Overexpression of 5-Lipoxygenase in Rat and Human Esophageal Adenocarcinoma and Inhibitory Effects of Zileuton and Celecoxib on Carcinogenesis

Xiaoxin Chen,[1] Su Wang,[1] Nan Wu,[1] Sandeep Sood,[1] Peng Wang,[1] Zhe Jin,[1] David G. Beer,[2] Thomas J. Giordano,[3] Yong Lin,[4] Wei-chung J. Shih,[4] Ronald A. Lubet,[5] and Chung S. Yang[1]

[1]Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey; [2]Section of General Thoracic Surgery, Department of Surgery and [3]Department of Pathology, University of Michigan, Ann Arbor, Michigan; [4]Division of Biometrics, University of Medicine and Dentistry of New Jersey School of Public Health, New Brunswick, New Jersey; and [5]Chemoprevention Branch, Division of Cancer Prevention, National Cancer Institute, Bethesda, Maryland

ABSTRACT

Purpose: **Aberrant arachidonic acid (AA) metabolism, especially through the cyclooxygenase (Cox) and 5-lipoxygenase (5-Lox) pathways, has been suggested to play an important role in the development of esophageal adenocarcinoma (EAC). The purpose of this study was to investigate the expression of 5-Lox in EAC of a rat model and in human samples as well as the chemopreventive effects of zileuton (a specific 5-Lox inhibitor) and celecoxib (a specific Cox2 inhibitor) in the rat EAC model.**

Experimental Design: **5-Lox expression in EAC of a rat esophagogastroduodenal anastomosis model and of humans was examined with immunohistochemistry. A chemoprevention study was designed to test whether zileuton and celecoxib could suppress aberrant AA metabolism and esophageal adenocarcinogenesis.**

Results: **With immunohistochemistry, we found that 5-Lox was overexpressed during esophageal adenocarcinogenesis in our rat model and in humans. In the chemoprevention study, EAC incidence was reduced in a dose-dependent manner from 68.8% (11 of 16) to 44.4% (8 of 18; $P > 0.05$) and 31.3% (5 of 16; $P < 0.05$) by 500 and 1,000 ppm zileuton, respectively, and to 33.3% (7 of 21; $P < 0.05$) and 20% (3 of 15; $P < 0.05$) by 500 and 1,000 ppm celecoxib, respectively. With isobolographic analysis, zileuton and celecoxib, both at a dose of 500 ppm, had an additive effect by reducing the tumor incidence to 16.7% (3 of 18, $P < 0.01$). Leukotriene B_4 and prostaglandin E_2 levels in the esophageal tissues were also significantly reduced by zileuton and celecoxib.**

Conclusions: **This study clearly demonstrated that 5-Lox and Cox2 play important roles in the development of EAC. Both zileuton and celecoxib had inhibitory effects on esophageal adenocarcinogenesis through inhibition on their respective enzymes of AA metabolism.**

INTRODUCTION

Esophageal adenocarcinoma (EAC) has received much attention in recent years because of its rapid increase in incidence. Between 1976 and 1990, the incidence rate of EAC in the United States tripled, with a yearly increase of approximately 10%. It is the fastest increasing cancer among all types, afflicting about 10,000 people per year (1). The sequence of events leading to EAC is thought to involve the development of gastroesophageal reflux disease, followed by multilayered epithelium and columnar-lined esophagus (CLE; also known as Barrett's esophagus), followed by multifocal dysplasia, and, eventually, invasive adenocarcinoma (2, 3).

Several lines of evidence suggested an important role of aberrant arachidonic acid (AA) metabolism in human esophageal adenocarcinogenesis. Intake of nonsteroidal anti-inflammatory drugs was associated with a lower risk of EAC (4), especially for those who overexpressed cyclin D1 (5). Cyclooxygenase (Cox) 2 was overexpressed in human EAC, and its high expression level was associated with poor clinical prognosis (6). Gastric acid and bile acids in gastroesophageal refluxate enhanced the expression of Cox2 expression (7, 8). As a result, prostaglandin E_2 (PGE_2) levels were increased in reflux esophagitis and suppressed by antacid therapy (9). Specific Cox2 inhibitors induced apoptosis in human EAC cell lines and primary esophageal epithelial cells from biopsy samples (10, 11). During rat esophageal adenocarcinogenesis, Cox2 was overexpressed and PGE_2 was overproduced (8, 12). Specific and nonspecific Cox2 inhibitors (sulindac and MF-tricyclic) significantly reduced the incidence of EAC in the rat models (12, 13).

Besides the Cox2 pathway, the lipoxygenase pathways are also important in carcinogenesis. The major AA metabolites of the 5-lipoxygenase (5-Lox) pathway, 5(*S*)-hydroeicosatetraenoic acid, leukotriene B4 (LTB_4), and cysteinyl leukotrienes, are known to recruit and activate inflammatory cells, increase vascular permeability, and induce contraction of smooth muscles (14). 5-Lox knockout mice are more resistant to inflammation

Received 4/28/04; revised 6/15/04; accepted 6/18/04.
Grant support: National Institutes of Health grants CA75683, DK63650, and N01-CN15117.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Xiaoxin Chen, Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 164 Frelinghuysen Road, Piscataway, NJ 08854. Phone: 732-445-3400, ext. 259; Fax: 732-445-0687; E-mail: xiaochen@rci.rutgers.edu.

and certain inflammation-associated diseases and more susceptible to infections than wild-type mice (15). Recently, 5-Lox has been found to be overexpressed in many human cancers, such as prostate (16), pancreatic (17), colon (18), bladder (19), and testicular cancer (20). In human colon cancer, 5-Lox overexpression was associated with poor clinical prognosis, especially for patients with Dukes' B stage disease (18). Inhibition of the 5-Lox pathway had antiproliferative and proapoptotic effects on different cancer cell lines (21). Chemopreventive effects of chemical compounds targeting enzymes and receptors on the 5-Lox pathway have been demonstrated in animal models of lung, skin, and pancreatic cancer (22–26).

