Published in final edited form as:

J Allergy Clin Immunol. 2014 November ; 134(5): 1100–1107.e4. doi:10.1016/j.jaci.2014.04.004.

TGFβ1-induced phospholamban expression alters esophageal smooth muscle cell contraction in eosinophilic esophagitis

Lisa Y. Beppu, B.S.^{1,2}, Arjun A. Anilkumar, B.S.^{1,3}, Robert O. Newbury, M.D.^{2,4}, Ranjan Dohil, M.D.^{2,5}, David H. Broide, M.B., Ch.B.^{1,3}, and Seema S. Aceves, M.D., Ph.D.^{1,2,3}

¹Division of Allergy, Immunology, University of California, San Diego

²Department of Pediatrics, University of California, San Diego

³Department of Medicine, University of California, San Diego

⁴Division of Pathology, University of California, San Diego

⁵Division of Gastroenterology, University of California, San Diego

Abstract

Background—Eosinophilic esophagitis (EoE) is a chronic antigen mediated disease characterized by esophageal eosinophilia, remodeling, and fibrosis. TGFβ1 is a central regulator of EoE remodeling and increases esophageal smooth muscle cell contraction.

Objective—In this study, we aimed to understand the molecular mechanisms by which TGFβ1 could induce esophageal smooth muscle cell contraction.

Methods—We used primary human esophageal smooth muscle (ESM) cells and EoE myofibroblasts (EMF) to assess the mechanisms of TGFβ1-induced contraction. We analyzed the expression, phosphorylation, and function of phospholamban (PLN), a sarcoendoplasmic reticulum regulatory protein which was induced by TGFβ1. Expression of PLN, phospho-PLN, and its regulatory pathway was analyzed in the esophageal smooth muscle of EoE and control biopsies. Gene silencing in EoE EMFs was utilized to understand the role of PLN in contraction.

Results—TGFβ1 induced and phosphorylated PLN in primary human ESM and EoE EMFs. PLN and phospho-PLN were elevated in EoE as compared to control subject smooth muscle *in vivo*. PLN inhibition significantly diminished TGFβ1 induced EoE EMF contraction. PLN expression and ESM/EMF contraction depended on TGFβ receptor I signals.

© 2014 American Academy of Allergy, Asthma and Immunology. Published by Mosby, Inc. All rights reserved.

Corresponding Author: Seema S. Aceves, MD, PhD, Division of Allergy, Immunology, Departments of Pediatrics and Medicine, University of California, San Diego, Rady Children's Hospital, San Diego, 9500 Gilman Drive, MC-0635, La Jolla, CA 92093, saceves@ucsd.edu, phone: 858-966-5961, fax: 858-966-6791.

The authors have no relevant conflicts of interest to declare.

The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conclusion—We describe a previously unrecognized mechanism for esophageal smooth muscle cell contraction that depends on TGFβ1, its receptors, and PLN. Since PLN is elevated in EoE smooth muscle and PLN silencing diminishes contraction, we provide a novel potential mechanistic framework and therapeutic target for esophageal smooth muscle dysfunction in EoE.

Keywords

Remodeling; Esophagus; Eosinophils; Smooth muscle; Transforming growth factor-beta; Phospholamban

Introduction

Eosinophilic esophagitis (EoE) is a chronic antigen mediated clinicopathologic disease of increasing worldwide prevalence (1). Transmural remodeling changes including epithelial basal zone hyperplasia, subepithelial fibrosis, and smooth muscle hypertrophy promote disease symptoms and complications such as esophageal narrowing, rigidity, strictures, and dysmotility (2–16). Dysphagia and food impactions are cardinal EoE features that can occur in the absence of esophageal narrowing or stricture (1, 17, 18), suggesting that additional mechanisms beyond fibrostenosis contribute to EoE pathogenesis (14). For example, pediatric EoE subjects have dysphagia and impactions but rarely have esophageal narrowing (1, 17). Patterns of dysmotility in EoE include increased contraction amplitude, nutcracker esophagus, achalasia, and ineffective, uncoupled contractions (13, 15, 16, 19–22). However, despite these multiple clinical observations, the molecular mechanisms driving esophageal smooth muscle remodeling and dysfunction in EoE remain largely undefined.

EoE remodeling depends on inflammatory cell infiltration (2, 10, 11, 23, 24) and TGFβ1 is significantly elevated in the esophagus of EoE subjects (2, 23, 25–27). Eosinophils and mast cells infiltrate the esophageal smooth muscle bundles in pediatric EoE, promote smooth muscle hyperplasia, and produce TGFβ1 which causes transcription dependent contraction of primary human esophageal smooth muscle (ESM) cells *in vitro* (10, 25). In order to understand EoE pathogenesis and elucidate novel TGFβ1 mechanisms, we utilized primary ESM and EoE myofibroblasts (EMF) to demonstrate that the contraction associated protein, phospholamban (PLN) is a TGFβ1 transcriptional target. PLN is required for proper cardiac muscle contraction and human PLN mutations can be lethal (28, 29). However, its role in EoE, in esophageal smooth muscle function and its induction by TGFβ1 has not been previously appreciated. We show for the first time that TGFβ1 induces and phosphorylates PLN in ESM cells and EoE EMFs. EoE subjects had significantly higher PLN and phospho-PLN expression in the esophageal smooth muscle *in vivo* as compared with normal control subjects. PLN down-regulation decreased TGFβ1-mediated contraction. PLN induction required the canonical TGFβ1 signaling pathway. As such, our findings reveal that PLN is a novel and previously unrecognized TGFβ1 target that likely alters esophageal smooth muscle function in the process of EoE associated tissue remodeling.

