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Free Communications: Cardiovascular/Kidney

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Chair: Livia Hool

Cardiac hypertrophy and oxidative stress are associated with insulin resistance in fructose fed mice

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Dietary fructose intake has increased considerably in recent decades, in parallel with an increase in the incidence of insulin resistance. Cardiovascular pathologies are highly prevalent in diabetic and pre-diabetic patients yet the impact of fructose on the heart is poorly understood. The aim of this study was to determine the specific cardiac effects of a 12 week 60% high fructose dietary intervention in C57Bl/6 male mice.

Blood pressure was measured by tail cuff method to establish that the effects of fructose were independent from hypertension. Systemic insulin sensitivity was estimated by glucose tolerance test and plasma insulin levels were determined by radioimmunoassay. Hearts were collected for measurement of ventricular weight index (VWI) and myocardial production of superoxide (lucigenin chemiluminescence). Phosphorylation states of signalling proteins in myocardial tissue were analysed by western blot and gene expression analysis of Thioredoxin 2 was performed using real time PCR.

VWI was increased by 22% in the fructose fed mice (p = 0.0006) which was associated with elevated superoxide production (fructose, 553 ± 28 counts/s/mg *vs* control, 489 ± 11 counts/s/mg, p = 0.049). This was not associated with an alteration in myocardial gene expression of the antioxidant, Thioredoxin 2. Fructose feeding suppressed phosphorylation of Akt and S6 indicative of a specific cardiac insulin resistance. Hyperglycaemia (fructose, 14.4 ± 0.6 mmol/L *vs* control, 12.1 ± 0.8 mmol/L) and impaired glucose tolerance were observed, but were not associated with hypertension or body weight gain. No change in plasma insulin levels was apparent. This study demonstrates that a 12 week dietary fructose intervention induces cardiac hypertrophy associated with oxidative stress. Fructose-induced insulin resistance is apparent both systemically and intrinsic to the myocardium suggesting that a specific cardiac insulin resistance may play a role in fructose induced cardiac pathologies. Importantly, these findings were observed in the absence of any volume or pressure loading effects from hypertension or obesity. This study demonstrates that excess consumption of fructose is detrimental to cardiac structure and signalling which may represent a primary pathology in insulin resistance.

Comparison of anoxic tolerance of isolated cardiac myocytes from male and female rats

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There is growing recognition that important cardiac-specific sex differences can impact on cardiac function, outcomes of cardiovascular disease, and the cardiac response to stresses such as ischemia. We have recently shown that intact *ex vivo* perfused hearts of male rats are more susceptible to ischemia/reperfusion (I/R) injury than those of female rats (Bell *et al.*, 2008). Ca^{2+} overload during early reperfusion has been shown to play a critical role in cardiac I/R injury and recent evidence has highlighted Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activation as a key factor in cellular apoptosis and necrosis in I/R injury. Whether sex-related differences in myocardial Ca^{2+} handling and/or CaMKII activation underlie the different vulnerability of male and female hearts to ischemic damage has, however, not been directly investigated. The goal of this study was to examine whether the enhanced susceptibility to I/R injury of male hearts could be attributable to differences in cardiac myocyte Ca^{2+} handling and/or CaMKII activity.

Hearts from 12-14 week old male and female Sprague Dawley rats were perfused in Langendorff mode with 50 mg/ml collagenase to enzymatically disperse the cardiac myocytes. The freshly isolated cells were loaded with the Ca^{2+} indicator fura-2 and placed in a superfusion chamber on the stage of an inverted fluorescence microscope. Fura-2 fluorescence (ratio 380/365 nm) and cell shortening (edge detection) were monitored using an Ionoptix system (Ionoptix Corporation, Maryland, USA). Cells were paced at 4Hz and subjected to a protocol designed to mimic ischemia and reperfusion. Following establishment of a steady-state in control solution the cells were superfused with a 'simulated ischemia' solution for 20 min followed by 'reperfusion' with control solution for 30 min. The composition of the control solution was (mM) NaCl (146), KCl (4.7), NaH_2PO_4 (0.35), $MgSO_4$ (1.05), $CaCl_2$ (2), glucose (11), HEPES (10), pH 7.4; while the 'simulated ischemia' solution contained (mM) NaCl (136), KCl (8), NaH_2PO_4 (0.35), $MgSO_4$ (1.05), $CaCl_2$ (2), Na-lactate (10), HEPES (10), pH 6.8. The 'simulated ischemia' solution was equilibrated with 100% N_2 and during the period of simulated ischemia the superfusion chamber was also maintained in a 100% N_2 environment. Cells were studied in either the absence or presence of the CaMKII inhibitor KN-93. For the KN-93 experiments cells were pre-treated with KN-93 for at least 15 min prior to use and KN-93 was present (5 μM) in all solutions used throughout the recording period.

