Hyper-Activation of Endoplasmic Reticulum Stress PERK/Calcineurin/RyR2 Signaling Pathway is Involved in AGEs-Exacerbated Post- Myocardial Infarction Ventricular Arrhythmias

Zhongwei Liu
Shaanxi Provincial People's Hospital
Yong Zhang
Shaanxi Provincial People's Hospital
Shuo Pan
Shaanxi Provincial People's Hospital
Chuan Qiu
Tulane University
Hao Jia
Affiliated Hospital of Northwest University
Yuan Wang (ooozkb@sina.com)
Shaanxi Provincial People's Hospital
Haitao Zhu (tarn1000@163.com)
Northwest Women's and Children's Hospital

Original investigation

Keywords: Diabetes, AGEs, Endoplasmic reticulum stress, Calcium, Ventricular arrhythmias

Posted Date: August 5th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-47561/v1

Abstract

Background: The current study was aimed to investigate the mechanisms of advanced glycation end products (AGEs) in exacerbating post- myocardial infarction (MI) ventricular arrhythmias (VAs).

Methods: Correlation between premature ventricular contractions (PVCs) and serum AGEs concentrations was analyzed in a cohort consisted of 101 STEMI patients with culprit vessel of left anterior descending artery (LAD). Established MI rat model were treated with AGEs and/or anti- receptor for AGE (RAGE) antibody. Electrocardiography was used to record VAs. Myocytes were isolated from adjacent area around infracted region. Immunofluorescent stains were used to evaluate association between FKBP12.6 (FK506-bindingprotein 12.6) and ryanodine receptor 2 (RyR2). Calcium sparks were evaluated by confocal microscope. Protein expression and phosphorylation were assessed by Western blotting. A colorimetric method was used to determine the enzymatic activity of calcineurin (CaN). [^{3}H]-ryanodine binding assay was carried out to detect the RyR2 channel activity.

Results: In the cohort study, significantly increased amount of (PVCs) were found in STEMI patients with diabetes ($P<0.05$). Serum AGEs concentration was significantly positively correlated with PVCs amount in STEMI patients ($r=0.416$, $P<0.001$). Multivariate analysis showed serum AGEs concentration was independently and positively related to frequent PVCs (adjusted hazard ratio, 1.86; 95% CI, 1.09-3.18, $P=0.022$). In the animal study, increased glucose regulated protein 78 (GRP78) expression, protein kinase RNA-like ER kinase (PERK) phosphorylation, CaN enzymatic activity, FKBP12.6-RyR2 disassociation, RyR2 channel opening and endoplasmic reticulum (ER) calcium releasing were found in MI animals exposed to AGEs, which were attenuated by anti-RAGE antibody treatment. This RAGE blocking also significantly lowered the VAs amount in MI animals exposed to AGEs.

Conclusions: Hyper-activation of ER stress- mediated PERK/CaN/RyR2 signaling participated in AGEs-exacerbated post-MI VAs.

Background

Higher incidence of lethal arrhythmias such as R-on-T premature ventricular contractions (PVCs), ventricular tachycardia and ventricular fibrillation were found in diabetes, which has been identified as a risk factor elevating the incidence of ventricular arrhythmia-related adverse events [1]. In our previous investigation, elevated amount of VAs was found in diabetic animals[2]. Ventricular arrhythmia (VA) is a common complication after myocardial infarction (MI). Increased risks of occurrence of life-threatening VAs were identified in acute MI (AMI) patients, which elevated in-hospital mortality. However, the underlying mechanisms of the exacerbating role of diabetes in post-MI VAs are still unclear.

Advanced glycation end products (AGEs) are characterized metabolites of DM which participated in many DM- associated pathological processes. AGEs are fostered by non-enzymatic reactions among nucleic acids, amino groups and lipids in DM and closely correlated with major adverse cardiovascular events (MACE) including lethal arrhythmias[3]. A recent study pointed out that inhibiting AGEs formation

attenuated the vulnerability to tachyarrhythmias in diabetic animals[4]. In this regard, we hypothesized that AGEs might be the cause facilitating post-MI VAs.

Calcium homeostasis is important for maintaining the normal electrophysiological integrity of myocytes. Previous investigation suggested intracellular calcium metabolism dys-regulation contributed to arrhythmias in diabetic hearts[5]. Delayed afterdepolarizations (DADs) have been recognized as the mechanism triggering VAs. Appearing posterior to the completion of repolarization, DADs are induced by oscillation of intracellular calcium released from endoplasmic reticulum (ER), which further trigger a greater amount calcium release from ER via channels such as ryanodine receptor 2 (RyR2), which was termed as calcium- induced calcium release (CICR) [6].

ER is the major cellular organelle maintaining intracellular calcium homeostasis[7]. ER stress is triggered when challenged by strong and sustained harmful stimuli [8]. Protein kinase RNA-like ER kinase (PERK) is a sensor transducting ER stress signals. By direct contacting, PERK activates calcineurin (CaN) which further facilitates ER calcium releasing via promoting the disassociation of FK506-binding protein 12.6 (FKBP12.6) from RyR2[9]. Our previous investigation indicated that ER stress PERK signaling was activated in diabetic hearts, inducing ventricular arrhythmias through the PERK/CaN signaling[10].

Notably, results from an investigation suggested that AGEs induced RyR2 channel hyperactivation-mediated ER calcium releasing after interacting with the receptor for AGE (RAGE) in cultured myocytes[11]. Moreover, interestingly, several previous studies pointed out ER stress PERK signaling activations were RAGE- dependent[12, 13]. Thus, it is reasonable for us to speculate that AGEs cause aggravation of post-MI VAs in DM. The RAGE- dependent ER stress mediated PERK/CaN/RyR2 signaling pathway could be possibly involved. We believe that results from this study would enrich our current understanding of the mechanisms of exacerbated post-MI VAs in DM.

Materials And Methods

Cohort study

In the period from May, 2017 to May, 2019, 115 patients diagnosed as STEMI with the culprit vessel of LAD at Shaanxi Provincial People's Hospital were initially included. The final cohort consisted of 101 STEMI patients. 3 patients were excluded due to identification of malignant cancer; 1 patient was excluded due to diagnosis of systemic lupus erythematosus; 10 patients were excluded because of rapid development of cardiac systolic dysfunction which were eventually supported by extra-corporeal membrane oxygenation (ECMO) or left ventricular assist devices. STEMI was diagnosed by the current guideline[14]. Culprit vessel was determined by ECG manifestation and coronary angiography. Diabetes was diagnosed according to current guideline[15]. Post-MI VAs were recorded by Holter ECG. Frequent premature ventricular contractions (PVCs) was defined as ventricular premature beats take ≥ 10% of total cardiac beats during 24 hour- Holter ECG recording[16]. The exclusion criteria were: age below 18 years or above 80 years; pregnant women; previous myocardial infarction; previous PCI/CABG history; New York Heart Association (NYHA) functional class III or IV, renal failure, hepatic dysfunction, known history of

cancer, immune-mediated disorders, mental disorders, recent/current use of anti-arrhythmic drugs. The peripheral venous blood samples were collected in a fasting state. Serum AGEs concentrations were determined by using a Human AGEs ELISA kit (CUSABIO, China) per the protocols provided by the manufacturer. All of the patients gave informed consent to participate in this study, which was approved by the ethics committee of Shaanxi Provincial Peoples' Hospital. Specifically, patients signed the informed consent and agreed the collection and research purposes of medical records for our investigation.