Normal esophageal squamous epithelium has limited 5-Lox activity and produces limited amounts of LTB_4. However, the levels of LTB_4 in human esophageal biopsy samples of reflux esophagitis and CLE increased markedly (27), and antacid therapy reduced LTB_4 levels (9). In human EAC samples and in a rat model, we found overexpression of leukotriene A_4 hydrolase (LTA_4H, the rate-limiting enzyme for synthesis of LTB_4) and an increased level of LTB_4. Bestatin, a LTA_4H inhibitor, suppressed LTB_4 level and carcinogenesis in our rat model (28). These results suggest that the 5-Lox pathway of AA metabolism plays an important role in esophageal adenocarcinogenesis. In this study, we examined the expression of 5-Lox in human and rat EAC. Zileuton, a specific 5-Lox inhibitor used clinically for asthma, was used alone or in combination with celecoxib, a specific Cox2 inhibitor, to determine its effect on carcinogenesis in our rat surgical model.

MATERIALS AND METHODS

5-Lipoxygenase Immunohistochemistry. The avidin-biotin-peroxidase complex method (Elite ABC kit; Vector Laboratories, Burlingame, CA) and monoclonal mouse anti-5-Lox antibody (5 μg/mL; Research Diagnostics Inc., Parsippany, NJ) were used for immunohistochemical staining of 5-Lox on archival formalin-fixed paraffin-embedded tissue sections of 20 esophagogastroduodenal anastomosis (EGDA) rats and the esophageal samples of 67 human patients (Table 1). The rat samples were obtained from a previous study (29). Each of the rat tissue sections contained different histology including normal esophagus, esophagitis, CLE, and EAC. Human samples were obtained from paraffin blocks from the pathology archives of the University of Michigan. They were used to construct tissue microarrays using standard techniques (30). Institutional review board approval (University of Michigan) was obtained to perform immunohistochemistry using archival tissues.

Histologic diagnosis was made according to the established criteria (31). The paraffin sections were pretreated with antigen unmasking fluid (BD PharMingen, San Diego, CA) before being incubated with the primary antibody. Cells expressing 5-Lox exhibited cytoplasmic and occasional nuclear dark brown staining. Two negative controls were set up to test the specificity of the antibody used for immunohistochemistry, omission of the first antibody and preadsorption with recombinant 5-Lox protein (Research Diagnostics Inc.). In both cases, no positive staining was observed. Staining was evaluated and graded by a pathologist (S. W.). Staining intensity in epithelial cells on human paraffin sections was rated as follows: −, no positive staining or slight staining close to background; +, weakly positive staining; ++, moderately positive staining; and +++, strongly positive staining. The area of maximal intensity was used for grading as long as it comprised >10% of the region of interest (esophageal epithelial tissues). We did not quantify the percentage of positively stained cells because the staining was homogeneous. Polymorphonuclear neutrophils, especially those in blood vessels, were used as internal positive controls. These cells express a high level of 5-Lox, with the staining intensity designated as ++.

Chemoprevention of EGDA-Induced Esophageal Adenocarcinoma in Rats by Zileuton and Celecoxib. A short-term experiment (4 weeks after surgery) was designed to find the proper doses of zileuton and celecoxib for EGDA rats. EGDA was performed on 8-week-old male Sprague-Dawley rats (Taconic, Germantown, NY) according to the procedure described previously (29), which was approved by the Animal Care and Facilities Committee at Rutgers University (Protocol 94-017). Five EGDA rats were fed with respective AIN93M-based diets containing the test agents (500 or 1,000 ppm zileuton, 500 or 1,000 ppm celecoxib, or 500 ppm zileuton + 500 ppm celecoxib) starting 1 day after EGDA surgery and continuing for 4 weeks. All of the diets were prepared by Research Diets (New Brunswick, NJ). Body weight was monitored once per week throughout the experiment. Food and fluid intake were measured at weeks 1, 2, and 4 after surgery. At each time point, three consecutive days were monitored. Liver and kidney were harvested and examined for possible toxic effects after hematoxylin and eosin staining of tissue sections.

In the long-term chemoprevention experiment (40 weeks after surgery), EGDA rats were randomly assigned to different groups and given the chemopreventive agents in the diet starting 1 day after surgery (Table 2). Forty weeks after surgery, the rats were sacrificed, and their esophagi were examined for gross abnormalities. Tumor volume was determined by measuring the height, length, and width of all visible tumors and by using the average of these three measurements as the diameter (volume = $4/3\pi r^3$). A small piece of esophageal tissue at the squamocolumnar junction (from the anastomosis line to 5 mm above) was also harvested and stored at −80°C for future analysis of LTB4 and PGE_2. The remaining esophageal tissue was fixed in 10% buffered formalin, Swiss rolled, and processed routinely. Histopathological analysis was carried out using the first, tenth, twentieth and thirtieth slides stained with hematoxylin and eosin. Dysplasia was diagnosed by the partial loss of cell polarity and maturation, nuclear atypia, and an increase in mitotic figures. EAC was diagnosed when dysplastic columnar epithelial cells invaded through the basement membrane (31).

