Title

Dysfunctional survival-signaling and stress-intolerance in aged murine and human myocardium

Journal

Experimental Gerontology, 50(1)

ISSN

0531-5565

Authors

Peart, Jason N
Pepe, Salvatore
Reichelt, Melissa E
et al.

Publication Date

2014-02-01

DOI

10.1016/j.exger.2013.11.015

Peer reviewed

Published in final edited form as:
Exp Gerontol. 2014 February ; 50: 72–81. doi:10.1016/j.exger.2013.11.015.

Dysfunctional survival-signaling and stress-intolerance in aged murine and human myocardium

Jason N. Peart[*,1], **Salvatore Pepe**[*,2], **Melissa E. Reichelt**[3], **Nikkie Beckett**[1], **Louise See Hoe**[1], **Victoria Ozberk**[1], **Ingrid R. Niesman**[5], **Hemal H. Patel**[4,5], and **John P. Headrick**[1,†]

[1]Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Southport, Australia

[2]Heart Research, Murdoch Children's Research Institute and Department of Paediatrics, University of Melbourne, Parkville, Australia

[3]Department of Physiology, University of Melbourne, Parkville VIC, Australia

[4]VA San Diego Healthcare System, San Diego, USA

[5]Department of Anesthesiology, University of California San Diego, USA

Abstract

Changes in cytoprotective signaling may influence cardiac aging, and underpin sensitization to ischemic insult and desensitization to 'anti-ischemic' therapies. We tested whether age-dependent shifts in ischemia-reperfusion (I-R) tolerance in murine and human myocardium are associated with reduced efficacies and coupling of membrane, cytoplasmic and mitochondrial survival-signaling. Hormesis (exemplified in ischemic preconditioning; IPC) and expression of proteins influencing signaling/stress-resistance were also assessed in mice. Mouse hearts (18 *vs*. 2–4 mo) and human atrial tissue (75±2 *vs.* 55±2 yr) exhibited profound age-dependent reductions in I-R tolerance. In mice aging negated cardioprotection via IPC, G-protein coupled receptor (GPCR) agonism (opioid, A_1 and A_3 adenosine receptors) and distal protein kinase C (PKC) activation (4 nM phorbol 12-myristate 13-acetate; PMA). In contrast, p38-mitogen activated protein kinase (p38-MAPK) activation (1 μM anisomycin), mitochondrial ATP-sensitive K^+ channel (mK_{ATP}) opening (50 μM diazoxide) and permeability transition pore (mPTP) inhibition (0.2 μM cyclosporin A) retained protective efficacies in older hearts (though failed to eliminate I-R tolerance differences). A similar pattern of change in protective efficacies was observed in human tissue. Murine hearts exhibited molecular changes consistent with altered membrane control (reduced caveolin-3, cholesterol and caveolae), kinase signaling (reduced p70 ribosomal s6 kinase;

[†]Corresponding Author: Prof. John P. Headrick, Heart Foundation Research Centre, Griffith University, Gold Coast Campus., Parklands Drive, Parklands 4222 QLD Australia, j.headrick@griffith.edu.au, Phone (Intl): +61 7555 28292, Facsimile (Intl): +61 7555 28908.
[*]Denotes equal first authorship

Conflicts of interest
The authors have no conflicts of interest

p70s6K) and stress-resistance (increased G-protein receptor kinase 2, GRK2; glycogen synthase kinase 3β, GSK3β; and cytosolic cytochrome *c*). In summary, myocardial I-R tolerance declines with age in association with dysfunctional hormesis and transduction of survival signals from GPCRs/PKC to mitochondrial effectors. Differential changes in proteins governing caveolar and mitochondrial function may contribute to signal dysfunction and stress-intolerance.

Keywords

Aging; Cardioprotection; Caveolae; G-Protein Coupled Receptors; Hormesis; Ischemia; Mitochondria; Protein Kinase; Stress-Resistance

1. Introduction

An understanding of the molecular basis of cardiac aging, and of the influences of age on myocardial responses to disease and therapy, are increasingly important goals. Ischemic heart disease (IHD) and associated stroke are the lead causes of mortality globally (Fuster & Kelly, 2010), with age a major risk factor in their development. While aged myocardium is most likely to suffer I-R insult (~75% of infarcts occur in those >65 yrs of age), it may possess reduced resistance to I-R injury (Headrick, 1998; Rosenfeldt *et al*., 1999; Mariani *et al*., 2000; Headrick *et al*., 2003; Lesnefsky *et al*., 2006) compounded by refractoriness to protective intervention (Ferdinandy *et al*., 2007; Boengler *et al*., 2009; Peart & Headrick, 2009). These clinically relevant changes could reflect mechanistic determinants of the poorly understood aging process itself: in the 'green hypothesis' the expression/functionality of intrinsic 'detoxification' systems eliminating molecular damage and governing cellular stress-resistance is forwarded as a primary determinant of aging and longevity (Gems & McElwee, 2005). Intrinsic resistance to diverse stressors is a common (potentially defining) feature of longevity phenotypes (Harper *et al.*, 2006), and this hypothesis is consistent with cytoprotective pathway induction with longevity extension (Shore *et al.*, 2012), and the anti-aging effects of hormesis (Calabrese *et al*., 2012). Hormesis refers to beneficial biological effects (including improved resistance to injury) arising from moderate sub-lethal stressors such as hypoxia or nutrient deprivation, and is exemplified in cardiac tissue by IPC. The functionality of signaling pathways underpinning cellular stress-resistance and hormesis responses may thus govern aging/longevity, and is also highly relevant to therapeutic manipulation of IHD outcomes.

Hormesis, autophagic damage-management, longevity pathway (*eg.* mammalian target of rapamycin, mTOR; and insulin-like growth factor, IGF) signaling and cellular stress-resistance are regulated by membrane GPCRs and receptor tyrosine kinases (RTKs), and altered growth-factor/RTK control has been implicated in replicative senescence and cellular aging (Cho & Park, 2005; Yu & Driscoll, 2011). However, there is also evidence of impaired GPCR-dependent stress-signaling in older myocardial tissue (Schulman *et al*., 2001; Headrick *et al*., 2003; Peart *et al*., 2007). The expression of proteins targeted by this signaling, such as GSK3β (Kostyak *et al*., 2006; Hunter *et al*., 2007) may also be modified with age, together with mitochondrial determinants of cell survival, including Ca^{2+}-sensitive K^+ channels and the mPTP (Heinen *et al*., 2008; Zhu *et al*., 2010, 2013). Such changes may collectively repress cellular resistance and adaptation to stress (a feature of aged

phenotypes), and in turn promote the aging process itself (Gems & McElwee, 2005; Calabrese et al., 2012; Shore *et al.*, 2012). Identifying intrinsic protective mechanisms that become dysfunctional or retain efficacy with age can thus unmask mechanistic aspects of biologic aging, and also reveal molecular targets for the manipulation of myocardial resistance to injury/disease.

In the present study we test whether age-related intolerance to stress (specifically, clinically relevant I-R) is associated with changes in membrane/GPCR sensitive cytoprotective signaling and hormesis, and emergence of a molecular profile favoring such dysfunction. Responses to the following stimuli were assessed to localize age-dependent changes in survival signaling, and to identify targets for manipulation of I-R outcomes in aged tissue: acute hormesis via IPC; agonism of membrane GPCRs mediating stress-resistance/hormesis (opioid and adenosine A_1 or A_3 receptors); activation of signal kinases (PKC and p38-MAPK) transducing GPCR/IPC responses (Headrick *et al.*, 2003; Peart & Gross, 2006; Fenton *et al.*, 2010); and modulation of mitochondrial effectors governing cell viability (mK_{ATP} channels and the mPTP). In murine tissue we also tested for shifts in determinants of membrane receptor signaling (caveolin-3, membrane cholesterol and GRK2), survival-kinase signal transduction (protein kinase B or AKT, p70s6K, p38-MAPK, and extracellular signal-regulated kinase 1/2 - ERK1/2), and mitochondrial dysfunction/cell death (GSK3β, GRK2, caspase-3, and cytochrome *c*).

2. Materials and methods

Investigations conformed to the guidelines of the Animal Ethics Committee of Griffith University. Male C57Bl/6 mice were sourced from the Animal Resources Centre (Canning Vale, WA, Australia). Young (2–4 mo; 24.6±0.5 g body weight) and old (18 mo; 37.1±1.9 g body weight) mice were studied, with hearts removed from terminally anaesthetized animals for Langendorff perfusion. The 18 mo group was studied as an ‘aged’ phenotype, midway between reproductive senescence (12–14 mo in females) and senescence based on median mortality (24–28 mo in C57Bl/6 mice). Onset of myocardial fibrosis is detectable at 18 mo (Willems *et al.*, 2005), consistent with an aged cardiac phenotype. Spontaneous mortality to this age (10–20%) is also equivalent to mortality by 70–75 yrs in human (developed country) populations - the older age group in human tissue analysis here.

2.1. Chemicals

All chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO), and antibodies sourced from Cell Signaling Technology (Danvers, MA) and Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Perfused murine heart model

Mice were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally), and hearts were excised and perfused in a Langendorff mode with modified Krebs-Henseleit buffer delivered via the aorta at a pressure of 80 mmHg, as described previously (Headrick *et al.*, 2003; Peart *et al.*, 2007). Hearts underwent 20 min of normoxic stabilization at intrinsic heart rates after ventricular balloon placement, followed by 10 min of pacing at 420 beats/

min. Hearts were then subjected to 20 min global normothermic ischemia followed by 60 min of aerobic reperfusion. Myocardial cell disruption/death was assayed by quantitating post-ischemic washout of cellular lactate dehydrogenase (LDH). We have previously established that LDH efflux correlates well with infarct size and mechanical dysfunction in this model (Peart & Headrick, 2003). Venous effluent was collected on ice throughout reperfusion before enzymatic determination of total LDH washout (IU/g tissue).