AGEs-BSA preparation

The protocol was in accordance with our previous investigations[17]. Briefly, 0.1 mmol/L glyceraldehydes (Sigma) and bovine serum albumin (BSA) were incubated in 0.2 mmol/L $NaPO_4$ buffer solution (pH = 7.4) at 37℃ at sterile condition for 7 days. BSA prepared without glyceraldehydes by the same protocol was used as control.

MI model establishment and animal treatments

SPF class Sprague-Dawley (SD) rats (9-week old, male/femal = 1, weighted 255 ± 6 g) were provided by Animal Experimental Center of Xi'an Jiaotong University. Animals were maintained in independent polypropylene cages under controlled conditions (12 h/12 h artificial light/dark cycle, humidity at 56% ±4%, temperature at 25℃±1℃). Animals were accessible to standard chow and sterilized water freely. Animals were accommodated 1 week prior to experiments.

Rats were anaesthetized by inhalation of isoflurane (2% for introducing and 4% for continuous anesthesia) in oxygen at 0.6L/min. MI model was induced by ligation of LAD following the protocol adopted from our previous investigation [18]. Before establishment of MI model, AGEs-BSA and/or anti-RAGE IgG_3 (Abcam) were administrated to animals via remaining needles implemented in tail veins. The dosages anti-RAGE antibody were selected according to previous reports[19, 20]. Treatments of each group were listed in Table 1.

Table 1
Animal treatments in each group

Groups (n = 10)	**Treatment1**		**Treatment2**	
	Reagent	Description	Reagent	Description
Control (sham operation)	BSA	chronic tail vein injection for consecutive 8 weeks (1 injection/d)	Physiological saline	chronic tail vein injection for 8 weeks (3 injection/w)
Myocardial infarction (MI) model	BSA	chronic tail vein injection for consecutive 8 weeks (1 injection/d)	Physiological saline	chronic tail vein injection for 8 weeks (3 injection/w)
MI model and AGEs treatments	AGEs-BSA (100 mg/Kg)	chronic tail vein injection for consecutive 8 weeks (1 injection/d)	Physiological saline	chronic tail vein injection for 8 weeks (3 injection/w)
MI model, AGEs exposure and RAGE antibody treatments	AGEs-BSA (100 mg/Kg)	chronic tail vein injection for consecutive 8 weeks (1 injection/d)	monoclonal anti-RAGE IgG_3 (1 mg/Kg)	chronic tail vein injection for 8 weeks (3 injection/w)

Table 1
Baseline clinical characteristics of the study patients

	Total (n = 101)	DM(-) (n = 67)	DM(+) (n = 34)	P value
Age (year)	53.54 ± 4.64	54.21 ± 4.36	52.21 ± 4.93	0.052
Male (%)	62(61.4)	42(62.7)	20(58.8)	0.829
BMI (kg/m^2)	24.12 ± 1.42	24.12 ± 1.45	24.10 ± 1.37	0.963
Smoking history (%)	38 (37.6)	27 (40.3)	11(32.4)	0.517
Mean heart rate/24hours (bpm)	69.56 ± 9.93	70.21 ± 9.15	68.29 ± 11.34	0.314
CRE (μmol/L)	60.60 ± 7.30	61.12 ± 7.06	59.56 ± 7.77	0.291
UA (μmol/L)	282.75 ± 41.31	288.16 ± 43.18	272.09 ± 35.58	0.071
ALT (U/L)	26.84 ± 11.11	27.53 ± 11.40	25.47 ± 10.55	0.495
AST (U/L)	81.39 ± 14.97	80.69 ± 13.98	82.76 ± 16.88	0.490
LDL (mmol/L)	1.98 ± 0.74	2.08 ± 0.74	1.78 ± 0.73	0.097
K^+ (mmol/L)	4.26 ± 0.31	4.25 ± 0.33	4.29 ± 0.26	0.433
Na^+(mmol/L)	138.10 ± 2.50	138.01 ± 2.30	138.26 ± 2.90	0.977
BNP(pg/ml)	68.15 ± 30.06	66.07 ± 31.63	72.24 ± 26.69	0.163
Troponin I(ng/ml)	5.36 ± 3.31	5.20 ± 3.02	5.67 ± 3.85	0.736
QTc interval (ms)	426.42 ± 11.26	426.51 ± 10.47	426.24 ± 12.84	0.838
AGEs (μg/mL)	18.45 ± 2.00	15.30 ± 0.96	24.60 ± 1.92	< 0.001
PVCs/24 hours	5685.62 ± 2940.11	4638.40 ± 2419.98	7749.27 ± 2804.95	< 0.001

Continuous variables are presented as mean ± SD; categorical variables are presented as numbers or percentages.

Abbreviations: ALT, glutamic-pyruvic transaminase; AST, glutamic-oxalacetic transaminease. BMI, body mass index; BNP, brain natriuretic peptide; CRE, creatinine; LDL, low density lipoprotein; UA, uric acid; AGEs, advanced glycation end products; PVCs, premature ventricular contractions.

Electrocardiography (ECG) evaluation

Anasthetized rats were fixed in a supine position, the standard limb leads were attached to the upper and lower limbs of the rats and connected to Powerlab 4/25 Biological Analysis System (AD Instruments)

which recorded the ECG. Occurring amount of VAs, including ventricular premature beats and tachycardia were recorded.

Primary myocytes isolation

The primary myocytes were isolated from the hearts harvested from the rats in accordance with the protocol described in our previous study[2]. Specifically, the myocytes were carefully extracted from the adjacent area around infracted region in the anterior left ventricular wall under a dissecting microscope. Myocytes were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented fetal bovine serum (15%, FBS, Hyclone) and antibiotic mix (Sigma) at 37℃ in an atmosphere composed of 95% fresh air and 5% CO_2. When cell populations reached confluence at 50%-60%, the cells were used for subsequent experiments.

Immunofluorescent staining

The protocols were carried out in accordance with our previous investigation[10]. Cultured primary myocytes were fixed by 4% paraformaldehyde for 15 min and permeabilized by 0.2% Triton on a cover glass. After incubation with blocking buffer (Abcam), fixed cells were incubated with primary antibody against RyR2 (Abcam, 1:200) and primary antibody against FKBP12.6 (Abcam, 1:100) at 4℃ for 12 hours. Nuclei were tagged by 4,6-Diamidino-2-phenylindole (DAPI, Sigma Aldrich). After washing, Alexa-488 conjugated secondary antibody (Invitrogen) and Alexa-555 conjugated secondary antibody (Invitrogen) were used to tag RyR2 and FKBP12.6 respectively. Fluorescence quenching was alleviated by using Antifade Kit (Molecular Probes). Axio Imager 2 inverted microscope (Zeiss) was used to capture the fluorescent images after excited at 488 nm and 594 nm respectively. Zeiss Physiological software (Zeiss, ver3.2) was used to analyze the co-localizations of RyR2 and FKBP12.6 based on these images.

Calcium spark detection

The calcium spark was detected in isolated myocytes by confocal optical calcium imaging according to the methods described previsouly[21]. Isolated myocytes were loaded with 10 μmol/l Fluo3/AM (Sigma-Aldrich) for 30 minutes at 37℃ in a humidified dark chamber. After washed by PBS, calcium sparks were observed with a SP8 STED confocal microscope (Leica) equipped with an argon laser at wave length at 488 nm. Liner scan was used to acquire the line scan images (512 pixels/line) at sampling rate of 2 ms/line. The scanning frequency was 600 Hz. Calcium sparks were analyzed by Image J with SparkMaster Plugin according to previous described method[22].