Methods

Cultured cells

(A) ESM cells: primary human esophageal smooth muscle (ESM) cells were cultured according to the manufacturer's instructions (ScienCell, San Diego, CA). (B) EoE fibroblasts: fibroblasts were isolated from pediatric EoE esophageal biopsies obtained during routine endoscopy with biopsy, dispersed using collagenase VIII (Sigma-Aldrich, St. Louis MO), and cultured in smooth muscle cell media (ScienCell, Carlsbad CA). Fibroblast phenotype was confirmed by the production of collagen I, fibronectin, and appropriate morphology. (C) EMF cells: esophageal myofibroblasts (EMF) were defined as EoE fibroblasts producing α -smooth muscle actin following treatment with recombinant human TGF β 1 (see below).

Treatment with TGF β 1 and inhibitors

ESM or EoE EMFs were grown to 90% confluency and placed in serum free media overnight prior to treatment with 10ng/ml recombinant human TGF β 1. Inhibitors of TGF β RI (SB431542, 10uM, Invivogen, San Diego CA), TGF β 1 activated kinase-1 (TAK1) (5Z-7-oxozeanol, 100nM Sigma-Aldrich, St. Louis MO), or the appropriate vehicle control were added 3 hours prior to TGF β 1. ESM cells were pre-treated with TAK1 inhibitor followed by treatment with recombinant IL-1 β for phosphorylated jun kinase assays.

Cell contraction assays

Gel contraction assays with ESM or EMF were done as previously described (10). Briefly, 1.25×10^5 cells were cultured in LPS free collagen gels (Advanced BioMatrix, San Diego, CA). Gels were treated with TGF β 1 and/or pharmacologic inhibitors and the area of the gels was quantified using a Chemidoc transilluminator and its accompanying software (Bio-Rad Laboratories, Hercules, CA). Experiments were done in triplicate on at least 3 separate days. For gene silencing experiments cells were transfected using an Amaxa Nucleofection system and siRNA for PLN or a control non-targeting (NTG) siRNA (ThermoScientific Dharmacon, Lafayette, CO) using the manufacturer's guidelines. NTG is a control siRNA that does not target any gene as controlled for by genome wide array (30).

Immunostaining and Pediatric EoE biopsy specimens

EoE was defined as 15 eosinophils per high power field (hpf) in an esophageal biopsy on 400x light microscopy and hematoxylin/eosin stain in the presence of typical EoE symptoms and endoscopic features. Control was defined as no esophageal endoscopic abnormalities and eosinophils of ≤ 2 per high power field. Archived biopsy specimens were screened for the presence of smooth muscle using our database of EoE subjects. Seven EoE subjects and 5 normal controls with adequate muscularis mucosa for analysis were selected randomly from the UCSD/Rady Children's Hospital, San Diego EoE database for PLN and phospho-PLN immunohistochemistry.

5um sections of tissue were deparaffanized and hydrated prior to immunostaining as previously described (2). Immunocytochemistry on cells was performed using paraformaldehyde fixation and detergent treatment prior to primary and secondary

antibodies. Esophageal biopsies, human LSM, and primary cells were processed for immunohistochemistry or immunofluorescence using primary antibodies (Table E1) specific for human PLN, phospho-serine-16-PLN, α -smooth muscle actin (α SMA), TGF β receptor I and II, Serca2a or appropriate isotype control. Vectastain ABC system (Vector Laboratories, Burlingame, CA) was used for immunohistochemistry as previously described (2). Double and single immunofluorescence was done as previously described (10). Images were captured and analyzed using image analysis and (ImagePro, Media Cybernetics, Bethesda MD) slides were all quantified and analyzed under identical light or fluorescence microscopic conditions, including magnification, gain, camera position, and background illumination.

Quantitative PCR analysis

RNA was isolated using chloroform extraction and isopropanol precipitation, converted to cDNA using Superscript II reverse transcriptase per the manufacturer's instructions (Invitrogen), and subjected to real time quantitative PCR (RT qPCR) using SYBR green (Quiagen) and PLN specific primers and normalized to housekeeping genes (Table E2). Data is presented as Ct normalized to housekeeping genes. All dissociation curves had single peaks.

Immunoblots

Cells were harvested on ice using RIPA lysis buffer (Teknova, Hollister, CA) with phosphatase and protease inhibitors (Roche Applied Science, Indianapolis, IN). Equivalent amounts of total protein was loaded into each well and electrophoresed on NuPAGE 4–12% Bis-Tris gels (Life Technologies, Grand Island, NY). Proteins were transferred to 0.2 μ m pore nitrocellulose membrane (Life Technologies, Grand Island, NY), blocked with 5% dry milk, incubated overnight with primary antibody (Table E1), and probed with the appropriate species specific HRP-linked secondary antibody. Immunoblots were developed with Immuno-Star WesternC Kit (BioRad, Hercules, CA), imaged, and analyzed using the Chemidoc transilluminator and its accompanying software (Bio-Rad Laboratories, Hercules, CA).

