Aberrant endoplasmic reticulum stress mediates coronary artery spasm through regulating MLCK/MLC2 pathway

Aimin Xue, Junyi Lin, Chunxing Que, Yijing Yu, Chunyan Tu, Han Chen, Baonian Liu, Xin Zha, Tianhao Wang, Kaijun Ma, Liliang Li

A Department of Forensic Medicine, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China
B Shanghai Key Laboratory of Crime Scene Evidence, Shanghai 200083, China
C Shanghai Institute of Cardiovascular Diseases, Department of Cardiology, Zhongshan Hospital, Fudan University, Shanghai 200032, China
D Department of General Practice, Zhongshan Hospital, Fudan University, Shanghai 200032, China

Keywords:
Coronary artery spasm
MLCK/MLC2 pathway
Vasoconstriction

ABSTRACT

Coronary artery spasm (CAS) is a pathophysiological phenomenon that may cause myocardial infarction and lead to circulatory collapse and death. Aberrant endoplasmic reticulum (ER) stress causes accumulation of misfolding proteins and has been reported to be involved in a variety of vascular diseases. The present study investigated the role of ER stress in the development of CAS and explored the possible molecular mechanisms. Initially, it was found that ER stress markers were elevated in response to drug-induced vascular smooth muscle cells (VSMCs) contraction. Pharmacologic activation of ER stress using Tunicamycin (Tm) persistently induced CAS and significantly promoted Pituitrin-induced CAS in mice as well as in a collagen gel contraction assay. On the contrary, pharmacologic inhibition of ER stress using 4-phenylacetic acid (4-PBA) completely blunted Pituitrin-induced CAS development in mice. Moreover, during the drug-induced VSMCs contraction, expression of ER stress markers were increased in parallel to those of myosin light chain kinase (MLCK) and phosphor-MLC2 (p-MLC2, at Ser19). After inhibiting MLCK activity by using its specific inhibitor ML-7, the ER stress activator Tm failed to activate the MLCK/MLC2 pathway and could neither trigger CAS in mice nor induce VSMCs contraction in vitro. Our results suggested that aberrant ER stress mediated CAS via regulating the MLCK/MLC2 pathway. ER stress activators might be more robust than the common drugs (Pituitrin or acetylcholine) as to induce vasoconstriction and thus may serve as potential therapeutics against chronic bleeding, while its inhibitor might be useful for treatment of severe CAS caused by other medication.

1. Introduction

Coronary artery spasm (CAS) is the transient contraction of coronary artery which could cause complete or incomplete occlusion of the vessel, leading to myocardial ischemia and subsequent angina pectoris, cardiac arrhythmia or even sudden cardiac death [1,2]. Two pathophysiological mechanisms are recognized as attributors to CAS development-endothelial dysfunction and vascular smooth muscle cells (VSMCs) hypersensitivity. Although endothelial dysfunction was thought to be critical, VSMCs hypersensitivity is the most influential factor that takes the lead in the course of arterial contraction [3].

Aberrant endoplasmic reticulum (ER) stress is derived from imbalance of the homeostasis inside endoplasmic reticulum and caused misfolding of proteins, aggregation of unfolded protein and irregularity of calcium concentration, thereby activating the unfolded protein re-action (UPR). Principally there are three parallel signal transductions via a class of transmembrane ER-resident signaling component-inositol requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Recent studies have shown the critical involvement of ER stress in cardiovascular diseases [4]. Cardiac contractile function was reported to be impaired by aberrant ER stress [5] via oxidative stress and autophagy pathways [6]. Aberrant ER stress has also been reported to induce vascular misfolding proteins and has been reported to be involved in a variety of vascular diseases. The present study investigated the role of ER stress in the development of CAS and explored the possible molecular mechanisms. Initially, it was found that ER stress markers were elevated in response to drug-induced vascular smooth muscle cells (VSMCs) contraction. Pharmacologic activation of ER stress using Tunicamycin (Tm) persistently induced CAS and significantly promoted Pituitrin-induced CAS in mice as well as in a collagen gel contraction assay. On the contrary, pharmacologic inhibition of ER stress using 4-phenylacetic acid (4-PBA) completely blunted Pituitrin-induced CAS development in mice. Moreover, during the drug-induced VSMCs contraction, expression of ER stress markers were increased in parallel to those of myosin light chain kinase (MLCK) and phosphor-MLC2 (p-MLC2, at Ser19). After inhibiting MLCK activity by using its specific inhibitor ML-7, the ER stress activator Tm failed to activate the MLCK/MLC2 pathway and could neither trigger CAS in mice nor induce VSMCs contraction in vitro. Our results suggested that aberrant ER stress mediated CAS via regulating the MLCK/MLC2 pathway. ER stress activators might be more robust than the common drugs (Pituitrin or acetylcholine) as to induce vasoconstriction and thus may serve as potential therapeutics against chronic bleeding, while its inhibitor might be useful for treatment of severe CAS caused by other medication.

