AuPS/ASB Meeting - Newcastle 2007

Symposium: Influencing factors in the fate of cardiac myocytes in heart disease

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Chair: Livia Hool, David Saint

Spatial and temporal patterns of cAMP production in cardiovascular physiology and pathophysiology using FRET

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The diffusible second messenger cAMP plays a central role in autonomic regulation of the electrical, mechanical, and metabolic activity of cardiac myocytes. This includes sympathetic responses involving the β_1-adrenergic receptor and parasympathetic responses involving the M_2 muscarinic receptor. It is often assumed that activation of these receptors produces either a uniform increase or decrease in cAMP levels throughout the entire cell. However, this does not easily explain some experimental observations, especially those associated with certain potentially pathologic responses. Therefore, we employed a systems biology approach to test the hypothesis that cAMP signaling in cardiac myocytes is compartmentalized. Live cell imaging of fluorescence resonance energy transfer (FRET)-based biosensors expressed in different subcellular locations was used to measure spatial and temporal changes in cAMP activity in adult ventricular myocytes. It was then determined if those results are consistent with the predictions of a quantitative computational model of compartmentalized cAMP signaling in a myocyte made up of two sub-membrane microdomains (caveolar and extra-caveolar) and one bulk cytosolic domain. This model was created using published information on the subcellular distribution and kinetic properties of β_1-adrenergic and M_2 muscarinic receptors, stimulatory and inhibitory G proteins, as well as different adenylyl cyclase and phosphodiesterase isoforms. Our findings support the conclusion that even under basal conditions there are significant differences in the concentration of cAMP found in various microdomains within cardiac myocytes. Furthermore, compartmentation of cAMP signaling can explain both simple and complex temporal responses associated with β_1-adrenergic and M_2 muscarinic receptor activation.

Multiple gene mutations altering cell fate and severity of heart disease

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Introduction: Familial hypertrophic cardiomyopathy (FHC) is characterised by both genetic and clinical heterogeneity: more than 400 mutations in at least 15 genes have been identified leading to a diverse phenotype ranging from no symptoms to heart failure and sudden cardiac death. Although previously believed to be caused by a single gene defect, our recent study demonstrated that in an Australian FHC cohort up to 5% families carry two FHC-causing mutations (Ingles *et al.*, 2005). The presence of a second mutation results in earlier disease onset, increased cardiac hypertrophy, and higher incidence of sudden death events. This observation suggests that the severity of human FHC may relate to the number of disease-causing gene mutations.

Methods: A double mutant mouse model has been established to investigate the mechanisms involved in the development of the severe FHC phenotype. Two single mutant mouse models of FHC - Gly203Ser cardiac troponin I (TnI-203) and Arg403Gln α-myosin heavy chain (MHC-403) - were crossed to obtain offspring with a double mutation genotype (TnI-203/MHC-403). Four groups of littermates (non-transgenic (NTG), TnI-203, MHC-403, and TnI-203/MHC-403) were characterised by survival, heart:body weight (HW:BW) and lung:body weight (LW:BW) ratios, histopathology, ECG and qRT PCR mRNA expression of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), cardiac L-type Ca^{2+} channel (LTCC), cardiac ryanodine receptor (RyR2), sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA2a) and phospholamban (PLB).

Results: Single mutant models have previously been shown to develop FHC by the age of 20-30 weeks with no effect on the life span (Semsarian *et al.*, 2002, Tsoutsman *et al.*, 2006). Unlike single mutant littermates, TnI-203/MHC-403 mice develop severe FHC phenotype resulting in 100% mortality by age 21 days. At age 14 days, TnI-203/MHC-403 mice developed significantly increased HW:BW ratio, marked interstitial myocardial fibrosis, and increased expression of the hypertrophy-related genes, ANF and BNP, compared to NTG and both single mutant littermates. This was associated with significant prolongation of PR, RR and corrected QT interval, consistent with the development of severe cardiomyopathy; marked down-regulation of mRNA levels of key regulators of intracellular Ca^{2+} homeostasis, RyR2, SERCA2a and PLB. At the age of 16-18 days LW:BW ratio in double mutant mice is significantly increased, mice rapidly developed a severe dilated cardiomyopathy leading to death from heart failure, most commonly between ages 16-20 days.