Cytosol and ER membrane fraction preparation and Western blotting

Subfraction isolation protocols were carried out according to ours and others' previous descriptions[10, 23]. With Protein Extraction kits (Beyotime), proteins were extracted from cytosol preparation and ER membrane preparation according to the protocol provided by the manufacturer respectively. A BCA kit (Beyotime) was used to determine the protein concentrations of the samples which were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were

then transferred to polyvinylidene fluoride (PVDF) membranes. After treated with blocking buffer (Abcam), primary antibodies against GRP78 (Abcam, 1:4000), phosphorylated PERK (p-PERK, Cell Signaling Tech, 1:2000), PERK (Cell Signaling Tech, 1:2000), FKBP12.6 (Invitrogen, 1:2000), Sigma receptor 1 (SigmaR1, Invitrogen, 1:1000) and GAPDH (Abcam, 1:1000) were used to incubate the membranes at 4℃ for 12 hours. After TBST washing, the membranes were incubated by corresponding HRP conjugated secondary antibodies (Abcam) at room temperature for 2 h. After developed with Super Signal West Pico chemiluminescence reagent (Thermo Scientific), the immunobands were visualized on X-ray films and then analyzed with ImageJ2x software (Rawak Software).

[H^3]-ryanodine bind assay

[H^3]-ryanodine bind assay was used to assess the channel activity of RyR2 according to the protocol described previously[10]. Cell lysate were incubated with [H^3]-ryanodine solution (PerkinElmer) at final concentration of 20 nmol/L in binding buffer (25 mmol/L Tris, 50 mmol/L HEPES, 100 μmol/L CaCl2, 1 mmol/Lbenzamidine, 0.5 mmol/L phenylmethanesulfonyl fluoride, 2 μg/mlpepstain A, 2 μg/ml leupeptin and 2 μg/ml aprotinin) at 37℃ for 3 h. After washed with washing buffer (25 mmol/L Tris and 250 mmol/L KCl) and filtered with washing buffer- soaked membrane filter (Millipore), the radioactivity of the filter was detected by liquid scintillation counter (Bioscan) which represented the binding [^{3}H]-ryanodine.

Calcineurin (CaN) activity detection

The CaN enzymatic activity was determined in total protein samples with a colorimetric method according to the protocol described previously[10]. A Calcineurin Activity Assay kit (Merck) was used per manufacturer's instructions.

Statistics

Data acquired in this study were presented in (mean ± standard deviations) or percentage manner. Number of independent experiments carried out was indicated as n. NSK tests were performed as post-hoc tests. Participants were divided into 2 groups (diabetes and non-diabetes). The baseline characteristics among the 2 groups were analyzed by t test for parametric variables, the Mann-Whitney U test for nonparametric variables, and the chi-square test for categorical variables. The Spearman rank correlation coefficient was computed to assess correlation between continuous variables. The association between serum AGEs and frequent PVCs were estimated with univariate and multivariate logistic regression models. Model 1 was unadjusted. Model 2 was adjusted for age, sex, body mass index, cigarette smoking. Model 3 was adjusted for age, sex, body mass index, cigarette smoking, creatinine, UA, low density lipoprotein, K^+, Na^+, troponin I, BNP, QTC interval. All statistical testing was 2-sided. Results were considered statistically significant at a level of $P < 0.05$. All analyses were performed with PASW Statistics 20.0 software.

Results

Serum AGEs concentration elevation correlated with frequent PVCs in STEMI patients

Data were successfully obtained from 101 STEMI patients. The baseline data was listed in Table 2. STEMI patients were divided into diabetes group and non-diabetes group. The amount of 24 h- PVCs was significantly higher in diabetes group than non-diabetes group (Fig. 1a). Spearman rank correlation was used to analyze the association between 24-h PVCs amount and serum AGEs concentrations. Results showed that 24-h PVCs amount and serum AGEs concentrations was significantly and positively related ($r = 0.461$, $P < 0.001$) (Fig. 1b). The univariate and multivariate logistic regression models were used to further evaluate the association between frequent PVCs and serum AGEs concentration in STEMI patients. As demonstrated in Table 3, in univariable Cox regression model (Model 1), serum AGEs concentration was positively correlated with frequent PVCs (OR = 1.90, 95% CI 1.36–2.65, $P < 0.001$). Results of multivariate analysis suggested serum AGEs concentration was positively and independently correlated with frequent PVCs (adjusted OR = 1.93, 95% CI 1.29–2.90, $P = 0.001$) after adjusting for age, sex, body mass index, cigarette smoking(Model 2). The results were similar in Model 3 (adjusted OR = 1.86, 95% CI 1.09–3.18, $P = 0.022$) after adjusting for age, sex, body mass index, cigarette smoking, creatinine, UA, low density lipoprotein, K+, Na+,troponin I, BNP, and QTC interval.

Table 2
Univariate and multivariate logistic analysis as a continuous variable of AGEs concentration

	Model 1*		Model 2†		Model 3‡	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
STEMI (n = 101)	1.90(1.36–2.65)	< 0.001	1.93(1.29–2.90)	0.001	1.86(1.09–3.18)	0.022
* Model 1: unadjusted.						
† Model 2: multivariate adjustment was made for age, sex, body mass index, cigarette smoking.						
‡ Model 3: multivariate adjustment was made for age, sex, body mass index, cigarette smoking, creatinine, UA, low density lipoprotein, K+, Na+,troponin I, BNP, QTC interval.						
Abbreviations: STEMI, ST- segment elevation myocardial infarction; CI, confidence interval; OR, odds ratio.						

RAGE antibody administration attenuated AGEs-exacerbated ventricular arrhythmias in MI rats

As demonstrated in Fig. 2a, VAs were presented as PVCs and ventricular tachycardias. In some particular cases, bigeminal rhythm, polymorphic PVCs and paroxysmal ventricular tachycardia could be observed.

Amount of VAs increased significantly in MI rats compared with control, which was further dramatically increased by AGEs exposure. However, the RAGE antibody treatment significantly reduced VAs amount in AGEs-exposed MI rats (Fig. 2b).

RAGE blocking suppressed activation of ER stress-mediated PERK/CaN signaling in AGEs-exposed MI rats

As demonstrated in Fig. 3, we found that the expression level of GRP78, phosphorylation level of PERK and enzymatic activity of CaN increased significantly in MI compared with control. AGEs exposure further caused significant elevation of GRP78 expression level, PERK phosphorylation level as well as enzymatic activity of CaN in myocytes isolated from MI hearts, which were dramatically decreased by RAGE antibody administration.

RAGE antibody treatment impaired ER membrane- cytosol translocation of FKBP12.6 and its disassociation with RyR2

The co-localization of FKBP12.6 and RyR2 was evaluated by calculating the Pearson's correlation coefficients based on analyzing the captured fluorescent images. The coefficients could indicate the degree of contact between two detected molecules. As demonstrated in Fig. 4a, lowered co-localization level between FKBP12.5 and RyR2 was found in MI compared with control. AGEs exposure further dramatically impaired the co-localization between FKBP12.5 and RyR2 in MI hearts, which was improved by RAGE antibody administration. CaN facilitates the disassociation between RyR2 and FKBP12.6 which would translocate from ER membrane to cytosol. As demonstrated in Fig. 4b, compared with control, significantly promoted ER membrane- cytosol translocation of FKBP12.6 was found in MI. AGEs facilitated more ER membrane-to-cytosol translocation of FKBP12.6. The RAGE antibody treatment, however, reduced the ER membrane-to-cytosol translocation in MI hearts exposed to AGEs.

