Digestive Diseases and Sciences, Vol. 44, No. 11 (November 1999), pp. 2283–2289

Gastroesophageal Reflux After Combined Lower Esophageal Sphincter and Diaphragmatic Crural Sling Inactivation in the Rat

SANDRA MONTEDONICO, MD, JUAN A. DIEZ-PARDO, MD, PhD, and JUAN A. TOVAR, MD, PhD

This study tests the hypothesis that either selective or combined destruction of the lower esophageal sphincter and the diaphragmatic crural sling should induce reflux in the rat. Pull-through perfusion manometry was performed before and after lower esophageal myectomy, crural myotomy, or both. pH monitoring was used to detect reflux. Unmanipulated rats served as controls. Paired *t* tests were used for comparison of pre- and postoperative pressure values and contingency tables with Fisher's tests for examining the association between the interventions and the appearance of reflux. Esophageal myectomy decreased only sphincteric pressure from 25.9 ± 15.5 to 9 ± 6 mm Hg ($P < 0.01$), whereas crural myotomy decreased only sling pressure from 26.2 ± 13.3 to 7.3 ± 3.9 mm Hg ($P < 0.01$). Simultaneous performance of both procedures decreased sphincteric and crural pressures from 20.4 ± 7.5 to 7.6 ± 4.3 mm Hg ($P < 0.01$) and from 45.9 ± 20.6 to 18.2 ± 7.4 mm Hg ($P < 0.01$), respectively. None of the control, myectomy, or myotomy animals showed reflux upon pH-metry but 5/8 rats in which both procedures were performed had prolonged acid exposure. No esophagitis was seen. In conclusion, normal rats do not have reflux. Selective destruction of either the sphincter or the crural sling does not induce reflux, despite causing flattening of their respective manometric profiles. Conversely, combined inactivation of both components is significantly associated with reflux.

KEY WORDS: gastroesophageal barrier; lower esophageal sphincter; crural sling; manometry; myectomy; myotomy; pH-metry; reflux; rat.

The gastroesophageal barrier against reflux consists of a double sphincteric mechanism formed by the lower esophageal sphincter (LES) and the diaphragmatic crural sling (1). We recently described the anatomic arrangement and the functional features of both components in the rat, in which they are widely separated by a long intraabdominal esophagus that facilitates their independent manometric assessment (2). We showed also that in this animal there is a striking correspondence of the anatomical structure of the U-shaped muscular bundles forming the LES (open toward the left) and the crural sling (open toward the right) with their respective manometric profiles that reflect pressures exerted on the right and left sides of the esophagus respectively (3). We proposed that their simultaneous and complementary actions result in a powerful and effective sphincteric

Manuscript received January 13, 1999; accepted May 15, 1999.
From the Department of Pediatric Surgery and Research Laboratory, Hospital Infantil Universitario La Paz, Madrid, Spain.
Supported in part by FIS grants 95/0498 and 97/00287.
Address for reprint requests: Prof. Dr. Juan A. Tovar, Departamento de Cirugía, Hospital Infantil Universitario La Paz, P. de la Castellana, 261 28046 Madrid, Spain.

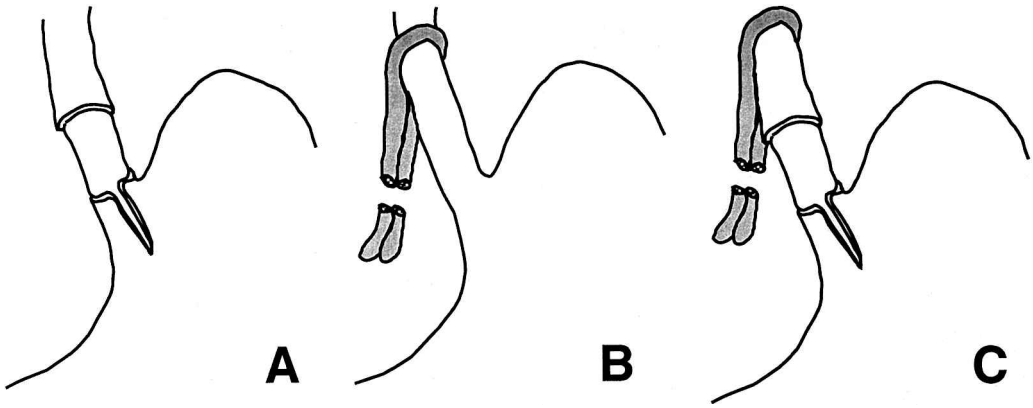

Fig 1. Schematic drawing of the interventions. For lower esophageal sphincter inactivation, a 10-mm circular extramucosal myectomy was associated with an anterior junctional myotomy (A). For diaphragmatic sling inactivation both crura were divided near their prevertebral insertion (B). Both procedures were combined in order to achieve complete inactivation (C).

