Sex-dependent alterations of Ca\textsuperscript{2+} cycling in human cardiac hypertrophy and heart failure

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Aims
Clinical studies have shown differences in the propensity for malignant ventricular arrhythmias between women and men suffering from cardiomyopathies and heart failure (HF). This is clinically relevant as it impacts therapies like prophylactic implantable cardioverter-defibrillator implantation but the pathomechanisms are unknown. As an increased sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} leak is arrhythmogenic, it could represent a cellular basis for this paradox.

Methods/Results
We evaluated the SR Ca\textsuperscript{2+} leak with respect to sex differences in (i) afterload-induced cardiac hypertrophy (Hy) with preserved left ventricular (LV) function and (ii) end-stage HF. Cardiac function did not differ between sexes in both cardiac pathologies. Human cardiomyocytes isolated from female patients with Hy showed a significantly lower Ca\textsuperscript{2+} spark frequency (CaSpF, confocal microscopy, Fluo3-AM) compared with men ($P < 0.05$). As Ca\textsuperscript{2+} spark width and duration were similar in women and men, this difference in CaSpF did not yet translate into a significant difference of the calculated SR Ca\textsuperscript{2+} leak between both sexes at this stage of disease ($P = 0.14$). Epifluorescence measurements (Fura2-AM) revealed comparable Ca\textsuperscript{2+} cycling properties (diastolic Ca\textsuperscript{2+} levels, amplitude of systolic Ca\textsuperscript{2+} transients, SR Ca\textsuperscript{2+} load) in patients of both sexes suffering from Hy. Additionally, the increased diastolic CaSpF in male patients with Hy did not yet translate into an elevated ratio of cells showing arrhythmic events (Ca\textsuperscript{2+} waves, spontaneous Ca\textsuperscript{2+} transients) ($P = 0.77$). In the transition to HF, both sexes showed an increase of the CaSpF ($P < 0.05$) and the sex dependence was even more pronounced. Female patients had a 69\% lower SR Ca\textsuperscript{2+} leak ($P < 0.05$), which now even translated into a lower ratio of arrhythmic cells in female HF patients compared with men ($P < 0.001$).

Conclusion
These data show that the SR Ca\textsuperscript{2+} leak is lower in women than in men with comparable cardiac impairment. Since the SR Ca\textsuperscript{2+} leak triggers delayed afterdepolarizations, our findings may explain why women are less prone to ventricular arrhythmias and confirm the rationale of therapeutic measures reducing the SR Ca\textsuperscript{2+} leak.

Keywords
Heart failure • Sex/gender • Calcium cycling • SR calcium leak • Arrhythmias

Introduction
The deterioration of left ventricular (LV) function in the development of heart failure (HF) goes along with an increased tendency towards malignant arrhythmias. This is also associated with a high incidence of sudden cardiac death in HF patients. A number of large trials demonstrated the benefits of the implantable cardioverter-defibrillator (ICD) therapy in patients with significantly reduced ejection fraction (EF).\textsuperscript{1} Accordingly, currently applicable guidelines recommend the implantation of an ICD device for primary prevention in patients with an EF of $\leq 35\%$.\textsuperscript{2} However, the propensity to life-threatening arrhythmias does not equally affect both sexes. A recent study revealed that women on ICD therapy due to HF are less likely to receive appropriate ICD shocks and generally have a
lower arrhythmic mortality than propensity-matched men. In women with HF due to coronary heart disease, the absolute risk of sudden cardiac death is just one-third of that in men. In general, women seem to be less prone to ventricular arrhythmias. It is known that women more often present with asystole and pulseless electrical activity while men usually showed ventricular tachycardia and ventricular fibrillation in out of hospital cardiac arrest. This ‘sex-paradox’, therefore, has significant impact on clinical practice. However, the pathomechanisms underlying this dissimilarity are largely unknown. The lower LV mass of women as well as hormonal effects on ion channels and the QT interval have been proposed to contribute. Of note, data from animal models have shown that there are also substantial sex-dependent differences in Ca$^{2+}$ cycling properties. Cardiomyocytes (CMs) of healthy female rats showed smaller spontaneous diastolic Ca$^{2+}$ release events (Ca$^{2+}$ sparks) and reduced diastolic Ca$^{2+}$ levels compared with males. This is important as a disruption of Ca$^{2+}$ homeostasis is known to represent a powerful substrate for arrhythmias. It has been repeatedly shown that the ryanodine receptor type 2 (RyR2), which physiologically acts as a powerful substrate for arrhythmias in HF due to severe aortic valve stenosis undergoing valve replacement and (ii) patients with end-stage HF undergoing heart transplantation. We sought to find out if the diastolic SR Ca$^{2+}$ leak shows a sex dependence, if this is present in both cardiac pathologies and if the SR Ca$^{2+}$ leak increases in both sexes in the transition from afterload-induced Hy to HF.