Florescence activated cell sorting analysis

After Fc block (Miltenyi Biotec, Auburn, CA), $0.5\text{--}1 \times 10^6$ ESM or EMF were stained for 30 minutes with TGF β RI, TGF β RII (Table E1), and species specific PE or APC-conjugated secondary antibody. Cells were resuspended and washed in FACS buffer (2% FBS 1M sodium azide in 1X dPBS) and used in standard indirect flow cytometry experiments. For TGF β RII surface staining cells were incubated with Fc receptor blocking antibody prior to primary and secondary antibody (APC-conjugated IgG, BioLegend, San Diego, CA). For TGF β RI intracellular staining, cells were treated with blocking antibody and then incubated with TGF β RI and species specific secondary (PE-conjugated, eBiosciences, San Diego CA). Mouse IgG1 (eBioscience, SD, CA) and rabbit IgG (GeneTex, Irvine, CA) were utilized as isotype control antibodies. The samples were analyzed with an AccuriC6 flow cytometer. Further analyses were performed with FlowJo software (Tree Star).

Statistics

All statistical analyses and graphing were carried out using GraphPad Prism (San Diego, CA) software. Comparisons between two groups were done using a student's t-test for unpaired variables. A two tailed p value <0.05 was considered statistically significant.

Human Subjects

All aspects of the study received IRB approval by the University of California, San Diego (UCSD protocol number 091485).

Results

TGF β 1 induces phospholamban in human esophageal smooth muscle cells

Our prior studies demonstrated that primary human ESM cells contract in a transcription dependent manner when cultured in the presence of TGF β 1 (10). However, potential contraction associated targets were not clear. We found that TGF β 1 treatment of ESM cells induced PLN mRNA 167-fold in human ESM cells (Figure 1A). PLN is a pentameric sarcoplasmic reticulum protein that regulates the function of Serca2a and is essential for proper cardiac contractility (31). As such, it was a potential candidate protein involved in TGF β 1-induced ESM contraction. PLN mRNA was undetectable at baseline and became increasingly abundant beginning 4 hours after and continuing through 48 hours of TGF β 1 treatment (data not shown). Similarly, induction of PLN mRNA was seen in EoE EMF following TGF β 1 treatment (Fig E1).

PLN protein was undetectable by immunoblotting at baseline in ESM cells but became detectable following 14 hours of TGF β 1 and continued to be expressed through 48 hours of treatment (Figure 1B). As primary esophageal smooth muscle from pediatric EoE subjects is difficult to culture directly from biopsy specimens, we utilized primary esophageal EoE fibroblasts that transform to myofibroblasts (EMF) in the presence of TGF β 1. EMF phenotype was verified by increased α -smooth muscle actin (α SMA) expression following 24 hours of TGF β 1 treatment (Figure 1C). EoE EMFs demonstrated increased PLN protein as well as co-expression of PLN and α -SMA following TGF β 1 treatment demonstrating that EoE fibroblast conversion to a EMF phenotype induces a protein cascade that includes PLN induction (Figure 1D, E1). Taken together, these data suggest a role for PLN in ESM and EMF cell function.

Pediatric EoE patient biopsies express PLN in the muscularis mucosa

Since TGF β 1 expressing mast cells and eosinophils infiltrate the muscularis mucosa in EoE patients (10) and since TGF β 1 induced PLN expression, we hypothesized that EoE patients would express increased PLN in their esophageal smooth muscle bundles as opposed to control subjects. EoE was defined as ≥ 15 eosinophils per hpf (mean=76/hpf). The majority of subjects (86%) had typical endoscopic findings (furrows, plaques, and/or pallor) and were allergic (71%). The mean age of the subjects whose biopsies were utilized was 4.8 years. Eighty-six percent had vomiting, 29% had regurgitation, 14% had gagging. While dysphagia can be difficult to discern in a young population, these symptoms could be clinical

manifestations of dysmotility. In addition, and 14% complained of dysphagia and 43% had abdominal pain and/or anorexia/early satiety with poor weight gain.

Immunohistochemistry and image analysis determined that EoE subjects had significantly more PLN in the muscularis mucosa as compared to control subjects (Figure 2A,B). PLN and α SMA were co-expressed in pediatric EoE biopsies confirming that the esophageal smooth muscle bundles were the cellular location of PLN expression in EoE (Figure 2C). These findings are consistent with the concept that TGF β 1 produced by smooth muscle infiltrating inflammatory cells in EoE can induce PLN expression and that the presence of PLN reflects a pathologic state in EoE.

PLN regulates contraction by altering sarcoplasmic reticulum calcium stores due to inhibition of the sarcoendoplasmic reticulum channel ATPase 2a (Serca2a) (31). Esophageal biopsies from EoE subjects showed co-localization of PLN and Serca2a in the esophageal smooth muscle (Figure 2D), supporting the hypothesis that PLN could regulate Serca2a function and calcium flux in an EoE inflammatory state.

Phospholamban is required for TGF β 1 induced contraction

Based on the known function of PLN, its expression in the pathogenic EoE state, and its induction by TGF β 1, we hypothesized that TGF β 1's effects on contraction would depend on PLN. We transfected TGF β 1-treated EoE EMFs with a non-targeting (NTG) control siRNA (30) or PLN specific siRNA in order to block the production of PLN protein in EoE EMFs (Figure 3A). Collagen gels impregnated with PLN-specific siRNA treated EMFs had significantly less TGF β 1-induced contraction as compared with EMFs that were transfected with control siRNA (Figure 3B, C). Thus, PLN could be a previously unrecognized mechanism for TGF β 1-induced esophageal smooth muscle contraction in EoE.

