

## Effects of lysophosphatidic acid on sling and clasp fibers of the human lower esophageal sphincter

*Lizofosfatidik asidin insan alt özofageal sfinkterinin sapan ve toka lifleri üzerine etkileri*

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### ABSTRACT

**Background:** This study aims to explore the role of lysophosphatidic acid receptors in the regulation mechanisms of contraction and relaxation of human lower esophageal sphincter.

**Methods:** Between July 2015 and March 2016, muscle strips were collected from a total of 30 patients (19 males, 11 females; mean age: 62±9.9 years; range, 52 to 68 years) who underwent an esophagectomy for mid-third esophageal carcinomas. The specimens were maintained in oxygenated Krebs solution. Muscle tension measurement technique *in vitro* was used to examine the effects of non-selective lysophosphatidic acid receptors agonists and antagonists, as well as selective lysophosphatidic acid receptors agonists on the clasp and sling fibers of human lower esophageal sphincter.

**Results:** The non-selective dopamine receptor agonist lysophosphatidic acid induced the contraction of the clasp and sling fibers of the human lower esophageal sphincter. The response induced by non-selective lysophosphatidic acid receptor agonist was inhibited completely by non-selective lysophosphatidic acid receptor antagonist. The selective lysophosphatidic acid 1 and 2 receptor agonist and the selective lysophosphatidic acid 3 receptor agonist induced a concentration-dependent contractile response of the clasp and sling fibers of the human lower esophageal sphincter. There was no significant difference in contraction rates between the clasp and sling fibers ( $p>0.05$ ).

**Conclusion:** This study indicates that lysophosphatidic acid regulates the lower esophageal sphincter through its receptor; the lysophosphatidic acid receptors may be involved in the contractile response of the human lower esophageal sphincter.

**Keywords:** Clasp fibers, human, lower esophageal sphincter, lysophosphatidic acid, sling fibers.

### ÖZ

**Amaç:** Bu çalışmada lizofosfatidik asit reseptörünün, insan alt özofagus sfinkterinin kasılma ve gevşeme düzenleyici mekanizmasındaki rolü araştırıldı.

**Çalışma planı:** Temmuz 2015-Mart 2016 tarihleri arasında özofagusun üçte ikisini tutan karsinomlar nedeniyle özofajektomi yapılan toplam 30 hastadan (19 erkek, 11 kadın; ort. yaş: 62±9.9 yıl; dağılım, 52-68 yıl) kas örnekleri alındı. Bu örnekler oksijenli Krebs solüsyonunda saklandı. Kas gerginliğinin *in vitro* ölçüm tekniği kullanılarak, seçici olmayan lizofosfatidik asit reseptörü agonistleri ve antagonistleri ile seçici lizofosfatidik asit reseptörü agonistlerinin insan alt özofagus sfinkter toka ve sapan lifleri üzerindeki etkileri incelendi.

**Bulgular:** Seçici olmayan lizofosfatidik asit reseptörü agonistleri, insan alt özofagus sfinkteri toka ve sapan liflerinin kasılmasına neden oldu. Seçici olmayan lizofosfatidik asit reseptör agonistleri tarafından indüklenen yanıt, seçici olmayan lizofosfatidik asit reseptörü antagonistleri tarafından tamamen inhibe edildi. Seçici lizofosfatidik asit 1 ve 2 reseptör agonistleri ve seçici lizofosfatidik asit 3 reseptör agonistleri, insan alt özofagus sfinkteri toka ve sapan liflerinin konsantrasyona bağlı daralmasının yanıtını indükledi. Toka ve sapan liflerinin büzülme oranında anlamlı bir fark yoktu ( $p>0.05$ ).

**Sonuç:** Bu çalışma, lizofosfatidik asidin reseptörü aracılığıyla alt özofagus sfinkterini düzenlediğini ve lizofosfatidik asit reseptörünün insan alt özofagus sfinkterinin kasılma yanıtında rol oynayabileceğini göstermektedir.

**Anahtar sözcükler:** Sapan lifleri, insan, alt özofagus sfinkteri, lizofosfatidik asit, toka lifleri.

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The lower esophageal sphincter (LES) is a special thickened annular muscle located at the esophagogastric junction (EGJ), which is approximately 2 to 3-cm wide. It is usually believed that the human LES consists of clasp fibers in the lesser curvature and sling fibers in the greater curvature.<sup>[1]</sup> The LES not only can make food enter the stomach harmoniously and release the gas in the stomach after meals, but also can prevent the stomach contents from potentially flowing back into the esophagus.<sup>[2]</sup> However, the characteristic of esophageal motor disorder is that swallowed food cannot enter the stomach completely.

