Gut 1995; 37: 168–173

PAPERS

Mucosal reactive oxygen species production in oesophagitis and Barrett's oesophagus

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Abstract

Reactive oxygen species (ROS) produced by inflammatory cells can contribute to tissue destruction. ROS have been implicated in various gastrointestinal abnormalities, including the acid related peptic diseases. Although the development of oesophagitis and Barrett's columnar epithelium is associated with prolonged reflux of gastric acid, the exact mechanism by which tissue damage occurs is not known. To discover if ROS are involved in damage to the oesophageal mucosa, this study measured in vitro the mucosal ROS concentrations of biopsied mucosal samples taken from patients with reflux oesophagitis using luminol enhanced chemiluminescence (LECL). Mucosal biopsy specimens were taken from 83 patients: 19 with normal oesophageal mucosa (group I); 20 with macroscopic oesophagitis (group II); 20 with biopsy confirmed Barrett's epithelium without macroscopic oesophagitis (group III); and 24 with Barrett's epithelium with macroscopic oesophagitis (group IV). The mucosa from patients exhibited significantly higher LECL values than the mucosa from controls. But, there were no significant differences between groups II, III, and IV. Addition of the myeloperoxidase inhibitor, azide, or the hydrogen peroxide scavenger, catalase, to the tissue suspension caused a decrease in LECL values of 32% and 45%, respectively, suggesting that neutrophils – although important – are not the only source of mucosal LECL. These data are consistent with the proposal that ROS play an important part in the tissue injury associated with oesophagitis and Barrett's columnar epithelium.

(*Gut* 1995; 37: 168–173)

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Accepted for publication 19 December 1994

Keywords: Barrett's oesophagus, oesophagitis, reactive oxygen species, oxygen free radicals, chemiluminescence, oesophageal cancer.

Oesophagitis and the pre-malignant condition of Barrett's oesophageal epithelium are common sequelae of gastro-oesophageal reflux.[1] Although reflux of gastric acid is the single most important initiating factor in the development of oesophagitis and subsequent Barrett's oesophagus,[2 3] the mechanism by which tissue injury occurs is not fully understood. Reactive oxygen species (ROS) can be produced by epithelial as well as phagocytic cells, including neutrophils.[4–7] Recent studies have shown increased mucosal concentrations of ROS in peptic disorders such as duodenal and gastric ulcer[7 8] and inflammatory disorders such as ulcerative colitis and Crohn's disease.[9–11] In these disorders, the high concentrations of tissue ROS may be responsible, in part, for the tissue injury. Additionally, ROS have been implicated in carcinogenesis and mutagenesis in malignancies of the stomach and colon.[12 13] Furthermore, Guilianelli *et al* showed that an iron containing mineral particle, nemalite, causes squamous metaplasia in rabbit primary cultures of tracheal epithelium.[14] Nemalite produced ROS, as measured by electron spin resonance, and damaged the cell lines. This study shows that ROS are capable of inducing epithelial metaplasia.

Thus, we hypothesise that ROS are present in high concentrations in the Barrett's epithelium and are important factors in the development of oesophageal mucosal tissue injury, metaplastic Barrett's oesophagus, and subsequent oesophageal adenocarcinoma. The purpose of our study, therefore, was to discover if the oesophageal mucosa of patients with oesophagitis and Barrett's oesophagus had increased tissue concentrations of ROS.

Methods

Subjects

Eighty three consecutive ambulatory outpatients undergoing upper gastrointestinal endoscopy in the outpatient endoscopy clinic between 1 September and 1 December 1993

TABLE I *Demographic characteristics of study groups*

Group (n)	Age (range)	Smoking			Alcohol			Treatment	
		Smoker	Stopped or never smoked	Pack years (mean)	Alcohol abuser	Stopped or never drank	Ounce years (mean)	H_2 receptor antagonists	Omeprazole
I Control (19)	63 (47–75)	9	10	50	9	10	206	4	0
II Oesophagitis (20)	62 (47–79)	6	14	49	13	7	105	7	0
III Inactive Barrett's (20)	67 (37–79)	5	15	63	12	8	216	3	0
IV Active Barrett's (24)	66 (43–82)	6	18	32	16	8	179	10	1

