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C10

Insulin directly remodels the glomerular filtration barrier of the kidney

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Background:
Albuminuria is a cardinal sign of disruption to the glomerular filtration barrier (GFB), and it is the earliest marker of renal involvement in diabetes and the hyperinsulinaemic metabolic syndrome, which are conditions secondary to a failure of the production, or cellular action, of insulin.
Methods/findings:
We have studied the direct effect of insulin on the healthy glomerular filtration barrier using in vivo and in vitro techniques. In vivo- Wistar rats were treated with high physiological doses of insulin (1000pM) and their blood glucose levels maintained with a glucose infusion. These were compared to control animals given a saline infusion. Within 30 minutes insulin treated rats increased their urinary albumin excretion by 60% ($p<0.01$). Animals were sacrificed and their kidneys perfusion fixed. Insulin treated animals showed widening of their podocyte foot processes with less filtration slits per standardised unit length of filtration area ($p<0.05$). There was also underlying swelling and vacuolation of endothelial cells. In vitro- Conditionally immortalised human glomerular podocyte and endothelial cell lines were examined. Human podocytes reorganised their actin cytoskeleton from stress fibre to cortical patterning within 15 minutes (phalloidin staining), with associated retraction of the fine processes of the cells (atomic force microscopy and real time imaging). Within 5 minutes, exclusively in podocytes, insulin switched on the small GTPase RhoA and switched off Rac1 and CDC42 (the molecular switches for actin reorganisation). Insulin also directly caused a functional loss of resistance across podocyte monolayers, but not endothelial cells (Electrical cell surface impedance sensing).
Conclusions:
Insulin has a previously unsuspected direct remodelling effect on the glomerular filtration barrier of the healthy kidney. As the earliest manifestation of renal involvement in diabetes and the metabolic syndrome is albuminuria, loss of mechanism could be of great importance and an early target for therapy.

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C11

A novel role for adiponectin in the glomerular filtration barrier?

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In diabetic nephropathy, the most important cause of renal failure in the developed world, overt albuminuria is a marker for a poor prognosis not only from renal disease itself but also from associated cardiovascular disease[1-3]. How systemic disorders of insulin action lead to detrimental effects on the glomerular filtration barrier GFB is surprisingly poorly understood. There is accumulating evidence that the podocyte is central to the development of diabetic nephropathy, although the mechanism is not known[4]. We have recently reported the novel observation that human podocytes are insulin sensitive cells[5]. It is now recognised that adipocyte derived factors are important in regulating insulin sensitivity of distant tissues. The levels of one such factor, adiponectin, have been shown to be altered in end stage renal disease

This laboratory has developed and characterised in detail human conditionally immortalised podocyte and endothelial cell lines[6,7] We have been studying the effect of adiponectin on these cell lines. Using RTPCR and western blotting techniques we have found that podocytes, but not endothelial cells, produce adiponectin and secrete it into the media (Fig 1). This is suprising since this factor was thought to be solely produced by adipocytes. Like adipocytes, adiponectin production is regulated by insulin (Fig 2.). Immunoflurescence analysis demonstrates that the adiponectin is localized within the podocyte to intracellular vesicles. Both cell types express adiponectin receptors suggesting that they both respond to adiponectin. Using phosphospecific antibodies we are at present looking at the activation of various cell signalling pathways known to be activated in other cell types by adiponectin namely the Protein Kinase B, MAPK and AMPK pathways.
Conclusion: Our results, demonstrating surprisingly that podocytes produce and secrete adiponectin, a key regulator of insulin sensitivity and tissue inflammation [8], point to potential new mechanisms in the regulation of the physiology of the cells of the glomerular filtration barrier. Furthermore these results may point to a novel component in the pathogenetic mechanism of early diabetic nephropathy.

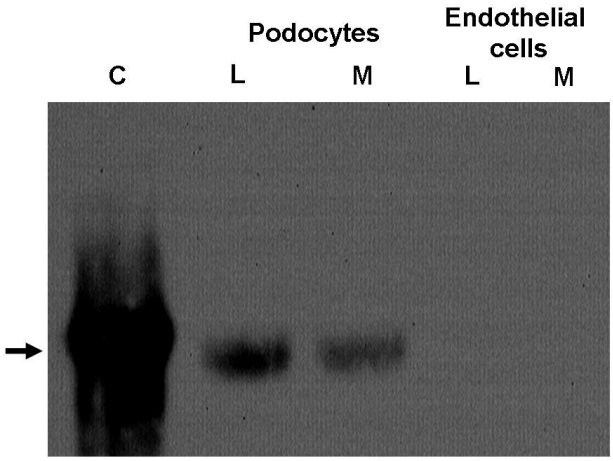

Fig.1 Analysis of adiponectin levels by Western blot of cell lysates (L) and culture media (M) from glomerular podocytes and endothelial cells. Position of adiponectin shown by arrow. C corresponds to a lane run using recombinant adiponectin as a control.

