, the animals were randomly placed in 10- × 16-inch plastic cages on Absorb Dri Litter and housed three per cage for the duration of the experiment. Six rats were found dead 48 h after DMBA administration. As outlined in Fig. 1, at 21 days post-DMBA administration, the rats were randomly divided into six groups of 29 rats each and fed six diets: (*a*) 20% corn oil diet (CO); (*b*) 20% CO diet containing 0.004% (w/w) indomethacin (CO + INDO); (*c*) 20% CO diet containing 0.5% (w/w) DFMO (CO + DFMO); (*d*) 20% CO diet containing 0.004% INDO and 0.5% DFMO (CO + INDO + DFMO); (*e*) diet containing 15% MO and 5% CO (MO + CO); and (*f*) diet containing 15% MO + 5% CO + 0.5% DFMO (MO + CO + DFMO). Sham-treated animals were divided into six groups of 10 rats each and fed the same diets. The DFMO level chosen was based on method and results reported by Thompson *et al.* (38). In their study, 0.5% DFMO in the drinking water delivered approximately 130 mg of DFMO/day/rat, and significantly reduced tumor incidence, average number of cancers and tumor weight per rat, and increased tumor latency without producing systemic toxic effects. In the present study, feeding 0.5% DFMO in the diet delivered approximately 75 mg of DFMO/day/rat, which is 57% of the dose given by Thompson *et al.* (38). The rats remained on their respective treatments without interruption until the experiment was terminated 112 days after DMBA administration.

Twenty kg batches of each of the 20% fat diets were prepared by ICN Nutritional Biochemical, Cleveland, OH. The diets were cold pressed into jumbo pellets, sealed under nitrogen, and shipped frozen. One kg bags of pellets were placed in Seal-N-Save bags, flushed with nitrogen, sealed, and stored frozen at −20°C until used. Measured amounts of frozen diet (20 g/rat/day) were placed in the cage food dispenser each morning after uneaten pellets were discarded. Food consumption was determined over an 8-week period beginning 6 weeks post-DMBA. Food intake was measured for one 24-h period each week by weighing the uneaten food per cage, from which the approximated food consumption per rat per day was calculated. Basic diet formulation was based on the AIN 76 semipurified rat diet and has been reported previously (40). The fatty acid composition of the oils in the diets is given in Table 1.

α-Tocopherol was added with the AIN vitamin mix so that each diet contained 310 IU of vitamin E/kg of diet. All diets were isocaloric and contained the recommended level of nutrients with a constant amount per kilocalorie of casein, fat, carbohydrate, salts, vitamins, and fiber. Corn oil (5%) was added to the menhaden oil diet to provide adequate

Table 1 *Fatty acid composition of dietary oils*

The values for corn oil were supplied by ICN Nutritional Biochemicals Corp., Cleveland, OH and the values for menhaden oil were supplied by Zapata Haynie Corp., Reedville, VA.

	% of total fatty acids	
Fatty acid	Corn oil[a]	Menhaden oil[b]
14:0[c]	11.2	8.35
16:0		15.17
16:1		11.62
16:2		2.37
16:3		1.96
18:0	2.1	2.67
18:1	25.0	9.5
18:2 n-6	59.9	1.81
18:3 n-3		1.82
18:4		3.47
20:1		1.32
20:4 n-6		2.30
20:5 n-3		16.03
22:5		3.92
22:5 n-3		10.83
Others	0.1	4.37

[a] Purchased from Seaway Foods, Cleveland, OH.
[b] Supplied by Zapata Haynie Corp., Reedville, VA.
[c] Carbon chain length: number of double bonds.

EFA. The 20% CO and the 15% MO + 5% CO diets contained 12% EFA and 3.62% EFA, respectively. The menhaden oil was provided by Dr. Tony Bimbo, Zapata Haynie Corp., Reedville, VA, and DFMO by Dr. Peter McCann, Merrell Dow Research Institute, Cincinnati, OH. Indomethacin was purchased from Sigma Chemical Co., St. Louis, Missouri. Corn oil was purchased from Seaway Foods, Inc., Cleveland, OH. Indomethacin and DFMO were added to the vitamin mix during diet preparation.