Enzyme Immunoassay of Leukotriene B_4 and Prostaglandin E_2. Frozen samples of the rat esophagoduodenal junction were analyzed immediately after being taken out of a −80°C freezer. After being pulverized and homogenized in a buffer containing indomethacin and zileuton, the tissue samples were aliquoted for determination of protein concentration and organic extraction. The organic extract was dried with nitrogen and reconstituted in enzyme immunoassay buffer. LTB_4 and PGE_2 were measured in triplicate using the experimental procedures provided by the manufacturer (Cayman Chemical Co.,

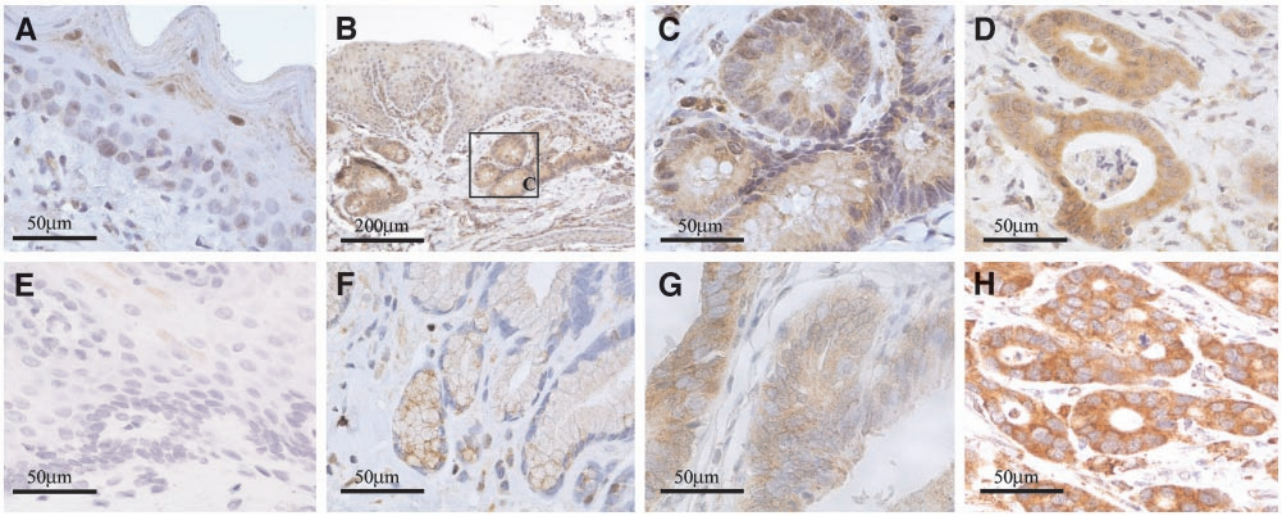

Fig. 1 Overexpression of 5-Lox in esophageal samples of EGDA rats (*A*−*D*) and humans (*E*−*L*). In rat normal esophagus (*A*), 5-Lox was barely detectable. In reflux esophagitis and CLE (*B* and *C*), 5-Lox was overexpressed in both epithelial cells and infiltrating inflammatory cells. In EAC (*D*), cancer cells strongly expressed 5-Lox. Similar to the rat model, normal human esophagus (*E*) had barely detectable 5-Lox staining, and staining intensity was designated as −. In human CLE (*F*), 5-Lox expression increased, and in this case the staining intensity was designated as +. In dysplasia (*G*; staining intensity, ++), 5-Lox was further overexpressed. In EAC (*H*), a staining intensity of +++ was seen. All of the *scale bars* represent 50 μm, except the *scale bar* of *B*, which represents 200 μm.

Ann Arbor, MI). The amounts of PGE_2 and LTB_4 were expressed as nanograms per milligram of protein.

Statistical Considerations and Analysis. A contingency table χ^2 test was performed to analyze the association of 5-Lox immunohistochemical staining intensity with histology in human esophageal tissue samples. The incidence of EAC was analyzed by Fisher's exact test, and the tumor size was analyzed by Wilcoxon Mann-Whitney test. The dose-response analysis on zileuton and celecoxib was performed with Mantel-Haenszel χ^2 test. Student's *t* test was used for analysis of LTB_4 and PGE_2.

The combination effect of zileuton and celecoxib on carcinogenesis was assessed using the method of Laska *et al.* (32). We took 500 and 1,000 ppm as the doses of zileuton and celecoxib. We assumed that the expected response with zileuton at a dose of 500 ppm and celecoxib at a dose of 500 ppm [g(500 ppm zileuton + 500 ppm celecoxib)] was a monotone, nondecreasing, and continuous function. Synergistic, additive, or antagonistic effects were defined if g(500 ppm zileuton + 500 ppm celecoxib) was greater than, equal to, or less than g(1,000 ppm zileuton) and g(1,000 ppm celecoxib). Here g was defined as the inhibition rate on the tumor incidence in our animal model.

RESULTS

Overexpression of 5-Lipoxygenase in Esophageal Adenocarcinoma of EGDA Rats and Humans. With immunohistochemistry, we examined the expression of 5-Lox in the esophageal tissues of EGDA rats. In the proximal esophagus, 5-Lox was barely detectable in the epithelial cells (Fig. 1*A*). In the areas of EGDA-induced esophagitis and CLE, 5-Lox was overexpressed in the squamous epithelial cells, especially in the columnar epithelial cells and infiltrating inflammatory cells (Fig. 1*B* and *C*). In EAC, 5-Lox was further overexpressed in both epithelial cells and infiltrating inflammatory cells (Fig. 1*D*). The esophageal samples from 20 EGDA rats showed the same pattern of 5-Lox expression.