Mouse hearts were either untreated (*n*=17 for young, 13 for aged), or subjected to IPC (3 × 90 s ischemia interposed with 120 s reperfusion; *n*=8 for young, 8 for aged) or 10 min pre-ischemic and 5 min post-ischemic treatment with: 10 μM of the broad-spectrum opioid receptor agonist morphine (*n*=9 for young, 7 for aged); 0.1 μM of the A_1 adenosine receptor agonist N^6-cyclopentyladenosine (CPA; *n*=10 for young, 8 for aged); 0.1 μM of the A_3 adenosine receptor agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-N-methyl-carboxamide (Cl-IB-MECA; *n*=9 for young, 7 for aged); 4 nM of the PKC activating phorbol ester PMA (*n*=9 for young, 8 for aged); 1 μM of the p38-MAPK activator anisomycin (*n*=8 for young, 8 for aged); 50 μM of the putative mK_{ATP} channel opener diazoxide (*n*=10 for young, 8 for aged); or 0.2 μM of the mPTP inhibitor cyclosporin A (*n*=9 for young, 8 for aged). Normoxic time-course experiments were undertaken in young (*n*=6) and aged (*n*=5) hearts subjected to 80 min of normoxic perfusion following initial stabilization.

2.3. Human atrial pectinate trabecular model

Right atrial myocardium was sampled from middle-aged and aged cardiac patients with mean ages of 55±2 (*n*=10) and 75±2 (*n*=10) yrs, respectively, and functionally assessed for I-R tolerance as detailed by us previously (Rosenfeldt *et al*., 1999; Mariani *et al*., 2000). Resected atrial appendage was acquired from patients undergoing elective coronary artery bypass graft or valve operations with cardiopulmonary bypass at the Alfred Hospital in Melbourne, under approval of the Human Research Ethics Committee for Discarded Tissue. Exclusion criteria included: re-operation; urgent or emergency procedures; current therapy with antioxidants; and recent myocardial infarction (≤6 weeks).

Atrial tissue was dissected under microscopy in Ringer's solution containing 30 mM 2,3 butanedione monoxime (BDM) to yield 5 pectinate trabeculae (~1 mm diameter, ~7 mm long) from each patient. Muscle strips were connected to a force transducer and held between 2 field-stimulation electrodes in normoxic BDM-free Ringer's solution (37°C, PO_2 ≥450 mmHg). Strips were initially maintained un-stretched and stimulated at 1 Hz for 30 min, before incremental stretching to lengths yielding maximal force development, and a further 30 min of stabilization. The 5 muscle strips per patient were then assessed as follows: one was assigned to a 60 min normoxic time-course while the remaining 4 strips were subjected to 30 min of simulated ischemia/30 min reperfusion, and were either untreated or pre-treated for 30 min with 10 μM morphine, anisomycin or diazoxide. Serial measures of contractile function were made every 10 min. As described previously (Mariani *et al*., 2000), the key elements of ischemia - restriction of O_2 and substrate delivery, and accumulation of tissue metabolites - are induced by draining sealed organ baths of oxygenated Ringer's (thus O_2 and metabolic substrate) and maintaining strips in humidified anoxic gas mix (95% N_2/5% CO_2) at 37°C for 30 min, with stimulation via punctate

electrodes. Reperfusion is effected by reintroduction of normoxic Ringer's solution (restoring O_2 and substrate delivery, washing out accumulated metabolites), with tissue paced at 1 Hz for a 30 min recovery period.

2.4. Protein changes in aging mouse heart

Additional to or facilitating changes in signal transduction, total quantities of proteins influencing cellular stress-resistance may change with age (total protein levels dictating maximal cellular activities, with post-translational events modulating this activity). To test for emergence of a stress-intolerant expression profile, we quantitated protein levels via immunoblot analysis of ventricular lysate samples from young and old hearts (n=6 for each), isolated from anesthetized animals as outlined previously (Peart *et al*., 2007). We assessed expression of the membrane orchestrator of survival-signaling caveolin-3, together with downstream kinases AKT, p70s6K, ERK1/2 and p38-MAPK implicated in tissue protection. In terms of proteins promoting cellular injury, we assessed expression of GRK2 and GSK3β which both induce mitochondrial dysfunction and cell death (Juhaszova *et al*., 2009; Brinks *et al.*, 2010; Chen *et al.*, 2013; Zhu *et al*., 2013), while GRK2 additionally inhibits GPCR signaling.

Lysate samples containing 30 μg of total protein were loaded onto 10% acrylamide gels and electrophoresed at 150 V for 1.5 hrs. Proteins were transferred to polyvinylidene difluoride membranes and blocked in 5% skim milk powder in Tris-buffered saline with 0.05% Tween-20 (TBST) for 60 min. Membranes were incubated with 1:1000 dilutions of primary antibodies overnight at 4°C (from Cell Signaling Technology Inc., Danvers, MA, USA: GRK2, #3982; AKT, #9272; p70s6K, #9202; ERK1/2, #9102; p38-MAPK, #9212; and GSK3β; #9315; from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA: caveolin-3, sc-5310). Following 3 washes in TBST, membranes were incubated with secondary antibody and visualized on a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Equal loading was confirmed by co-analysis of β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For purposes of comparison protein expression levels were normalized to values for young hearts.

Active caspase-3 and cytosolic cytochrome *c* content were assayed as measures of apoptotic potential. Caspase-3 activity was measured via a commercial kit (Clontech Laboratories Inc., Mountain View, CA, USA) for fluorometric detection of 7-amino-4-trifluoromethyl coumarin (AFC) cleaved from a synthetic DEVD (Asp-Glu-Val-Asp) substrate. Relative caspase-3 activities determined from fluorescence change/mg protein were normalized to values for young hearts. Cytochrome *c* was assayed in crude cytosolic fractions from ventricular lysate samples using an immunoassay kit (R&D Systems, Minneapolis MN), with levels expressed per mg protein. Finally, total cholesterol content was assayed in crude membrane fractions from cardiac lysates using an Invitrogen Amplex Red Cholesterol kit (Life Technologies Australia Pty Ltd, VIC, Australia). Fluorescence change was measured on a microplate reader (Tecan Australia Pty Ltd, VIC, Australia) with excitation and detection at 560 nm and 590 nm, respectively. Cholesterol content per mg protein was determined from standard curves acquired with each analysis, and data normalized to values for young hearts.

To further explore effects of age on the membrane compartment, immunofluorescence and electron microscopy (EM) were employed to examine membrane ultrastructure, caveolin-3 expression/localization, and caveolar density. For EM, tissue from young and aged mice was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h, post-fixed in 1% OsO4 in 0.1 M cacodylate buffer (1 h), and embedded in LX-112 (Ladd Research, Williston, VT, USA), as outlined recently (Fridolfsson *et al*., 2012). Sections were stained in uranyl acetate and lead citrate and assessed via EM (Jeol 1200 EX-II; Jeol Ltd., Akishima, Japan). For immunohistochemistry, fixed tissue was sectioned and processed as previously described (Head et al., 2005).

2.5. Statistical analysis

All data are expressed as means ± the standard error of the mean (SEM). Age and treatment effects were statistically identified via 2-way ANOVA (employing repeated-measures for functional responses assessed in Fig. 1), with a post-hoc Newman-Keuls test employed for specific comparisons. Significance in all tests was accepted for $P<0.05$.

3. Results

3.1. Age-dependence of I-R injury in murine and human myocardium

Heart mass increased by ~50% with age, while a modest rise in the heart:body weight ratio to 18 mo was not statistically significant (Table 1). Normoxic contractile function did not vary substantially between young and aged hearts (Table 1), although we observed a significant 10% fall in spontaneous beating rate and an insignificant trend towards reduced dP/dt (by ~15%, suggestive of emerging hypo-contractility). Despite comparable mechanical function, normoxic coronary perfusion rate was significantly reduced in older myocardium (Table 1). Stability of contractile function over 80 min of normoxic perfusion was also comparable in young and aged hearts, with no change in diastolic pressure (Fig. 1A) and similar maintenance of force development (86±6% and 83±7% of baseline force, respectively; Fig. 1B). A 20 min ischemic insult resulted in sustained contractile dysfunction throughout 60 min of reperfusion (Fig. 1A & 1B), in association with significant cellular damage as indicated by LDH efflux (Fig. 1A inset). Contractile dysfunction and cellular injury were markedly exaggerated in aged *vs.* young hearts.

Isolated human atrial trabecular strips from 55 and 75 yr age groups did not differ in terms of size or baseline contractile and relaxation properties (Table 2). The functional stability of normoxic trabeculae over a 90 min period was also similar for the 55 and 75 yr age groups (90±9% and 87±10% of basal force development, respectively; Fig. 1C). Atrial tissue subjected to simulated ischemia failed to recover fully during a subsequent 30 min reperfusion period, with relative functional recovery in old tissue approximately half that for younger trabeculae (Fig. 1C).

3.2. Age-dependence of protective stimuli in murine myocardium

Induction of IPC together with pre-ischemic agonism of opioid and adenosine A_1 and A_3 GPCRs significantly enhanced I-R tolerance in young hearts, whereas these stimuli all failed to modify outcomes in aged hearts (Fig. 2). Protection in young hearts was evident as

improvements in diastolic function (Fig. 2A), pressure development (absolute and % of pre-ischemia; Figs. 2B and C) and LDH efflux (Fig. 2D). The relative protective efficacies of these receptor-dependent stimuli within young hearts were comparable. The cardioprotective effects of distal signaling kinase activation varied with age (Fig. 3). As for GPCR agonism, pre-ischemic treatment with the PKC activator PMA markedly improved post-ischemic outcomes in young but not aged hearts (Fig. 3). In contrast, p38-MAPK activation with anisomycin was effective in both ages, though the effect in older hearts was not as robust as in younger tissue. Targeted modulation of mitochondrial end-effectors proved effective in both young and aged mouse hearts, though responses appeared moderately reduced with age (Fig. 4). The putative mK_{ATP} channel opener diazoxide improved contractile recoveries by 30–50% in young and aged hearts, while the mPTP inhibitor cyclosporin A exerted slightly less improvement in outcomes. Since mK_{ATP} channel activation and mPTP inhibition are protective in aged hearts, failed responsiveness to PKC activation (Fig. 3) likely originates upstream of these mitochondrial effectors. The impaired efficacy of PMA, and of cyclosporin A, did not appear dose-dependent: trials of higher concentrations (10 nM PMA, 2 μM cyclosporin A; n=4–5) failed to further improve outcomes (data not shown). Despite profound protection via mK_{ATP} activation and mPTP inhibition in both age groups, myocardial ischemic tolerance was not normalized across ages with a substantial 2-fold difference in cellular damage persisting in aged *vs*. young hearts (Fig. 4).