Administration of RAGE antibody alleviated calcium releasing by inhibiting RyR2 channel activity in AGEs-exposed MI hearts

As demonstrated in Fig. 6a, results from [^{3}H]-ryanodine binding assay indicated that the RyR2 channel activity increased significantly in myocytes isolated from MI hearts. The AGEs exposure was proved to further increase the RyR2 channel activity which was reduced by administration of RAGE antibody. The opening of RyR2 channel would result in calcium releasing from ER which was assessed by calcium sparks evaluations (Fig. 6b). Compared with control, promoted calcium sparks were found in MI. The AGEs exposure further promoted calcium sparks in myocytes isolated from MI hearts. The administration of RAGE antibody, however, dramatically attenuated calcium sparks in myocytes isolated from MI hearts exposed to AGEs.

Discussion

VAs are critical clinical manifestations of MI and contribute to the major adverse cardiovascular events (MACE). Indeed, MI rat model established in this study was characterized by increased amount of VAs. MACE was reported to be closely associated with AGEs content in high-risk patients with atherosclerotic cardiovascular diseases [24]. In the present study, our cohort analysis suggested that post-MI PVCs were more frequent in STEMI patients complicated with diabetes. More importantly, we found that serum AGEs concentration was significantly and independently associated with post-MI PVCs in STEMI patients. Thus, AGEs forged in diabetes could be possibly identified as the cause exacerbating post-MI VAs.

AGEs are sets of biochemical byproducts generated by non-enzymatic glycosylations *in vivo*, contributing to diabetic complications via interacting with its receptor RAGE located on the targeted cells[25]. As a member of immunoglobulin superfamily of cell surface receptors, RAGE is a trans-membrane protein involved in multiple cell functions including proliferation, redox adjustment, inflammation and migration[25]. An investigation suggesting the correlation between RAGE and VAs aroused our interest: RAGE knockdown by specific small interfering RNA (siRNA) effectively reduced VAs in cardiac ischemia and reperfusion model [26]. Moreover, it has been proved that ER stress activation was RAGE- dependent [27, 28]. Therefore, our hypothesis that AGEs could induce VAs through RAGE- dependent ER stress activation was formed and further testified in the current study.

Evidenced by GRP78 expression and PERK phosphorylation elevation, ER stress- mediated PERK signaling was activated in myocytes from MI rats which exhibited increased post-MI VAs. The AGEs administration resulted in further elevation of GRP78 expression and PERK phosphorylation, indicating the hyper-activation of ER stress- mediated PERK signaling in AGEs- exposed MI rats which were featured with exacerbated post-MI VAs. Similar to several previous works, we used anti-RAGE IgG_3 antibody to block AGEs-RAGE interactions [11]. Evidenced by decreased GRP78 expression and PERK phosphorylation, RAGE blockage alleviated ER stress- mediated PERK signaling hyper-activation, resulting in attenuation of post-MI VAs in MI rats exposed to AGEs. These results indicated that RAGE- dependent ER stress- mediated PERK signaling played a critical role in exacerbating post-MI VAs after AGEs exposure. As an ER membrane localized protein, phosphorylated PERK could increase enzymatic activity of its down-stream effecter CaN by direct contact [6, 10]. CaN would further promote the disassociation between RyR2 and its inhibitor FKBP12.6 on ER membrane to facilitate RyR2 channel opening [23]. Our data showed that RAGE blockage reduced CaN activity, leading to impaired disassociation between FKBP12.6 and RyR2 which was evidenced by reduced ER membrane-to-cytosol translocation behavior of FKBP12.6. As a result, RyR2 channel activity was compromised.

Calcium released through RyRs from ER raised calcium concentration in subspace between plasma membrane and ER membrane, which is visualized as calcium sparks. It has been well established that DADs are mediated by ER calcium release[29]. DADs are voltage oscillations at diastole period causing triggered activity (TA) which is manifested as arrhythmias, typically PVCs. We found that due to elevated RyR2 channel activity, calcium sparks were enhanced significantly in a spatio-temporal manner in myocytes isolated from MI hearts, which was further boosted by AGEs exposure. The RAGE blockage

effectively suppressed calcium sparks in myocytes isolated from AGEs-exposed MI hearts. These results further supported our theory that AGEs mediated ER RyR2 calcium releasing via RAGE.

Conclusion

Notably, we also found that ER stress was also activated in MI heart not exposed to AGEs. This phenomenon we observed was in consistence with the established opinions [30]. We innovatively reported ER stress was hyper-activated via RAGE/ROS pathway in MI heart exposed to AGEs which made MI individuals more vulnerable to VAs. Our further investigation suggested AGEs-induced hyper-activation of PERK/CaN/RyR2 signaling was the underlying mechanism of exacerbated VAs. Taken together, to some extent, the data we acquired in the present study interpreted the clinical scenario that post-MI VAs are more frequent in patients with diabetes.

Abbreviations

AGEs	advanced glycation end products
MI	myocardial infarction
VAs	ventricular arrhythmias
PVCs	premature ventricular contractions
STEMI	ST segment elevated myocardial infarction
LAD	left anterior descending artery
RAGE	receptor for AGEs
FKBP12.6	FK506-bindingprotein 12.6
RyR2	ryanodine receptor 2
CaN	calcineurin
PERK	protein kinase RNA-like ER kinase
GRP78	glucose regulated protein 78
ER	endoplasmic reticulum
DADs	delayed afterdepolarizations

Declarations

Ethics approval and consent to participate

All of the patients gave informed consent to participate in this study, which was approved by the ethics committee of Shaanxi Provincial Peoples' Hospital. Specifically, patients signed the informed consent and agreed the collection and research purposes of medical records for our investigation.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

Original data and materials were available on reasonable requests.

Competing interests

None

Funding

The study was supported by Health Research Foundation of Shaanxi Province (2018E011); Innovative Talents Promotion Project of Shaanxi Province (2019KJXX-019).

Authors' contributions

Zhongwei Liu and Haitao Zhu implemented experiments and wrote the manuscript; Chuan Qiu and Yuan Wang accomplished statistic analysis; Shuo Pan, Hao Jia and Yong Zhang participated in implementing experiments; Zhongwei Liu and Yuan Wang reviewed and revised the manuscript.

Acknowledgements

None

References

1. Kromhout D, Geleijnse JM, de Goede J, Oude Griep LM, Mulder BJM, de Boer M-J, Deckers JW, Boersma E, Zock PL, Giltay EJ. n-3 fatty acids, ventricular arrhythmia-related events, and fatal myocardial infarction in postmyocardial infarction patients with diabetes. Diabetes Care. 2011;34(12):2515–20.
2. Liu Z-W, Zhu H-T, Chen K-L, Dong X, Wei J, Qiu C, Xue J-H. Protein kinase RNA-like endoplasmic reticulum kinase (PERK) signaling pathway plays a major role in reactive oxygen species (ROS)-mediated endoplasmic reticulum stress-induced apoptosis in diabetic cardiomyopathy. Cardiovascular diabetology. 2013;12:158–8.