In the esophageal samples of 67 human patients, 5-Lox immunoreactivity was negative in most samples of normal esophagus (17 of 22; Fig. 1*E*). 5-Lox expression was markedly increased in CLE and dysplasia, with most cases positively stained (Fig. 1*F* and *G*). In EAC, overexpression was obvious in 50% of the cases (28 of 56), with staining intensity at the level of ++ and +++. There were six cases of EAC showing very strong staining of 5-Lox (Fig. 1*H*; Table 1). Positive staining was observed mainly in the cytoplasm. With a contingency table χ^2 test, there was a significant association between staining intensity and histology ($P < 0.0001$). The results showed that in both our rat model and human tissue samples, there was a

Table 1 Expression of 5-Lox in the esophageal samples of 67 patients

		5-Lox staining intensity			
Histology	No.	−	+	++	+++
Normal	22	17	5	0	0
CLE	7	0	6	1	0
Dysplasia	9	2	6	1	0
EAC	56	3	25	22	6

NOTE. In this collection of tissue samples, some patients contributed samples of more than one type of histology. Seven cases contributed samples of all four types of histology, but none contributed more than one tissue sample with the same histology. Diagnosis was based on the established criteria (31). Staining intensity in epithelial cells was scored as follows: −, no positive staining; +, weakly positive staining; ++, moderately positive staining; and +++, strongly positive staining. With a contingency table χ^2 test, there was a significant association between staining intensity and histology ($P < 0.0001$).

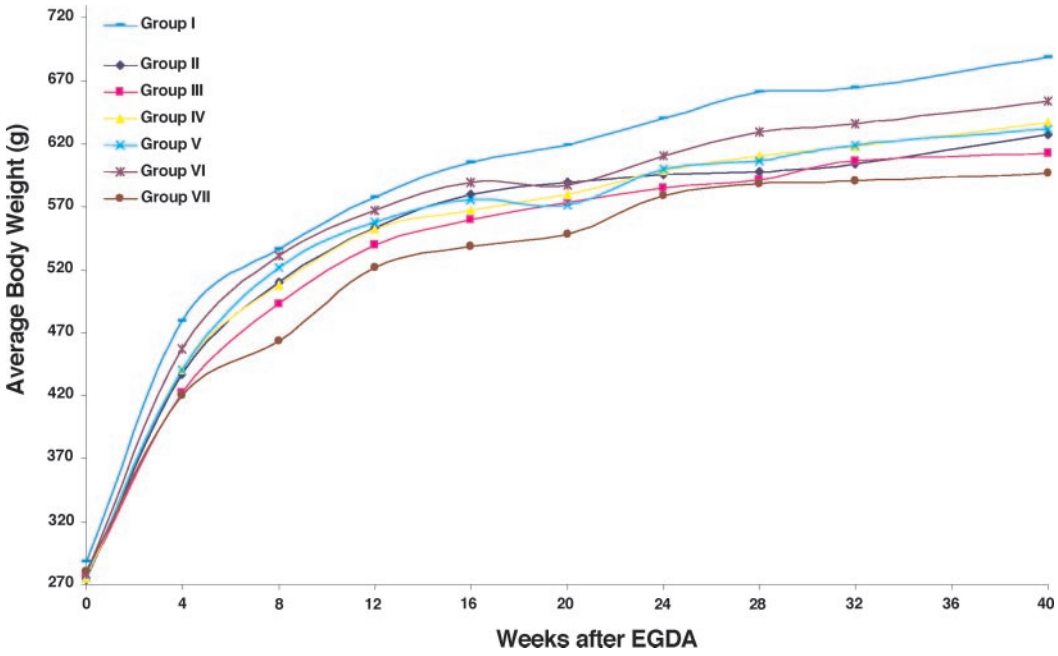

Fig. 2 Body weight of EGDA rats during the long-term chemoprevention experiment.

progressive increase in 5-Lox expression during histologic progression to EAC. More importantly, 5-Lox was overexpressed at the stage of CLE, an early stage of esophageal adenocarcinogenesis.

Chemopreventive Effects of Zileuton and Celecoxib. In the short-term (4 weeks after surgery) dose-finding experiment with five rats in each group, all of the animals survived both surgery and treatment with zileuton or celecoxib. We did not detect a statistically significant difference in food or fluid intake at 1, 2, and 4 weeks after surgery in groups receiving zileuton (500 or 1,000 ppm), celecoxib (500 or 1,000 ppm), or the combination of agents (500 ppm each agent). No significant difference in body weight among all of the groups was observed. No obvious toxicity was observed when the histology of liver and kidney of the five rats from each group was examined.

In the long-term experiment (40 weeks after surgery), 104 of 110 (94.6%) animals survived the surgery and remained healthy until the end of the experiment. During the experiment, these animals were active and healthy. In the early time points (up to 16 weeks after surgery), body weights of the experimental groups (groups II–VII) were slightly lower than those of the nonoperated control (group I), but the difference was not significant ($P > 0.05$). After 16 weeks, all of the experimental groups had significantly lower body weights than the nonoperated control group ($P < 0.05$). However, treatment with zileuton and/or celecoxib had no significant effect on body weight as compared with the EGDA group (group II; $P > 0.05$; Fig. 2).

The incidence of visible tumors was significantly reduced by 1,000 ppm zileuton ($P < 0.01$), 500 or 1,000 ppm celecoxib ($P < 0.01$ for both), and the combination of agents ($P < 0.001$). The combination (group VII) dramatically suppressed the formation of visible tumors by 91.9%. As compared with the EGDA control (group II), zileuton and celecoxib at all of the test doses did not significantly reduce tumor volume in this study ($P > 0.05$, Wilcoxon Mann-Whitney test; Fig. 3). This was due to the large variation in tumor sizes even within the same group. It suggested that factors other than 5-Lox and Cox2 may play important roles in tumor growth once a tumor is developed.