Lysophosphatidic acid (LPA) is an intercellular phospholipid messenger with biological activity,<sup>[3,4]</sup> and it realizes its biological functions, including cell proliferation, cell survival, cell migration, the promotion of wound healing, platelet aggregation, vascular remodeling, axon retraction, inhibition/reversal differentiation, membrane depolarization, the formation of adhesion spots and stress fibers,<sup>[5-10]</sup> blood pressure regulation and smooth muscle contraction,<sup>[11-15]</sup> by binding to G protein-coupled receptors (GPCRs) on its specific cell membrane surface. To date, six GPCRs have been identified as special LPA receptors, LPA1-6,<sup>[16-22]</sup> Among them, LPA1-3 has high homology and is identified as a member of the GPCR subfamily of endothelial differentiation genes (EDGs).<sup>[23]</sup> The difference in LPA fragments implies that there are at least two different LPA receptor subtypes: smooth muscle type and platelet type.<sup>[24]</sup> All EDG family LPA receptors are more likely to be activated by acyl than alkyl-LPA, and they are smooth-muscle-type LPA receptors.<sup>[23]</sup>

We have previously shown the expression of LPA1-6 receptors in sling and clasp fibers from the human LES.<sup>[25]</sup> In the present study, we aimed to examine the effects of non-selective LPA receptor agonists and antagonists, as well as selective LPA receptor agonists, on sling and clasp fibers of the LES.

## PATIENTS AND METHODS

### Patients and patient tissue

This randomized controlled study was conducted at Department of Thoracic Surgery of Fourth Hospital of Hebei Medical University between July 2015 and March 2016. Muscle strips were collected from a total of 30 patients (19 males, 11 females; mean age: 62±9.9 years; range, 52 to 68 years) who underwent an esophagectomy for mid-third esophageal carcinomas. Patients with a history of gastroesophageal reflux

disease or esophageal motor disorders were excluded from the study. Each specimen was resected *en bloc* in the operating room and placed immediately in ice-cold Krebs solution. Specimens were not included in this study, if the segment contained a macroscopically visible tumor.

In the laboratory, fresh EGJ specimens collected in the operating room were immediately placed in 4°C Tris-buffered saline (TBS). Following washing with 37°C Krebs solution, the specimens were pinned on a wax plate containing TBS with a continuous mixed gas of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The mucosa and submucosa were, then, gently removed by sharp dissection. The sling fibers and clasp fibers were separated and cut into 2×10 mm muscle strips.

The gastric sling and clasp fibers were identified as thickened bands of circular oriented smooth muscle in the gastric cardia, adjacent to the greater and lesser curvature of the stomach, respectively. The sling and clasp muscle strips were prepared using a method described previously.<sup>[26]</sup>

### Measurement of muscle strip tension

#### The most suitable initial length

Both ends of the muscle strips were fastened with silk, placed in a 10 mL bath containing Krebs liquid, maintained at a constant temperature of 37°C and perfused with gas containing 5% CO<sub>2</sub> and 95% O<sub>2</sub>. The upper muscles and JZ101-type muscle tension transducer (Xinhang Electric Apparatus, Gaobeidian, China) were fastened together, and tension was recorded using MedLab 6.0 software (MedEase, Nanjing, China). Each muscle strip was stretched slightly and rapidly, until 200 mg of force was generated. This was taken as the initial length (L<sub>0</sub>). The muscle strips were, then, sequentially stretched to 200% of the L<sub>0</sub><sup>[27]</sup> at increments of 25% of the L<sub>0</sub> each time.<sup>[28]</sup> This was taken as the most suitable initial length.

To avoid the influence of other factors, the receptor subtypes with excitation effects on muscle strips were compared with those treated with NG-nitro-L-arginine (Sigma, Chemical Co., St. Louis, MO, USA).

