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TRPV4 increases cardiomyocyte calcium cycling and contractility yet contributes to damage in the aged heart following hypoosmotic stress

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Aims Cardiomyocyte Ca^{2+} homeostasis is altered with aging via poorly-understood mechanisms. The Transient Receptor Potential Vanilloid 4 (TRPV4) ion channel is an osmotically-activated Ca^{2+} channel, and there is limited information on the role of TRPV4 in cardiomyocytes. Our data show that TRPV4 protein expression increases in cardiomyocytes of the aged heart. The objective of this study was to examine the role of TRPV4 in cardiomyocyte Ca^{2+} homeostasis following hypoosmotic stress and to assess the contribution of TRPV4 to cardiac contractility and tissue damage following ischaemia–reperfusion (I/R), a pathological condition associated with cardiomyocyte osmotic stress.

Methods and results TRPV4 protein expression increased in cardiomyocytes of Aged (24–27 months) mice compared with Young (3–6 months) mice. Immunohistochemistry revealed TRPV4 localization to microtubules and the t-tubule network of cardiomyocytes of Aged mice, as well as in left ventricular myocardium of elderly patients undergoing surgical aortic valve replacement for aortic stenosis. Following hypoosmotic stress, cardiomyocytes of Aged, but not Young exhibited an increase in action-potential induced Ca^{2+} transients. This effect was mediated via increased sarcoplasmic reticulum Ca^{2+} content and facilitation of Ryanodine Receptor Ca^{2+} release and was prevented by TRPV4 antagonism (1 μmol/L HC067047). A similar hypoosmotic stress-induced facilitation of Ca^{2+} transients was observed in Young transgenic mice with inducible TRPV4 expression in cardiomyocytes. Following I/R, isolated hearts of Young mice with transgenic TRPV4 expression exhibited enhanced contractility vs. hearts of Young control mice. Similarly, hearts of Aged mice exhibited enhanced contractility vs. hearts of Aged TRPV4 knock-out (TRPV4-/-) mice. In Aged, pharmacological inhibition of TRPV4 (1 μmol/L, HC067047) prevented hypoosmotic stress-induced cardiomyocyte death and I/R-induced cardiac damage.

Conclusions Our findings provide a new mechanism for hypoosmotic stress-induced cardiomyocyte Ca^{2+} entry and cell damage in the aged heart. These finding have potential implications in treatment of elderly populations at increased risk of myocardial infarction and I/R injury.

Keywords Aging • TRP channel • Calcium overload • Calcium sparks • Ischaemia–reperfusion

1. Introduction

The functional properties of the heart change with advancing age in part due to alterations in cardiomyocyte excitation–contraction coupling (ECC).[1] ECC is a precisely co-ordinated series of cellular processes initiated by cardiomyocyte depolarization, which opens L-Type Ca^{2+} channels (LTCC) on the surface sarcolemma and triggers Ca^{2+} release from Ryanodine Receptor (RyR) channels of the sarcoplasmic reticulum (SR) Ca^{2+} store (for review, see ref.[2]). The subsequent elevation in $[Ca^{2+}]_i$ initiates myofilament force production, myocyte shortening, and

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cardiac systole. Cytosolic Ca^{2+} removal, and ensuing cardiac relaxation, occurs by SR Ca^{2+} reuptake via the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) and Ca^{2+} extrusion (in exchange for Na^{+}) via the Na^{+}/Ca^{2+} exchanger (NCX). During the cardiac cycle the amount of Ca^{2+} which enters the myocyte is typically extruded via NCX, and the amount of Ca^{2+} released from the SR is typically re-sequestered via SERCA. Enhanced Ca^{2+} influx mechanisms (e.g. via increased activity of LTCC during β-adrenergic stimulation) shift Ca^{2+} flux balance towards cellular and SR Ca^{2+} accumulation, with enhanced Ca^{2+} release during ECC and positive inotropic effects. Unfortunately, the increased cellular Ca^{2+} stress induced by such manoeuvres often associates with detrimental effects including increased risk of pro-arrhythmic SR Ca^{2+} release[3] and Ca^{2+}-dependent cell death.[4]

The Transient Receptor Potential (TRP) superfamily of non-selective cation channels has emerged as an important Ca^{2+} entry pathway in the cardiovascular system, and includes TRP members Ankyrin (TRPA), Canonical (TRPC), Melastatin (TRPM), Mucolipin (TRPML), Polycystin (TRPP), and Vanilloid (TRPV). Although not considered to be a prominent ion entry pathway during cardiomyocyte ECC, cation entry via TRPC,[5] TRPM,[6,7] and TRPV[8,9] family members alters Ca^{2+} homeostasis, cellular electrophysiology, bioenergetics, and contractile function. However, in diseased states and/or following neurohormonal activation excessive cardiomyocyte TRP channel activity induces pathological effects including cellular Ca^{2+} overload, myocyte death, hypertrophic remodelling, and arrhythmia.[10–14] The TRPV4 ion channel functions primarily as a Ca^{2+} influx channel (6:1: Ca^{2+}:Na^{+} permeability ratio[15]) and was originally described as a cellular osmosensor responsive to hypoosmotic stress.[16] Subsequent studies revealed a more diverse role for TRPV4 in cellular Ca^{2+} homeostasis, and TRPV4 is now believed to be a multi-modal ion channel responsive to cellular stimuli including mechanical stretch, temperature, shear stress, and intracellular signalling molecules.[17]

Considering that TRPV4 has a high single-channel conductance (50–100 pS[15]) and exhibits coupled-gating behaviour,[18] it represents an attractive ion channel target to prevent cellular Ca^{2+} overload and cell death.[19–21] Despite the expansive literature on TRPV4 in many muscle cell types, TRPV4 expression and function in adult cardiomyocytes remains unclear.[22,23] In this investigation, we test the hypothesis that TRPV4 contributes to cardiomyocyte Ca^{2+} entry and enhances cardiomyocyte ECC following hypoosmotic stress in the aged heart. We show that TRPV4 protein expression increases in cardiomyocytes of Aged (24–27 months) mice. Elevated expression of TRPV4 in cardiomyocytes, either endogenously in Aged mice or in Young mice with transgenic expression of TRPV4, enhances Ca^{2+} transients following hypoosmotic stress. Further, following ischaemia–reperfusion (I/R) injury (a pathological condition associated with pronounced osmotic stress on cardiomyocytes), TRPV4 contributes to hypercontractility and tissue damage. The TRPV4 ion channel may therefore represent a critical regulator of Ca^{2+} homeostasis in cardiomyocytes of the aged heart, and TRPV4 inhibition may provide benefit following myocardial infarction in elderly populations. Previous reports of this work have been presented in abstract form.[24,25]

2. Methods

2.1 Animals

Procedures were approved by the Animal Care and Use Committee at the University of Missouri and complied with all US and UK regulations involving animal experimentation. C57BL/6, TRPV4(-/-),[26] transgenic MerCreMer[27] × Tg(αMHC-loxP-mCherrySTOP-loxP-TRPV4)1td, and MerCreMer × Tg(CAG-loxP-STOP-loxP-TRPV4-mCherry)1td mice were studied at ages of 3–6 (defined as *Young*) or 24–27 months (defined as *Aged*). Mice were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg), and hearts were rapidly (∼30 s) excised for subsequent experimentation.

2.2 Human tissue samples

Myocardial tissue was obtained from the basal septum of the left ventricle in patients undergoing surgical aortic valve replacement for aortic stenosis. Immunohistochemistry was performed on formalin fixed, paraffin embedded specimens. Written informed consent was obtained from all patients under an Institutional Review Board approved protocol,[28] and conform to the declaration of Helsinki.