1. Introduction

Coronary artery spasm (CAS) is the transient contraction of coronary artery which could cause complete or incomplete occlusion of the vessel, leading to myocardial ischemia and subsequent angina pectoris, cardiac arrhythmia or even sudden cardiac death [1,2]. Two pathophysiological mechanisms are recognized as attributors to CAS development-endothelial dysfunction and vascular smooth muscle cells (VSMCs) hypersensitivity. Although endothelial dysfunction was thought to be critical, VSMCs hypersensitivity is the most influential factor that takes the lead in the course of arterial contraction [3].

Aberrant endoplasmic reticulum (ER) stress is derived from imbalance of the homeostasis inside endoplasmic reticulum and caused misfolding of proteins, aggregation of unfolded protein and irregularity of calcium concentration, thereby activating the unfolded protein re-action (UPR). Principally there are three parallel signal transductions via a class of transmembrane ER-resident signaling component-inositol requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Recent studies have shown the critical involvement of ER stress in cardiovascular diseases [4]. Cardiac contractile function was reported to be impaired by aberrant ER stress [5] via oxidative stress and autophagy pathways [6]. Aberrant ER stress has also been reported to induce vascular
contractile dysfunction [7,8] and cause hypertension, whilst inhibition of ER stress reduces hypertension through the preservation of resistance blood vessel structure and function [9]. Induction of ER stress also impairs insulin-stimulated vasomotor relaxation in rat aortic rings [10]. However, whether ER stress exerts any functional role in the development of CAS remains to be elucidated.

Myosin light chain kinase (MLCK) is the upstream regulator of myosin II regulatory light chain (MLC2) and important for microfilament reorganization as well as normal myocyte contraction [11,12]. In smooth muscle cells, the binding of calmodulin with Ca2+ activates MLCK by removing an autoinhibitory domain from the kinase catalytic site, so that the activated MLCK further phosphorylates MLC2 at Ser19 to cause cascade reaction [13]. MLCK-dependent phosphorylation of MLC2 is an essential intermediate for physiological regulation [14]. Inactivation of MLCK/MLC2 pathway in intestinal smooth muscle was involved in bowel motility disorder [15], while epithelial MLCK activity was associated with respiratory diseases, atherosclerosis and pancreatitis [16].

Our previous study showed that phosphorylated MLC2 (p-MLC2) levels could serve as an indication for antemortem CAS in sudden cardiac deaths [17]. The present study investigated whether ER stress mediated the development of CAS both in vivo and in vitro. To this end, a specific activator of ER stress, Tunicamycin (Tm), and an inhibitor 4-phenyl acetic acid (4-PBA) was respectively adopted to modulate ER stress. In addition, we also explored the molecular mechanisms that contributed to ER stress-mediated CAS development using VSMCs. Our data showed that activation of ER stress promoted, while inhibition of ER stress completely suppressed drug-induced CAS development. Pharmacologic inhibition of MLCK activity abolished ER stress-induced increases in the p-MLC2 level and rescued ER stress-induced CAS development both in vivo and in vitro. These observations suggested that ER stress mediated CAS through regulation of MLCK/MLC2 pathway. The ER stress activators might serve as potential therapeutics against vasodilatation, while its inhibitor might be useful for treatment of severe side effects on coronary artery caused by other medication.