3. Ajith TA, Vinodkumar P. Advanced Glycation End Products: Association with the Pathogenesis of Diseases and the Current Therapeutic Advances. Curr Clin Pharmacol. 2016;11(2):118–27.
4. Chang G-J, Yeh Y-H, Chen W-J, Ko Y-S, Pang J-HS, Lee H-Y. Inhibition of Advanced Glycation End Products Formation Attenuates Cardiac Electrical and Mechanical Remodeling and Vulnerability to Tachyarrhythmias in Diabetic Rats. J Pharmacol Exp Ther. 2019;368(1):66–78.
5. Monnerat G, Alarcón ML, Vasconcellos LR, Hochman-Mendez C, Brasil G, Bassani RA, Casis O, Malan D, Travassos LH, Sepúlveda M, et al. Macrophage-dependent IL-1β production induces cardiac arrhythmias in diabetic mice. Nature communications. 2016;7:13344–4.
6. Scoote M, Williams AJ. The cardiac ryanodine receptor (calcium release channel): emerging role in heart failure and arrhythmia pathogenesis. Cardiovascular research. 2002;56(3):359–72.
7. Marchi S, Patergnani S, Missiroli S, Morciano G, Rimessi A, Wieckowski MR, Giorgi C, Pinton P. Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. Cell calcium. 2018;69:62–72.
8. Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. Annu Rev Pathol. 2015;10:173–94.
9. Yuan M, Meng X-W, Ma J, Liu H, Song S-Y, Chen Q-C, Liu H-Y, Zhang J, Song N, Ji F-H, et al. Dexmedetomidine protects H9c2 cardiomyocytes against oxygen-glucose deprivation/reoxygenation-induced intracellular calcium overload and apoptosis through regulating FKBP12.6/RyR2 signaling. Drug Des Devel Ther. 2019;13:3137–49.
10. Liu Z, Cai H, Zhu H, Toque H, Zhao N, Qiu C, Guan G, Dang Y, Wang J. Protein kinase RNA-like endoplasmic reticulum kinase (PERK)/calcineurin signaling is a novel pathway regulating intracellular calcium accumulation which might be involved in ventricular arrhythmias in diabetic cardiomyopathy. Cellular signalling. 2014;26(12):2591–600.
11. Yan D, Luo X, Li Y, Liu W, Deng J, Zheng N, Gao K, Huang Q, Liu J. Effects of advanced glycation end products on calcium handling in cardiomyocytes. Cardiology. 2014;129(2):75–83.
12. Luo Y, Li S-J, Yang J, Qiu Y-Z, Chen F-P. HMGB1 induces an inflammatory response in endothelial cells via the RAGE-dependent endoplasmic reticulum stress pathway. Biochem Biophys Res Commun. 2013;438(4):732–8.
13. Chen W, Chan Y, Wan W, Li Y, Zhang C. Aβ(1–42) induces cell damage via RAGE-dependent endoplasmic reticulum stress in bEnd.3 cells. Exp Cell Res. 2018;362(1):83–9.
14. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, Caforio ALP, Crea F, Goudevenos JA, Halvorsen S, et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). European heart journal. 2018;39(2):119–77.
15. Davies MJ, D'Alessio DA, Fradkin J, Kernan WN, Mathieu C, Mingrone G, Rossing P, Tsapas A, Wexler DJ, Buse JB. Management of Hyperglycemia in Type 2 Diabetes, 2018. A Consensus Report by the

American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetes Care. 2018;41(12):2669–701.

16. Park K-M, Im SI, Park S-J, Kim JS, On YK. Risk factor algorithm used to predict frequent premature ventricular contraction-induced cardiomyopathy. Int J Cardiol. 2017;233:37–42.
17. Liu Z, Wang Y, Zhu H, Qiu C, Guan G, Wang J, Guo Y. Matrine blocks AGEs- induced HCSMCs phenotypic conversion via suppressing Dll4-Notch pathway. Eur J Pharmacol. 2018;835:126–31.
18. Zhao N, Mi L, Zhang X, Xu M, Yu H, Liu Z, Liu X, Guan G, Gao W, Wang J. Enhanced MiR-711 transcription by PPARgamma induces endoplasmic reticulum stress-mediated apoptosis targeting calnexin in rat cardiomyocytes after myocardial infarction. J Mol Cell Cardiol. 2018;118:36–45.
19. Grauballe MB, Østergaard JA, Schou S, Flyvbjerg A, Holmstrup P. Blockade of RAGE in Zucker obese rats with experimental periodontitis. J Periodontal Res. 2017;52(1):97–106.
20. Yang D, Liu W, Ma L, Wang Y, Ma J, Jiang M, Deng X, Huang F, Yang T, Chen M. Profilin–1 contributes to cardiac injury induced by advanced glycation end–products in rats. Mol Med Rep. 2017;16(5):6634–41.
21. Tjondrokoesoemo A, Li N, Lin P-H, Pan Z, Ferrante CJ, Shirokova N, Brotto M, Weisleder N, Ma J. Type 1 inositol (1,4,5)-trisphosphate receptor activates ryanodine receptor 1 to mediate calcium spark signaling in adult mammalian skeletal muscle. J Biol Chem. 2013;288(4):2103–9.
22. Picht E, Zima AV, Blatter LA, Bers DM. SparkMaster: automated calcium spark analysis with ImageJ. American journal of physiology Cell physiology. 2007;293(3):C1073–81.
23. Liao B, Zheng Y-M, Yadav VR, Korde AS, Wang Y-X. Hypoxia induces intracellular Ca2 + release by causing reactive oxygen species-mediated dissociation of FK506-binding protein 12.6 from ryanodine receptor 2 in pulmonary artery myocytes. Antioxid Redox Signal. 2011;14(1):37–47.
24. Cavero-Redondo I, Soriano-Cano A, Álvarez-Bueno C, Cunha PG, Martínez-Hortelano JA, Garrido-Miguel M, Berlanga-Macías C, Martínez-Vizcaíno V: **Skin Autofluorescence-Indicated Advanced Glycation End Products as Predictors of Cardiovascular and All-Cause Mortality in High-Risk Subjects**: **A Systematic Review and Meta-analysis**. *Journal of the American Heart Association* 2018, **7**(18):e009833-e009833.
25. Fukami K, Yamagishi S-I, Okuda S. Role of AGEs-RAGE system in cardiovascular disease. Curr Pharm Design. 2014;20(14):2395–402.
26. Park H, Ku SH, Park H, Hong J, Kim D, Choi B-R, Pak H-N, Lee M-H, Mok H, Jeong JH, et al. RAGE siRNA-mediated gene silencing provides cardioprotection against ventricular arrhythmias in acute ischemia and reperfusion. J Control Release. 2015;217:315–26.
27. Wang B, Cai Z, Liu B, Liu Z, Zhou X, Dong N, Li F. RAGE deficiency alleviates aortic valve calcification in ApoE(-/-) mice via the inhibition of endoplasmic reticulum stress. Biochimica et biophysica acta Molecular basis of disease. 2017;1863(3):781–92.
28. Liu J, Huang K, Cai G-Y, Chen X-M, Yang J-R, Lin L-R, Yang J, Huo B-G, Zhan J, He Y-N. Receptor for advanced glycation end-products promotes premature senescence of proximal tubular epithelial cells

via activation of endoplasmic reticulum stress-dependent p21 signaling. Cellular signalling. 2014;26(1):110–21.