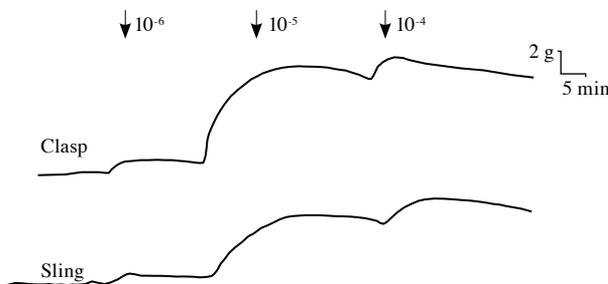
#### Effects of non-selective LPA receptor agonists, antagonists, and selective LPA receptor agonists on human LES

The optimal initial length of the muscle was stabilized for approximately 40 min and, then, a non-selective LPA receptor agonist (LPA) (Cayman Chemical, Ann Arbor, MI, USA) was added into a thermostatic bath to activate each

LPA receptor subtype in a cumulative manner from  $10^{-9}$  to  $10^{-4}$  mol/L. Each concentration of the drug was added after the reaction of the previous concentration reached a maximum. Cumulative administration concentration-response dose-response curves were established based on the above results. The maximum effect after dosing and its corresponding concentration were calculated. While observing the effects of a non-selective antagonist (tetradecyl-phosphonate) (Cayman Chemical, Ann Arbor, MI, USA), the concentration of the antagonist was found to be the same as the concentration of agonist-induced muscle maximal effect. The administration of a selective LPA1 and LPA2 receptor agonist (L- $\alpha$ -LPA) (Sigma, Chemical Co., St. Louis, MO, USA) and a selective LPA3 receptor agonist (OMPT) (Cayman Chemical, Ann Arbor, MI, USA) was performed using the same method. The responses in all of the experiments were quantified based upon the percentage of the baseline value of muscle strip tone relative to the nadir of the response.

### Statistical analysis

Statistical analysis was performed using the IBM SPSS version 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Descriptive data were expressed in mean $\pm$ standard error (SEM). Drug-induced muscle strip response was based on mean $\pm$ SEM of contraction or relaxation percentage of muscle strips. Two-way analysis of variance (ANOVA) was used to compare the two drug concentration-response curves. A *p* value of <0.05 was considered statistically significant.



**Figure 1.** Example curves of the responses of the sling and clasp muscles to LPA. The non-selective lysophosphatidic acid receptor agonist LPA induced the contraction of the human LES at concentrations of  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  mol/L.

LPA: Lysophosphatidic acid; LES: Lower esophageal sphincter.

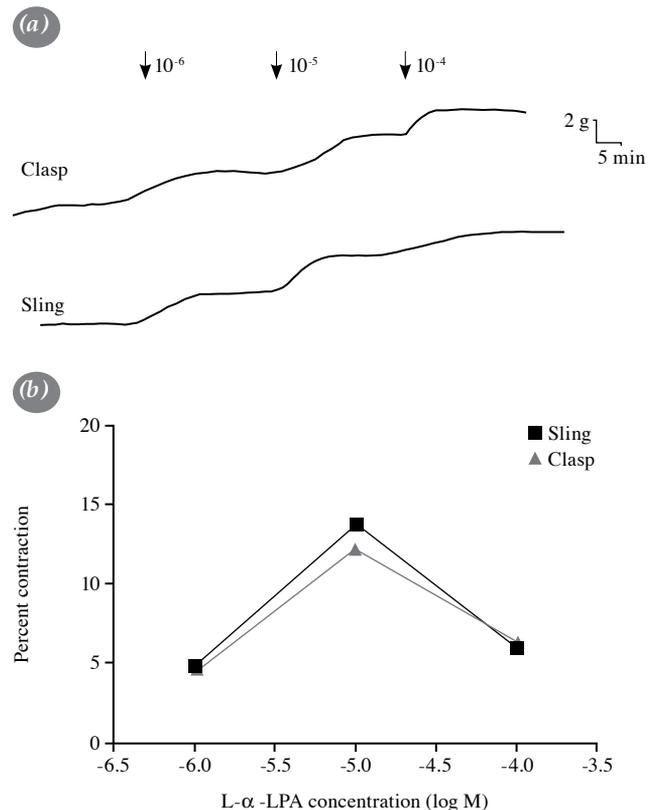
## RESULTS

### Effect of non-selective LPA receptor agonist and antagonist on the human LES

The non-selective LPA receptor agonist LPA induced the contraction of the clasp and sling fibers of the human LES at the concentration of  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  mol/L. The response induced by non-selective LPA receptor agonist tetradecyl-phosphonate at a concentration of  $10^{-5}$  mol/L was inhibited completely by the non-selective LPA receptor antagonist tetradecyl-phosphonate ( $10^{-5}$  mol/L) (Figure 1).