Since polyunsaturated oils may oxidize during preparation, storage and feeding of experimental diets, peroxidation and anisidine values were measured in the stored frozen diets and after 24 h at room temperature. The oils were extracted from 100-g samples of each diet with glacial acetic acid/chloroform (3:2, v/v). The extract was flash-evaporated at 45°C to give pure oils which were then measured for peroxidation and anisidine values (41, 42). All peroxide and anisidine values were within the acceptable range for commercial oils as used by Zapata Haynie Corporation, Reedville, VA.[4]

Antemortem Protocol, Necropsy, and Histopathology. Animals were monitored daily for general health, and no signs of toxicity for either indomethacin or DFMO were observed. The rats were weighed weekly and palpated for the presence of tumors. The location of each tumor was noted. One rat died during the experiment. At 16 weeks post-DMBA, all surviving rats were killed using CO_2 gas. A complete necropsy examination was performed on each rat. Particular attention was paid to internal organs for signs of toxicity from DMFO or indomethacin. Mammary tumors were removed, weighed and coded as to location. Two sections from each tumor were placed in 10% buffered formalin. The tumors were then embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin. Each tumor was described histologically and a diagnosis was ascertained according to the criteria of Van Zweiten (43). Only histologically confirmed malignant mammary tumors were used in data analysis.

Eicosanoid Analyses. Malignant mammary tumors from DMBA-treated rats and fat pads of sham-treated animals were finely minced and an appropriate tissue aliquot was incubated in 1 ml Krebs buffer (with millimole/liter concentrations of NaCl, 118.1; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.9; KH_2PO_4, 1.2; $NaHCO_3$, 2.5; and dextrose, 5.6; pH 7.4) for 1 h at 37°C under 95% O_2/5% CO_2. The reaction was stopped by acidification to pH 3 with 0.8 M phosphoric acid and the tissue-buffer mixture was extracted once with 4 volumes of ethyl acetate by vigorous shaking (44). The organic phase was removed, and 50 μl of 0.1 M Tris-HCl buffer (pH 7.4) was added. The organic phase was evaporated under nitrogen, and the residue was stored sealed under nitrogen at −80°C. The samples were appropriately diluted in assay buffer and the stable metabolite of prostaglandin E, bicyclic PGE, and leukotriene B_4 (LTB_4) were analyzed by radioimmunoassay using kits purchased from Amersham Corp., Arlington Heights, IL. The sensitivities of the antibodies are 1.6 pg for LTB_4 and 43 pg for PGE. The PGE_2 antibody from Amersham showed 100% cross-reactivity with PGE_1, thus PGE_2 is designated as PGE indicating that both PGE_2 and PGE_1 were assayed in the tissue extracts when Amersham PGE_2 radioimmunoassay kit was used. The LTB_4 antibody as provided by Amersham was specific for LTB_4. Eicosanoid values are expressed as nanograms of eicosanoid synthesized per gram of tissue per hour. Eicosanoid production was proportional to incubation time up to 90 min.

Ornithine Decarboxylase Assay. The tissue was quickly excised, placed on ice and homogenized in 3 volumes of 50 mM phosphate buffer, pH 7.2, containing 1 mM dithiothreitol and 1 mM EDTA using a Potter-Elvehjem homogenizer. Postmicrosomal cytosol fractions were prepared by differential centrifugation, and ODC was assayed by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine hydrochloride, essentially as described by Russell and Snyder (45). Briefly, the assay mixture contained 50 mM phosphate buffer, pH 7.2, 0.3 mM pyridoxal phosphate, 4.0 mM dithiothreitol, 1.0 mM EDTA, 0.4 mM L-ornithine, 0.5 μCi of L-[1-^{14}C]ornithine hydrochloride (58.6 mCi/mmol) and 0.1–0.6 ml of tissue supernatant in a final volume of 1 ml. All components except substrate were incubated at 37°C for 10 min prior to the addition of substrate. Incubations were routinely carried out for 60 min at 37°C. The reaction was stopped by addition of 0.4 ml 2 M citric acid and incubations were continued for an additional 60 min to insure complete absorption of $^{14}CO_2$ by β-phenylethylamine. All enzyme activities were corrected against a blank prepared by heating the tissue supernatants at 100°C for 10 min. Activity was expressed as nanomoles CO_2 released/h/mg protein. Protein was determined by the method of Lowry *et al.* (46) using serum albumin as the standard.