Under control conditions the baseline amplitude of the Ca^{2+} transient and cell shortening were significantly lower in the female myocytes compared to the male myocytes. During simulated ischemia cell shortening initially decreased substantially, together with some diastolic cell lengthening, but then recovered somewhat with continued exposure to the 'simulated ischemia' solution. Upon 'reperfusion' with control solution cells exhibited diastolic contracture and variable recovery of contractile function. There was a marked difference in cell mortality during 'reperfusion' with only a 33% survival rate in male cells as compared to nearly 90% survival in female cells. Among the surviving cells no significant differences were detected in contractile recovery or Ca^{2+} handling between male and female myocytes during the I/R protocol. CaMKII inhibition attenuated diastolic contracture during 'reperfusion' in both male and female myocytes but did not significantly improve contractile recovery. CaMKII inhibition was, however, associated with markedly improved preservation of cell viability during 'reperfusion'.

These results suggest that male vulnerability to I/R injury may be due to an enhanced susceptibility of male myocytes to I/R-induced necrosis rather than depressed contractile recovery of surviving cells. Enhanced CaMKII activity in male cardiac myocytes, possibly due to higher steady-state cell Ca^{2+} levels, may contribute to their greater vulnerability to I/R damage compared to female cardiac myocytes.

Bell JR, Porello ER, Huggins CE, Harrap SB & Delbridge LM. (2008) *American Journal of Physiology: Heart and Circulatory Physiology*, **294:** H1514-22.

The Hypertrophic Heart Rat (HHR) exhibits enhanced myocardial PI3-K mediated signalling in the neonate, but not in the adult

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Hypertrophy of the heart represents a significant cardiovascular risk, independent of blood pressure. There is growing appreciation that adult cardiovascular disease states may be 'programmed' in early life, though the influence of perinatal growth on the development of adult cardiac hypertrophy has not been established. We have previously identified a genetic locus (*Lvm-1*) in the spontaneously hypertensive rat (SHR) associated with heart size but not blood pressure, and developed the Hypertrophic Heart Rat (HHR) as a normotensive model of adult primary cardiac hypertrophy (Harrap *et al.*, 2002). As phenotypic characterization revealed neonatal cardiac growth restriction relative to the control Normal Heart Rat (NHR), this study compared the expression and activation of selected intermediates of growth signalling pathways in young and adult HHR and NHR.

Neonatal (post-natal day 2, n =7) and adult (12 week old, n = 10) male NHR/HHR were anaesthetized with halothane inhalation, and hearts excised and immediately placed in liquid nitrogen. Ventricles were homogenized at 4°C in a HEPES/sucrose buffer using an Ultra-Turrax tissue grinder, with samples centrifuged at 3,000g for 5 minutes at 4˚C to recover the cytosolic fraction. Equal amounts of protein were subsequently loaded onto polyacrylamide gels (10%) for SDS-PAGE/Western blot analysis (quantified as relative expression units).

Marked hypertrophy was observed in adult HHR hearts, however, no changes in the phosphorylation status of Akt (1.14 ± 0.09 *vs* 1.00 ± 0.05, HHR *vs* NHR, p = ns), GSK3β (1.14 ± 0.12 *vs* 1.00 ± 0.12, p = ns) or ERK1/2 (1.03 ± 0.04 *vs* 1.00 ± 0.04, p = ns) were observed (*vs* NHR). Total calcineurin expression was similarly unchanged (1.19 ± 0.10 *vs* 1.00 ± 0.10, p = ns). In contrast to the adult, neonatal HHR hearts were smaller than NHR controls. This was associated with an increase in Akt phosphorylation (2.31 ± 0.53 *vs* 0.99 ± 0.07, p = 0.029), and a decrease in phosphorylation of both GSK3β (0.46 ± 0.06 *vs* 1.00 ± 0.17, $p < 0.01$) and ERK1/2 (0.82 ± 0.04 *vs* 1.00 ± 0.05, $p < 0.01$). Calcineurin expression was unchanged (0.98 ± 0.09 *vs* 1.00 ± 0.05, p = ns).

These differential activities are consistent with augmented PI3-K mediated 'physiological' growth signalling in the neonatal HHR. These findings indicate that where there is a genetic pre-disposition for hypertrophy, transient growth signalling perturbation in the neonate is observed and may represent an important modeling event in determining the occurrence of adult hypertrophy.

Harrap SB, Danes VR, Ellis JA, Griffiths CD, Jones EF, Delbridge LM (2002). *Physiological Genomics*, **9:** 43-8.