### Effect of selective LPA1 and LPA2 receptor agonist on the human LES

The selective LPA1 and LPA2 receptor agonist L- $\alpha$ -LPA induced a concentration-dependent



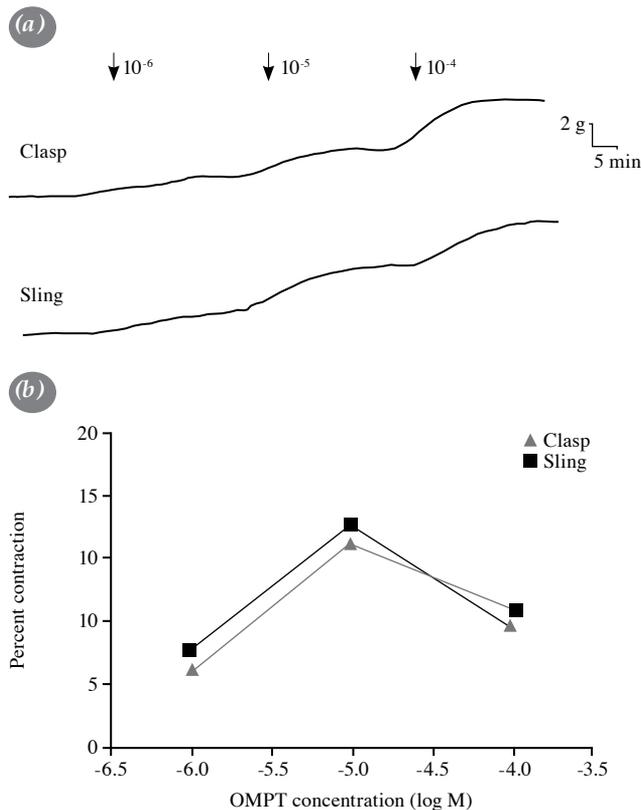
**Figure 2.** (a) Concentration-tension curves of the selective LPA1 and LPA2 receptor agonist. There was no significant difference in contraction between sling fibers and clasp fibers ( $p>0.05$ ). The optimal concentration leading to maximum contraction was  $10^{-5}$  mol/L. (b) Example curves of the responses of the two muscle strips to the selective LPA1 and LPA2 receptor agonist. The selective LPA1 and LPA2 receptor agonist induced a concentration-dependent contractile response of the human LES at concentrations of  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  mol/L.

LPA: Lysophosphatidic acid; LES: Lower esophageal sphincter.

contractile response of the clasp and sling fibers of the human LES at concentrations of  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  mol/L. There was no significant difference in contraction between the clasp and sling fibers ( $F=3.26$ ,  $p=0.72$ ). The optimal concentration leading to the maximum contraction percentage was  $10^{-5}$  mol/L. The mean maximum contraction percentage of clasp fibers was  $12.6\pm0.4\%$ . The mean maximum contraction percentage of sling fibers was  $13.1\pm0.4\%$ . There was no significant difference in the maximum contraction percentage of clasp and sling fibers ( $F=0.02$ ,  $p=0.90$ ) (Figure 2a, b).

### Effect of selective LPA3 receptor agonist on the human LES

The selective LPA3 receptor agonist OMPT also induced the contraction of the human LES



**Figure 3.** (a) Concentration-tension curves of the selective LPA3 agonist. There was no significant difference in contraction between the two muscle strips ( $p>0.05$ ). The optimal concentration leading to maximum contraction was also  $10^{-5}$  mol/L. (b) Example curves of the responses of the two muscle strips to the selective LPA3 receptor agonist. The selective LPA3 receptor agonist induced the contraction of the human LES at concentrations of  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  mol/L in a manner that was also concentration-dependent.

LPA: Lysophosphatidic acid; LES: Lower esophageal sphincter.

at concentrations of  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  mol/L in a concentration-dependent manner. There was no significant difference in contraction between the clasp and sling fibers ( $F=1.98$ ,  $p=0.16$ ). The optimal concentration leading to the maximum contraction percentage was  $10^{-5}$  mol/L. The mean maximum contraction of clasp fibers was  $6.5\pm0.5\%$ , and the mean maximum relaxation of sling fibers was  $6.9\pm0.6\%$ , indicating no significant difference ( $F=0.13$ ,  $p=0.72$ ) (Figure 3a, b).

### DISCUSSION

Lysophosphatidic acid has a variety of biological functions in tissues and cells, and these functions are mediated by LPA receptors. The LPA receptors are widely present in the cardiovascular system and gastrointestinal tract and are important physiological regulators.