Statistical Analysis. Differences in cancer incidences among groups were evaluated by the Chi-square procedure without continuity correction. Comparisons of body weights, tumor latency, eicosanoid synthesis and ODC activity among groups were evaluated by one-way analysis of variance, and significant differences were accepted at $P < 0.05$. Comparisons of number of tumors and tumor burden per tumor-bearing rat among groups were made by one-way analysis of variance following square-root transformation. Statistical significance levels were set at $P < 0.05$.

RESULTS

Animals and Diets. The effects of high fat diets, DFMO, and indomethacin on weight gain and final body weights are shown in Fig. 2 and Table 2. The addition of indomethacin and/or DFMO to diets containing corn oil or menhaden oil had no significant effects on food consumption. DMBA-treated rats fed MO + CO ate less than those fed CO alone but their weight gains (Fig. 2) and final body weights (Table 2) were similar. However, body weight gain in DMBA-exposed animals were uniformly depressed by simultaneous incorporation of DFMO in the diet (Fig. 2). The weight gain of sham-treated rats was similar to those treated with DMBA in that the final body weights of both DMBA- and sham-treated rats were less in rats fed DFMO than in animals not fed DFMO (Table 2).

Diet and Tumorigenesis. Palpable mammary tumor incidences during the experiment are shown in Fig. 3. Rats fed either CO or CO + INDO had significantly higher palpable mammary tumor incidences than rats fed diets containing 0.5% DFMO or the MO + CO diet. The final tumor incidence at 16-weeks post-DMBA was highest in the CO and CO + INDO groups, 86 and 83%, respectively, while the lowest incidences were noted in rats receiving 0.5% DFMO in their diet (Table 3). Feeding the diet containing 15% MO + 5% CO reduced tumor incidence by 31% compared to feeding the 20% CO diet. The addition of 0.5% DFMO reduced tumor incidence 72% in animals fed MO and 67% in rats fed CO compared to feeding

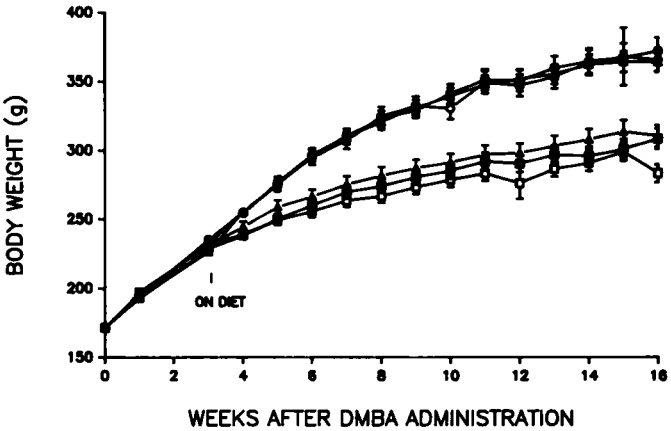

Fig. 2. Effect of diets and treatments on body weight gain of DMBA-treated rats. Rats were maintained on a Purina Lab Chow regimen until 71 days of age. At 50 days of age, each rat was given 10 mg of DMBA i.g. Lab chow was continued until 71 days (3 weeks post-DMBA) at which time the tumor-promoting high fat diets and treatments were begun and continued until 162 days (experiment terminated). ○, 20% corn oil (CO); ●, 20% CO + 0.004% INDO (CO + INDO); △, 20% CO + 0.5% DFMO (CO + DFMO); □, 20% CO + 0.004% INDO + 0.5% DFMO (CO + INDO + DFMO); ▲, 15% menhaden oil plus 5% corn oil (MO + CO); ■, 15% MO plus 5% CO + 0.5% DFMO (MO + CO + DFMO).