3.3. Age-dependence of protective stimuli in human myocardium

Similar to observations in murine hearts, opioid receptor agonism with morphine failed to protect older human atrial tissue while exerting significant benefit in younger tissue (Fig. 5). Moreover, the p38-MAPK activator anisomycin and the mK_{ATP} opener diazoxide both improved recoveries in young and aged human tissue (Fig. 5), as observed in murine studies. Thus, as for perfused murine hearts, GPCR activation was ineffective in older human atrial tissue while p38 and mitochondrial effector modulation conferred benefit across ages. Moreover, as in mouse hearts the age-dependent difference in human atrial ischemic tolerance was reduced but not eliminated by mK_{ATP} (or p38-MAPK) activation (Fig. 5).

3.4. Age-dependent shifts in myocardial molecular makeup

Analysis of protein expression in murine hearts (Table 3) revealed that older tissue expresses less caveolin-3 (a key orchestrator of membrane receptor signaling) and p70s6K (a pro-survival signal kinase that phospho-inhibits GSK3β), and increased levels of GRK2 (an inhibitor of GPCR signaling that promotes mitochondrial injury/cell death) and GSK3β (which also promotes mitochondrial dysfunction and cell death). On the other hand, levels of the survival kinases AKT, ERK1/2 and p38-MAPK did not differ significantly between young and aged hearts (Table 3). Cytosolic cytochrome *c* content was increased in aged hearts, whereas active caspase-3 content was comparable in both groups. Membrane cholesterol content, critical to caveolar formation and control, was also significantly reduced in aged tissue (Table 3).

Consistent with altered cholesterol and caveolin-3 levels, immunohistochemical and EM assessment revealed significant alterations in sarcolemmal architecture in aged *vs*. young hearts (Fig. 6). As shown in prior work (Head *et al*., 2005), in young myocardium

caveolin-3 is expressed in a punctate pattern within the sarcolemma and in transverse striations running across the cell interior (Fig. 6A). This defined pattern was not evident in aged tissue, which exhibited reduced total caveolin-3 expression and a reduction in structural membrane invaginations consistent with caveolar depletion (Fig. 6B).

4. Discussion

Given prevalence of heart disease in older individuals, it is critical we understand the influence of age on myocardial responses to injurious insult and protective therapies (and unravel poorly understood mechanisms of cell aging). Myocardial I-R tolerance was markedly reduced in older rodent and human tissue, in agreement with reports of age-related changes in stress-resistance in animal (Headrick, 1998; Headrick *et al*., 2003; Lesnefsky *et al*., 2006) and human tissue (Rosenfeldt *et al*., 1999; Mariani *et al*., 2000). This is consistent with the view of declining stress-resistance as a hallmark of cellular aging, however the genesis of this decline remains obscure. Reduced myocardial I-R tolerance is manifest as worsened ionic and energetic imbalances (Headrick, 1998), oxidative-stress (Liu *et al*., 2004; Oudot *et al*., 2006), apoptotic activation (Azhar *et al*., 1999), mitophagy dysregulation (Dutta *et al*., 2012) and mitochondrial dysfunction (Pepe *et al*., 1999; Lesnefsky *et al*., 2006; Boengler *et al*., 2007; Marzetti *et al.*, 2013). Importantly, these processes are all modulated by cytoprotective signaling paths, whose functionality could influence the aging process itself (Gems & McElwee, 2005; Shore *et al.*, 2012). The current study shows that I-R intolerance in aged tissue is associated with repression of hormesis and cytoprotective signaling, with impaired transduction of signals from a modified membrane environment to mitochondrial effectors (Fig. 7). This dysfunctional stress-intolerant phenotype may involve differential shifts in determinants of caveolar control (caveolin-3, membrane cholesterol), GPCR signaling (GRK2), pro-survival signal transduction (p70s6K), and mitochondria-dependent cell death (GSK3β, GRK2, cytosolic cytochrome *c*). These changes may underpin the negative effects of age on myocardial ischemic injury and responsiveness to therapeutic intervention, and potentially promote myocardial aging.

4.1. Impaired cytoprotective signaling from membrane to mitochondria

Protective interventions to limit I-R injury with myocardial infarction or surgery must be efficacious in older hearts. Unfortunately, aging may induce dysfunction within the pathways targeted by experimental therapies, with evidence of shifts in multiple protective signaling networks (Tani *et al*., 2001; Headrick *et al*., 2003; Boengler *et al*., 2007; Peart *et al*., 2007; Boengler et al., 2009; Peart & Headrick, 2009). Such changes may contribute to disparities between moderate benefit in clinical trials of conditioning stimuli (in middle-aged/aged patients) *vs*. profound protection in experimental (typically young) animal models (Peart & Headrick, 2009; Ludman *et al*., 2010). The age-dependent pattern of cardioprotective efficacies and the molecular profile of older myocardium support impaired initiation and transduction of protective signals from cell membrane to mitochondria (Fig. 7).

Membrane- and GPCR-dependent cardioprotection—While changes in growth-factor/RTK signaling have been implicated in aging and senescence of non-cardiac cells

(Cho & Park, 2005; Yu & Driscoll, 2011), the protective functionality of GPCR signaling may also decline in older myocardium (Schulman et al., 2001; Headrick et al., 2003; Peart et al., 2007; Boengler et al., 2009). We show aged hearts are insensitive to agonism of opioid and adenosine receptors mediating cardiac conditioning responses (hormesis) and stress-resistance. This is congruent with impairment of acute IPC in older mouse hearts. Whether initiated by stress and endogenous ligands (IPC) or via exogenously applied agonists, GPCR-dependent cytoprotection is clearly impaired in older tissue. These findings are consistent with age-dependent repression of pre- (Schulman *et al*., 2001; Przyklenk *et al*., 2003; Boengler *et al*., 2007) and post-conditioning responses (Przyklenk *et al*., 2008) mediated by these GPCR families, and indicate that the anti-aging influences of hormesis (Calabrese *et al*., 2012) may wane with age.

Insensitivity to IPC and multiple GPCRs supports a broad-spectrum dysfunction that could arise locally, via altered membrane micro-domain control of receptor signaling (Fridolfsson *et al*., 2012, 2013), and/or distally within convergent signaling pathways (Boengler et al., 2009). The former possibility is supported by reductions in membrane cholesterol and caveolin-3 expression in older hearts, the latter in agreement with the findings of Kawabe *et al*. (2009) in old rat hearts. Moreover, sarcolemmal ultrastructure is substantially modified with age, with altered localization of caveolin-3 and a paucity of caveolae (Fig. 6). These changes are predicted to impair caveolar control of transmembrane signaling and stress-resistance (Head et al., 2005; Tsutsumi *et al*., 2008; Fridolfsson et al., 2012, 2013). Caveolae are also important in cell-tension homeostasis and responses to mechanical stress (Sinha *et al.*, 2011), and caveolin-3 represses cardiac myoblast growth (Fujita et al., 2007), and myocardial hypertrophy (Horikawa *et al.*, 2011) that is a feature of the aged phenotype. Age-dependent reductions in caveolin-3 and caveolae may thus not only impact stress-signaling but also alter responses to mechanical loading and facilitate age-dependent hypertrophy. These reductions in myocardial caveolin-3 and caveolae (Figs. 6–7), together with evidence of caveolin-dependent inhibition of neurodegeneration and aging *in vivo* (Head et al., 2010), and caveolin-dependent senescence in replicating cells *in vitro* (Cho & Park, 2005), support a potentially overarching role for caveolins/caveolae in governing cellular aging, albeit in a tissue-specific manner.

Survival-kinase dependent protection—Broad-spectrum dysfunction in cytoprotection may also arise within common signaling downstream of membrane receptors. Pro-survival kinases such as PKC, phosphoinositide 3-kinase (PI3K), AKT, MAPKs and protein kinase G transduce signals from GPCRs to targets such as GSK3β, mitochondrial K_{ATP} channels and the mPTP. PKC is critical in opioid (Peart & Gross, 2006) and adenosine receptor responses (Headrick *et al*., 2003; Fenton *et al.*, 2010) and IPC (Boengler et al. 2009), and induces mitochondrial protection. However, as for IPC and GPCR agonism, PKC activation fails to limit I-R injury in aged hearts. Age-related changes in PKC signaling have been identified in rat hearts, including altered expression/regulation of PKC isoforms (Takayama *et al*., 2001) and protection via PMA (Tani *et al*., 2001). Przyklenk *et al.* (2003) also report that age modifies the PKC-dependence of conditioning stimuli in rabbits. Age-dependent dysfunction in cardiac PKC signaling is thus conserved across species, and likely participates in impaired protective signal transduction.

Cardiac 38-MAPK signaling may also contribute to protection via opioid and adenosine GPCRs (Peart & Gross, 2006; Fenton *et al.*, 2010), limiting mitochondrial damage and apoptosis (Whittaker *et al.*, 2009). Beneficial effects of pre-ischemic p38-MAPK activation in both young and older murine and human myocardium agrees with prior findings (Hassouna *et al*., 2006; Peart *et al*., 2007), and localizes age-dependent signal dysfunction upstream of p38-MAPK. These observations also support acute MAPK manipulation as a potential protective strategy in aged hearts.