2. Material and methods

2.1. Reagents

Primary antibodies against p-MLC2 (Ser19) (Catalog No.: 3675), PERK (Catalog No.: 3192), phospho-eIF2α (p-eIF2α, Ser51) (Catalog No.: 3597), and XBPs1s (Catalog No.: 12782) were purchased from Cell Signaling Technology (Boston, MA, USA). Specific anti-MLCK antibody was purchased from Abcam (Catalog No.: ab76092, Cambridge, UK). Anti-ATF6 was purchased from Novus Biologicals (Catalog No.: NBP1-40256, Littleton, CO, USA). Primary antibody against GRP78 was purchased from Santa Cruz Biotechnology (Catalog No.: sc-376768, Santa Cruz, CA, USA). The following drugs were obtained from commercial sources as specified below: Pituitrin (Catalog No.: H31022259, Shanghai Pharma No. 1 Biochemical & Pharmaceutical Co., Ltd.), Tunicamycin (Tm) (Catalog No.: T7765, Sigma Co., Billerica, MA, USA), 4-Phenybutyric acid (4-PBA) (Catalog No.: P21005, Sigma Co.), Acetylcholine (ACh) (Catalog No.: A2661, Sigma Co.), ME-7 (Catalog No.: S8388, Selleck Chemicals, Houston, TX, USA.).

2.2. Animal experiments

To set up the in vivo CAS model, Pituitrin is used to trigger coronary spastic activity. Pituitrin is extracted from pituitary gland that contains vasopressin and oxytocin. It has been evidenced that Pituitrin is able to affect circulation, blood pressure and cause cardiac ischemia by inducing vascular contraction [18], and therefore, Pituitrin has been widely used to establish CAS model [19,20].

For the in vivo experimental procedures, male C57BL/6 mice at the age of 6–8 week old (weighing 15–20 g) were purchased from Shanghai SLAC Co. (Shanghai, China) and housed in a temperature-controlled room at 20–22 °C, under a 12-h light/dark cycle and with free access to food and water. All mice were allowed to acclimate for 1 week before any treatment. To evaluate the effects of ER stress on coronary artery constriction, mice were randomly subgrouped as vehicle-injected, Pituitrin (5.1 U/kg)-injected, Tm (10.0 mg/kg)-injected, Pituitrin + Tm injected groups, or vehicle-injected, Pituitrin (5.1 U/kg)-injected, 4-PBA (77.5 mg/kg)-injected, Pituitrin + 4-PBA-injected groups, respectively (n = 5 per group). Vehicle-treated mice were intraperitoneally injected with 200 μL of saline as the control group. For Tm treatment, mice were pretreated from tail vein before electrophysiological recording (ECG) monitoring and for 4-PBA treatment, mice were injected of 4-PBA from the tail vein 24 h ahead of time. Before ECG monitoring, all mice were anesthetized with 10% chloral hydrate (133 mg/kg, i.p.) and received vehicle or Pituitrin treatments, followed by lead II assessment. The ECG was monitored in a 9-min length unless otherwise stated. ECG (lead II) of mice were recorded using a multi-channel physiological signal system (RM-6240BD, Chengdu Instrument Factory, China) at a speed of 20 ms/div. In normal mouse ECG, S-wave is immediately followed by a J-wave which then inverts into a negative T-wave. Hence, J-wave starts from the end of S-wave and goes to the point where positive J-wave changes into negative T-wave. It is suggestive of CAS-induced local ischemia when an ECG shows prominently elevated J-wave and is absent from S-wave [21]. Therefore, we measured the J-wave amplitude before and after drug treatments and assessed the mean elevation of J-wave amplitude to assess the level of CAS in each group.

To evaluate the effects of MLCK/MLC2 pathway inactivation on ER stress-mediated CAS development, a specific MLCK activity inhibitor ML-7 (0.085 mg/kg, i.p.) was used. Briefly, mice were randomly divided as vehicle-treated, Tm-treated, ML-7-treated, Tm + ML-7 treated groups, respectively (n = 5 per group). Monitoring of ECG was specified as above mentioned. All experimental procedures were approved by the Institutional Animal Care and Use Committees at the School of Basic Medical Sciences, Fudan University. All efforts were made to minimize sufferings.

2.3. Cells and cell culture

Human vascular smooth muscle cells (VSMCs) were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in F-12K Medium (Gibco, Los Angeles, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Cells were grown to a confluence of approximate 70–80% before drug treatments.

