Calcium Receptor and Nitric Oxide Synthase Expression in Circular Muscle of Lower Esophagus from Patients with Achalasia

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To the Editor: Achalasia is a primary esophageal motility disorder.[1] The etiology of achalasia remains unknown, but it is associated with degeneration, reduction, or absence of neurons in the esophageal muscular layer (Auerbach's plexus), leading to nerve–muscle conduction abnormalities.[2] It has been demonstrated that excitatory neurons induce calcium ion (Ca^{2+}) activity and contract smooth muscles by releasing acetylcholine[3] in esophagus, and nitric oxide (NO) and vasoactive intestinal peptide (VIP) induce smooth muscle relaxation by inhibitory neurons.[4] Hydrogen sulfide synthases may also be involved.[5] We measured expression of L-type calcium channels (LTCCs),[6] ryanodine receptors (RyRs),[7] inositol 1,4,5-trisphosphate receptors (IP_3Rs),[7] and NO synthases (NOSs)[4,8] in circular muscles of the lower esophagus of achalasia patients. The result provides a better understanding for the pathogenesis of achalasia.

Circular muscles from the lower esophagus were obtained from achalasia patients undergoing Heller's myotomy (the experimental group) and esophageal cancer patients undergoing esophagectomy for the upper esophageal carcinoma (the control group) between January 2015 and October 2017. Achalasia patients underwent preoperative esophageal manometry routinely. Specimens were removed during surgery as previous procedures.[3] Tissues for immunohistochemistry (IHC) were immersed in 10% neutral formalin immediately and for RNA-related experiments were immersed in RNAlater (Thermo Scientific, USA) and stored at −80°C. Hematoxylin and eosin staining was conventional in controls to ensure that there was no invasion of tumor cells.

RNA was obtained according to the protocol of TriQuick total RNA extraction kit (R1100, Solarbio, China). Each real-time PCR reaction included 2 μl reverse transcription product, 5 μl SYBR Green qPCR SuperMix (2×) (11744100, Invitrogen, USA), 0.8 μl mixture of forward and reverse primers in 100-fold dilution, and 2.2 μl nuclease-free water. Reactions were carried out in ABI 7500 Real-Time PCR System (Thermo Scientific, USA) for 40 cycles (95°C for 30 s, the annealing temperature for 30–40 s, and 72°C for 30–40 s). Primer information is as follows (5′-3′): Cav1.2-(F) ATCACCGAGGTAAACCCAGC, (R) CCGATCACCGCGTAGATGAA, annealing temperature (AT) 59°C, product (bp) 236; Cav1.3-(F) TGCGATAGGATGGGAATGGC, (R) TCCACCAGCACCAGAGACTT, AT 61°C, product (bp) 365; Cav1.1-(F) GACTGTATTGCCTGGGTGGAG, (R) TGCCGATGACAGCGTAGATG, AT 59°C, product (bp) 225; IP_3R1-(F) TAACCCAGGCTGCAATGAGG, (R) CACTGAGGGCTGAAACTCCA, AT 61°C, product (bp) 394; IP_3R2-(F) TTATGTGCACAGGACCAGAAGC, (R) ATGATGGCAATTGCGGGACT, AT 58°C, product (bp) 82; IP_3R3-(F) GCGTCCCGAGATGACAAGAA(R) CCAGGCTGACCACCTCAAAA, AT 58°C, product (bp) 139; RyR1GGGAGAACGGTGAAGCTGAA, (R) CTGGCGATTGATGACAGTGC, AT 59°C, product (bp) 389; RyR2-(F) TGAAAGCATCAAACGCAGCA, (R) TCCACCACACAGCCAATCTC, AT 59°C, product (bp) 105; RyR3-(F) AGGAGCAGTTGAAAGCCGAT, (R) CCAGACTTTACTTGCATGGC, AT 59°C, product (bp) 334; iNOS-(F) GAGCTTCTACCTCAAGCTATC, (R) CCTGATGTTGCCATTGTTGGT, AT 58°C, product (bp) 312; nNOS-(F) CCCTTCAGTGGCTGGTACAT, (R) ACCGCGATATTGATCTCCAC, AT 58°C, product (bp) 164; eNOS-(F) ACTGAAGGCTGGCATCTGGAA, (R) ACCTCCCAGTTCTTCACACGA, AT 58°C, product (bp) 329; GAPDH-(F) CGCTGAGTACGTCGTGGAGTC, (R)