mechanism, but since the occurrence of reflux and its possible mechanisms have not been investigated in this animal, convincing demonstration of the efficiency of the barrier was still lacking.

The present study demonstrates that normal rats have no reflux and tests the hypothesis that either individual or combined inactivation of the components of the gastroesophageal barrier—the LES and the diaphragmatic crural sling—will abolish its function and allow free reflux of gastric contents into the esophagus.

MATERIALS AND METHODS

Animals. Adult male Wistar rats ($N = 34$) weighing 300–500 g (Criffa, Barcelona, Spain) were housed in our animal quarters under controlled temperature and humidity conditions with 12-hr light cycles. Prior to the experiments they received standard rat chow and tap water *ad libitum*. All these conditions were approved by the local institutional research committee and met the requirements established by the current regulations for animal care and research in Europe (EC 86/L 609).

Experimental Design. Rats were randomly divided into four groups: Group 1 ($N = 9$) was used to test pH-metrically the absence of reflux in unmanipulated animals. Group 2 rats ($N = 8$) underwent pull-through esophageal manometry immediately before and 24 hr after esophagogastric junction myectomy. In group 3 ($N = 8$), manometric studies were performed before and after crural diaphragm myotomy. Finally, in Group 4 ($N = 9$), the rats had both esophagogastric junction myectomy and crural diaphragm myotomy. Twenty-four hours after these interventions, all animals underwent esophageal pH monitoring with repeated abdominal compressions to assess whether or not they had gastroesophageal reflux. A fifth group originally planned for pH studies after sham operation was discarded after the results of groups 2 and 3 were known. Four rats from each group were killed 30 days after the experiment and their esophagi were histologically investigated for esophagitis.

Operative Procedures. For esophagogastric junction myectomy, the distal esophagus was exposed and isolated through a midline abdominal incision using a clean but not sterile surgical technique and 10 mm of the distal esophageal muscular wall were excised extramucosally. This procedure was accompanied by anterior myotomy of the lower esophagogastric junction performed with microsurgery scissors and knife under surgical microscope (Wild M-650, Herbrugg, Switzerland). For diaphragmatic crural sling myotomy, the crura were exposed on the left side of the greater curvature of the stomach and underneath the spleen. A transverse incision extending through the whole thickness of the crura was carried out until they were completely detached from their insertions in the prevertebral plane. For combined myectomy–myotomy, both procedures were carried out simultaneously in the same animal. After these interventions, the incisions were closed with a two-layer running suture and the animals were allowed to recover for 24 hr. The operative procedures are depicted in Figure 1.

Esophageal Manometry. All measurements were taken in overnight-fasted animals in the supine position and under intraperitoneal anesthesia (6.25 mg/100 g ketamine hydrochloride and 0.5 mg/100 g diazepam) and spontaneous breathing according to previously described techniques (4, 5). Briefly, a tip-occluded single-lumen catheter (1 mm OD, 0.5 mm ID) with a distal side hole (1.0 × 0.5 mm) connected to an external transducer (HP 1280; Hewlett Packard, Palo Alto, California) and continuously perfused with bubble-free distilled water (0.4 ml/min) using a high-pressure, low-compliance pneumohydraulic pump (Mui Scientific, Mississauga, Ontario, Canada) was advanced into the stomach within which the pressure was registered by a monitor (Schiller) with a screen display and on-line printout at a paper speed of 2.5 mm/sec. The atmospheric pressure at the level of the atrium served as the zero reference. The recording orifice of the catheter was oriented towards the anterior wall of the stomach—the plane where both the LES and the crural diaphragm show higher pressure values (3)—and it was subsequently withdrawn into the esophagus

