Changes in STIM Isoforms Expression and Gender-Specific Alterations in Orai Expression in Human Heart Failure

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Summary

Store-operated calcium entry (SOCE) is one of regulatory mechanisms which regulates Ca^{2+} cycling in the heart. SOCE alterations in pathological conditions contribute to progression of heart failure and cardiac hypertrophy by multiple signaling pathways such as Cn/NFAT and CaMKII/MEF2. Several components mediating SOCE have been identified, such as STIM and Orai. Different isoforms of both Orai and STIM have been detected in animal studies, exhibiting distinct functional properties. This study is focused on the analysis of STIM and Orai isoforms expression in the end-stage human failing myocardium. Left ventricle samples isolated from 43 explanted hearts from patients undergoing heart transplant and from 5 healthy donor hearts were used to determine the mRNA levels of Orai1, Orai2 and Orai3, STIM1, STIM2 and STIM2.1 by qRT-PCR. The expression was further analyzed for connection with gender, related co-morbidities, pathoetiology, clinical data and biochemical parameters. We show that Orai1 expression is decreased by 30 % in failing myocardium, even though we detected no significant changes in expression of Orai2 or Orai3. Interestingly, this decrease in Orai1 was gender-specific and was present only in men, with no change in women. The ratio Orai1/Orai3 was significantly lower in males as well. The novel STIM2.1 isoform was detected both in healthy and failing human myocardium. In the end-stage heart failure, the expression of STIM2.1 was significantly decreased. The lower ratio of STIM2.1/STIM2 in failing hearts indicates a switch from SOCE-inhibiting STIM2.1 isoform to stimulatory STIM2.2. STIM1 mRNA levels were not significantly changed. These observed alterations in Orai and STIM expression were independent of functional heart parameters, clinical or biochemical patient characteristics. These results provide detailed insight into the alterations of SOCE regulation in human failing myocardium. Gender-specific change in Orai1 expression might represent a possible mechanism of cardioprotective effects of estrogens. The switch from STIM2.1 to STIM2.2 indicates an amplification of SOCE and could contribute to the hypertrophy development in the filing heart.

Key words

Cardiac hypertrophy • Calcium • SOCE • Orai • STIM

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Introduction

Store-operated Ca^{2+} entry (SOCE) is one of the principal mechanisms which regulates Ca^{2+} cycling in the heart and which can convey Ca^{2+} signals (Parekh and Putney 2005). Even though it has been described more than 20 years ago, only recently have the molecular regulatory and functional components of SOCE been identified (Cahalan 2009, Lewis 2011). Underlying SOCE activity are Ca^{2+} release-activated Ca^{2+} channels (CRAC) which can be formed by essential proteins Orai and stromal interaction molecule (STIM) (Feske *et al.* 2006, Soboloff *et al.* 2006). There are indications that

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other proteins, such as TRPC channels, may be also involved in SOCE (Pani *et al.* 2009). However, the Ca^{2+} sensor STIM (Stathopulos *et al.* 2008) and the channel pore forming subunit Orai (Vig *et al.* 2006) are sufficient for SOCE (Lewis 2011).

SOCE is activated by depletion of endoplasmic reticulum (ER) Ca^{2+} stores, usually after activation of cell membrane (CM) receptors. Store depletion triggers oligomerization (Covington *et al.* 2010) and conformation changes of STIM (Muik *et al.* 2011). These conformational rearrangements allow the accumulation of STIM proteins in the ER-CM junctions (Wu *et al.* 2006, Park *et al.* 2009), where they directly bind to Orai through their CRAC activation domains (also known as SOAR, STIM1 Orai1 activation region; Park *et al.* 2009). STIM binding to Orai opens the channel and activates SOCE (Li *et al.* 2011).

Since cardiomyocytes have large Ca^{2+} influx with each heartbeat, SOCE in cardiomyocytes was largely ignored and, even today, its contribution to normal cardiac physiology is still under debate (Bootman and Rietdorf 2017). However, compelling experimental evidences have shown that SOCE plays a central role in the activation of prohypertrophic signaling pathways such as calcineurin(Cn)/NFAT during the development of pathological cardiac remodeling (Avila-Medina *et al.* 2018).