TGF β RI signals are required for PLN induction

In order to understand if PLN expression depended on the canonical TGF β 1 signaling pathway, we utilized pathway specific pharmacological inhibitors. FACS analysis demonstrated that TGF β RI and RII are expressed on both EoE fibroblasts and ESM cells (Figure 4A) and double immunofluorescence using TGF β RI and RII specific antibodies showed co-localization on EoE smooth muscle *in vivo* and on EoE EMFs, verifying that both receptors were expressed together to allow for proper TGF β 1 signals (Figure 4B, C). The TGF β RI specific inhibitor SB431542 caused complete abolishment of PLN expression induced by TGF β 1 (Figure 4D). Blockade of TGF β RI signaling also completely abrogated TGF β 1-induced contraction of both ESM and EMF impregnated collagen gels (Figure 4F, E2). By contrast, pharmacologic inhibition of the non-canonical TGF β 1 activated kinase 1 (TAK1) pathway did not decrease PLN expression (Figure 4D) although it did inhibit JNK phosphorylation (Figure 4E), providing proof that non-canonical TGF β 1 pathways are functional in ESM cells. As would be predicted by an inability to block TGF β 1 induction of PLN, TAK1 inhibition did not alter TGF β 1-mediated ESM or EMF contraction (Figure 4F, E2).

Serine phosphorylation alters the function of PLN. Therefore, we evaluated the phosphorylation state of PLN in EoE SM. Phospho-serine-PLN and phospho-threonine-PLN specific antibodies and extracts from TGF β 1 treated and untreated EMF and ESM cells (cardiac lysates were utilized as a positive control) demonstrated that phospho-serine-16 PLN was detectable following TGF β 1 treatment but phospho-threonine-17-PLN was not (Figure 5A, B). Phospho-serine-16-PLN was also detected in the muscularis mucosa of EoE subjects but not in non-diseased controls thereby verifying the presence of phosphorylated PLN in the EoE diseased state (Figure 5C).

Discussion

In this study we demonstrate a number of novel findings. This study is the first to document a potential mechanism for TGF β 1-induced esophageal smooth muscle contraction in EoE. PLN induction and phosphorylation by TGF β 1 have not been previously documented. In addition, we show the TGF β 1 signaling is necessary to induce PLN in ESM and EoE EMF cell cultures. It is possible that TGF β 1 could alter both smooth muscle contraction and general tissue contraction during a process akin to wound healing through its effects on ESM and EMFs (Figure 6). Since the human PLN gene contains two well conserved Smad consensus binding sequences, PLN is a feasible direct transcriptional TGF β 1 target and may provide a new therapeutic target in EoE. Since TGF β 1 also uses non-canonical signaling pathways in other systems, for example JunB for uterine smooth muscle contraction (32), it will be interesting to understand the pSmad dependent and independent mechanisms for TGF β 1-mediated contraction and PLN induction.

Understanding the mechanisms underlying smooth muscle changes in EoE is important since both dysphagia and non-stricture associated food impactions likely occur due to alterations in the smooth muscle (6, 13, 14, 18, 19, 33–35). While esophageal fibrosis with resultant fixed esophageal strictures/narrowing contribute to dysphagia (3, 4, 8, 9), a body of work supports a role for altered motor function in EoE (14). Structural analyses demonstrate that eosinophils and mast cells are present in the deep muscular layers (36–38) and murine models demonstrate effects of mast cells on smooth muscle proliferation (11). Indeed, animal studies have shown that the mechanisms that promote motor abnormalities can be distinct from those that drive esophageal strictures (24). In pediatric EoE, 24 hour monitoring of esophageal motility demonstrated that transient motility disturbances reflected clinical dysphagia (18). Studies using stationary manometry, which is limited to an isolated assessment of circular muscle function, have determined variable patterns of esophageal dysmotility in subsets of EoE patients including sustained, irregular, and/or ineffective contractions, achalasia, and nutcracker esophagus (13, 16, 19, 20). In contrast, studies that assess both the concentric and longitudinal esophageal layers demonstrated uniform, ineffective longitudinal muscle function and muscle layer asynchrony with no changes in concentric smooth muscle function (15). As such, there are clearly EoE subjects who suffer from dysmotility. Thus, the definition of a dysmotile EoE phenotype will be critical as it may indicate the opportunity to target novel molecules such as PLN.

Elevated TGF β 1 levels are likely to induce a cascade of genes that can regulate ESM and EMF function. The basal expression of esophageal smooth muscle TGF β 1 receptors coupled

with the influx of TGF β 1 producing mast cells and eosinophils into the EoE smooth muscle provides a cellular mechanism explaining the physiologic and pathogenic consequences of TGF β 1 (10, 11, 36). Since smooth muscle inflammation occurs in EoE, PLN induction would not occur in normal non-inflamed smooth muscle. Indeed, PLN expression is readily detected in EoE muscularis mucosa and is very low in control subjects. Since PLN blockade significantly decreases TGF β 1-induced contraction, we provide evidence that PLN is likely a necessary part of TGF β 1's effects on smooth muscle. However, since PLN blockade partially reduces contraction, it appears to be an integral but not the only component for TGF β 1 effects. Indeed deciphering PLN dependent and independent pathways on EoE smooth muscle function will be of interest to better understand disease pathogenesis.