2.3 Solutions

Isosmotic (∼300 mOsm/L, *ISO*) Physiological Saline Solution (PSS) for isolated cardiomyocyte experimentation contained (in mmol/L): 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, 10 Hepes, pH 7.4 with NaOH. In experiments monitoring cardiomyocyte Ca^{2+} homeostasis, hypoosmotic (∼250 mOsm/L, *HYPO*) PSS contained the same (in mmol/L) but with a 25 mmol/L reduction in NaCl to 110 mmol/L, and hypoosmotic stress was induced by 40 min of pre-treatment with hypoosmotic PSS solution prior to return to isosmotic PSS for experimental procedures. For cardiomyocyte damage experiments, sustained hypoosmotic stress was achieved via a switch from a ∼300 mOsm/L solution containing (in mmol/L) 110 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, 10 Hepes, 50 Mannitol, pH 7.4 with NaOH, to a ∼250 mOsm/L solution of the same composition lacking Mannitol. Krebs-Henseleit buffer for Langendorff heart experimentation contained (in mmol/L): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11.1 Glucose, 0.4 Caprylic Acid, 1 Pyruvate, 0.5 Na EDTA, and 1.8 $CaCl_2$.

2.4 Immunoassays

Western blotting and immunohistochemistry were performed with anti-TRPV4 (1:100–1:500 Biorbyt, orb215251), anti-Caveolin-3 (1:200, Santa Cruz Biotech, SC-5310), and/or anti-α-tubulin (1:200, Cell Signalling 3873S) according to standard approaches (see Supplementary material online). An anti-CSQ primary antibody (1:1000 Thermo Fisher Scientific, PA1-913) was utilized for western blot normalization, while normal rabbit IgG (1:80, SC-2027, Santa Cruz Biotech) was utilized as a control for Immunohistochemistry. Antibody performance in immunoassays was validated using cardiomyocyte expression of a transgenic TRPV4-mCherry fusion protein in MerCreMer × Tg(CAG-loxP-STOP-loxP-TRPV4-mCherry)1td mice, with anti-TRPV4 (1:100, Biorbyt) and anti-mCherry (1:200, 16D7, #M11217, Thermo Fisher Scientific) antibodies. Live-cell fluorescence imaging was utilized to co-localize mCherry fluorescence with SiR Tubulin (Cytoskeleton, #CY-SC006) or CellMask Deep Red (Thermo Fisher Scientific, #C10046).

2.5 Cardiomyocyte functional experiments

Intracellular Ca^{2+} (fluo-4/AM or fluo-5F/AM) was monitored in electrically-stimulated (0.5 Hz) isolated cardiomyocytes at 25°C using laser-scanning confocal fluorescence microscopy.[29] For the cardiomyocyte damage assay, cells were exposed to hypoosmotic stress and electrically stimulated (1 Hz, 37°C) for 1 h to assess the percent of cells

exhibiting irreversible contracture and damage (see Supplementary material online for additional details).

2.6 Isolated heart experiments

Left ventricular pressure development was monitored using a 1 F tip Millar catheter in Langendorff perfused hearts (60 mmHg afterload, 37°C). Pressure development and rate were assessed at sinus rhythm under control conditions, during global ischaemia (45 min), and during reperfusion (2 h). A subset of hearts was perfused with 1% triphenyltetrazolium chloride (TTC) following reperfusion to assess metabolically active (red) vs. inactive (white) tissue. Pressure waveform selection criteria are described in detail in Supplementary material online.

2.7 Statistical analysis

All data are reported as individual observations or means ± standard error. Summary data were analysed using *t*-tests, two-way ANOVA, or two-way repeated measures ANOVA (Bonferroni *post hoc*) where appropriate based on experimental design. Data are reported as statistically significant at $P < 0.05$ (* or #), $P < 0.01$ (** or ##), and $P < 0.01$ (*** or ###) levels.

3. Results

TRPV4 protein expression was monitored using western blot analysis of isolated left-ventricular cardiomyocyte homogenates from Young and Aged C57BL/6 mice, and indicated a significant increase in TRPV4 protein in Aged (*Figure 1A and B*). Immunocytochemistry (*Figure 1C*) revealed subcellular localization of TRPV4 in perinuclear regions and along the microtubule network (colocalization with α-tubulin, *Figure 1D*), as well as within the t-tubule network (colocalization with Caveolin-3, *Figure 1E*). TRPV4 localization was confirmed in live cells via transgenic expression of a TRPV4-mCherry fusion protein (see Supplementary material online, *Figure S1A and B*), and colocalization was observed between mCherry and SiR Tubulin and mCherry and the membrane dye CellMask Deep Red (see Supplementary material online, *Figure S1C*). Similar to findings observed in Aged mice (*Figure 1F*), TRPV4 protein was also detected in left ventricular tissue sections of elderly patients ($n = 14$, 57–87 years of age) obtained from the basal septum during surgical aortic valve replacement (*Figure 1G*).

TRPV4 is well-described as a Ca^{2+} influx channel responsive to hypoosmotic stress.[16,17] We, therefore, explored the functional role of TRPV4 on action-potential induced Ca^{2+} transient amplitude (fluo-4/AM) following hypoosmotic stress. Cardiomyocytes of Aged (*Figure 2A and B*), but not Young (see Supplementary material online, *Figure S2*), responded to hypoosmotic stress with an enhancement in Ca^{2+} transient amplitude. The hypoosmotic stress-induced increase in Ca^{2+} transient amplitude observed in Aged associated with faster Ca^{2+} reuptake kinetics (see Supplementary material online, *Table S1*), and these effects were prevented by the TRPV4 antagonist HC067047 (HC, 1 μmol/L). The TRPV4 antagonist did not affect Ca^{2+} transient parameters in Aged in the absence of hypoosmotic stress, and was without effect in Young under any experimental condition.

In mouse ventricular cardiomyocytes the majority (~90%) of the Ca^{2+} transient is due to Ca^{2+} release from the SR via RyR release channels, which in turn is highly dependent on SR Ca^{2+} content.[30] To assess the mechanisms by which TRPV4 augments Ca^{2+} transients in Aged following hypoosmotic stress, we monitored the frequency of spontaneous RyR Ca^{2+} sparks following rest from action-potential stimulation. Ca^{2+} spark frequency was low under isosmotic conditions (*Figure 2C*, upper) but increased following hypoosmotic stress (*Figure 2C*, lower). The increase in Ca^{2+} sparks observed following hypoosmotic stress was prevented by TRPV4 inhibition with HC067047 (*Figure 2D*). We next assessed if SR Ca^{2+} content contributes to enhanced SR Ca^{2+} release by monitoring the amplitude of the 10 mmol/L caffeine-induced Ca^{2+} transient in Aged cardiomyocytes loaded with the moderate-affinity Ca^{2+} indicator fluo-5F. The amplitude of the caffeine-induced fluo-5F Ca^{2+} transient (*Figure 2E*, denoted by bars) was elevated following hypoosmotic compared with isosmotic conditions, and this effect was prevented by TRPV4 inhibition with HC067047 (*Figure 2F*). Taken together, these data suggest that TRPV4 contributes to cellular and SR Ca^{2+} loading following hypoosmotic stress, with a subsequent increase in SR Ca^{2+} release during ECC.