Table 1. Manometric Effects of Selective Destruction of Gastroesophageal Barrier Components*

	GE junction myectomy (N = 8)		*Crural sling myotomy (N = 7)*		*GE myectomy + sling myotomy (N = 9)*	
	Pre	*Post*	*Pre*	*Post*	*Pre*	*Post*
LESP	25.9 ± 15.5	9 ± 6†	12.9 ± 9.6	11.7 ± 8	20.4 ± 7.5	7.6 ± 4.3†
CSP	26.8 ± 23.5	24.2 ± 19.4	26.2 ± 13.3	7.3 ± 3.9†	45.9 ± 20.6	18.2 ± 7.4†

* Values are expressed as means ± SD and units are millimeters of mercury. LESP = lower esophageal sphincter pressure; CSP = diaphragmatic crural sling pressure.
† $P < 0.01$ against baseline within the same group.

at a constant speed (1 mm/sec) with a purposely made mechanical device in order to measure the pressures in the gastroesophageal barrier. The values recorded are the average of three successive pull-throughs. The variables analyzed were the lower esophageal sphincter pressure (LESP) or difference between intragastric pressure and the peak of the more distal component of the pressure profile, and the crural sling pressure (CSP) or difference between baseline pressure and the peak of the more proximal component of the pressure profile.

Esophageal pH Monitoring. The anesthesized animals were orotracheally intubated to avoid pharyngeal obstruction by the pH probe and left under spontaneous breathing. With the rat in the supine position, the trachea was illuminated through the neck with a fiberoptic illuminator (Raypa S.L. F-150), and the lighted larynx was observed directly through the mouth using a veterinary otoscope. A guide wire was then advanced into the trachea and, after removing the otoscope, a 16-G radiopaque intravenous cannula was placed as a tracheal tube and secured to the snout. A radiopaque catheter was advanced into the stomach under fluoroscopy and 0.5 ml of 0.1 N HCl was injected in the stomach to ensure that its content was acidic. The catheter was subsequently withdrawn within another slightly larger catheter to avoid acid contamination of the esophagus. A 2.5-mm pH antimony electrode (Synectics, Stockholm, Sweden) was advanced through the mouth and the tip placed approximately 2 vertebral bodies above the diaphragm under x-ray control. A skin electrode taped to the chest of the rat served as a ground electrode for the pH probe and pH signals were recorded on a Synectics Mark IV digitrapper (Synectics) and later analyzed using Multigram software (Gastrosof, Irving, Texas). The total duration of pH metering was 30 min: 10 min in the resting condition followed by a period during which abdominal compression lasting 60 sec was applied three times using a blood pressure cuff placed around the abdomen inflated up to pressures of 200 mm Hg (we knew from preliminary experiments that this maneuver induces intraabdominal pressure increases of around 30 mm Hg. The pH electrodes were calibrated before and after the experiment according to the equivalent clinical routines, and acid reflux into the esophagus was defined as any decrease of the intraesophageal pH below the arbitrarily chosen limit of 4.

Histological Studies. Four weeks after the operative procedure, four rats from each experimental group and four control rats were killed and their esophagi were resected. The specimens were fixed in 10% formalin for 24 hr and serial transversal 5-μm sections from paraffin blocks were stained with Masson trichrome and H&E and microscopically assessed by a blinded observer. The following criteria were accepted for the diagnosis of esophagitis: destruction of the outer keratinized mucosal layer with frank ulceration, hyperkeratosis, acanthosis, basal layer hyperplasia, and leukocyte infiltration (6).

Data Analysis. Manometric values are described as means ± standard deviations and the pressure units are millimeters of mercury. The normality of their distribution was assessed by comparing the actual values with the theoretical ones for the same means using the Kolmogorov-Smirnov test. After ascertaining that parametric tests could be used, comparisons among pre- and postoperative situations for both variables (LESP and CSP) in each experimental group were done with paired parametric tests (Student's *t*). The association of the presence of gastroesophageal reflux with the operative procedures was tested by two-way contingency tables and chi-square tests with continuity correction when indicated or Fisher's tests. When P value was <0.05, the null hypothesis was rejected and the difference was considered significant.

