

**AuPS/ASB Meeting - Newcastle 2007**

**Free communications: Cardiovascular/heart**

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Chair: Saxon White

## Evidence for direct regulation of the cardiac L-type Ca<sup>2+</sup> channel during changes in thiol redox state

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It has previously been demonstrated in isolated cardiac myocytes that the current through the L-type Ca<sup>2+</sup> channel (I<sub>Ca-L</sub>) can be altered by changes in the redox state of the cell. Mild oxidative stress induced by application of 30 μM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), increases basal I<sub>Ca-L</sub> while acute hypoxia (that is associated with a decrease in cellular H<sub>2</sub>O<sub>2</sub>) decreases basal I<sub>Ca-L</sub>. However, these effects were demonstrated in mammalian cells where the channel may be regulated by a number of other proteins that can undergo modifications in function as a result of changes in redox state. Therefore, the aim of this study was to determine whether the function of the cardiac L-type Ca<sup>2+</sup> channel can be altered as a result of direct modification of thiol groups on the channel protein.

HEK cells were transiently transfected with the cDNA for the long N terminal isoform of the α<sub>1C</sub> subunit of the human L-type Ca<sup>2+</sup> channel and the purified protein reconstituted into liposomes using the dehydration-rehydration method. Functional examination of channels was performed by patch-clamp method with pipette and external solutions containing (in mM): BaCl<sub>2</sub> 100 and KCl 50 (pH adjusted to 7.4). Bay K 8644 (50 μM) was added to facilitate channel opening. The current produced by the reconstituted protein was confirmed as the L-type Ca<sup>2+</sup> channel current according to the amplitude and slope conductance of the current and sensitivity to the L-type Ca<sup>2+</sup> channel blocker nisoldipine.

Exposure of liposomes to the thiol-specific oxidising agent 5,5'-dithio-bis[2-nitrobenzoic acid] (DTNB, 200 μM) significantly increased channel open probability (Po) by 98% (*p* = 0.033, *n* = 9) while exposure to the thiol-specific reducing agent dithiothreitol (1 mM) significantly decreased Po by 48% (*p* = 0.003, *n* = 7). Furthermore, addition of H<sub>2</sub>O<sub>2</sub> (30 μM) mimicked the effect of DTNB by significantly increasing Po by 67% (*p* = 0.049, *n* = 6). However, exposure of the liposomes to acute hypoxia by decreasing oxygen tension from 150 mmHg to 17 mmHg, had no significant effect on Po (*p* = 0.604, *n* = 7). Of the various thiol redox modifications there is increasing evidence that glutathionylation predominates in cells because glutathione is the most abundant low molecular mass thiol in the human cell. We examined the effect of oxidised glutathione (GSSG) on channel function. Addition of 5 mM GSSG increased the open probability of the channel 5 fold (*p* < 0.001, *n* = 5).

This study indicates that the thiol redox state of the cardiac L-type Ca<sup>2+</sup> channel is an important determinant of channel function. In addition inhibition of the basal current during hypoxia in cardiac myocytes does not occur as a result of a direct effect of oxygen on the channel protein. Changes in the glutathionylation state of the channel are likely to account for altered channel function during oxidative stress.

## “Battle of the Clocks” – Sinoatrial pacemaking through plasmalemmal ionic currents or intracellular Ca<sup>2+</sup> release

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The sinoatrial node (SAN) initiates and regulates cardiac pacemaking. Traditionally, the mechanism underlying generation of pacemaking in the SAN cells (SANCs) has been considered to arise through a “clock”, termed the plasmalemma clock, which resides in the cell membrane and involves cyclical interaction of various voltage-dependent channels. Recently a rival intracellular pacemaker mechanism, termed the store clock, has been proposed to dominate cardiac pacemaking. This mechanism is proposed to arise from rhythmic Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> stores which trigger or drive the plasmalemma clock. We have used experimental and modeling techniques to study the interaction between these two clocks underlying SAN pacemaking.