29. Wasserstrom JA, Shiferaw Y, Chen W, Ramakrishna S, Patel H, Kelly JE, O'Toole MJ, Pappas A, Chirayil N, Bassi N, et al. Variability in timing of spontaneous calcium release in the intact rat heart is determined by the time course of sarcoplasmic reticulum calcium load. Circulation research. 2010;107(9):1117–26.
30. Bozi LHM, Takano APC, Campos JC, Rolim N, Dourado PMM, Voltarelli VA, Wisløff U, Ferreira JCB, Barreto-Chaves MLM, Brum PC: **Endoplasmic reticulum stress impairs cardiomyocyte contractility through JNK-dependent upregulation of BNIP3**. *International journal of cardiology* 2018, **272**:194–201.

Figures

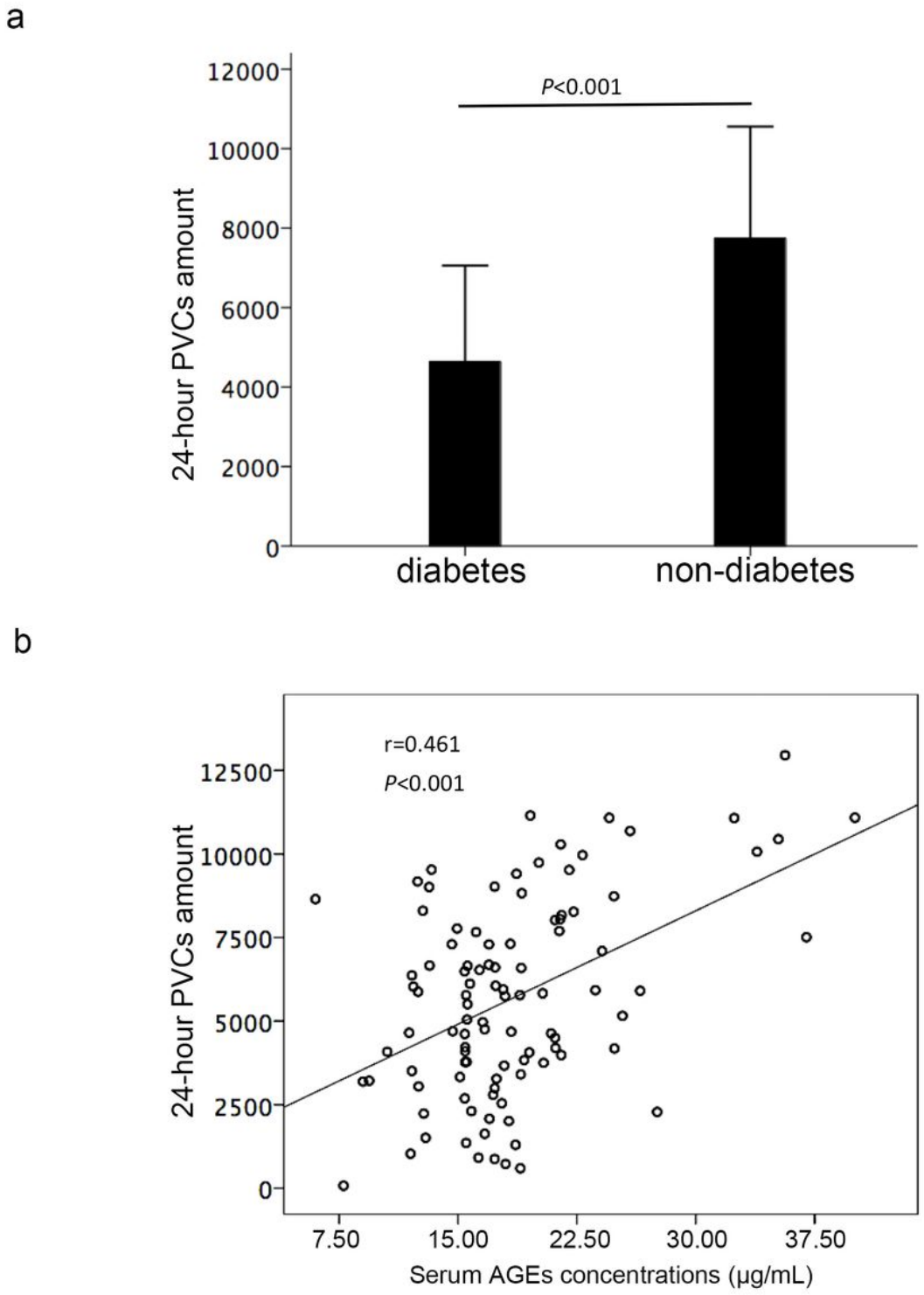

Figure 1

a columns indicated the amount of premature ventricular contractions (PVCs) during 24-hour recording in STEMI patients complicated with or without diabetes. b the correlation analysis between serum AGEs concentrations and 24-hour PVCs amount in STEMI patients. (n=101)