RESULTS

Effect of Esophagogastric Myectomy and Crural Myotomy on Manometric Tracings. In group 2 all rats tolerated well the myectomy of the gastroesophageal junction. All survived, and after the period of fasting they resumed normal feeding. After operation, the LESP pressure significantly decreased from 25.9 ± 15.5 mm Hg to 9 ± 6 mm Hg ($P < 0.01$) while CSP remained unchanged (Table 1, Figure 2A and B). In group 3 one rat died of pneumothorax after crural diaphragm myotomy whereas the remaining seven animals did well after the operation. This maneuver decreased significantly the CSP from 26.2 ± 13.3 mm Hg to 7.3 ± 3.9 mm Hg ($P < 0.01$) while LESP did not change (Table 1, Figure 2C and D). Finally, in group 4, in which gastroesophageal myectomy and crural myotomy were performed simultaneously, both LESP and CSP significantly decreased after operation from 20.4 ± 7.5 to 7.6 ± 4.3 mm Hg ($P < 0.01$) and from 45.9 ± 20.6 to 18.2 ± 7.4 mm Hg ($P < 0.01$) respectively (Table 1, Figure 2E and F).

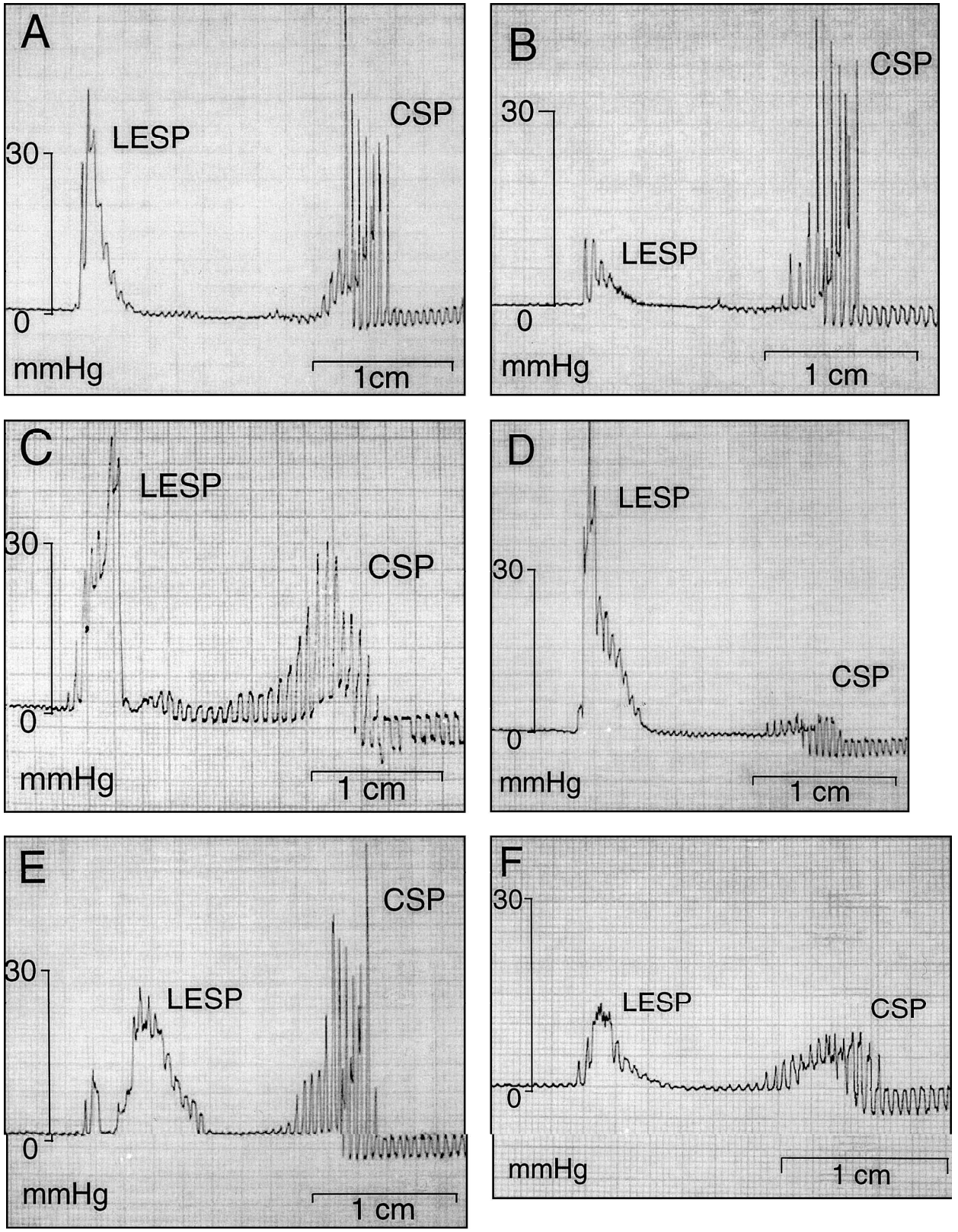

Fig 2. Constant-speed pull-through manometric profiles of the gastroesophageal junction in three experimental groups. The first or distal pressure component corresponds to the lower esophageal sphincter pressure (LESP) and is separated by a long intraabdominal segment from the more proximal phasic one corresponding to the crural sling pressure (CSP), after which intrathoracic pressures are recorded. The tracings are almost identical before the operation in animals of the three groups (A, C, and E), but the sphincteric component, the crural component, or both are obviously flattened after esophagogastric myectomy (B), crural myotomy (D), or both (F).