Mechanisms maintaining kidney tissue oxygenation during renal ischaemia in anaesthetised rabbits

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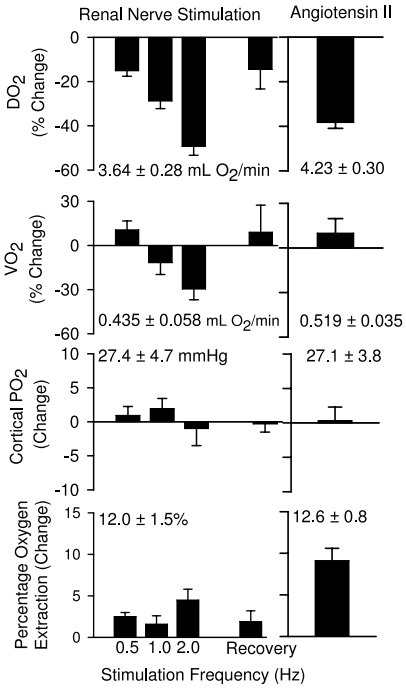
We have recently shown that the kidney has a remarkable ability to maintain stable tissue oxygen tension (PO_2) in the face of changes in renal blood flow (RBF) within the physiological range (O'Connor *et al.*, 2006; Leong *et al.*, 2007). According to the conventional view of kidney oxygenation, maintenance of homeostasis of kidney oxygenation is thought to be achieved almost exclusively through the 'flow limited' nature of kidney oxygen consumption (VO_2). That is, because most oxygen consumption by kidney tissue is attributable to sodium reabsorption, which in turn also drives reabsorption of other solutes, renal VO_2 often varies in proportion with glomerular filtration rate and thus RBF. However, this mechanism could only completely maintain homeostasis of kidney oxygenation if there is no mis-match between changes in renal oxygen delivery (DO_2) and VO_2. Therefore, we investigated the potential for percentage oxygen extraction by the kidney to increase during renal ischaemia, even in the absence of reduced tissue PO_2.

Rabbits were anaesthetised with pentobarbitone (90-150 mg plus 30-50 mg/h) and artificially ventilated. Catheters were placed in the ear arteries and renal vein and a transit-time ultrasound flow probe was placed around the renal artery. DO_2 and VO_2 were calculated from the oxygen content of renal venous and/or arterial blood and RBF. Cortical tissue PO_2 was determined by fluorescence optode. Urine was collected from the catheterized ureter. For electrical stimulation of the renal nerves (RNS, n = 15), the renal nerves were sectioned cranially and placed on stimulating electrodes (O'Connor *et al.*, 2006). For renal arterial infusion of angiotensin II (n = 12), a catheter was placed in the renal artery (Leong *et al.*, 2007).



The figure shows responses to RNS and angiotensin II infusion. Baseline values are within each panel. RNS caused frequency-dependent reductions in RBF (-44 ± 4% at 2 Hz) and DO_2 (-49 ± 4%), but a smaller reduction in VO_2 (-30 ± 7%). Angiotensin II reduced RBF (-37 ± 3%) and DO_2 (-38 ± 3%), but not VO_2 (+10 ± 10%). Despite mis-matched changes in DO_2 and VO_2, cortical tissue PO_2 did not fall. Percentage oxygen extraction increased 1.4-fold during 2 Hz RNS and 1.8-fold during angiotensin II. Renal venous PO_2 fell by -5.7 ± 1.7 mmHg during 2 Hz RNS and by -11.2 ± 2.0 mmHg during angiotensin II infusion. Neither renal venous blood PCO_2 nor pH changed in response to these ischaemic stimuli.

We conclude that during mild renal ischaemia, induced by RNS or angiotensin II infusion, reductions in DO_2 are not matched by reductions in VO_2. But tissue hypoxia does not occur, because the kidney extracts a greater proportion of DO_2, even in the absence of an increase in the PO_2 gradient between arterial blood and tissue. We speculate that increased percentage oxygen extraction could be driven by changes in the counter-current exchange of oxygen and/or carbon dioxide between renal arteries and veins. For example, diffusional shunting of carbon dioxide from intrarenal veins to arteries may *increase* during ischemia, reducing the pH of blood in peritubular capillaries. This should reduce the affinity of haemoglobin for oxygen (the Bohr effect) within renal peritubular capillaries, so increasing delivery of oxygen to tissue. There is also a theoretical basis for diffusional shunting of oxygen to *decrease* during renal ischemia (Evans *et al.*, 2008), which should increase delivery of oxygen to tissue.