Tissue preparations containing the SAN and associated muscle were isolated from mouse heart freshly removed from young adult mice (age 5-8 weeks). Mice were euthanased by overexposure to the inhalation anaesthetic isoflurane (5-10% in air), a procedure approved by the Animal Care and Ethics Committee at the University of Newcastle. Tissues were loaded with a calcium indicator (Oregon Green/AM) and viewed with a high speed confocal imaging system (Perkin-Elmer Ultraview). In some cases intracellular microelectrode recordings were made simultaneously with the imaging. Numerical simulations were performed using a single SAN cell model containing two types of oscillators; 1) a plasmalemma oscillator composed of pacemaker, Na<sup>+</sup>, K<sup>+</sup>, and L-type Ca<sup>2+</sup> currents, 2) an intracellular Ca<sup>2+</sup> oscillator composed of a Ca<sup>2+</sup> store with cyclical release and uptake and Ca<sup>2+</sup> induced Ca<sup>2+</sup> release capability. These two oscillators were linked by a Na-Ca exchanger current that transformed the Ca<sup>2+</sup> oscillations into membrane potential oscillations. Thus the two clocks interacted through 1) Na-Ca exchanger and 2) Ca<sup>2+</sup> entry through L-type Ca<sup>2+</sup> channels. A multi-cellular model was also simulated using the single model cells now coupled by gap junctions.

The main results of our study were: 1) blockade of ryanodine receptors (RyRs) decreased pacemaker frequency but did not abolish pacemaking, thus pacemaking can continue in the absence of RyR-mediated store Ca<sup>2+</sup> release; 2) action potentials continued in the presence of Ca<sup>2+</sup>-free solution containing EGTA (0.1 mM) but now without associated changes in intracellular Ca<sup>2+</sup>; and 3) Ca<sup>2+</sup> increases were at times observed to precede action potentials during recovery from pacemaker blockade by either 2,3-buanedione monoxime (BDM) or high K<sup>+</sup> containing solutions. These results demonstrate that SAN pacemaking can continue in the absence of intracellular store clock but Ca<sup>2+</sup> released from intracellular Ca<sup>2+</sup> stores can advance the plasmalemma clock and effectively set the pacemaking frequency of the SAN (see also Lakatta *et al.*, 2003). Our modelling studies suggest that each clock is capable of dominating the other depending on the mutual frequency relationship. Importantly, in a group of gap junction connected pacemaker model cells the store clock can induce some cells to become the dominant pacemaker and *vice versa*.

We predict that in this symbiotic relationship the winner between these two clocks may vary according to various influences placed on the heart. Further studies are needed to show how these mechanisms interact to respond to such changing demands.

Lakatta EG, Maltsev VA, Bogdanov KY, Stern MD & Vinogradova TM. (2003) *Circulation Research*, **92**: e45-50.

## Impaired post-ischemic functional recovery in primary cardiac hypertrophy is accentuated in female rats

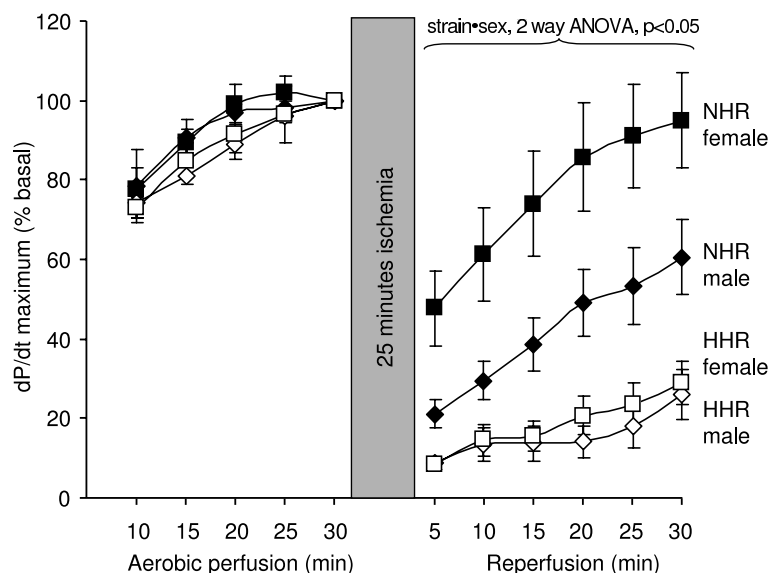
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Pathological cardiac hypertrophy is associated with impaired cardiac function and a heightened vulnerability to ischemia/reperfusion injury. Sex differences have been reported in the characteristics of left ventricular remodeling and the susceptibility of these hearts to ischemia/reperfusion injury. Much of the evidence in this field derives from animal models exhibiting adaptive cardiac hypertrophy secondary to genetic hypertension or experimental haemodynamic loading. This study investigated the susceptibility of the hypertrophic heart rat (HHR), a normotensive model of primary cardiac hypertrophy, to post-ischemic cardiac dysfunction and evaluated sex-specific effects.