Advancing age associates with numerous alterations in Ca^{2+} handling protein expression and function. We, therefore, evaluated the effect of increased TRPV4 expression on cardiomyocyte Ca^{2+} homeostasis, independent of advancing age, using a double-transgenic (DTg), Tg(αMHC-loxP-mCherrySTOP-loxP-TRPV4)1td × αMHC-MerCreMer mouse (*Figure 3A*) with tamoxifen-inducible, cardiac-specific TRPV4 expression. Induction of TRPV4 transgene expression in Young mice resulted in TRPV4 protein levels ~2-fold greater than those observed endogenously in Aged mice (*Figure 3D*). Consistent with observations in Aged C57BL/6 mice, cardiomyocytes of Young DTg mice exhibited an increase in Ca^{2+} transient amplitude following hypoosmotic stress, which was prevented by TRPV4 inhibition (*Figure 3E and F*). These effects were absent in Young DTg mice not fed tamoxifen, and absent in tamoxifen-fed αMHC-MerCreMer single transgenic mice (combined controls $\Delta F/F_0$, ISO: 1.78 ± 0.11 vs. $\Delta F/F_0$, HYPO: 1.78 ± 0.07).

A pathological scenario associated with pronounced cardiomyocyte hypoosmotic stress is I/R injury.[31] Therefore, we examined the role of TRPV4 in pressure development and contractility (dP/dt_{Max}) following global I/R in isolated perfused hearts of Young C57BL/6 vs. Young DTg mice, as well as Aged C57BL/6 vs. Aged TRPV4(-/-) mice. Hearts of Young C57BL/6 and Young DTg mice had similar baseline contractile performance (*Figure 4*, *Control*), and as expected each ceased pressure development during global ischaemia (*Figure 4A and B*, *Ischaemia*). However, in the early phases of reperfusion (<30 min), hearts of Young DTg mice exhibited enhanced contractile function vs. Young C57BL/6 mice (*Figure 4*, *Reperfusion*). Hearts of Aged C57BL/6 and Aged TRPV4(-/-) mice also had similar baseline contractile performance (*Figure 5*, *Control*) and ceased pressure development during global ischaemia (*Figure 5A and B*, *Ischaemia*). In early reperfusion (<30 min) hearts of Aged C57BL/6 mice exhibited enhanced contractile function vs. Aged TRPV4(-/-) mice (*Figure 5*, *Reperfusion*). In addition, the reperfusion-induced change in contractile function in Aged C57BL/6 was prevented by TRPV4 inhibition with 1 μmol/L HC067047 (see Supplementary material online, *Figure S3*). The enhanced contractile function observed in early reperfusion in Young DTg and Aged mice was not sustained 2 h after reperfusion (see Supplementary material online, *Table S2*). Taken together, these data identify cardiomyocyte TRPV4 as a novel mediator of enhanced contractile function early in I/R.

Excessive Ca^{2+} entry into cardiomyocytes is an established cause of cardiomyocyte necrosis and tissue damage following I/R.[4] We, therefore, examined if TRPV4 inhibition reduces hypoosmotic stress-induced damage in isolated cardiomyocytes and I/R-induced cardiac damage in Langendorff-perfused hearts of Aged mice (corresponding to the population at high risk of myocardial infarction). In isolated cardiomyocytes of Aged mice, application of hypoosmotic stress resulted in 50% of cells exhibiting damage within one hour. When cardiomyocytes were treated

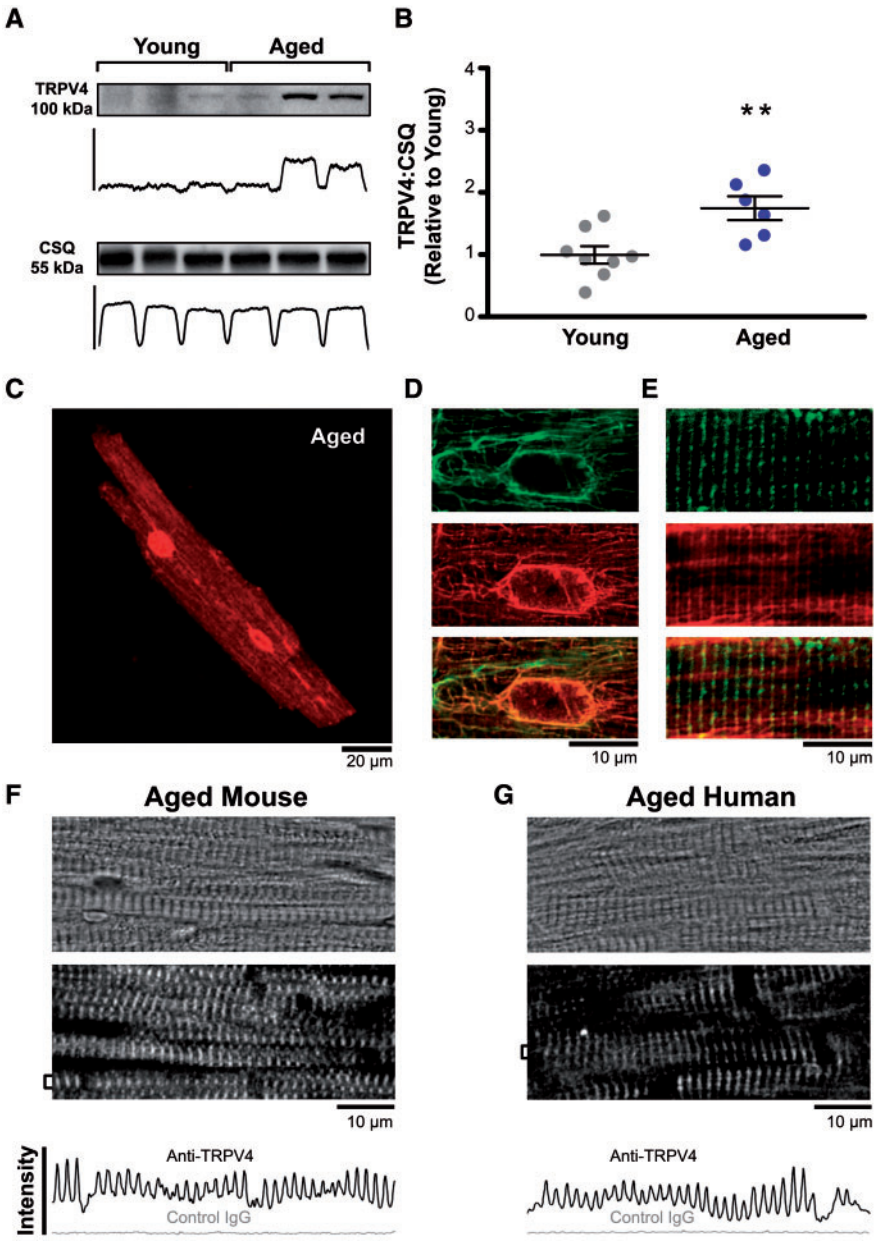

Figure 1 TRPV4 expression in cardiomyocytes of the aged heart. (*A*) Example western blots of isolated cardiomyocyte homogenates of 3 Young (left 3 samples) and 3 Aged (right 3 samples) mice, probed with anti-TRPV4 (~100 kDa, upper) and anti-calsequestrin (CSQ, ~55 kDa, lower) antibodies. Densitometry profiles (background-subtracted using regions below bands of interest, with plots inverted for presentation, scale bar = 150 AU) are presented below images. (*B*) TRPV4:CSQ ratio (relative to levels in Young) in $n = 8$ Young mice (gray) and $n = 6$ Aged mice (blue). (*C–E*) Representative immunocytochemistry image of cardiomyocyte (*C*) or subcellular cardiomyocyte regions (*D*, *E*, microscope zoom) of Aged mice using antibodies for TRPV4 (*C–E*, red), α-tubulin (*D*, upper panel in green from perinuclear region) and Caveolin-3 (*E*, upper panel in green from sub-sarcolemmal region). Merge of green and red channels in *D–E* are presented in bottom panels, with yellow indicating colocalization. (*F–G*) Transmitted light (upper panels) and TRPV4 immunofluorescence (lower panels) images of fixed left ventricular tissue from an Aged Mouse (*F*) and a 68-year-old patient undergoing aortic valve replacement surgery (*G*). TRPV4 fluorescence intensity (black traces, from region denoted by bracket to left of images) is shown below images, alongside fluorescence intensity of IgG control obtained from duplicate section on each slide (gray traces). Intensity scale bar = 2000 arbitrary units. A similar staining pattern was observed in $n = 13$ additional left ventricular samples from both male and female patients, ages 57–87, with a range in ejection fraction between 34% and 76%. $^{**}P<0.01$ Aged vs. Young.