Both STIM and Orai represent a wider family of proteins. Two genes encoding STIM1 and STIM2 were described (Liou *et al.* 2005) and three genes encoding Orai1, Orai2 and Orai3 proteins (Feske *et al.* 2006, Vig *et al.* 2006). Although STIM1 and STIM2 have highly conserved structure and respond similarly to store depletion, they seem to perform different functions. Both genes give rise to several splicing variants: next to the conventional SOCE-activating STIM1 and STIM2.2 variants, STIM1L responsible for rapid SOCE activation mostly in skeletal muscle was described, and recently (Rana *et al.* 2015), a SOCE-inactivating STIM2.1 was detected (for review see Rosado *et al.* 2016). The function of STIM2 was considered controversial and unclear for a long time (Avila-Medina *et al.* 2018), possibly because two splicing variants with opposing functions were subsumed into one entity.

In this study, we analyze the expression of Orai and STIM variants in human failing myocardium to assess their involvement in the cardiac remodeling and their relevance as possible targets for pharmacological intervention.

Methods

Human heart samples

For this study, 43 patients were chosen who were diagnosed with heart failure in NYHA III-IV class (HF group). These patients were indicated for heart transplant surgery and underwent the surgery in National Institute for Cardiovascular Diseases from August 2009 to June 2013. The samples of heart tissue were isolated from explanted heart left ventricle within 30 min after the explantation, avoiding the scared, fibrotic and adipose tissues, as a cross-section of the whole wall of the ventricle. They were afterwards rinsed, dried and snap-frozen in liquid nitrogen, transported to lab and kept at -80 °C until further processing. Tissue samples of nonfailing controls (CTR; n=5) were similarly obtained from donor hearts that were prepared for transplantation, but the surgery was not performed. Clinical and biochemical data were obtained from patient documentation and were not older than 3-6 months before transplantation, except the heart weight, which was measured after the surgery. Because the clinical data was not available from all patients, the number of data points for statistical evaluation can differ across the data groups and is noted in respective tables. The whole project and experimental procedures were approved by the Ethics committee of the National Institute for Cardiovascular Diseases in Bratislava, Slovak republic. The study conformed to the principles outlined in the Declaration of Helsinki, all patients (or their legal representatives) were informed about and gave informed consent prior to participation in this project.

RNA isolation and real-time polymerase chain reaction

Total RNA was isolated from left ventricle samples using Tri-Reagent® (Sigma-Aldrich, USA) according to the recommended manufacturer's protocol. The quality of isolated RNA was verified with electrophoresis on 2 % agarose gel. Quality and quantity of the RNA was measured with spectrophotometric analysis (NanoDropND-1000, Thermo Scientific, USA). Reverse transcription was performed using High-Capacity cDNA KIT with RNAse inhibitor (Applied Biosystems, USA). SYBR Green detection (SYBR Select Master Mix, Life Technologies, USA) was used to perform quantitative RT-PCR on StepOnePlus® Real-Time PCR System (Life Technologies, USA) according to manufacturer's instructions. Primers (Sigma-Aldrich, USA) were designed to amplify human Orai1, Orai2,

Table 1. HF group characteristics

	ALL	Female	Male
Number	43	8	35
Age [years]	50.8 ± 1.3 (43)	46.9 ± 2.6 (8)	51.7 ± 1.4 (35)
BMI [kg/m^2]	27.1 ± 0.7 (42)	24.8 ± 1.8 (8)	27.65 ± 0.7# (34)
SBP [mmHg]	109.3 ± 2.0 (42)	103.8 ± 5.8 (8)	110.6 ± 2.0 (34)
DBP [mmHg]	70.9 ± 1.2 (42)	69.0 ± 2.8 (8)	71.4 ± 1.3 (34)
HR [min^{-1}]	80.0 ± 1.754 (43)	78.9 ± 2.7 (8)	80.2 ± 2.1 (34)
NT-proBNP [ng/l]	4666 ± 632 (40)	5132 ± 1152 (8)	4549 ± 742 (32)
Troponin T [ng/l]	30.2 ± 5.2 (31)	38.5 ± 18.0 (6)	28.1 ± 4.9 (25)
Cholesterol [mmol/l]	4.1 ± 0.2 (32)	4.5 ± 0.3 (5)	4.1 ± 0.2 (27)
TAG [mmol/l]	1.68 ± 0.24 (31)	1.10 ± 0.14 (6)	1.835 ± 0.29$^{\#}$ (25)
HW [g]	635.8 ± 24.1 (30)	415.8 ± 43.0 (4)	669.7 ± 20.0$^{\#}$ (26)
LVEF [%]	22.6 ± 1.4 (42)	30.9 ± 5.4 (8)	20.7 ± 1.0 (34)
LVEDD [mm]	69.4 ± 1.8 (40)	54.5 ± 3.7 (8)	73.1 ± 1.3$^{\#}$ (32)
RVEDD [mm]	33.9 ± 0.7 (39)	32.3 ± 1.1 (8)	34.3 ± 0.9 (31)
QRS [s]	0.137 ± 0.007 (36)	0.142 ± 0.017 (7)	0.136 ± 0.007 (27)
QT [s]	0.422 ± 0.007 (34)	0.426 ± 0.012 (7)	0.421 ± 0.009 (27)