Pack years – number of cigarette packs daily×number of years. Ounce years – ounces of alcohol daily×number of years. One ounce was equivalent to (a) one 12 ounce can be beer, (b) one ounce (30 cc) of spirits, or (c) four ounces of wine. Efforts were made to obtain estimates based on patient recall and chart review.

were included in this study. Four groups of patients were studied (Table I).

Group 1 (control) consisted of 19 male patients with no oesophageal symptoms and a macroscopically normal oesophageal mucosa. These patients received upper gastrointestinal endoscopy as part of a clinical evaluation for a variety of non-reflux related conditions such as abdominal pain, anaemia, unexplained faecal blood, and peptic ulcer disease follow up. Group 2 (oesophagitis without Barrett's) consisted of 20 male patients with erosive oesophagitis. All patients had heartburn and regurgitation. Group 3 (inactive Barrett's) consisted of 20 male patients with previously confirmed Barrett's oesophagus and no macroscopic oesophagitis. These patients received upper gastrointestinal endoscopy either as part of a Barrett's surveillance programme or for evaluation of reflux symptoms. Group 4 (active Barrett's) consisted of 24 male patients with previously confirmed Barrett's oesophagus and macroscopic oesophagitis. These subjects received upper gastrointestinal endoscopy as part of the Barrett's surveillance programme or for evaluation of reflux symptoms.

Barrett's epithelium was defined as the presence of specialised columnar epithelium of any length in the tubular oesophagus. The mean length of Barrett's epithelium was 1·8 cm (range <1·0 cm to 10 cm). Tongues of Barrett's less than 2·0 cm were present in 31 (70·4%) patients; tongues greater or equal to 2·0 cm in six (13·6%) patients; and circumferential with or without tongues in seven (16·0%) patients.

Upper gastrointestinal endoscopy, biopsy, and handling of specimens

All subjects underwent upper gastrointestinal endoscopy using a standard Olympus gastroscope. All endoscopies were performed by either of two endoscopists using predefined criteria. Multiple mucosal biopsy specimens were obtained under direct vision from both the distal oesophagus (at the squamocolumnar junction) and the proximal oesophagus. In groups I and II, biopsy samples were taken 1 cm above the gastro-oesophageal junction and in the proximal oesophagus at 25 cm from the incisors. In groups III and IV biopsy samples were taken of the Barrett's epithelium from about 1 cm above the gastro-oesophageal junction (if Barrett's segment was less than 2·0 cm) and from the centre of the Barrett's if the segment was greater than 2·0 cm; and from the squamous epithelium 25 cm from the incisors. In addition, specimens were taken of the gastric mucosa from 1–2 cm below the gastro-oesophageal junction.

TABLE II *Endoscopic classification of oesophagitis*

Grade 0 – Normal mucosa with no abnormalities
Grade 1 – Erythema or hyperaemia of the oesophageal mucosa, with no macroscopic erosions
Grade 2 – Superficial ulceration or erosions involving <10% of the last 5 cm of the oesophageal squamous mucosal surface
Grade 3 – Superficial ulceration or erosions involving >10–50% of the last 5 cm of the oesophageal squamous mucosal surface
Grade 4 – Deep ulceration anywhere in the oesophagus or confluent erosion of more than 50% of the last 5 cm of the oesophageal squamous mucosal surface

Taken from Hetzel *et al.*[15]

The oesophageal mucosa was scored from grade 1 to 4 based on the endoscopic (not histological) appearance (Table II) as previously described.[15] The mucosa was considered normal (control group) if there was no macroscopic oesophagitis (grade 0). Oesophagitis was diagnosed if there was any break in the mucosa (erosions or ulcerations, or both) with or without exudate, as seen during endoscopy (grades 2–4). Barrett's oesophagus, with or without oesophagitis, was diagnosed only if there was columnar epithelium of the specialised (intestinal) type, obtained by biopsy from any level of the tubular oesophagus. The presence of columnar epithelium of the gastric type only was not considered to be Barrett's oesophagus. Histological examination of the oesophageal mucosa was used only to establish the presence of Barrett's oesophagus and not the diagnosis of oesophagitis.

