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Electrophysiological basis of metabolic syndrome-induced cardiac dysfunction

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Abstract

Myocardial contractility is controlled by intracellular Ca^{2+} -cycling with the contribution of sarcoplasmic reticulum (SR). In this study we aimed to investigate the role of altered SR function in defective regulation of intracellular Ca^{2+} levels in rats with metabolic syndrome (MetS) induced by a 16-week high-sucrose drinking-water diet. Electric-field stimulated transient intracellular Ca^{2+} -changes in MetS-cardiomyocytes exhibited significantly reduced amplitude (~30%) and prolonged time courses (2-fold) as well as depressed SR- Ca^{2+} loading (~55%) with increased basal Ca^{2+} level. Consistent with these data, altered ryanodine receptor (RyR2) function and SERCA2a activity were found in MetS-cardiomyocytes through Ca^{2+} spark measurements and caffeine application assay in a state in which sodium calcium exchanger was inhibited. Furthermore, tetracaine application-assay results and hyperphosphorylated level of RyR2 also support the “leaky RyR2” hypothesis. Moreover, altered phosphorylation levels of phospholamban (PLN) support the depressed SERCA2a-activity thesis and these alterations in the phosphorylation of Ca^{2+} -handling proteins are correlated with altered protein kinase and phosphatase activity in MetS-cardiomyocytes. In conclusion, MetS-rat heart exhibits altered Ca^{2+} signaling largely due to altered SR function via changes in RyR2 and SERCA2a activity. These results point up RyR2 and SERCA2a as potential pharmacological targets for restoring intracellular Ca^{2+} -homeostasis and thereby combatting dysfunction in MetS-rat heart.

Key words: SERCA, heart function, calcium sparks, calcium transients, insulin resistance, high sucrose diet, diabetes.

Introduction

Metabolic syndrome (MetS) is becoming a world-wide matter for every risk factors for diseases such as diabetes and pre-diabetes, abdominal obesity, high cholesterol and high blood pressure that mostly induce platform for several cardiac disorders (Grundy et al. 2004). This syndrome includes also glucose intolerance and insulin resistance with or without obesity. All these anomalies associated with MetS are mostly leading to the loss of cardiomyocytes, cardiac dysfunction and ultimately heart failure (Hansen 1999; Kassi et al. 2011; Matsuzawa et al. 2011). It should be also very important to emphasize the fact of the significant differences in parameters of cardiac structure and the left ventricular function of the patients with MetS compared to those of patients with diabetes although underlying reasoning is not clear yet (Suh and Lee 2014).

Insulin resistance is one of the important risk factors for several pathological conditions including hypertension (Guo et al. 2005; Xi et al. 2004), and type-2 diabetes (Fontes-Carvalho et al. 2015; Lee and McDonald 2015; Lopez-Jaramillo et al. 2014; Streja 2004) while it even predicts coronary heart disease in either adult or elderly non-diabetic subjects (Kim et al. 2013; Lempiainen et al. 1999). Furthermore, it has been demonstrated that high dietary sucrose triggers hyperinsulinemia, increases myocardial β -oxidation, reduces glycolytic flux and delays post-ischemic contractile recovery. In a clinical study, it had been evaluated the association between left ventricle diastolic dysfunction with insulin resistance, MetS and diabetes, across the diabetic continuum and demonstrated that the changes in the diastolic function were already present before the onset of diabetes, being mainly associated with the state of insulin resistance (Fontes-Carvalho et al. 2015). Furthermore, in another clinical study, authors examined the role of insulin resistance versus hyperglycemia in the risk for coronary heart disease and explore the interaction of insulin resistance with hyperglycemia. Interestingly, their data show that insulin resistance to be a greater risk for

coronary heart disease than diabetes, indicating how non-diabetics can have a higher risk for coronary heart disease than diabetics when insulin resistance is elevated (Kim et al. 2013).

Since myocardial contractility is primarily controlled by Ca^{2+} -cycling into/out of cytoplasm and sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2a) plays important role for its removal following muscular contraction, in here, we aimed to show a possible contribution of altered SERCA2a function to defective regulation of Ca^{2+} -cycling in cardiomyocytes from MetS-rats with defective cardiac contractility.

Research Design and Methods

Animals

Metabolic syndrome (MetS) was induced in the rats as described previously (Okatan EN 2015). Shortly, male Wistar rats were fed with drinking water containing 32% sucrose besides their standard chow ad libitum daily. Following 16-week feeding, MetS in animals were validated by using oral glucose tolerance test, blood glucose and serum insulin levels and the development of insulin resistance was determined by the HOMA index. The MetS group exhibited significant weight-gain (~20%) compared to that of age-matched control group (Okatan EN 2015). Additionally, the serum triglyceride level of MetS group was significantly higher than of the control group.

The experimental protocol and handling of animals during experiments were approved by the Ankara University ethics committee (No: 2012-5-35).

