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Uraemic cardiomyopathy is characterised by loss of the cardioprotective effects of insulin

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Running Title:

Uraemic cardiomyopathy and insulin resistance

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Abstract

Chronic kidney disease is associated with a unique cardiomyopathy, characterised by a combination of structural and cellular remodelling, and an enhanced susceptibility to ischaemia-reperfusion injury. This may represent dysfunction of the reperfusion injury salvage kinase pathway, due to insulin resistance.

Aims: The susceptibility of the uraemic heart to ischaemia-reperfusion injury and the cardioprotective effects of insulin and rosiglitazone were investigated.

Methods and Results: Uraemia was induced in Sprague-Dawley rats by subtotal nephrectomy. Functional recovery from ischaemia was investigated in vitro in control and uraemic hearts ±insulin ±rosiglitazone. The response of myocardial oxidative metabolism to insulin was determined by 13C NMR spectroscopy. Activation of reperfusion injury salvage kinase pathway intermediates (Akt and GSK3β) were assessed by SDS-PAGE and immuno-precipitation. Insulin improved post-ischaemic rate pressure product in control but not uraemic hearts, (recovered rate pressure product (%), control 59.6±10.7 vs 88.9±8.5, $p<0.05$; uraemic 19.3±4.6 vs 28.5±10.4, p=ns). Rosiglitazone resensitised uraemic hearts to insulin-mediated cardio-protection (recovered rate pressure product (%) 12.7±7.0 vs. 61.8±15.9, $p<0.05$). Myocardial carbohydrate metabolism remained responsive to insulin in uraemic hearts. Uraemia was associated with increased phosphorylation of Akt (1.00±0.08 vs. 1.31±0.11, $p<0.05$) in normoxia, but no change in post-ischaemic phosphorylation of Akt or GSK3β. Akt2 isoform expression was decreased post-ischaemia in uraemic hearts ($p<0.05$).

Conclusion: Uraemia is associated with enhanced susceptibility to ischaemia-reperfusion

injury and a loss of insulin-mediated cardio-protection, which can be restored by administration of rosiglitazone. Altered Akt2 expression in uraemic hearts post ischaemia-reperfusion and impaired activation of reperfusion injury salvage kinase pathway may underlie these findings.

Key Words

Chronic kidney disease; ischaemia reperfusion injury; RISK pathway

Introduction

Chronic kidney disease (CKD) is an independent risk factor for cardiovascular mortality. (85) Although 'traditional' cardiovascular risk factors identify high risk populations, multiple 'non-traditional' risk factors which may be specific to CKD have also been identified. (71) Experimental and clinical investigations have provided evidence for unique properties of the uraemic heart at both cellular and structural levels which amount to a distinct uraemic cardiomyopathy (UCM). (3) An emerging feature of UCM is an enhanced susceptibility of the heart to ischaemia-reperfusion injury (IRI). (23)

IRI is a complex process in which the final common pathway for cellular damage is opening of the mitochondrial permeability transition pore (mPTP), (31) itself the focus of an endogenous protective cascade, termed the reperfusion injury salvage kinase (RISK) pathway. (33) Diverse strategies and ligands, including insulin, have been identified which activate this cascade and confer significant cardio-protection in experimental models. (33) In addition, small clinical trials and retrospective analyses of larger clinical studies suggest favourable outcomes when RISK activating interventions are given early. (4,59,60)

Insulin resistance remains an independent risk factor for cardiac death in CKD stages 3-5 and end stage renal failure. (6,75,82) The insulin signalling cascade converges on the RISK pathway at protein kinase Akt (also known as protein kinase B), and insulin administration is cardioprotective in non-uraemic experimental models of IRI. (37) Both experimental and clinical CKD have been associated with insulin resistance due to a post-receptor defect in skeletal muscle, (46) raising the possibility of impaired activation of the RISK pathway as a mechanism to explain the enhanced susceptibility to IRI. Experimental uraemia is

associated with increased chronic Akt phosphorylation and activation, (49) which has been experimentally demonstrated to inhibit RISK pathway cardio-protection in non-hypertrophied hearts. (56)

Thiazolidiones (TZDs) enhance glycaemic control in insulin resistance states, although their widespread clinical use is limited by adverse effects on heart failure. (18,58,77) However, experimentally pre-treatment with thiazolidiones offers cardioprotection in models of IRI. (48,89) In particular, rosiglitazone exerts cardioprotective effects in other non-uraemic but insulin resistant states, (90) an action mediated, at least in part, through the Akt and the RISK pathway, (89) via cardiac peroxisome proliferator-activated receptor γ (PPARγ). (87)

There is a great clinical need to better understand the pathophysiology of UCM. Although CKD is a chronically progressive condition, the risk of progression to end stage renal failure is overshadowed at all stages by a greater risk of death from cardiovascular causes. (32,42) This excessive cardiovascular risk continues after the initiation of renal replacement therapy and half of all deaths in the dialysis population are a result of cardiovascular events. (25,47) Further, in the haemodialysis population, there is increasing evidence that haemodialysis itself results in repeated cardiac IRI episodes that adversely affect cardiac function and prognosis. (10)

To date there are no data on the functional consequences of IRI, or the efficacy of the cardio-protective RISK pathway in experimental uraemia. Using the surgical remnant kidney model of chronic uraemia, this study investigated the hypothesis that the myocardial insulin resistance evident in UCM enhances the susceptibility of the uraemic heart to ischaemic reperfusion injury through impaired response of the RISK pathway. Further, we tested the hypothesis that pre-treatment with the insulin sensitising agent, rosiglitazone,

would improve cardioprotection in UCM.

Methods

Experimental model of uraemia

All animal experiments conformed to the UK Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Uraemia was induced in male Sprague-Dawley rats (approximately 250g) (Charles River, Sussex, UK), via a one-stage 5/6th nephrectomy as described previously. (79) Briefly, animals were anaesthetised using a mixture of isoflurane in oxygen (2.5% in 1L), a laparotomy was performed, the left kidney exposed, and at least two-thirds removed. This was immediately followed by a total right nephrectomy. Care was taken to ensure no damage was done to the adrenal glands. For control animals, a sham operation was performed whereby both kidneys were decapsulated and replaced intact.

Animals were maintained for 12 weeks post induction of uraemia, housed individually and pair-fed with control animals. Water was available ad libitum. Cardiac hypertrophy was assessed at the time of sacrifice by determining wet heart weight/tibia length (HW:TL).

Isolated heart perfusion

Animals were fasted for 12 hours, anaesthetised with sodium thiopentone (100 mg/Kg body weight) and the hearts excised. Hearts were perfused via the aorta in an isovolumic Langendorff mode, as described previously, (79) using Krebs-Henseleit buffer containing 3% fatty acid free Bovine serum albumin (BSA) and the following components (mM) NaCl (118.5), $NaHCO_3$ (25), KCl (4.8), KH_2PO_4 (1.2), MgSO4 (1.2), $CaCl_2$ (1.25-2.5), glucose (5),

113 sodium pyruvate (0.1), sodium lactate (1), sodium palmitate (0.3), glutamine (0.5) ± 0.1
114 mU/ml insulin. The buffer was gassed with 95% O_2, 5% CO_2 and maintained at 37°C.

115 Cardiac function was recorded continuously via a fluid filled balloon (inserted into the left
116 ventricle) and a physiological pressure transducer (SensoNor, Norway) connected to a bridge
117 amplifier and Powerlab 4/30 (20). Data were recorded using Chart 5.5 software (AD
118 Instruments, Hastings UK). The end diastolic pressure (EDP) was set to 5-7 mmHg by
119 adjusting the balloon volume and hearts were paced at 300 bpm. Effluent samples were
120 collected and oxygen content measured using a blood gas analyser (ABL77 Radiometer,
121 Copenhagen, Denmark). Oxygen consumption (MVO_2) was calculated as the product of
122 arterio-venous oxygen content difference and coronary flow rate (ml/min) normalised to wet
123 heart weight. (57) Heart rate (HR), left ventricular peak systolic and end diastolic pressures
124 (PSP and EDP), and rate of change of left ventricular pressure (+/-dP/dt) were recorded. As
125 a measure of cardiac work, rate pressure product (RPP) was calculated from (PSP-EDP) ×
126 HR and the ratio RPP/MVO_2 used as an indicator of cardiac efficiency.

127 **Steady state perfusion for assessment of myocardial metabolism.**
128 After a 20-minute equilibration period, the perfusion medium was switched to an identical
129 buffer replacing unlabelled substrates with 1-^{13}C labelled glucose and U-^{13}C palmitate for 45
130 minutes. Hearts were then freeze-clamped using Wollenberger tongs and extracted using 6%
131 perchloric acid for Nuclear Magnetic Resonance (NMR) spectroscopy. (72)

132 **Induction of ischaemia reperfusion injury.**
133 After a 20-minute equilibration period or normoxic perfusion with insulin free buffer, hearts
134 were immersed in perfusion buffer at 37^{o}C and perfusion ceased (warm total global
135 ischaemia) for 25 minutes. On reperfusion, the ventricular balloon was deflated for 5

minutes to minimise the 'no re-flow' phenomena. (27) The ventricular balloon as then re-inflated to produce an EDP of 5-7mmHg and indices of cardiac function measured as above for 25 minutes.