PLN is most completely characterized in the heart where it is a component of the Serca2a-PLN calcium regulatome (31). Both null and missense mutations in the human phospholamban gene cause cardiomyopathy, heart failure, cardiomyocyte enlargement, decreased contractile function, and electrophysiological remodeling (28, 39, 40). PLN has been described in the murine gastric smooth muscle as well as in the airway of beta receptor overexpressing mice. The expression patterns and effects of PLN on smooth muscle function may vary by disease state and inflammatory milieu. For example, non-asthmatic human airway smooth muscle cells express PLN mRNA but not protein while isolated airway smooth muscle cells have a reduction in Serca2a following treatment with IL-13 and TNF α . In addition, transgenic mice overexpressing the β 2-adrenergic receptor have decreased levels of PLN and PLN deficient mice have reduced airway smooth muscle contractile response to methacholine (41, 42). Given our current study on EoE, it would be of interest to know if PLN levels are altered in human asthmatic airways and if TGF β 1 can regulate PLN levels in the airway smooth muscle cells.

Prior studies also support a role for PLN in gastrointestinal motility. In murine gastric smooth muscle, phosphorylation of PLN by CamKinase II is associated with relaxation and PLN deficient mice showed increased slow waves demonstrating that PLN is involved in the electrical activity of gastric smooth muscle (43, 44). In the neonatal guinea pig gallbladder, decreased levels of PLN are associated with decreased contraction as compared with the adult gallbladder and CCK causes both PLN phosphorylation and increased contraction (45). In a DSS induced colitis model, decreased colonic contractility was associated with decreased levels of PLN (46). While a study of subjects with achalasia demonstrated an inability to detect PLN by immunoblotting in human biopsy tissues, it did demonstrate decreased levels of the calcium handling proteins calreticulin and calsequestrin in subjects with achalasia (47). Given that eosinophil products can alter esophageal contractility (38), it is possible that subjects with esophageal eosinophilia and achalasia could also have alterations in PLN. Although animal models may not entirely align with human PLN function (for example, human subjects with PLN deficiency have heart failure while mice do not exhibit a lethal phenotype (40)), it is clear that PLN and its alterations are important in smooth muscle calcium handling, electrical activity, and contraction.

TGF β 1-induced PLN phosphorylation on serine-16 was dependent on TGF β RI but not on the non-canonical TAK1 pathway. This is consistent with the serine phosphorylation signals that occur via TGF β RI receptor. Both the levels and phosphorylation state of PLN affect its

inhibitory effects on Serca2a (31). Baseline Serca2a expression coupled with the onset of PLN expression in EoE could provide a significant functional insult on calcium homeostasis in EoE. It is also important to consider that by altering calcium flux, which regulates a number of physiologic processes, PLN could play a more global role in the EoE structural remodeling process in the smooth muscle. In addition, since TGF β 1 constitutes a pathway to fibrosis and remodeling in asthma and heart and renal failure, our findings could have important implications for other disease states. Indeed, current clinical trials in patients with abnormal PLN expression are aimed at reducing not only cardiac dysfunction but also cardiac fibrosis.

In conclusion, our data proposes a new paradigm for esophageal remodeling in which inflammatory cell derived TGF β 1 acts on the esophageal smooth muscle to induce and phosphorylate PLN and alter calcium flux as regulated via Serca2a. The fact that the effects of TGF β 1 on PLN induction and phosphorylation occur slowly is consistent with the chronic disordered contraction and smooth muscle remodeling in EoE. The relationship of this mechanism to other diseases as well as to other structural remodeling features in EoE warrants further investigation and could provide novel and currently unappreciated therapeutic options for chronic allergic esophagitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support: NIH/NIAID AI 092135 (S.A.), DOD FA100044 (D.H.B., S.A.), NIH/NIAID AI 107779 (D.H.B.), AI 70535 (D.H.B.), AI 72115 (D.H.B.), Hearst Foundation (R.D.)

We thank Kevin Alford, B.A. and Naseem Khoram, B.A. for technical assistance and Dr. Taylor Doherty for assistance with the flow cytometry studies. We thank Drs. Stacie Jones and Richard Kurten for their insights. The project described was partially supported by the National Institutes of Health, Grant ULRR031980 for years 1&2 or CTSA funding and/or UL1TR0001000 during year 3 and beyond of CTSA funding (support of the EoE database).

Abbreviations

EoE	Eosinophilic esophagitis
TGFβ1	Transforming growth factor beta-1
PLN	Phospholamban
LP	Lamina propria
SM	Smooth muscle
ESM	Esophageal smooth muscle
EMF	Esophageal myofibroblast