Male and female HHR and control Normal Heart Rats (NHR), aged 12 weeks ( $n = 8-10$  per group), were anaesthetized (halothane). Hearts were rapidly excised and retrogradely perfused in the Langendorff mode with oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) bicarbonate buffer (37°C) at a constant pressure of 73mmHg. Left ventricular pressure was monitored continuously with an isovolumic, intraventricular balloon inflated to give an end diastolic pressure of 4mmHg. Hearts were aerobically perfused for 30 min prior to 25 min global ischemia and 30 min reperfusion.

In HHR hearts, ventricular weight index was significantly higher than NHR controls ( $4.6 \pm 0.1$  vs.  $5.6 \pm 0.2$  mg/g, male NHR vs HHR, and  $4.8 \pm 0.1$  vs.  $7.9 \pm 0.5$  mg/g, female, both  $p < 0.001$ ). No significant differences were observed between NHR and HHR in basal cardiac function (left ventricular developed pressure (LVDP), coronary flow, heart rate, rate pressure product (RPP), dP/dt max and min), though females in both strains had reduced dP/dt max and min compared with males. HHR hearts exhibited a substantial decrease in contractile recovery during post-ischemic reperfusion compared with NHR controls in both males (LVDP at end of reperfusion:  $31.7 \pm 7.1\%$  vs.  $70.3 \pm 8.32\%$ ,  $p < 0.005$ ) and females (LVDP:  $38.2 \pm 7.4\%$  vs.  $84.7 \pm 9.2\%$ ,  $p < 0.005$ ). Heart rates were not different. Analysis of ectopy in the first 10 minutes of reperfusion also revealed increased incidence of arrhythmias in HHR hearts for males ( $59.4 \pm 7.6\%$  vs.  $28.6 \pm 6.0\%$ , HHR vs NHR,  $p < 0.01$ ) and females ( $46.9 \pm 10.0\%$  vs.  $15.8 \pm 5.0\%$ , HHR vs. NHR,  $p < 0.05$ ).

In NHR females, a significantly greater improvement in post-ischemic recovery of dP/dt max compared with males was observed. This sex difference in post-ischemic recovery was not evident in HHR (strain\*sex, 2 way ANOVA,  $p < 0.05$ , see Figure). A similar pattern was observed in other parameters measured, including LVDP, RPP, and dP/dt min.



In conclusion, primary cardiac hypertrophy exacerbates the left ventricular dysfunction associated with ischemia/reperfusion and obviates the cardioprotection observed in non-hypertrophic females. Further studies are required to elucidate the signaling mechanisms responsible for this functional difference and to examine the role of sex steroids in the apparent loss of cardioprotection in the female HHR.

## Diet high in N6 PUFA lowers cardiac membrane N3:N6 fatty acid ratio and increases atrial mass and cardiomyocyte size

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Cardiac hypertrophy is an independent risk factor for cardiac morbidity and mortality (Levy *et al.*, 1990). Human trials have shown that omega-3 (N3) polyunsaturated fatty acids (PUFA) used in secondary prevention post myocardial infarction, are associated with a reduction in cardiovascular events (Marchioli *et al.*, 2005). Experimental evidence suggests that increasing myocardial phospholipid omega3:omega6 (N3:N6) PUFA ratio is cardioprotective (McLennan & Abeywardena, 2005). It is not known whether dietary N3 PUFA can protect against pathological cardiac growth. The aim of this study was to determine the effects of high PUFA diet (N3 & N6) on cardiac membrane fatty acid composition, growth and cardiomyocyte size.

