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• *BRIEF REPORTS* •

Metabolic changes in the lower esophageal sphincter influencing the result of anti-reflux surgical interventions in chronic gastroesophageal reflux disease

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Abstract

AIM: With the availability of a minimally invasive approach, anti-reflux surgery has recently experienced a renaissance as a cost-effective alternative to life-long medical treatment in patients with gastroesophageal reflux disease (GERD). We are not aware of the fact whether reflux episodes causing complaints for a long time i.e., at least for one year are associated with metabolic changes in the lower esophageal sphincter, and if so, whether these may influence functional results achieved after anti-reflux surgery.

METHODS: Between 1 January 2001 and 31 December 2002 we performed anti-reflux surgery on 79 patients. Muscle samples were taken from the lower esophageal sphincter (LES) in 33 patients during anti-reflux surgery. Inclusion criteria were: LES resting pressure below 10 mmHg and a marked, pH proven acid exposure to the esophagus of at least one year's duration, causing subjective complaints and requiring continuous proton pump inhibitor treatment. Control samples were obtained from muscle tissue in the gastroesophageal junction that had been removed from 17 patients undergoing gastric or esophageal resection. Metabolic and lysosomal enzyme activities and special protein concentrations 16 parameters in total were evaluated in tissue taken from control specimens and tissue taken from patients with GERD. The biochemical parameters of these intra-operative biopsies were used to correlate the results of anti-reflux operations (Visick I and II-III).

RESULTS: In the reflux-type muscle, we found a significant increase of the energy-enzyme activities e.g., creatine kinase, lactate dehydrogenase, β-hydroxybutyrate dehydrogenase, and aspartate aminotransaminase-. The concentration of the structural protein S-100 and the myofibrillar protein troponin I were also significantly increased. Among lysosomal enzymes, we found that the activities of cathepsin B, tripeptidyl-peptidase I, dipeptidyl-peptidase II, β-hexosaminidase B, β-mannosidase and β-galactosidase were significantly decreased as compared to the control LES muscles. By analyzing the activity values of the 9 patients in Visick groups II and III at two months post-surgery, we found a significant increase in the activity of the so-called energy-enzyme values and in the concentration of structural and myofibrillar proteins as compared to the rest of the reflux patients.

CONCLUSION: Our results call attention to the metabolic changes that occurred in the LES muscles of reflux patients. The developing hypertrophy-like changes of LES muscles may be a reason for complaints after anti-reflux surgery, which consisted mainly of reports of persisting dysphagia.

Key words: LES muscle; Metabolic enzymes; Lysosomal enzymes; Anti-reflux surgery; Hypertrophy; Dysphagia; Gastroesophageal reflux

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INTRODUCTION

Clinical medicine has advanced by retrospective analysis, relating symptoms to anatomic or structural lesions and using this relationship prospectively to diagnose disease. In time, biochemical or histological abnormalities were identified as having a high probability of being caused by a disease process, such as metabolic alterations, neoplasia, inflammation, or ischemia. Consequently, biochemical and histological patterns are now used to recognize and identify specific diseases in symptomatic patients.

Functional disorders of the esophagus are abnormalities that can exist for a period of time without causing morphologic

changes even though considerable symptoms develop, such as heartburn, regurgitation, and dysphagia.

However, we are not aware of whether reflux episodes causing complaints for a long time at least for a year - induce any metabolic changes in the lower esophageal sphincter (LES), and if so, whether these may influence functional results achieved after anti-reflux operations.

It is true that with the availability of a minimally invasive approach, anti-reflux surgery has recently experienced a renaissance as a cost-effective alternative to lifelong medical treatment in patients with gastroesophageal reflux disease (GERD). The number of anti-reflux procedures performed has virtually exploded. The laparoscopic approach does not, however, reduce the prevalence of side effects usually associated with anti-reflux surgery even in experienced hands[1-3].