References

1. Liacouras CA, Furuta GT, Hirano I, Atkins D, Attwood SE, Bonis PA, et al. Eosinophilic esophagitis: updated consensus recommendations for children and adults. *The Journal of allergy and clinical immunology*. 2011; 128:3–20. [PubMed: 21477849]
2. Aceves SS, Newbury RO, Dohil R, Bastian JF, Broide DH. Esophageal remodeling in pediatric eosinophilic esophagitis. *The Journal of allergy and clinical immunology*. 2007; 119:206–12. [PubMed: 17208603]
3. Kwiatek MA, Hirano I, Kahrilas PJ, Rothe J, Luger D, Pandolfino JE. Mechanical properties of the esophagus in eosinophilic esophagitis. *Gastroenterology*. 2011; 140:82–90. [PubMed: 20858491]
4. Nicodeme F, Hirano I, Chen J, Robinson K, Lin Z, Xiao Y, et al. Esophageal Distensibility as a Measure of Disease Severity in Patients with Eosinophilic Esophagitis. *Clinical gastroenterology and hepatology*. 2013; 11:1101–7. [PubMed: 23591279]
5. Straumann A. The natural history and complications of eosinophilic esophagitis. *Gastrointestinal endoscopy clinics of North America*. 2008; 18:99–118. [PubMed: 18061105]
6. Lucendo AJ. Motor disturbances participate in the pathogenesis of eosinophilic oesophagitis, beyond the fibrous remodelling of the oesophagus. *Alimentary pharmacology & therapeutics*. 2006; 24:1264–7. [PubMed: 17014589]
7. Lucendo AJ, Arias A, De Rezende LC, Yague-Compadre JL, Mota-Huertas T, Gonzalez-Castillo S, et al. Subepithelial collagen deposition, profibrogenic cytokine gene expression, and changes after prolonged fluticasone propionate treatment in adult eosinophilic esophagitis: a prospective study. *The Journal of allergy and clinical immunology*. 2011; 128:1037–46. [PubMed: 21880354]
8. Schoepfer AM, Safroneeva E, Bussmann C, Kuchen T, Portmann S, Simon HU, et al. Delay in Diagnosis of Eosinophilic Esophagitis Increases Risk for Stricture Formation, in a Time-Dependent Manner. *Gastroenterology*. 2013; 145:1230–6. [PubMed: 23954315]
9. Dellon ES, Kim HP, Sperry SL, Rybnicek DA, Woosley JT, Shaheen NJ. A phenotypic analysis shows that eosinophilic esophagitis is a progressive fibrostenotic disease. *Gastrointestinal endoscopy*. 2013; 13:02460–7.
10. Aceves SS, Chen D, Newbury RO, Dohil R, Bastian JF, Broide DH. Mast cells infiltrate the esophageal smooth muscle in patients with eosinophilic esophagitis, express TGF-beta1, and increase esophageal smooth muscle contraction. *The Journal of allergy and clinical immunology*. 2010; 126:1198–204. [PubMed: 21047675]
11. Niranjana R, Mavi P, Rayapudi M, Dynda S, Mishra A. Pathogenic role of mast cells in experimental eosinophilic esophagitis. *American journal of physiology Gastrointestinal and liver physiology*. 2013; 304:G1087–94. [PubMed: 23599040]
12. Chehade M, Sampson HA, Morotti RA, Magid MS. Esophageal subepithelial fibrosis in children with eosinophilic esophagitis. *Journal of pediatric gastroenterology and nutrition*. 2007; 45:319–28. [PubMed: 17873744]
13. Roman S, Hirano I, Kwiatek MA, Gonsalves N, Chen J, Kahrilas PJ, et al. Manometric features of eosinophilic esophagitis in esophageal pressure topography. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 2011; 23:208–14. [PubMed: 21091849]
14. Read AJ, Pandolfino JE. Biomechanics of esophageal function in eosinophilic esophagitis. *Journal of neurogastroenterology and motility*. 2012; 18:357–64. [PubMed: 23105995]
15. Korsapati H, Babaei A, Bhargava V, Dohil R, Quin A, Mittal RK. Dysfunction of the longitudinal muscles of the oesophagus in eosinophilic oesophagitis. *Gut*. 2009; 58:1056–62. [PubMed: 19136515]
16. Landres RT, Kuster GG, Strum WB. Eosinophilic esophagitis in a patient with vigorous achalasia. *Gastroenterology*. 1978; 74:1298–301. [PubMed: 648822]
17. Noel RJ, Putnam PE, Rothenberg ME. Eosinophilic esophagitis. *The New England journal of medicine*. 2004; 351:940–1. [PubMed: 15329438]
18. Nurko S, Rosen R, Furuta GT. Esophageal dysmotility in children with eosinophilic esophagitis: a study using prolonged esophageal manometry. *The American journal of gastroenterology*. 2009; 104:3050–7. [PubMed: 19755968]