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GCTGATGATCTTGAGGCTGTTGTC, product (bp) 172. The fold-change in the expression of each gene was calculated using the $2^{-\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. mRNA expression of these proteins in the achalasia group was analyzed by linear correlation with integrated relaxation pressure (IRP) of esophageal manometry. Reverse transcription polymerase chain reaction (RT-PCR) for each sample was detected by DreamTaq Green PCR Master Mix (2×) (K1081, Thermo Scientific, USA) in the AB Applied Biosystems-Veriti 96-well Thermal Cycler (Thermo Scientific, USA), with the same amplification procedure as real-time RT-PCR. Base sequences of the products were detected by Invitrogen Company (Thermo Fisher Ltd., Beijing) to determine the corresponding mRNA expression.

IHC and scoring were conducted as described.[9] Primary antibodies of inducible NOS (iNOS) (mouse, Abcam, ab129372), neuronal NOS (nNOS) (rabbit, Abcam, ab3511), Cav1.2 (mouse, Abcam, ab84814), Cav1.3 (mouse, Abcam, ab85491), IP_3R1 (rabbit, Abcam, ab125076), IP_3R2 (rabbit, Millipore, ab9074), IP_3R3 (rabbit, Millipore, ab9076), and RyR2 (rabbit, Chemicon, ab9080) were diluted at 1:100. Three pathologists, blinded to patients' details, measured the extent of expression. Expression was scored as follows: 9–12 = strong, 5–8 = moderate, 1–4 = weak, and 0 = negative. To identify the components of smooth muscles effectively, four specific indicators were selected to assist the identification. The vascular endothelial cells were identified by CD31 (mouse, Zsbio, ZM-0044, 1:100); the hematopoietic cells (lymphocytes and leukocytes, etc.) were identified by CD45 (mouse, Zsbio, ZM-0183, 1:100); the lymphatic vessels were identified by D2-40 (mouse, DAKO, IS07230-2, ready to use); and the nervous tissues were identified by S-100 (mouse, Servicebio, GB13361, 1:200).

Statistical analysis was conducted with SPSS version 13.0 (IBM Corp., USA). Measurement data for real-time RT-PCR that was neither normal nor homogeneous was recorded as (median [interquartile range]), followed by a non-parametric test (Kruskal-Wallis Test). Count data in IHC experiments, in which there were fewer than 40 cases, were evaluated using an exact probability method (Fisher's exact test). For the achalasia group in linear correlation analysis, IRP and mRNA were analyzed using the Spearman's correlation coefficient. Differences were considered statistically significant when $P < 0.05$.

Ten patients (seven females, aged 48.0 ± 3.5 years, age range: 23–62 years) undergoing Heller's myotomy for achalasia and 17 patients in controls were included. In achalasia patients, the preoperative IRP was 23.05 (16.83) mmHg (range 13.00–96.10 mmHg). According to the Chicago Classification, one patient was Type I, eight were Type II, and one was Type III. The duration of symptoms was 36 (110.6) months (range 1–180 months).

From automatic plotting of dissolution and amplification curves in ABI 7500 and base sequence results of Invitrogen, Cav1.2, Cav1.3, three subtypes of IP_3Rs, RyR2, iNOS, and nNOS were positive in the achalasia and control groups (esophageal circular muscle, EC), but other subtypes were negative [Figure 1a]. Compared with controls, relative mRNA expression of iNOS (0.039 [0.145] vs. 0.000 [0.000], $P = 0.000$), nNOS (0.001 [0.040] vs. 0.000 [0.001], $P = 0.000$), Cav1.3 (0.001 [0.087] vs. 0.000 [0.000], $P = 0.000$), IP_3R1 (0.007 [0.029] vs. 0.013 [0.017], $P = 0.022$), and IP_3R2 (0.041 [0.055] vs. 0.012 [0.012], $P = 0.000$) was greater in the achalasia group ($P < 0.05$), but with no statistical differences for Cav1.2 (0.003 [0.008] vs. 0.002 [0.003], $P = 0.318$), RyR2 (0.021 [0.020] vs. 0.017 [0.018], $P = 0.119$), and IP_3R3 (0.001 [0.001] vs. 0.001 [0.002], $P = 0.675$) between the two groups [Figure 1b].