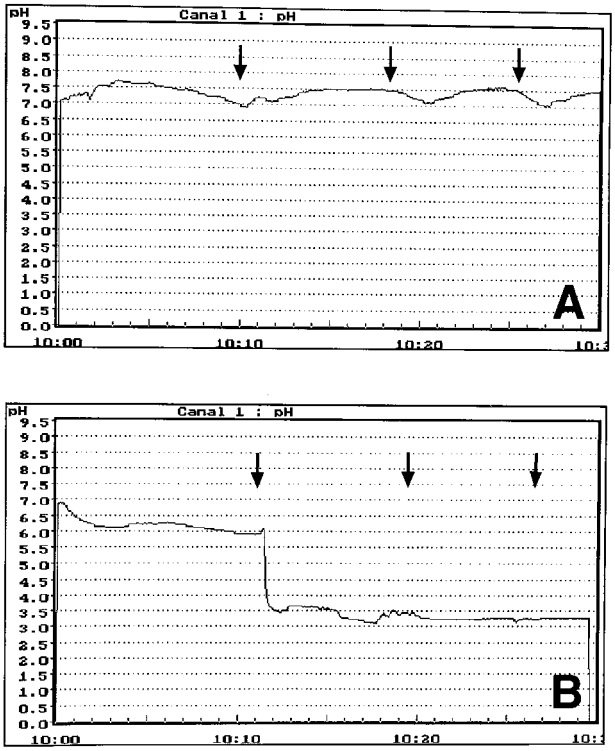

Fig 3. Thirty-minute pH-meter tracings in a control rat (A) and in an animal subjected to combined esophagogastric myectomy and crural myotomy (B). pH remains close to 7 in A despite three 60-sec abdominal compressions (arrows), whereas it falls below 4 after the first compression in B and remains on the acid side from then on.

Effect of Esophagogastric Myectomy and Crural Myotomy on Antireflux Barrier Function. Upon pH monitoring, none of the nine rats from the control group 1 had a single episode of gastroesophageal reflux either in baseline conditions or during or after abdominal compression (Figure 3). In the gastroesophageal myectomy group, pH monitoring was possible in seven rats and in the crural myotomy group, it could be performed in five animals. No acid reflux could be documented in any of them in the resting condition or after abdominal compression. Conversely, 5/9 animals in which both lower esophageal sphincter myectomy and crural myotomy had been performed (55.5%) had falls of the intraesophageal pH below pH 4 after the first abdominal compression was applied, and the episodes were not cleared for the subsequent 20 min (Figure 3). The association of combined myectomy and myotomy with gastroesophageal reflux was statistically significant ($P < 0.01$). The outer keratinized layer was intact in all histologically examined esophagi and neither inflammatory infiltration nor changes in epithelial thickness could be seen.

