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Effects of bile acids on COX2 in rat model of duodenoesophageal anastomosis

Hashimoto N. Effects of bile acids on COX2

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**Abstract**

**AIM**: To examine the expression of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) in rat esophageal lesions induced by duodenal contents reflux.

**METHODS**: Thirty 8-wk-old male Wistar rats were exposed to duodenal content esophageal reflux. All animal underwent an esophagoduodenal anastomosis (EDA) with total gastrectomy to elicit chronic esophagitis. In ten rats the sham (Control). The rats were sacrificed at the 40th week, their esophagi were examined for hematoxylin eosin (HE), COX2, PGE2, and proliferating cell nuclear antigen (PCNA), and total bile acids in the esophageal lumen was measured.

**RESULTS**: After 40wk of reflux, columnar dysplasia, squamous cell carcinoma and adenocarcinoma were observed. Total bile acid in the esophageal lumen was significantly increased in the EDA group compared with the sham operated rats. PCNA LI and esophageal tissue PGE2 levels were higher in dysplastic and cancer tissue than those of the control. Overexpression of COX2 was observed in dysplastic and cancer tissue.

**CONCLUSION**: In this study, we demonstrated that bile reflux of duodenal contents induces COX2 and increases prostaglandin synthesis in dysplastic and cancer tissue. This result suggests a possible mechanism by which bile acids could promote esophageal cancer.

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**Key words**: Bile acids, COX2, Prostaglandin E2, Esophageal cancer, Esophagoduodenal anastomosis

**Core tip**: It is known that reflux of duodenal contents (bile acids) can induce mucosal injury, stimulate cell proliferation, and promote tumorigenesis. We examined the expression of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) in rat esophageal lesions induced by duodenal contents reflux. All animals underwent an esophagoduodenal anastomosis with total gastrectomy to elicit produce chronic esophagitis. In this study, we demonstrated that bile reflux of duodenal contents induces COX2 and increases prostaglandin synthesis in dysplastic and cancer tissue. This result suggests a possible mechanism by which bile acids could promote esophageal cancer.

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**INTRODUCTION**

Reflux of duodenal contents appears to contribute to the development of esophagitis and Barrett’s esophagus[1,2]. This idea is supported by several observations. In patients with gastroesophageal reflux disease, the concentration of bile acids in the esophageal refluxate correlates with the degree of esophageal mucosal injury[3]. In experimental animals, induction of a duodenoesophageal anastomosis led to esophagitis, Barrett’s esophagus and esophageal cancer[4]. The precise mechanism by which duodenal reflux causes esophageal injury and predisposes to neoplasia is uncertain. However, there is considerable evidence to suggest that bile acids contribute to this mechanism. Bile acids can induce mucosal injury[5], stimulate cell proliferation[6] and promote tumorigenesis[7].

Two isoforms of cyclooxygenase (COX) ,designated COX1 and COX2, catalyze the synthesis of prostaglandins (PGs) from arachidonic acid. COX-1 is a housekeeping gene that is expressed constitutively in most tissues. COX2 is an immediate –early response gene that is induced by a variety of mitogenic and inflammatory stimuli[8]. Elevated levels of COX2 have been detected in both inflammatory[9] and neoplastic conditions[10]. For example, COX2 is up-regulated, in peptic ulcer disease, Barrett’s esophagus and esophageal cancer. Taking this information together, it seems likely that COX2 plays a role in the pathogenesis of duodenal reflux-related diseases of the esophagus.

 In this study, we investigated the effects of bile acids and duodenal reflux on COX2 expression in a rat model of duodenoesophageal anastomosis.