with the TRPV4 antagonist HC067047 only 10% of cells exhibited significant damage (*Figure 6A and B*). Langendorff-perfused hearts were subjected to global I/R (45 min ischaemia, 2 h of reperfusion) and perfused with TTC to differentiate metabolically active vs. inactive tissue (*Figure 6C*), and under these conditions hearts of Aged mice exhibited a 20% loss of viable cardiac tissue. In contrast, hearts of Aged mice treated with the TRPV4 antagonist HC067047 exhibited only a 6% loss of viable tissue (*Figure 6D*). Taken together, these data are consistent with TRPV4

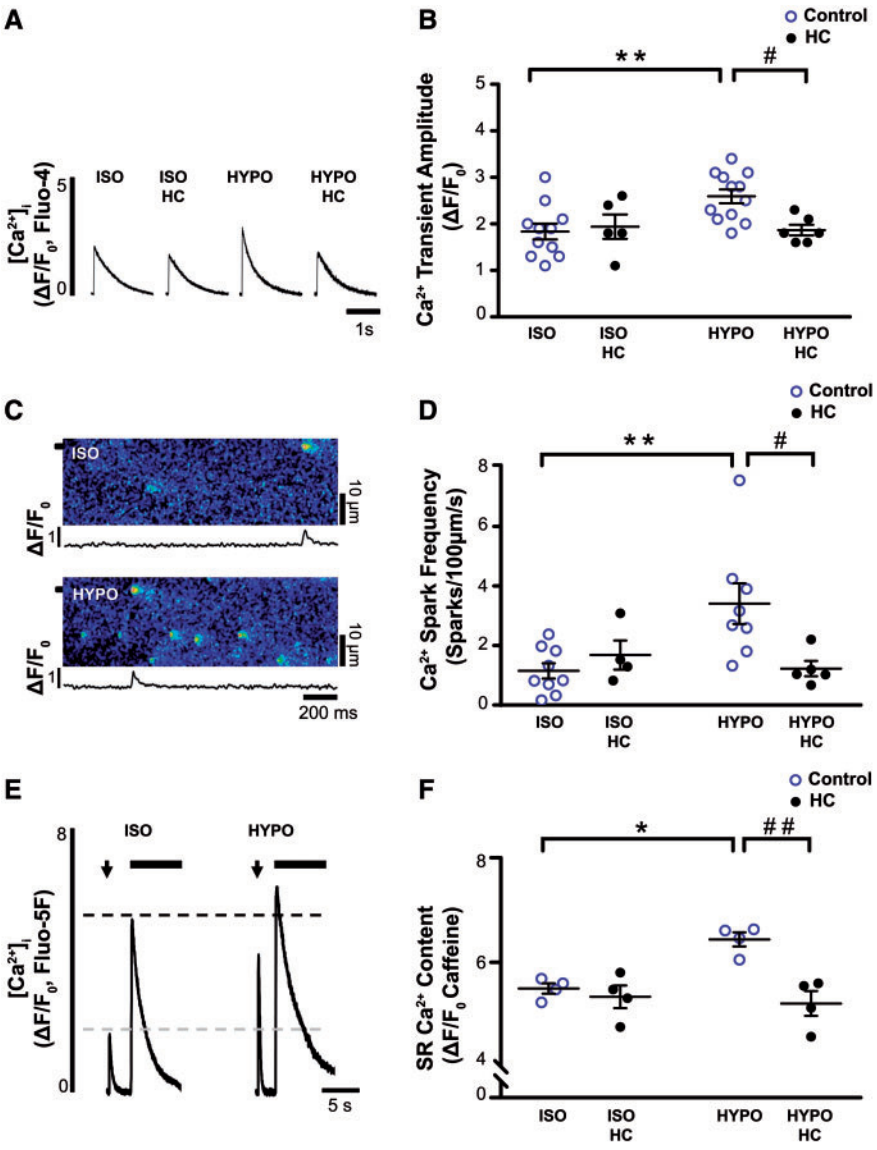

Figure 2 TRPV4 activation enhances Ca^{2+} transients via increased SR Ca^{2+} content and activation of RyR Ca^{2+} release. (*A*) Example Ca^{2+} transient traces (*A*) and summary data of Ca^{2+} transient amplitude (*B*) of electrically stimulated (0.5 Hz) cardiomyocytes of Aged mice. Ca^{2+} transients were examined under isomotic conditions (ISO: ~300 mOsm/L) and following transient hypoosmotic stress (HYPO: ~250 mOsm/L, followed by a return to isosmotic conditions for criterion measurement) in the absence (blue, open) and presence (black, closed) of the TRPV4 antagonist HC067047 (HC, 1 μmol/L). (*C*) Example line-scan Ca^{2+} spark images in a cardiomyocyte of Aged under isosmotic conditions (ISO, upper panel) and following hypoosmotic stress (HYPO, lower panel). Fluorescence profiles of Ca^{2+} sparks (from regions indicated by bars to left of images) are presented below images. (*D*) Summary data of Ca^{2+} spark frequency (sparks × 100 μm^{-1} × s^{-1}) under control conditions (ISO) and following transient hypoosmotic stress (HYPO, followed by a return to isosmotic conditions for criterion measurement) in the absence (blue, open) and presence (black, closed) of the TRPV4 antagonist HC067047 (HC, 1 μmol/L). (*E*) Example fluo-5F action potential-induced Ca^{2+} transient (arrow) followed by 10 mmol/L caffeine-induced Ca^{2+} transient (bar) in cardiomyocytes of Aged under isosmotic conditions (ISO, left) and following hypoosmotic stress (HYPO, right). (*F*) Summary data of SR Ca^{2+} content ($\Delta F/F_{0,\,Caffeine}$) under control conditions (ISO) and following transient hypoosmotic stress (HYPO, followed by a return to isosmotic conditions for criterion measurement) in the absence (blue, open) and presence (black, closed) of the TRPV4 antagonist HC067047 (HC, 1 μmol/L). Two-way ANOVA revealed a significant interaction between osmotic conditions and antagonist treatment in Aged cardiomyocytes ($P < 0.05$). $^{*}P < 0.05$ or $^{**}P < 0.01$ HYPO vs ISO within control; $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ antagonist treatment vs. control within HYPO. Number of cells (*n*) from number of animals (*N*) are as follows: Aged ISO $n = 23/N = 11$; Aged ISO + HC: $n = 18/N = 5$; Aged HYPO: $n = 36/N = 12$; Aged HYPO + HC: $n = 20/N = 6$ (Ca^{2+} transients), Aged ISO $n = 11/N = 9$; Aged ISO + HC: $n = 13/N = 4$; Aged HYPO: $n = 13/N = 8$; Aged HYPO + HC: $n = 13/N = 5$ (Ca^{2+} sparks), and Aged ISO $n = 12/N = 4$; Aged ISO + HC: $n = 10/N = 4$; Aged HYPO: $n = 15/N = 4$; Aged HYPO + HC: $n = 12/N = 4$ (SR Ca^{2+} content).