Mitochondria-dependent protection—Preservation of mitochondrial function is central to cellular stress-resistance, while dysfunction is considered important in cell aging (Marzetti *et al.*, 2013). Preconditioning, protective GPCRs, and PKC and p38-MAPK activities converge on mK_{ATP}- and mPTP-dependent control of mitochondrial function. Beneficial effects of diazoxide, which activates mK_{ATP} channels to increase mitochondrial reactive oxygen species (ROS) signaling and inhibit the mPTP (Pain *et al*., 2000; Facundo *et al*., 2007), supports preserved functionality of mK_{ATP} channels in aging human and murine myocardium. Moreover, since PKC activation is ineffective in aged hearts, the protective actions of mK_{ATP} opening do not appear to require downstream PKC activation in older tissue. The influence of mPTP inhibition on cell survival was also preserved in aged hearts. A number of studies confirm protection via mPTP inhibition in young tissue (Halestrap *et al*., 1997; Liu *et al*., 2011), and cyclosporin A protects human myocardium *in vitro* (Schneider *et al*., 2003) and *in vivo* (Piot *et al*., 2008). However, Liu *et al.* recently reported cyclosporin A is ineffective in aged rat hearts (Liu *et al*., 2011), and its effects may also be reduced by obesity or diabetes (Huhn *et al*., 2010). Interestingly, although mPTP inhibition protects aged myocardium, ischemic tolerance remains profoundly depressed in older *vs*. younger hearts. This could reflect increased activity and altered control of the mPTP (Zhu *et al*., 2010). Since cyclosporin A limits mPTP opening by reducing cyclophilin D binding (Halestrap *et al*., 1997), increased cyclophilin D expression together with resistance of the interaction to inhibition with age (Zhu *et al.*, 2013), could render the mPTP resistant to modulation. While preliminary data suggests a 10-fold higher concentration of cyclosporin A does not exert greater effects in aged tissue, this possibility warrants further study.

Preservation of protective responses to p38-MAPK and mitochondrial modulators despite failed protection via IPC, GPCRs and PKC activation, supports dysfunction in protective signal transduction from membrane GPCRs (and PKC) to mitochondrial effectors (Fig. 7). Whether this change involves a profound decline in cellular sensitivity to hormesis stimuli, GPCR agonism and PKC activation *vs.* complete abrogation of responses is unclear (though data indicates a 2.5-fold elevation in PMA does not induce protection in aged hearts). In either case, the phenotypic outcome is insensitivity to protective stimuli that near maximally enhance stress-resistance in younger tissue. From a clinical perspective, reduced sensitivities to protective interventions might be overcome by strategic combinations of stimuli, whereas unresponsive molecular targets might be by-passed in favor of downstream effectors. From a fundamental perspective, these major changes in the functionality of membrane-to-mitochondrial signaling - an evolutionarily conserved feature of cellular stress-adaptation (Fridolfsson *et al*., 2012) - could reflect an important mechanistic component of myocardial

aging, given evidence the expression and function of cytoprotective signaling paths may govern aging and longevity (Gems and McElwee, 2005; Shore et al., 2012).

4.2. Emergence of a stress-intolerant molecular profile

Whether alterations in signaling and stress-resistance reflect stochastic and/or active (*eg.* via mTOR or IGF pathways) changes in myocardial protein expression is unclear. We show that additional to repression of caveolar components, aged hearts express increased levels of proteins favoring mitochondrial dysfunction and cell death (Table 3, Fig. 7). These differential changes are consistent with evolving stress-intolerance, and hint at active control (though this requires further study). Increased GRK2 expression may inhibit GPCR function (Jurado-Pueyo *et al*., 2008) and promote mPTP opening and cell death (Brinks *et al.*, 2010; Chen *et al*., 2013). Elevated GSK3β levels are predicted to enhance mPTP activity and effects of pro-apoptotic proteins (Juhaszova *et al*., 2009; Zhu *et al*., 2013), and are in accord with observations in male and female Fisher 344 rats (Kostyak *et al*., 2006; Hunter *et al*., 2007). On the other hand Zhu *et al.* (2010, 2013) found no age-dependent shift in GSK3β in the same model. Increased cytosolic cytochrome *c* levels may also sensitize tissue to apoptotic death. The reduced expression of p70s6K may further undermine stress-resistance and signaling, based on involvement in cardioprotection via GPCRs (Gross *et al*., 2004; Förster *et al*., 2006) and pre- and post-conditioning (Tsang *et al*., 2004; Zhu *et al*., 2006). Since p70s6K phospho-inhibits GSK3β (Sutherland *et al*., 1993), declining expression will amplify detrimental effects of elevated GSK3β in aged tissue. These changes are consistent with data for middle-aged hearts (Peart *et al*., 2012), and demonstrate that age induces an expression profile favoring signal dysfunction, mitochondrial injury and cell death. The basis of the differential changes in pro-survival (caveolin-3, p70s6K) *vs*. pro-injury proteins (GSK3β, GRK2) remains to be established.

5. Conclusions

This study confirms age-dependent repression of I-R tolerance in human and rodent myocardium, and reveals these changes are associated with impaired hormesis and dysfunctional transduction of cytoprotective signals from membrane GPCRs and PKC to mitochondrial targets (Fig. 7). Impaired membrane micro-domain orchestration of survival-signaling (Fridolfsson *et al*., 2012; Fridolfsson & Patel, 2013), together with shifts in determinants of signal transduction and mitochondrial preservation may collectively limit resistance and adaptation to damaging stressors (which may, in turn, promote the cardiac aging process). Further studies are warranted to evolve our understanding of mechanisms of cellular- and organ-specific aging, to assess the relevance of age-related myocardial changes reported here, and to rationally evolve strategies to promote resistance to injury in the ‘at-risk’ aged heart.

6. Limitations

A number of study limitations deserve attention. First, while we do not directly compare distinct murine and human tissue models (as our aim was to test whether age commonly impairs stress-responses and protective signaling in different species), it is relevant to highlight that we assessed whole myocardial responses in mice, and isolated atrial muscle

strip responses in humans. As discussed previously (Mariani *et al*., 2000), structural and functional differences may limit the applicability of atrial tissue as a surrogate for ventricular myocardium. However, human ventricular samples are rare to access, with biopsies generally too small to assess functionally and problematic to study as discrete muscle bundles. In contrast, atrial tissue is more readily available and amenable to functional assessment, with the isolated trabeculae model validated and applied in pharmacological and functional analyses (Guo *et al*., 1983; Keon *et al*., 1991; Labow *et al*., 1991), and in the interrogation of ischemic or hypoxic responses (Rosenfeldt *et al*., 1999; Mariani *et al*., 2000). Even so, availability of human (particularly younger) tissue precluded the more extensive analysis of protective responses and molecular changes achievable in murine hearts (limiting stimuli assessed in human tissue to a GPCR agonist, and p38-MAPK and mK_{ATP} channel activation). Sensitivity of the LDH assay also prevented accurate assessment of cell death in this preparation. What is nonetheless remarkable is that despite differing species, tissues and experimental models, data from human and murine analyses remain internally consistent, demonstrating that aging profoundly impairs intrinsic I-R resistance and GPCR-dependent protection, without negating responses to distal p38-MAPK or mK_{ATP} channel activity.

Additional to examining aspects of cardiac aging, a goal of this work was to contrast the functionality of different molecular determinants of I-R tolerance (thus identifying potential cardioprotective ‘targets’) in aged *vs*. young tissue. Informed by the changes in membrane-dependent survival signaling and regulatory proteins observed here, future studies are warranted to unravel the basis of these alterations, and rationally refine experimental strategies to bolster I-R tolerance in the aged heart. Since proteins expressed in greater or lower levels in aged hearts are post-translationally modified, additional analysis of this control is also needed. Significant changes in phosphorylation, for example, will either exaggerate or limit effects of altered protein expression. In this regard, increased phosphatase activity detected in aged hearts (Fenton *et al*., 2005) is likely to exaggerate the functional impacts of reduced p70s6K and increased GSK3β expression documented here.

Acknowledgments

This study was supported by grants from the NHF and NHMRC of Australia (481922). JNP was supported by a Future Fellowship from the Australian Research Council. VO is the recipient of an Australian Postgraduate Award scholarship. JPH was supported by an international fellowship from the QLD state government. HHP is supported by grants from the National Institutes of Health, USA (HL091071 and HL107200) and VA Merit BX001963.

References

Azhar G, Liu L, Zhang X, Wei JY. Influence of age on hypoxia/reoxygenation-induced DNA fragmentation and bcl-2, bcl-xl, bax and fas in the rat heart and brain. Mech Ageing Dev. 1999; 112:5–25. [PubMed: 10656180]

Boengler K, Konietzka I, Buechert A, Heinen Y, Garcia-Dorado D, Heusch G, Schulz R. Loss of ischemic preconditioning’s cardioprotection in aged mouse hearts is associated with reduced gap junctional and mitochondrial levels of connexin 43. Am J Physiol Heart Circ Physiol. 2007; 292:H1764–H1769. [PubMed: 17142336]

Boengler K, Schulz R, Heusch G. Loss of cardioprotection with ageing. Cardiovasc Res. 2009; 83:247–261. [PubMed: 19176601]

Brinks H, Boucher M, Gao E, Chuprun JK, Pesant S, Raake PW, Huang ZM, Wang X, Qiu G, Gumpert A, Harris DM, Eckhart AD, Most P, Koch WJ. Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms. Circ Res. 2010; 107:1140–1149. [PubMed: 20814022]

Calabrese EJ, Iavicoli I, Calabrese V. Hormesis: why it is important to biogerontologists. Biogerontology. 2012; 13:215–235. [PubMed: 22270337]

Chen M, Sato PY, Chuprun JK, Peroutka RJ, Otis NJ, Ibetti J, Pan S, Sheu SS, Gao E, Koch WJ. Prodeath signaling of G protein-coupled receptor kinase 2 in cardiac myocytes after ischemic stress occurs via extracellular signal-regulated kinase-dependent heat shock protein 90-mediated mitochondrial targeting. Circ Res. 2013; 112:1121–1134. [PubMed: 23467820]

Cho KA, Park SC. Caveolin-1 as a prime modulator of aging: a new modality for phenotypic restoration? Mech Ageing Dev. 2005; 126:105–110. [PubMed: 15610768]

Dutta D, Calvani R, Bernabei R, Leeuwenburgh C, Marzetti E. Contribution of impaired mitochondrial autophagy to cardiac aging: mechanisms and therapeutic opportunities. Circ Res. 2012; 110:1125–1138. [PubMed: 22499902]

Facundo HT, de Paula JG, Kowaltowski AJ. Mitochondrial ATP-sensitive K^+ channels are redox-sensitive pathways that control reactive oxygen species production. Free Radic Biol Med. 2007; 42:1039–1048. [PubMed: 17349931]