19. Moawad FJ, Maydonovitch CL, Veerappan GR, Bassett JT, Lake JM, Wong RK. Esophageal motor disorders in adults with eosinophilic esophagitis. *Digestive diseases and sciences*. 2011; 56:1427–31. [PubMed: 21416245]
20. Savarino E, Gemignani L, Zentilin P, De Bortoli N, Malesci A, Mastracci L, et al. Achalasia with dense eosinophilic infiltrate responds to steroid therapy. *Clinical gastroenterology and hepatology*. 2011; 9:1104–6. [PubMed: 21839711]
21. Bassett J, Maydonovitch C, Perry J, Sobin L, Osgard E, Wong R. Prevalence of esophageal dysmotility in a cohort of patients with esophageal biopsies consistent with eosinophilic esophagitis. *Diseases of the esophagus*. 2009; 22:543–8. [PubMed: 19302212]
22. Hejazi RA, Reddymasu SC, Sostarich S, McCallum RW. Disturbances of esophageal motility in eosinophilic esophagitis: a case series. *Dysphagia*. 2010; 25:231–7. [PubMed: 19707817]
23. Mishra A, Wang M, Pemmaraju VR, Collins MH, Fulkerson PC, Abonia JP, et al. Esophageal remodeling develops as a consequence of tissue specific IL-5-induced eosinophilia. *Gastroenterology*. 2008; 134:204–14. [PubMed: 18166354]
24. Mavi P, Rajavelu P, Rayapudi M, Paul RJ, Mishra A. Esophageal functional impairments in experimental eosinophilic esophagitis. *American journal of physiology Gastrointestinal and liver physiology*. 2012; 302:G1347–55. [PubMed: 22361731]
25. Abonia JP, Franciosi JP, Rothenberg ME. TGF-beta1: Mediator of a feedback loop in eosinophilic esophagitis--or should we really say mastocytic esophagitis? *The Journal of allergy and clinical immunology*. 2010; 126:1205–7. [PubMed: 21134572]
26. Straumann A, Spichtin HP, Grize L, Bucher KA, Beglinger C, Simon HU. Natural history of primary eosinophilic esophagitis: a follow-up of 30 adult patients for up to 11.5 years. *Gastroenterology*. 2003; 125:1660–9. [PubMed: 14724818]
27. Straumann A, Conus S, Degen L, Felder S, Kummer M, Engel H, et al. Budesonide is effective in adolescent and adult patients with active eosinophilic esophagitis. *Gastroenterology*. 2010; 139:1526–37. [PubMed: 20682320]
28. Schmitt JP, Kamisago M, Asahi M, Li GH, Ahmad F, Mende U, et al. Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. *Science*. 2003; 299:1410–3. [PubMed: 12610310]
29. Haghighi K, Kolokathis F, Gramolini AO, Waggoner JR, Pater L, Lynch RA, et al. A mutation in the human phospholamban gene, deleting arginine 14, results in lethal, hereditary cardiomyopathy. *Proceedings of the National Academy of Sciences*. 2006; 103:1388–93.
30. Baum P, Fundel-Clemens K, Kreuz S, Kontermann RE, Weith A, Mennerich D, et al. Off-target analysis of control siRNA molecules reveals important differences in the cytokine profile and inflammation response of human fibroblasts. *Oligonucleotides*. 2010; 20:17–26. [PubMed: 20038253]
31. Kranias EG, Hajjar RJ. Modulation of cardiac contractility by the phospholamban/SERCA2a regulome. *Circulation research*. 2012; 110:1646–60. [PubMed: 22679139]
32. Ramachandran A, Gangopadhyay SS, Krishnan R, Ranpura SA, Rajendran K, Ram-Mohan S, et al. JunB mediates basal- and TGFbeta1-induced smooth muscle cell contractility. *PloS one*. 2013; 8:e53430. [PubMed: 23308222]
33. Fox VL, Nurko S, Teitelbaum JE, Badizadegan K, Furuta GT. High-resolution EUS in children with eosinophilic “allergic” esophagitis. *Gastrointestinal endoscopy*. 2003; 57:30–6. [PubMed: 12518127]
34. Straumann A, Conus S, Degen L, Frei C, Bussmann C, Beglinger C, et al. Long-term budesonide maintenance treatment is partially effective for patients with eosinophilic esophagitis. *Clinical gastroenterology and hepatology*. 2011; 9:400–9. [PubMed: 21277394]
35. Remedios M, Campbell C, Jones DM, Kerlin P. Eosinophilic esophagitis in adults: clinical, endoscopic, histologic findings, and response to treatment with fluticasone propionate. *Gastrointestinal endoscopy*. 2006; 63:3–12. [PubMed: 16377308]
36. Saffari H, Peterson KA, Fang JC, Teman C, Gleich GJ, Pease LF 3rd. Patchy eosinophil distributions in an esophagectomy specimen from a patient with eosinophilic esophagitis: Implications for endoscopic biopsy. *The Journal of allergy and clinical immunology*. 2012; 130(3): 798–800. [PubMed: 22502795]