Insulin (0.1 mU/ml) was added to the reperfusion buffer immediately on reperfusion if indicated by experimental group.

13C- NMR spectroscopy

High-resolution ^{1}H decoupled ^{13}C NMR spectra were collected at 101 MHz using an 11.7 Tesla ultra- shielded superconducting vertical wide bore magnet and 5mm broadband probe interfaced with a Bruker spectrometer. Free induction decays (FIDs) were acquired over 32000 scans with a 90° pulse (9.95 us pulse duration and 1 s inter-pulse delay) and fourier transformed for analysis using Bruker Topspin (1.3) software. The relative contributions of glucose and palmitate to oxidative metabolism were determined using the TCAcalc program provided by Dr Mark Jeffrey (University of Texas, Southwestern Medical centre, TX). (79)

Haematocrit and serum metabolite analysis

Fasting venous tail blood samples for analysis of serum insulin concentration were obtained prior to terminal anaesthetisation, separated by centrifugation (3000g $4^{o}C$) and analysed using an ultra-sensitive rat specific insulin ELISA kit as per the manufacturer's instructions (Mercodi, Sweden). Immediately after excision of the heart, blood samples were collected from the chest cavity into heparinised syringes for determination of haematocrit using the blood gas analyser or centrifuged at 4000g for 10 minutes at 4°C. Serum was removed and stored at -20°C for metabolite analysis. Serum urea and creatinine were analysed at the Clinical Biochemistry Department, Hull Royal Infirmary, Hull and East Yorkshire Hospitals

158 NHS Trust, UK. HOMA-IR has been used previously to assess insulin resistance in rat
159 models (1,15) and was calculated by the following equation: HOMA-IR=[fasting serum
160 glucose]*[fasting serum insulin]/22.5

161 Protein expression

162 Expression of total Akt, pAkt, GSK3β, phospho GSK3β (pGSK3β), ANP and β-Actin in
163 uraemic and control hearts were determined by western blotting as described previously. (79)
164 Briefly, samples containing 20μg protein were separated on 10% sodium dodecyl sulphate
165 polyacrylamide gel electrophoresis (SDS PAGE) and transferred onto nitrocellulose
166 membranes. Membranes were incubated with primary antibodies (rabbit monoclonal
167 anti-Akt, anti-pAkt(ser473), anti- GSK3β anti-pGSK3β(ser9) and anti-β-Actin at 1:1000
168 dilution, New England Biolabs, USA, rabbit polyclonal anti-ANP, Santa Cruz Biotech USA)
169 followed by secondary antibody (1:2000 dilution goat anti-rabbit, Santa Cruz Biotech, USA).
170 Protein bands were visualised using enhanced chemiluminescence (ECL) (Amersham,
171 Uppsala, Sweden) and quantified using scanning densitometry and ImageJ software.
172 β-Actin was used as the loading control.

173 Expression of total and phosphorylated Akt1 and Akt2 were determined by first
174 immuno-precipitating the isomer of interest from a crude extract using anti-Akt isomer
175 monoclonal antibodies (1:50, anti-Akt1 and anti-Akt2, New England Biolabs, USA) with
176 agarose beads, before separation of proteins by 10% SDS-PAGE and Western blotting with
177 anti-Akt and anti-pAkt(ser473) as above.

178 Rosiglitazone administration

179 Rosiglitazone (Avandia^{tm}, Glaxo-Smith-Kline, UK) was administered to animals by oral

gavage at a dose of 3mg/kg/day for eight days prior to experimentation as described in (91). Treatment control animals received identical volumes (by weight) of vehicle (PEG).

Experimental groups were control (C) vs. uraemic (U), rosiglitazone untreated (-R) and treated (+R), and non-insulin (-I) and insulin (+I) treated, resulting in eight groups in total.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was determined using an unpaired *t* test (for single mean comparisons) or two-way ANOVA when testing 2 independent variables (using the Scheffe post-hoc test). Pearsons analysis was used to determine the significance of bi-variate correlations. Statistical analysis was performed using SPSS software (16.0) and level of significance was set at *p* less than 0.05.

Results

Uraemia is associated with compensated ventricular hypertrophy independently of cardiac or systemic insulin resistance. The magnitude of elevation of both serum creatinine and urea in the uraemic group at 12 weeks were comparable with previous studies. (1,66) Uraemia was associated with anaemia, but not with impaired growth (preserved tibia length) (table 1).

Cardiac hypertrophy (determined by increased HW:TL) developed in uraemic animals (table 1) and correlated with serum creatinine (HW:TL $r=0.37$, $p<0.001$). However, there was no increase in percentage lung water and uraemic hearts exhibited no evidence of dysfunction with no change in rate pressure product (RPP), contractility (dP/dt_{max}), relaxation (dP/dt_{min}) or cardiac efficiency (table 2), findings consistent with compensated ventricular hypertrophy.

Fasting serum glucose, free fatty acids, insulin concentrations and HOMA-IR did not differ between control and uraemic animals (table 1). In fact there was a trend towards improved

HOMA-IR scores in uraemic animals, providing little evidence of systemic insulin resistance in this model. Furthermore, subsequent assessment of insulin mediated substrate utilisation in isolated hearts by ^{13}C NMR spectroscopy, did not detect cardiac insulin resistance (table 2). Uraemia altered substrate utilisation in a manner consistent with our previous findings in this rat strain (79) and remained sensitive to insulin (table 2).

Uraemia is associated with decreased functional recovery and a loss of insulin mediated cardio-protection after ischaemia reperfusion injury. The effects of IRI were assessed during *ex vivo* cardiac perfusion. Twenty-five minutes of total global ischaemia produced a significantly greater impairment of cardiac function in uraemic hearts (max recovered RPP (%) 59.6±10.7 vs. 19.3±4.6, n=10, $p<0.05$; figure 1) than in controls. Furthermore, while insulin demonstrated a cardioprotective effect in control hearts (max recovered RPP (%) 59.6±10.7 vs 88.9±8.5, n=10, $p<0.05$), consistent with other published studies (29,37,39,52,92), such an effect was not seen in uraemic hearts (max recovered RPP (%) 19.3±4.6 vs 28.5±10.4, n=10, p=ns) (figure 1).

Rosiglitazone therapy is associated with restoration of the cardioprotective effects of insulin in the uraemic heart. The ability of the oral thiazolidione rosiglitazone to re-sensitise the uraemic heart to the protective effects of insulin was investigated in the uraemic model. Administration of rosiglitazone at a dose of 3mg/kg/day for 8 days had no effect on weight gain, tibia length, renal function, anaemia in either group (table 1). Nor did it affect hypertrophy of the remnant kidney or heart in uraemic animals. Overall, rosiglitazone reduced fasting glucose and insulin concentrations, and thus HOMA-IR (table 1), although this effect did not reach statistical significance in uraemic animals. Rosiglitazone had little effect on serum fatty acid levels. Baseline *ex vivo* cardiac function was also unaffected by

225 rosiglitazone administration (table 3).

226 In control hearts rosiglitazone appeared to improve post IRI function, but did not modify the
227 cardioprotective effect of insulin (figure 2 and table 4). In uraemic hearts, however, an
228 overall positive effect on cardiac recovery was associated with rosiglitazone treatment (figure
229 3 and table 5). The combination of rosiglitazone and insulin treatment was also linked with
230 significant improvements in recovery of cardiac function, as evidenced by increased RPP,
231 dP/dt_{max},dP/dt_{min}, and was associated with greater recovery than either treatment alone.

232 *Uraemia is associated with altered activation of the common insulin signalling and RISK*
233 *pathway intermediate Akt, but not the common intermediate GSK3β.* Protein expression in
234 ventricular muscle (prior to ischaemia) of total Akt, phospho Akt, Akt1 and Akt2 isoforms,
235 total GSK3β and phospho GSK3β was assessed by immuno-blotting and
236 immuno-precipitation. Ventricular muscle phospho Akt was significantly increased in the
237 uraemic hearts (relative optical density 1.00±0.08 vs. 1.3±0.11; n=12, $p<0.05$; figure 4),
238 which was predominantly attributable to phosphorylation of Akt2 rather than Akt1 (figure 5).
239 However, there was no change in phospho and total GSK3β associated with uraemia
240 (pGSK3β 1.00±0.05 vs. 0.90±0.03, n=23, p=ns; total GSK3β 1.00±0.03 vs. 0.88±0.06,
241 n=23, p=ns).