When the tissues were analyzed under a microscope, zileuton (500 ppm) reduced the incidence of EAC from 68.8% (11 of 16) to 44.4% (8 of 18), but the difference was not statistically significantly ($P > 0.05$). At the dose of 1,000 ppm zileuton, the incidence of EAC was significantly reduced to 31.3% (5 of 16; $P < 0.05$). Using the extended Mantel-Haenszel χ^2 test, we found that zileuton inhibited tumor incidence in a dose-depen-

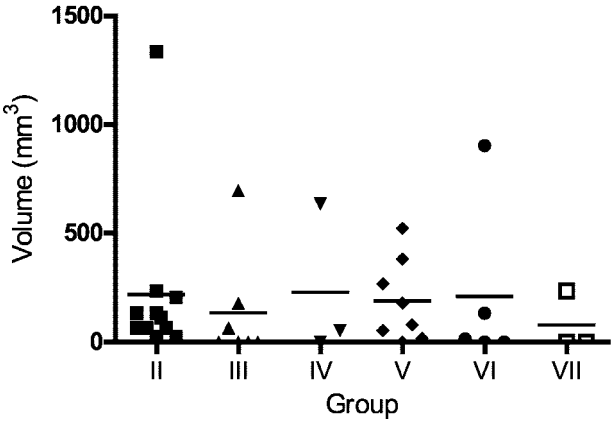

Fig. 3 Tumor size of EGDA rats at 40 weeks after surgery. With the Wilcoxon Mann-Whitney test, there was no significant difference between the treatment groups and the EGDA control (group II; $P > 0.05$). *Bars* indicate the average tumor size of that specific group.

Table 2 Chemopreventive effects of zileuton and celecoxib on esophageal adenocarcinogenesis (visible and histological tumor), LTB4, and PGE_2 in EGDA rats

Group	Treatment	No.	Visible tumor		Histological tumor		LTB_4 (ng/mg protein)	PGE_2 (ng/ml protein)
			Incidence	Inhibition	Incidence	Inhibition		
I	Nonoperated control	10					1.68 ± 0.34*	24.15 ± 20.43*
II	EGDA	16	68.8		68.8		18.48 ± 10.32	424.89 ± 90.43
III	EGDA + 500 ppm zileuton	18	38.9	43.5	44.4	35.5	13.94 ± 10.11	287.27 ± 136.52†
IV	EGDA + 1,000 ppm zileuton	16	18.8‡	72.7	31.3†	54.5	10.48 ± 3.67†	170.13 ± 149.08‡
V	EGDA + 500 ppm celecoxib	21	14.3‡	79.2	33.3†	51.6	16.30 ± 9.24	369.16 ± 160.27
VI	EGDA + 1,000 ppm celecoxib	15	13.3‡	80.7	20.0†	70.9	10.42 ± 5.20†	249.76 ± 173.33†
VII	EGDA + 500 ppm celecoxib + 500 ppm zileuton	18	5.6*	91.9	16.7‡	75.7	10.17 ± 6.28†	308.82 ± 98.96†

NOTE. EAC incidence rate is shown as a percentage of the number of tumor-bearing animals divided by the total number of animals in that group. Inhibition rate was calculated as the percentage reduction from the positive control group (group II). Diagnosis of the visible tumors was confirmed by histopathological analysis. A histological tumor was diagnosed based on histopathology and when a visible tumor was not seen at sacrifice. All *P* values were based on comparison with group II. Fisher's exact test was used for analysis of the incidences. Student's *t* test was used for analysis of LTB_4 and PGE_2 levels.

* Significantly different from group II ($P < 0.001$).

† Significantly different from group II ($P < 0.05$).

‡ Significantly different from group II ($P < 0.01$).

dent manner ($P < 0.05$). The chemopreventive effect of celecoxib was slightly stronger than that of zileuton. At the dose of 500 ppm, celecoxib had a significant inhibitory effect on carcinogenesis with a tumor incidence of 33.3% (7 of 21; $P < 0.05$). At the dose of 1,000 ppm, the incidence of EAC was further significantly reduced to 20% (3 of 15; $P < 0.05$). Similar to zileuton, celecoxib also inhibited tumor incidence in a dose-dependent manner ($P < 0.01$; Table 2).

When zileuton and celecoxib were administered in combination (500 ppm each), the incidence of EAC was dramatically reduced to 16.7% (3 of 18; $P < 0.01$; Table 2). To determine the combination effect between zileuton and celecoxib, we used the method of Laska *et al.* (32). At a dose of 1000 ppm, zileuton inhibited tumorigenesis by 54.5% and celecoxib inhibited tumorigenesis by 70.9%, respectively. When both were at doses of 500 ppm, the combination of zileuton and celecoxib inhibited tumorigenesis by 75.7% (Table 2). The combination 500 ppm zileuton + 500 ppm celecoxib was only slightly [but not statistically significantly ($P > 0.05$)] more effective than 1,000 ppm zileuton alone and 1,000 ppm celecoxib alone. It indicated an additive inhibitory effect of 500 ppm zileuton + 500 ppm celecoxib on esophageal adenocarcinogenesis in our animal model.