Fenton RA, Dickson EW, Dobson JG Jr. Inhibition of phosphatase activity enhances preconditioning and limits cell death in the ischemic/reperfused aged rat heart. Life Sci. 2005; 77:3375–3388. [PubMed: 16098993]

Fenton RA, Shea LG, Doddi C, Dobson JG Jr. Myocardial adenosine A_1-receptor-mediated adenoprotection involves phospholipase C, PKC-epsilon, and p38 MAPK, but not HSP27. Am J Physiol Heart Circ Physiol. 2010; 298:H1671–H1678. [PubMed: 20363896]

Ferdinandy P, Schulz R, Baxter GF. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. Pharmacol Rev. 2007; 59:418–458. [PubMed: 18048761]

Förster K, Paul I, Solenkova N, Staudt A, Cohen MV, Downey JM, Felix SB, Krieg T. NECA at reperfusion limits infarction and inhibits formation of the mitochondrial permeability transition pore by activating p70S6 kinase. Basic Res Cardiol. 2006; 101:319–326. [PubMed: 16604438]

Fridolfsson HN, Kawaraguchi Y, Ali SS, Panneerselvam M, Niesman IR, Finley JC, Kellerhals SE, Migita MY, Okada H, Moreno AL, Jennings M, Kidd MW, Bonds JA, Balijepalli RC, Ross RS, Patel PM, Miyanohara A, Chen Q, Lesnefsky EJ, Head BP, Roth DM, Insel PA, Patel HH. Mitochondria-localized caveolin in adaptation to cellular stress and injury. FASEB J. 2012; 26:4637–4649. [PubMed: 22859372]

Fridolfsson HN, Patel HH. Caveolin and caveolae in age associated cardiovascular disease. J Geriatr Cardiol. 2013; 10:66–74. [PubMed: 23610576]

Fujita T, Otsu K, Oshikawa J, Hori H, Kitamura H, Ito T, Umemura S, Minamisawa S, Ishikawa Y. Caveolin-3 inhibits growth signal in cardiac myoblasts in a Ca^{2+}-dependent manner. J Cell Mol Med. 2006; 10:216–224. [PubMed: 16563233]

Institute of Medicine (US) Committee on Preventing the Global Epidemic of Cardiovascular Disease. Meeting the Challenges in Developing Countries. Promoting Cardiovascular Health in the Developing World: A Critical Challenge to Achieve Global Health. In: Fuster, V.; Kelly, BB., editors. Epidemiology of Cardiovascular Disease. Washington (DC): National Academies Press (US); 2010. p. 2

Gems D, McElwee JJ. Broad spectrum detoxification: the major longevity assurance process regulated by insulin/IGF-1 signaling? Mech Ageing Dev. 2005; 126:381–387. [PubMed: 15664624]

Gross ER, Hsu AK, Gross GJ. Opioid-induced cardioprotection occurs via glycogen synthase kinase beta inhibition during reperfusion in intact rat hearts. Circ Res. 2004; 94:960–966. [PubMed: 14976126]

Guo Z, Levi R, Aaronson L. The isolated human pectinate muscle: a reliable preparation of human cardiac tissue. J Pharmacol Toxicol Methods. 1983; 9:127–135.

Halestrap AP, Connern CP, Griffiths EJ, Kerr PM. Cyclosporin A binding to mitochondrial cyclophilin inhibits the permeability transition pore and protects hearts from ischaemia/reperfusion injury. Mol Cell Biochem. 1997; 174:167–172. [PubMed: 9309682]

Harper JM, Salmon AB, Chang Y, Bonkowski M, Bartke A, Miller RA. Stress resistance and aging: influence of genes and nutrition. Mech Ageing Dev. 2006; 127:687–694. [PubMed: 16713617]

Hassouna A, Loubani M, Matata BM, Fowler A, Standen NB, Galinanes M. Mitochondrial dysfunction as the cause of the failure to precondition the diabetic human myocardium. Cardiovasc Res. 2006; 69:450–458. [PubMed: 16330008]

Head BP, Patel HH, Roth DM, Lai NC, Niesman IR, Farquhar MG, Insel PA. G-protein-coupled receptor signaling components localize in both sarcolemmal and intracellular caveolin-3-associated microdomains in adult cardiac myocytes. J Biol Chem. 2005; 280:31036–31044. [PubMed: 15961389]

Head BP, Peart JN, Panneerselvam M, Yokoyama T, Pearn ML, Niesman IR, Bonds JA, Schilling JM, Miyanohara A, Headrick J, Ali SS, Roth DM, Patel PM, Patel HH. Loss of caveolin-1 accelerates neurodegeneration and aging. PLoS One. 2010; 5(12):e15697. [PubMed: 21203469]

Headrick JP. Aging impairs functional, metabolic and ionic recovery from ischemia-reperfusion and hypoxia-reoxygenation. J Mol Cell Cardiol. 1998; 30:1415–1430. [PubMed: 9710809]

Headrick JP, Willems L, Ashton KJ, Holmgren K, Peart J, Matherne GP. Ischaemic tolerance in aged mouse myocardium: the role of adenosine and effects of A_1 adenosine receptor overexpression. J Physiol. 2003; 549:823–833. [PubMed: 12717009]

Heinen A, Winning A, Schlack W, Hollmann MW, Preckel B, Frässdorf J, Weber NC. The regulation of mitochondrial respiration by opening of mKCa channels is age-dependent. Eur J Pharmacol. 2008; 578:108–113. [PubMed: 17936270]

Horikawa YT, Panneerselvam M, Kawaraguchi Y, Tsutsumi YM, Ali SS, Balijepalli RC, Murray F, Head BP, Niesman IR, Rieg T, Vallon V, Insel PA, Patel HH, Roth DM. Cardiac-specific overexpression of caveolin-3 attenuates cardiac hypertrophy and increases natriuretic peptide expression and signaling. J Am Coll Cardiol. 2011; 57:2273–2283. [PubMed: 21616289]

Huhn R, Heinen A, Hollmann MW, Schlack W, Preckel B, Weber NC. Cyclosporine A administered during reperfusion fails to restore cardioprotection in prediabetic Zucker obese rats in vivo. Nutr Metab Cardiovasc Dis. 2010; 20:706–712. [PubMed: 19819119]

Hunter JC, Kostyak JC, Novotny JL, Simpson AM, Korzick DH. Estrogen deficiency decreases ischemic tolerance in the aged rat heart: Roles of PKCdelta, PKCepsilon, Akt, and GSK3beta. Am J Physiol Regul Integr Comp Physiol. 2007; 292:R800–R809. [PubMed: 17008461]

Juhaszova M, Zorov DB, Yaniv Y, Nuss HB, Wang S, Sollott SJ. Role of glycogen synthase kinase-3β in cardioprotection. Circ Res. 2009; 104:1240–1252. [PubMed: 19498210]

Jurado-Pueyo M, Campos PM, Mayor F, Murga C. GRK2-dependent desensitization downstream of G proteins. J Recept Signal Transduct Res. 2008; 28:59–70. [PubMed: 18437630]

Kawabe JI, Grant BS, Yamamoto M, Schwencke C, Okumura S, Ishikawa Y. Changes in caveolin subtype protein expression in aging rat organs. Mol Cell Endocrinol. 2001; 176:91–95. [PubMed: 11369447]

Keon WJ, Labow RS, Hendry PJ, Barry YA. The human atrial trabecula: effects of calcium and temperature. J Mol Cell Cardiol. 1991; 23:357–364. [PubMed: 1880817]

Kostyak JC, Hunter JC, Korzick DH. Acute PKCδ inhibition limits ischaemia-reperfusion injury in the aged rat heart: role of GSK-3β. Cardiovasc Res. 2006; 70:325–334. [PubMed: 16542646]

Labow R, Desjardins S, Keon W. Validation of human trabecular preparation for evaluation of inotropic substances. J Pharmacol Toxicol Methods. 1991; 26:257–268.

Lesnefsky EJ, He D, Moghaddas S, Hoppel CL. Reversal of mitochondrial defects before ischemia protects the aged heart. FASEB J. 2006; 20:1543–1545. [PubMed: 16793872]

Liu P, Xu B, Cavalieri TA, Hock CE. Attenuation of antioxidative capacity enhances reperfusion injury in aged rat myocardium after MI/R. Am J Physiol Heart Circ Physiol. 2004; 287:H2719–H2727. [PubMed: 15358609]

Liu L, Zhu J, Brink PR, Glass PS, Rebecchi MJ. Age-associated differences in the inhibition of mitochondrial permeability transition pore opening by cyclosporine A. Acta Anaesthesiol Scand. 2011; 55:622–630. [PubMed: 21827445]

Ludman AJ, Yellon DM, Hausenloy DJ. Cardiac preconditioning for ischaemia: lost in translation. Dis Model Mech. 2010; 3:35–38. [PubMed: 20075380]

Mariani J, Ou R, Bailey M, Rowland M, Nagley P, Rosenfeldt F, Pepe S. Tolerance to ischemia and hypoxia is reduced in aged human myocardium. J Thoracic Cardiovasc Surg. 2000; 120:660–667.