2.4. Real time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNAs from VSMCs were extracted using Trizol reagent (Catalog No. 15596-026, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality and concentration were assessed with a NanoDrop 2000 (Erlangen, Germany). cDNA copies of total RNAs were obtained using a Roche Transcriptor First Strand cDNA Synthesis Kit (Catalog No.: 4897030001, Roche, CA, USA). Then, the cDNA product was diluted to 1:10 for quantitative real-time PCR which was performed using Quantifast SYBR Green PCR Kit (Catalog No.: 204057, QIAGEN, Germany) by 7500 Fast Real-Time PCR Systems (Applied Biosystems, USA). Glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as an internal control and primer sequences for quantitative real-time PCR were provided in Table 1.

2.5. Western blot analysis

VSMCs in culture dish were lysed at 4 °C using RIPA lysis buffer (Catalog No.: P0013C, Beyotime Biotechnology, Nantong, China) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ML leupeptin, 1 μg/ML pepstatin, 1 μg/ML aprotinin). An
equal amount of proteins were loaded onto a 12% SDS-PAGE gel and transferred to the nitrocellulose membrane (Millipore, NY, USA). After blocking in 5% fat-free milk for 1 h, the membrane was incubated with specific primary antibody with a 1:1000 dilution at 4 °C overnight. The bound antibody was visualized using respective HRP-conjugated secondary antibody and ECL kit (Catalog No.: 34096, Thermo Fisher, Shanghai, China). The intensity was quantified using the Image J v1.50 (National Institutes of Health, Bethesda, MD, USA) and normalized to β-actin expression.

2.6. Evaluation of VSMCs contractility

To evaluate VSMCs contractility, the collagen gel contraction assay was conducted. To prepare the gel, an aliquot of 7.5 μL NaOH (0.1 mol/L) was added to 200 μL collagen solution (Catalog No.: 11179179001, Roche, CA, USA) on ice, followed by 12.5 μL 10× PBS. Then 280 μL of VSMCs suspension (1.2×10^5 cells/ML) was added to get the gel-cell mixture which was subsequently transferred to 24-plate wells to let further incubation for 30 min at 37 °C. After gelation, 500 μL of culture medium were added to each well and cells were cultured for another 3 days at 37 °C in a 5% CO2 atmosphere. After 3-day incubation, the gel was dissociated from the well wall with a pinhead and treated with reagents as specified in each assay. Contraction of the collagen gel reflected the VSMCs contractility and was monitored for a 24-h period. Images were photographed for each group using Tanon 6100 imaging system (Tanon, Shanghai, China). Area of each collagen gel was calculated from the images in each group using Image J v1.50 (National Institutes of Health, Bethesda, MD, USA). Contraction efficacy was measured by the equation (So-St)/So×100%, where So means the gel area at 0 h and St means the gel area at each indicated experimental time.

2.7. Statistical analysis

Data was expressed as mean ± standard deviation (SD). All experiments were repeated at least three times. The Student’s t-test was used for comparison of means between groups. One-way analysis of variance (ANOVA) was used for comparison of means among multiple (≥3) groups, followed by a LSD post-hoc analysis when necessary. A p value < 0.05 (labeled with “**”, or “#”) in two-tailed tests was considered as statistically significant; “***” was used for labeling differences with p value < 0.01 and “****” for p value < 0.0001.

3. Results

3.1. ER stress was triggered in drug-induced contraction of VSMCs

ACh and 5-HT are classical stimulators that induce CAS in atherosclerotic as well as normal human coronary arteries [22,23]. It is unclear yet how vessel wall responds to the stimulation. To this end, human VSMCs were treated with ACh or 5-HT. ACh and 5-HT treatments caused significant increases in the level of phosphor-MLC2 (p-MLC2) (p = 0.014 for time points in Fig. 1A and p = 0.049 for time points in Fig. 1B), which was consistent with our previous report [17] and confirmed the drug-induced VSMCs contraction. More importantly, GRP78, a marker of ER stress, was significantly increased with ACh treatment time extended (p = 0.04). Phosphor-eIF2α (p-eIF2α) and spliced XBP1 (XBP1s) which reflected the activation of ER stress were also concordantly up-regulated during ACh treatments (p = 0.048 and p = 0.026, respectively). Interestingly, ACh and 5-HT-induced increases of p-MLC2 and major phosphorylation-related ER stress markers (i.e. PERK and p-eIF2α) were observed in earlier minutes (Figs. 1A and 1B), while expression of XBP1s peaked until 12 h after ACh treatment (Fig. 1A, upper panel). Quantification of the western blots from three independent assays also confirmed the above observations (Fig. 1A, lower panels). The time difference in the alteration of ER stress markers might implicate the 3 ER stress signaling might be step-wise activated and our observations were basically consistent with previous reports [24–26]. Similar to ACh treatment, it was found that ER stress markers (GRP78, ATF6, p-eIF2α) were also elevated in response to short-term 5-HT treatments (p = 0.042, 0.017 and 0.041, respectively, Fig. 1B). These results implicated that ER stress was triggered in drug-induced contraction of VSMCs.