### Methods

**Human myocardial tissue**

All procedures were conducted in compliance with the local ethics committee and written informed consent was received from all participants prior to inclusion.

Left ventricular myocardial tissue was taken from the LV septum of 24 patients (n = 12 women, 12 men) with severe aortic valve stenosis, who had pronounced cardiac Hy but still preserved EF and from corresponding regions of explanted hearts of 13 patients (n = 5 women, 8 men) with end-stage HF (New York Heart Association HF classification IV) undergoing heart transplantation. The myocardial tissue was acquired directly in the operating room during surgical procedures and immediately placed in precooled cardioplegic solution (in mmol/L: NaCl 110, KCl 16, MgCl$_2$ 16, NaHCO$_3$ 16, CaCl$_2$ 1.2, and glucose 11). The heart tissue was stored for cell isolation in cooled cardioprotective solution containing (in mmol/L): Na$^+$ 156, K$^+$ 3.6, Cl$^-$ 135, HCO$_3^-$ 25, Mg$^{2+}$ 0.6, H$_2$PO$_4^-$ 1.3, SO$_4^{2-}$ 0.6, Ca$^{2+}$ 2.5, glucose 11.2, 2,3-butanedionemonoxime 10, and aerated with 95% O$_2$ and 5% CO$_2$.

**Myocyte isolation**

Human myocardium was rinsed, cut into small pieces and incubated at 37°C in a spinner flask filled with Joklik-MEM solution (JMEM: Applied Chem, Darmstadt, Germany) containing 1.0 mg/mL collagenase (Worthington type 1, 185 U/mg) and trypsin (2.5 g/L, Life Technologies, Carlsbad, California, USA). After 45 min, the supernatant was discarded and fresh JMEM solution containing only collagenase was added. The solution was incubated for 10–20 min until myocytes were disaggregated using a Pasteur pipette. The supernatant containing disaggregated cells was removed and centrifuged (600 rpm, 3 min). Fresh JMEM with collagenase was added to the remaining tissue. This procedure was repeated four to five times. After every step, the centrifuged cells were resuspended in KB medium containing (mmol/L): taurine 10, glutamic acid 70, KCl 25, KH$_2$PO$_4$ 10, dextrose 22, EGTA 0.5, bovine calf serum 10% (pH 7.4, KOH, room temperature). Only cell solutions from afterload-induced Hy to HF in both genders.

### Intracellular Ca$^{2+}$ imaging

**Confocal microscopy (measurement of sarcoplasmic reticulum Ca$^{2+}$ sparks)**

Isolated CMs were incubated at room temperature for 30 min with a Fluo3-AM loading buffer (10 μmol/L, Molecular Probes, Eugene, Oregon, USA). Experimental solution contained (mmol/L): NaCl 136, KCl 4, NaH$_2$PO$_4$ 0.33, NaHCO$_3$ 4, CaCl$_2$ 2, MgCl$_2$ 1.6, HEPES 10, glucose 10, isotropicrolen 0.01 (pH 7.4, NaOH, room temperature). Cells were continuously superfused during experiments. To wash out the loading buffer and remove any extracellular dye as well as to allow enough time for complete de-esterification of Fluo3-AM, cells were superfused with experimental solution for 5 min before experiments were started. Ca$^{2+}$ sparks measurements were performed with a laser scanning confocal microscope (LSM 5 Pascal, Zeiss, Oberkochen, Germany).
Epifluorescence microscopy (systolic Ca\(^{2+}\) transients and sarcoplasmic reticulum Ca\(^{2+}\) content)