Marzetti E, Csiszar A, Dutta D, Balagopal G, Calvani R, Leeuwenburgh C. Role of mitochondrial dysfunction and altered autophagy in cardiovascular aging and disease: from mechanisms to therapeutics. Am J Physiol Heart Circ Physiol. 2013; 305:H459–H476. [PubMed: 23748424]

Oudot A, Martin C, Busseuil D, Vergely C, Demaison L, Rochette L. NADPH oxidases are in part responsible for increased cardiovascular superoxide production during aging. Free Radic Biol Med. 2006; 40:2214–2222. [PubMed: 16785035]

Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM. Opening of mitochondrial K_{ATP} channels triggers the preconditioned state by generating free radicals. Circ Res. 2000; 87:460–466. [PubMed: 10988237]

Peart J, Headrick JP. Adenosine-mediated early preconditioning in mouse: protective signaling and concentration dependent effects. Cardiovasc Res. 2003; 58:589–601. [PubMed: 12798432]

Peart JN, Headrick JP. Clinical cardioprotection and the value of conditioning responses. Am J Physiol Heart Circ Physiol. 2009; 296:H1705–H1720. [PubMed: 19363132]

Peart JN, Gross GJ. Cardioprotective effects of acute and chronic opioid treatment are mediated via different signaling pathways. Am J Physiol Heart Circ Physiol. 2006; 291:H1746–H1753. [PubMed: 16731654]

Peart JN, Gross ER, Headrick JP, Gross GJ. Impaired p38-MAPK/HSP27 signaling underlies aging-related failure in opioid-mediated cardioprotection. J Mol Cell Cardiol. 2007; 42:972–980. [PubMed: 17407780]

Peart JN, See Hoe L, Pepe S, Johnson P, Headrick JP. Opposing effects of age and calorie restriction on molecular determinants of myocardial ischemic tolerance. Rejuvenation Res. 2012; 15:59–70. [PubMed: 22236144]

Pepe S, Tsuchiya N, Lakatta EG, Hansford RG. PUFA and aging modulate cardiac mitochondrial membrane lipid composition and Ca^{2+} activation of PDH. Am J Physiol Heart Circ Physiol. 1999; 276:H149–H158.

Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, André-Fouët X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, Ovize M. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med. 2008; 359:473–481. [PubMed: 18669426]

Przyklenk K, Li G, Simkhovich BZ, Kloner RA. Mechanisms of myocardial ischemic preconditioning are age related: PKC-epsilon does not play a requisite role in old rabbits. J Appl Physiol. 2003; 95:2563–2569. [PubMed: 12909609]

Przyklenk K, Maynard M, Darling CE, Whittaker P. Aging mouse hearts are refractory to infarct size reduction with post-conditioning. J Am Coll Cardiol. 2008; 51:1393–1398. [PubMed: 18387442]

Rosenfeldt FL, Pepe S, Ou R, Mariani JA, Rowland MA, Nagley P, Linnane AW. Coenzyme Q10 improves the tolerance of the senescent myocardium to aerobic and ischemic stress: studies in rats and in human atrial tissue. Biofactors. 1999; 9:291–299. [PubMed: 10416043]

Schneider A, Ad N, Izhar U, Khaliulin I, Borman JB, Schwalb H. Protection of myocardium by cyclosporin A and insulin: *in vitro* simulated ischemia study in human myocardium. Ann Thorac Surg. 2003; 76:1240–1245. [PubMed: 14530018]

Schulman D, Latchman DS, Yellon DM. Effect of aging on the ability of preconditioning to protect rat hearts from ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol. 2001; 281:H1630–H1636. [PubMed: 11557553]

Shore DE, Carr CE, Ruvkun G. Induction of cytoprotective pathways is central to the extension of lifespan conferred by multiple longevity pathways. PLoS Genet. 2012; 8:e1002792. [PubMed: 22829775]

Sinha B, Köster D, Ruez R, Gonnord P, Bastiani M, Abankwa D, Stan RV, Butler-Browne G, Vedie B, Johannes L, Morone N, Parton RG, Raposo G, Sens P, Lamaze C, Nassoy P. Cells respond to mechanical stress by rapid disassembly of caveolae. Cell. 2011; 144:402–413. [PubMed: 21295700]

Sutherland C, Leighton IA, Cohen P. Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. Biochem J. 1993; 296:15–19. [PubMed: 8250835]

Takayama M, Ebihara Y, Tani M. Differences in the expression of protein kinase C isoforms and its translocation after stimulation with phorbol ester between young-adult and middle-aged ventricular cardiomyocytes isolated from Fischer 344 rats. Jpn Circ J. 2001; 65:1071–1076. [PubMed: 11768000]

Tani M, Honma Y, Hasegawa H, Tamaki K. Direct activation of mitochondrial K_{ATP} channels mimics preconditioning but protein kinase C activation is less effective in middle-aged rat hearts. Cardiovasc Res. 2001; 49:56–68. [PubMed: 11121796]

Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of “modified reperfusion” protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res. 2004; 95:230–232. [PubMed: 15242972]

Tsutsumi YM, Horikawa YT, Jennings MM, Kidd MW, Niesman IR, Yokoyama U, Head BP, Hagiwara Y, Ishikawa Y, Miyanohara A, Patel PM, Insel PA, Patel HH, Roth DM. Cardiac-specific overexpression of caveolin-3 induces endogenous cardiac protection by mimicking ischemic preconditioning. Circulation. 2008; 118:1979–1988. [PubMed: 18936328]

Whittaker R, Glassy MS, Gude N, Sussman MA, Gottlieb RA, Glembotski CC. Kinetics of the translocation and phosphorylation of alphaB-crystallin in mouse heart mitochondria during ex vivo ischemia. Am J Physiol Heart Circ Physiol. 2009; 296:H1633–H1642. [PubMed: 19252088]

Willems L, Zatta A, Holmgren K, Ashton KJ, Headrick JP. Age-related changes in ischemic tolerance in male and female mouse hearts. J Mol Cell Cardiol. 2005; 38:245–256. [PubMed: 15698831]

Yu S, Driscoll M. EGF signaling comes of age: promotion of healthy aging in C. elegans. Exp Gerontol. 2011; 46:129–134. [PubMed: 21074601]

Zhu M, Feng J, Lucchinetti E, Fischer G, Xu L, Pedrazzini T, Schaub MC, Zaugg M. Ischemic postconditioning protects remodeled myocardium via the PI3K-PKB/Akt reperfusion injury salvage kinase pathway. Cardiovasc Res. 2006; 72:152–162. [PubMed: 16901477]

Zhu J, Rebecchi MJ, Tan M, Glass PS, Brink PR, Liu L. Age-associated differences in activation of Akt/GSK-3β signaling pathways and inhibition of mitochondrial permeability transition pore opening in the rat heart. J Gerontol A Biol Sci Med Sci. 2010; 65:611–619. [PubMed: 20427381]

Zhu J, Rebecchi MJ, Glass PS, Brink PR, Liu L. Interactions of GSK-3β with mitochondrial permeability transition pore modulators during preconditioning: age-associated differences. J Gerontol A Biol Sci Med Sci. 2013; 68:395–403. [PubMed: 23070879]

Highlights

- Aging repressed ischemic tolerance in human atrial tissue and mouse hearts
- Aging reduced cardioprotection via preconditioning (hormesis) and GPCR/PKC activation
- Protection via modulation of key mitochondrial effectors of protection was preserved
- Survival signal transduction from receptors to mitochondria is dysfunctional, in association with membrane/caveolar changes
- Protein expression in aged mouse heart further favors mitochondrial injury/cell death

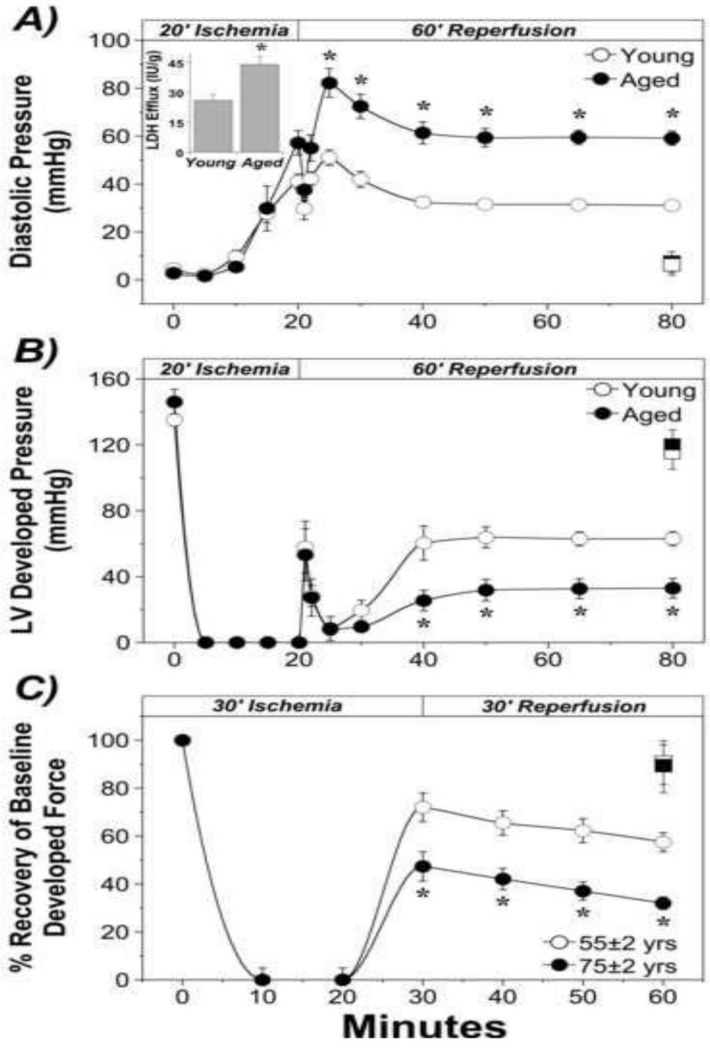

Fig. 1.

Effects of I-R on contractile dysfunction and cell damage in young to senescent murine (**A** and **B**) and human (**C**) myocardium. Functional outcomes and LDH efflux (inset to **A**) were assessed in Langendorff hearts subjected to 20 min ischemia/60 min reperfusion. Final diastolic and developed pressures are shown for normoxic hearts (time-course non-ischemic controls) from young and aged mice (open and closed squares, respectively). Functional outcomes were assessed in human trabeculae subjected to 30 min ischemia and 30 min or longer reperfusion (**C**). Recovery of LV end-diastolic pressure (mmHg) and LV developed pressure (mmHg) is shown, together with post-ischemic LDH efflux (see panel within **A**). Stability of function in normoxic control (non-ischemic) tissue is also shown for the 55 and 75 yr old groups (open and closed squares, respectively). * $P<0.05$ vs. Young. Values are reported as mean ± SEM (murine: n=13–17 per group; human: n=10 per group).