BMI - body mass index, SBP - systolic blood pressure, DBP - diastolic blood pressure, HR - heart rate, NT-proBNP - N-terminal pro B-type natriuretic peptide and TAG - triacylglyceride blood concentrations. HW - heart weight, LVEF - left ventricle ejection fraction, LVEDD - left ventricle end-diastolic diameter, RVEDD - right ventricle end-diastolic diameter. Number of patients where data was available shown in brackets; $^{\#}$ $p<0.05$ *vs.* Female

Orai3, STIM1, all splicing variants of STIM2, and STIM2.1 variant only. All primers were verified to yield a single PCR product with the correct molecular weight, and the absence of signal was verified when reverse transcription was omitted. The results were normalized to the expressions of endogenous reference genes (HPRT1, hypoxanthine phosphoribosyltransferase 1; B2M, beta-2-microglobulin). The primer sequences used were as follows:

B2M: forward: TCCGTGGCCTTAGCTGTGCTT, reverse: TCCATTCTCTGCTGGATGACGTGAG;
HPRT1: forward: AGCCCTGGCGTCGTGATTAGTGA, reverse: GGTCACAATGTGATGGCCTCCCA;
OraiI1: forward: CGCCAAGCTTAAAGCCTCCA, reverse: CTGATCATGAGCGCAAACAGG;
Orai2: forward: CAGCTCCGGGAAGGAACGTC, reverse: TAGGCACGTTAAGCTCAGCACT;
Orai3: forward: CCACGTACCGGGAGTTCG, reverse: GTACTCGTGGTCACTCTCCAG;
STIM1: forward: GACTGACGACGTGGATGACA, reverse: TACCCGGCTTGTCAAGAAGT;
STIM2: forward: TGTCACTGAGTCCACCATGC, reverse: TCTCTGTGCAGATGGCTGTG;
STIM2.1: forward: AGGTTCGCATGGCTCTGAAA, reverse: TGAATCAGATATGAAGCAGCAACC.

Computational and statistical analysis

PCR efficacy and quantification cycle values for each sample were determined with LinRegPCR software (version 2017.1). Shapiro-Wilk test was used as normality test. To compare two groups with normally distributed data, Student's t-test was used. For non-normally distributed data, we performed the Mann-Whitney test. ANOVA was used to assess the differences among multiple groups. Statistical correlation between two data groups was determined by Pearson correlation coefficients. All data were handled by GraphPad Prism (GraphPad Software Inc., version 6). All data are presented as average ± standard error of the mean.

Results

Clinical and biochemical characteristics of patients with heart failure

The wet weight of the explanted hearts was higher than the physiologic range (250-350 g) and this was observed both in the sex-specific group of women and men. The diagnosed heart failure was in last stages as