Cardiomyocytes were isolated and plated as described above and incubated with a Fura2-AM loading buffer (10 \(\mu\)mol/L, Molecular Probes, Eugene, Oregon, USA) for 15 min. After staining, the CMs were superfused with experimental solution. Measurements were performed with a Nikon Eclipse TE2000-U microscope provided with a fluorescence detection system (ION OPTIX Corp.) at room temperature. Cells were excited at 340 and 380 nm and the emitted fluorescence was collected at 510 nm. The intracellular Ca\(^{2+}\) level was measured as the ratio of fluorescence at 340 and 380 nm (F340/F380 nm in ratio units, r.u.). Systolic Ca\(^{2+}\) transients were recorded at steady state conditions under constant field stimulation (0.5 Hz, 20 V). To assess the SR Ca\(^{2+}\) content, we measured the amplitude of caffeine-induced Ca\(^{2+}\) transients. Two seconds after stopping the stimulation during steady state conditions, caffeine (10 mmol/L) was applied directly onto the cell leading to immediate and complete SR Ca\(^{2+}\) release. The recorded Ca\(^{2+}\) transients were analysed with the software IONWizard\(^{\text{b}}\) (ION OPTIX Corp.).

Statistics

All descriptive statistics (Table 1) are presented as mean ± SEM (software: GraphPad Prism). For the confocal and epifluorescence measurements, statistical testing was done according to a repeated measures ANOVA (software: proc mixed, SAS 9.3), to account for the clustering of measurements within one patient. The respective figures represent estimates ± standard error. Owing to the exchangeability of measurements, a compound symmetry covariance structure was assumed and unequal variances were allowed in different subgroups regarding disease as well as gender. Pairwise comparisons between Hy and HF (stratified between gender) and male vs. female (stratified by disease) are reported, considering \(P\)-values < 0.05 as statistically significant. For Figures 1A, 3H, 4H, and 5H, Fisher’s exact test was used.
Sex-dependent differences of the sarcoplasmic reticulum Ca$^{2+}$ leak in afterload-induced cardiac hypertrophy

Severe afterload-induced cardiac Hy due to aortic valve stenosis is known to come along with a high risk for ventricular arrhythmias. To find out if there is a sex-dependent difference already at this stage of cardiac disease, we isolated ventricular CMs from myocardial tissue of female and male patients undergoing aortic valve replacement ($n = 12$ women, 12 men). We only included patients with preserved EF to exclude mechanisms occurring in the development of systolic HF. Key characteristics of the patients included are shown in Table 1 (left columns, Hy). Women and men had a similar age ($74.2 \pm 2.2$ vs. $69.2 \pm 2.3$ years, $P = 0.13$), an equally good EF $55.4 \pm 2.5$ vs. $55.2 \pm 1.9$, $P = 0.94$), and a similarly narrowed aortic valve area (AVA, $0.6 \pm 0.1$ vs. $0.8 \pm 0.1$ cm$^2$, $P = 0.19$). This resulted in a comparable mean pressure gradient over the aortic valve ($P_{mean}$, $52.8 \pm 2.4$ vs. $56.3 \pm 8.9$ mmHg, $P = 0.76$) and a similar degree of interventricular septum hypertrophy (IVS, $13.3 \pm 1.2$ vs. $15.0 \pm 1.1$ mm, $P = 0.37$) in female and male patients. Cardiac dimensions measured as LV end-diastolic diameter (LVEDD) were also comparable ($45.9 \pm 1.3$ vs. $50.1 \pm 2.9$ mm, $P = 0.21$).