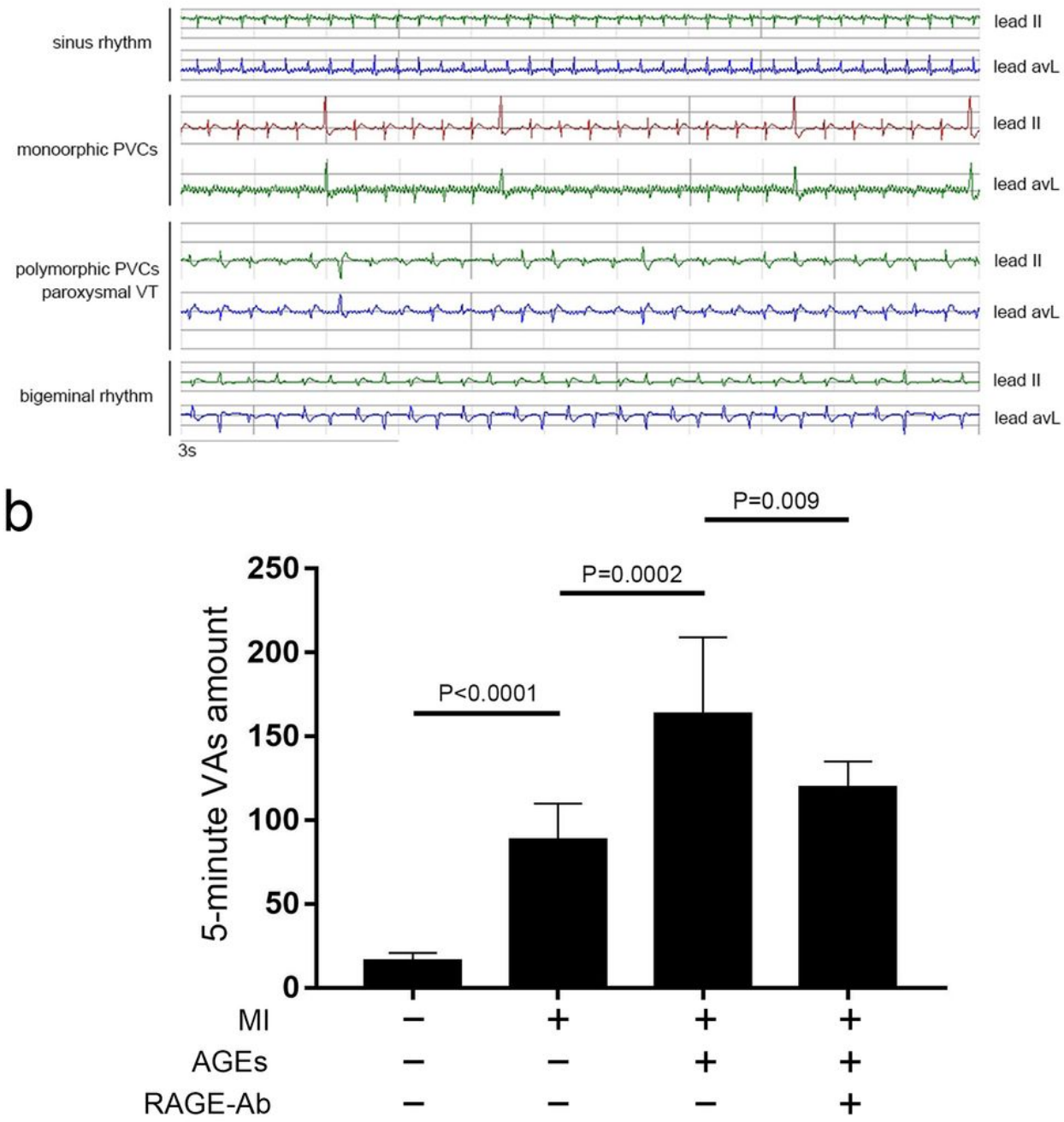

Figure 2

a ECG recorded patterns of ventricular arrhythmias in rats. b columns indicated amounts of ventricular arrhythmias (VAs) during 5-minute recording in rats. (n=10)

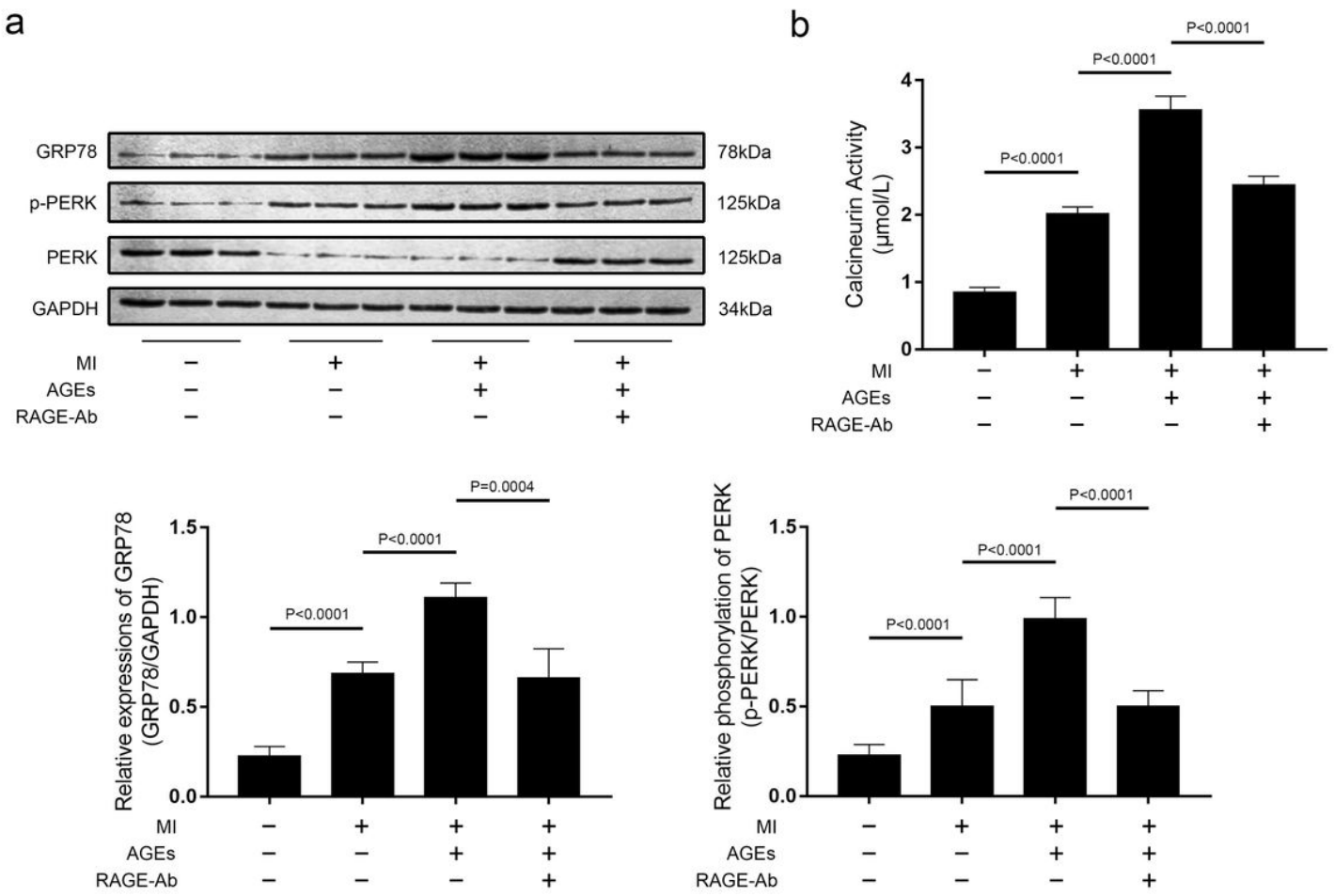

Figure 3

a Demonstrated the immunoblots of GRP78, phosphorylated PERK (p-PERK), PERK and GAPDH in myocytes isolated from rats. Columns indicated the relative expression levels of GRP78 and phosphorylation levels of PERK in isolated myocytes. b Columns indicated the detected enzymatic activity of calcineurin (CaN) in myocytes isolated from rats. (n=6)