Conclusions: The presence of two disease-causing mutations may predispose individuals to higher risk of developing a severe cardiac phenotype, heart failure and early death compared to single-gene mutation. The TnI-203/MHC-403 model is an important molecular tool to facilitate the understanding of FHC pathogenesis and its complications, including the end-stage heart failure.

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Heart cell attrition early in life – the beginning of the end?

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There is considerable evidence that perinatal trophic influences have a long-term impact on defining adult growth patterns. An inverse relationship has been demonstrated between birth weight and adult susceptibility to cardiovascular disease. The fetal origins of disease have been most extensively studied with respect to early vascular and renal development, antecedent to the later occurrence of hypertension, kidney disease, diabetes and metabolic syndrome. Cardiac enlargement at maturity is an important and independent predictor of cardiovascular morbidity and mortality. However, relatively little is known about the early developmental origins of the hypertrophic heart. Angiotensin II (AngII) mediates both cardiomyocyte hypertrophy and apoptosis, and appears to be of special importance in regulating neonatal heart growth. The cardiac renin-angiotensin system is particularly active in the fetus and neonate, and is up-regulated in the growth-restricted fetus.

Most of the known physiological functions of AngII are mediated by the AT1 receptor, which is ubiquitously expressed in adult tissues and signals via the classical Gq- pathways. The AT2 receptor is predominantly expressed in fetal and neonatal tissues, and its function is less well delineated. AT2-mediated cardiovascular responses have been characterized as generally opposing AT1-mediated effects, exerting vasodilatory, antigrowth, anti-proliferative, and pro-apoptotic actions. However, some studies of genetically manipulated rodents indicate that the AT2 receptor is also involved in modulating myocyte enlargement and is necessary for left ventricular hypertrophy. Thus, the role of the AT2 receptor remains ambiguous. How the AT1 and AT2 receptor balance regulates cardiac growth and apoptosis in the neonate and predisposes for abnormal adult myocardial growth is not known.

In our experimental studies of genetically determined cardiac hypertrophy in rodents, we have identified a link between cardiac growth restriction in the neonate and the development of cardiac hypertrophy at maturity. Neonatal heart growth suppression occurs in association with a high AT2/AT1 receptor expression ratio, and neonatal cardiomyocytes appear to be predisposed to a significantly increased incidence of AT1A receptor-mediated AngII-induced apoptosis. We have also used adenoviral constructs to manipulate AT1/AT2 receptor expression stoichiometry *in vitro* to characterise growth and apoptotic responses of neonatal cardiomyocytes of normal and hypertrophy pre-destined hearts.

Our studies identify a novel function of the AT2 receptor in modulating cardiac growth and development, and suggest that myocardial cell loss early in life may program for the later development of hypertrophic myocardial pathology.

RyR2 peptides mimic Ca^{2+} dysfunction associated with disease mutations and suggest greater susceptibility in atrial than ventricular myocytes

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(Introduced by Derek Laver)

Missense mutations in RyR2 underlie a number of cardiomyopathies including cathecholaminergic polymorphic ventricular tachycardia (CPVT) and type-2 arrhythmogenic right ventricular dysplasia (ARVD2). These conditions are associated with tachyarrhythmias during exercise or stress and affected patients are at increased risk of sudden cardiac death (Francis *et al.*, 2005). The mutations associated with CPVT or ARVD2 occur in 3 clusters, termed the N-terminal, central and the pore-forming regions of RyR2 (or regions 1, 2 & 3 respectively). This clustering of mutations and the qualitative similarity in phenotype led to the proposal of a common underlying molecular mechanism: Initially, it was proposed that the N-terminal and central regions represent interacting subdomains, which serve to stabilise the channel in the closed configuration (Yamamoto & Ikemoto, 2002). More recently, evidence has been presented that the large cluster of mutations in region 3 reflects the presence of a second pair of interacting subdomains, which comprise amino acid residues 3778-4220 and the cytosolic regions of the transmembrane domain located between residues 4498-4959 (Hamada *et al.*, 2007).

