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Chair: Lea Delbridge and John Headrick

Age-associated changes in cardiac excitation-contraction coupling in ventricular myocytes isolated from male and female Fischer 344 rats

S.E. Howlett, Dalhousie University, Department of Pharmacology, 5850 College Street, Halifax, Nova Scotia, Canada B3H 1X5. (Introduced by Lea Delbridge)

Mammalian hearts show an age-related decline in contractile function, in particular when heart rates are rapid or when hearts are stimulated by catecholamines (Lakatta & Levy, 2003). Similar age-related deficits in contractile function also are present in ventricular myocytes isolated from aged hearts (Xiao *et al.*, 1994; Lim *et al.*, 2000). However, most previous studies of excitation-contraction (EC)-coupling in aged myocytes have been conducted in cells from male animals only, or from animals where the sex has not been specified. We recently showed that age-associated changes in EC-coupling are more prominent in myocytes from male mice compared to cells from female mice (Grandy & Howlett, 2006). The goal of this study was to determine whether age-related alterations in EC-coupling were affected by the sex of the animal in the Fischer 344 rat, a commonly used animal model of ageing.

Young adult (approximately 3 months) and aged (approximately 24 months) male and female Fischer 344 rats were anaesthetized with an intra peritoneal injection of sodium pentobarbital (220 mg/kg). Ventricular myocytes were isolated by enzymatic dissociation as described previously (O'Brien & Howlett, 2008). All studies were performed at 37°C. Voltage clamp experiments were conducted with high resistance microelectrodes. Cells were paced with a series of conditioning pulses delivered at a frequency of 2 Hz prior to a test step from -40 to 0 mV. Myocytes were loaded with fura-2 AM to measure intracellular calcium concentrations. Unloaded cell shortening was measured simultaneously with a video edge detector. Sarcoplasmic reticulum (SR) calcium content was assessed by rapid application of 10 mM caffeine.

Results showed that myocyte length increased with age in males, but the amplitude of contraction normalized to cell length (fractional shortening) declined with age in the male group (mean contractions were 6.7 ± 0.6 *vs* 2.4 ± 0.4 % for young adult and aged males; n = 19-23 cells/group). Calcium current density also declined with age in cells from males. Calcium transient amplitudes and rates of rise were significantly smaller in aged male myocytes compared to young adult cells (mean calcium transient amplitudes were 47.7 ± 4.6 *vs* 28.1 ± 2.1 nM for young adult and aged males; $p < 0.05$). The SR calcium content did not change with age in male myocytes, but the rate of calcium released per unit calcium current density (an estimate of EC-coupling gain) declined with age in male myocytes (values were 322.8 ± 54.8 *vs* 186.8 ± 23.7 (nM/s)/(pA/pF) for young adult and aged males; $p < 0.05$). Furthermore, the fractional release of SR calcium was reduced in myocytes from aged males compared to younger cells. These results indicate that there is a marked age-related decline in cardiac contractile function in ventricular myocytes from male rats. Results in myocytes isolated from female rat hearts were markedly different. In contrast to results in male animals, cell length was unaffected by age and the degree of fractional shortening was similar in young adult and aged myocytes from female hearts (mean contractions were 4.9 ± 0.7 *vs* 4.9 ± 0.5 % for young adult and aged females; n = 16/cells/group). Furthermore, calcium transient amplitudes and rates of rise were unaffected by age in female myocytes (mean calcium transient amplitudes were 37.5 ± 5.5 *vs* 30.9 ± 3.7 nM for young adult and aged females). Calcium current density did decline with age in cells from females. Still, SR calcium content actually increased markedly in aged female myocytes compared to cells from younger animals (mean values were 49.0 ± 7.5 *vs* 147.3 ± 28.5 nM for young adult and aged females; $p < 0.05$), while fractional SR calcium release declined. However, the gain of EC coupling was not affected by age in myocytes from female rats (mean values were 158.7 ± 18.9 *vs* 202.4 ± 33.3 (nM/s)/(pA/pF) for young adult and aged females).

Together with results of our earlier work, these findings show that age-associated changes in cardiac EC-coupling are profoundly influenced by the sex of the animal. Age-related changes in EC-coupling are more prominent in myocytes from males than in cells from females in two different rodent models. This suggests that there may be a female advantage that limits detrimental effects of age on cardiac EC-coupling. As EC coupling gain was not affected by age in cells from female hearts, the increased SR calcium content might compensate for the decrease in calcium current to maintain contraction.