Contractile activity of papillary muscle strips

Papillary muscle strips with similar sizes were isolated from left ventricles and placed in a chamber and pinned down from one end with a stimulating electrode and the second end was connected to a force-displacement transducer (FT-03, Grass Instruments) connected to a preamplifier (P16, Grass Instruments). Recording chamber was perfused with Krebs solution

(as mM: 119 NaCl, 4.8 KCl, 1.8 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 20 NaHCO₃, and 10 glucose with pH 7.4) gassed with 95% O₂ – 5% CO₂ and maintained at 37 °C. The muscle strips were stimulated by using rectangular electrical pulses with 3-ms duration at a frequency of 0.2 Hz (S48, Grass Instruments). Twitch activities of the strips were transferred to a PC through an A/D converter and evaluated by a homemade-programme. Spatio-temporal parameters of twitch activity such as peak tension (contraction), time to peak twitch amplitude (TP), and time to half relaxation from peak twitch activity (DT₅₀) were measured.

Cardiomyocyte isolation

In all groups, cardiomyocytes from rat hearts were isolated as described previously (Turan et al. 1997). Briefly, the heart was first perfused at 37°C with a Ca²⁺-free, HEPES-buffered solution containing (in mM); NaCl 123, KCl 5.4, NaHCO₃ 5, NaH₂PO₄ 2, MgCl₂ 1.6, glucose 10, taurine 20, HEPES 20, and bubbled with 100% O₂. The pH was adjusted to 7.1 with NaOH. After 5 min, fresh buffer supplemented with 1-1.3 mg/ml collagenase (Type A, Boehringer) was perfused (6-8 mL/min) for 20-30 min. The heart was then removed from the perfusion system and apex was cut off and stirred to disperse the cardiomyocytes. The cells were then suspended in HEPES buffer with 1 mM Ca²⁺ and 0.5 % bovine serum albumin (pH 7.4). Cardiomyocytes were kept at 37°C until used for experiments in the same day. For our experiments, we only used Ca²⁺ tolerant rod-shaped cells.

Measurement of cytosolic transient Ca²⁺ changes

Freshly isolated cardiomyocytes were loaded with the fluorescent Ca²⁺ indicators Fura-2 (4-μM Fura-2 AM). Transient global cytosolic free Ca²⁺ changes under electric-field stimulation were measured from Fura-2 loaded cardiomyocytes at room temperature (21±2°C). Fluorescence was recorded using micro-spectrophotometer and FELIX software (Photon Technology International, Inc., NJ USA). Cells were sequentially excited at 340 nm/380 nm

and emission was measured at 510 nm under electric-field stimulation with electrical pulses with 10-ms duration, at a frequency of 0.2 Hz. The ratio of the fluorescence at 340 nm excitation to the fluorescence at 380 nm was calculated and peak amplitudes of fluorescence changes (difference between basal and peak $F_{340/380}$) used as an indicator of cytosolic free Ca^{2+} changes were determined from Ca^{2+} transients evoked by electrical-field stimulation. Background fluorescence measured from a cell-free field was subtracted from all recordings prior to calculation of the fluorescence ratios.

Measurement of SERCA function and Ca^{2+} leak from ryanodine receptors

SERCA2-mediated Ca reuptake was evaluated in isolated cardiomyocytes as described previously (Bassani et al. 1992). Briefly, after field stimulation, Fura-2 AM loaded cells were perfused with modified Tyrode solution ($0\text{Na}^+/0\text{Ca}^{2+}$, $[\text{Na}(0)\text{Ca}(0)]$; NaCl and CaCl_2 replaced with equimolar NMDG or 10 mmol/L EGTA, respectively) and then cells were stimulated with a brief pulse of 10 mmol/L caffeine for 1-s to induce sarcoplasmic reticulum (SR) Ca^{2+} release. It is known that (Bassani et al. 1992), $\text{Na}^+/\text{Ca}^{2+}$ exchanger, NCX is disabled in nominally Na^+ and Ca^{2+} free solution thus increased $[\text{Ca}^{2+}]_i$ is primarily declined by SERCA2 function. The $[\text{Ca}]_i$ measured in freshly isolated cardiomyocytes loaded with Fura-2 AM in the presence of tetracaine and $[\text{Na}(0)\text{Ca}(0)]$ containing modified Tyrode solution. Following the pre-pulses in 1.8 mM Ca^{2+} containing bath solution, the solution switch to tetracaine (1 mmol/L) containing nominally $[\text{Na}(0)\text{Ca}(0)]$ solution and decrease in the basal fluorescence intensity was evaluated as Ca^{2+} leak from ryanodine receptors. The results were normalized to corresponding caffeine responses to compare each datum (Curran et al. 2007).

Ca^{2+} -sparks measurement

Cardiomyocytes were set into the recording chamber set the stage of an inverted microscope equipped with a laser scanning confocal microscope (200-M, LSM-Pascal, Zeiss, Germany). In the experiments, 40X oil immersion objectives (NA 1.3) were used for imaging

cardiomyocytes located over cover glass base of the recording chamber. A 488-nm laser line from an argon laser (25-mW) was used to excite Fluo-3 (AM) and the emitted fluorescence was collected at 505 nm. Intracellular free Ca^{2+} changes in the isolated cells as changes in the fluorescence intensity were recorded with line-scan mode (spatial [x], vs. temporal [t], 1.9 ms/line) in the confocal microscopy. To prevent photo-bleaching and cell damage, laser line was kept 4-6 % of maximal intensity and the confocal pinhole was set to 1-1.5 airy units to achieve the best resolution and emission intensity.