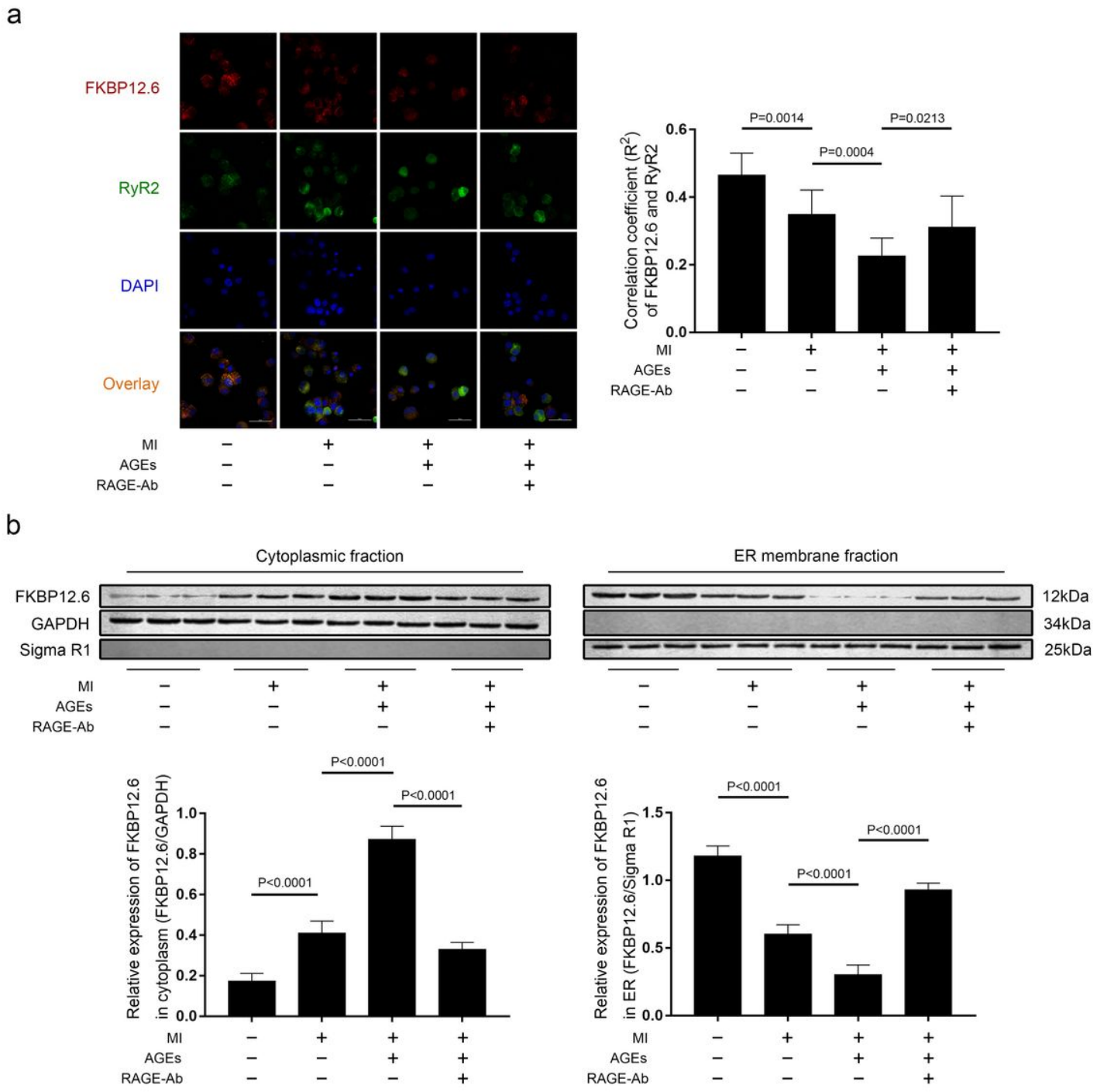

Figure 4

a captured images of immune-fluorescent staining FKBP12.6, RyR2 and DAPI of isolated myocytes. Columns indicated the quantification of the degree of colocalization of FKBP12.6 and RyR2 by calculating coefficients analysis. b immunoblots of FKBP12.6, GAPDH and Sigma R1 extracted from cytoplasmic fraction and ER membrane fraction respectively. Columns below indicated the relative expression levels of FKBP12.6 in cytolasmic and ER membrane fractions in isolated myocytes. (n=6)

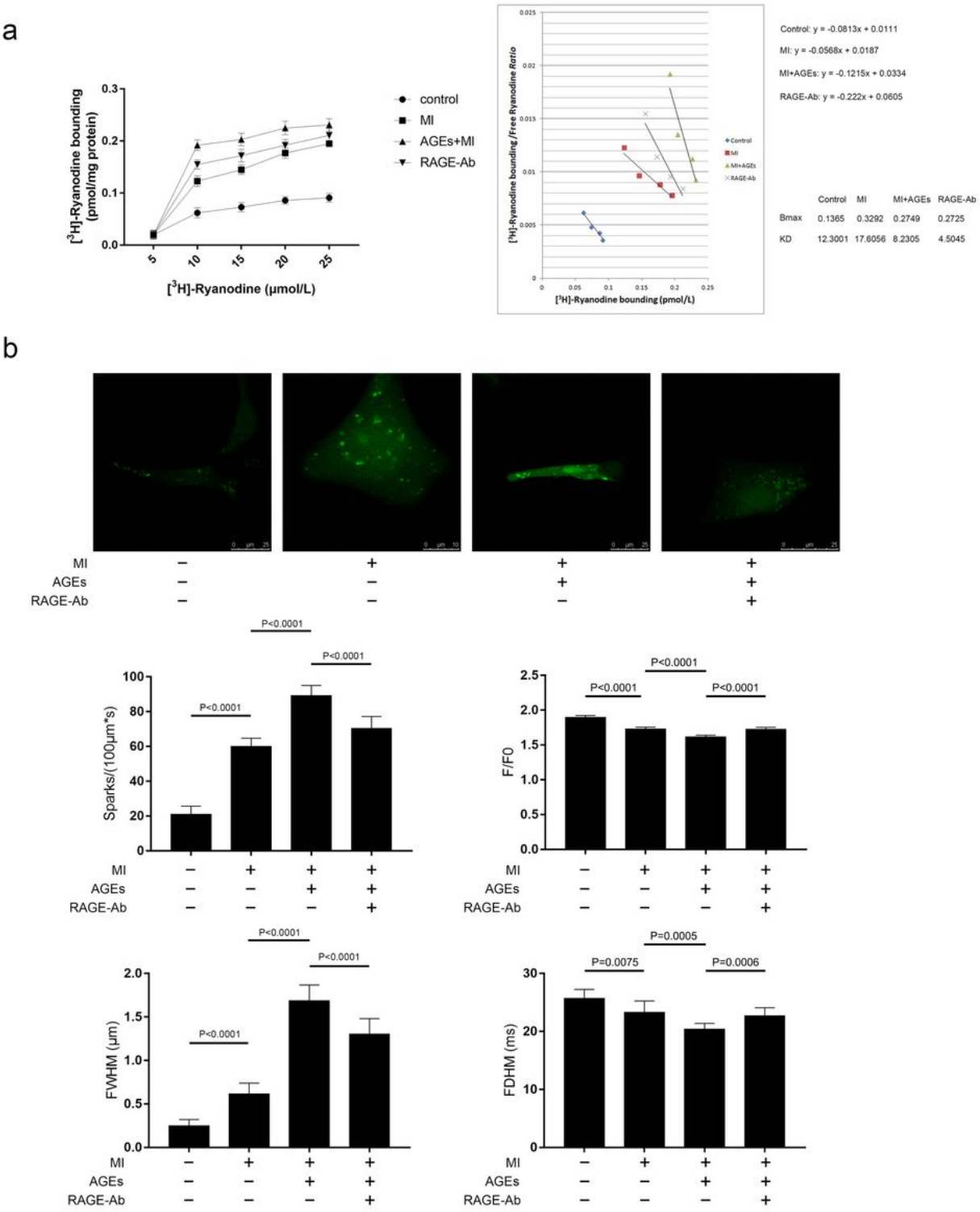

Figure 5

a The left chart represented the results of [3H]-ryanodine binding assay was conducted in cell lysate from isolated myocytes. Chart on the right side demonstrated the results of Scatchard analysis of [3H]-ryanodine binding assay. Equation of each group was indicated. Bmax and dissociation constant (KD) were calculated according to equation of each group. b Captured images of calcium sparks were demonstrated. Columns indicated the incidence of calcium sparks, spark amplitude (F/F0), full width at

half maximum (FWHM) and the full duration of half maximum (FDHM) in isolated myocytes respectively. (n=6)