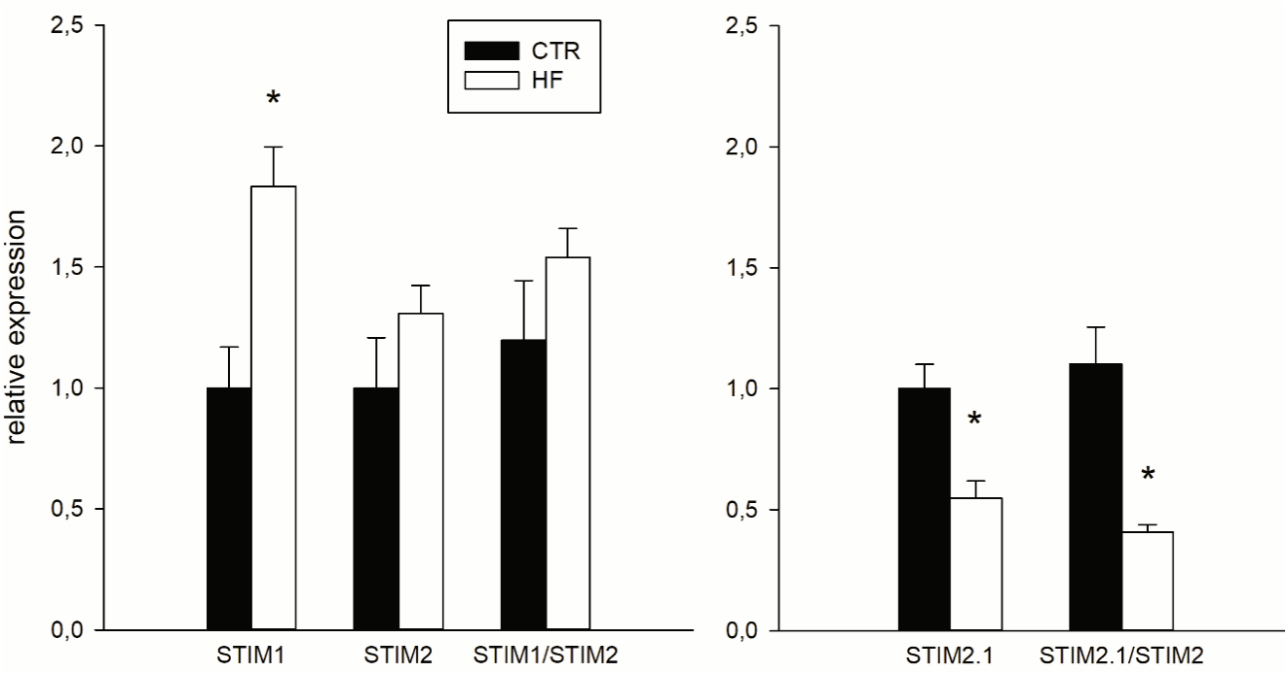

Fig. 1. Relative gene expression of STIM in the left ventricle of patients with heart failure (HF) and non-failing (CTR) controls. Shown is the expression of STIM1 and all STIM2 variants (left), and the expression of splicing variant STIM2.1 together with the ratio of STIM2.1 to all STIM2 variants (right). *p<0.05 *vs.* CTR.

shown by the left ventricle ejection fraction (LVEF), that was decreased to 22.64±1.407 %. The failing hearts of the women displayed higher ejection fraction than men. Both average LVEDD and RVEDD was increased. QT interval values were within the physiological range (<0.44 s) and QRS complex values were slightly elevated. The average age of patients was 50.84 years, where women were insignificantly younger than men. The patients' average BMI falls within the overweight range. Blood pressure values were within the physiological limits, although the heart rate was elevated. Table 1 also summarizes the biochemical parameters of the HF group. Plasmatic concentrations of N-terminal pro B-type natriuretic peptide (NT-proBNP) and troponin T were in the range of values that confirm end-stage heart failure. We detected no sex-specific differences in these concentrations. Levels of cholesterol and TAG were not increased.

Expression of STIM splicing variants

As shown in Figure 1, the expression of STIM1 was significantly increased in the LV of HF patients. Despite certain trend, there were no significant changes in the expression of STIM2 variants, nor in the ratio of STIM1/STIM2. However, the expression of splicing variant STIM2.1 was decreased to 55 % in HF myocardium and the ratio of this variant to all STIM2 forms (STIM2.1/STIM2) was decreased to 41 %. We detected no gender-specific differences in either parameter. Interestingly, the expression of either STIM isoform was not influenced by the functional state of the LV (no significant correlation to functional LV parameters were detected), comorbidities or by the patients´ biochemical characteristics (data not shown).