Effects of Zileuton and Celecoxib on the Levels of Leukotriene B_4 and Prostaglandin E_2. Both LTB_4 and PGE_2 increased dramatically in all of the EGDA rats (group II) as compared with the nonoperated control (group I; $P < 0.0001$; Table 2). Treatment with 500 ppm zileuton (group III) or 500 ppm celecoxib (group V) did not significantly reduce the levels of LTB_4 ($P > 0.05$). A dose of 500 ppm zileuton, but not 500 ppm celecoxib, reduced the level of PGE_2 ($P < 0.05$). When the doses were increased to 1,000 ppm, both zileuton and celecoxib were significantly effective in reducing the levels of both LTB_4 and PGE_2 ($P < 0.05$). In the combination group (group VII), the levels of both LTB_4 and PGE_2 were inhibited significantly ($P < 0.05$).

DISCUSSION

This study clearly demonstrated that 5-Lox was overexpressed in EAC of humans and EGDA rats. Both zileuton and celecoxib inhibited the development of EGDA-induced rat EAC in a dose-dependent manner, and such chemopreventive effects correlated with inhibition of LTB_4 and PGE_2 levels. Zileuton and celecoxib, both at a dose of 500 ppm in the diet, together had an additive effect in preventing esophageal adenocarcinogenesis in EGDA rats.

There are two major sources of 5-Lox in the esophagi of humans and EGDA rats: resident or infiltrating inflammatory cells, and epithelial cells (33). As shown in Fig. 1, 5-Lox was expressed not only in inflammatory cells but also in esophageal epithelial cells, in both rat and human samples. This finding was consistent with our observation of 5-Lox expression in human esophageal epithelial cells (HET1A, SEG-1, FLO-1, BIC-1, SKGT4, and BE3) by reverse transcription-polymerase chain reaction and Western blotting (data not shown). As we and others have shown in previous studies (8, 12, 28), other AA-metabolizing enzymes (such as Cox2 and LTA_4H) were also expressed in esophageal epithelial cells of human and rat tissue samples. These findings suggested that, besides inflammatory cells, esophageal epithelial cells actively participated in local inflammation by producing AA metabolites, such as LTB_4 and PGE_2. In this study, we found overexpression of 5-Lox during esophageal adenocarcinogenesis in both human samples and animal models. 5-Lox was overexpressed at the early stages of carcinogenesis, esophagitis, and CLE (Fig. 1; Table 1), similar to the Cox2 expression pattern (12). This observation provides a mechanistic rationale for targeting 5-Lox at the early stage of esophageal adenocarcinogenesis to prevent this deadly cancer.

5-Lox metabolites are important mediators of inflammation and inflammation-associated carcinogenesis. Recent studies in cell lines showed that LTB_4 stimulated the proliferation of human cancer cells, enhanced oxidative stress, and stimulated cell spreading (34). Receptors of LTB_4 and cysteinyl leukotrienes were overexpressed in some human pancreatic and colon

cancers (17, 18). Consistent with the proinflammatory and carcinogenic effects of 5-Lox metabolites, 5-Lox inhibitors had chemopreventive effects in several animal models of carcinogenesis. In a carcinogen-induced pancreatic cancer model in hamsters, zileuton (28 mg/day) and a combination of zileuton (28 mg/day) and celecoxib (7 mg/day) significantly inhibited tumor incidence and tumor size (25). A combination of zileuton and celecoxib also significantly reduced the incidence, number, and size of liver metastases (26). Similar chemopreventive effects of 5-Lox pathway inhibitors have been demonstrated in carcinogen-induced models of lung (22, 23), skin (24), and oral cancer.[6] In the esophagus, we previously demonstrated LTA_4H as a chemopreventive target, and a LTA_4H inhibitor, bestatin, significantly suppressed carcinogenesis in the rat EGDA model (28). In the present study, zileuton alone was effective in preventing EAC in the EGDA rats, and the preventive effect was dose dependent.

Consistent with previous results by others and us (12, 13) using nonspecific or specific Cox inhibitors (sulindac and MF-tricyclic), celecoxib at doses of 500 and 1,000 ppm significantly inhibited esophageal adenocarcinogenesis in our rat EGDA model. With the calculation of nutrient density, we estimated these doses at about 0.13 to 0.26 mg/kcal (35), similar to the commonly used doses of celecoxib in humans (200–400 mg/day or 0.1–0.2 mg/kcal). With a clinical trial on the chemopreventive effect of celecoxib (200 mg, by mouth, twice a day) in human Barrett's esophagus patients ongoing (36), it would be interesting to find out whether the animal study might help dose selection for human studies.

An interesting finding of this study was that zileuton and celecoxib were not only effective alone in inhibiting carcinogenesis in the EGDA rats but also exerted an additive effect when used in combination (each at a dose of 500 ppm). The incidence of EAC was reduced significantly from 68.8% to 16.7% by the combination. Similarly, inhibition of both 5-Lox and Cox pathways was more effective than inhibition of either pathway alone in a lung cancer model (22). In our study of the hamster oral cancer model, the combination of zileuton and celecoxib was also additive in inhibiting carcinogenesis. We believe targeting both the 5-Lox and Cox2 pathways might be more potent in suppressing inflammation and carcinogenesis in patients at risk for EAC than targeting either one of these pathways alone.

The 5-Lox and Cox2 pathways interact closely. Previous studies have shown that genetic knockout of 5-Lox (37) or Cox1/Cox2 (38) resulted in activation of the other pathway. Chemical inhibitors of one pathway may also activate other AA-metabolizing pathways (39). In this study, we examined the levels of LTB_4 and PGE_2 in esophageal epithelial tissues of EGDA rats at 40 weeks after surgery. When zileuton or celecoxib alone was administered to the EGDA rats, the levels of LTB_4 and PGE_2 decreased concurrently at the high doses, and there was no obvious activation of the other pathway (Table 2). It was possible that in this long-term experiment, the levels of LTB_4 and PGE_2 reflected the extent of inflammation. Thus, the levels of both LTB_4 and PGE_2 were reduced by 1,000 ppm zileuton or celecoxib, as well as by the combination of these agents at 500 ppm. At 500 ppm, neither agent reduced the levels of LTB_4 and PGE_2 significantly (except that zileuton reduced the level of PGE_2), although celecoxib inhibited EAC formation. The large variations in LTB_4 and PGE_2 among the samples from different animals hindered our effort to correlate inhibition of carcinogenesis with inhibition of AA metabolism.