242 The presence of insulin at reperfusion was associated with an increase in both phospho Akt
243 and phospho GSK3β independently of uraemia (Control: pAkt 1.00±0.6 vs. 17.4±3.5, n=12,
244 $p<0.05$; pGSK3β 1.0±0.1 vs. 2.5±0.3, n=12, p=ns; Uraemic: pAkt 3.5±1.8 vs. 14.5±7.1,
245 n=12, $p<0.05$; pGSK3β 1.0±0.2 vs. 4.7±2.2, n=12, $p<0.05$). Uraemia was not related to a
246 change in post ischaemic Akt1 phosphorylation or expression, but both insulin and uraemia
247 produced independent reductions in total Akt2 (tAkt2) expression post-IRI (figure 6).

Discussion

UCM is characterised by increased susceptibility to IRI and a failure of insulin mediated cardio-protection - potential role for altered RISK pathway activation. Uraemic hearts displayed significantly reduced functional recovery during reperfusion (figure 1). These observations complement those of Dikow *et al* (22,23), who demonstrated increased infarct size following temporary occlusion of the left coronary artery in uraemic rats *in vivo* and are consistent with the clinical picture of adverse outcomes in patients with IHD and CKD. (76,86) One other study by Raine *et al (64)* investigated the susceptibility of the uraemic heart to IRI and observed increased inosine release, a measure of ATP catabolism and thus indicative of enhanced myocyte damage. In the study presented here, total global ischaemia in an *ex vivo* setting has been employed to assess ischaemic injury. The consistent observation of enhanced susceptibility of the uraemic heart to IRI under these *in vitro* conditions confirms that this is a function of a uraemic cardiomyopathy, rather than purely a consequence of the *in vivo* uraemic milieu.

The continuing responsiveness of cardiac metabolism to insulin in uraemia during *in vitro* perfusion (table 2) indicates that the increased susceptibility to IRI here is not a result of reduced metabolic flexibility. (2,26) However, the lack of the cardioprotective effect of insulin (figure 1) and the alterations in Akt phosphorylation in uraemic hearts (figures 5 and 6) demonstrated in this study indicate that an underlying defect in the RISK pathway is more likely responsible.

The lack of insulin mediated cardioprotection in uraemic hearts is in contrast to experimental studies on normal hearts and non-uraemic models of cardiac hypertrophy (37,67). This therefore may be a unique finding of UCM. However, Dikow *et al* (23) demonstrated a reduction in infarct size in uraemic hearts exposed to hyperinsulinaemic euglycaemic

clamping (for 45 minutes prior to ligation of the left anterior descending coronary artery and continued throughout ischaemia and reperfusion). Two significant differences in study design may account for the apparent contradiction here. Firstly, Dikow *et al* administered insulin prior to the ischaemic insult, in effect a pre-conditioning stimulus. The protective effects of pre-conditioning and post-conditioning may be linked by the RISK pathway. (33) However, neither process is fully characterised and it is possible for aspects of the pre-conditioning pathway to remain effective while the post-conditioning pathway is not. Secondly, the concurrent administration of a significant glucose load can enhance myocardial glucose metabolism, an intervention known to be cardioprotective. (50) However, both of these potential mechanisms should have improved the outcome in the control group, which was not the case, suggesting that in Dikow's study cardioprotection was not conferred through previously identified mechanisms such as the RISK pathway.

The cardio-protective effects of insulin have been widely studied utilising a range of cardiac preparations. (29,39,52,92) Insulin has been shown to reduce cell death and improve function during the reperfusion period. These effects are critically dependent on both Akt and GSK3β phosphorylation. (33,40) The loss of insulin-mediated cardioprotection observed in the uraemic heart may therefore reflect impaired signalling through these key components of the RISK pathway. In the absence of IRI, uraemia was associated with increased phospho Akt expression (figure 4). While this might be predicted to be cardio-protective, the findings of Nagoshi et al (56) suggest that chronic activation of the RISK pathway can also lead to down regulation of key intermediates and a loss of the cardio-protective phenotype. While the data presented here do not show a deficit in insulin stimulated phosphorylation of Akt or GSK3β in unfractionated cellular extracts, closer investigation of Akt isoform expression post-IRI suggests alterations in Akt2 expression

296 (figure 6). DeBosch et al (21) have previously demonstrated that Akt 2 rather than Akt1
297 underlies RISK-mediated cardioprotection. Data here support the concept that insulin
298 stimulation alters Akt2 expression post-IRI in control and uraemic hearts with an additional
299 independent reduction in post-IRI levels of Akt2 in uraemic hearts. Thus the Akt signalling
300 axis is modified in the post-ischaemic uraemic heart raising the possibility of its involvement
301 in the increased IRI susceptibility.

302 *Rosiglitazone therapy re-sensitised the uraemic heart to the cardio-protective effects of*
303 *insulin.* In uraemic hearts rosiglitazone treatment improved functional recovery (RPP) at all
304 time points in addition to all measures of function and efficiency. The addition of insulin
305 post IRI to rosiglitazone-treated hearts had an additive effect, achieving significant increases
306 in RPP at all time points (figure 3 and table 5). These results demonstrated that, despite the
307 lack of effect of insulin alone, IRI damage in the uraemic heart is amenable to salvage by
308 selected interventions, and further that rosiglitazone treatment is capable of 're-sensitising'
309 the uraemic heart to the pro-survival effects of insulin. Rosiglitazone demonstrated modest
310 pro-survival effects in control hearts in keeping with previous studies, (54) but did not
311 provide addition benefit to insulin treatment alone (figure 2 and table 4). This may represent
312 a maximal effect as control hearts exposed to insulin at reperfusion are already achieving a
313 recovery of RPP of approximately 85%.

314 These results complement those of Taniguchi *et al* who have demonstrated pioglitazone
315 mediated IRI cardioprotection in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a
316 non-uraemic model on insulin resistance. (83) They also identified pioglitazone mediated
317 enhancement of stress induced Akt phosphorylation, suggesting that this is the likely path of
318 action. The re-sensitisation to the effects of insulin in the study presented here also suggests

that rosiglitazone is acting through the RISK-Akt pathway as has been demonstrated in other experimental models utilising both rosiglitazone and the related TZD, pioglitazone. Yue *et al* (91), utilising diabetic rats and rosiglitazone administration as in this study, demonstrated rosiglitazone treatment conferred a similar degree of cardio-protection, with enhanced post-IRI Akt phosphorylation. Cardioprotection and Akt phosphorylation were almost completely abolished by inhibition of the upstream RISK pathway intermediate phosphatidylinositol 3-kinase (PI3K), with Wortmannin. Lie *et al* (48) demonstrated in hypercholesterolaemic rabbit hearts rosiglitazone-mediated reductions in post-IRI apoptosis, a well recognised effect of Akt activation, although that was not directly assessed in their study. Cao *et al* (16) confirmed pioglitazone-mediated reductions in post-IRI cardiomyocyte apoptosis in the rat heart associated with reduced caspase 3 and Bax expression (pro-apoptotic) and increased Bcl-2 expression (anti-apoptotic), alterations normally associated with Akt activation. (38,63,88)

More recently Yasuda *et al* (89) have demonstrated pioglitazone-mediated protection against *in vivo* myocardial infarction of rabbit hearts, linked to increased phospho Akt and phospho eNOS. eNOS is a downstream target of Akt (28,52,81) and Akt mediated pro-survival effects are dependent on generation of NO. (28) Protection was abrogated in Yasuda's study by the application of inhibitors of PPARγ, PI3K and NOS inhibition, providing strong evidence for a TZD-PI3K-Akt-eNOS mediated mechanism of cardio-protection post IRI.