**MATERIAL AND METHODS**

Eight week old male Wistar rats with body weight of approximately 300 g were used for the experiments. They were allowed to acclimatize for 2 wk prior to surgery. Solid food was withdrawn 1 d before and for 1 d after surgery. Esophagoduodenal nastomosis (EDA) was performed in 30 rats under general anesthesia (pentobarbital 50mg/kg body wt ip) through an upper midline incision. The gastroesophageal junction was ligated, and the distal esophagus was transected 2 mm above the ligature. Furthermore, the gastroduodenal junction was also ligated, and the proximal duodenum was transected 3 mm distal to the pylorus. A total gastrectomy was performed with the removal of the entire stomach, and end-to-end anastomosis of the esophagus and duodenum. The abdominal incision was closed in two layers　(Figure 1). In 10 rats, sham operations with only a midline laparotomy (control group) were performed. Postoperatively, the rats were allowed to drink water after six hours and were fed the following day. This procedure was approved by the Animal Care and Facilities Committee, Kinki University.

All of the rats were killed as described previously[11]. Special care was taken to separate the esophagus from the duodenum based on the suture line. For the animals killed at the 40 th week, all of the esophagi were cut longitudinally, and were fixed in 10% buffered formalin. The formalin-fixed esophagus was Swiss-rolled, processed and embedded in paraffin. Five-micron sections were mounted on glass slides and used for pathological, and immunohistochemical analysis.

***Immunohistochemical analysis***

**COX2**: Localization of COX2 protein was determined by immunohistochemical staining with specific antibodies. The DAKO EnVision system (Dako Cytomation Japan Co. Ltd., Kyoto, Japan) was used with autoclave acceleration. After blocking endogenous peroxidase, deparaffinized sections were covered with a protein block and serum-free medium (Dako) and were incubated overnight at 4°C with individual primary antibodies: anti-mouse COX2 (1:50, mouse monoclonal; BD Transduction Laboratories, San Jose, CA). Sections were treated with a secondary biotinylated antibody (Dako), 3,3’-diamonobenzidine tetrahydrochloride was used as the chromogen, and the sections were counterstained with hematoxylin.

**Proliferating cell nuclear antigen**: Immunohistochemical detection of proliferating cell nuclear antigen (PCNA)was performed by the avidin-biotin complex method using mouse monoclonal anti-human PCNA antibody and the appropriate Histostain Gold AEC kit. The PCNA labeling index has been widely used to assess of cell proliferation. In this study, the index was defined as the number of squamous epithelial cells with a PCNA-positive nucleus (or nuclei)/100 squamous epithelial cells (%).

***Measurement of PGE2 Production***

Each tissue sample frozen at -80°C was weighed and homogenized using a Polytron homogenizer PT-MR2100 in 0.5 mL of homogenization buffer (0.1 mol/L phosphate, pH 7.4, containing 1mmol/L ethylenediamine tetraacetic acid and 10 μmol/L indomethacin) to 100mg tissue. Then, 2 volumes of acetone were added to the samples and vigorously spun. The samples were incubated at room temperature for 5 min, and centrifuged at 1500 × *g* for 10 min to remove precipitated proteins. The supernatant was transferred into a clean tube, and dried to remove the acetone using a gentle stream of nitrogen. One mL of 1.0 mol/L acetate buffer, pH 7.4 was added to dissolve the samples, which were immediately affinity purified with an SPE cartridge (Cayman Chemical, Ann Arbor, MI). The samples were assayed using a PGE2 EIA kit (Cayman Chemical), according to the manufacturer’s instructions. PGE2 levels were shown as pg/mg of tissue.

***Measurement of bile acid in the esophageal lumen***

After the each rat was sacrificed, its esophagus was removed and lavaged with 0.5ml of saline. The saline used for the lavage was centrifuged at 1500 × *g* at 4°C for 5min. The supernatant was frozen and stored. Total bile acid concentration was measured with an ENZa BILE kit (Daiichi Chemical, Tokyo).

***Statistical analysis***

Data are expressed as the mean ± SD of each group. Student’s *t*-test was used for statistical analysis. *P <* 0.05 was considered statistically significant.

RESULTS

***General observations***

A total of 37 of 40 (92.5%) rats completed the study. In the EDA group, 27 (90%) rats completed the study, and 3 rats died from complications, such as malnutrition, and pneumonia. In the control group, 10 (100%) rats completed the study.