[4] A. P. Bimbo, personal communication.

Table 2 *Effect of diets and treatments on food intake during the experiment and final body weights at 16 weeks post-DMBA of sham- and DMBA-treated animals*

Diet[a]	Sham-treated			DMBA-treated		
	No. of rats	Body weight (g)	Food intake (g/rat/day)[b]	No. of rats	Body weight (g)[c]	Food intake (g/rat/day)
20% CO	10	366.4 ± 9.2[d]	14.2 ± 0.1[d]	29	365.5 ± 8.4[d]	14.8 ± 0.3[b]
20% CO + INDO	10	385.7 ± 20.7[d]	15.5 ± 0.2[e]	29	372.0 ± 9.6[d]	14.4 ± 0.4[d, e, f]
20% CO + DFMO	10	316.9 ± 9.2[e]	15.0 ± 0.3[e, f]	29	310.4 ± 7.9[e]	14.0 ± 0.2[d, e, f]
20% CO + INDO + DFMO	10	291.2 ± 10.3[e]	13.8 ± 0.3[d]	28	293.2 ± 6.4[e]	13.4 ± 0.6[e, f]
15% MO + 5% CO	10	379.7 ± 9.5[d]	14.5 ± 0.2[d, f]	29	363.8 ± 7.4[d]	13.2 ± 0.7[f]
15% MO + 5% CO + DFMO	10	300.9 ± 8.6[e]	14.3 ± 9.4[d]	29	308.4 ± 7.5[e]	14.5 ± 0.4[d, e]

[a] CO, corn oil; INDO, 0.004% indomethacin; DFMO, 0.5% D,L-2-difluoromethylornithine; MO, menhaden oil.
[b, c] Food intake values are the average of eight measurements over an 8-week period beginning 6 weeks post-DMBA. The final body weight values are inclusive of tumor weight.
[d, e, f] Mean ± SEM. Comparisons among the dietary groups were made using one-way analysis of variance. Significant differences ($P < 0.05$) are noted by different superscripts.

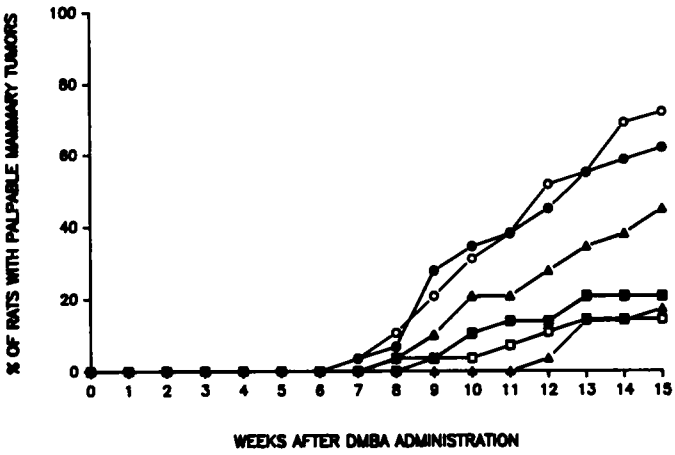

Fig. 3. Effect of diets and treatments on palpable mammary tumor incidences. Virgin female Sprague-Dawley rats were given 10 mg DMBA i.g. at 50 days of age. Mammary tumor-promoting high fat diets were begun 3 weeks post-DMBA and were continued until 162 days of age (experiment terminated). ○, 20% CO (CO); ●, 20% CO + 0.04% INDO (CO + INDO); △, 20% CO + 0.5% DFMO (CO + DFMO); □, 20% CO + 0.04% INDO + 0.5% DFMO (CO + INDO + DFMO); ▲, 15% Menhaden oil + 5% CO (MO + CO); ■, 15% MO + 5% CO + 0.5% DFMO (MO + CO + DFMO).