There is a great clinical need to modify the functional consequences of UCM. In the haemodialysis population in particular strategies to improve the tolerance of the heart to ischaemic insult are urgently required. McIntyre *et al* have assessed cardiac function and damage during haemodialysis using serum troponin T concentrations, serial

echocardiography and serial positron emission tomography scans. (12,13,20) They have demonstrated repeated episodes of myocardial ischaemia (myocardial stunning) and regional ventricular dysfunction associated with haemodialysis, and further, that the presence of such defects predicts deterioration of cardiac function in the subsequent 12 months. (12) As haemodialysis is a predictable event it is amenable to prophylactic strategies, avoiding the pitfall of interventions for acute cardiac ischaemia when the protective intervention is often administered too late. (4) Pre-treatment protective interventions have already been successful in other clinical situations, such as coronary artery bypass grafting and acute myocardial infarction (AMI). (14,35,44,60)

Clinically rosiglitazone treatment has been associated with exacerbation of heart failure and an enhanced incidence of myocardial infarction in non-CKD populations. (9,32,42) However, as discussed above, pioglitazone also has experimental data to support a role in activation of the RISK pathway. Furthermore, there is no excess risk of myocardial infarction or heart failure associated with its use in clinical practice. (30) There is also theoretical reason to suspect clinically significant differences in outcomes in the end stage renal failure population, where the effects of rosglitazone on the distal tubule (41) will be diminished. The safety of TZDs in renal failure has been tested in a number of *post hoc* or retrospective studies. Schneider *et al* (70) performed a *post hoc* analysis of the PROactive trial and demonstrated reduced all-cause mortality, myocardial infarction and stroke in patients with CKD (GFR <60ml/min/1.73m^2) treated with pioglitazone. Subsequently Ramirez *et al* (65) demonstrated increased all-cause mortality in rosiglitazone treated patients on haemodialysis enrolled in the Dialysis Outcomes and Practice Patterns Study (DOPPS). Yet Brunelli *et al* (11) have shown reduced all-cause mortality for haemodialysis patients receiving either pioglitazone or rosiglitazone, with no significant difference between the two

366 agents. The safety of TZDs in CKD therefore remains unclear. (8) Rosiglitazone treatment
367 in this study was utilised in a way previously shown to be cardio-protective in experimental
368 IRI. (91) The duration of treatment was too short to have a significant effect on cardiac
369 hypertrophy. However, a trend towards reduced cardiac function and a statistically
370 significant increase in % lung water were noted (tables 1 and 3).

371 These findings and the clinical data limit the direct 'translatability' of the positive findings in
372 this study. However, the core finding of successful improvement in functional outcomes in
373 uraemic hearts after IRI should provoke further investigation of alternative protective
374 strategies involving the Akt-eNOS pathway. Many such alternatives have been investigated
375 in non-uraemic models. (7,33) Disappointingly the extensive pre-clinical data is not yet
376 matched by corresponding success in clinical trials. Adenosine or the synthetic Adenosine
377 receptor agonist AmP579 have been examined in 3 clinical trials (45,53,68), the results of
378 which are mixed and essentially flawed by aspects of study design (59). However, complete
379 re-analysis of the largest of these trials, AMISTAD-II, has shown benefit (reduced early and
380 late survival, and reduced death or heart failure composite endpoint at 6 months) to the use of
381 adenosine as an adjunct to reperfusion in acute myocardial infusion in those with a short
382 duration of ischaemic symptoms. (44)

383 Another alternative mechanism of RISK pathway activation with early positive outcomes in
384 human studies is 'ischaemic post-conditioning', an extension of the original discovery by
385 Murry *et al* (55) of reduce IRI after repeated episodes of brief ischaemia prior to the index
386 ischaemic event (ischaemic pre-conditioning). Ischaemic post-conditioning was first defined
387 by Kin *et al* (43) and the mechanism has since been extensive studied and found to involve
388 activation of the RISK pathway. (78) Since 2005 there have been several translational

clinical studies demonstration significant reductions in infarct size and improved functional parameters when utilising ischaemic post-conditioning in the treatment of AMI. (19,80,84) However, the general utility of this method is limited by the need for access to the coronary circulation. More attractive might be the concept of remote ischaemic conditioning, in which repeated brief ischaemia to an organ remote from the heart either pre-ischaemia, or pre-reperfusion, confers protection from IRI. (34,62) Two human studies, one in human volunteers and patients with coronary artery disease (51) and one in the setting of acute myocardial infarction (14) have shown that repeated brief (5 minute) limb ischaemia, induced using an inflatable cuff, improve endothelial function and reduce infarct size following reperfusion. Further, investigation is required to confirm these results, but the technique is attractive in instances of predictable IRI, such as haemodialysis.

In an alternative approach significant cardio-protection in experimental and human studies has been demonstrated through inhibition of the final end effector of IRI, the mitochondrial permeability transition pore (mPTP). (5) This large non-selective pore forms in the mitochondrial inner membrane during reperfusion resulting in cell death through, dissipation of the mitochondrial membrane potential, inhibition of ATP production, release of pro-apoptotic ligands and swelling and rupture of mitochondria. Inhibition of mPTP opening is the primary effect through which RISK pathway activation reduces IRI. (17,36) The long established immunosuppressive drug cyclosporin A directly inhibits opening on the mPTP, and has previously been shown to be cardio-protective in experimental models. (73) Recently however, a small scale study has confirmed this effect in humans. Piot *et al* demonstrated reduced infarct size with the use of a single bolus of cyclosporin prior to reperfusion in 58 patients undergoing primary percutaneous coronary intervention for acute myocardial infarction. (60)

413 Therefore, although TZDs may not be utilised in clinical practice as cardio-protective agents
414 in renal failure, multiple other RISK pathway activating manoeuvres have been identified in
415 experimental studies and there is a growing body of clinical evidence for their utility in the
416 non-uraemic population. Such avenues should be the focus of future studies in the CKD
417 population.

418 *The reduction in HOMA-IR scores by rosiglitazone was reduced in uraemic animals.*
419 Overall rosiglitazone improved insulin sensitivity as evidenced by reduce HOMA-IR scores
420 (table 1). However, whilst exhibiting the same trend, this effect was not statistically
421 significant in subgroup analysis of uraemic animals. TZD treatment has previously been
422 reported to have no effect on serum glucose and insulin concentrations in non-diabetic rats.
423 (74) However, rosiglitazone did significantly reduce HOMA-IR values in control animals in
424 this model. The lack of statistically significant effect in the uraemic group appears to stem
425 from a dilution of the effect due to a additional non-significant trend for lower HOMA-IR
426 values in ureamic animals.

427 *The metabolic and pleiotropic effects of insulin can be altered independently in CKD.*
428 Insulin resistance as it is typically attributed in clinical and experimental studies relates
429 specifically to one of insulin's many effects, namely that of serum glucose control. Using
430 this definition, insulin resistance, which remains an independent risk factor for cardiovascular
431 death in CKD (6,75), has been implicated in the pathogenesis of pathological cardiac
432 hypertrophy (24,69). However, the data presented here clearly reveal resistance to the
433 pleiotropic effects of insulin, in the absence of systemic metabolic insulin resistance. This is
434 consistent with data from Potenza *et al* (61)who have demonstrated defects in discrete
435 pathways of the insulin signalling cascade which leave signal transduction unaffected through

436 other routes.

437 Resistance to the metabolic effects of insulin results in hyperinsulinaemia, and imbalance in
438 the various pleiotropic effects of insulin at the level of the endothelium, gene transcription
439 and protein synthesis that favour cardiac hypertrophy (24,69). In particular it appears that
440 hyperinsulinaemia can exacerbate pathological cardiac hypertrophy in the presence of other
441 factors. This has been demonstrated in aortic banded rats (24), where the combination of
442 hyperinsulinaemia and hypertension produced significantly more cardiac hypertrophy than
443 hypertension alone. Here we demonstrate that uraemia is associated with a 'primary' defect
444 in one of the pleiotropic effects of insulin, independent of metabolic insulin resistance.

445 Conclusions

446 This is the first study to demonstrate significantly reduced function of the *ex vivo* uraemic
447 heart after IRI. Further, the loss of insulin mediated cardio-protection and alterations in Akt
448 expression and phosphorylation suggest an underlying deficit in the RISK pathway.

449 The insulin sensitising agent rosiglitazone restored the cardio-protective effects of insulin, in
450 the uraemic heart.

451 Enhanced IRI damage and pathological cardiac hypertrophy occurred in the absence of either
452 systemic or cardiac 'metabolic' insulin resistance, despite the utility of insulin resistance as a
453 risk factor for cardiovascular disease in the CKD.

454 The role of the RISK pathway in the development of UCM and the cardio-protective
455 potential of its manipulation warrant further investigation.