Male Sprague-Dawley rats aged 8 weeks, were fed a fabricated diet high in either N3 (N3D, Nu-Mega fish oil), N6 (N6D, sunflower oil) or saturated fatty acids (SFD, cocoa butter) for 4 weeks. Other PUFA levels were low and caloric intake controlled among diet groups (16.8 MJ/ Kg). At feeding completion, rats were anaesthetized using halothane and killed by decapitation. Hearts were removed and dissected to measure tissue mass (atria, right ventricle, left ventricle) and tissues were snap frozen in liquid nitrogen for biochemical analysis ( $n = 8$ / diet group). Membrane phospholipid fatty acid (FA) composition was determined in left ventricle (LV) tissue by gas chromatography. In another group of diet treated rats ( $n = 4$ / diet group) hearts were excised and perfused in Langendorff mode for cardiomyocyte isolation by collagenase digestion for measurement of cardiomyocyte size. Length and width measurement were made for 100 LV myocytes per heart. All data are presented as mean  $\pm$  SEM and analyzed by one-way ANOVA.

Somatic growth over the dietary treatment period was equivalent for the diet groups and at feeding completion there were no significant differences in body mass (N3D  $485 \pm 10$  g, N6D  $491 \pm 9$  g, SFD  $492 \pm 10$  g). No significant differences were detected for whole heart mass (N3D  $1338 \pm 34$  mg, N6D  $1360 \pm 45$  mg, SFD  $1394 \pm 33$  mg) or LV mass (N3D  $958 \pm 28$  mg, N6D  $957 \pm 33$  mg, SFD  $980 \pm 25$  mg) between the three dietary groups. Atrial mass was greater in the N6D group, relative to N3D and SFD (N6D  $121 \pm 5$  mg vs. N3D  $102 \pm 4$  mg, SFD  $114 \pm 4$  mg,  $p = 0.046$ ), and was also greater when normalized atria to body mass (N6D  $0.25$  mg/g  $\pm 0.1$  vs. N3D  $0.2$  mg/g  $\pm 0.01$ , SFD  $0.2 \pm 0.01$  mg/g,  $p = 0.031$ ). Relative to N3D and SFD groups, the N6D group had a significantly increased LV cardiomyocyte width (N6D  $12.9 \pm 0.5$   $\mu$ m vs. N3D  $12.0 \pm 0.4$   $\mu$ m, SFD  $12.6 \pm 0.3$   $\mu$ m,  $p = 0.031$ ). LV cardiomyocyte length was not significantly different between treatment groups.

Long chain PUFA membrane incorporation reflected the differences in diet composition. Hearts of PUFA fed rats (N3D & N6D) showed significantly higher levels of membrane total PUFA compared to hearts of SFD rats. N6D rats had a significantly greater level of membrane N6 compared to N3D and SFD rats. The N3:N6 LV membrane ratio in the N6D and SFD rats was one-fifth the level measured in N3D rats (Table).

Fatty Acid Class	% total membrane fatty acids		
	SF Diet	N6 Diet	N3 Diet
Total Saturated FA	$34.7 \pm 0.1$	$34.4 \pm 0.1$	$34.3 \pm 0.3$
Total PUFA	$53.8 \pm 0.2$	$57.2 \pm 0.2^{\#}$	$54.9 \pm 0.2^*$
Total N6	$43.7 \pm 0.2$	$48.5 \pm 0.2^{\#}$	$27.5 \pm 0.5^*$
Total N3	$10.6 \pm 0.3$	$9.1 \pm 0.2$	$27.7 \pm 0.5^*$
N3:N6	$0.2 \pm 0.1$	$0.2 \pm 0.1$	$1.0 \pm 0.1^*$

\* $p < 0.05$  vs N6D and SFD;  $\#p < 0.05$  vs SFD

These findings demonstrate that an N6 diet promotes atrial and LV cardiomyocyte growth. While no change was observed in LV mass, the increase in cell width suggests that dietary treatment may result in LV hypertrophy with a prolonged feeding period. Dietary N3 FA incorporation into the membranes may act as a protective stimulus suppressing whole heart and cardiomyocyte hypertrophy.

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Marchioli R, Levantesi G, Macchia A, Magginoi AP, Masrfisi RM, Silletta MG, Tavazzi L, Tognoni G, Valagussa F on behalf of the GISSI-Prevenzione Investigators (2005) *The Journal of Membrane Biology*, **206**:117-28.