Image analysis was performed using LSM Image-examiner, as described previously (Yaras et al. 2005). The fluorescence intensity of the images was calculated by averaging pixels comparing potential spark areas and then $\Delta F/F_0$ image was created. The Ca^{2+} sparks were manually detected and converted to temporal lines by averaging fluorescence intensity of 2-3 pixels aligning the peak of fluorescence intensity over time. The signals were filtered using Butterworth digital filter. Then the temporal profiles were fitted to gamma-function to analyze the spatial and time course of the fluorescence changes such as the peak amplitude of fluorescence intensity, the time to peak intensity, TP and the decay time to half-maximum of the intensity, DT_{50} .

Western blot analysis

Western blot analysis was performed to determine the relative expression or phosphorylation levels of RyR2 (pRyRSer²⁸⁰⁸), FKBP12.6, SERCA2a, PLN (pPLNSer¹⁶), PKA (pPKAThr¹⁹⁸) and PP1 (pPP1Thr³²⁰) in high sucrose-induced MetS-rat hearts. For preparation of tissue homogenates, frozen heart-samples from left ventricle were crushed at liquid N₂ temperature and homogenized in ice cold lysis buffer as described previously (Okatan et al. 2015). The membranes were then incubated overnight with the antibodies against on autoradiograms as protein bands were visualized using the ECL plus detection system.

Chemicals, data analysis and statistics

Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All antibodies were obtained from Santa Cruz Biotechnology (USA). Goat anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (USA). Molecular weight markers and PVDF membranes were purchased from Bio-Rad. ECL plus was purchased from GE healthcare.

The results are expressed as means $\pm$ SEM. Statistical significance was evaluated by one-way ANOVA followed by Tukey post-test. The probability level of $p < 0.05$ was considered statistically significant.

Results

Effect of high sucrose-intake on mechanical derangement of papillary muscle strips

Isolated papillary muscle strips from metabolic syndrome (MetS)- and control (CON)-groups were contracted regularly by electrical stimulation and representative twitch traces under isometric condition recorded at 37°C (± 2) are given in Fig. 1A (inset) ($n_{\text{CON}}=11$, $n_{\text{MetS}}=14$). Bar-graphs show the effect of MetS on the amplitude of peak tension (maximum contractile performance; left), the time to peak twitch amplitude (TP) and the time to half relaxation from peak twitch activity (DT_{50}) (right). Metabolic syndrome induced marked depression in the peak tension (152 ± 20 mg) together with very significant slowing down in both TP (103 ± 6 ms) and DT_{50} (76 ± 4 ms) compared to those of controls (291 ± 29 mg, 83 ± 4 ms and 60 ± 3 ms, respectively).

Effect of high sucrose-intake on Ca^{2+} handling in isolated cardiomyocytes

In literature, several previous studies demonstrated existence of cardiac dysfunction via defective Ca^{2+} handling in different MetS models in animals (Balderas-Villalobos et al. 2013; Ceylan-Isik et al. 2013; Davidoff et al. 2004; Mellor et al. 2012). To evaluate whether this

relationship exists in our MetS-model rats, we first recorded global Ca^{2+} transients in electrical field-stimulated and Fura-2 AM loaded left ventricular cardiomyocytes from MetS- and CON-rats by using micro-spectrofluorescence microscopy. Fig. 1B shows representative fluorescence images and the corresponding time-dependent Ca^{2+} transient profiles elicited by electrical stimulation in cardiomyocytes under basal conditions from both groups (cell number, $n_{\text{CON}}=24$, $n_{\text{MetS}}=28$). The original representative fluorescence changes recorded at room temperature ($22\pm 2^\circ\text{C}$) are given in Fig. 1B (inset). The maximum amplitude of Ca^{2+} transients (Fig. 1B, left) was significantly lower in MetS-group ($\Delta F_{340/380} = 0.19\pm 0.02$ a.u.) than in CON-group ($\Delta F_{340/380} = 0.28\pm 0.02$ a.u.). Additionally, we analyzed the time course of fluorescence changes under electrical-stimulation with parameters such as TP and DT_{50} . The DT_{50} values of electrically-stimulated Ca^{2+} transients were found to be markedly slow in MetS-group (0.37 ± 0.02 s vs. 0.59 ± 0.02 s) than in CON-group (0.25 ± 0.02 s vs. 0.42 ± 0.02 s) (Fig. 1B, right). Therefore, overall our average data associated with global transient Ca^{2+} changes in MetS-rats point out a presence of defective SR function, at least, due to less Ca^{2+} release as well as slowed down in cytosolic Ca^{2+} release and removal from SR. These data are consistent with previous studies with different MetS models, as well (Balderas-Villalobos et al. 2013; Ceylan-Isik et al. 2013; Davidoff et al. 2004; Dutta et al. 2001; Hintz et al. 2003; Wold et al. 2005).