A review of the literature shows that between 5% and 20% of patients who had an anti-reflux operation would experience some form of recurrent or persistent symptoms, requiring continued or renewed medical attention. Today recurrent reflux is the most common reason for failure of anti-reflux surgery. This is followed by dysphagia and by a combination of dysphagia with reflux symptoms. The so-called "gas bloat syndrome" or gastric denervation symptoms are rare after anti-reflux surgery[4].

Although most patients with recurrent, persistent or new symptoms after an anti-reflux procedure can be managed medically, some will require revisional or salvage surgery and it is well-known that the rate of success exponentially decreases in proportion to the number of re-operations[5].

In the present study, we have analyzed the effect of persistent reflux on LES muscle metabolism based on the biochemical analysis of muscle samples taken from LES during 46 operations performed in the cardiac region.

The biochemical analyses used in this study included measurement of enzymes with functions in anabolic processes "energy enzymes": creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (HBDH), aspartate aminotransaminase (AST), lysosomal enzymes that play a role in catabolic processes, namely carbohydrate hydrolysis α-mannosidase (AMAN), β-mannosidase (BMAN), β-galactosidase (BGAL), β-glucuronidase (GCU), β-hexosaminidase B (HEX), and enzymes involved with protein degradation cathepsin B (CB), tripeptidyl-peptidase I (TPP I), dipeptidyl-peptidase II (DPP II). In addition, the myoglobin (MYO) and troponin I (TNI) proteins are important in skeletal muscle function and S-100 is a highly acidic calcium-binding protein found in various organs in the body.

MATERIALS AND METHODS

Between January 1 2001 and December 31 2002, we performed anti-reflux surgery on 79 patients. Out of the 75 cases we performed a laparoscopic operation, in 4 cases the intervention was conventional Nissen or Belsey-Mark IV fundoplication. In 33 patients the muscle sample was taken from the LES with scissors, without cauterization, during the operation from a standard site on the right side of the anterior vagus, on the intra-abdominal part of the esophagus, where the floppy-Nissen wrap was placed. First the mucosa was brought into the visual field similar to cardiomyotomy in order to ensure that the muscle samples, to be analyzed, represented both longitudinal and circular muscle fibers and the excision was made in this way. Inclusion criteria for patients in the reflux group were as follows: LES resting pressure below 1.33 kPa (10 mmHg), and a marked, pH proven acid exposure to the esophagus that has persisted for at least a year, causing subjective complaints and requiring continuous proton pump inhibitor (PPI) treatment. Those patients whose contraction amplitude was less than 2.66 kPa (20 mmHg) in one or more of the three lowest esophageal segments and/or for whom more than 20% simultaneous waves in these segments had been verified were excluded.

The control group contained muscle samples, obtained from the gastroesophageal junction, that was removed during 17 gastric or esophageal resections. Only patients with no reflux complaints in the history were included in the control group. Since in the majority of these patients the surgical intervention was necessary because of a tumor, we checked the muscle sample histology for tumor infiltrates.

The data of 46 patients, out of the 50 included (92%), were evaluated. Reasons for exclusion included histologically confirmed tumor infiltration (n = 2), progressive muscular dystrophy and congenital esophageal atresia (n = 1 each). Thus, GERD was represented with samples from 31 patients, while normal LES with samples from 15 patients. The anti-reflux operation was in each case a floppy-Nissen type reconstruction. The same surgeon performed the operations as well as muscle sampling.

Complaints of operated patients were evaluated according to the following Visick-classification: I: symptom-free. II: mild symptoms, requires no treatment. III: can be treated with medication or with dilation. IV: symptoms that cannot be controlled with conservative treatment, reoperation needed.

The muscle samples were frozen on dry ice immediately after dissection and stored at -70 ℃ prior to use.

Samples were thawed on ice, placed in 50 volumes (w/v) of 0.15 mol/L NaCl, 0.1% Triton X-100 and homogenized with a Brinkmann Polytron homogenizer. A soluble supernatant was prepared by centrifugation at 12 000 g at 4 ℃ for 25 min.