Numerous esophageal motility disorders are all associated with motor disorders of the LES. Gastroesophageal reflux is the most important cause of the development of Barrett esophagus.<sup>[29]</sup> Previous studies have shown that the regulatory mechanism of the LES involves a variety of receptors.<sup>[26,30]</sup>

Lysophosphatidic acid receptors are widely distributed in gastrointestinal smooth muscle and are closely related to gastrointestinal motility and secretion. The pharmacological effects of LPA on different parts of the gut have been confirmed.<sup>[13-15]</sup> Toews et al.<sup>[15]</sup> found that LPA both strengthened the contraction and inhibited the relaxation of tracheal smooth muscle. Xu et al.<sup>[31]</sup> confirmed that the LPA receptor induced the contraction of vascular smooth muscle and increased blood pressure. Markiewicz et al.<sup>[32]</sup> found that direct activation of LPA1, LPA2, and LPA3 receptors could enhance the contraction of the smooth muscle of pig uterus in early pregnancy, and the three receptor subtypes work together. Sriwai et al.<sup>[33]</sup> confirmed that the LPA3 receptor acted in the contraction of the smooth muscle of rabbit stomach. The use of LPA receptor agonists and antagonists has shown that the LPA1 receptor blocks the relaxation of the LES.<sup>[34]</sup> These results suggest that LPA receptors may play a key role in the contraction of smooth muscle.

Our study is based on evidence from the above studies and used *in vitro* muscle tension measurement technology to analyze the regulatory role of a non-selective LPA receptor agonist (LPA) and a non-selective LPA receptor antagonist (tetradecyl-phosphonate) in the contraction and relaxation responses of clasp and sling fibers in

the LES. In addition, agonists of selective LPA receptors were used to further clarify the role of different subtypes of LPA receptors in the regulatory mechanism of human LES.

We found that the non-selective LPA agonist LPA could induce contraction in the clasp and sling fibers of the LES. This suggests that LPA can induce the contraction of the LES. In addition, a non-selective LPA receptor antagonist can completely inhibit the contraction of clasp and sling fibers induced by LPA. It is suggested that LPA may regulate the contraction of the human LES through the LPA receptor. The results preliminarily confirm that the LPA receptor plays an important role in enhancing the contraction of smooth muscle in the human LES. These results are consistent with those of domestic and foreign scholars.<sup>[13-15,34,35]</sup> Moreover, our study found that LPA1, LPA2, and LPA3 receptors were all involved in contraction reactions in the human LES. This result is similar to that found by Markiewicz et al.<sup>[32]</sup> However, some studies have confirmed that only the LPA3 receptor acts in the contraction reaction in the smooth muscle of rabbit stomach, and the experiment does not find the existence of LPA1 and LPA2 receptors.<sup>[33]</sup> The LPA inhibits the relaxation of the LES in cats by blocking the nitric oxide-mediated signal transduction pathway of the LPA1 receptor.<sup>[34]</sup> This result may be caused by different species and different sites of tissue action, and the exact reason is not clear and needs to be further understood.

The main limitations to the present study must be recognized. First, this is a single-center study, and we need to further carry out multicenter research. Second, this study is an *in vitro* study, and the results may not be in agreement with the results of *in vivo* studies. Third, some receptors have no specific agonists, which may have affected the results. Finally, this is the first report on the role of LPA receptors in modulating the human LES.

In conclusion, we found that all three lysophosphatidic acid receptor subtypes play a contractile role in the human lower esophageal sphincter, but the possible signal transduction pathway of the lysophosphatidic acid receptor in this process needs further investigation. In this way, the regulatory role of the lysophosphatidic acid receptor in human lower esophageal sphincter can be more clearly defined, which may play an important role in the future treatment of esophageal motor function diseases.

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**Ethics Committee Approval:** The project was approved by the Research Ethics Committee of the Fourth Hospital of Hebei Medical University, Shijiazhuang, China (2015mECD35). All patients gave written informed consent. The study was conducted in accordance with the principles of the Declaration of Helsinki.

**Patient Consent for Publication:** A written informed consent was obtained from each patient.

**Data Sharing Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author Contributions:** Designed the study: Y.F., J.F.L.; Analysis and interpretation of data: W.W., L.C.; Drafting the article: Y.F., W.W.; Revising it critically for important intellectual content: J.F.L., L.C.; All authors final approval of the version to be published.

**Conflict of Interest:** The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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