It should be noted that zileuton and celecoxib might also inhibit carcinogenesis by mechanisms other than inhibition of AA metabolism. For example, 500 ppm zileuton significantly reduced PGE_2 levels, but not LTB_4 levels. Carcinogenesis was inhibited slightly, but not significantly. As a hydroxyurea compound, zileuton is an iron chelator (40). In our animal model of EAC, iron has been shown to promote carcinogenesis by inducing oxidative damage (31). Cox2-independent anticarcinogenic actions of celecoxib have been well documented in the literature. In a cell culture study, similar levels of apoptosis were observed in Cox2-positive and Cox2-negative epithelial cancer cells after treatment with celecoxib (41). Genetic up- or down-regulation of Cox2 in a colon cancer cell line significantly altered the effects of celecoxib on cell survival and apoptosis, but not cell cycle arrest (42). A study is ongoing in our laboratory to test whether zileuton and celecoxib can suppress tumorigenesis in 5-Lox or Cox2 knockout mice. With both the pharmacological and the genetic approaches, we may clarify the roles of 5-Lox and Cox2 in the development of EAC.

In summary, this study clearly demonstrated that 5-Lox and Cox2 played an important role in the development of EAC, and both zileuton and celecoxib prevented esophageal adenocarcinogenesis through inhibition on their respective AA-metabolizing enzymes. Currently, there are two ongoing clinical trials assessing the effectiveness of zileuton in the treatment and prevention of lung cancer.[7] New 5-Lox inhibitors and dual Cox/5-Lox inhibitors with more potent anti-inflammatory effects are also under development or in clinical trials (39). It would be interesting to test whether a combination of a 5-Lox inhibitor and a Cox2 inhibitor might be more effective than a single agent on esophageal adenocarcinogenesis in clinical trials.

REFERENCES

1. Powell J, McConkey CC, Gillison EW, et al. Continuing rising trend in oesophageal adenocarcinoma. Int J Cancer 2002;102:422–7.

2. Chen X, Yang CS. Esophageal adenocarcinoma: a review and perspectives on the mechanism of carcinogenesis and chemoprevention. Carcinogenesis (Lond) 2001;22:1119–29.

3. Su Y, Chen X, Klein M, et al. Phenotype of columnar lined esophagus in rats with esophagogastroduodenal anastomosis: similarity with human Barrett's esophagus. Lab Investig 2004;84:753–65.

4. Corley DA, Kerlikowske K, Verma R, et al. Protective association of aspirin/NSAIDs and esophageal cancer: a systematic review and meta-analysis. Gastroenterology 2003;124:47–56.

[6] N. Li, S. Sood, S. Wang, M. Fang, P. Wang, Z. Sun, C. S. Yang, and X. Chen. Overexpression of cyclooxygenase and 5-lipoxygenase in hamster and human oral cancer and chemopreventive effects of celecoxib and zileuton, manuscript in preparation.

[7] http://clinicaltrials.gov/.

5. Gammon MD, Terry MB, Arber N, et al. Nonsteroidal anti-inflammatory drug use associated with reduced incidence of adenocarcinomas of the esophagus and gastric cardia that overexpress cyclin D1: a population-based study. Cancer Epidemiol Biomark Prev 2004; 13:34–9.

6. Buskens CJ, Van Rees BP, Sivula A, et al. Prognostic significance of elevated cyclooxygenase 2 expression in patients with adenocarcinoma of the esophagus. Gastroenterology 2002;122:1800–7.

7. Shirvani VN, Ouatu-Lascar R, Kaur BS, et al. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: ex vivo induction by bile salts and acid exposure. Gastroenterology 2000;118:487–96.

8. Zhang F, Altorki NK, Wu YC, et al. Duodenal reflux induces cyclooxygenase-2 in the esophageal mucosa of rats: evidence for involvement of bile acids. Gastroenterology 2001;121:1391–9.

9. Marcinkiewicz M, Sarosiek J, Edmunds M, et al. Monophasic luminal release of PGE2 in patients with reflux esophagitis under the impact of acid and acid/pepsin solutions. J Clin Gastroenterol 1995;21:268–74.

10. Souza RF, Shewmake K, Beer DG, et al. Selective inhibition of cyclooxygenase-2 induces apoptosis in human esophageal adenocarcinoma cells. Cancer Res 2000;60:5767–72.

11. Buttar NS, Wang KK, Anderson MA, et al. The effect of selective cyclooxygenase-2 inhibition in Barrett's esophagus epithelium: an in vitro study. J Natl Cancer Inst (Bethesda) 2002;94:422–9.

12. Chen X, Li N, Wang S, et al. Aberrant arachidonic acid metabolism in esophageal adenocarcinogenesis, and the effects of sulindac, nordihydroguaiaretic acid, and a-difluoromethylornithine on tumorigenesis in a rat surgical model. Carcinogenesis (Lond) 2002;23:2095–102.

13. Buttar NS, Wang KK, Leontovich O, et al. Chemoprevention of esophageal adenocarcinoma by COX-2 inhibitors in an animal model of Barrett's esophagus. Gastroenterology 2002;122:1101–12.