Fresh mucosal biopsy specimens were placed in oxygenated Krebs-Ringer buffer and transported to the laboratory for measurement of ROS. A second specimen was placed in formalin saline for subsequent histological analysis.

Luminol enhanced chemiluminescence (LECL)

Mucosal ROS concentrations were estimated by LECL as previously described by us[9 10] by an operator who was unaware of the subject's group. Light was detected by an EMI 9813 B photomultiplier in an EMI FACT 50 MK III cooler, cooled to −20°C, and operated at

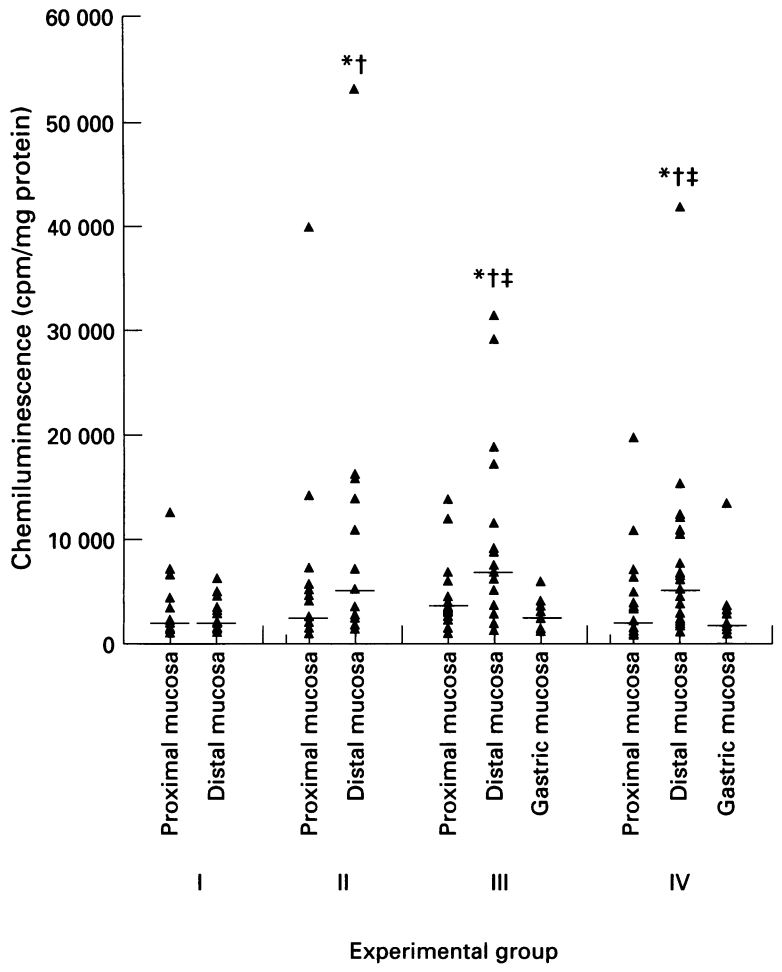

*Figure 1: LECL in the proximal, distal oesophageal mucosa and the gastric mucosa. In group I subjects, values of mucosal LECL in the distal oesophageal mucosa were similar to those in the proximal oesophageal mucosa, while groups II, III, and IV patients all had distal LECL values that were significantly higher than those in the proximal oesophageal mucosa. *=p<0·05 proximal v distal oesophagus; †=distal oesophagus v control group; ‡=distal oesophagus v gastric. Horizontal line=median. Group I – control (normal oesophageal mucosa); group II – oesophagitis without Barrett's; group III – inactive Barrett's (no macroscopic oesophagitis); group IV – active Barrett's (macroscopic oesophagitis).*

1375 volts. Single photon pulses were detected with an EMI APED amplifier discriminator (Thorn EMI, Ruislip, England), and these pulses were recorded with a frequency counter. The photomultiplier was operated in the single photon counting mode.