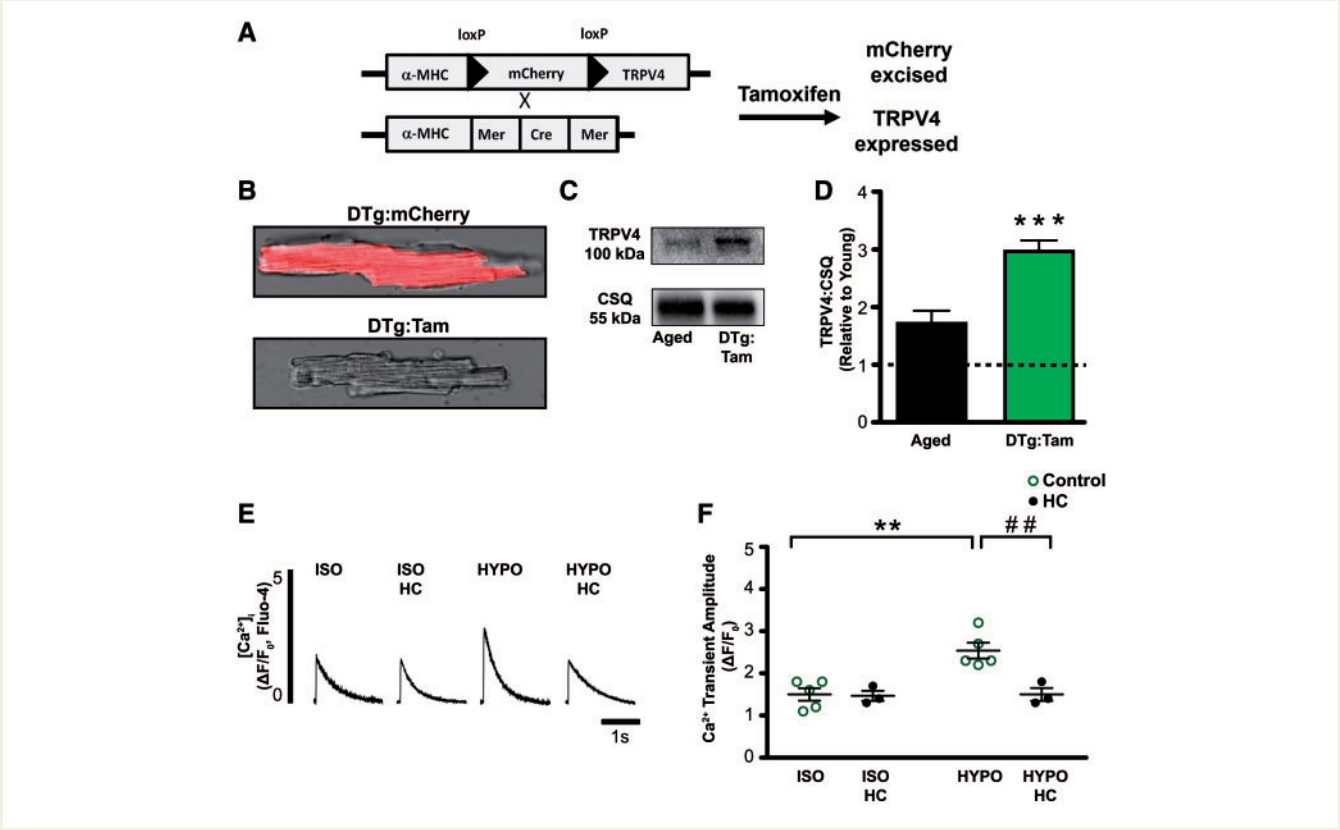

Figure 3 Transgenic TRPV4 expression in Young mice enhances cardiomyocyte Ca^{2+} transients following hypoosmotic stress. (*A*) Schematic of double-transgenic (DTg) mouse line with the Tg(αMHC-loxP-mCherrySTOP-loxP-TRPV4)1td (upper) and αMHC-MerCreMer (lower) transgenes. In the absence of tamoxifen, the α-MHC promoter drives mCherry expression (with STOP sequence) within cardiomyocytes (*B*, upper, *DTg:mCherry*, overlay of mCherry fluorescence and transmitted light image). With tamoxifen treatment, Cre-recombinase excises the mCherry(STOP) sequence thereby eliminating mCherry expression (*B*, lower, *DTg:Tam*, overlay of mCherry fluorescence and transmitted light image), and inducing cardiac specific TRPV4 expression. (*C*) Example western blots of isolated cardiomyocyte homogenates of an Aged and DTg:Tam mouse, probed with anti-TRPV4 (~100 kDa, upper) and anti-calsequestrin (CSQ, ~55 kDa, lower) antibodies. (*D*) Summary data of TRPV4 expression in cardiomyocytes of DTg:Tam mice (green bar, $n = 3$) compared with levels observed endogenously in Aged mice (black bar, $n = 6$, data from *Figure 1B*). Expression level in Young C57BL/6 mice used for normalization is indicated by dashed line. $^{***}P < 0.001$ DTg:Tam vs. Aged. (*E–F*) Example Ca^{2+} transient traces (*E*) and summary data of Ca^{2+} transient amplitude (*F*) of electrically stimulated (0.5 Hz) cardiomyocytes of Young DTg:Tam mice examined under control conditions (ISO: ~300 mOsm/L) and following transient hypoosmotic stress (HYPO: ~250 mOsm/L, followed by a return to isosmotic conditions for criterion measurement) in the absence (green, open) and presence (black, closed) of the TRPV4 antagonist HC067047 (1 μmol/L). Control groups associated with the MerCreMer system exhibited Ca^{2+} transient amplitudes ($\Delta F/F_0$, HYPO: 1.78 ± 0.07 and $\Delta F/F_0$, ISO: 1.78 ± 0.11) similar to those observed in Young C57BL/6 mice (see Supplementary material online, *Figure S2*). Two-way ANOVA revealed a significant interaction between osmotic conditions and antagonist treatment in Young DTg:Tam cardiomyocytes ($P < 0.05$). $^{**}P < 0.01$ HYPO vs. ISO within control; $^{\#\#}P < 0.01$ antagonist treatment vs. control within HYPO. Number of cells (n) from number of animals (N) are as follows: Dtg:Tam 300 $n = 13/N = 5$; Dtg:Tam 300 + HC: $n = 4/N = 3$; Dtg:Tam 250: $n = 17/N = 5$; Dtg:Tam 250 + HC: $n = 11/N = 3$.

exerting an initial beneficial effect of enhanced Ca^{2+} cycling and contractility, with a secondary detrimental effect of excessive cardiomyocyte Ca^{2+} stress and damage following I/R in the aged heart.

4. Discussion

4.1 TRPV4 channel expression in cardiomyocytes

The TRP ion channel superfamily has recently been appreciated as a prominent mediator of signal transduction within cardiomyocytes, and the present investigation adds to this emerging literature by providing the first description of the functional role of TRPV4 in cardiomyocytes of the aged heart. While organ-level TRPV4 expression in the heart is well-established, cell types known to express TRPV4 (including cardiac fibroblasts, vascular endothelial cells, and vascular smooth muscle cells) collectively far outnumber cardiomyocytes within the heart, and therefore, expression of TRPV4 in cardiomyocytes remains unclear.[22,32] Recent data in whole-heart homogenates of young mice subjected to pressure overload[33] or ischaemia–reperfusion injury[34,35] show an increase in TRPV4 expression following pathological stimuli. While these findings suggest a change in TRPV4 expression in cardiomyocytes, changes in TRPV4 expression in other cell types (most notably fibroblasts[36]) may also underlie such findings. Our data reveal that with advancing age TRPV4 expression and function increases and exerts significant effects on cardiomyocyte Ca^{2+} homeostasis and contractile function. Further,