14. Peters-Golden M, Brock TG. 5-Lipoxygenase and FLAP. Prostaglandins Leukot Essent Fatty Acids 2003;69:99–109.

15. Funk CD, Chen XS, Johnson EN, et al. Lipoxygenase genes and their targeted disruption. Prostaglandins Other Lipid Mediat 2002;68–69:303–12.

16. Gupta S, Srivastava M, Ahmad N, et al. Lipoxygenase-5 is overexpressed in prostate adenocarcinoma. Cancer (Phila) 2001;91:737–43.

17. Hennig R, Ding XZ, Tong WG, et al. 5-Lipoxygenase and leukotriene B4 receptor are expressed in human pancreatic cancers but not in pancreatic ducts in normal tissue. Am J Pathol 2002;161:421–8.

18. Ohd JF, Nielsen CK, Campbell J, et al. Expression of the leukotriene D4 receptor CysLT1, COX-2, and other cell survival factors in colorectal adenocarcinomas. Gastroenterology 2003;124:57–70.

19. Yoshimura R, Matsuyama M, Tsuchida K, et al. Expression of lipoxygenase in human bladder carcinoma and growth inhibition by its inhibitors. J Urol 2003;170:1994–9.

20. Yoshimura R, Matsuyama M, Mitsuhashi M, et al. Relationship between lipoxygenase and human testicular cancer. Int J Mol Med 2004;13:389–93.

21. Steele VE, Holmes CA, Hawk ET, et al. Lipoxygenase inhibitors as potential cancer chemopreventives. Cancer Epidemiol Biom Prev 1999; 8:467–83.

22. Rioux N, Castonguay A. Inhibitors of lipoxygenase: a new class of cancer chemopreventive agents. Carcinogenesis (Lond) 1998;19:1393–400.

23. Gunning WT, Kramer PM, Steele VE, et al. Chemoprevention by lipoxygenase and leukotriene pathway inhibitors of vinyl carbamate-induced lung tumors in mice. Cancer Res 2002;62:4199–201.

24. Jiang H, Yamamoto S, Kato R. Inhibition of two-stage skin carcinogenesis as well as complete skin carcinogenesis by oral administration of TMK688, a potent lipoxygenase inhibitor. Carcinogenesis (Lond) 1994;15:807–12.

25. Wenger FA, Kilian M, Achucarro P, et al. Effects of Celebrex and Zyflo on BOP-induced pancreatic cancer in Syrian hamsters. Pancreatology 2002;2:54–60.

26. Wenger FA, Kilian M, Bisevac M, et al. Effects of Celebrex and Zyflo on liver metastasis and lipid peroxidation in pancreatic cancer in Syrian hamsters. Clin Exp Metastasis 2002;19:681–7.

27. Triadafilopoulos G, Kaczynska M, Iwane M. Esophageal mucosal eicosanoids in gastroesophageal reflux disease and Barrett's esophagus. Am J Gastroenterol 1996;91:65–74.

28. Chen X, Li N, Wang S, et al. Leukotriene A4 hydrolase in rat and human esophageal adenocarcinomas and inhibitory effects of bestatin. J Natl Cancer Inst (Bethesda) 2003;95:1053–61.

29. Chen X, Yang G-Y, Ding W, et al. An esophagogastroduodenal anastomosis model for esophageal adenocarcinogenesis in rats and enhancement by iron overload. Carcinogenesis (Lond) 1999;20:1801–7.

30. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844–7.

31. Lewin KJ, Appelman HD. Barrett's esophagus, columnar dysplasia, and adenocarcinoma of the esophagus. In: Lewin KJ, Appelman, HD, editors. Tumors of the esophagus and stomach. Washington DC: Armed Forces Institute of Pathology; 1995. p. 99–144.

32. Laska EM, Meisner M, Siegel C. Simple designs and model-free tests for synergy. Biometrics 1994;50:834–41.

33. Werz O. 5-Lipoxygenase: cellular biology and molecular pharmacology. Curr Drug Targets Inflamm Allergy 2002;1:23–44.

34. Chen X, Wang S, Wu N, et al. Leukotriene A4 hydrolase as a target for cancer prevention and therapy. Curr Cancer Drug Targets 2004;4: 267–83.

35. Newmark HL. Nutrient density: an important and useful tool for laboratory animal studies. Carcinogenesis (Lond) 1987;8:871–3.

36. Heath EI, Canto MI, Wu TT, et al. Chemoprevention for Barrett's esophagus trial. Design and outcome measures. Dis Esophagus 2003; 16:177–86.

37. Goulet JL, Snouwaert JN, Latour AM, et al. Altered inflammatory responses in leukotriene-deficient mice. Proc Natl Acad Sci USA 1994; 91:12852–6.

38. Gavett SH, Madison SL, Chulada PC, et al. Allergic lung responses are increased in prostaglandin H synthase-deficient mice. J Clin Investig 1999;104:721–32.

39. Fiorucci S, Meli R, Bucci M, et al. Dual inhibitors of cyclooxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy? Biochem Pharmacol 2001;62:1433–8.

40. Carter GW, Young PR, Albert DH, et al. 5-Lipoxygenase inhibitory activity of zileuton. J Pharmacol Exp Ther 1991;256:929–37.

41. Waskewich C, Blumenthal RD, Li H, et al. Celecoxib exhibits the greatest potency amongst cyclooxygenase (COX) inhibitors for growth inhibition of COX-2-negative hematopoietic and epithelial cell lines. Cancer Res 2002;62:2029–33.

42. Maier TJ, Schilling K, Schmidt R, et al. Cyclooxygenase-2 (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. Biochem Pharmacol 2004;67:1469–78.