Expression of Orai channels

Orai1 mRNA levels in the failing myocardium were significantly decreased by 30 % (Fig. 2). The expression of Orai2 and Orai3 in HF were not significantly different from the CTR group and the ratio of Orai1 to Orai3 expression was not changed either in the whole HF group. However, further analysis showed distinct differences between male and female patients. As shown in Figure 3, the decreased Orai1 expression was limited to the male patients only (HF female: 92 % of CTR, HF male: 65 % of CTR expression). The Orai1/Orai3 ratio in HF male patients was significantly lower than in the HF female group. We detected no influence of the functional state of the LV, comorbidities or by the patients´ biochemical characteristics on the Orai expressional patterns (data not shown).

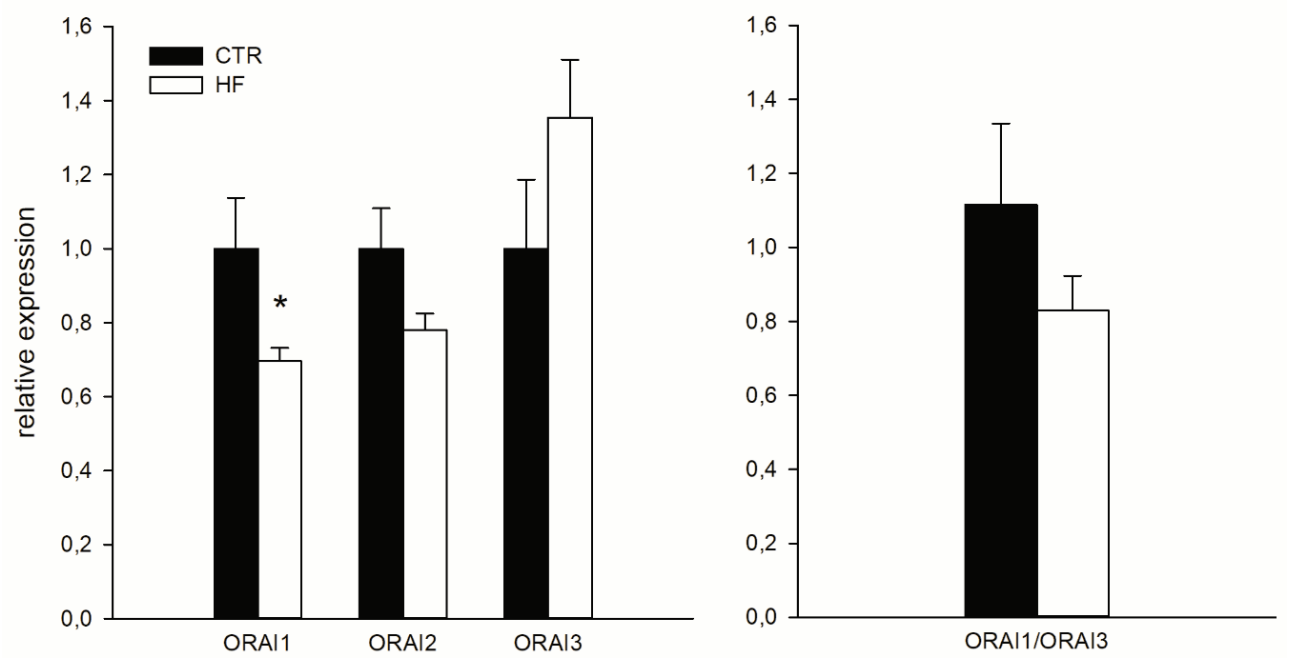

Fig. 2. Relative gene expression of Orai channels in the left ventricle of patients with heart failure (HF) and non-failing (CTR) controls. Shown is the expression of individual Orai isoforms (left), and the ratio of Orai1 to Orai3 expression (right). *p<0.05 *vs.* CTR.