Isolated CMs were stained with Fluo3-AM, paced at 1 Hz for 10 s to ensure appropriate SR Ca$^{2+}$ load and then scanned for diastolic Ca$^{2+}$ sparks. Interestingly, we detected a lower frequency of diastolic Ca$^{2+}$ sparks in women compared to men already at $0.16$ s vs. $0.80$ s, $n = 340/9$ vs. $380/6$, $P = 0.74$, and the same extent of spontaneous Ca$^{2+}$ transients, Figure 4C), did not yet differ between both sexes in cardiac Hy with preserved LV function (34 of 119 in women vs. 31 of 99 in men, $P = 0.77$, Figure 1F).

Sex-dependent differences of systolic Ca$^{2+}$ transients and sarcoplasmic reticulum Ca$^{2+}$ load in afterload-induced cardiac hypertrophy

As the diastolic SR Ca$^{2+}$ leak is not only dependent on RyR2-gating properties but also on diastolic Ca$^{2+}$ levels (trigger for RyR2-opening) and SR Ca$^{2+}$ load, we additionally evaluated Ca$^{2+}$ cycling parameters in female and male patients with afterload-induced cardiac Hy. Freshly isolated human CMs were stained with Fura2-AM and paced at 0.5 Hz. Systolic Ca$^{2+}$ transients were recorded upon reaching steady state levels (epifluorescence microscopy). Sarcoplasmic reticulum Ca$^{2+}$ load was evaluated by caffeine application. As shown in Figure 2A and 8, the amplitude of systolic Ca$^{2+}$ transients ($F_{340}/F_{380}$, $0.18 \pm 0.08$ vs. $0.21 \pm 0.03$, n cells/patients = 8/3 vs. 33/6, $P = 0.74$) and diastolic Ca$^{2+}$ levels ($F_{340}/F_{380}$ women vs. men = $0.34 \pm 0.03$ vs. $0.36 \pm 0.03$, n = 8/3 vs. 33/6, $P = 0.61$, Figure 2C) was not significantly different between women and men. Furthermore, SR Ca$^{2+}$ load (amplitude of caffeine-induced Ca$^{2+}$ transients, $F_{340}/F_{380}$, $0.23 \pm 0.04$ vs. $0.33 \pm 0.04$, n = 10/3 vs. 18/6, $P = 0.14$, Figure 2A and E) and Ca$^{2+}$ elimination kinetics of systolic Ca$^{2+}$ transients (Ca$^{2+}$ elimination time 50%, RT50$\text{sys}$: $0.62 \pm 0.05$ s vs. $0.80 \pm 0.16$ s, n = 33/6 vs. 8/3, $P = 0.38$, Figure 2D) as well as caffeine-induced Ca$^{2+}$ transients (RT50$\text{cuff}$: $2.59 \pm 0.89$ vs. $2.50 \pm 0.36$ s; n = 18/6 vs. 10/3, $P = 0.94$, Figure 2F) did not reveal a distinct sex dependence.

Sex-dependent difference of the sarcoplasmic reticulum Ca$^{2+}$ leak in end-stage heart failure

It has been repeatedly shown that the SR Ca$^{2+}$ leak increases during the development of HF and that this may contribute to the high incidence of arrhythmias in patients suffering from HF. To elucidate whether the diastolic SR Ca$^{2+}$ leak increases equally in both sexes and whether the sex dissimilarity further aggravates in the