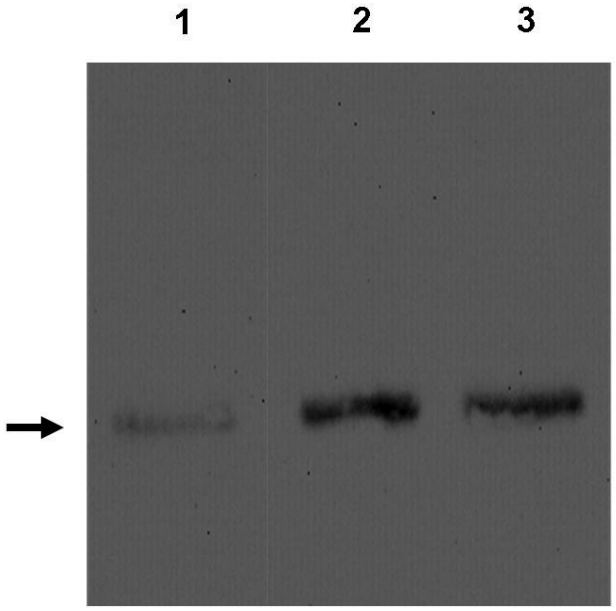

Fig 2. Analysis of adiponectin levels byWestern blot of podocyte cell lysates grown in the presence (1) or absence of insulin for 24 (2) or 48 hours (3). Position of adiponectin shown by an arrow.

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C12

Interleukin-6 causes insulin resistance in human podocytes

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Introduction:
Insulin resistance states are increasing in the developed world at alarming rates with recent studies suggesting that between 12% and 25% of the population are affected. Albuminuria is the earliest sign that the kidney is affected in these conditions. The podocyte is critical for preventing albumin loss into the urine and we have recently shown this cell is insulin sensitive cell with kinetics similar to that of muscle (1). Interleukin-6 (IL-6) is shown to be elevated in the insulin resistant metabolic syndrome. We have studied the effects of co-incubating podocytes with IL-6 on the insulin responsiveness of this cell and it's affect on the know insulin signalling pathways described in insulin responsive cells (PI3-kinase and MAP-kinase phosphorylation).
Methods:
Conditionally immortalised human podocytes were either grown in standard culture conditions with or without high physiological doses of IL-6, TNF-alpha or both (100pg/ml). Cells were then starved of insulin for 12 hours and 2-Deoxyglucose uptake assays performed to assess insulin responsiveness (15 minutes 100nM) in respect to glucose uptake. Insulin signalling was also assessed using Western blotting with phosphospecific antibodies to assess activation of the PI3-kinase and MAP-kinase pathways.
Results:
Co-incubating podocytes with IL-6 abrogates the glucose uptake response completely ($p<0.05$). Interestingly IL-6 does not affect insulin stimulated PI3-kinase or MAPK pathways, upstream of Akt (PKB) or p44/42 MAPK. In IL-6 treated podocytes phospho-Akt [Ser473] ($p<0.05$) increased by 90% in response to 5 minutes exposure to 100nM insulin ($p<0.05$) and p44/42 MAP Kinase increased by 108% ($p<0.05$) under the same conditions. We have also shown that insulin stimulated glucose uptake in the cells is dependent on intact PI3-kinase and MAP-kinase pathways by the use of LY294002 and UO126 inhibitors, (inhibition of either pathway completely abrogates glucose uptake). Statistical analysis was performed using ANOVA with post hoc Bon Ferroni, with between 3-6 independent experiments carried out for each result.
Conclusions:
IL-6 directly induces insulin resistance in human podocytes. Mechanistically its effect is not through inhibition of proximal PI3-kinase or MAP-kinase pathways which are critical for podocyte insulin signalling. We conclude that IL-6 is having its effect either through the distal parts of these pathways or through another signalling pathway. Understanding the mechanism by which IL-6 renders the podocyte insulin resistant may lead to new therapeutic targets in insulin resistant states.