3.2. Activation of ER stress induced coronary artery spasm in mice

Pituitrin is a common reagent to induce CAS in vivo [19,20]. The typical manifestation of Pituitrin-induced CAS in mice is the remarkable elevation of J-wave amplitude [21]. The present study recorded ECG at indicated time after injection and showed that the J-wave amplitude in Pituitrin-injected mice was significantly higher than the vehicle-treated control group (Fig. 2A and B). This observation confirms that Pituitrin is a critical inducer of CAS. We then injected Tunicamycin (Tm), one specific activator of ER stress, into mice. The ECG records showed that Tm treatment also elevated the J-wave amplitude and its effect was more persistent than Pituitrin in the setting of inducing CAS. Co-treatment of Tm and Pituitrin led to even higher J-wave elevation (Figs. 2A and 2B). These data suggested that Tm could cooperate with Pituitrin to induce CAS. On the contrary, 4-PBA, one specific inhibitor of ER stress, significantly blunted Pituitrin-induced J-wave elevation. Co-treatment of 4-PBA and Pituitrin decreased the J-wave amplitude to a level that was comparable to the control mice (Figs. 2C and 2D). These results strongly suggested that ER stress might be robust to induce CAS and activation of ER stress had even stronger effects on coronary spastic activity than the known inducer Pituitrin.

3.3. Activation of ER stress promoted VSMCs contractile activity in vitro

To mimic the in vitro CAS, a collagen gel contraction assay was performed to visualize the contraction of VSMCs. Compared with control VSMCs that developed slow gel contraction, either Tm or ACh treatment led to more remarkable contraction and reached its plateau at approximately 3 h after stimulation. Two hours after Tm treatments, VSMCs exhibited a remarkable contraction that was approximately 1.6-fold of the control VSMCs (Fig. 3A and B). Comparatively, Tm had a stronger impact than ACh treatment on gel contraction in the initial 5 h (Fig. 3B). After 5 h, VSMCs exhibited decreases in contractile activity even under either ACh or Tm treatments, and the gel sizes among the three distinct treatments were indifferent at the end of monitored time (Figs. 3A and 3B). The decreased contractile activity of VSMCs in late hours mirrored the nature of contraction fatigue after long-term exposure. But in consideration that the contractile activity of VSMCs was remarkably promoted by Tm treatment in the early hours, it was strongly suggestive that activation of ER stress accentuated VSMCs.
Fig. 1. ER stress was triggered in drug-induced contraction of VSMCs. ACh and 5-HT were used as stimulators to induce VSMCs contraction. (A) Key molecules of ER stress were detected at the indicated time of ACh-treated VSMCs by western blot (upper panel). The intensity was quantified using Image J software from three independent analyses (lower panels).

(B). Western blots detected expression of GRP78, ATF6, p-eIF2α and p-MLC2 in 5-HT-treated VSMCs at indicted time. ACh, acetylcholine. 5-HT, 5-hydroxytryptamine. VSMCs, vascular smooth muscle cells. *p < 0.05 vs. control group.
On the contrary, while ACh promoted VSMCs contraction in the collagen gel assay (Figs. 3C and 3D, ACh group vs. control), inhibition of ER stress by its specific inhibitor 4-PBA significantly blunted ACh-promoted cell contraction (Figs. 3C and 3D, ACh+4-PBA group vs. ACh group). Together with the above data, it was conclusive that aberrant ER stress regulated VSMCs contractile activity.