DISCUSSION

Both the smooth muscle of the lower esophageal sphincter (LES) and the skeletal muscle of the diaphragmatic crural sling that embraces the terminal esophagus contribute to the gastroesophageal barrier that prevents reflux of material from the stomach into the esophagus (1). The LES was the first component to be identified in the 1950s as a manometric high-pressure zone (7), although no actual anatomic structure corresponding to this pressure effect had been demonstrated at this site. Only later detailed studies on the human gastroesophageal junction showed that the muscular equivalent of the LES corresponds to a thickened inner muscle layer straddling the lesser curvature and consisting of a combination of clasp fibers and long oblique slinglike bundles straddling the greater curvature oriented almost perpendicular to the former ones (8). Nowadays, the muscular architecture and the manometric three-dimensional pressure image of the LES are better known (9).

Since its description, the smooth muscle of the LES was thought to be mainly responsible for the high-pressure zone at the esophagogastric junction, and for years the role of the diaphragmatic crural sling in the valvular mechanism of the gastroesophageal barrier was ignored. In 1985, Boyle et al (10) anchored a pressure recording device to the LES in order to avoid transhiatal axial displacements caused by respiratory movements. They were able to show that the pressure increases observed in the cat LES during inspiration were caused by the rhythmic sphincterlike activity of the crural sling of the diaphragm, since they were abolished by neuromuscular paralysis and were proportional to the depth of the respiratory excursions (10). Later on, a high-pressure zone located at the thoracoabdominal junction was demonstrated in patients who had undergone gastroesophageal junction resection and sphincter ablation (11), and it was also confirmed that crural pressure was abolished in patients with hiatal hernia and that crural repair reestablished them (12). However, manometric assessment of the individual contributions of the sphincteric and crural components of the barrier remains difficult in both animals and human individuals because of the wide overlapping of the anatomical structures that account for their effects in them (13). Our group has recently shown the advantages of the rat as an experimental model for the independent

manometric study of the diaphragmatic and crural components of the barrier because in this animal they are widely separated by a long intraabdominal esophagus. The striated muscle of the crural sling, consisting of a powerful U-shaped bundle inserted in the anterior surface of the vertebral bodies, is inactivated by muscular relaxation, whereas the lower esophageal sphincter, consisting of an assembly of two U-shaped smooth muscles perpendicularly arranged on the gastroesophageal junction, remain tonic in the same conditions (2). In this animal, there is a striking correspondence of the anatomical structure with the manometric profile of both components of the barrier that act in opposite directions: the LES is open toward the left and exerts its pressure on the right side, whereas the crural diaphragm opens posteriorly and to the right and exerts its pressure on the left and anterior quadrants (3). In our previous studies we proposed that the simultaneous and complementary action of both components could result in a particularly powerful and effective sphincteric mechanism, but we did not provide evidence of this mechanism representing an actual defense against acid reflux in this model.

Our present results show that normal rats do not have acid reflux in resting conditions and that intraabdominal pressure increases are not able to induce it in them. We also show that neither sphincteric myectomy nor crural diaphragm myotomy alone induce reflux into the esophagus in spite of significantly decreasing pressures of their respective manometric profiles. It appears that inactivation of the barrier is only possible by simultaneous destruction of both components because this double intervention was capable of causing increased esophageal acid exposure after abdominal compression. In spite of this, no esophageal lesions were seen after one month of reflux, but this was expected because the esophageal epithelium in rats is keratinized and resistant to acid challenge. In fact, esophagitis secondary to purely acid reflux has never been demonstrated in this animal (20).

The harmful effects of LES inactivation are well known because gastroesophageal reflux can be induced in achalasia patients by myotomy and in pigs by myectomy (14) and also because individuals with severe reflux disease have often low or absent LES pressures (15). Conversely, the contribution of the crural sling fibers to reflux has been explored only recently in experimental and clinical settings: Mittal et al found acid reflux after crural myotomy in cats in resting conditions but not after abdominal compression (16). It has been pointed out that most reflux episodes occur during periods of transient LES relaxation accompanied by inhibition of the crural diaphragm (15, 17). Mittal et al suppressed the gastroesophageal barrier in normal subjects by either atropine or stimulation of pharyngeal receptors and found that gastroesophageal reflux occurred only during periods of transient inhibition of both the LES and the crural diaphragm (18, 19). These findings indicate that the absence of LES pressure in normal subjects does not necessarily induce reflux by itself if contraction of the crural diaphragm is preserved and, therefore, that the diaphragmatic contribution to the defense against reflux might be more important than previously thought. Our demonstration of the relative contributions of both LES and crural diaphragm to the antireflux barrier in rats and the facts that their independent inactivation is unable to elicit reflux and that this is only observed after the combined destruction of both components demonstrates that this animal is an affordable and reproducible model for further studies on various aspects of barrier dysfunctions.