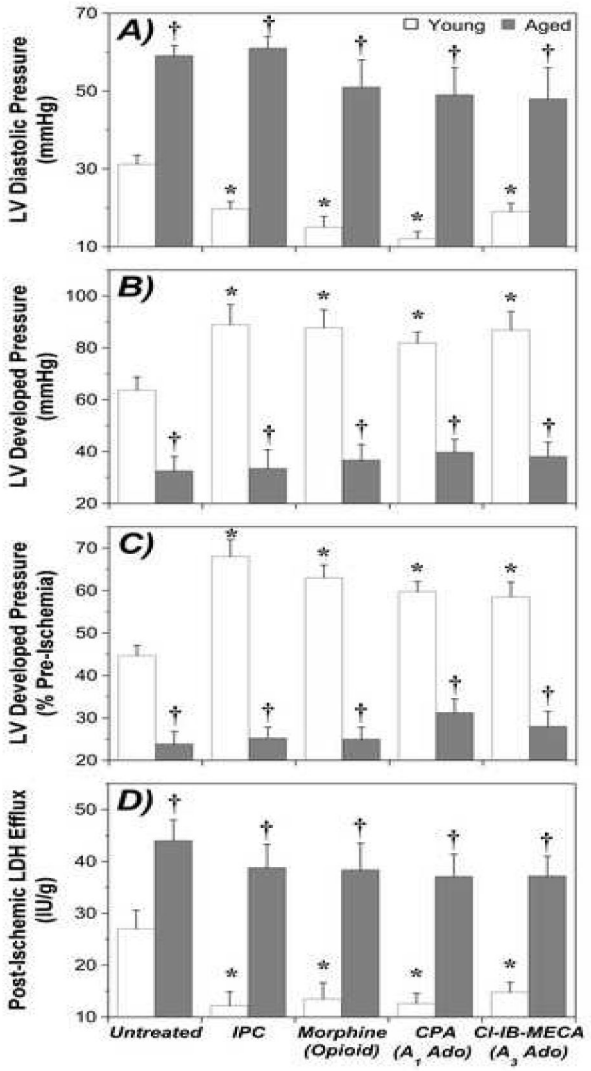

Fig. 2.

Protective efficacies of IPC and GPCR stimuli in young (open bars) and aged (shaded bars) murine hearts. Effects of IPC, and opioid (morphine) and A_1 and A_3 adenosine receptor agonism (CPA and Cl-IB-MECA, respectively) are shown on: **A)** post-ischemic recovery of LV end-diastolic pressure; **B)** post-ischemic recovery of LV developed pressure; **C)** % post-ischemic recovery of LV developed pressure; and **D)** post-ischemic efflux of LDH. † $P<0.05$ *vs*. Young; * $P<0.05$ *vs*. Untreated. Values are reported as mean ± SEM (n=7–17 per group).

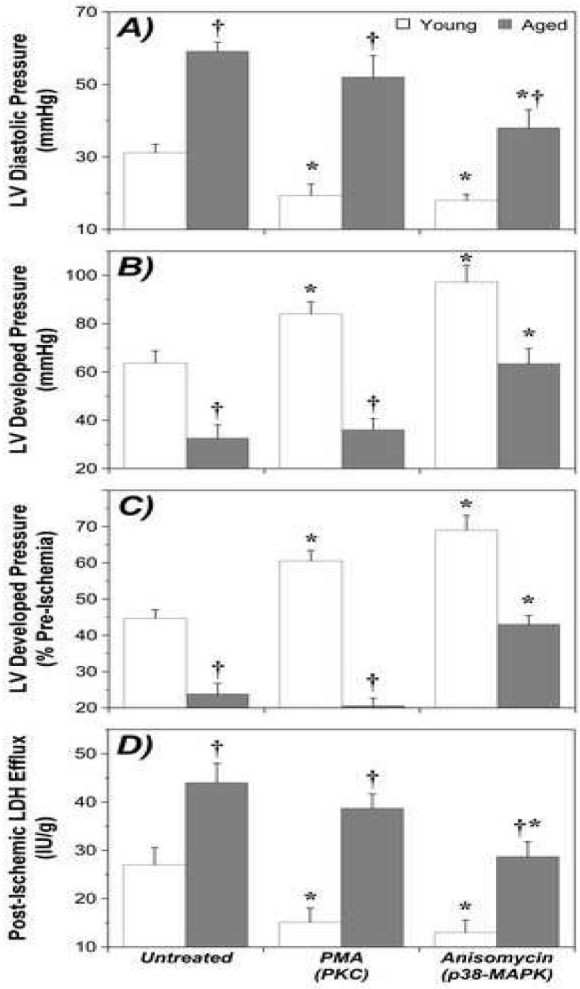

Fig. 3.

Protective efficacies of survival kinase stimuli in young (open bars) and aged (shaded bars) murine hearts. Effects of PKC (PMA) and p38-MAPK (anisomycin) activation are shown on: **A)** post-ischemic recovery of LV end-diastolic pressure; **B)** post-ischemic recovery of LV developed pressure; **C)** % post-ischemic recovery of LV developed pressure; and **D)** post-ischemic efflux of LDH. † $P<0.05$ *vs.* Young; * $P<0.05$ *vs*. Untreated. Values are reported as mean ± SEM (n=8–17 per group).

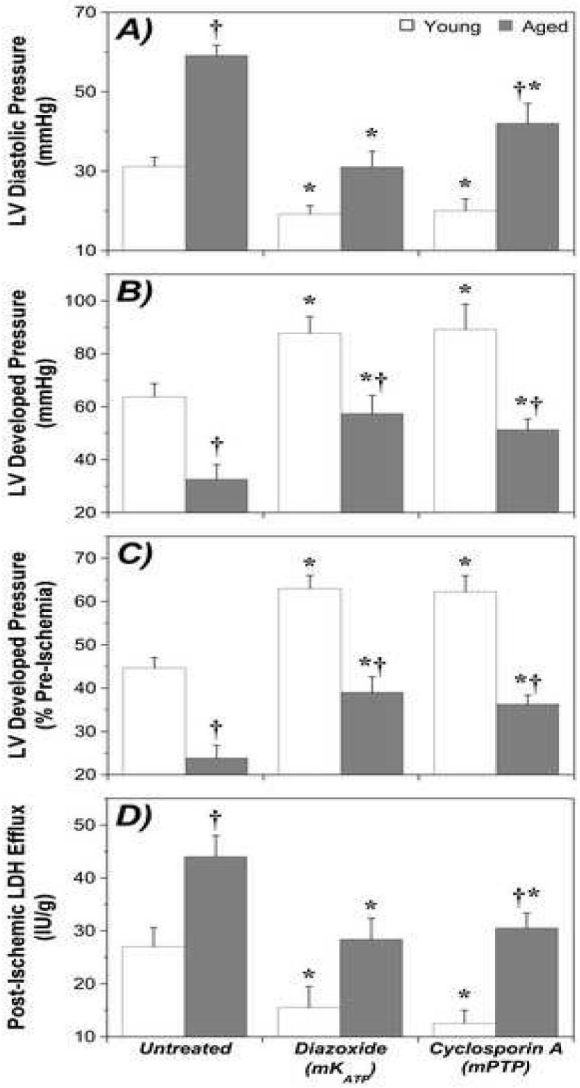

Fig. 4.

Protective efficacies of mitochondria-dependent stimuli in young (open bars) and aged (shaded bars) murine hearts. Effects of mK_{ATP} channel activation (diazoxide) and mPTP inhibition (cyclosporin A) are shown on: **A)** post-ischemic recovery of LV end-diastolic pressure; **B)** post-ischemic recovery of LV developed pressure; **C)** % post-ischemic recovery of LV developed pressure; and **D)** post-ischemic efflux of LDH. † P<0.05 *vs*. Young; * P<0.05 *vs*. Untreated. Values are reported as mean ± SEM (*n*=8–17 per group).

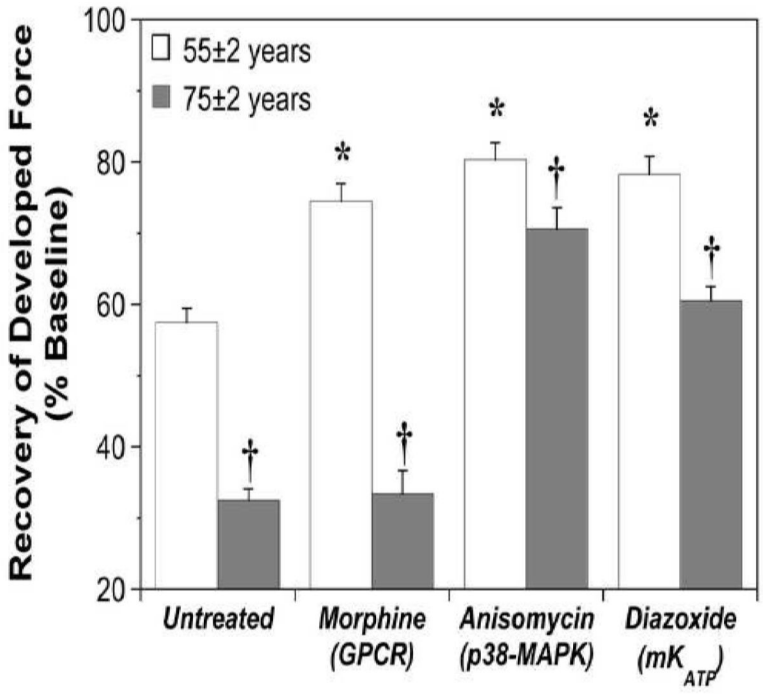

Fig. 5.

Functional recoveries from 30 min I-R in human atrial trabeculae preparations from 55±2 and 75±2 yrs cohorts. Data are shown for the % recovery of baseline normoxic (pre-ischemic) developed force in the absence and presence of pretreatment with a GPCR stimulus (10 μM morphine), a protein kinase activator (10 μM anisomycin) and a mitochondrially targeted stimulus (10 μM diazoxide). † $P<0.05$ vs. younger age group; * $P<0.05$ *vs*. Untreated. Values are reported as mean ± SEM (n=10 per group).