Characterization of cellular mechanisms underlying cardiomyocyte dysfunction in MetS: Evaluation of SR Ca^{2+} content

As we and others have already shown, previously (Okatan EN 2015; Okatan et al. 2015; Vasanji et al. 2006; Wold et al. 2005), herewithin, we have demonstrated that the marked heart dysfunction in both tissue and cellular levels including papillary muscle strips and isolated cardiomyocytes, developed in MetS rats (Fig. 1A and B). To characterize the cellular mechanisms underlying cardiac dysfunction in rats with MetS, we first measured the Fura-2

fluorescence values for MetS- and CON-groups to demonstrate the increased basal levels of cytosolic Ca^{2+} . As shown in Fig. 2B (left), under basal conditions in resting cells at room temperature ($22 \pm 2^\circ\text{C}$), the intensity of fluorescence ratio ($\Delta F_{340/380}$) was slightly but significantly higher in MetS-group (0.39 ± 0.01 a.u.) compared to that of CON-group (0.34 ± 0.02 a.u.) ($n_{\text{CON}}=16$ and $n_{\text{MetS}}=14$).

To explore the contribution of these above possibilities to defective SR function, we first focused on to investigate the effect of MetS on SR Ca^{2+} content by measuring (at $22 \pm 2^\circ\text{C}$) the amplitude of caffeine-induced Ca^{2+} transients in cardiomyocytes from MetS- and CON-group, due to the fact on the amplitude of the caffeine-induced Ca^{2+} transient as an index of the SR Ca^{2+} content. As shown in Fig. 2B (right), under basal conditions, the amplitude of caffeine-induced (10 mM) Ca^{2+} transients (as $\Delta F_{340/380}$) was significantly smaller in MetS-group (0.43 ± 0.03 a.u.) than CON-group (0.54 ± 0.03 a.u.; left). Additionally, the time courses of caffeine responses (as fluorescence intensity changes; Fig. 2C) in terms of TP and DT_{50} values were significantly prolonged in MetS-group (1.05 ± 0.09 s and 3.65 ± 0.39 s; left and right, respectively) than CON-group (0.75 ± 0.05 s and 2.38 ± 0.17 s) ($n_{\text{CON}}=16$ and $n_{\text{MetS}}=14$). Representative fluorescence changes for the groups are given in Fig. 2A.

Depressed SERCA2a activity in cardiomyocytes from MetS-rats

Amplitude of the caffeine-induced Ca^{2+} transient is an index of the SR Ca^{2+} content, especially in nominally Na^+ and Ca^{2+} free solution ($[\text{Na}(0)\text{Ca}(0)]$) solution, where $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) is inhibited (Dutta et al. 2002). On the other hand, it has been shown that SR Ca^{2+} -ATPase (SERCA2a) abnormalities have important role on decreased cytosolic Ca^{2+} removal assessed in cardiomyocytes by caffeine-induced Ca^{2+} transients, which were evoked by a rapid caffeine-application under prevention of NCX activity (Dutta et al. 2002). From these already known facts, in here, we aimed to evaluate role of NCX activity in SR for Ca^{2+} handling of cardiomyocytes from rat with MetS, which will confirm if the slower decay of the

Ca^{2+} transients could be due to diminished SERCA2a activity. Cardiomyocytes during electrical-stimulation, first global Ca^{2+} transients were established in control buffer, and then, the superfusate was rapidly switched to a Ca^{2+} -free buffer [$\text{Ca}^{2+}(0)$] for 10 s, then to a [Na(0)Ca(0)] solution for an additional 10 s, recording at room temperature ($22 \pm 2^\circ\text{C}$). The SR Ca^{2+} release was evoked by a rapid (100 ms; due to the fact of a short application of caffeine is sufficient to stimulate SR Ca^{2+} release without interfering with Ca^{2+} uptake (Dutta et al. 2002; Wold et al. 2005)) caffeine application. Representative fluorescence changes for the groups are given in the inset of Fig. 3A. As can be seen in Fig. 3B, application of caffeine (10 mM) dissolved in [Na(0)Ca(0)] solution, which prevented Ca^{2+} extrusion through NCX, induced less response in MetS-group (0.48 ± 0.05 a.u.) compared to CON-group (0.66 ± 0.06 a.u.) ($n_{\text{CON}}=12$ and $n_{\text{MetS}}=11$). The time course of caffeine-induced SR Ca^{2+} release (in terms of TP and DT_{50}) was significantly prolonged in cardiomyocytes from MetS-group (1.34 ± 0.12 s and 9.13 ± 0.42 s; left and right, respectively) compared to those of controls (0.91 ± 0.12 s and 6.18 ± 0.39 s) ($n_{\text{CON}}=12$ and $n_{\text{MetS}}=11$) (Fig. 3C). These data can explain both depressed SERCA2a activity and less SR Ca^{2+} content in cardiomyocytes from MetS-rats, which, in turn, may explain the reduction in the amplitude of electrically stimulated Ca^{2+} transients, as well.