Biopsied oesophageal mucosal samples were suspended in 2 ml of oxygenated Krebs-Ringer solution. The tissue suspension was mixed with the incubation mixture (0·1 M NaCl, 0·05 phosphate, 0·04 mM luminol, pH 7·6), transferred to 12 mm×75 mm test tubes (total volume=2 ml), and placed in the chemiluminescence spectrophotometer where light production was measured for one minute. At this point, various vehicles or agents, including azide (100 μM) and catalase (2 μg/tube), were added to the incubation media. Chemiluminescence was again measured for one minute.

Tissue was then stored at −70°C for subsequent measurement of protein content and myeloperoxidase activity. Protein content of the tissue was measured by the Bradford method.[16] Data were expressed as counts per minute per milligram of protein. Myeloperoxidase activity was measured as previously described.[17]

Statistical analysis

Analysis of variance and χ^2 analysis were used to compare demographic data between groups. The Mann-Whitney U test was used to assess differences in chemiluminescence values between groups. A value for p of less than 0·05 was considered significant. Spearman correlation analysis was used to determine a possible correlation between mucosal chemiluminescence values and other indicators of inflammation.

These studies were approved by the Institutional Review Board for Human Studies at Hines Veterans Affairs Hospital and were performed after written consent.

Results

All four groups were similar with regard to age, and use of acid inhibiting agents such as omeprazole and H_2 receptor antagonists (Table I). There was also no statistically significant differences (χ^2 analysis) between groups with regard to smoking or ethanol abuse.

LECL in the proximal oesophagus

Median and (mean) values of mucosal LECL (cpm/mg protein) in the proximal oesophageal mucosa of control subjects (1500 (2874)), of oesophagitis without Barrett's patients (1842 (5373)), or inactive Barrett's patients (2925 (4028)), and of active Barrett's patients (1079 (2966)) were not significantly different (Fig 1). There were no significant differences in LECL values between smokers (1684) and non-smokers (2582). Furthermore, there was no significant correlation between packs/year smoking and LECL values (r=0·12) in smokers. Similarly, there was no significant difference in LECL values between smokers and non-smokers in each of the four groups. Alcoholism also had no significant effect on mucosal LECL values.

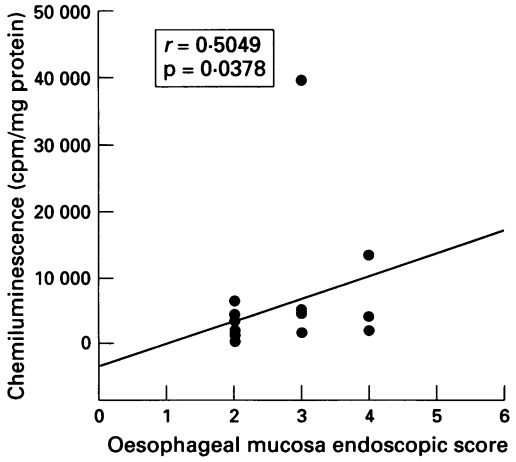

Figure 2: Correlation between mucosal LECL values and the severity of oesophagitis in patients with oesophagitis. There was a modest but significant correlation found between mucosal LECL values and oesophageal mucosa endoscopic score in patients with oesophagitis.

There was no significant difference in LECL values between heavy drinkers and non-drinkers. There was no correlation ($r=0{\cdot}04$) between ethanol consumption and LECL values.