***Macroscopic findings***

The middle and lower esophagus of animals in the EDA group was wide and thickened. There was gross evidence of severe esophageal mucosal injury in the EDA group, which included epithelial thickening and extensive hyperplasia of the lower two thirds of the esophagus. Ulceration was frequently present in the area above the anastomosis (Figure 2).

There was a small polyploid tumor in the lower esophagus in the EDA group. The tumor was squamous cell carcinoma and adenocarcinoma. Most of the nodular lesions were also associated with carcinomas, the others with esophagitis. In addition, there was grossly normal tissue in the control group.

***Microscopic findings***

The esophagi of the control rats did not reveal any pathological findings, but various squamous cell lesions were observed in the middle and lower esophagus in the EDA group (Figure 3).

All animals from the EDA group showed histological features of esophagitis, including marked hyperplastic changes with increased thickness of the squamous epithelium, hyperkeratosis and regenerative changes with papillomatosis, and basal cell hyperplasia. These features were not found in the control group. Columnar lined epithelium (CLE) and epithelial ulceration were frequently present adjacent to the anastomosis. CLE was observed in 40% at the 40th week. Sever dysplasia in the lower esophagus occurred in 100%, squamous cell carcinoma (SCC) was observed in 40% and adenocarcinoma (ADC) was observed in 30% at the 40th week.

 To assess the biological behavior of various squamous lesions, we performed immunohistochemical staining for PCNA because the proliferative index is often increased in dysplastic and cancerous tissues. The PCNA labeling index of dysplasia and cancer (75 ± 5%) was higher than that of control (30 ± 5%) (*P <* 0.05) (Figure 4A).

***Total bile acid in the esophageal lumen (μmol/L)***

Total bile acid in the esophageal lumen was significantly increased in the EDA group (175 ± 5) compared with the sham operated rats (35 ± 5) (*P <* 0.05).

***Immunohistochemistry of COX2***

Every animal that suffered from reflux demonstrated COX2 protein expression in the lower esophagus. COX2 was abundantly expressed in both inflammatory and proliferative esophageal mucosa of rats exposed to chronic duodenoesophageal reflux (EDA). Some SCC and ADC epithelial cells strongly expressed the COX2 protein (Figure 4B).

***Measurement of PGE2 production (pg/TPmg) in esophageal tissue***

PGE2 synthetic activity was significantly increased in the EDA group (691 ± 50) compared with the sham operated group (25 ± 5) (*P <* 0.01).

**DISCUSSION**

Overexpression of COX2 has been linked to a variety of inflammatory and neoplastic conditions. Hence, it is logical to postulate that endogenous inducers of COX2 could predispose to inflammation and malignancy. Previously, Song et al[12] reported that unconjugated bile acids induced COX2.

Recent evidence suggests that bile acids, major constituents of the duodenogastroesophageal refluxate, can also promote the development of Barrett’s esophagus and esophageal cancer. Bile reflux is particularly common in individuals with gastroesophageal reflux disease who subsequently develop Barrett’s esophagus[13,14]. Barrett’s esophagus also develops in patients who have undergone total gastrectomy: a situations in which bile reflux is common. Development of Barrett’s esophagus and subsequently esophageal cancer occurs in a rat model that uses esophagoduodenostomy. The present study demonstrates that it is duodenal contents, and not gastric contents, that induce esophageal carcinogenesis through reflux. Because this carcinogenesis required no administration of carcinogens, and because spontaneous esophageal carcinoma is rare in animals, duodenal contents are most likely carcinogenic in the development of esophageal carcinoma.

The histological pattern of esophageal carcinoma induced in the present study was classified into 2 types; adenocarcinoma (ADC), and squamous cell carcinoma (SCC).

ADC always occurred near the esophagoduodenostomy and always in the columnar lined epithelium. Human esophageal ADC mostly arises in the lower third of the esophagus, and when it does occur, it is usually associated with Barrett’s esophagus. The majority of Barrett’s esophagus cases result from chronic gastro-esophageal reflux. SCC was observed distant from the site of the anastomosis, and was surrounded by chronic squamous esophagitis with features of basal-cell hyperplasia and regenerative thickening.