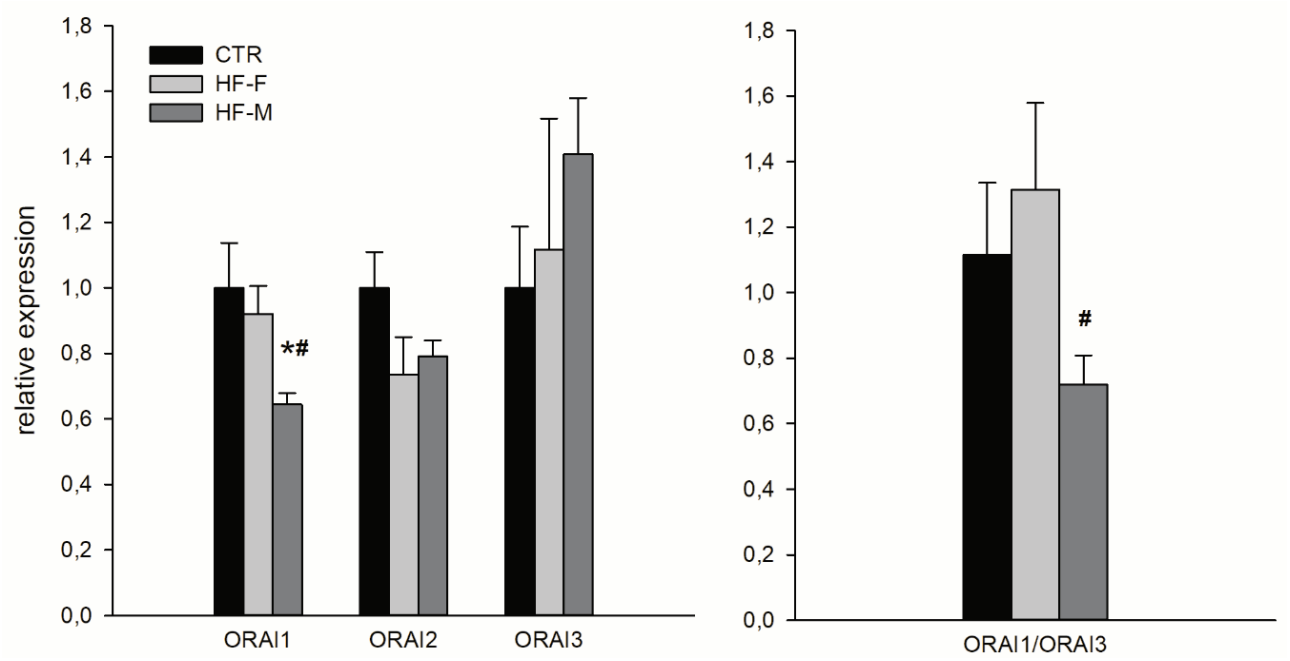

Fig. 3. Gender-specific gene expression of Orai channels in the failing left ventricle (HF) and non-failing myocardium (CTR). The decrease in Orai1 expression was detected in LV of male patients (HF-M), but not in LV of female patients (HF-F, left). The ratio of Orai1 to Orai3 expression was decreased in male left ventricle (right). *p<0.05 *vs.* CTR, # p<0.05 *vs.* HF-F.

Discussion

Clinical and biochemical data

Analysis of the clinical and biochemical data indicate severe heart damage. According to National Institute for Health and Care Excellence, patients with NT-proBNP levels above 2000 ng/l are suspected for heart failure and need echocardiographic examination (NICE 2018). Increased NT-proBNP and troponin T levels of HF patients included in this study, together with the very low average LVEF confirm the end-stage of HF. The increased heart weight over the expected

physiological values indicates probable cardiac hypertrophy development. As expected, in male sex-specific group, the heart weight was significantly higher compared to women.

Interestingly, we detected no significant correlation between either clinical, functional or biochemical parameters of HF patients and the expression of followed genes. This might be explained by the end-stage of the heart failing (requiring heart transplant due to compensatory mechanisms reaching their limits). This would require follow-up of this question in earlier stages of HF.

Expression of STIM splicing variants

Proteins of the STIM family are generally recognized to have two functions: act as a sensor of Ca^{2+} concentration in ER and to regulate CRAC activity in response to this concentration. The identification of STIM1 in 2005 as a ER-membrane protein responsible for activation of SOCE was an important step to understanding this mechanism (Roos *et al.* 2005). The role of STIM2 was controversial, however; some studies described his inhibiting effect on SOCE, while other have shown that STIM2 can activate Orai channels in a constitutive and Ca^{2+}-independent manner (Soboloff *et al.* 2006). More insight into this problem brought a 2015 study which identified a new splicing variant of STIM2 (Miederer at al. 2015). This new variant STIM2.1 keeps the exon 9 during splicing, which leads to additional sequence of 8 amino acids in the SOAR domain. In contrast to STIM1 and STIM2.2 (which can bind to and activate CRACs through the SOAR domain), STIM2.1 cannot activate Orai channels and has inhibitory effect on SOCE.