The activity of CK, LDH, HBDH, AST enzymes and CK-MB were measured with reagents produced by A.L. Instruments (Diachem Kft.; Budapest, Hungary), with kinetic UV photometry method, on an Olympus AU 600 chemical analyzer (Olympus Diagnostica GmbH; Hamburg, Germany).

Protein S-100 determination was performed with a two-step immunoluminometric sandwich assay (ILMA) technique, by applying LIAISON® Sangtec® 100 (AB Sangtec Medical; Bromma, Sweden) in a LIAISON® immunochemical automat (BYK SANGTEC Diagnostica; Dietzenbach, Germany).

The concentration of TNI and MYO were determined with the Microparticular Enzyme Immunoassay (MEIA) method (Lumi-Phos 530 is measured) in a Beckman Access immunochemical automat (Beckman Coulter Access

Immunoassay System; Fullerton, USA).

Glycosidase activities were measured using 4-methylumbelliferyl (4-MU) substrates described by Sleat *et al*[6] Protease assays using amino-4-methylcoumarin (AMC) substrates have been described by Sleat *et al*[7] and Sohar *et al*[8] Reactions were initiated by adding 40 μL substrate (various concentration 20 μmol/L), buffer (100 mmol/L) solution to 5 μL (CB) or 10 μL (other enzymes) of sample (after centrifugation, supernatants were diluted 2-, 4- and 8-fold in homogenization buffer in duplicate), incubated at 37 ℃, and terminated by the addition of 100 μL of 0.5 mol/L glycine, pH10.5 (at 4-MU substrates) or 0.1 mol/L monochloroacetic acid in 0.1 mol/L acetate, pH4.3 (AMC substrates). Buffers consisted of 0.1 mol/L citric acid or 0.1 mol/L sodium acetate adjusted to the indicated pH using sodium hydroxide, acetic acid, or HCl, respectively, and contained 0.15 mol/L NaCl with 0.1% Triton-X-100. Substrates were purchased from Sigma Chemical Company (St. Louis, MO, USA) and were prepared as stocks in dimethyl sulfoxide that were added to the reaction buffer immediately prior to assay. Samples added to substrate solutions after addition of termination buffer were used as blanks. Fluorescent reaction products were measured using a CytoFluor II (PerSeptive Biosystems, Framingham, MA) fluorescence multiwell plate reader with excitation at 360 nm and emission at 460 nm.

Statistical analyses were performed using a one-factor variance-analysis and interval estimate method by applying MS Excel Analysis Toolpack program. Each subject enrolled into the study signed an informed consent form. Permission for the investigations was sought and obtained from the appropriate local Ethical Committee.

RESULTS

In 12 out of the 16 measured parameters, we found significant biochemical differences between reflux-type and control LES muscle. The activities of CK, LDH, HBDH, and AST enzymes were significantly elevated in the reflux-type LES muscle compared with the control group (Table 1). A significant increase was also found for the concentration of S-100 and TNI proteins in the reflux-type muscle. There were no statistically significant differences in myoglobin concentration or in CK-MB activity between the two examined groups, although seemingly marked individual differences were noted.

We found definite differences in relation to the lysosomal enzymes, since the activity of CB, TPP I, DPP II, HEX, BMAN and BGAL were significantly lower in the reflux-type LES as compared to the normal LES (Table 2).