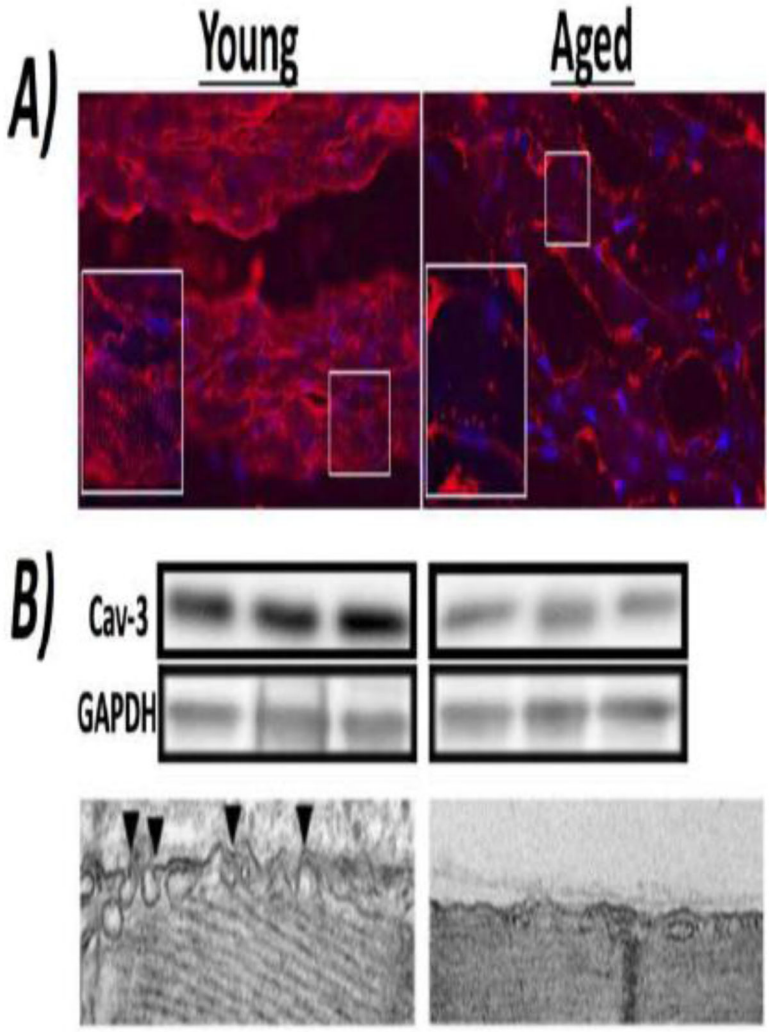

Fig. 6.

Aging alters the membrane, reducing caveolae and caveolin-3 (Cav-3) expression. **A)** Immunohistochemistry of young and aged heart tissue for caveolin-3. Large inset is higher zoom of smaller inset. Aged hearts show reduced Cav-3. **B)** Immunoblots confirming reduced Cav-3 protein expression, together with evidence of reduced caveolae formation (assessed by EM) in aged relative to young hearts.

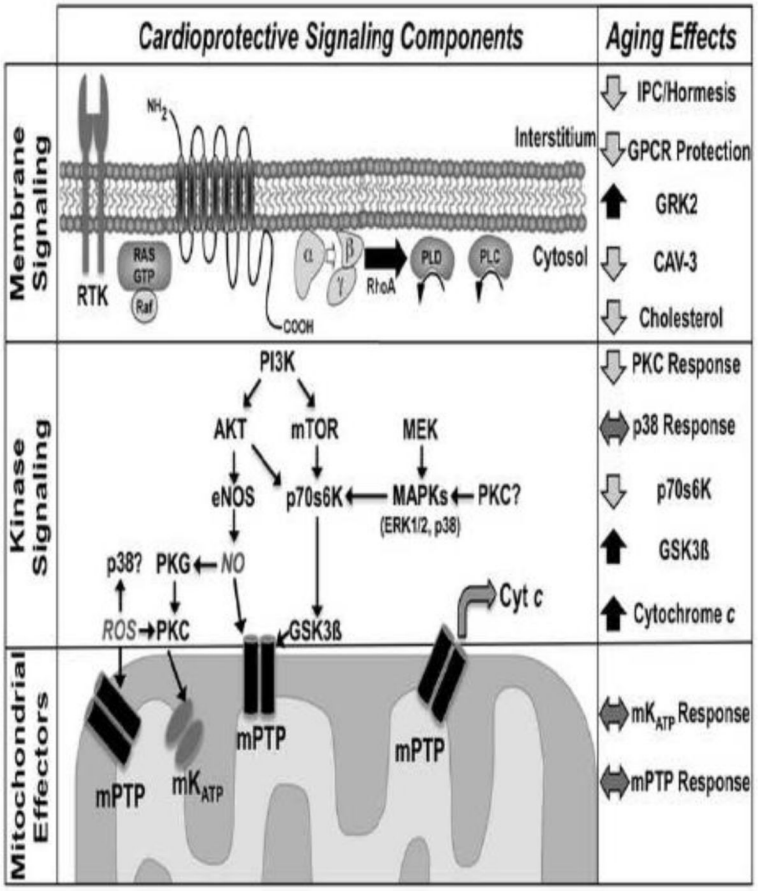

Fig. 7.

Schematic of myocardial protective signaling, highlighting effects of age (up/down arrows: increased/reduced responsiveness or expression; sideways arrows: no change). Multiple membrane receptors (G-protein coupled and growth factor receptors) are activated by local ligands to initiate pro-survival signaling during stress, involving diverse 2nd messengers and kinase signaling (select kinases are shown, with specific interactions between kinase paths, the precise order of signaling events, and mechanisms coupling kinases to mitochondria yet to be fully elucidated). Age negatively impacts the membrane signal compartment and local aspects of kinase signaling, while mK_{ATP} channels and the mPTP retain functionality (localizing dysfunction between GPCRs/PKC and p38-MAPK/mitochondria). Impaired stress-resistance and survival signaling may involve shifts in determinants of membrane signaling (caveolin-3, cholesterol), GPCR function (GRK2), kinase signaling (p70s6K, PKC), mitochondrial injury (GSK3β, GRK2) and apoptosis (cytochrome *c*).

Abbreviations: AKT, protein kinase B; CAV-3, Caveolin-3; Cyt *c*, cytochrome *c*; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; GRK2, G-protein coupled receptor kinase 2; GSK3β, glycogen synthase kinase 3β; MEK, MAPK/ERK kinase; mK_{ATP}, mitochondrial ATP-gated K^+ channels; mPTP, mitochondrial permeability transition pore; mTOR, mechanistic target of rapamycin; p38, p38-mitogen activated protein kinase; p70s6K, p70 ribosomal protein S6 kinase; PI3K, phosphoinositide

3-kinase; PKC, protein kinase c; PKG, protein kinase G; PLC, phospholipase C; PLD, phospholipase D; RhoA, Ras homolog family member A; RTK, receptor tyrosine kinase.

Table 1

Baseline cardiac function in perfused hearts from young and aged mice.

Group	Heart Wt (mg)	Heart:Body Weight	EDP (mmHg)	LVDP (mmHg)	Heart Rate (beat/min)	+dP/dt (mmHg/s)	−dP/dt (mmHg/s)	Coronary Flow (ml/min/g)
Young (*n*=17)	122±5	4.87±0.12	4±2	135±4	385±10	5876±367	−4573±413	23.0±1.6
Aged (*n*=13)	184±7*	5.08±0.10	3±1	146±8	344±11*	5118±395	−3677±248	16.9±1.2*

Heart rate is the spontaneous rate prior to commencement of pacing at 420 bpm. All other values were recorded immediately prior to onset of ischemia. EDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; + and −dP/dt, peak differentials of LV pressure development and relaxation, respectively.

*P<0.05 *vs*. Young. Values are reported as mean ± SEM (*n*=13–17 per group).

Table 2

Baseline properties of human atrial tissue segments from 55 and 75 yr old cohorts.

Age	Weight (mg)	L_{max} (mm)	CSA (mm^2)	DT (mg)	RT (mg)	tPT (ms)	tR50% (ms)	CD (ms)
55±2 (*n*=10)	6.8±1.2	7.4±1.1	0.8±0.1	1147±150	294±77	90±13	96±11	187±11
75±2 (*n*=10)	7.0±1.1	7.8±1.1	0.9±0.2	1215±151	357±81	95±8	101±11	196±11

Tissue dimensions are provided (weights and cross-sectional areas, CSA) together with baseline contractile parameters assessed immediately prior to simulated ischemia (or parallel normoxic control conditions). Contractile parameters measured and reported include: L_{max}, the tissue length yield maximal force or tension development; DF, developed tension (peak tension minus resting tension); RT, resting tension; tPT, time taken to develop peak tension per contraction; tR50%, time taken to reach 50% relaxation post-contraction; CD, contraction duration.

*P<0.05 *vs*. 55 yrs. Values are reported as mean ± SEM (*n*=10 per group).

Table 3

Stress-intolerant molecular profile in aged *vs.* young murine hearts.

	SARCOLEMMAL		***PRO-SURVIVAL***				***PRO-INJURY***		***PRO-APOPTOSIS***	
Group	**CAV-3**	**CHOL**	**AKT**	**p70s6K**	**ERK**	**p38**	**GRK2**	**GSK3β**	**CASP-3**	**Cyt-*C***
Young (*n*=6)	100±3	100±4	100±8	100±6	100±9	100±12	100±11	100±8	100±25	740±24
Aged (*n*=6)	75±5*	77±5*	110±9	78±7*	106±13	109±8	162±14*	142±10*	110±19	973±38*

Myocardial protein expression and membrane cholesterol levels normalized to values in young murine hearts. Cytosolic cytochrome *c* levels are expressed as ng/μg protein. CAV-3, caveolin-3; CHOL, cholesterol; AKT, protein kinase B; p70s6K, p70 ribosomal protein S6 kinase; ERK, extracellular signal-regulated kinase 1/2; p38, p38-mitogen activated protein kinase; GRK2, G-protein coupled receptor kinase 2; GSK3β, glycogen synthase kinase 3β; CASP-3, caspase-3; Cyt-*C*, cytochrome *c*.

*P<0.05 *vs*. Young. Values are reported as means ± SEM; *n*=6 per group.