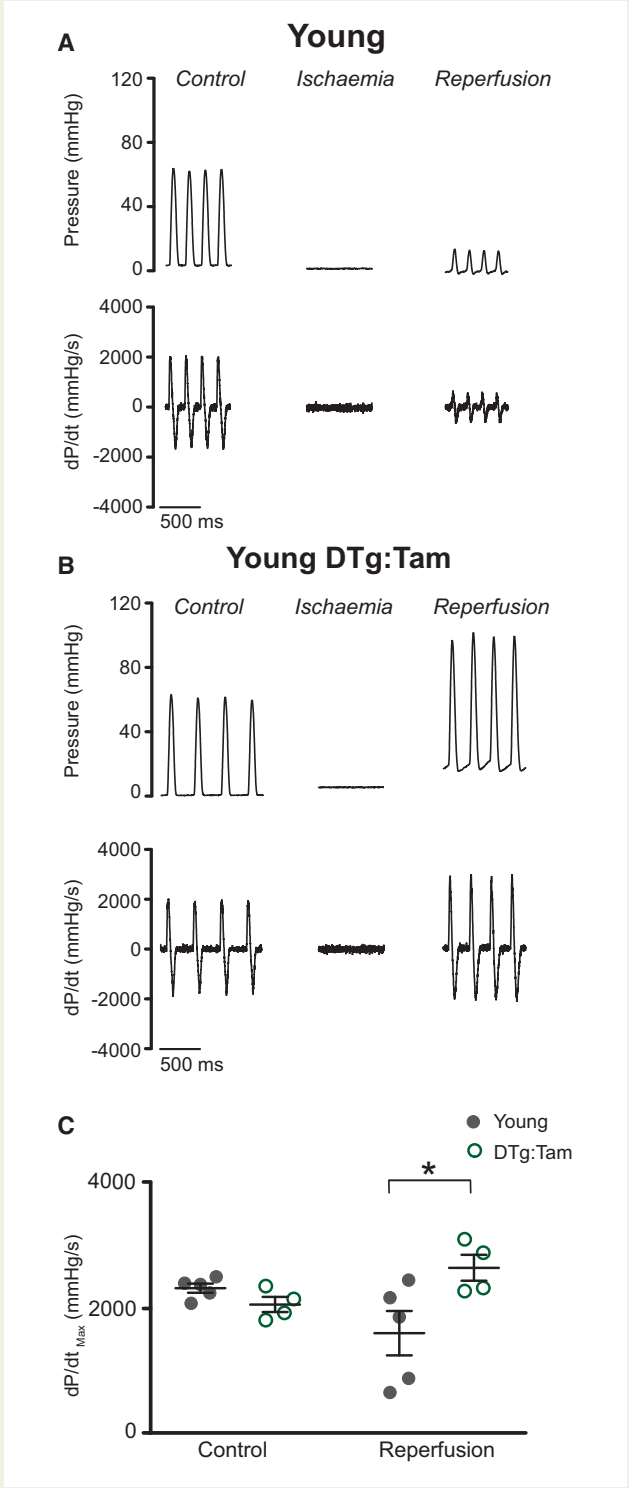

Figure 4 Transgenic TRPV4 expression enhances contractility following I/R in Young hearts. (*A* and *B*) Example traces of left ventricular pressure (upper) and rate of pressure change (d*P*/dt, lower) in hearts of Young (*A*) and Young tamoxifen-fed DTg (DTg:Tam) mice (*B*) under control conditions, during ischaemia, and following reperfusion. Contractility (dP/dt_{Max}, *C*) was elevated in hearts of Young tamoxifen-fed DTg mice (green, open) vs. Young mice (gray, closed) during reperfusion. Two-way repeated measures ANOVA revealed a significant interaction between conditions and genotype ($P < 0.05$). $^{*}P < 0.05$ Young DTg:Tam vs. Young within reperfusion. Young $n = 5$; Young DTg:Tam $n = 4$.

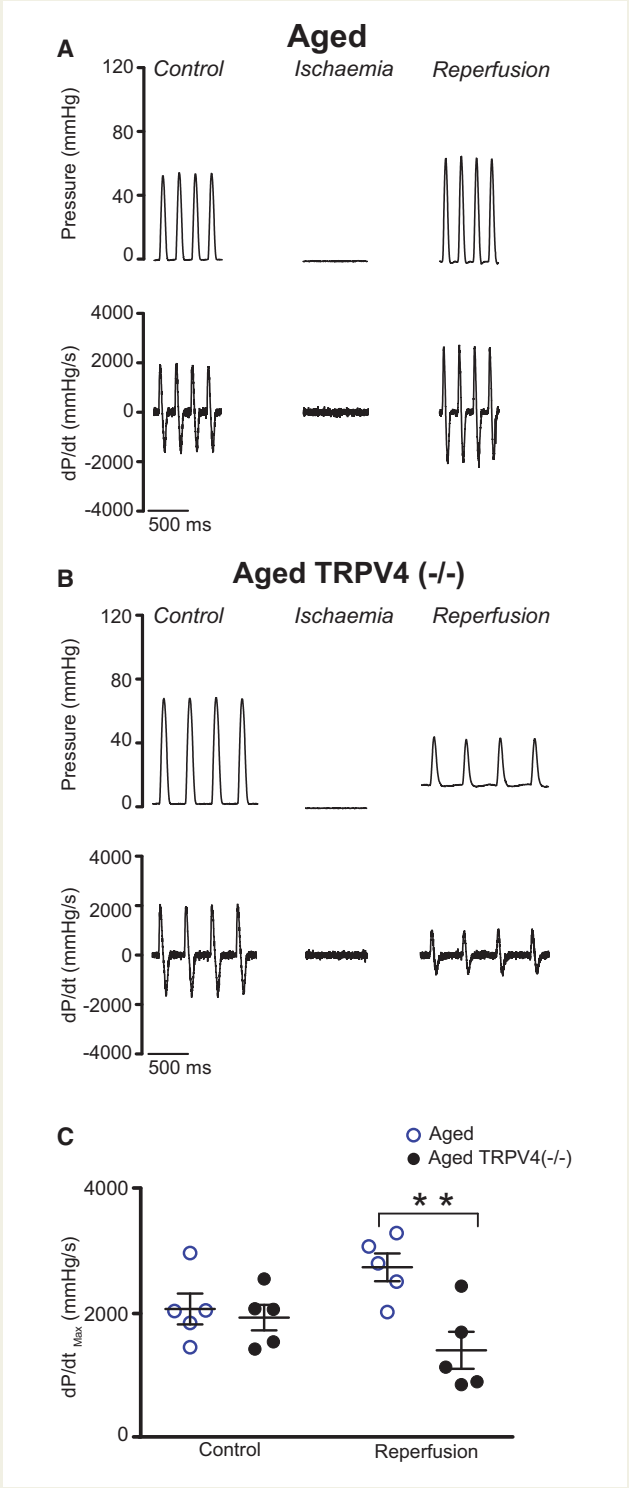

Figure 5 TRPV4 enhances contractility following I/R in Aged hearts. (*A* and *B*) Example traces of left ventricular pressure (upper) and rate of pressure change (d*P*/dt, lower) in hearts of Aged (*A*) and Aged TRPV4(-/-) mice (*B*) under control conditions, during ischaemia, and following reperfusion. Contractility (dP/dt_{Max}, *C*) was elevated in hearts of Aged (blue, open) vs. Aged TRPV4(-/-) mice (black, closed) during reperfusion. Two-way repeated measures ANOVA revealed a significant interaction between conditions and genotype ($P < 0.05$). $^{**}P < 0.01$ Aged TRPV4(-/-) vs. Aged within reperfusion. Aged C57BL/6 $n = 5$; Aged TRPV4(-/-) $n = 5$.