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PC14

Inhibition of the CFTR Cl^- channel by loop diuretics

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Loop diuretics are widely used to inhibit the Na^+-K^+-2Cl^- cotransporter (Haas & Forbush, 2000). However, Venglarik (1997) demonstrated that loop-diuretics inhibit the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. To understand better how loop diuretics inhibit CFTR, we studied furosemide, bumetanide, and two other agents xipamide and piretanide, which are structurally related to furosemide and bumetanide, respectively. We recorded CFTR Cl^- currents in inside-out membrane patches excised from C127 cells expressing wild-type human CFTR. The pipette (external) solution contained 10 mM Cl^- and the bath (internal) solution contained 147 mM Cl^-, 0.3 mM ATP and 75 nM PKA at 37°C; voltage was –50 mV. Data are means ± SEM of n observations and statistical analyses were performed using Student's paired t test.

When added to the internal solution, loop diuretics caused a reversible, concentration-dependent decrease in CFTR Cl^- current. For all agents tested, the concentration-response relationship was well fitted by the Hill equation with Hill coefficients of ~1. The rank order of potency for CFTR inhibition was xipamide (K_i = 45 ± 4 μM) ≥ bumetanide (K_i = 56 ± 11 μM) = piretanide (K_i = 58 ± 18 μM) ≥ furosemide (K_i = 71 ± 15 μM) (n = 5 for all agents).

To investigate further channel block, we used noise analysis. In the absence of furosemide, power density spectra of CFTR Cl^- currents were best fitted with two Lorentzian components with corner frequencies f_{c1} and f_{c2} of 1.17 ± 0.6 and 81 ± 10 Hz (n = 4), whereas in the presence of furosemide (100 μM), power density spectra were best fitted with three Lorentzian components with f_{c1}, f_{c2} and f_{c3} of 2.64 ± 1.38, 63 ± 25 and 312 ± 107 Hz (n = 4), respectively, suggesting that f_{c3} corresponds to the rapid binding and dissociation of furosemide to and from individual CFTR Cl^- channels. Consistent with this idea, furosemide (100 μM) caused a flickery block of CFTR decreasing both open probability (P_o: control, 0.41 ± 0.03; furosemide (100 μM), 0.17 ± 0.02; n = 6; $p < 0.01$) and single-channel current amplitude (i: control, –0.76 ± 0.01 pA; furosemide (100 μM), –0.63 ± 0.03 pA; n = 6; $p < 0.01$). Thus, our data demonstrate that loop diuretics inhibit CFTR, their potency approaches that of the widely used CFTR blocker glibenclamide and that furosemide acts an open channel blocker of CFTR.

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PC15

Anion transport across planar lipid membranes by an artificial anionophore

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Natural and artificial ion transporters consisting of transmembrane channels or carriers are well known. Most of the synthetic transporters concern cation transport, whereas less interest has been shown in anion transport so far. In previous work (Koulov et al. 2003), we demonstrated that a family of small molecules derived from cholic acid termed 'cholapods' bind anions with high-affinity, promote Cl- efflux from liposomes and ion transport across polarised MDCK epithelia (Koulov et al. 2003). Using excised inside-out membrane patches from giant liposomes, we compared the activity of cholapods with that of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl- channel under the same conditions. Addition of cholapods caused a concentration-dependent increase in Cl- current, but no unitary events were observed.

We present here results obtained with a series of cholapods studied in planar lipid membranes (PLM). Membranes were formed by painting a mixture of POPE/Cholesterol (7/3) plus a known amount of cholapod. Using PLM, we have access to both sides of the membrane and thus to modify its environment. Thus, it has been possible to measure cholapod-mediated current under a series of conditions. Addition of cholapods in DMSO to the cis side of the membrane increased the observed current at an applied voltage (e.g. -100 mV). As the ion concentration was elevated the magnitude of cholapod-induced current saturated. Permeability experiments have been carried out and the transporters show good anion vs. cation selectivity, and also selectivity between anions. We interpret these data to suggest that cholapods can mediate anion transport across artificial lipid membranes by a carrier mechanism. Access to both sides of the membrane makes the PLM a potent technique to analyse the mechanism of action of synthetic anion transporters.

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PC20

Evidence for a role for ERK5 in EGF-induced renal epithelial cell survival - a possible role for MEF2C?