456

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Competing Financial Interests

None to declare

Author Contributions

David Semple - Primary investigator. Primary author of manuscript

Sunil Bhandari - Supervision of investigation. Editoral review of manuscript

Anne-Marie Seymour - Supervision of investigation. Editoral review of manuscript

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779

780

781

Figures

Figure 1: Recovery of cardiac function over time in control and uraemic hearts

Upper panel: absolute rate pressure product (RPP) pre and post 25 minutes warm total global ischaemia. Lower panel: recovery of RPP as a percentage of normoxic value. Uraemic hearts exhibited poorer functional recovery after the ischaemic insult. Insulin, which improved functional recovery in control hearts, failed to improve functional recovery in uraemic hearts. *$p<0.05$ C-I vs. C+I; #$p<0.05$ C+I vs. U+I; §$p<0.05$ C-I vs. U-I. All groups n=5

Figure 2: Effect of insulin and rosiglitazone on recovery of RPP after ischaemia reperfusion injury in control hearts

Mean ± SEM of % recovery of baseline rate pressure product (RPP) in control hearts after 25min warm total global ischaemia. -I: no insulin, +I: with insulin, -R: no rosiglitazone, +R: with rosiglitazone. Rosiglitazone appeared to improve recovery post IRI in control hearts, but did not add any benefit to insulin treatment. *$p<0.05$ -I-R vs. +I-R, §$p<0.05$ -I+R vs. +I+R. C-I-R n=4; C+I-R n=5; C-I+R n=5; C+I+R n=5

Figure 3: Effect of insulin and rosiglitazone on recovery of RPP after ischaemia reperfusion injury in uraemic hearts

802 Mean ± SEM of % recovery of baseline rate pressure product (RPP) in uraemic hearts after
803 25min warm total global ischaemia. -I: no inuslin, +I: with insulin, -R: no rosiglitazone, +R:
804 with rosiglitazone. Rosiglitazone and insulin treatment produced significant improvements
805 in post IRI recovery greater than either therapy alone. #$p<0.05$ +I-R vs. +I+R. U-I-R n=4;
806 U+I-R n=5; U-I+R n=5; U+I+R n=5

807

808 **Figure 4: Total and phospho Akt and GSK3β protein expression in control and uraemic**
809 **hearts prior to ischaemia reperfusion injury**

810 Representative immunoblot of total and phospho Akt and GSK3β expression in uraemic and
811 control hearts prior to either ischaemia or reperfusion. Total Akt expression is unchanged
812 between control and uraemic hearts. However, phospho Akt expression is increased in
813 uraemic hearts. (relative optical density 1.00±0.08 vs. 1.3±0.11; n=12, $p<0.05$) Total and
814 phospho GSK3β remain unchanged in uraemic animals.

815

816 **Figure 5: Akt1 and Akt2 protein expression in control and uraemic hearts prior to**
817 **ischaemia reperfusion injury**

818 Akt1 and Akt2 isoform expression in whole cell extracts determined by immuno-precipitation
819 and immunoblotting. The increase in overall pAkt expression in uraemic hearts (see text)
820 related predominantly to pAkt2 expression, although this did not reach statistical
821 significance. A - Representative immunoblot of phospho and total Akt 1 and Akt 2
822 expression in control and uraemic hearts. B - Mean±SEM values of relative Akt1 and Akt2
823 expression. All values normalised to the respective control. *$p<0.05$ uraemic vs. control;

824 Control n=10, Uraemic n=10

825

826 **Figure 6: Effect of uraemia and insulin on Akt1 and Akt2 phosphorylation post**
827 **ischaemia reperfusion injury**

828 Akt1 and Akt2 isoform expression in whole cell extracts after 25min warm total global
829 ischaemia determined by immuno-precipitation and immunoblotting. Insulin produced
830 increases in pAkt1 and pAkt2. Both insulin and uraemia were associated with reduced Akt2
831 expression post ischaemia. A - Representative immunoblot of phospho and total Akt 1 and
832 Akt 2 expression in control and uraemic hearts with and without insulin. B - Mean±SEM
833 values of relative Akt1 and Akt2 expression post IRI in the presence of absence of insulin.
834 All values normalised to the respective control. *$p<0.05$ vs. no insulin; #$p<0.05$ vs.
835 respective control; All groups n=5

836

837

Tables

Table 1: Effect of uraemia and rosiglitazone on renal function, anaemia, anthropometric measurements and serum metabolic substrate concentrations

Mean±SEM values for serum urea, creatinine and haematocrit values, anthropometric measures and serum metabolic substrate concentrations in control and uraemic animals. Rosiglitazone had no significant effect on either renal function or cardiac hypertrophy but was associated with lower serum glucose and insulin concentrations, and thus HOMA-IR values. No change was detected in serum free fatty acid concentrations. C-R: control - rosiglitazone, C+R: control + rosiglitazone, U-R: uraemic - rosiglitazone, U+R: uraemic + rosiglitazone. p values given for two way ANOVA; subgroup analysis by Scheffé *post hoc* test. §$p<0.05$ vs. no rosiglitazone; #$p<0.05$ vs. respective control; a - C-R n=8, C+R n=10, U-R n=9, U+R n=10; b - C-R n=5, C+R n=7, U-R n=7, U+R n=6

Table 2: Effect of uraemia and insulin on *in vitro* baseline cardiac function, efficiency and metabolism in control and uraemic hearts

Mean±SEM values for measures of cardiac function and relative substrate contribution of Acetyl-CoA to the Krebs cycle in control and uraemic hearts perfused in the absence or presence of insulin. Uraemia was not associated with decline in measures of cardiac function. Insulin acted to increase cardiac contractility (dP/dt) and efficiency, independently of uraemia. Uraemia was associated with a significant shift from fatty acid to carbohydrate metabolism, yet substrate selection remained sensitive to insulin. C-I - Control no insulin; U-I - Uraemic no insulin; C+I - Control plus insulin; U+I - Uraemic plus insulin. p values given for two way ANOVA; subgroup analysis by Scheffé *post hoc* test, *$p<0.05$ vs. no

insulin; #p<0.05 vs. respective control

Table 3: Effect of rosiglitazone on *in vitro* baseline cardiac function

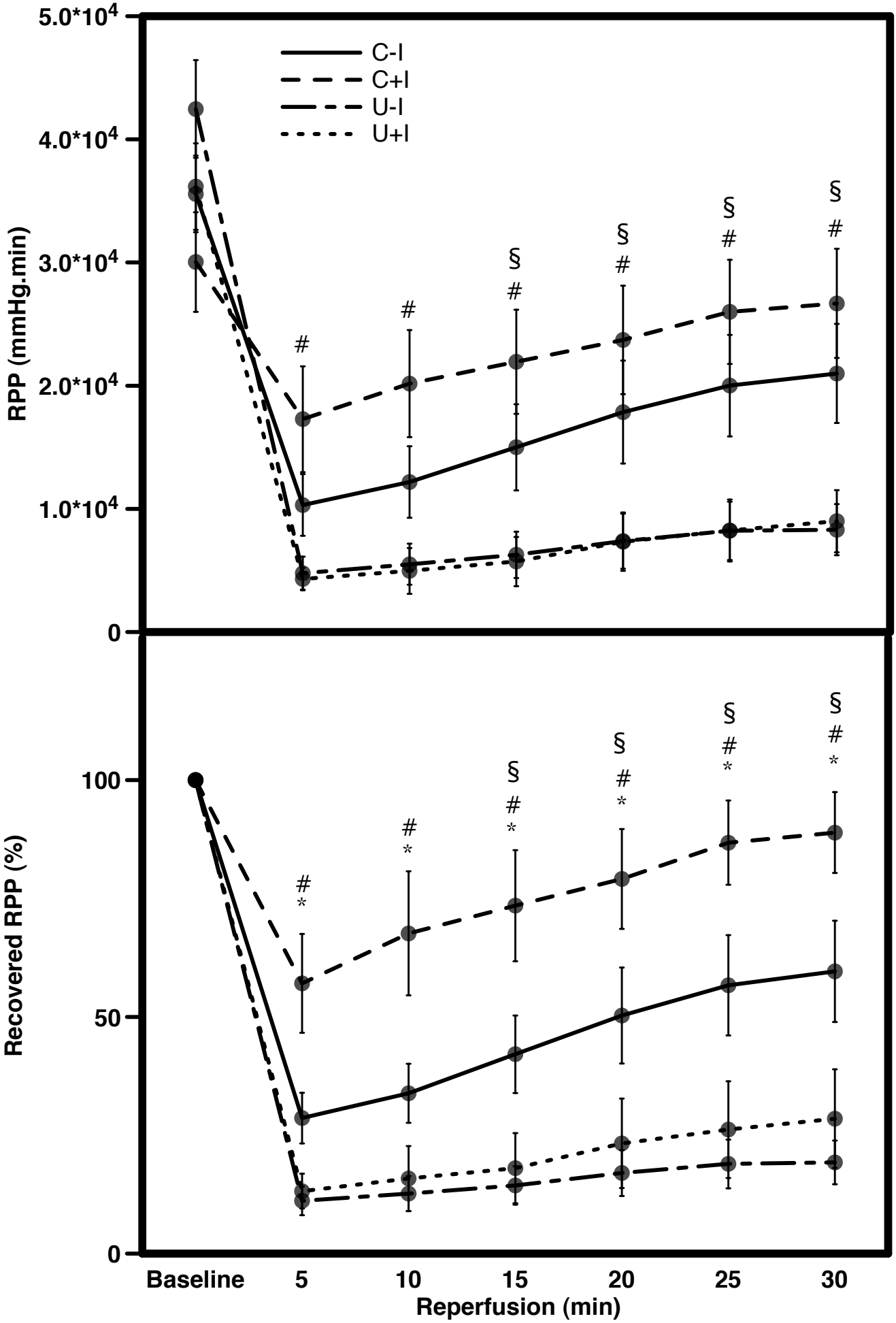
Mean±SEM for rate pressure product (RPP), contractility (dP/dt_{max}), relaxation (dP/dt_{min}), myocardial oxygen consumption (MVO_2) and efficiency in control and uraemic animals ± rosiglitazone therapy prior to ischaemia reperfusion. C-R: control - rosiglitazone, C+R: control + rosiglitazone, U-R: uraemic - rosiglitazone, U+R: uraemic + rosiglitazone. p values given for two way ANOVA; subgroup analysis by Scheffé *post hoc* test. a - C-R n=8, C+R n=10, U-R n=6, U+R n=10