It is widely accepted in humans that regurgitation of duodenal contents is closely linked to Barrett’s esophagus and to the development of esophageal ADC; however, esophageal SCC is not reported to be related to reflux[15], but is strongly associated with tobacco smoking and alcohol consumption. Gastroesophageal reflux does not appear to be an independent risk factor for esophageal SCC, but it may enhance the acknowledged risk factors such as tobacco smoking and alcohol consumption. In contrast, results from several studies using rat duodenal contents reflux models have shown that development of esophageal carcinomas includes SCC[16]. In this study, the incidence of pure ADC is lower than that of SCC. It is unclear what factors lead to the formation of carcinomas of specified histology.

The precise mechanisms by which duodenal reflux causes esophageal injury and predisposes to esophageal cancer are uncertain. There is considerable evidence, however, that bile acids contribute to this process. Total bile acid　in　the　esophageal　lumen　was　significantly　increased　in　the　EDA　group　compared　with　the　control　group. Bile acids induce AP-1-mediated gene transcription[17―19] and enhance the activity of protein kinase C[20]. Recent evidence has linked bile acid induced tumorigenesis with increased activity of COX2. It is also unclear which bile acids in the refluxate contribute to COX2 induction.

As discussed above, bile acids represent one of the important constituents of duodenal fluid that has been implicated in esophageal mucosal injury[21]. Bile acids strongly induce COX2 by either transcriptional or post-transcriptional mechanisms in multiple gastrointestinal tract cancers, including cancer of the colon. pancreas, stomach, liver, esophagus and bile duct [22].

An animal model was used to determine whether duodenoesophageal reflux caused induction of COX2. We observed markedly enhanced expression of COX2 in dysplastic and cancerous mucosa obtained from rats in which an esophagoduodenal anastomosis had been created. In contrast, COX2 was undetectable in esophageal and duodenal mucosa from the control rats. Esophageal tissue PGE2 levels were significantly increased in rats that developed dysplasia and cancer. This result suggests a possible mechanism by which bile acids could promote esophageal cancer.

Bile acids increase cellular proliferation and the number of mitotic events in colonic mucosa[23]. Enhanced DNA synthesis has been demonstrated in the epithelium of the large intestine of rats treated with bile acids [24] . Reduced susceptibility to apoptosis occurs in animal and human models of colon cancer following bile acid treatment[25]. Taken together, the data suggest that bile acids are important mediators of carcinogenesis.

In conclusion, our findings suggest that reflux of bile acids induced the growth of esophageal cancers. COX2 induced by bile acids might be responsible for tumor angiogenesis, an important process in the development of esophageal cancers.

**COMMENTS**

***Background***

It is known that reflux of duodenal contents (bile acids) can induce mucosal injury, stimulate cell proliferation, and promote tumorigenesis.

***Innovations and breakthroughs***

In this study, bile reflux of duodenal contents induce COX2 and increase prostaglandin synthesis in dysplastic and cancer tissue. This result suggests a possible mechanism by which bile acids could promote esophageal cancer.

***Terminology***

Hematoxylin eosin, COX2, PGE2, and proliferating cell nuclear antigen.

***Peer review***

This is an excellent experimental study which probably adds to the existing literature.

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**Figure 1 Esophagoduodenal anastomosis model: Esophagoduodenal anastomosis with total gastrectomy.**

**Figure2 Macroscopic appearance of a resected esophagus from esophagoduodenal anastomosis and control rats.**

**Figure 3 Microscopic findings in the distal portion of the esophagus from esophagoduodenal anastomosis rats.**

**Figure 4 Immunohistochemical findings.** A: For proliferating cell nuclear antigen in esophagoduodenal anastomosis (EDA) and control rats; B: For cyclooxygenase-2 in squamous cell carcinoma and adenocarcinoma in EDA rats.