Because of nonnormal distribution data for each mRNA, Spearman's correlation coefficients were calculated to detect the linear correlation between esophageal IRP and the corresponding mRNA in achalasia patients. mRNA of iNOS and Cav1.3 was correlated with IRP positively ($P = 0.008$ and 0.036, respectively) and r_s was 0.344 and 0.261, respectively, but no linear relations for other parameters ($P > 0.05$) [Figure 1c].

In Figure 1d, specific indicators showed the different expression, respectively. CD31 was strongly stained in endothelial cells of blood capillaries, with no significant difference between the two groups. CD45 was positive in lymphocytes and leukocytes, D2-40 was strong in lymphatic endothelial cells, and S-100 showed strong staining in nervous tissues and cytoplasm of SMCs in the two groups. However, positive cells of CD45, D2-40, and S-100 in achalasia were less than that in controls. With these auxiliary diagnosis, expression of parameters of this study was easy to be defined in esophageal smooth muscles. Expression of iNOS was negative, but nNOS, Cav1.2, Cav1.3, IP_3R1, IP_3R2, IP_3R3, and RyR2 could be detected positively in IHC in both achalasia and control groups [Figure 1e]. nNOS was distributed in nervous tissues of smooth muscles with strong staining in both groups, but in controls, it was also widely distributed in cytoplasm of SMCs. Cav1.2 and Cav1.3 were distributed on cellular membrane and cytoplasm as IP_3R1, IP_3R2, IP_3R3, and RyR2 in both groups, but positive staining of Cav1.2, IP3R1, IP3R2, and RyR2 were not uniform in achalasia. SMCs of small vessel wall in both groups could be stained positively by Cav1.2 and Cav1.3.

In terms of protein and mRNA expression, IP_3R2 was greater in the achalasia group compared with the controls ($P = 0.042$), but there was no significant difference among the other parameters, which was not consistent with their respective mRNA expression [Table 1].

Most classic studies of achalasia etiology focused on the effects of neurotransmitters such as NO or VIP.[4] NOS is the key rate-limiting enzyme to induce NO *in vivo*. Its isoenzymes include nNOS, epidermal NOS (eNOS), and iNOS.[4,8]

In achalasia patients, studies suggested that loss of NO-secreting esophageal myenteric plexus neurons caused imbalance of excitatory and inhibitory neurons, leading to altered manometry results.[4] We detected iNOS and nNOS mRNA in both achalasia and control tissues as positive, but only nNOS was positive in IHC. nNOS staining was almost pale in cytoplasm of SMCs in achalasia, different to the widely distribution in controls. This result showed that nNOS still existed in the smooth muscles of patients' lower esophagus, but the decreased distribution of the nervous tissues accompanied the decreased nNOS expression in the SMCs reduced the effective regulation of the SMCs in the NOS-NO conduction pathway to NO-induced relaxation.

In the achalasia group, there was a positive linear correlation between esophageal IRP and iNOS mRNA ($P < 0.05$) [Figure 1c]. There are few literatures about the effect of iNOS on the function of esophageal smooth muscles, but iNOS often appears in the pathological conditions though the damage to cells.[10] With the increased iNOS mRNA in achalasia patients, the role of NO