CO alone. However, feeding 0.004% indomethacin did not change tumor incidence compared to feeding 20% CO diet, nor did adding 0.004% INDO along with 0.5% DFMO to the 20% CO diet enhance the inhibition afforded by DFMO.

The tumor multiplicity (number of tumors/tumor bearing rat) and tumor burden (weight) were lowest in the rats which received 0.5% DFMO in their diets (Table 3). The average time (in weeks) for the appearance of the first tumor (tumor latency) in the CO + DFMO group was significantly longer than in the CO and CO + INDO groups (Table 3).

Eicosanoid and ODC Analyses. The effects of the high fat diets, with or without inhibitors, on eicosanoid synthesis and ODC activity of sham- and DMBA-treated rats are shown in Tables 4 and 5. PGE production rates in mammary tumors were highest in CO-fed rats. Indomethacin and DFMO each inhibited PGE synthesis, and when both were included in the CO diet, the inhibition was additive. Incorporation of MO in the diet inhibited PGE synthesis to a greater extent than either indomethacin or DFMO, and this inhibition was enhanced by incorporation of DFMO into the MO diet. The addition of indomethacin to the CO diet appeared to enhance LTB_4 synthesis while depressing PGE synthesis and ODC activity. In contrast, DFMO and MO significantly depressed LTB_4 synthesis of mammary tumors.

Ornithine decarboxylase activity was not detected in the mammary fat pads of sham-treated animals (Table 4). However, ODC activity in the tumors of CO-fed rats (Table 5) was significantly higher ($P < 0.05$) than in the tumors of rats fed any of the other five diets. ODC activity was depressed by 36 and 25% in tumors of rats fed indomethacin or menhaden oil, respectively. The incorporation of DFMO suppressed ODC activity by 75%, 81%, 91% for CO + DFMO, CO + INDO + DFMO and MO + DFMO, respectively, suggesting that suppression was additive with that induced by indomethacin or menhaden oil (Table 5). When ODC activities were compared among all dietary groups, no significant differences were observed among the DFMO dietary groups. However, when ODC activities of only tumors from rats fed diets containing DFMO were compared, the MO + CO + DFMO diet significantly ($P < 0.05$) reduced ODC activity compared to feeding CO + DFMO or CO + INDO + DFMO (Table 5). When the Pearson correlation coefficient was determined between mammary tumor incidences and ODC activities, the value was $r = 0.92$ ($P < 0.01$). A correlation also exists between PGE level and ODC activity ($r = 0.652$, $P < 0.001$).

Table 3 *Effects of 20% fat diets and treatments on mammary tumor development at 16 weeks after DMBA administration*

Diet and treatment[b]	Tumor incidence	Total no. of tumors[c]	Latency period[a] (week)	No. of tumors/ tumor-bearing rat[a]	Tumor burden/ tumor-bearing rat (g)[a]
20% CO	25/29 (86%)[d]	83	11.9 ± 0.6[g]	1.79 ± 0.10[g]	2.36 ± 0.28[g]
20% CO + INDO	24/29 (83%)[d]	75	12.0 ± 0.6[g]	1.75 ± 0.12[g]	2.29 ± 0.35[g]
20% CO + DFMO	8/29 (28%)[e]	13	14.3 ± 0.6[h]	1.23 ± 0.13[h]	1.50 ± 0.46[g, h]
20% CO + INDO + DFMO	9/28 (32%)[e]	14	13.8 ± 1.0[g, h]	1.23 ± 0.07[h]	0.68 ± 0.14[h]
15% MO + 5% CO	17/29 (59%)[f]	54	12.6 ± 0.7[g, h]	1.72 ± 0.11[g]	1.92 ± 0.26[g]
15% MO + 5% CO + DFMO	7/29 (24%)[e]	13	12.7 ± 1.0[g, h]	1.28 ± 0.19[h]	1.40 ± 0.38[g, h]