SR Ca^{2+} leakage in MetS cardiomyocytes

In order to evaluate and compare the SR Ca^{2+} content, which will then show the role of defective SR function in MetS cardiomyocytes, we also examined SR Ca^{2+} leakage, as described previously (Belke and Pierce 2014). An example of measurement of SR Ca^{2+} leakage performed in cardiomyocytes at room temperature ($22 \pm 2^\circ\text{C}$) is given in Fig. 4A. In the protocol, tetracaine application (1 mM) to the perfusion medium to block the release of Ca^{2+} through the Ca^{2+} -release channels/ryanodine receptor (RyR2)-channels, permits the uptake of Ca^{2+} by SERCA2a. Following the stimulation of cells with electrical pulses at 0.2

Hz (to obtain baseline fluorescence intensity), the cells were perfused with [Na(0)Ca(0)] solution containing 1 mM tetracaine which was followed with 10 mM caffeine application. The measured average ($\pm$ SEM) values for tetracaine responses and caffeine responses following tetracaine application are given in Fig. 4B (left and right, respectively) for groups ($n_{\text{CON}}=8$, $n_{\text{MetS}}=11$)(0.044 ± 0.008 a.u. and 0.532 ± 0.033 a.u. for MetS-group; 0.019 ± 0.003 a.u. and 0.642 ± 0.032 a.u. for CON-group, respectively).

The recorded drop of fluorescence following tetracaine application in [Na(0)Ca(0)] solution is corresponding to the Ca^{2+} leakage from SR. Then, the total SR Ca^{2+} load was subsequently determined by a 5-s exposure of the cell to modified [Na(0)Ca(0)] solution containing caffeine. Consequently, we normalized the extent of Ca^{2+} leakage from the SR to the total SR Ca^{2+} load and then the final values were compared between MetS- and CON-groups. As can be seen from Fig. 4C, the average ($\pm$ SEM) value of SR Ca^{2+} leakage is over 3-fold higher in MetS-group (0.087 ± 0.017 a.u.) compared to CON-group (0.026 ± 0.004 a.u.) ($n_{\text{CON}}=8$, $n_{\text{MetS}}=11$).

Functional examination of SR Ca^{2+} release channels, RyR2 in cardiomyocytes from MetS-rats

To further test the hypothesis that the RyR2 channels in MetS cardiomyocytes are leaky, we recorded spontaneous releases of SR Ca^{2+} (Ca^{2+} sparks) in quiescent Fluo-3 (AM) loaded cells. It is known that the parameters of Ca^{2+} sparks represent SR Ca^{2+} release from clusters of RyR2. There is also a close relation between the amount of Ca^{2+} release from SR and the amplitude of cytosolic Ca^{2+} transients, being associated with SR Ca^{2+} content as well as basal level of cytosolic Ca^{2+} in cardiomyocytes. In addition, it has been demonstrated that SR Ca^{2+} load is determined by the balance between SR Ca^{2+} leak, mediated by clusters of RyR2 and SR Ca^{2+} reuptake via SERCA2a in heart (Eisner et al. 2000). Therefore, in here, we examined the function of RyR2 by measuring Ca^{2+} sparks in resting cardiomyocytes and comparing

their parameters among MetS-group and CON-group. Representative line-scan images of the cardiomyocytes from both groups (individual Ca^{2+} spark image x vs. t) are displayed in the inset of Fig. 5A (left). The spatial and time courses were extracted as the mean of all recorded spatial or temporal pixels centered at the peak of the $\Delta F/F$ of Ca^{2+} sparks. These transients were then fitted by a gamma distribution function to obtain objective kinetic and spatial measures of the events as described previously (Ozdemir et al. 2005).

All quantitative data of both groups for Ca^{2+} spark characteristics are summarized in Fig. 5A (middle and right). The mean ($\pm$ SEM) values for the peak fluorescence amplitude determined as $\Delta F/F$ at the peak of Ca^{2+} sparks and the frequency of Ca^{2+} sparks are given in Fig. 5A (middle and right, respectively). The average $\Delta F/F$ value in MetS-group was significantly smaller than that of CON-group (0.799 ± 0.023 a.u. and 0.954 ± 0.035 a.u. in MetS-group and CON-group, respectively) while spontaneous Ca^{2+} sparks frequency was markedly higher in MetS-group (0.587 ± 0.085 $100 \text{ s}^{-1} \mu\text{m}^{-1}$) than in that of CON-group (0.386 ± 0.065 $100 \text{ s}^{-1} \mu\text{m}^{-1}$; right) ($n_{\text{CON, cell}}=71$, $n_{\text{MetS, cell}}=101$ from 5 hearts/group).

Effects of MetS on intracellular Ca^{2+} regulatory protein levels

We examined whether the alterations in Ca^{2+} release pattern in terms of both global and local Ca^{2+} changes in MetS-rat cardiomyocytes could be due to a defect in RyR2 function following its phosphorylation and/or any change in proteins associated with RyR2 macromolecular complex such as an accessory protein FKBP12.6 and/or PKA, as documented previously in streptozotocin-associated heart dysfunction (Yaras et al. 2005). We measured very high phosphorylated RyR2 (phospho-RyR2 at Ser²⁸⁰⁸) levels in heart homogenates from MetS-group while this value was very small in CON-group, as estimated from the Western-blot bands (Fig. 5B). Total protein level of RyR2 in MetS-group was slightly but not significantly lower than that of CON-group. In here, we presented the ratio of phospho-RyR2

to total RyR2 as bar graphs in the lower part of the same figure. As can be seen from this figure, this ratio is about 6-fold higher in MetS-group than CON-group (left) ($n_{\text{CON, heart}}=6$, $n_{\text{MetS, heart}}=6$).