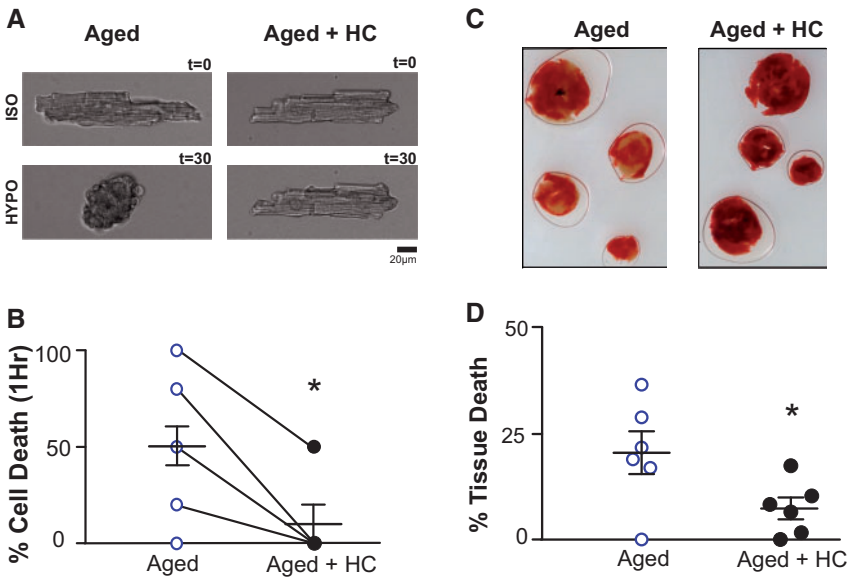

Figure 6 Pharmacological TRPV4 inhibition prevents cardiomyocyte damage following hypoosmotic stress and cardiac damage following I/R in Aged mice. (*A*) Example images of cardiomyocytes of Aged prior to (ISO, $t = 0$) and 30 min following sustained hypoosmotic stress (HYPO, $t = 30$), in the absence (left) and presence (right) of the TRPV4 inhibitor HC067047 (HC, 1 μmol/L). (*B*) Percent of cardiomyocytes that exhibited irreversible contracture and damage within one hour following hypoosmotic stress in the absence (blue, open) and presence (black, closed) of HC. Values for both untreated and treated groups were obtained from each animal using a paired design ($n = 5$, animal indicated by solid line); *P<0.05 Aged + HC vs. Aged (paired t-test). (*C*) Representative images of TTC-stained (red colour = live tissue) cardiac sections of hearts of Aged mice following I/R in the absence (left) and presence (right) of HC (1 μmol/L). (*B*) Summary data of percent tissue death in sections of Aged mice in the absence (blue, open) and presence (black, closed) of HC. *P < 0.05 Aged + HC vs. Aged (unpaired t-test), $n = 6$ per group.

our data in Young mice with transgenic TRPV4 expression indicate that increased TRPV4 expression in itself (i.e. independent of aging or disease processes) contributes to enhanced cardiomyocyte Ca^{2+} transients following hypoosmotic stress and hypercontractility following I/R.

4.2 TRPV4 enhances SR Ca^{2+} content and SR Ca^{2+} release during ECC

Although phylogenetically part of the TRP family of non-selective cation channels, TRPV4 functions primarily as a Ca^{2+} influx channel with a Ca^{2+}:Na^{+} permeability ratio of ~6:1.[15] Ca^{2+} influx via TRPV4 is substantial, due to both high single channel conductance (~90 pS) and Ca^{2+} signal amplification via co-operative gating behaviour.[18] Increased TRPV4 activity may also lead to cytosolic Na^{+} accumulation, either directly through the channel or secondary to the elevation in Ca^{2+} via enhanced diastolic forward-mode NCX activity. During systole, elevated cytosolic Na^{+} favours reverse-mode NCX Ca^{2+} entry at the peak of the action-potential, which increases the amplitude of the Ca^{2+} transient,[37] and such a mechanism has been proposed to underlie augmentation of ECC following TRPC channel activation.[38] TRPV4 therefore, represents a significant mode of Ca^{2+} entry that, similar to other TRP channel members,[9,39] may shift net Ca^{2+} flux towards cell and SR Ca^{2+} accumulation (*Figure 2F*). In turn, increased SR Ca^{2+} enhances Ca^{2+} transient amplitude (*Figure 2B*) according to the fundamental relationship between SR Ca^{2+} content and SR Ca^{2+} release[30] via SR luminal Ca^{2+} regulation of RyR activity.[40] Although we only examined acute activation of TRPV4 in the present investigation, sustained TRP channel activity may also lead to phosphorylation of RyR by Ca^{2+}/calmodulin-dependent protein kinase II,[39] resulting in SR Ca^{2+} leak and a cellular phenotype of enhanced RyR Ca^{2+} spark frequency with no change (or even a decrease) in SR Ca^{2+} content. Therefore, TRP channel activation may lead to complex time-dependent changes, with initial enhancement of ECC followed by a secondary deterioration in function.

4.3 TRPV4-mediated contractile function and cardiomyocyte death following I/R

Excessive cardiomyocyte Ca^{2+} entry and adverse Ca^{2+} overload during I/R are classically believed to be pH-driven processes (for reviews, see refs[41,42]). During ischaemia, cardiomyocytes shift energy production from oxidative phosphorylation to anaerobic glycolysis with concomitant lactic acid accumulation and intracellular acidification.[43] During reperfusion the extracellular environment is rapidly restored creating a large outward H^{+} gradient which drives sequential Na^{+}/H^{+} and Na^{+}/Ca^{2+} exchange and an elevation in intracellular Ca^{2+} (*Figure 7*, right). Our data in Aged mice reveal a novel osmolarity-induced Ca^{2+} signalling pathway via TRPV4 in cardiomyocytes (*Figure 7*, left). We propose that during ischaemia, both the intracellular and extracellular environment gradually develop an increase in osmolarity[31] yet osmotic stress on the cardiomyocyte membrane is minimal due to equilibration between the intracellular and extracellular compartments. During reperfusion, however, rapid washout of the extracellular fluid creates a marked hypoosmotic stress on the cardiomyocyte sarcolemma which activates TRPV4 and additional Ca^{2+} entry. The combination of pH-driven and osmolarity-driven Ca^{2+} entry leads to enhanced Ca^{2+} cycling and contractility in the early stages of reperfusion. However, the pronounced