### Table 1: Characteristics of patients included in the different groups

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<tr>
<td>n</td>
<td>12</td>
<td>12</td>
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<tr>
<td>Age (years)</td>
<td>74.2 $\pm$ 2.2</td>
<td>69.2 $\pm$ 2.3</td>
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<tr>
<td>EF (%)</td>
<td>55.4 $\pm$ 2.5</td>
<td>55.2 $\pm$ 1.9</td>
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<tr>
<td>AVA (cm$^2$)</td>
<td>0.6 $\pm$ 0.1</td>
<td>0.8 $\pm$ 0.1</td>
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<tr>
<td>$P_{mean}$ (mmHg)</td>
<td>52.8 $\pm$ 2.4</td>
<td>56.3 $\pm$ 8.9</td>
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<tr>
<td>IVS (mm)</td>
<td>13.3 $\pm$ 1.2</td>
<td>15.0 $\pm$ 1.1</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>45.9 $\pm$ 1.3</td>
<td>50.1 $\pm$ 2.9</td>
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EF, ejection fraction; AVA, aortic valve area; $P_{mean}$, mean pressure gradient over aortic valve; IVS, interventricular septum; LVEDD, left ventricular end diastolic diameter.
development of HF, we additionally isolated CMs from patients with end-stage HF undergoing heart transplantation ($n = 5$ women, 8 men). The respective patient characteristics are annotated in Table 1 (right columns, HF). Women and men showed similar systolic cardiac functions ($EF = 22.7 \pm 2.9$ vs. $18.4 \pm 1.8\%$, $P = 0.21$) and comparable cardiac dimensions ($IVS = 6.7 \pm 1.2$ mm vs. $9.2 \pm 0.8$ mm, $P = 0.22$; $LVEDD = 62.4 \pm 8.4$ vs. $69.1 \pm 4.0$, $P = 0.44$). Additionally, men and women were of comparable age at the time of heart transplantation ($55.3 \pm 3.2$ vs. $43.4 \pm 6.6$ years, $P = 0.10$).

When we assessed the diastolic SR Ca$^{2+}$ leak in these CMs, we found the sex dependence to be even more pronounced in HF. Women with end-stage HF had a significantly lower CaSpF ($1.14 \pm 0.22$ vs. $2.25 \pm 0.33$, $n$ cells/patients = $85/5$ vs. $44/8$, $P < 0.05$, Figure 3B) as well as a lower spark amplitude ($F340/F380 = 1.64 \pm 0.01$ vs. $1.82 \pm 0.06$, $P < 0.05$, Figure 3C). The width of Ca$^{2+}$ sparks ($3.02 \pm 0.27$ vs. $3.33 \pm 0.24$ $\mu$m, $P = 0.39$, Figure 3D) and their duration ($43.6 \pm 5.5$ vs. $57.2 \pm 3.9$, Figure 3E, $P = 0.08$; $n$ sparks/patients = $229/5$ vs. $253/8$ each) were not different between both sexes. In sum, the SR Ca$^{2+}$ leak of female patients only reached approximately one-third of the Ca$^{2+}$ leak of men with equally compromised cardiac function (difference $69 \pm 10\%$, $n$ cells/patients = $85/5$ vs. $44/8$, $P < 0.05$, Figure 3F). Importantly, this pronounced difference translated into a markedly increased ratio of arrhythmic cells from male patients compared with female patients with HF (36 of 80 male vs. 17 of 95 female, $P < 0.001$, Figure 3H).

**Sex-dependent development of the sarcoplasmic reticulum Ca$^{2+}$ leak in the transition from cardiac hypertrophy to heart failure**

The data presented above reveal distinct differences in the dysregulation of diastolic RyR2 closure between women and men. To find out, how the diastolic SR Ca$^{2+}$ leak changes in the transition from Hy to HF in both sexes, we compared both cardiac pathologies in women and men. As shown in Figure 4, women with end-stage HF are more affected by diastolic RyR2 leakiness than women with cardiac Hy. While the CaSpF is significantly increased in female HF compared with Hy (1.14 ± 0.22 vs. 0.40 ± 0.08 $\times$ $100 \mu$m$^{-1}$s$^{-1}$, $n$ cells/patients = $85/5$ vs. $83/9$, $P < 0.05$, Figure 4A and B), Ca$^{2+}$ spark duration, amplitude, and width are not significantly different between HF and Hy ($n = 229/5$ vs. $88/9$ each, Figure 4C–E).
**Figure 3** Sex dependence of the SR Ca$^{2+}$ leak in end-stage HF. (A) Representative confocal line scans, upper lane: female patient, lower lane: male patient. Women with end-stage HF showed (B) a significantly lower CaSpF ($P < 0.05$) than men ($n$ cells/patients = 85/5 vs. 44/8). The detected sparks had (C) a lower amplitude ($P < 0.05$), while (D) the width ($P = 0.39$) and (E) duration ($P = 0.08$) did not differ significantly ($n$ sparks/patients = 229/5 vs. 156/7). (F) The calculated SR Ca$^{2+}$ leak ($\text{CaSpF} \times \text{amplitude} \times \text{width} \times \text{duration}$) was markedly lower in women than in men with HF ($P < 0.05$, $n$ cells/patients = 85/5 vs. 44/8) translating into a lower ratio of CMs showing arrhythmic events (G) in men compared with women with HF ($H, P < 0.001$). The absolute column height represents the total number of measured cells. The height of the coloured part of this column represents the number of arrhythmic cells.