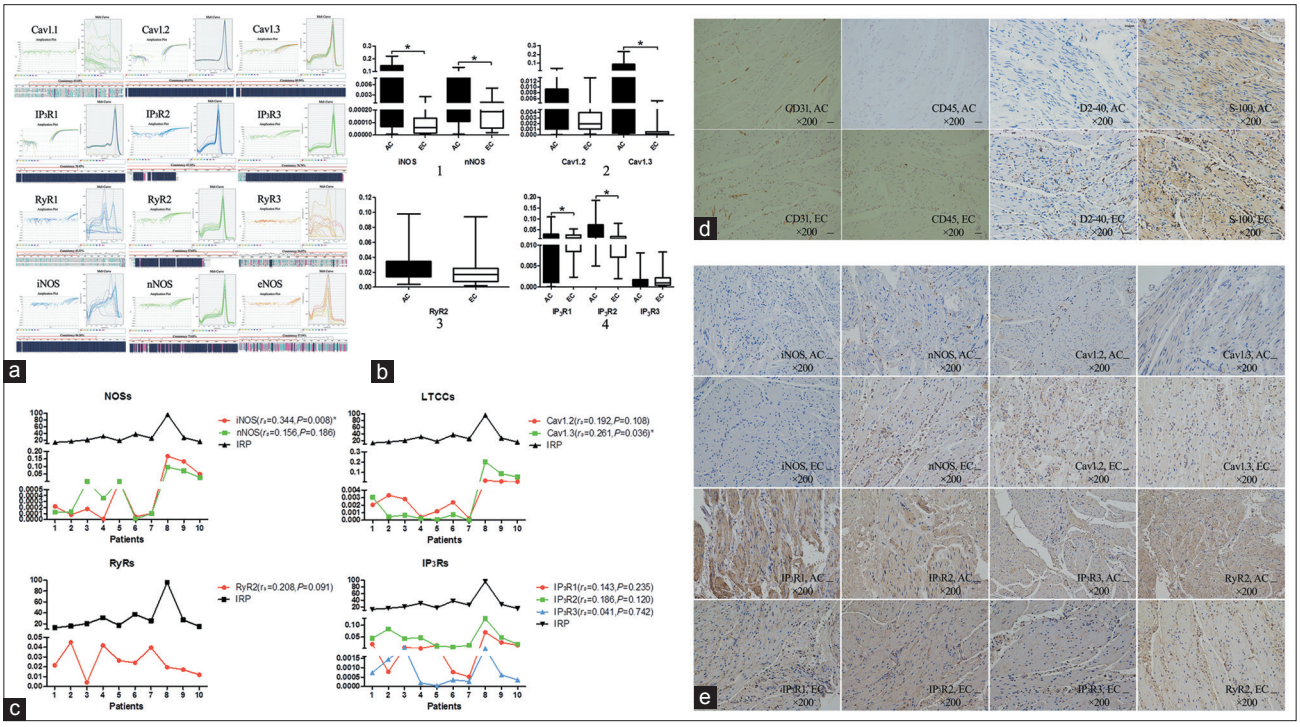

Figure 1: Research results of calcium receptors and nitric oxide synthases in achalasia patients. (a) Relative mRNA expression of calcium receptors and nitric oxide synthases. In achalasia and control groups, Cav1.2, Cav1.3, IP_3R1, IP_3R2, IP_3R3, RyR2, inducible nitric oxide synthase, and neuronal nitric oxide synthase could be detected, but Cav1.1, RyR1, RyR3, and epidermal nitric oxide synthase were negative. (b) Comparison of Ca^{2+}-related proteins and nitric oxide synthases between achalasia and control groups by real-time reverse transcription polymerase chain reaction (*$P < 0.05$). Relative mRNA expression of inducible nitric oxide synthase, neuronal nitric oxide synthase, Cav1.3, IP_3R1, and IP_3R2 in achalasia was increased compared with controls. (c) The linear correlation between esophageal-integrated relaxation pressure and corresponding mRNA in achalasia patients (*$P < 0.05$). mRNA of inducible nitric oxide synthase and Cav1.3 correlated with integrated relaxation pressure positively, but there were no linear relations for other parameters ($P > 0.05$). (d) Immunohistochemistry of CD31, CD45, D2-40, and S-100 in the lower esophageal smooth muscles (SP, original magnification ×200) (AC: Achalasia circular muscle; EC: Esophageal circular muscle in control group; Bar = 100 μm). (e) Immunohistochemistry in achalasia and controls (SP, original magnification ×200) (AC: Achalasia circular muscle, EC: Esophageal circular muscle in control group; Bar = 100 μm).

in the regulation of esophageal motility and its mechanism of damage to SMCs remained to be studied. Furthermore, we did not detected the same or similar phenomenon that myenteric neurons were replaced by collagen and inflammatory cells as described,[11,12] because inflammatory cells as lymphocytes and leukocytes stained by CD45 were not gathered or common in the lower esophageal smooth muscles in both achalasia and control groups [Figure 1d]. We might conclude that the dysfunction of lower esophageal relaxation with achalasia not only caused by local neuropathological changes but also associated with local pathological manifestations, such as congenital (primary) or acquired (secondary) lesions, should be considered.