In another set of experiments, we examined an accessory protein of FK506 binding protein (FKBP12.6) in heart homogenates. The amount of FKBP12.6 measured with respect to β -actin (at 42 kDa) was decreased about 40% in MetS-group compared with CON-group ($n_{\text{CON, heart}}=6$, $n_{\text{MetS, heart}}=6$) (original Western-blot bands in upper part of Fig. 5B, right). As can be seen from Fig. 5B (lower part), the mean values ($\pm$ SEM) of this ratio is significantly lower in MetS-group compared to CON-group.

To test whether this apparent phospho-RyR2 level in MetS-group corresponds to changes in the PKA activity, we analyzed the phosphorylation and protein levels PKA in the same heart homogenates. MetS caused marked increase in the phosphorylation level of PKA (phospho-PKA) without altering its total protein level (Fig. 6A). A ratio of phospho-PKA to total PKA in MetS-group was higher as about 40% compared to the CON-group (left) ($n_{\text{CON, heart}}=6$, $n_{\text{MetS, heart}}=6$). We also examined a phosphatase from RyR2 macromolecular complex, protein phosphatase 1 (PP1). The phospho-PP1 level was significantly higher in MetS-group than CON-group while total protein levels were similar between these two groups ($n_{\text{CON, heart}}=6$, $n_{\text{MetS, heart}}=6$) (Fig. 6A, right). Therefore, the ratio of phospho-PP1 to total PP1 was found significantly higher in MetS-group compared the CON-group.

Furthermore, since it has been previously shown that high-fat diet intake and obesity have been shown to alter the expression of the SERCA2a en route to altered cardiac remodeling and pump function (Ceylan-Isik et al. 2013), in here, we also examined the total protein level of SERCA2a in heart homogenates. We found that MetS-rats had slightly but not significantly lower cardiac levels of SERCA2a compared to the hearts from CON-group ($n_{\text{CON, heart}}=6$, $n_{\text{MetS, heart}}=6$) (Fig. 6B, left). Since SERCA2a activity is regulated by phospholamban by

reducing its affinity for Ca^{2+} and inhibition of SERCA2a by phospholamban, PLN is relieved by phosphorylation of phospholamban, we examined also the phospholamban phosphorylation level in MetS-group heart homogenates compared to CON-group. As can be seen from Fig. 6B (right), MetS induced significantly decreased phosphorylation of phospholamban (phospho-PLN) in heart homogenates compared to the CON-group without altering its total protein level. Furthermore, when we analyzed the ratio of phospho-PLN to total SERCA2a protein level, the differences between these two groups turned to not statistically significant among the groups ($n_{\text{CON, heart}}=6$, $n_{\text{MetS, heart}}=6$).

Discussion

In the present study, we have shown that the contractile activity of isolated papillary muscle strips and Ca^{2+} handling events in isolated myocytes (either in electric field stimulation or in quiescence condition) from left ventricle of rats with metabolic syndrome (MetS) are significantly different from those of age-matched controls. Our electrophysiological data confirmed the intracellular Ca^{2+} -dyshomeostasis with both lower amplitude and slower kinetics of Ca^{2+} transients as well as the decreased SR Ca^{2+} load, of interest, they are very similar to those of findings from STZ-diabetic samples (Yaras et al. 2005). Consistent with these findings, increased basal Ca^{2+} level was measured in left ventricular cardiomyocytes as well as altered SERCA2a and RyR2 function. In order to investigate the underlying molecular mechanism of the altered Ca^{2+} -homeostasis in our disease model, protein levels of Ca^{2+} -handling and SERCA2a or RyR2 modulating proteins were evaluated in cardiac homogenates. The findings were reduced amount of RyR2 or FKBP12.6 levels and PKA-dependent hyperphosphorylation of RyR2 which are supposed to be responsible for most of the altered Ca^{2+} -homeostasis.

The defective SR function is consistent with previous findings in overweight and insulin resistant-rats (Balderas-Villalobos et al. 2013; Davidoff et al. 2004; Li et al. 2006; Vasanji et

al. 2006; Wold et al. 2005). These studies address the altered SERCA2a function in their MetS-disease models. In this study we also confirmed depressed SERCA2a function under inhibited $\text{Na}^+/\text{Ca}^{2+}$ -exchanger, NCX, condition in MetS-group. In here, for the first time, we define RyR2 aspect of the SR dysfunction in high sucrose diet induced-MetS.