**Figure 4** Development of the SR Ca$^{2+}$ leak in the transition from Hy to HF in women. (A) Representative confocal line scans of isolated CMs from women with either Hy (upper lane) or HF (lower lane). (B) The CaSpF was increased in HF compared with Hy ($n$ cells/patients = 85/5 vs. 83/9, $P < 0.05$), while (C) spark amplitude ($P = 0.06$), (D) width ($P = 0.33$), and (E) duration ($P = 0.09$) were not significantly altered ($n$ sparks/patients = 229/5 vs. 88/9). The resulting difference of calculated SR Ca$^{2+}$ leak ($\text{CaSpF} \times \text{amplitude} \times \text{width} \times \text{duration}$) did not reach significance ($F, n$ cells/patients = 85/5 vs. 83/9, $P = 0.06$). (G) Representative confocal line scans of spontaneous Ca$^{2+}$ waves in female patients with Hy (upper lane) and HF (lower lane). The ratio of arrhythmic cells was not significantly different ($H, P = 0.08$). The absolute column height represents the total number of measured cells. The height of the coloured part of this column represents the number of arrhythmic cells.
sum, the calculated diastolic SR Ca\textsuperscript{2+} leak does not significantly increase in the transition from afterload-induced Hy to HF in women (n cells/patients = 83/9 vs. 85/5, P = 0.06, Figure 4F). Furthermore, the ratio of cells showing major arrhythmic events is not significantly elevated in female HF compared with Hy (34 of 119 in Hy vs. 17 of 95 in HF, P = 0.08, Figure 4H).

In contrast, a more pronounced increase of diastolic SR Ca\textsuperscript{2+} leak could be observed in the group of male patients. Cardiomyocytes from men with HF displayed a significantly higher CaSpF than in compensated Hy (2.25 ± 0.33 vs. 1.14 ± 0.22 × 100 µm\textsuperscript{-1}s\textsuperscript{-1}, n cells/patients = 44/8 vs. 75/6, P < 0.01, Figure 5A and B). Furthermore, the amplitude of Ca\textsuperscript{2+} sparks was significantly increased in HF (1.82 ± 0.06 vs. 1.64 ± 0.01, n sparks/patients = 253/8 vs. 190/6, P < 0.05, Figure 5C) while spark width and duration were not significantly changed (n sparks/patients = 253/8 vs. 190/6, Figure 5D and E). This translates into a robust increase of the diastolic SR Ca\textsuperscript{2+} leak in male patients in the transition from Hy to HF by ∼233% (n cells/patients = 75/6 vs. 44/8, P < 0.05, Figure 5F). The ratio of arrhythmic cells (Figure 5G) was also prominently increased in HF compared with Hy although this did not reach statistical significance (36 of 80 vs. 31 of 99, P = 0.06, Figure 5G and H).

**Discussion**

The current study reveals for the first time that there is a pronounced difference between both sexes with regard to the dysregulation of diastolic RyR2 closure in human cardiac disease. We show that (i) there is a lower diastolic CaSpF in women than in men already at the stage of afterload-induced cardiac Hy with still preserved EF and that (ii) this sex dissimilarity is even more pronounced in HF. This leads to (iii) a markedly lower SR Ca\textsuperscript{2+} leak in women with end-stage HF compared with men translating into a smaller ratio of CMs showing major arrhythmic events in HF (H) did not reach significance (P = 0.06). The absolute column height represents the total number of measured cells. The height of the coloured part of this column represents the number of arrhythmic cells.