Table 1 Metabolic enzyme activities and special protein concentrations in normal LES and in LES operated due to gastroesophageal reflux, as well as in patients with and without complaints in the postoperative period

	LES					LES in GERD				
	Normal		GERD			Visick I		Visick II–III		
Enzymes	Mean	SE	Mean	SE	*P*	Mean	SE	Mean	SE	*P*
CK	73 027.3	32 312.4	398 220.7	210 587.2	*P*<0.05	111 904.3	29 131.0	1 098 105.3	514 604.5	*P*<0.001
LDH	23 805.7	9 561.9	127 786.9	62 210.7	*P*<0.05	44 229.0	7 850.8	332 039.5	54 804.5	*P*<0.001
HBDH	7 960.3	3 159.9	41 400.3	20 129.8	*P*<0.05	13 906.0	2 588.4	105 553.7	47 629.0	*P*<0.001
AST	2 869.5	1 453.0	13 397.2	6 825.7	*P*<0.05	3 697.0	923.0	36 030.9	14 938.4	*P*<0.001
CK-MB	269.1	103.9	1 580.3	1 332.6	NS	597.5	185.3	3 655.2	4 374.7	NS
S-100	1 252.5	844.0	10 569.1	5 038.1	*P*<0.05	3 986.5	2 123.0	26 018.5	11 485.7	*P*<0.001
MYO	750.3	538.3	11 339.8	14 029.3	NS	2 395.8	1 268.1	32 581.8	52 349.6	*P*<0.05
TNI	0.17	0.08	1.56	0.78	*P*<0.05	0.68	0.24	3.93	2.25	*P*<0.001

LES: lower esophageal sphincter; GERD: gastroesophageal reflux disease; Visick I: symptom-free; Visick II-III: mild symptoms can be treated with medication; SE: standard error; *P*: significance; NS: non-significant; CK: creatine kinase; LDH: lactate dehydrogenase; HBDH: α-hydroxybutyrate dehydrogenase; AST: aspartate aminotransaminase; CK-MB: creatine kinase MB (U/g soluble protein); S-100; MYO: myoglobin; TNI: troponin I (μg/g wet weight).

Table 2 Lysosomal enzyme activities in normal LES and in LES operated due to gastroesophageal reflux, as well as in patients with and without complaints in the postoperative period

	LES					LES in GERD				
	Normal		GERD			Visick I		Visick II–III		
Enzymes	Mean	SE	Mean	SE	*P*	Mean	SE	Mean	SE	*P*
CB	14 177.5	3 441.9	7 727.6	1 913.9	*P*<0.001	6 442.8	2 242.7	10 439.8	3 548.1	*P*<0.05
TPP I	74 318.0	10 742.6	47 509.9	5 339.3	*P*<0.001	47 541.6	6 760.4	47 438.5	1 1067.2	NS
DPP II	3 970.4	1 189.3	2 513.3	512.0	*P*<0.001	2 860.7	647.1	1 779.7	744.1	*P*<0.05
HEX	17 253.7	4 457.9	9 185.0	1 604.7	*P*<0.001	9 528.2	2 267.2	8 460.4	2 054.9	NS
GCU	17 345.0	5 145.4	13 123.5	2 307.5	NS	13 980.1	3 263.8	11 315.2	2 541.3	NS
BMAN	4 207.5	1 246.1	2 922.9	587.7	*P*<0.05	3 248.4	824.1	2 235.9	481.4	NS
AMAN	962.4	726.8	469.9	172.2	NS	567.4	232.8	238.5	108.9	NS
BGAL	17 714.3	3 561.0	11 396.7	1 567.3	*P*<0.001	11 733.9	2 241.9	10 684.9	1 821.4	NS

LES: lower esophageal sphincter; GERD: gastroesophageal reflux disease; Visick I: symptom-free; Visick II–III: mild symptoms can be treated with medication; SE: standard error; *P*: significance; NS: non-significant; CB: cathepsin B; TPP I: tripeptidyl-peptidase I; DPP II: dipeptidyl-peptidase II; HEX: β-hexosaminidase B; GCU: β-glucuronidase; BMAN = β-mannosidase; AMAN: α-mannosidase; BGAL: β-galactosidase (pmoL/h·mg).

The HBDH/LDH ratio was significantly lower in the reflux-type LES muscle as compared to the control group, while the CK-MB/CK ratio showed no statistical differences (Table 3).