It is known that left ventricular diastolic dysfunction is a common clinical event of myocardial alterations in patients with MetS, similar to diabetes (Dinh et al. 2011; Schannwell et al. 2002). MetS-induced cardiac dysfunction, with pronounced left ventricular diastolic and systolic dysfunctions can lead to increased incidence in morbidity and mortality in humans (Bell 2003). Coincident with the alteration of cardiac function (both electrical and mechanical), there are a variety of metabolic and biochemical abnormalities that include change in the contractile apparatus, including activity of SERCA2a besides other actors associated with intracellular Ca^{2+} -homeostasis (Bouchard and Bose 1991; Choi et al. 2002; Ganguly et al. 1983; Malhotra et al. 1981). Overall, the data exhibit that MetS-induced cardiac dysfunction, having a few but important similarities with both experimental diabetes and heart failure in animals as well as in humans, arises at most as a consequence of elevated diastolic Ca^{2+} following altered RyR2 properties and depressed SERCA function (Fauconnier et al. 2005; Yaras et al. 2005). All of the above studies, as ours, were conducted using a defective heart function model and show alteration in spatio-temporal properties of the Ca^{2+} -sparks as well as the caffeine responses under normal and $[\text{Na}(0)\text{Ca}(0)]$ conditions, indicating depressed SERCA2a-activity as suggested by a decrease in SERCA2a-mediated Ca^{2+} -reuptake that is associated with a slower rate of cytosolic Ca^{2+} -removal. Interestingly, we observed no change in protein level of SERCA2a with depressed phosphorylation level in PLN (phospho-PLN). This provides further evidence that the ratio of phospho-PLN to SERCA2a does not change significantly in our disease model. Our present data are supported by the study of the Vasanji -team, who studied the heart function of rats that were sucrose-fed

for 10 weeks (Vasanji et al. 2006). The rats with cardiac dysfunction had depressed SR function with a significant reduction in SR Ca^{2+} uptake. Furthermore, in a recent study, rats fed with high sucrose for 16 weeks showed a decreased SERCA2a activity, which was mediated by elevated oxidative stress produced in the MetS heart (Tang et al. 2010). It should be taken into consideration some important differences in underlying mechanisms of cardiac dysfunction in MetS-rats, most probably due to variation of the metabolic changes associated with different strains of rat used, as well as the mode, amount, and duration of sucrose feeding, although all animals in these studies have common parameters such as increased body weight and blood glucose level, insulin resistance and similar structural alterations in heart tissue (Aguilera et al. 2004; Chen et al. 2011; Misra et al. 1999; Panchal and Brown 2011).

It is known that the main events demonstrating the excitation-contraction coupling in cardiomyocytes are Ca^{2+} sparks, short-lived Ca^{2+} release events from SR. Briefly, contraction is started with a small amount of Ca^{2+} entering via L-type Ca^{2+} -channels into the cell following membrane depolarization, which in turn triggers a larger amount of Ca^{2+} release from SR (Cheng et al. 1993). On the other hand, it is generally accepted that any alteration of Ca^{2+} signaling could be a main source of cardiomyopathy under different pathologies (Wehrens and Marks 2003). Although we have not provided any data on L-type Ca^{2+} channel current (I_{CaL}) and action potential parameters in here, our unpublished observation in MetS-cardiomyocytes, similar to STZ-diabetic cardiomyocytes (Yaras et al. 2005), is that the characteristics of I_{CaL} are not different between MetS- and control-rats. By contrast the cardiomyocytes in the present study demonstrated marked alterations in Ca^{2+} -homeostasis, including increases in rise time and half-decay time of Ca^{2+} transients together with decrease in Ca^{2+} transient amplitude and SR Ca^{2+} load as well as increase in diastolic Ca^{2+} . All these changes can be attributed to the alterations in both RyR2 and SERCA2a functions. In order to

evaluate the contribution of RyR2 to these changes, we measured protein and phosphorylation levels and found very high phosphorylation level in RyR2 with depressed protein levels in both total protein of RyR2 and a coordinating protein FKBP12.6. Furthermore, the results of our tetracaine application assay, which showed a 2.5-fold increase in Ca^{2+} leakage and increased Ca^{2+} -spark frequency support the “leaky-RyR2” thesis in MetS-rat hearts.

In summary, our data demonstrated that SR function-associated mechanisms are responsible for the defective regulation of Ca^{2+} -cycling in MetS-rat cardiomyocytes and they are mainly due to leaky-RyR2 and depressed SERCA2a function in cardiomyocytes. These results point up RyR2 and SERCA2a as potential pharmacological targets for restoring intracellular Ca^{2+} -homeostasis and thereby combatting dysfunction in MetS-rat heart. In view of the fact that MetS incidence is significantly increasing across the world in our century with obviously serious symptoms including heart diseases, identifying the molecular mechanisms underlying MetS induced cardiac dysfunction is important for developing pharmacological treatment methods.

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Conflicts of interest

There are no conflicts of interest to declare for any of the authors.