These findings gain clinical relevance as the differences in the SR Ca\textsuperscript{2+} leak may underline the fact that women with HF less frequently suffer from malignant ventricular arrhythmias than men. This has been observed in several clinical studies\textsuperscript{3,7,15} but the underlying mechanisms have not been identified. Data from animal models showed that CMs from healthy female rats have smaller systolic Ca\textsuperscript{2+} transients and smaller and shorter diastolic Ca\textsuperscript{2+} sparks than their male littermates.\textsuperscript{8} However, the differences in Ca\textsuperscript{2+} cycling properties seem to be species-dependent and remain controversial. Whereas some studies did not detect sex-dependent differences as to the SR Ca\textsuperscript{2+} load in rat and rabbit ventricular CMs,\textsuperscript{8,16} other studies revealed a higher SR Ca\textsuperscript{2+} load in CMs of male healthy cats compared with females.\textsuperscript{17} Furthermore, it was shown that early ovariectomy in mice leads to increased SR Ca\textsuperscript{2+} loading and an elevated SR Ca\textsuperscript{2+} leak suggesting sex hormone-related mechanisms.\textsuperscript{18} In line with lower systolic Ca\textsuperscript{2+} transients in females, weaker systolic contractions were detected in several studies using rat ventricular CMs.\textsuperscript{8,19} However, other...
studies contradicted these results and observed similar contractions of CMs from female and male rats and mice. It was postulated that an increased myofibrillar ATPase activity in female rats might compensate for lower systolic Ca\textsuperscript{2+} transients. Of note, the experimental data evaluating sex differences in cellular Ca\textsuperscript{2+} homeostasis (see above) have been derived from healthy animals. To the best of our knowledge, functional data from diseased animals or human pathologies have not yet been performed. Our results only in part confirm the data derived from healthy animals and shed new light on sex-dependent differences in Ca\textsuperscript{2+} cycling in human diseased myocardium. We also detected a lower diastolic CaSpF in women, already at the stage of cardiac Hy with still preserved EF. This suggests that women may, indeed, harbour a very robust diastolic RyR2 closure at baseline which cannot easily be disturbed. Of note, we did not detect significant differences in diastolic Ca\textsuperscript{2+} levels or systolic Ca\textsuperscript{2+} transients between both sexes in Hy. The SR Ca\textsuperscript{2+} load was nominally higher in male patients, although this did not reach statistical significance (P = 0.14). One might speculate that a sex-dependent difference of SR Ca\textsuperscript{2+} loading could be present but the limited number of measurements and the inherent distribution of values did not allow definite results. A sex-dependent shift of SERCA2a/NCX balance towards SERCA2a activity may enhance SR Ca\textsuperscript{2+} storage in men with Hy and thereby facilitate the arrhythmogenic SR Ca\textsuperscript{2+} leak. However, it has repeatedly been shown in animal models that an increased activity of SERCA2a may also exert antiarrhythmic effects, possibly by a sequestration of spontaneous Ca\textsuperscript{2+} release events and an inhibition of cell wide Ca\textsuperscript{2+} wave propagation. This mechanism may also contribute to the finding that the increased CaSpF in male patients with Hy, where SERCA2a expression is presumably conserved, does not translate into an increased frequency of cellular arrhythmias. If and how differences in SERCA2a activity contribute to the sex-dependent differences in SR Ca\textsuperscript{2+} leak and the different propensity towards arrhythmias merits further investigation. Similar diastolic Ca\textsuperscript{2+} levels in both sexes in Hy despite marked differences in the SR Ca\textsuperscript{2+} leak confirm the capacity of SERCA2a to quickly eliminate cytoplasmic Ca\textsuperscript{2+} and render the hypothesis that the increased diastolic open probability of RyR2-clusters in male patients might be explained by an enhanced trigger of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (diastolic Ca\textsuperscript{2+} levels) unlikely. As the cytosolic Ca\textsuperscript{2+} elimination is also dependent on the re-influx via voltage dependent L-type Ca\textsuperscript{2+} channels and the electrogenic ion exchange via NCX is known to be dependent on membrane potential, the sex-dependent differences in repolarization dynamics and action potential duration may also play a role. As we detected similar amplitudes of systolic Ca\textsuperscript{2+} transients in CMs of female and male patients with Hy, our study does not confirm the assumption of a reduced Ca\textsuperscript{2+} turnover in women as it was postulated on the basis of studies performed on healthy animals. Our data further show that the CaSpF increases in both sexes in the transition from afterload-induced Hy to HF, which may consequently lead to a depletion of SR Ca\textsuperscript{2+} stores and thus be a crucial factor for the deterioration of LV function. Although women likewise are affected by an increasing CaSpF in the progression of heart disease, this does by far not reach the absolute level observed in men. In line with this, the less robustly increased CaSpF of female patients with HF does not seem to be sufficient to significantly boost the occurrence of major cellular arrhythmic events compared with Hy (see Figure 3H). Of note, the diastolic CaSpF and the CaSpSs of CMs isolated from women with HF in our study were comparable with those observed in CMs from men with cardiac Hy and still preserved LV function (see Figures 3 and 4). In other words, women with end-stage HF undergoing heart transplantation had a ‘leak-phenotype’ of men with preserved systolic function. As it is known that LV function is a crucial factor determining the risk of patients to develop life-threatening arrhythmias, it can be stated that they had a ‘leak-phenotype’ of patients with a much lower arrhythmic burden. The detected differences in the SR Ca\textsuperscript{2+} leak may be an important cellular mechanism that protects women from arrhythmias and contributes to the ‘sex-paradox’. Our observations therefore support the rationale to develop therapeutic measures that reduce the SR Ca\textsuperscript{2+} leak as these could attenuate arrhythmogenicity.