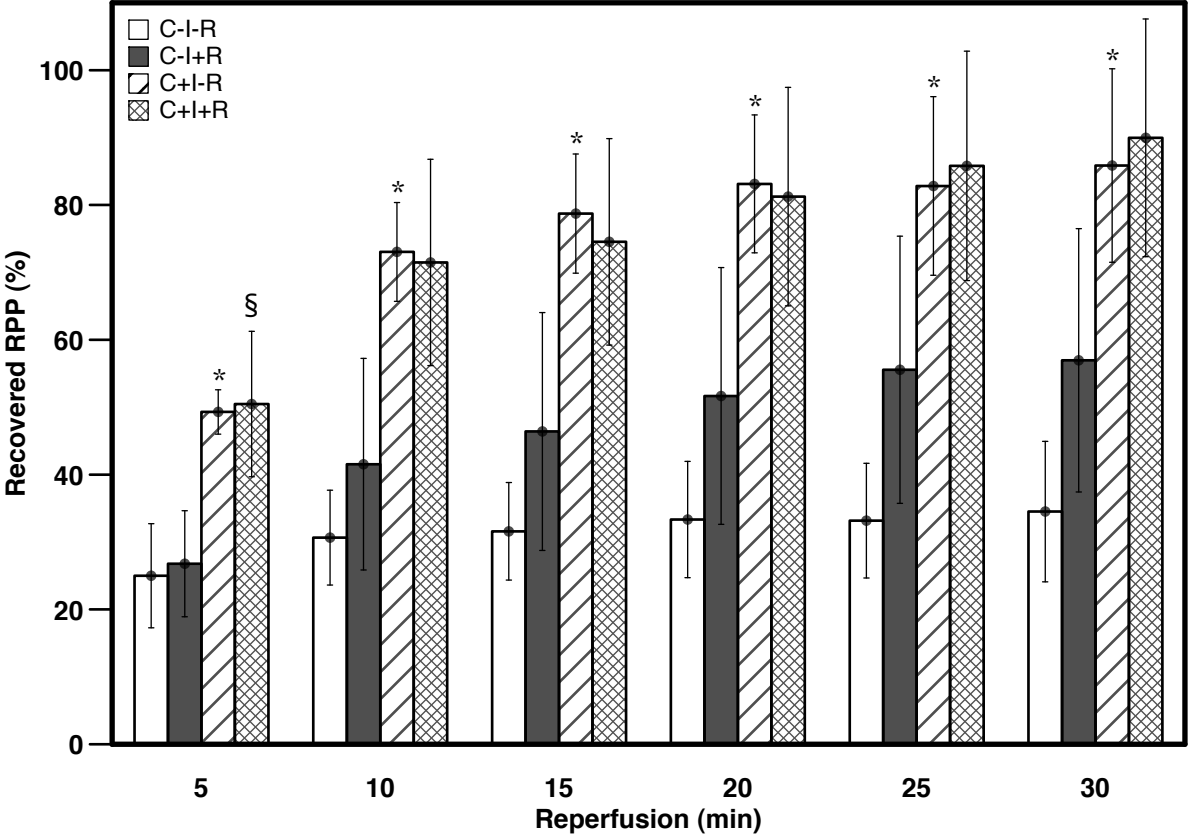
Table 4: Effect of insulin and rosiglitazone on maximal recovery of control hearts after ischaemia reperfusion injury

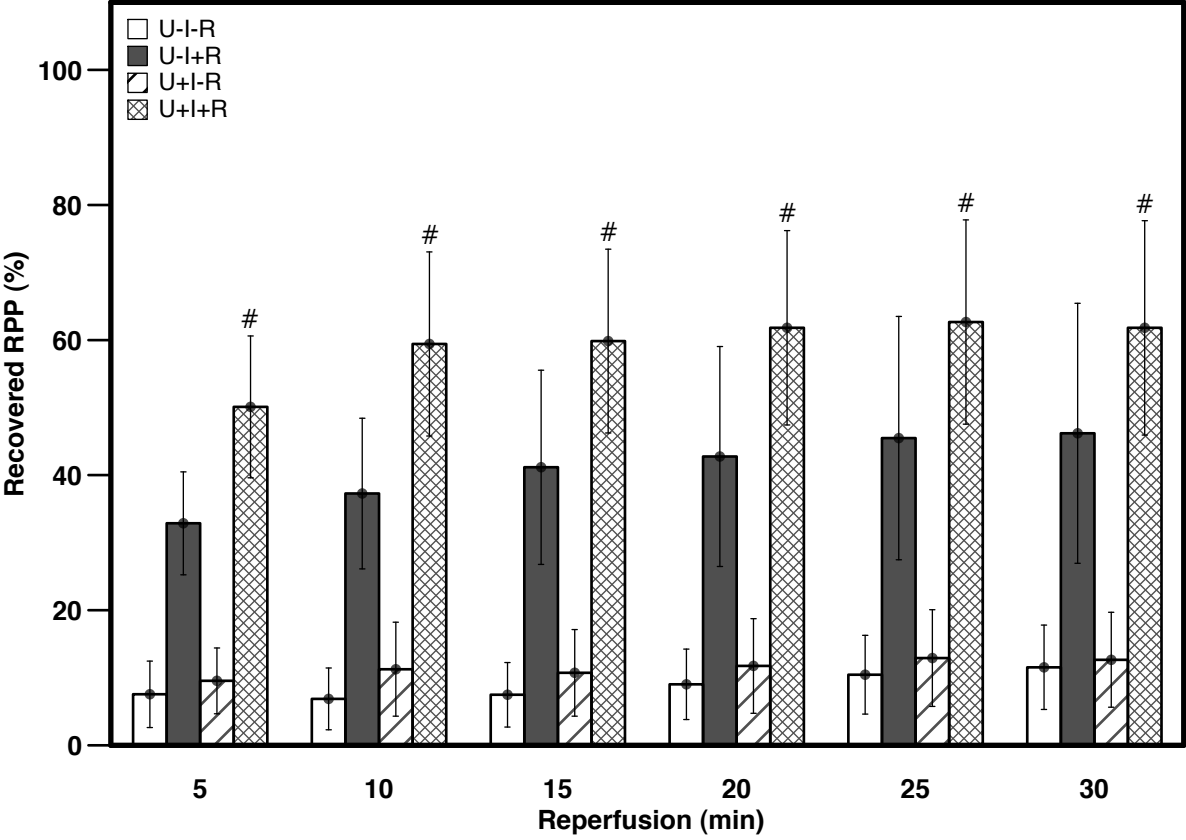
Mean ± SEM of maximal % recovery of baseline cardiac function in control hearts after 25min warm total global ischaemia. -I: no inuslin, +I: with insulin, -R: no rosiglitazone, +R: with rosiglitazone. *p<0.05 -I-R vs. +I-R, §p<0.05 -I+R vs. +I+R.