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Cardioprotective signalling in aging myocardium: failure of receptor-triggered protection

J.P. Headrick, J.N. Peart and K.J. Ashton, Heart Foundation Research Centre, Griffith University, Gold Coast, QLD 4217, Australia. (Introduced by Lea Delbridge)

The intrinsic ability of hearts to withstand injury during ischaemia-reperfusion (I/R) appears to decline with age. This may be linked to shifts in functionality of endogenous cardioprotective responses. Studies in young to senescent C57/Bl6 mice and Wistar rats reveal age-dependent reductions in functional recovery from I/R, together with increases in cell death and worsened metabolic/bioenergetic recoveries. This ischaemic 'intolerance' emerges prior to senescence, being near fully manifest by middle-age (12 mth) in mice (Willems *et al.*, 2005). This also precedes morphologic changes such as hypertrophy and fibrosis. Further analysis in mice reveals ischaemic tolerance actually begins to decline within 16-20 wk from birth, with changes slightly delayed in female *vs* male hearts.

Ischaemic intolerance coincides with failed protection in response to the G-protein coupled receptor (GPCR) ligands adenosine (1-50 μM) or morphine (10-30 μM), or with ischaemic pre- or postconditioning (Headrick *et al.*, 2003; Peart & Gross, 2004). cDNA microarray interrogation identifies substantial age-dependent shifts in ventricular myocardial gene expression that may play some role in the intolerant phenotype, with particular alterations in functional groups involved in energy/substrate metabolism, apoptosis, transcription and translation, and cell signalling pathways. In terms of the latter, changes were evident in the mTOR, Akt, TGF-β, Wnt, NFκB, and MAPK/Erk paths, and in GPCR signalling.

From a signalling perspective, aging was found to abrogate phorbol 12-myristate 13-acetate (PMA) mediated (PKC-dependent) protection, whereas activation of mitochondrial ATP-sensitive K^+ channels (50 μM diazoxide) or inhibition of the mitochondrial permeability transition pore (0.3 μM cyclosporin A) is protective in young to aged hearts. These findings implicate failed protective signalling distal to GPCRs and PKC, but proximal to mitochondrial targets. This is consistent with measured shifts in protective kinase activation (Peart *et al.*, 2007), which supports normal activation of signalling elements including GRK2, Akt, Erk1/2 and p70S6 kinase, but impaired phosphorylation/activation of p38 MAPK and its downstream targets (*e.g.* HSP27) in aged hearts. Pharmacological activation of p38 MAPK with 1 μM anisomycin affords protection in aged tissue, a response sensitive to p38 inhibition (1 μM SB203580). The basis of failed kinase signalling in these GPCR triggered, conventional forms of protection is unclear, though preliminary data support altered mRNA expression for: MKK3 upstream of p38 MAPK; MAPKAP2, the immediate target of p38; and Dusp1, a phosphatase that may counter p38 activation.

In contrast to conventional protective responses involving the abovementioned kinase pathways (and mitochondrial targets), novel protection in response to sustained activation of δ-opioid receptors is effective in young to aged hearts (Peart *et al.*, 2004). This may reflect the novel nature of signalling involved, which appears to be PI3-kinase/Akt, PKC and mito K_{ATP} independent, but PKA dependent. Further unraveling the basis of ischaemic intolerance and altered kinase signalling with age may facilitate development of protective strategies effective in the 'at-risk' aged myocardium.

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Phosphoinositide 3-kinase (PI3K, p110α) and adaptive growth in the heart

J.R. McMullen, Cardiac Hypertrophy, Baker IDI Heart & Diabetes Institute, Melbourne, VIC 8008, Australia.
(Introduced by L. M. Delbridge)

PI3Ks are important signalling proteins in numerous cell types. PI3Ks catalyse the phosphorylation of lipids in the cell membrane, leading to the generation of second messengers such as phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). There are three major classes of PI3Ks (classes I-III). These are determined based on amino acid sequence, homology of the lipid-kinase domains, and specificity for substrate binding. Class I PI3Ks consist of a 110kDa catalytic subunit (p110) complexed with a regulatory subunit, and can be divided into two subclasses: I_A and I_B. Class I_A PI3Ks (p110α, p110β and p110δ) associate with the regulatory proteins p85α, p85β and p55γ (as well as spliced variants of p85α), while p110γ (class I_B PI3K) is regulated by p101. p110α, β and γ are expressed in the heart and vasculature, while p110δ is found predominantly in leukocytes.