[a] Means ± SEM ($N = 7$–25). The values for number of tumors and tumor burden per tumor bearing rat are shown after square root transformation and compared among dietary groups using one-way analysis of variance.
[b] CO, corn oil; INDO, 0.004% w/w indomethacin; DFMO, 0.5% (w/w) D,L-2-difluoromethylornithine; MO, menhaden oil.
[c] Tumors were diagnosed as tubulopapillary carcinoma (TPC), cystic TPC, solid tubular carcinoma and tubular carcinoma.
[d, e, f] Tumor incidences were compared using Chi-square procedure without continuity correction. Incidences which are significantly ($P < 0.001$–0.05) different are followed by different superscripts.
[g, h] Comparison among the dietary groups was made using one-way analysis of variance. Means which are significantly ($P < 0.05$) different are followed by different superscripts.

Table 4 *Influence of diets and treatments on eicosanoid synthesis and ornithine decarboxylase (ODC) activity in mammary fat pads of sham-treated animals after 13 weeks of feeding high fat diets*

Diet[a]	PGE[a] (ng/g tissue/h)	LTB_4[a] (ng/g tissue/h)	ODC activity[a] (nmoles CO_2/h/mg protein)
20% CO	90.0 ± 6.7[b]	2.3 ± 0.3[b, c]	ND[e]
20% CO + INDO	39.7 ± 2.7[c]	4.2 ± 0.03[d]	ND
20% CO + DFMO	38.3 ± 2.3[c]	3.0 ± 0.4[b]	ND
20% CO + DFMO + INDO	27.8 ± 2.0[d]	2.8 ± 0.3[b]	ND
15% MO + 5% CO	24.5 ± 1.9[d]	2.3 ± 0.4[b, c]	ND
15% MO + 5% CO + DFMO	18.3 ± 2.2[d]	1.7 ± 0.1[c]	ND

[a] Means ± SEM (N = 7–10). CO, corn oil; INDO, 0.004% (w/w) indomethacin; DFMO, 0.5% (w/w) D,L-2-difluoromethylornithine; MO, menhaden oil.
[b, c, d] Comparisons among the dietary groups were made using one-way analysis of variance. Means which are significantly ($P < 0.05$) different are followed by different superscripts.
[e] ND, not detected.

Table 5 *Influence of diets and treatments on eicosanoid synthesis and ODC activity in mammary tumors at 16 weeks post-DMBA*

Diet[a]	PGE[a] (ng/g tissue/h)	LTB_4[a] (ng/g tissue/h)	ODC activity[a] (nmol CO_2/h/mg protein)
20% CO	953.2 ± 26.9[b]	15.8 ± 1.1[b]	57.19 ± 6.8[b]
20% CO + INDO	526.7 ± 25.2[c]	25.2 ± 3.1[c]	36.39 ± 5.5[c]
20% CO + DFMO	429.0 ± 20.4[d]	8.1 ± 0.9[d]	14.44 ± 1.8[d]
20% CO + DFMO + INDO	282.3 ± 14.7[e]	19.3 ± 1.6[b]	10.96 ± 0.8[d]
15% MO + 5% CO	114.7 ± 6.5[f]	9.0 ± 1.1[d]	42.7 ± 4.6[c]
15% MO + 5% CO + DFMO	88.8 ± 3.0[f]	7.6 ± 0.9[d]	5.24 ± 0.5[d]

[a] Means ± SEM (N = 6–10). CO, corn oil; INDO, 0.004% indomethacin; DFMO, 0.5% D,L-2-difluoromethylornithine; MO, menhaden oil.
[b, c, d, e, f] Comparisons among the dietary groups were made using one-way analysis of variance. Means which are significantly ($P < 0.05$) different are followed by different superscripts.