LECL in the distal oesophagus

In control subjects, median (mean) values of mucosal LECL in the distal oesophageal mucosa (1257 (1951)) were similar to those in the proximal oesophageal mucosa (Fig 1). In contrast, patients with oesophagitis without Barrett's (4436 (9708)), inactive Barrett's (6022 (8638)), and active Barrett's (4500 (6390)) all had mucosal LECL values that were significantly higher than the values in the proximal oesophageal mucosa (Fig 1). Furthermore, there was a significant ($p=0{\cdot}037$) correlation between mucosal LECL values and the severity of oesophagitis in patients with oesophagitis ($r=0{\cdot}51$, Fig 2).

Values of mucosal LECL in the distal oesophageal mucosa of patients with Barrett's oesophagus, regardless of the presence of oesophagitis, were significantly higher than those in control mucosa (Fig 1). Additionally, in patients with Barrett's oesophagus, LECL values in the distal oesophageal mucosa were significantly higher than those in gastric mucosa in patients without (1850 (2020)) and with (1245 (2080)) endoscopic oesophagitis (Fig 1).

Neither smoking or alcohol abuse had significant effects on LECL values in any of the four groups. Mucosal LECL values in smokers (2838) were not significantly different from values in non-smokers (4240). Additionally, there was no significant correlation ($r=0{\cdot}03$) between pack years smoking and LECL. Similarly, mucosal LECL values in heavy drinkers (4166) were similar to non-alcoholic subjects (2500).

Effect of inhibitors on mucosal chemiluminescence

Azide, a specific myeloperoxidase enzyme inhibitor at 100 μM, and catalase, a hydrogen peroxide scavenger, both decreased the LECL values (mean (SEM)) in the distal oesophageal mucosa by 31·8 (6·5)%, $n=13$ and 44·6 (13·3)%, $n=11$, respectively. These results suggest that in patients with oesophagitis and Barrett's oesophagus, less than 50% of mucosal chemiluminescence originates from mucosal granulocytes.

Myeloperoxidase concentrations in the oesophageal mucosa

As expected, myeloperoxidase concentrations (mg protein) were increased in the distal oesophageal mucosa in patients with oesophagitis (113·6 (41·7), $n=9$, mean (SEM)), inactive Barrett's (148·2 (42·7), $n=12$), and active Barrett's (221·7 (80), $n=13$) when compared with controls (59·3 (15·4), $n=8$). There was no significant correlation between mucosal LECL values and myeloperoxidase values ($r=0{\cdot}17$, $p=0{\cdot}29$).

Discussion

The initiating factor for development of oesophagitis and Barrett's oesophagus is reflux of gastric contents into the oesophagus.[2 3] The most probable injurious factor is gastric acid,[18–20] but other compounds such as bile acids and pancreatic enzymes have also been implicated.[21–24] Regardless of the initiating factor, inflammatory mediators such as ROS can play an important part in maintaining the inflammatory process and subsequent tissue damage.

ROS have indeed been implicated as an important factor in tissue damage in a variety of diseases.[25 26] ROS can be produced by phagocytic cells, including neutrophils[4–6] and epithelial cells.[7] In inflammatory disorders such as ulcerative colitis and Crohn's disease, ROS can either initiate or sustain the inflammatory process, which in turn can result in tissue damage.[9 10 27 28] Hence, in oesophageal disorders, ROS can participate in oesophageal mucosal damage by maintaining the inflammatory processes.

Our data clearly show that mucosal ROS concentrations are increased in patients with oesophagitis and Barrett's oesophagus. Our results can only suggest but cannot prove a pathogenetic role of ROS. It is not unreasonable to speculate, however, that ROS are involved in the damage to the oesophageal mucosa. A positive and significant correlation between mucosal chemiluminescence values and the severity of oesophagitis support this hypothesis.

One potentially useful method that may show a pathogenetic role for ROS in the development of oesophagitis is the prevention of mucosal damage or promotion of mucosal healing, or both, by addition of antioxidant agents. The combination of catalase (a hydrogen peroxide scavenger) and superoxide dismutase did not affect experimentally induced oesophagitis in animals.[29] It is important to note, however, that catalase and superoxide dismutase are poorly absorbed and have limited tissue penetration. The lack of response to these compounds, therefore, does not exclude a possible role for ROS in oesophagitis. Further studies on the use of more suitable antioxidants in human oesophagitis are needed.