Here, we show that the human myocardium does express the STIM2.1 variant together with STIM1 and (presumably) STIM2.2. As it was not possible to design primers detecting only STIM2.2, we have quantified the ratio of STIM2.1 expression to the expression of all STIM2 variants. We have shown that the expression of SOCE-activating STIM1 is increased in HF myocardium, while the expression of SOCE-inhibiting STIM2.1 is decreased in HF. These data suggest that in human HF, distinct expressional changes lead to increased SOCE, increased intracellular $[Ca^{2+}]$ and stimulation of prohypertrophic pathways such as Cn/NFAT. This would confirm the results seen in animal experiments, where SOCE and nuclear translocation of NFAT was amplified during the development of cardiac remodeling (Eder 2017).

Expression of Orai channels

In mammals, Orai1, Orai2 and Orai3 isoforms are present and form highly Ca^{2+}-selective channels which are a principal component of SOCE (Ruhle and Trebak 2013). In myocytes, the predominant isoforms are Orai1 and Orai3 (Saliba *et al.* 2015). Our analysis of gene amplification C_t from human samples confirmed highest expression of Orai1, while Orai2 and Orai3 were expressed with comparable intensity (data not shown). The role of different Orai isoforms under physiological condition in the heart is not clear. These channels are probably not involved in the normal excitation-contraction coupling, however, Orai1 and Orai3 maintain electromechanical stability of the myocardium and Ca^{2+} homeostasis (Ruhle and Trebak 2013, Uehara *et al.* 2002).

In contrast, Orai3 channels play a critical role in pressure-induced hypertrophic process in cardiomyocytes (Saliba *et al.* 2015). Orai3 knockdown prevents cardiomyocyte hypertrophy, similar to STIM1 knockdown models (Hulot *et al.* 2011). It has been suggested that Orai3 is recruited into Orai1/STIM complexes during hypertrophy development, which leads to increased Orai3-dependent Ca^{2+} entry (Saliba *et al.* 2015). Results shown here would support this mechanism, as the ratio of Orai1/Orai3 was decreased in failing hearts. In addition to SOCE, Orai3-driven store-independent Ca^{2+} influx and arachidonic acid (AA)-induced Ca^{2+} current were described that can contribute to NFAT activation (Shuttleworth 2012). In pressure-overload hypertrophy, an elevation of AA in total phospholipids was reported (Reibel *et al.* 1986) and an increased AA-induced Ca^{2+} current (Saliba *et al.* 2015), which seems to be in agreement with more STIM/Orai1/Orai3 complexes.

Interestingly, we detected these alterations in Orai1/Orai3 expression only in male patients. As the expressional changes described here indicate an increased Ca^{2+} influx and prohypertrophic effect, the absence of altered Orai1/Orai3 can possibly be interpreted as a cardioprotective affect. This gender-specific expressional profile of Orai channels in HF might be one of the mechanisms underlying well-described cardioprotective effects of estrogen in women. Indeed, Orai3 has been shown to be regulated by estrogen, at least in breast cancer cells (Motiani *et al.* 2013).

Study limitations

Main limitation of the study is the reliance on mRNA levels to assess the gene expression. The results presented here need to be expanded with data on protein expressions, posttranslational modifications, and function, which should provide deeper insight into the role of SOCE in HF. Another limitation is generally low number of patients in the CTR and HF-F groups. This contributes to a relatively high variability which is already inherently high in clinical setting and makes the interpretation of the correlation analysis particularly complicated. Targeted investigation should be performed in near future to confirm the presented findings. Finally, end-stage HF represents a time point, where most of the damage to the heart is done, and similar analysis of earlier stages of human HF would help to identify the underlying pathoetiological mechanisms.

Conclusions

Altogether, these results provide detailed insight into the alterations of SOCE regulation in human failing myocardium. Gender-specific change in Orai1/Orai3 expression might represent a possible mechanism of cardioprotective effects of estrogens. The switch from STIM2.1 to STIM2.2 indicates an amplification of SOCE and could contribute to the hypertrophy development in the filing heart.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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