DISCUSSION

These data suggest that eicosanoid and polyamine syntheses are involved in tumor promotion by high fat diets. When compared to feeding a 20% corn oil diet: (*a*) Feeding a diet containing a n-3/n-6 fatty acid ratio of 1.2 (15% menhaden oil and 5% corn oil) reduced tumorigenesis, PGE and LTB_4 syntheses, and ODC activity by 31%, 88%, 43%, and 25%, respectively. (*b*) Feeding a 20% CO diet containing 0.5% DFMO reduced tumorigenesis, PGE and LTB_4 syntheses, and ODC activity by 67%, 55%, 49%, and 75%, respectively. (*c*) Although feeding 0.004% indomethacin did not inhibit tumorigenesis, it inhibited PGE synthesis and ODC activity by 45% and 36%, respectively, and shunted eicosanoid synthesis toward lipoxygenase products as shown by a 60% increase in LTB_4 synthesis. (*d*) Feeding a diet containing an n-3/n-6 fatty acid ratio of 1.2 plus 0.5% DFMO (MO + CO + DFMO) reduced tumorigenesis, PGE, LTB_4 and ODC by 72%, 91%, 52%, and 96% respectively. Although feeding diets that contained 0.5% DFMO inhibited tumorigenesis, the role of DFMO in this inhibition is clouded by the fact that rats receiving DFMO containing diets failed to gain as much weight as those not receiving DFMO.

Thompson *et al.* (38) showed that DFMO administered by drinking water containing from 0.125% to 0.5% DFMO inhibited the incidence of mammary gland adenocarcinomas induced by 1-methyl-1-nitrosourea. When they (38) gave 0.5% DFMO in the drinking water, the average daily intake of DFMO per rat was 130 mg, and no deleterious effect of DFMO on final body weights was observed. In the present study, when 0.5% DFMO was given in the diet, the average daily intake of DFMO per rat was 75 mg. The final body weights of rats fed CO + DFMO or MO + CO + DFMO were 15% lower than in rats fed CO diets without DFMO, and when CO + INDO + DFMO were fed, body weights were 20% less than in CO-fed rats. Although rats fed CO + INDO + DFMO gained even less weight than rats fed CO + DFMO, tumorigenesis was not less in the CO + INDO + DFMO group compared to the CO + DFMO fed group. No significant differences in food intake between the CO fed, CO + DFMO fed or CO + INDO + DFMO fed groups were observed. The failure to gain weight may have resulted from an enhanced systemic toxicity of DFMO associated with the level of fat in the diet. Since caloric density determines the quantity of food consumed by rats and since food consumption was unchanged when diets containing DFMO were fed, it appears that the absorption of nutrients and calories was not affected by DFMO. Nevertheless, feeding 0.5% DFMO in a 20% fat diet led to a reduction in body weight not observed when DFMO is given in the drinking water to rats fed a lab chow diet. Thompson *et al.* (37) reported that higher levels of DFMO administered in drinking water reduced body weights but they concluded that the inhibitory effect on mammary carcinogenesis could not be accounted for on the basis of effect on somatic growth. Ongoing studies in our laboratory are being conducted to determine if the decreased tumorigenesis was a specific effect of the treatment or secondary to failure to gain weight.

Feldman *et al.* (16) studied the effect of 0.004% indomethacin in a 20% corn oil diet on R3230AC mammary tumor growth. They found an 89% reduction in both tumor and plasma PGE_2 levels. However, feeding indomethacin did not reduce tumor growth when indomethacin was started 3 days prior to tumor implantation. In the present study, DMBA was used to induce mammary cancer, and indomethacin was started 3 weeks post-DMBA administration. No effect by indomethacin on tumor incidence was observed although tumor PGE production was reduced by 45%. These observations are similar to those seen by Feldman *et al.* (16) despite differences in the tumor model and the experimental protocol.