Ca^{2+}-related signal molecules have been most closely studied in the cardiovascular system. There are few studies regarding Ca^{2+} and Ca^{2+}-related receptors in the esophagus of achalasia patients.[13] In physiological conditions, extracellular Ca^{2+} enters cells via membrane channels including LTCC to activate IP_3Rs and RyRs through serial signal transduction. LTCC was characterized according to their α1 subunits (160–240 kDa) as Cav1.2 (α1C), Cav1.3 (α1D), and Cav1.1 (α1S).[6,14] Kovac *et al*.[6] studied α1C expression in human LES by RT-PCR; their study showed that LTCC participated in the regulation of esophageal tone. RyRs and IP_3Rs are primarily distributed in organelle membrane to regulate $[Ca^{2+}]_i$ in cellular activities.[15] RyRs is one of the largest known ion channels, with each subunit about 560 kDa.[15] Similar to RyRs, IP_3Rs is a homotetramer-binded IP_3 and Ca^{2+} channel, and each subunit is about 240–300 kDa.[16]

Expression of LTCC, RyRs, and IP_3Rs subtypes in the same tissue might occur in various combinations.[15,17,18] We measured mRNA and protein expression of Cav1.2, Cav1.3, IP_3R1, IP_3R2, IP_3R3, and RyR2 in lower esophageal smooth muscles [Figure 1a and 1e]. Compared with controls, mRNA expression of Cav1.3, IP_3R1, and IP_3R2 was greater [Figure 1b] in achalasia, but only IP_3R2 in IHC was greater than that of the controls. We showed that there was positive correlation between esophageal IRP and Cav1.3 mRNA expression [Figure 1c]. This suggests that Ca^{2+}-related proteins involved in the regulation of IRP in achalasia patients. Ca^{2+} regulation as a myogenic factor appeared to be important in the pathogenesis of achalasia.

In this study, mRNA of nNOS, Cav1.3, and IP_3R1 was greater in the achalasia group without statistical difference in corresponding IHC. This might be related to the small samples in both groups. Statistical samples of achalasia were difficult to calculate because the corresponding epidemiological data in China were not available, while the achalasia incidence was 1.6/100,000 in North America.[19] In the future, we will expand our samples to define the possible differences of nNOS, Cav1.3, IP_3R1, and other lesions in achalasia patients.

Table 1: Statistical data in IHC

Items	Positive IHC (*n*)			Negative IHC (*n*)			*P*
	Strong	Moderate	Total	Weak	Negative	Total	
iNOS							
AC			0		10	10	–
EC			0		17	17	
nNOS							
AC	5	2	7	3		3	1.000
EC	8	5	13	4		4	
Cav1.2							
AC		7	7	2	1	3	0.678
EC	9	3	12	5		5	
Cav1.3							
AC		4	4	5	1	6	0.224
EC	8	4	12	5		5	
IP_3R1							
AC	6	2	8	2		2	0.124
EC		8	8	9		9	
IP_3R2							
AC	8	1	9	1		1	0.042*
EC	5	3	8	9		9	
IP_3R3							
AC		4	4	6		6	1.000
EC		8	8	9		9	
RyR2							
AC	3	2	5	5		5	0.687
EC	2	9	11	6		6	

*P<0.05. NOS: Nitric oxide synthases; iNOS: Inducible NOS; eNOS: Epidermal NOS; IHC: Immunohistochemistry; AC: Achalasia circular muscle; EC: Esophageal circular muscle.

In conclusion, we should focus on Ca^{2+} regulation as myogenic factors and NOSs in pathogenesis of achalasia. These mechanisms and how they relate to Auerbach's plexus lesions require further studies.

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Conflicts of interest

There are no conflicts of interest.

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