Transgenic and knockout mouse models have provided a powerful approach for understanding the specific roles of different PI3K isoforms in the heart. Studies in cardiac-specific PI3K transgenic mice have demonstrated that the p110α isoform of PI3K is critical for developmental and exercise-induced heart growth (physiological cardiac hypertrophy). Unlike pathological cardiac hypertrophy (heart growth in response to chronic pressure or volume overload e.g. hypertension, valve disease), physiological hypertrophy is characterised by normal cardiac structure and function, and does not lead to heart failure. Mice expressing a cardiac-specific constitutively active (ca) form of PI3K(p110α) displayed a 6.5 fold increase in PI3K(p110α) activity, which was associated with a 20% increase in heart size compared with control mice (non-transgenics). Mice expressing a dominant negative (dn) PI3K mutant displayed a 77% decrease in PI3K activity, and had 20% smaller hearts compared with non-transgenics. Importantly, caPI3K and dnPI3K mice showed no signs of cardiomyopathy (such as fibrosis) and had normal cardiac function and lifespan under basal conditions. dnPI3K mice showed a blunted response to exercise (a stimulus that induces physiological heart growth), but not to pressure overload (a pathological stimulus that leads to maladaptive heart growth, cell death and fibrosis), suggesting that PI3K(p110α) is critical for physiological, but not pathological, induced cardiac growth. These studies were later confirmed using a knockout approach. Deletion of class I_A PI3Ks from cardiac myocytes in mice led to a reduction in heart size that was similar in magnitude to that observed in dnPI3K mice. Knockout mice also showed a blunted cardiac hypertrophic response to exercise training.

Genetic mouse models have also highlighted the potential of targeting the PI3K(p110α) pathway in a setting of cardiac disease. This pathway is important for maintaining cardiac function and has anti-fibrotic and anti-apoptotic actions. In general, heart failure research and therapy has concentrated on identifying and inhibiting pathological processes. An alternative approach may be to identify and activate genes elevated during physiological cardiac hypertrophy, such as PI3K(p110α). Of note, given PI3K(p110α) has numerous actions in numerous cell types an on-going challenge is to find a means by which the PI3K(p110α) pathway can specifically be activated in cardiac myocytes.

Metabolic challenges for cardiac mitochondria: from womb to tomb

S. Pepe, Murdoch Children's Research Institute, Department of Cardiology, Royal Children's Hospital, Flemington Rd., Melbourne, VIC 3052, Australia. (Introduced by Lea M.D. Delbridge)

Mitochondrial metabolism has long been recognized as central to sustaining the energy requirements of myocardial work and other cellular processes, with mitochondrial volume varying from 30% to 60% depending on the type of heart cell. Key elements of our latest understanding of how mitochondrial delivery of energy is intertwined with intracellular signaling between all cell compartments, indicate that the role of mitochondria extends well beyond mere energy delivery and includes regulatory roles in the cell cycle, growth and development, maintenance of cell and organ level homeostasis, apoptosis and cell death.

The most current view is that mitochondria form a crucial nexus of multiple intracellular signal transduction pathways, participating in bi-directional exchange of signaling between intracellular components. Such centrality not only ensures that changes in energy demand and metabolism are rapidly and efficiently met, it constantly permits adaptation to a lifetime of stress due to high work demand, acute and chronic disease, and senescence.

Mitochondria form a contiguous integrated reticulum which is morphologically plastic and undergoes continuous remodeling/movement, especially in development and adaptation. Mitochondrial morphology is regulated by fission, fusion and motility of the reticulum in response to changes in myocardial state set by function and energy demand. Mitochondrial respiration and metabolism is closely regulated according to mitochondrial shape, location and signaling. Thus the location, function and biogenesis of mitochondria closely reflect critical mitochondrial response to the demands of change on the cell such as in growth, cell division, development, pathological stress, ageing or cell death.

It is not surprising then that during development, advanced age or cardiovascular disease, heart dysfunction is associated with abnormal mitochondrial structure and function. Mitochondrial dysfunction may involve genetic defects and/or the consequences of post-metabolic perturbations. The severity of heart dysfunction will depend on the adaptive reserve capacity of mitochondria and their ability to activate or suppress alternative metabolic pathways and related gene expression.