Carter *et al.* (12), however, reported that feeding 0.004% indomethacin in a 20% corn oil diet inhibited DMBA-induced mammary tumor incidence. Several differences in protocol between their work and the present study may explain the apparent discrepancy in findings. In their study, a 5-mg dose of DMBA was used in contrast to the present study which used 10 mg to induce mammary tumors. It is possible that indomethacin is a less effective inhibitor of mammary tumorigenesis when a high carcinogen dose is used. An additional protocol difference concerns the optimal time at which indomethacin treatment should be started. Carter *et al.* (12) started indomethacin 3 days post-DMBA while in the present study indomethacin was started 3 weeks post-DMBA. The significant protective activity of indomethacin against chemically induced mammary tumors may be best achieved when indomethacin is started shortly after carcinogen administration, during the initiation period while DMBA is being metabolized (12). Moreover, it appears that indomethacin is a less effective inhibitor of mammary tumorigenesis when a high response mammary tumor model is used, *i.e.*, a high dose of chemical carcinogen combined with promotion by a high fat diet.

Leukotriene LTB_4 was measured as a marker for lipoxygenase activity since it is of special significance in inflammatory responses. It is also a mediator of T-lymphocyte function, regulating the balance between helper and suppressor T-lymphocytes, tipping response toward the suppressor side (47). In

the present study, LTB_4 was higher in tissues of rats fed the high fat diet containing indomethacin than in the other groups fed diets without indomethacin. Inhibition of cyclooxygenase appears to shunt arachidonic acid metabolism toward the lipoxygenase pathway. Fischer *et al.* (30) suggested that in the SENCAR mouse skin tumor model, one or more lipoxygenase products may mediate events such as ODC, DNA synthesis and finally tumor promotion by TPA. In the DMBA-induced mammary tumor model of this study, inhibition of cyclooxygenase as well as lipoxygenase accomplished by feeding either MO or DFMO significantly reduced tumor incidence compared to feeding a 20% corn oil diet; inhibition of cyclooxygenase alone by indomethacin did not change tumorigenesis.

It is well established that tumor promoters, such as phorbol esters, induce ODC activity as well as products of cyclooxygenase (19–30), and that irreversible inhibition of polyamine synthesis inhibits DNA synthesis, cell proliferation, and tumorigenesis (31–39). In a recent review, Pegg (31) suggested that combinations of polyamine synthesis inhibitors along with biological modifiers such as retinoids, fish oil, or antioxidants may have application not only in cancer chemotherapy (as in combinations of DFMO and interferon) but also in the inhibition of carcinogenesis and tumor promotion. In the present study, the combination of DFMO with a diet containing an n-3/n-6 fatty acid ratio of 1.2 profoundly inhibited (72%) tumor promotion. It is interesting to note that in this study simultaneous inhibition of the arachidonic acid cascade (both cyclooxygenase and lipoxygenase) as well as polyamine synthesis were necessary to achieve the greatest inhibition of tumorigenesis.

In summary, this study shows that mammary tumor promotion by a 20% n-6 fatty acid diet can be inhibited by simultaneous inhibition of cyclooxygenase, lipoxygenase, and ODC activities. Mammary tumor promotion by a 20% CO diet was not reduced by inhibition of cyclooxygenase and ODC activities without inhibition of the lipoxygenase pathway. Moreover, mammary tumor promotion elicited by a high corn oil diet was more effectively inhibited when the diet contained a natural dietary eicosanoid inhibitor (as found in menhaden oil) and a polyamine synthesis inhibitor (0.5% DFMO). This is particularly noted when the ratio of n-3 to n-6 fatty acids in the diet is near to 1.0. If, as this study suggests, the 5-lipoxygenase product LTB_4 is involved in mammary tumor promotion, the inhibition of tumorigenesis by menhaden oil may be due to inhibition of 5-lipoxygenase rather than cyclooxygenase. Polyamine and eicosanoid syntheses appear to be related to mammary tumor promotion by high n-6 fat diets. However, additional work will be required to determine the mechanistic relationship of these parameters of tumor promotion.

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