REFERENCES

1. Mittal RK, Balaban DH: The esophagogastric junction. N Engl J Med 336:924–932, 1997
2. Soto C, Qi B, Diez-Pardo JA, Tovar JA: Identification of the diaphragmatic crural component of the gastro-esophageal barrier in the rat. Dig Dis Sci 42:2420–2425, 1997
3. Montedonico S, Godoy J, Mate A, Possoegel A, Diez-Pardo JA, Tovar JA: Muscular architecture and manometric image of the gastroesophageal barrier in the rat. Dig Dis Sci (submitted)
4. Wang WL, Tovar JA, Eizaguirre I, Aldazabal P: Airway obstruction and gastroesophageal reflux. An experimental study of the pathogenesis of this association. J Pediatr Surg 28:995–998, 1993
5. Qi B, Soto C, Diez-Pardo JA, Tovar JA: An experimental study on the pathogenesis of gastroesophageal reflux after repair of diaphragmatic hernia. J Pediatr Surg 32:1310–1313, 1997
6. Ismael-Beigi F, Horton PF, Pope CE: Histological consequences of gastroesophageal reflux in man. Gastroenterology 58:163–174, 1970
7. Fyke FE, Code CF, Schlegel JF: The gastroesophageal sphincter in healthy human beings. Gastroenterologia (Basel) 86:135–150, 1956
8. Liebermann-Meffert D, Allgwer M, Schmid P, Blum AL: Muscular equivalent of the lower esophageal sphincter. Gastroenterology 76:31–38, 1979
9. Stein H, Liebermann-Meffert D, DeMeester T, Siewert JR: Three-dimensional pressure image and muscular structure of the human lower esophageal sphincter. Surgery 117:692–698, 1995
10. Boyle JT, Altschuler SM, Nixon TE, Tuchman DN, Pack AI, Cohen S: Role of the diaphragm in the genesis of lower esophageal sphincter pressure in the cat. Gastroenterology 88:723–730, 1985

11. Klein WA, Parkman HP, Dempsey DT, Fisher RS: Sphincter-like thoracoabdominal high pressure zone after esophagogastrectomy. Gastroenterology 105:1362–1369, 1993
12. Peck N, Callander N, Watson A: Manometric assessment of the effect of the diaphragmatic crural sling in gastro-oesophageal reflux: Implications for surgical management. Br J Surg 82:798–801, 1995
13. Mittal RK: The crural diaphragm, an external lower esophageal sphincter: A definitive study (editorial). Gastroenterology 105:1565–1577, 1993
14. Schopf BW, Blair G, Dong S, Troger K: A porcine model of gastroesophageal reflux. J Invest Surg 10:105–114, 1997
15. Mittal RK, Holloway RH, Penagini R, Blackshaw LA, Dent J: Transient lower esophageal sphincter relaxation. Gastroenterology 109:601–610, 1995
16. Mittal RK, Sivri B, Schirmer B, Heine K: Effect of crural myotomy on the incidence and mechanism of gastroesophageal reflux in cats. Gastroenterology 105:740–747, 1993
17. Martin CJ, Dodds WJ, Liem HH, Dantas RO, Layman RD, Dent J: Diaphragmatic contribution to gastroesophageal competence and reflux in dogs. Am J Physiol 263:G551–G557, 1992
18. Mittal RK, Chiarelli C, Liu J, Shaker R: Characteristics of lower esophageal sphincter relaxation induced by pharyngeal stimulation with minute amounts of water. Gastroenterology 111:378–384, 1996
19. Mittal RK, Holloway R, Dent J: Effect of atropine on the frequency of reflux and transient lower esophageal sphincter relaxation in normal subjects. Gastroenterology 109:1547–1554, 1995
20. Levrat M, Lambert R, Kirshbaum G: Esophagitis produced by reflux of duodenal contents in rats. Am J Dig Dis 7:564–573, 1962