Our study did not identify the exact source of oesophageal mucosa ROS. It did, however, suggest that neutrophils are not the sole source of ROS in oesophagitis and Barrett's oesophagus, as azide and catalase only partially inhibit ROS values. In contrast, azide and catalase inhibited more than 80% of mucosal ROS values in ulcerative colitis.[9 10] Moreover, there was no significant correlation between mucosal myeloperoxidase concentrations, a sensitive index of mucosal polymorphonuclear neutrophil infiltration, and mucosal chemiluminescence values. None the less, our data suggest that polymorphonuclear neutrophils

are still an important source of mucosal ROS, as a myeloperoxidase enzyme inhibitor, azide, inhibited mucosal ROS concentrations. It should be pointed out, however, that our data do not necessarily indicate that oesophageal mucosa LECL is myeloperoxidase dependent, as azide can also inhibit other haemoproteins and can scavenge singlet oxygen.[30–32] Azide at concentrations less than 100 μM, however, which we used in our experiments, seems to be a specific myeloperoxidase inhibitor.[30–32] Hence, it seems that at least one third of the ROS in the inflamed oesophageal mucosa originates from mucosal granulocytes.

Our biochemical findings in Barrett's oesophagus and oesophagitis are consistent with well established histological findings in that only a limited number of infiltrated neutrophils are present in the mucosa of patients with oesophagitis.[33 34] In fact, our myeloperoxidase data suggested that although neutrophils are present in the inflamed Barrett's oesophagus and oesophagitis, the magnitude of infiltration is significantly less than would be normally seen in patients with ulcerative colitis.[10]

As the oesophageal mucosa contains enzymes such as xanthine oxidase,[35] which is capable of producing ROS, it is not unreasonable to suggest that epithelial cells are an important source of mucosal ROS in oesophagitis. Indeed, in such diseases as reperfusion ischaemic injury of the small intestine,[7] the ROS that result in tissue injury are produced by the epithelial cells. It is therefore not surprising that ROS concentrations are increased in oesophagitis and Barrett's oesophagus. Other sources of ROS, such as endothelial cells and mucosal macrophages, should also be considered.

We found that both myeloperoxidase activity and ROS production were increased in endoscopically uninflamed distal oesophageal mucosa in patients with Barrett's oesophagus. These findings show the lack of reliability and low sensitivity of the endoscopic appearance for diagnosing oesophageal injury. Our findings are consistent with previous experience that also showed a low sensitivity for normal appearing oesophageal mucosa in assessing the presence of oesophagitis.[34]

The potential importance of ROS in carcinogenesis in Barrett's oesophagus is intriguing, especially as ROS can result in damage to DNA.[13 36] Indeed, ROS have been implicated as a carcinogenic factor in various malignant disorders including colon cancer.[12 13] Thus, increased concentrations of ROS may play a part in carcinogenesis of Barrett's oesophagus. Our study was not designed to show a difference in ROS values between the benign condition of oesophagitis and the premalignant condition of dysplastic Barrett's epithelium. As none of our patients with Barrett's oesophagus had high grade dysplasia, the lack of significant difference in mucosal chemiluminescence values between patients with Barrett's epithelium and those with oesophagitis should not be interpreted to mean that ROS do not play an important carcinogenic part in Barrett's epithelium. Further studies are needed in patients with Barrett's and high grade dysplasia to answer these questions.

In conclusion, our data suggest that ROS may play a part in the tissue injury of oesophagitis and Barrett's oesophagus. The contribution of ROS to carcinogenesis in Barrett's epithelium remains to be shown.

The authors are grateful to Ms Jean Seidel and Ms Heidi Heintz for preparing this manuscript. The data were presented in part at Digestive Disease Week 1993 and published in abstract form in *Gastroenterology* 1993; **104:** A778.

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