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

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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 yrs) 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; 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.

1. Introduction

An understanding of the molecular basis of cardiac aging, and of the influences of age on myocardial responses to disease and therapy, is an increasingly important goal. Ischemic heart disease (IHD) and associated stroke are the lead causes of mortality globally (Fuster and 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; Headrick et al., 2003; Lesnefsky et al., 2006; Mariani et al., 2000; Rosenfeldt et al., 1999) compounded by refractoriness to protective intervention (Boengler et al., 2009; Ferdinandy et al., 2007; Peart and 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 and McElwee, 2005). Intrinsic resistance to diverse stressors is a common (potentially defining) feature of longevity phenotypes (Harper et al., 2006), and this hypothesis is

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.

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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 (*e.g.* mechanistic 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 and Park, 2005; Yu and Driscoll, 2011). However, there is also evidence of impaired GPCR-dependent stress-signaling in older myocardial tissue (Headrick et al., 2003; Peart et al., 2007; Schulman et al., 2001). The expression of proteins targeted by this signaling, such as GSK3β (Hunter et al., 2007; Kostyak et al., 2006) 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 (Calabrese et al., 2012; Gems and McElwee, 2005; Shore et al., 2012). Identifying intrinsic protective mechanisms that become dysfunctional or retain efficacy with age can thus unmask mechanistic aspects of biologcial 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 (Fenton et al., 2010; Headrick et al., 2003; Peart and Gross, 2006); 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 anesthetized 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 mm Hg, 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 and 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 (Mariani et al., 2000; Rosenfeldt et al., 1999). 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 the 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, $PO_2 \geq 450$ mm Hg). 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