3.4. ER stress positively regulated the MLCK/MLC2 pathway

Our previous study showed that the phosphorylated level of MLC2 was elevated in early CAS tissues and pMLC2 might serve as a biomarker indicating antemortem CAS-induced sudden cardiac death [17]. In view of ER stress-induced CAS above, we hypothesized that ER stress may regulate the MLCK/MLC2 pathway. In the Tm-treated VSMCs, it was found that along with the activation of ER stress, expression of MLCK as well as its substrate p-MLC2 were increasingly elevated ($p=0.028$ and 0.0005, respectively for all time points), which was in parallel to the significant up-regulation of ER stress markers GRP78 ($p=0.0102$ for all time points) and p-eIF2α ($p=0.0268$ for all time points) (Fig. 4A). Of note, Tm took effect at relatively later hours as compared with ACh or 5-HT. Our observation was consistent with previous reports that only long-term Tm exposure (on the scale of hours) increased the levels of ER stress markers [27,28], and might suggest that Tm is a slow-acting activator of ER stress. MLCK and pMLC2 levels showed an initial increase and decreased in late hours of Tm treatments (Fig. 4A). In turn, when ER stress was inactivated by 4-PBA, the expression of MLCK and p-MLC2 were significantly decreased as compared with control VSMCs (Fig. 4B). Moreover, 4-PBA significantly reversed ACh-induced elevation of MLCK and pMLC2 in VSMCs (Fig. 4B). These results suggested that ER stress positively regulated the MLCK/MLC2 pathway in vitro.

3.5. Inhibition of MLCK activity blunted ER stress-induced activation of MLCK/MLC2 pathway

To confirm the regulatory role of ER stress in MLCK/MLC2 pathway, we treated VSMCs with ML-7, a specific inhibitor of MLCK. As shown in Fig. 5A, the protein levels of MLCK were decreased by ML-7 treatments and the least expression of MLCK was present 4 h after ML-7 treatment.
In addition, with the concentration of ML-7 increased, the protein level of MLCK was dose-dependently decreased in VSMCs (Fig. 5B). Moreover, RT-qPCR analysis showed that ML-7 treatment significantly decreased the mRNA level of MLCK without affecting that of GRP78 in VSMCs, suggesting that MLCK/MLC2 pathway had no inverse regulation of ER stress. While the mRNA level of MLCK was significantly promoted by Tm treatment alone, it was significantly decreased by Tm and ML-7 co-treatments ($p = 0.028$, Tm+ML-7 vs. Tm, Fig. 5C) and was comparable to that of control VSMCs after Tm and ML-7 co-treatments (Fig. 5C). More importantly, though the ER stress markers were altered by Tm treatment, Tm minimally elevated the protein level of MLCK and failed to increase the pMLC2 level after ML-7 co-treatments (Fig. 5D). In fact, after ML-7 treatments, the pMLC2 level was remarkably lower than the control cells even under Tm stimulation, suggesting that MLCK activity is dispensable for the phosphorylation and activation of MLC2. In addition, ML-7 treatment did not affect the ER stress markers as compared with the control group (Fig. 5D), reinforcing that there was no inverse regulation of ER stress by MLCK/MLC2 pathway. These results suggested that ER stress was the upstream regulator of MLCK expression at both protein and mRNA levels. Inhibition of MLCK suppressed ER stress-induced activation of MLCK/MLC2 pathway.