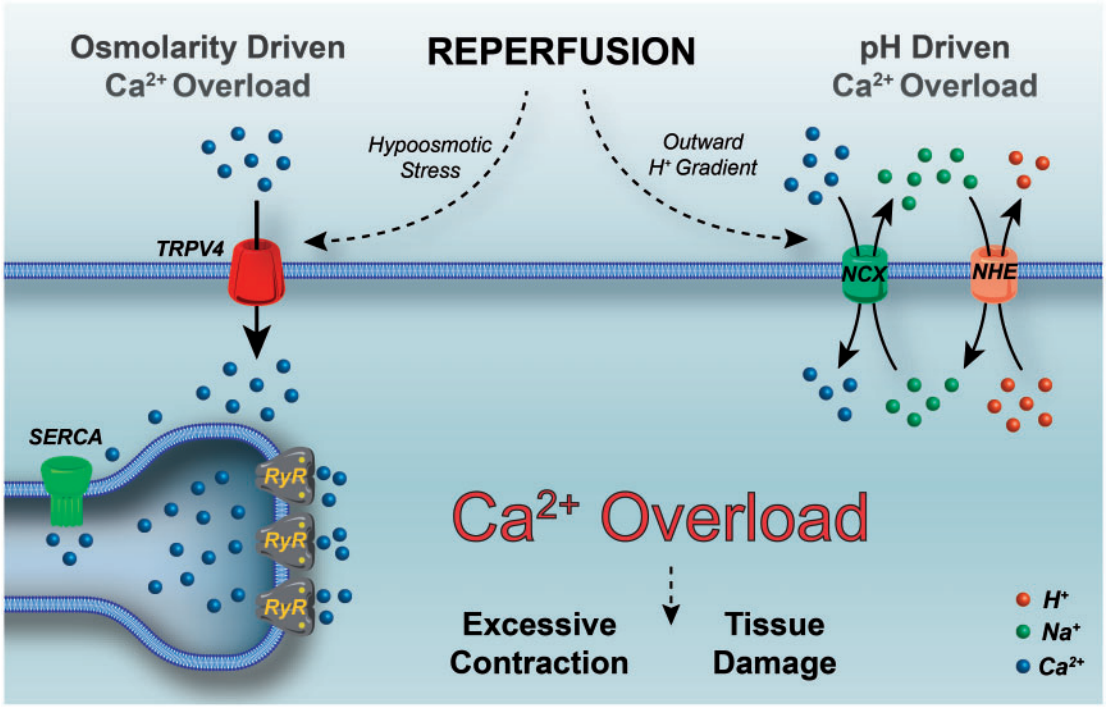

Figure 7 Model of Cardiomyocyte Ca^{2+} overload following I/R in the aged heart. Ca^{2+} overload in the Aged heart induced by combined osmolarity-driven (left) and pH driven (right) processes. During ischaemia intracellular acidosis, blunted ATP generation, and oxidative stress alters the function of ion channels, transporters, and ATPases. Upon reperfusion accumulated metabolites in the extracellular fluid are eliminated, producing both hypoosmotic stress (left) and a large gradient for cellular H^+ extrusion (right). Resulting TRPV4-mediated Ca^{2+} influx in combination with NHE/NCX coupled Ca^{2+}-entry leads to Ca^{2+} overload, excessive contractility, and irreversible myocyte damage.

cardiomyocyte Ca^{2+} overload and excessive contractility render the aged heart highly susceptible to cardiomyocyte damage following I/R. Exacerbating the excessive plasma membrane Ca^{2+} entry is dysfunctional SR Ca^{2+} cycling due to oxidative modification of SERCA and RyR, which makes SERCA less effective at re-sequestering Ca^{2+} and leads to SR Ca^{2+} leak via RyR channels. Collectively, enhanced Ca^{2+} entry processes and dysfunctional SR Ca^{2+} handling create a vicious cycle of Ca^{2+} overload, during which mitochondria accumulate excessive amounts of Ca^{2+} within the mitochondrial matrix leading to mitochondrial permeability transition (MPT), rapid dissipation of the mitochondrial proton gradient, and mitochondrial depolarization.[42] Further, Ca^{2+} overload-induced MPT ceases ATP production, leads to generation of reactive oxygen species, promotes mitochondrial swelling and rupture, and ultimately leads to cardiomyocyte necrosis and short-term cardiac dysfunction.[4] Consistent with this working model, TRPV4-mediated Ca^{2+} entry was recently shown to mediate reactive oxygen species production, MPT, mitochondrial depolarization, and cell death in cultured H9C2 cells following hypoxia-reoxygenation challenge.[20] Therefore, in the intact heart an initial increase in contractility is observed during early reperfusion due to a TRPV4-mediated enhancement of cardiomyocyte Ca^{2+} cycling (*Figure 2*) and contractile strength (*Figure 5*). However, the enhanced contractility is not maintained (see Supplementary material online, *Table S2*) due to cardiomyocyte death (*Figure 6A and B*) and cardiac damage (*Figure 6C and D*). In addition to the acute effects on cardiac dysfunction, cardiomyocyte death also activates an inflammatory response leading to a secondary phase of maladaptive remodelling and long-term cardiac dysfunction. This working model is consistent with the literature on short-term benefit/long-term harm with positive inotropes such as catecholamines. β-Adrenergic stimulation enhances Ca^{2+} cycling and contractility (a beneficial short-term effect) yet activation of this signalling pathway increases myocardial oxygen demand and leads to Ca^{2+}-dependent cardiomyocyte necrosis.[4] For this reason, β-inotropic support is only utilized to treat acute cardiogenic shock following myocardial infarction, and once patients are haemodynamically stable they are prescribed the reciprocal β-blocker treatment which provides long-term clinical benefit. TRPV4 inhibitors may therefore, represent a novel pharmacological approach to prevent excessive contractility, cardiomyocyte Ca^{2+} overload, and cardiomyocyte death following I/R.

4.4 Acute vs. chronic effects of TRPV4 inhibition following I/R

Our data indicate that TRPV4 is expressed in cardiomyocytes of the aged heart and exerts effects on cardiomyocyte viability in the acute phase (min to h) of I/R. Interestingly, multiple recent investigations report that hearts of young mice exhibit a secondary increase in TRPV4 expression following I/R[20,35] that may contribute to cardiomyocyte apoptosis, myofibroblast differentiation, fibrosis,[36,44] adverse remodelling, and cardiac dysfunction.[20,35,45] Taken together, these data suggest that pharmacological therapies which inhibit TRPV4 will exert beneficial effects in both the acute and chronic stages of I/R injury. However, our data highlight the need for immediate treatment with TRPV4 antagonists during reperfusion therapy to prevent acute Ca^{2+}-dependent

cardiomyocyte damage, and the therapeutic window to obtain maximum clinical benefit may be shorter than previously reported.

4.5 Study limitations

Cardiac I/R associates with complex changes in tissue osmolarity, with a gradual increase in osmotically active particles in both the intracellular and extracellular compartments, followed by reperfusion and a rapid reversion of the extracellular environment to normal osmotic conditions with associated hypoosmotic stress on the cardiomyocyte. Given the difficulty in precisely replicating this form of stress in the isolated cell environment, we utilized a defined reduction in osmotically active particles in the extracellular solution to induce hypoosmotic stress on cardiomyocytes. A global I/R protocol was used to examine contractile function and tissue damage in isolated perfused hearts. Unfortunately, this protocol associated with frequent supraventricular tachycardia and other complex arrhythmias, which precluded rigorous classification and quantification of ventricular arrhythmia burden in our experiments. Thus, while Ca^{2+}-dependent arrhythmia is well-established following I/R,[41] the specific role of TRPV4 in ventricular arrhythmia post I/R remains to be appropriately tested. Heteromeric assembly of TRPV4 with other TRP channel family members is well-established,[46] including with members known to be expressed in cardiomyocytes such as TRPV2 and TRPC6. It is, therefore, plausible that TRPV4 inhibition may prevent ion flux through multiple TRP channels, as has been shown with dominant-negative TRPC expression in cardiomyocytes.[10,39] TRPV4 antagonists exhibit excellent pharmacological properties *in vivo*[47] and are currently in clinical trials to improve pulmonary function in heart failure (NCT02119260, NCT02497937). Heteromeric assembly of TRPV4 with other TRP channel members would therefore, be advantageous, as it would allow TRPV4 inhibitors to prevent adverse effects of several TRP channel family members.

5. Conclusions

TRPV4 is now appreciated as a critical cellular signal integrator. Our working model is that increased TRPV4 expression in the aged heart is a physiological adaptation that enhances Ca^{2+} cycling and contributes to processes such as cardiac mechanotransduction, cell volume regulation, or Ca^{2+}-dependent hypertrophic remodelling. However, during I/R, physiological TRPV4-mediated Ca^{2+} signal transduction transitions into pathological Ca^{2+} signal overload. When combined with pH-driven Ca^{2+} entry processes, TRPV4-mediated Ca^{2+} entry leads to excessive contractility and adverse tissue damage. Over 50% of all hospital admissions and 80% of deaths due to myocardial infarction occur in elderly individuals. TRPV4 may therefore, represent a novel ion channel target to prevent tissue damage and mortality following myocardial infarction.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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