The interacting subdomain hypothesis suggests that it should be possible to mimic the effects of specific mutations by designing RyR2 peptides, which interact competitively to disrupt the subdomain interactions (Yamamoto & Ikemoto, 2002), *e.g.*, a peptide comprising a section of region 2, with the necessary amino acid sequence to bind to the corresponding recognition site on region 1, should interact competitively to weaken the subdomain interaction, thereby mimicking the disease state. However, a similar peptide containing a disease mutation should lack the structural characteristics necessary to interact competitively with the corresponding region 1 binding site, and have little or no effect on channel gating. In support of this hypothesis, we have shown that in permeabilized rat ventricular myocytes, a peptide corresponding to residues 2460-2495 of region 2 (DPc10) causes: i) a sustained increase the SR Ca^{2+} leak; ii) a transient increase in Ca^{2+} spark frequency; and iii) a decrease in the cytosolic [Ca^{2+}] threshold for spontaneous Ca^{2+} waves (Yang *et al.*, 2006). However, a similar peptide containing a disease mutation (A2474S) linked to CPVT had no effect on RyR2 function.

Here we report preliminary findings regarding the effects of DPc15 and DPc15-mut: novel region 3 peptides, corresponding to residues 4752-4773 of rabbit RyR2. DPc15mut contains the H4762P mutation of human RyR2, linked to CPVT. Experiments were done on saponin-permeabilized rat myocytes isolated from either the ventricle or the atrium. Rats (Wistar, 200g) were humanely killed in accordance with UK legislation. During and after permeabilization, cells were exposed to a mock intracellular solution containing (mM) ATP 5, phosphocreatine 10, HEPES 15 (free Ca^{2+} 200 nM, Mg^{2+} 1 mM. pH 7.1, 21^{o}C) Solutions also contained fluo-3 (10 μM), allowing changes in cytosolic [Ca^{2+}] to be detected using confocal microscopy. Under these conditions, addition of DPc15 to ventricular myocytes had no significant effect on: 1) Ca^{2+} sparks; 2) Ca^{2+} waves or the background fluorescence, an index of the RyR2 mediated Ca^{2+} leak (n = 6). However, in atrial myocytes, DPc15 caused: 1) a transient increase in spark frequency; 2) a sustained rise in background fluorescence and complex changes in the properties of spontaneous Ca^{2+} waves (n = 6). Consistent with the interacting subdomain hypothesis, DPc15mut had no significant effect on Ca^{2+} regulation in atrial or ventricular myocytes. Assuming the active peptide accurately mimics the CPVT mutation, these findings suggest that with less severe changes in RyR2 function, the atrium may be more susceptible than the ventricle.

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The effect of mechanical stimulation on cardiac myocytes: The acute and chronic effects of stress and strain

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Mechanical stimulation has an important regulatory function in the healthy heart, influencing both contractility (The Frank-Starling Law) and rhythm (The Bainbridge effect) on a beat to beat basis. However, chronic increases in stress, via pressure- or volume- overload, are associated with heart disease. Initially the response of myocytes to hypertension is compensatory. These include hypertrophy, a prolongation of the action potential duration (APD) and an increase in the intracellular Ca^{2+} transient with an associated increase in contractility. In rat, these effects seem common to both systemic (*e.g.* the spontaneously hypertensive rat, SHR) and pulmonary (*e.g.* monocrotaline-induced pulmonary hypertension, MCT) models. Possibly as a result of the complex fibre and sheet structure of the left ventricle there are gradients of stress and strain and of gene expression and function of proteins modulated by mechanical stimulation within the normal heart (Kelly *et al.*, 2006; Stones *et al*., 2007). It is therefore interesting, but perhaps not surprising, that the structural and functional response to hypertension is likewise, not uniform across the ventricular wall (*e.g.* as shown in the SHR (McCrossan *et al*., 2004). There is evidence that hypertrophied hearts are more susceptible to arrhythmogenic stimuli, including mechanical stimuli, than normal hearts. Acute increases in left ventricular dilation cause increased arrhythmias in the SHR (Evans *et al.*, 1995). A possible reason for this pre-disposition to arrhythmia is the APD lengthening and alteration in APD dispersion which is combined with increased activity of mechanosensitive channels (Kamkin *et al*., 2000). The generation of arrhythmias can rapidly lead to myocyte death due to the uncontrolled release of intracellular Ca^{2+}. If left unchecked compensatory hypertrophy usually progresses to heart failure where there is depressed contractile function despite the presence of hypertrophy. In addition to changes that affect contractility at a cellular level, there is evidence that increased apoptosis plays a role in the depression of whole heart pump function in both SHR (Bing *et al*., 2002) and MCT (Buermans *et al.*, 2005) hypertensive heart failure, where there is a switch from an anti-apoptotic to pro-apoptotic gene expression profile in the transition from a compensated to failing state.

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