3.6. Inactivation of MLCK/MLC2 pathway rescued ER stress-induced CAS in vivo and in vitro

In the in vitro collagen gel contraction assay, it was observed that while Tm induced higher contractile activity of VSMCs, inhibition of MLCK activity by ML-7 led to no VSMCs contraction throughout the monitored time. Co-treatment of Tm and ML-7 also failed to induce observable gel contraction within 24 h (Fig. 6A). Inactivation of MLCK activity also blunted ACh-induced CAS in the mouse model (Supplementary Fig. 1), verifying the efficiency of ML-7 to inactivate MLCK activity in vivo. These observations were supportive of the in vitro data (Fig. 5D) and reinforced that MLCK/MLC2 pathway activation is inevitable for VSMCs contraction. In addition, in the in vivo mice CAS model, it was observed that Tm alone elevated significantly the J-wave amplitude throughout the recorded time periods (Fig. 6B). There was no ECG abnormality recorded on ML-7 treated mice, reflecting no significant adverse effects of ML-7 on mouse heart. More importantly, when mice were co-treated with both Tm and ML-7, the elevation of J-wave was no longer recorded in the early minutes, while mice still presented with a trend of increasing J-wave amplitude in late minutes (Figs. 6B and 6C). All these results demonstrated that inhibition of MLCK/MLC2 pathway rescued ER stress-induced CAS in vivo and in vitro.
Fig. 4. ER stress positively regulated the MLCK/MLC2 pathway in VSMCs. (A) Upper panels: western blot analysis of GRP78, p-eIF2α, MLCK, p-MLC2 levels in Tm-treated VSMCs at the indicated time. Lower panels: intensity of the blots were quantified from at least three independent assays. (B, C) Under the treatments with 4-PBA and/or ACh, the protein levels of GRP78, p-eIF2α, MLCK and p-MLC2 were analyzed by western blot. *p < 0.05 vs. control group. #, p < 0.05 ACh+4-PBA vs. ACh as indicated. Tm, Tunicamycin; 4-PBA, 4-phenylacetic acid; ACh, Acetylcholine.
The MLCK/MLC2 pathway-dependent mechanism might dominate the early period in the ER stress-induced CAS development.

4. Discussion

Coronary artery spasm (CAS) is one common entity in the cardiovascular system. Current knowledge has attributed endothelial dysfunction and VSMC hyperreactivity to the induction of CAS with the VSMCs hyperreactivity being regarded as the most influential factor [3]. However, the intracellular mechanisms of VSMCs hyperreactivity remain to be elucidated and diagnosis of death by CAS is still challenging and questionable due to the lack of specific diagnostic biomarkers.

It has been widely reported that aberrant endoplasmic reticulum stress (ER stress) is involved in vascular contraction [7,8]. Tunicamycin, an ER stress activator, induced VSMCs contraction and caused vasoconstriction of rat aortic ring [10]. Inversely, an ER stress inhibitor 4-PBA was reported to diminish vessel contractility and augment vasodilation [9]. Consistent with previous reports, we found that ER stress markers, including GRP78, PERK, phospho-eIF2α (p-eIF2α) and XBP1s were elevated in drug-induced VSMCs hyperreactivity. In the mice CAS model which was established by infusion of the specific inducer Pituitrin, it was found that treatment of an ER stress activator alone significantly elevated the J point, and even promoted the Pituitrin-induced J point elevation, suggesting the synergistic effect of ER stress activator and Pituitrin. In turn, pharmacologic inhibition of ER stress using an ER stress inhibitor lowered the J point to the normal level, and more importantly, completely blunted the Pituitrin-induced CAS as evidenced by the electrocardiogram. Moreover, we took use of gel contractility model which enabled the measurement of visualized VSMCs contraction. In the in vitro cell contractility assay, ACh, a common inducer of cell contraction, induced VSMCs contraction. Activation of ER stress induced VSMCs contraction more robustly than ACh, while inhibition of ER stress using 4-PBA completely suppressed ACh-induced VSMCs contraction. All these observations are suggestive...
that aberrant ER stress is an important mediator of CAS genesis.

Interestingly, we observed that expression of ER stress biomarkers and MLCK were synchronously elevated in ACh-exposed VSMCs. In the Tm-treated VSMCs, it was detected that MLCK and p-MLC2 levels were correspondingly modulated with Tm exposure time extended. Early exposure to Tm led to increased MLCK expression as well as its substrate, whilst long-term exposure decreased both expression. The time-dependent regulation of MLCK/MLC2 pathway mirrored the nature of MLC2 phosphorylation which only occurred in the early periods and substituted by dephosphorylation in late time [17]. In addition, the ER stress inhibitor 4-PBA significantly blunted ACh-induced MLCK expression and MLC2 phosphorylation. Therefore, our results suggested that ER stress positively regulated MLCK/MLC2 pathway. It was the first report to our best knowledge that ER stress exerted positive regulatory effects on MLCK/MLC2 pathway.