Limitations

(i) Sex-dependent measurements of Ca\textsuperscript{2+} cycling properties were performed in Hy but could not be performed in human HF. As distinct alterations of Ca\textsuperscript{2+} cycling proteins, like a decreased expression and activity of SERCA2a and an increased phosphorylation of RyR2, occur in the course of a deterioration of cardiac function, different mechanisms may contribute to sex-dependent Ca\textsuperscript{2+} alterations in HF. Furthermore, the differences in Ca\textsuperscript{2+} buffer capacity between both sexes may influence caffeine-induced Ca\textsuperscript{2+} transients serving as a measure of SR Ca\textsuperscript{2+} load in this manuscript. (ii) A decreased inward rectifying current (IK\textsubscript{1}) synergistically interacts with compromised RyR2 closure in HF in respect to the initiation of ventricular arrhythmias. Furthermore, influences of sex hormones on L-type Ca\textsuperscript{2+} currents have been described. The evaluation of sex-dependent differences in IK\textsubscript{1} and L-type Ca\textsuperscript{2+} currents may further elucidate the mechanisms underlying the different propensity of both sexes to life-threatening arrhythmias. (iii) Some of the female patients included in HF group were pre-menopausal. Confounding influences of cyclically increasing hormone concentrations cannot be excluded. (iv) Experiments were performed at room temperature as isolated human diseased CMs revealed to be more stable under this condition. The lower temperature compared with in vivo conditions might, however, influence Ca\textsuperscript{2+} levels and elimination kinetics. A validation of the presented results at body temperature may be useful to improve translatability. (v) The measured SR Ca\textsuperscript{2+} leak in the present study can be regarded as an accepted cellular arrhythmogenic trigger that can give rise to DADs and spontaneous action potentials. However, a direct correlation between SR Ca\textsuperscript{2+} leak and cellular proarrhythmia or whole heart arrhythmias has not been performed in the present study.

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References