McLennan PL & Abeywardena MY (2005) *The Journal of Membrane Biology*, **206**: 85-102.

## Effect of hexarelin on transient outward potassium current in rat ventricular myocytes

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Hexarelin is a synthetic peptide growth hormone secretagogue (GHS), which possesses a variety of cardiovascular protective effects mediated by the GHS receptor (GHSR), including improving cardiac dysfunction and remodeling (Xu *et al.*, 2005). It has been reported that GHRP increased  $\text{Ca}^{2+}$  influx *via* voltage-gated  $\text{Ca}^{2+}$  channels in cultured neonatal rat cardiomyocytes (Xu *et al.*, 2003). This increase in  $\text{Ca}^{2+}$ -influx can be achieved by a direct increase in  $\text{Ca}^{2+}$  channel conductance and/or a decrease in  $\text{K}^{+}$  channel conductance with prolonged depolarization. Modification of ion channels and the intracellular signaling pathways by GHS are currently unknown and this study aims to fill this gap. Ventricular myocytes were enzymatically isolated from adult male Sprague-Dawley rats and kept in Tyrode solution. Nystatin-perforated whole-cell patch-clamp recording was performed on isolated cells within 6 hours after successful cell preparation. Hexarelin (100 nmol, 10 nmol, 1 nmol, 0.1 nmol, and 0.01 nmol) inhibited transient outward potassium current ( $I_{\text{to}}$ ) in a dose-dependent manner. The inhibition appeared at doses above 0.01 nmol. Ghrelin evoked similar inhibition on this  $\text{K}^{+}$  current. The inhibition was abolished in the presence of the GHS-R1a specific antagonist BIM28163. In term of triggered action potential duration (APD), ghrelin and hexarelin significantly prolonged APD in the presence of the calcium channel blocker  $\text{CoCl}_2$  (2 mmol) and the delayed rectifier  $\text{K}^{+}$  channel blocker tetraethylammonium (50 mmol/l) in bath solution. The hexarelin-induced  $I_{\text{to}}$  inhibition was abolished by the protein kinase C (PKC) inhibitor, Gö-6983, but not by the PKA inhibitor, H89. We therefore conclude that hexarelin and ghrelin activate PKC system through the stimulation of GHS-R1a, resulting in a decrease in the  $I_{\text{to}}$  amplitude, prolongation of action potential duration, and increase in  $\text{Ca}^{2+}$ -influx.

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Xu XB, Pang JJ, Cao JM, Ni C, Xu RK, Peng XZ, Yu XX, Guo S, Chen MC & Chen C. (2005) *American Journal of Physiol*, **289** H1643-51.

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## **Antibody Microarrays: A new tool for testing leukocytes for inflammation**

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Currently, no blood-based test can rapidly and objectively distinguish between stable angina pectoris (SAP - chest pain when increased myocardial oxygen demand is not satisfied by an appropriate coronary blood flow), and unstable angina pectoris (UAP - where inadequate coronary flow produces pain at rest). In the search for appropriate identifying biomarkers, most methods have focused on serum-based tests. However, since leukocytes play an active role in the progression of coronary artery disease, we hypothesize that these cells can provide novel markers of SAP and UAP and may indeed be able to distinguish between them. Here we use antibody microarrays containing 82 cluster of differentiation (CD) antibodies (plus isotype controls) that selectively immobilize specific types of leukocytes from a suspension of applied peripheral blood mononuclear cells. This differential capture depends on the expression patterns of CD antigens expressed on their surface membranes. We find that the pattern of immobilization of leukocytes from both SAP and UAP patients with coronary artery disease (CAD) significantly differs from age- and gender-matched healthy subjects (Australian Red Cross Blood Service blood donors). Within the CAD group, 15 SAP patients exhibited significant ( $p < 0.05$ ) changes in the intensity of 10 of the 82 CD antibody spots in the array compared to 19 healthy blood donors. In the UAP group, the intensity of these 10 changes increased and an additional eight CD antigens differed significantly ( $p < 0.05$ ) between the blood donors and UAP patients. These preliminary data suggest that it is now appropriate to engage a larger clinical trial to test the hypothesis that these antibody arrays can be used to diagnose CAD and can monitor the progression from SAP to UAP.