MLCK in intestinal smooth muscle was involved in bowel motility disorder [15], while epithelial MLCK was associated with respiratory diseases, atherosclerosis and pancreatitis [16]. MLCK also participated in actin-myosin contractility and was responsible for the shift in cell shape and cell motility [29]. In all of these pathologies, MLCK functions by phosphorylating MLC2, the regulatory chain of myosin, at Ser19 to cause cascade reaction [13]. The phosphorylated MLC2 subsequently led to the constitutional change of myosin, making its ATPase exposed. Decomposition of ATP energized the shifting of thick and thin filaments, causing the contraction of VSMCs and thereby contributing to CAS by vasoconstriction [30,31]. In view of the regulation of MLCK/MLC2 pathway by ER stress, we then blocked the activity of MLCK using pharmacologic inhibitor ML-7. It was found that while ER stress activator Tm caused long-lasting manifestation of CAS in mice, blockade of MLCK activity significantly blunted Tm-induced CAS in vivo. Pharmacologic inhibition of MLCK/MLC2 pathway in VSMCs also abrogated Tm-induced VSMCs contraction in vitro. All these findings suggested that ER stress mediated CAS by regulating MLCK/MLC2 pathway. It remains yet unclear which pathway of ER stress specifically dominates the MLCK/MLC2 activity in order to induce CAS. The present data provided evidence that all the three pathway markers of ER stress were altered in response to ACh treatment of VSMCs with each pathway activated in different time. It merits further investigation of the functional role of specific pathway in CAS induction.

Of note, it was observed in animal experiment that inhibition of MLCK activity could completely suppressed ER stress-induced CAS at an early stage but tend to lose the ability of suppressing ER stress-mediated effects in late hours, suggesting that ER stress induced CAS in a MLCK/MLC2 pathway-dependent way in the early stage. In other words, ER stress-mediated CAS induction was possibly MLCK-independent in late stages and there might be other pathways involved in this process. Several studies demonstrated that Rho-kinase enhanced pMLC2 level in a direct way or via inhibiting its phosphatase activity [32–34]. A Rho-kinase inhibitor Fasudil efficiently abolished CAS induced by ACh [35,36] and Tm [37]. Aberrant ER stress also increased vascular contractility in an AMPK-dependent way [7]. Hence, it could be conceived that the augmentation of CAS at later stage might be possibly mediated by other mechanisms independent of MLCK/MLC2 pathway.

It is noteworthy that the ER stress activator Tm exerted a more robust promotion effect on vascular contraction than Pituitrin. Pituitrin acts on hepato-splanchnic vascular bed and effects to reduce portal pressure and flow by producing a potent vasoconstriction in patients with portal hypertension [38]. During liver transplantation, Pituitrin...
has been shown to reduce blood inflow [39, 40]. Pituitrin has also been used to control bleeding in clinic [41]. We provided evidence that ER stress activator Tm had a more persistent vasoconstriction effect than Pituitrin. The vasoconstriction effect by Tm suggested that Tm might also a potential therapeutic against chronic bleeding. In addition, several studies have reported severe cardiovascular complications such as hypertension, bradycardia and cardiac arrest following Pituitrin use [19, 41]. Oral administration of Pituitrin provoked coronary spasitic angina in a patient with unstable angina [42]. Our in vivo experiment found that the ER stress inhibitor 4-PBA completely abolished Pituitrin-induced CAS in mice. This finding indicated that ER stress inhibitors may be potential drugs to alleviate CAS in clinic.

5. Conclusion

The present study investigated the role of ER stress in the induction of CAS. Activation of ER stress promoted, while inhibition of ER stress attenuated CAS both in vivo and in vitro. ER stress mediated CAS genesis via regulating the MLCK/MLC2 pathway. Our results suggested that ER stress activators might be useful therapeutics against vasoconstriction diseases and its specific inhibitor might aid in treating CAS in clinic.

Acknowledgments

This work was financially supported by the Opening Project of Shanghai Key Laboratory of Crime Scene Evidence (No. 2014XCMZK13), the Open Project Program of Shanghai Key Laboratory of Forensic Medicine (No.KF1306) and the Zhengyi Scholar Foundation of School of Basic Medical Sciences, Fudan University (No. S18-14).

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

A.X., J.L. and C.Q. performed equally the work. A.X., J.L. and C.Q. performed equally the project. L.L. revised the manuscript. B.L. conducted part of the western blot analysis. H.C. helped in the animal ECG monitoring. K.M., X.Z. and T.W. helped in data analysis and provided part of the reagents. A.X. and L.L. conceived and supervised the project. L.L. revised the final manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jeycr.2018.01.032.

References


学霸图书馆

www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页  文献云下载  图书馆入口  外文数据库大全  疑难文献辅助工具