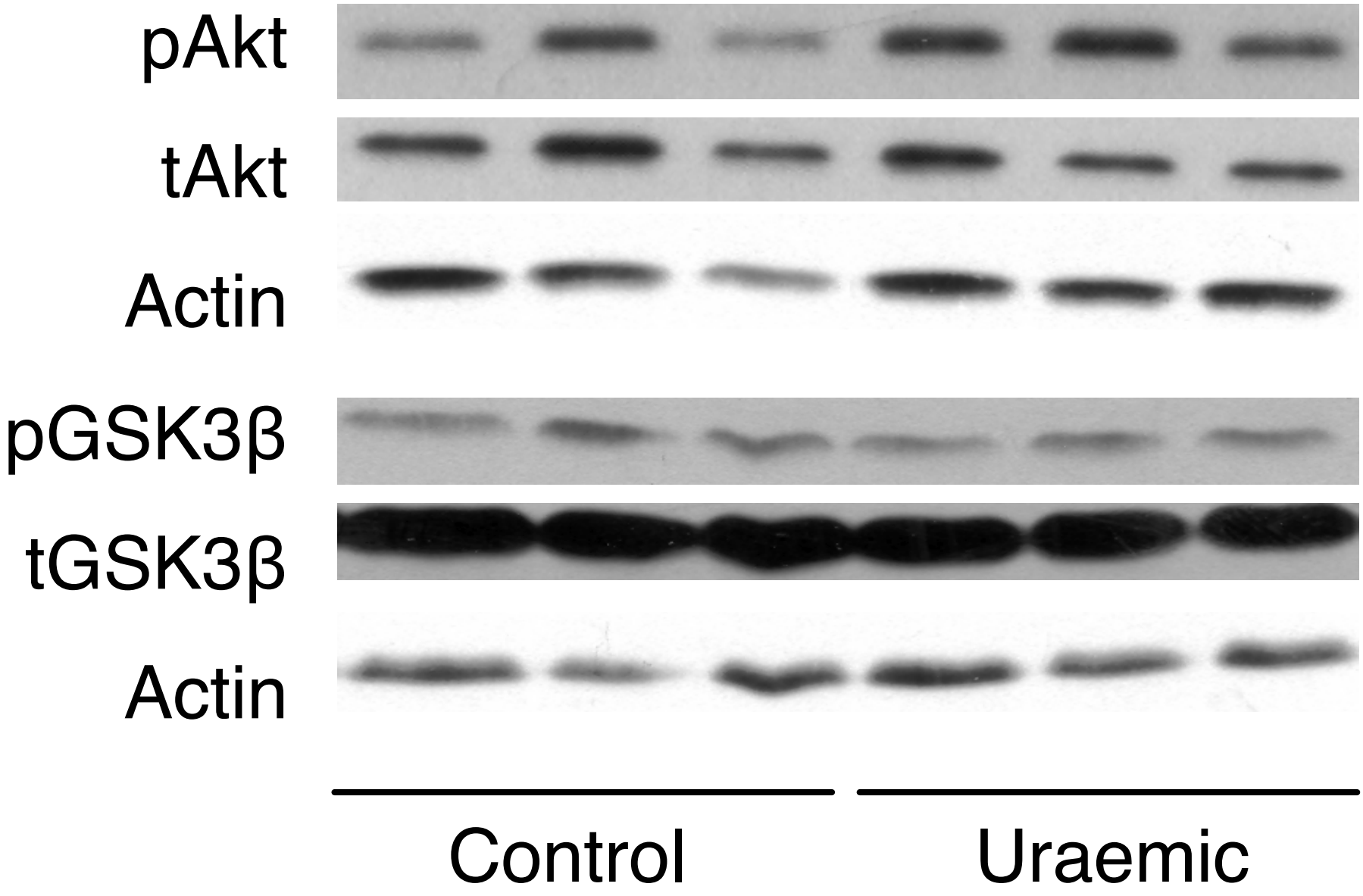
Table 5: Effect of insulin and rosiglitazone on maximal recovery of uraemic hearts after ischaemia reperfusion injury

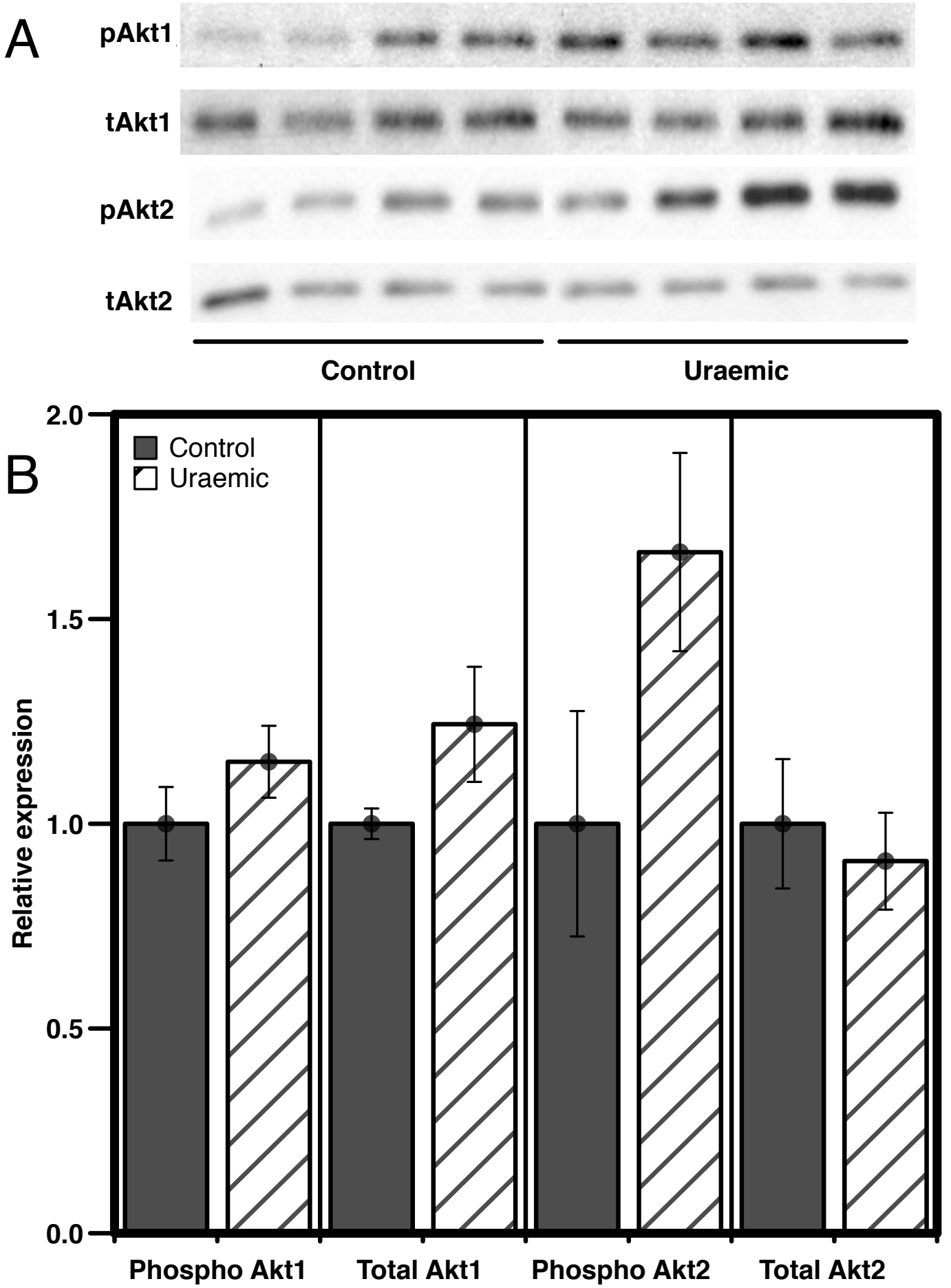
Mean ± SEM of maximal % recovery of baseline cardiac function in uraemic hearts after 25min warm total global ischaemia. -I: no inuslin, +I: with insulin, -R: no rosiglitazone, +R: with rosiglitazone. #p<0.05 +I-R vs. +I+R