Evans RG, Gardiner BS, Smith DW, O'Connor PM. (2008) *American Journal of Physiology – Renal Physiology* doi:10.1152/ajprenal.90230.2008

Leong C-L, Anderson WP, O'Connor PM, Evans RG. (2007) *American Journal of Physiology – Renal Physiology* **292:** F1726-33.

O'Connor PM, Kett MM, Anderson WP, Evans RG. (2006) *American Journal of Physiology – Renal Physiology* **290:** F688-94.

Computational modelling of oxygen transport in the whole kidney

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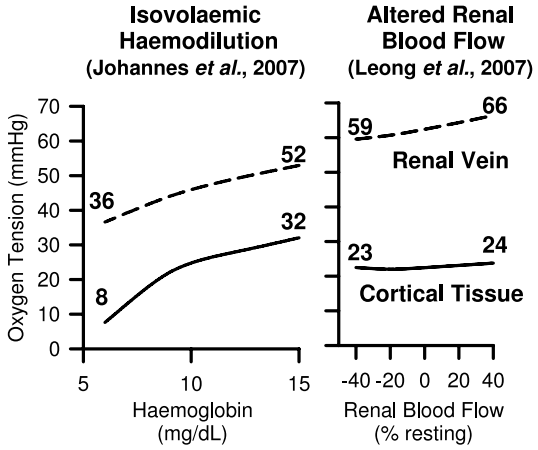
The traditional view is that oxygen homeostasis is achieved in the kidney by matching changes in renal oxygen delivery with proportionate changes in kidney oxygen consumption (VO_2). We have amassed evidence that this view of oxygen regulation is incomplete (Evans *et al.*, 2008), and that the parallel architecture of the renal vasculature facilitates dynamic regulation of kidney oxygenation by enabling oxygen shunting between arteries and veins, such that a significant portion of oxygen in renal arterial blood bypasses kidney tissue. To integrate this new evidence into the conventional model of renal oxygen homeostasis and so achieve a deeper understanding of the processes involved, we have developed a computational model of the parallel-branched vessel architecture for vessel sizes between 10-450 μm (in radius).

In our model the renal vasculature is represented by eight compartments of counter-current systems connected in series. That is, within each compartment an artery and a vein are in parallel configuration with opposing fluid velocities. These compartments correspond directly to the average vessel dimensions at eight of the eleven Strahler orders (branch level in vasculature) identified by Nordsletten *et al.*, (2006) in their structural analysis of the renal vasculature. Further, to account for the intimate association of the arteries and veins at each branch level (the vein is often seen to wrap around the artery) we use a concentric tube with an artery running down the centre of a vein. The Navier-Stokes equation is used to describe blood flow in each vessel and the reaction-advection-diffusion equations are used to model oxygen transport within and between vessels. These equations are numerically solved within each compartment and information on oxygen concentration is fed forward to the neighbouring branch. The binding of oxygen to haemoglobin is included in the model, as is VO_2.



As shown in the Figure, our computational model is able to predict changes in the venous PO_2 and tissue PO_2 for a range of RBF and haemoglobin concentrations consistent with available experimental data (Johannes *et al.*, 2007; Leong *et al.*, 2007). The model predicts a venous PO_2 greater than tissue PO_2 (consistent with oxygen shunting), relatively stable tissue PO_2 in the face of moderate changes in RBF, and that oxygen shunting renders the kidney susceptible to hypoxia during haemodilution. Further, our simulations have revealed that shunting provides a system level robustness which helps to ensure renal tissue PO_2 remains stable in the face of changes in RBF for wide range of combinations of VO_2 to RBF. That is the matching of changes in VO_2 and RBF required by the conventional model of renal oxygen regulation is relaxed by oxygen shunting to allow for a wider range of combinations of VO_2 and RBF.

In summary, the computational model of the parallel countercurrent renal vasculature we have developed has allowed us to integrate several complex processes involved in oxygen transport in the mammalian kidney. This model is able to reproduce existing experimental data and has provided a platform for testing and formulating hypotheses and development of a new understanding of renal oxygen regulation.

Evans RG, Gardiner BS, Smith DW, O'Connor PM. (2008) *American Journal of Physiology*, doi:10.1152/ajprenal.90230.2008.

Johannes T, Mik EG, Nohe B, Unertl KE, Ince C. (2007) *American Journal of Physiology*, **292:** F796-803.

Leong C-L, Anderson WP, O'Connor PM, Evans RG. (2007) *American Journal of Physiology*, **292:** F1726-33.

Nordsletten DA, Blackett S, Bentley MD, Ritman EL, Smith NP. (2006) *American Journal of Physiology* **291:** H296-309.