Patients were followed up in the 2nd and 12th month after the operation. Stratification of patients by Visick classification at two months post-surgery showed more patients with Visick I (n = 22) than with Visick II (n = 8) or III (n = 1). The biochemical parameters of Visick II-III patients were combined for comparison with Visick I patients. The leading symptom in the Visick II-III group was difficulty in swallowing (Table 4). In the Visick II-III group, the activities of CK, LDH, HBDH, AST, CB enzymes were significantly higher while DPP II lower than in the Visick I group. Similarly, the concentrations of S-100, TNI, and myoglobin were also significantly higher in Visick II-III patients as compared to Visick I. At the one-year control examination, four of these patients were dissatisfied with the results of the operation. We performed objective imaging and functional examinations for these four patients, and found that the complaints of only one patient could be explained by clear anatomical reasons, where recurring hiatal hernia developed as a consequence of latent brachy-esophagus.

DISCUSSION

With the renaissance of anti-reflux surgery, patients with persistent, recurrent, or newly developed symptoms following an anti-reflux procedure are likely to become a more common problem in the near future. Recurrent reflux is usually due to a breakdown of the repair and can frequently be treated medically or by repeating the procedure. In contrast, post-operative dysphagia with or without accompanying reflux symptoms may be due to a myriad of causes, which include a slipped wrap, a wrap that has been placed around the stomach rather than the esophagus, a too tight or too long wrap, the development of a stricture, the presence of a motor disorder of the esophageal body, hitherto unknown factors, or a combination of these.

So far, no adequately designed clinical trials have shown any benefit with a tailored approach to anti-reflux surgery, where motor function of the esophagus and in the gastroesophageal junction is assessed pre-operatively to determine the exact surgical procedure to be followed.

Lundell *et al*[9] supports this opinion, which found that pre-operative manometric observations had no predictive value regarding the outcome of either form of fundoplication i.e., Nissen-Rosetti total fundic wrap and the 180° partial wrap. An important question is, therefore, whether patients with chronic GERD benefit from anti-reflux surgery and if not, whether it is possible to define the patient profiles of these potential failures.

We looked for an answer by performing the biochemical analysis of LES muscles of those suffering from chronic gastroesophageal reflux disease. In the reflux-type muscle we found a significant increase in energy-enzyme activities CK, LDH, HBDH, AST, as well as, the concentrations of the S-100 protein and TNI. Among lysosomal enzymes, we found that the activities of CB, TPP I, DPP II, HEX, BMAN and BGAL were significantly decreased as compared to the control LES muscles. This is not in conflict with the observation that, in the early stage of hypertrophy, the protein synthesis and the activity of lysosomal glycosidases and proteases temporarily increase, since proteins and carbohydrates, that became unnecessary during muscle transformation, have to be eliminated. However, after the

Table 3 Metabolic enzyme ratios in normal LES and in LES operated due to gastroesophageal reflux, as well as in patients with and without complaints in the postoperative period

	LES					LES in GERD				
	Normal		GERD			Visick I		Visick II–III		
Enzymes	Mean	SE	Mean	SE	*P*	Mean	SE	Mean	SE	*P*
HBDH/LDH	0.337	0.009	0.319	0.006	*P*<0.001	0.321	0.009	0.318	0.008	NS
CK-MB/CK	0.005	0.003	0.006	0.003	NS	0.006	0.003	0.005	0.007	NS

LES: lower esophageal sphincter; GERD: gastroesophageal reflux disease; Visick I: symptom-free; Visick II–III: mild symptoms can be treated with medication; SE: standard error; *P*: significance; NS: non-significant; HBDH: α-hydroxybutyrate dehydrogenase; LDH: lactate dehydrogenase; CK-MB: creatine kinase MB; CK: creatine kinase.