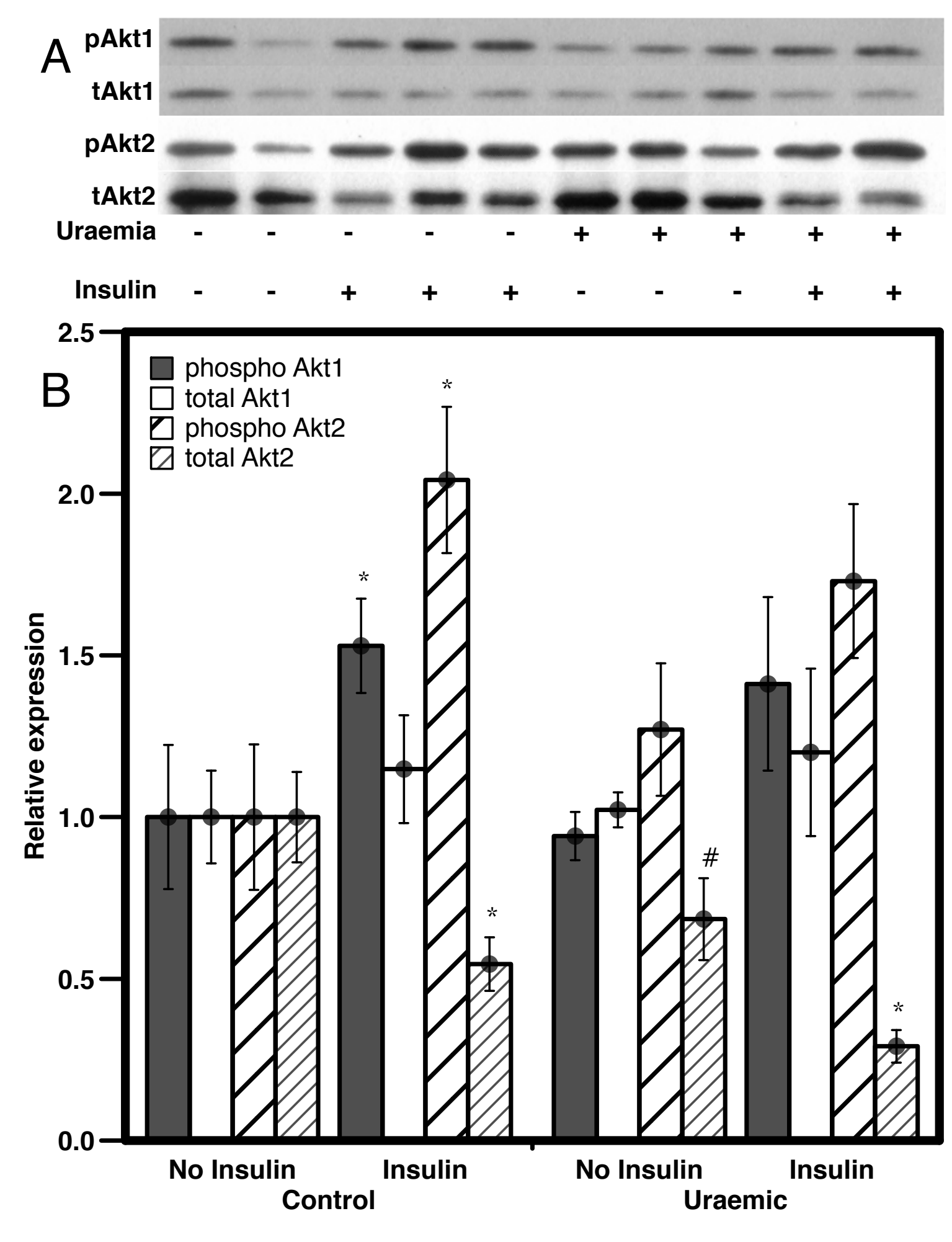

C-I
C+I
U-I
U+I
RPP (mmHg.min)
5.0*10^4
4.0*10^4
3.0*10^4
2.0*10^4
1.0*10^4
0
Recovered RPP (%)
100
50
0
Baseline
5
10
15
20
25
30
Reperfusion (min)


C-I-R
C-I+R
C+I-R
C+I+R
Recovered RPP (%)
0
20
40
60
80
100
5
10
15
20
25
30
Reperfusion (min)
*
§


U-I-R
U-I+R
U+I-R
U+I+R
Recovered RPP (%)
0
20
40
60
80
100
#
5
10
15
20
25
30
Reperfusion (min)


pAkt
tAkt
Actin
pGSK3β
tGSK3β
Actin
Control
Uraemic


A
pAkt1
tAkt1
pAkt2
tAkt2
Control
Uraemic
B
Control
Uraemic
Relative expression
2.0
1.5
1.0
0.5
0.0
Phospho Akt1
Total Akt1
Phospho Akt2
Total Akt2


A
pAkt1
tAkt1
pAkt2
tAkt2
Uraemia - - - - - + + + + +
Insulin - - + + + - - - + +
B
phospho Akt1
total Akt1
phospho Akt2
total Akt2
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
#
*
No Insulin
Insulin
No Insulin
Insulin
Control
Uraemic

					p value	
	C-R (n=10)	**C+R (n=15)**	**U-R (n=10)**	**U+R (n=15)**	**C vs. U**	**-R vs. +R**
Weight gain (g)	268±13	266±9	266±15	253±12	ns	ns
Tibia length (cm)	4.48±0.03	4.47±0.03	4.51±0.04	4.48±0.02	ns	ns
Heart weight (g)	1.57±0.04	1.56±0.08	1.79±0.08[#]	1.80±0.53[#]	<0.05	ns
HW:TL (g/cm)	0.35±0.01	0.35±0.02	0.40±0.02	0.40±0.01[#]	<0.05	ns
% Lung water	75.8±0.3	76.7±0.5	76.4±0.3	76.9±0.1	ns	<0.05
Left kidney (g)	1.40±0.08	1.46±0.04	1.55±0.08	1.54±0.09	ns	ns
Liver (g)	15.2±1.3	13.1±0.9	14.0±1.1	12.8±0.6	ns	ns
Urea (mM)	4.6±0.5	4.9±0.4	11.5±1.1[#]	13.3±1.1[#]	<0.05	ns
Creatinine (µmol/l)	28.9±2.3	29.0±1.1	71.6±6.5[#]	75.0±9.3[#]	<0.05	ns
Hct (%)	41±1	41±1	35±1[#]	35±1[#]	<0.05	ns
Glucose (mM)[a]	8.4±0.6	6.2±0.4[§]	7.2±0.5	5.9±0.5	ns	<0.05
Insulin (µg/l)[b]	1.33±0.32	0.89±0.17	1.07±0.13	0.89±0.2 6	ns	ns
HOMA-IR (mmol/L x µU/ml)[b]	12.1±2.3	6.4±1.4[§]	8.6±1.5	6.3±2.2	ns	<0.05
Free fatty acids (mM)	0.31±0.04	0.36±0.04	0.32±0.05	0.35±0.05	ns	ns

					p value	
	C-I (n=8)	**U-I (n=8)**	**C+I (n=6)**	**U+I (n=8)**	**C vs. U**	**-I vs. +I**
RPP $x10^3$ (mmHg.min)	45±3	49±2	45±5	51±3	ns	ns
dP/dt_{max} (mmHg/s)	4480±756	5426±561	5960±400	6579±368	ns	<0.05
dP/dt_{min} (mmHg/s)	-2929±160	-3131±142	-3127±216	-3566±193	ns	<0.05
MVO_2 (µmol/g/min)	0.85±0.06	0.82±0.06	0.65±0.10	0.65±0.06	ns	ns
Efficiency $x10^4$ (mmHg/µmol/g wet wt)	5.4±0.4	6.4±.07	7.6±1.0	8.3±0.9	ns	<0.05
Glucose (%)	10.6±1.2	12.9±1.8	12.3±0.3	17.3±1.4*#	<0.05	<0.05
Palmitate (%)	48.8±2.1	37.8±2.2#	31.4±1.8*	16.1±0.9*#	<0.05	<0.05
Unlabelled (%)	40.6±1.9	49.3±2.2#	56.3±1.9*	66.6±1.9*#	<0.05	<0.05

					p value	
	C-R	**C+R**	**U-R**	**U+R**	**C vs. U**	**-R vs. +R**
	(n=9)	**(n=10)**	**(n=9)**	**(n=10)**		
RPP x 10^3 (mmHg.min)	30.1±1.9	28.1±1.7	32.5±1.4	29.1±1.5	ns	ns
dP/dt_{max} (mmHg/s)	3371±217	3122±238	3402±234	3074±204	ns	ns
dP/dt_{min} (mmHg/s)	-1926±87	-1903±101	-2002±81	-1856±66	ns	ns
MVO_2 (µmol/g/min)[a]	0.93±0.05	0.95±0.03	0.98±0.03	0.93±0.04	ns	ns
Efficiency x 10^3 (mmHg/µmol/g)[a]	32.1±2.4	29.9±1.8	33.8±1.8	31.6±1.8	ns	ns

					p value	
	C-I-R (n=4)	**C+I-R (n=5)**	**C-I+R (n=5)**	**C+I+R (n=5)**	**-I vs. +I**	**-R vs. +R**
RPP (% recovery)	35.5±10.4	85.9±14.4*	57.0±19.5	90.0±17.6	<0.05	ns
dP/dt_{max} (% recovery)	34.4±8.1	65.9±24.5	55.9±19.2	87.7±17.8§	ns	ns
dP/dt_{min} (% recovery)	70.0±20.8	79.1±24.6	65.6±23.4	93.2±20.5	ns	ns
MVO_2 (μmol/g/min)	0.91±0.01	0.94±0.08	0.98±0.03	0.89±0.04	ns	ns
Cardiac Efficiency (% recovery)	36.4±12.4	82.3±12.3	57.1±19.6	83.0±17.1	<0.05	ns

					p value	
	U-I-R (n=4)	U+I-R (n=5)	U-I+R (n=5)	U+I+R (n=5)	-I vs. +I	-R vs. +R
RPP (% recovery)	11.5±6.2	12.7±7.0	46.2±19.2	61.8±15.9#	ns	<0.05
dP/dt_{max} (% recovery)	22.0±5.8	42.5±17.1	31.0±25.6	62.1±16.9#	ns	ns
dP/dt_{min} (% recovery)	9.5±3.5	27.6±7.9	50.7±20.1	70.7±17.6#	ns	<0.05
MVO_2 (µmol/g/min)	0.80±0.07	0.83±0.02	0.86±0.03	0.94±0.07	ns	ns
Cardiac Efficiency (% recovery)	15.8±9.4	15.8±8.8	47.3±19	65.2±15.4#	ns	<0.05