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Figure legends

Fig. 1. The parameters of contractile activity measured in papillary muscle strips and electrically-stimulated transient intracellular Ca^{2+} changes in isolated cardiomyocytes from MetS rats. The tension development in papillary muscle strips from metabolic syndrome (MetS) rats comparison to controls (CON) (A, left) and representative tension traces are also given in the inset (middle). The time course of twitch activity as time to peak (TP) and relaxation time at half maximum (DT_{50}) are given in (A, right). The amplitude changes of intracellular Ca^{2+} transients were obtained under electric-field stimulation (electrical pulses of 20-30 V with 10 ms duration at a frequency of 0.2 Hz) in MetS-group compared to CON-group (B, left) and representative traces of twitches in the inset (middle), and the time course of intracellular Ca^{2+} transients expressed as the time to peak fluorescence

increase (TP) and the time at half maximum of fluorescence decay (DT₅₀) (B, right). Data are presented as mean ($\pm$ SEM). *P<0.05 vs. CON ($n_{\text{CON}}=11$, $n_{\text{MetS}}=14$ papillary muscles for tension measurements and $n_{\text{CON}}=24$, $n_{\text{MetS}}=28$ cells for Ca²⁺ transient measurements).

Fig. 2. Measurement of sarcoplasmic reticulum, SR Ca²⁺ load. The representative traces given in (A). The basal level of intracellular free Ca²⁺ (as $\Delta F_{340/380}$) in (B; right) and the responses to caffeine application (10-mM) measured in Fura-2 (AM) loaded cardiomyocytes from metabolic syndrome (MetS)-group and control (CON)-group in (B; left). The time courses of the caffeine-induced Ca²⁺ transients as the time to peak fluorescence increase (TP; left) and relaxation time at half maximum (DT₅₀) of fluorescence decay (right). All data are presented as mean ($\pm$ SEM). *P<0.05 vs. CON-group with unpaired Student's t-test (number of cells used in experiments as $n_{\text{CON}}=16$ and $n_{\text{MetS}}=14$).

Fig. 3. SERCA2a function in cardiomyocytes from MetS-rats via Na⁺/Ca²⁺ exchanger inhibition. (A) Representative Ca²⁺ transients obtained following caffeine application (10-mM) measured at nominally Na⁺ and Ca²⁺ free solution [Na(0)Ca(0)] in MetS- and CON-group. The maximum values of caffeine responses (as $\Delta F_{340/380}$) (B) and time course of the transients (C) as the time to peak fluorescence, TP (right) and the time at half maximum of fluorescence decay (DT₅₀) (right). All data herein are presented as mean ($\pm$ SEM). *P<0.05 vs. CON-group with unpaired Student's t-test (number of cells in experiments as $n_{\text{CON}}=12$ and $n_{\text{MetS}}=11$).

Fig. 4. Measurement of SR Ca²⁺ leakage in cardiomyocytes from MetS rats. (A) An example for experimental protocol used to measure SR Ca²⁺ leakage. (B) Changes in fluorescence intensity with 1-mM tetracaine addition to block the RyR2 at rest (left) and caffeine-induced Ca²⁺ transients measured in CON- and MetS-groups (right). SR Ca²⁺ leakage, calculated by tetracaine response via proportional to corresponding caffeine response (C). All data are presented as mean ($\pm$ SEM). *P<0.05 vs. CON-group with unpaired Student's t-test (number of cells in experiments as $n_{\text{CON}}=8$, $n_{\text{MetS}}=11$).

Fig. 5. Measurements of RyR2 function and phosphorylation level in cardiomyocytes from MetS-rats with monitoring local Ca^{2+} release events. (A) The representative line-scan images of local Ca^{2+} releases (or Ca^{2+} sparks) are monitored by using confocal microscopy in Fluo-3 loaded quiescent cells (left). The peak fluorescence amplitude, $\Delta F/F_0$ (middle) and the frequency of spontaneous Ca^{2+} releases (right) calculated in the cardiomyocytes from metabolic syndrome (MetS)-group and control (CON)-group. Data are presented as mean ($\pm$ SEM). * $P < 0.05$ vs. CON with unpaired Student's t-test (the number of sparks analyzed; $n_{\text{CON, cell}} = 71$, $n_{\text{MetS, cell}} = 101$ from 5 hearts/group). (B) Phosphorylated levels of RyR2 (pRyR2) calculated relative to total RyR2 (RyR2) protein levels (left) and RyR2 stabilizing protein FKBP12.6 (FKBP) levels (right) relative to β -actin protein level. Data are presented as mean ($\pm$ SEM). * $P < 0.05$ vs. CON, with unpaired Student's t-test (the number of hearts for experiments as $n_{\text{CON, heart}} = 6$, $n_{\text{MetS, heart}} = 6$).

Fig. 6. Effects of MetS on the activity and protein levels of some SR-function associated proteins and kinases. (A) Phosphorylated levels of protein kinase A (pPKA) relative to total PKA protein levels (left) and phosphorylated levels of protein phosphatase 1 (pPP1) relative to total PP1 protein levels (right) measured in the heart homogenates. (B) SERCA2a protein levels measured relative to β -actin protein level (left) and phosphorylated levels of phospholamban (pPLN) relative to total PLN protein levels (right). Data are presented as mean ($\pm$ SEM). * $P < 0.05$ vs. CON, with unpaired Student's t-test (the number of hearts for experiments; $n_{\text{CON, heart}} = 6$, $n_{\text{MetS, heart}} = 6$).

A **B**

Fig. 2

A **B** **C**

A

B

C

Fig. 4

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Fig. 6