37. Nicholson AG, Li D, Pastorino U, Goldstraw P, Jeffery PK. Full thickness eosinophilia in oesophageal leiomyomatosis and idiopathic eosinophilic oesophagitis. A common allergic inflammatory profile? *The Journal of pathology*. 1997; 183:233–6. [PubMed: 9390039]
38. Rieder F, Nonevski I, Ma J, Ouyang Z, West G, Protheroe C, et al. T-helper 2 Cytokines, Transforming Growth Factor beta1, and Eosinophil Products Induce Fibrogenesis and Alter Muscle Motility in Patients with Eosinophilic Esophagitis. *Gastroenterology*. 2014 Epub.
39. Wang HS, Arvanitis DA, Dong M, Niklewski PJ, Zhao W, Lam CK, et al. SERCA2a superinhibition by human phospholamban triggers electrical and structural remodeling in mouse hearts. *Physiological genomics*. 2011; 43:357–64. [PubMed: 21266500]
40. Haghighi K, Kolokathis F, Pater L, Lynch RA, Asahi M, Gramolini AO, et al. Human phospholamban null results in lethal dilated cardiomyopathy revealing a critical difference between mouse and human. *The Journal of clinical investigation*. 2003; 111:869–76. [PubMed: 12639993]
41. Sathish V, Thompson MA, Bailey JP, Pabelick CM, Prakash YS, Sieck GC. Effect of proinflammatory cytokines on regulation of sarcoplasmic reticulum Ca²⁺ reuptake in human airway smooth muscle. *American journal of physiology Lung cellular and molecular physiology*. 2009; 297:L26–34. [PubMed: 19395670]
42. McGraw DW, Fogel KM, Kong S, Litonjua AA, Kranias EG, Aronow BJ, et al. Transcriptional response to persistent beta2-adrenergic receptor signaling reveals regulation of phospholamban, which alters airway contractility. *Physiological genomics*. 2006; 27:171–7. [PubMed: 16849635]
43. Kim M, Hennig GW, Park K, Han IS, Smith TK, Koh SD, et al. Modulation of murine gastric antrum smooth muscle STOC activity and excitability by phospholamban. *The Journal of physiology*. 2008; 586:4977–91. [PubMed: 18755751]
44. Kim M, Hennig GW, Smith TK, Perrino BA. Phospholamban knockout increases CaM kinase II activity and intracellular Ca²⁺ wave activity and alters contractile responses of murine gastric antrum. *American journal of physiology Cell physiology*. 2008; 294:C432–41. [PubMed: 18045856]
45. Camello-Almaraz C, Macias B, Gomez-Pinilla PJ, Alcon S, Martin-Cano FE, Baba A, et al. Developmental changes in Ca²⁺ homeostasis and contractility in gallbladder smooth muscle. *American journal of physiology Cell physiology*. 2009; 296:C783–91. [PubMed: 19211915]
46. Qureshi S, Song J, Lee HT, Koh SD, Hennig GW, Perrino BA. CaM kinase II in colonic smooth muscle contributes to dysmotility in murine DSS-colitis. *Neurogastroenterology and motility*. 2010; 22(2):186–95. [PubMed: 19735476]
47. Fischer H, Fischer J, Boknik P, Gergs U, Schmitz W, Domschke W, et al. Reduced expression of Ca²⁺-regulating proteins in the upper gastrointestinal tract of patients with achalasia. *World journal of gastroenterology*. 2006; 12:6002–7. [PubMed: 17009399]

Clinical Implications

EoE is accompanied by smooth muscle remodeling and functional alterations but the mechanisms are not known. We show that PLN is regulated by TGF β 1 and, as such, it may provide a novel therapeutic target to improve esophageal dysmotility and clinical dysphagia.

Figure 1. TGFβ1 induces PLN in ESM and EoE EMFs

PLN mRNA (A) and protein (B) is detected in primary ESM cells after TGFβ1 treatment (H-cardiac extract positive control). Primary EoE fibroblasts express alpha-smooth muscle actin (αSMA, green; blue-nuclear DAPI) (C) and PLN (green) which co-localizes (yellow) with αSMA (red) upon TGFβ1 treatment (D). Protein molecular weight ladder shows PLN monomer below the 5kD marker. Isotype control (insets) shows no stain.

Figure 2. PLN is expressed in EoE smooth muscle and localizes with Serca2a

Representative image (A) (EPI-epithelium, LP-lamina propria, SM- smooth muscle) and quantification (B) of smooth muscle PLN in Control and EoE esophageal biopsies. PLN (green) co-localizes (yellow) with α SMA (red) (C). PLN (green) co-localizes (yellow) with Serca2a (red) (D) in pediatric EoE. Inset shows nuclear DAPI

Figure 3. PLN down-regulation significantly decreases esophageal EoE EMF contraction
 PLN siRNA inhibits TGFβ1 induced of PLN expression (A, representative western blot).
 TGFβ1- induced contraction of EoE EMFs (representative contraction gel, B) is significantly
 decreased by PLN siRNA but not by control siRNA (C). Graphs represent means and
 standard errors.

Figure 4. TGF β receptors I and II are expressed on esophageal smooth muscle and required for TGF β 1 mediated contraction as well as PLN phosphorylation

FACs analysis (black plot- isotype control, grey plot- specific antibody) demonstrates that ESM cells and EoE fibroblasts express TGF β 1RI and TGF β 1RII (A). TGF β 1RI (red) and TGF β 1RII (green) are co-expressed (yellow) on EoE fibroblasts (B). Representative image of EoE biopsy shows co-localization (yellow) of TGF β 1RI (red) and TGF β 1RII (green) receptors in esophageal smooth muscle (insets show isotype control) (C). TGF β 1RI inhibitor but not TAK1 inhibitor abolishes PLN expression induced by TGF β 1 (D) but TAK1 inhibitor does block JNK phosphorylation by IL-1 β (E). TGF β 1RI inhibitors blocks ESM contraction but TAK1 inhibitor does not (F).

Figure 5. TGFβ1 causes PLN phosphorylation

Phospho-serine-16 is detectable following TGFβ1 treatment (A) but phospho-threonine-17 is not (B). Heart (H) was used a positive control to detect phospho-threonine-17-PLN. A representative image demonstrates that phospho-serine-16 is expressed in EoE but not in Control esophageal smooth muscle (C).

Figure 6.

Model of the pathway by which TGFβ1 could regulate alter esophageal smooth muscle and myofibroblast function. TGFβ1 produced by mast cells and eosinophils binds to its receptor (TGFβRI) causing PLN transcription via the phosphorylated Smad pathway and co-localization of PLN with the calcium channel Serca2a. PLN phosphorylation (P-PLN) and de-phosphorylation changes calcium flux into the sarcoplasmic reticulum via Serca2a. Intracellular calcium changes could cause esophageal smooth muscle cell and myofibroblast contraction to cause esophageal dysmotility and tissue contraction.