Table 4 Patient satisfaction index according to Visick in the 2nd and 12th postoperative months of 31 patients who had laparoscopic floppy Nissen type operation due to gastroesophageal reflux disease

Visick	2 (mo, %)			12 (mo, %)		
I Symptoms-free	22 (71)			27 (87)		
II Mild symptoms	8 (26)	- Dysphagia	6/8	3 (10)	- Dysphagia	2/3
		- Bloating	3/8		- Bloating	2/3
		- Epigastric pain	2/8		- Heartburn	1/3
III Can be treated with medication or with dilation	1 (3)	- Dysphagia		1 (3)	- Dysphagia	
		- Heartburn			- Heartburn	
		- Belching			- Belching	
IV Reoperation needed		-			-	

alteration of muscle structure and metabolism, there is no need for increased protein degradation, as we found in a previous study of muscle hypertrophy with stimulation of muscle cells[10]. In our study the energy-enzyme activity and the specific protein content in the muscle are proportional to the protein synthesis processes, and the lysosomal enzyme activities are proportional to the protein degradation processes in the cardia muscle. The changes in lysosomal enzyme activities have been found to be similar to those found after treatment of rats with gamma irradiation; both irradiation and reflux disease produced oxygen free radicals[11,12].

The observation that increased muscular activity leads to muscle hypertrophy has been published for a long time. But only in the last decade, have newly developed models helped to identify the factors that play a role in the development of muscle hypertrophy. With continued mechanical stimulation in differentiated avian skeletal muscle cells, total protein degradation rate and several protease activities have been seen to increase in the first 2-3 h and return to control levels after several days, with total protein degradation rates falling to levels below those seen in static controls. Decreased protein degradation and the faster protein synthesis contributed to stretch-induced cell growth. Secretion and production of prostaglandin E2, F2 alpha[13] and insulin-like growth factor 1[14] were found to increase with the mechanical stimulation. Recent studies have also demonstrated that the calcium-activated transcription factor NFATC2 controls myoblast fusion by secretion of IL-4 and prostaglandin F2 alpha[15,16].

In reflux disease there is an increase in frequency of transient lower esophageal sphincter relaxations (TLESR) after a meal, which may be related to a greater acid reflux. Thus, an alteration in the triggering of TLESRs is now accepted as one of the key features in the development of gastroesophageal reflux disease[17].

This increased frequency of TLESR may be in the background of the statistically evaluable decrease of HBDH/LDH ratio found by us in chronic reflux-type LES muscles. The shift of oxidative metabolism into glycolytic i.e., anaerobic - direction can be explained by the greater overstrain.

Dysphagia following fundoplication is a common problem and generally occurs in all patients during the first week after surgery. In the great majority of patients the problem rapidly resolves, but in some patients it persists. In the review by Pope[18], dysphagia was reported in 2-44% in 6 different series. This wide variation is attributed to differing patient populations, differing techniques and differing methods of evaluation.

This brings the question of why dysphagia occurs in the first place. In the early post-operative days it is easy to imagine a certain degree of swelling associated with lower esophageal dysfunction, causing some difficulty in swallowing. In the longer term, the simplest explanation for dysphagia would be that the fundoplication is too tight. Several studies, however, suggest that the degree to which the LES region can be opened does not correlate with dysphagia[19-21].

By analyzing the activity values of the 9 patients in Visick groups II and III at two months after surgery, we found a significant increase in the activity of the so-called energy-enzyme values and in the concentration of structural and myofibrillar proteins as compared to the Visick group I patients. This fact also calls attention to the great individual differences of metabolic changes in the LES muscles of reflux patients. The developing hypertrophy of LES muscles may be a reason for complaints after anti-reflux surgery, persistent dysphagia in particular. It is not yet known when these metabolic changes begin to develop following reflux periods, and indeed whether these changes are reversible. It is readily evident, however, that interpatient variability in these metabolic/biochemical changes' rates of occurrence represents a prognostic barrier in the treatment of GERD, and that a "standard" mechanical wrap is not sufficient to recover the highly complex, neurohormonally controlled function of the LES in all patients.

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