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Extracellular signal-regulated kinase-5 (ERK5) (previously known as BMK1) is an atypical MAP kinase. The exact role of ERK5 remains unknown however it is critically important in cell survival and differentiation as highlighted by the embryonic lethality of the ERK5 knock-out (Yan et al., 2003). ERK5 possess a transactivation domain and may act as a transcriptional coactivator by recruiting basal transcriptional machinery. ERK5 has been shown to induce its translocation to the nucleus, where it can activate the transcription factor, Myocyte enhancing factor-2C (MEF2C), inducing c-Jun expression (Kato et al. 1997). Hence the functions of MEF2 could be regulated through ERK5. ERK5 has been shown to be anti-apoptotic in endothelial cells (Pi et al. 2004) and to mediate the survival of neurones in the CNS via the activation of MEF2 (Liu et al., 2003; Shalizi et al. 2003). Inhibitors of the ERK1/2 pathway (PD 98059 or UO126) have been reported to inhibit ERK5. However previously we have shown that neither inhibitor reduce EGF-induced ERK5 in proximal tubule epithelial cells (PTECs). We therefore used siRNA to selectively knock down ERK5 and investigate the role of ERK5 in PTEC apoptosis.

Lysates of HKC-8 cells treated for 5 min with either EGF (10ng/ml) or vehicle (0.1% BSA) were used for Western blotting or subjected to Immunoprecipitation (for MEK5 and ERK5) prior blotting using antibodies targeting proteins of interest. ERK5 siRNA was optimised and a consistent knock-down of 60% was achieved. ERK5 siRNA (100nM) transfected cells were similarly treated but for 24h and caspase-3/7 activity measured as a measure of apoptosis.

Here we show that EGF-induced activation of ERK5 in PTEC is associated with increased association of MEK5 with not only ERK5 but also MEF2C. In addition, EGF inhibited staurosporine-induced apoptosis. siRNA knockdown of ERK5 significantly ($P<0.001$) increased apoptosis and EGF did not alter apoptosis in the presence of ERK5 siRNA.

Our work provides evidence that ERK5 may mediate EGF-induced cell survival in human PTEC. We present the first demonstration of MEF2C expression in PTEC and propose that it has a role in EGF-ERK5 cell survival.

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PC21

Differential dose-dependent BMP-7 signalling in PTECs and implication in fibrosis

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Bone Morphogenic protein-7 (BMP-7) appears to maintain proximal tubule epithelial cells (PTECs) morphology in human adult kidney at levels which activates Smad 1. Reduced levels of BMP-7 exist in diabetic animal model of fibrosis and the effects of low dose BMP-7 which does not activate Smad 1 may correlate with the progression of this and other fibrotic diseases. Hence high dose BMP-7 which activates Smad 1 may reverse TGF-β induced fibrotic outcomes. We have previously described novel BMP-7-induced signalling in PTECs, at concentrations below those known to activate the Smad pathway. We have also investigated upstream, downstream signalling and cell survival and proliferation in response to low dose BMP-7. In addition this work investigates BMP-7 regulation of Smads and p38-MAP kinase signalling and modulation of TGF-β-induced fibrotic outcomes in adult human PTECs. We hypothesized that activation of Smad and p38 may be mutually exclusive.

Human Kidney Clone-8 (HKC-8) cells were treated with BMP-7 (2.5 and 200 ng/ml) at 5, 15 and 60 mins. Activation of p38, MKK3/6, MAPKAPK-2 and Smad signalling was studied by Western blot. Immunocytochemistry was used to look at the intracellular localisation of phoshpo-p38, MAPKAPK-2 and Smad 1 after treatment with 2.5 and 200 ng/ml of BMP-7. The effect of BMP-7 and p38 on cell apoptosis and proliferation of PTECs was measured with Caspase-Glo 3/7 and BrdU assay respectively. The effect of BMP-7 on TGF-β induced signalling and fibronectin (Fn) production was measured by ELISA.

BMP-7 activated Smad 1 at high concentration (200 ng/ml), but activated p38 at lower concentration (2.5 ng/ml). Nuclear localization of both Smad 1 and phospho-p38 is dependent on the concentration of BMP-7, 2.5 ng/ml resulting in nuclear phospho-p38 and 200ng/ml in nuclear Smad 1. We have also shown MKK3/6 to be the upstream of p38 and MAPKAPK-2 to be downstream following treatment with low dose BMP-7. BMP-7 (2.5ng/ml) did not significantly affect cell proliferation nor did it induce or modify staurosporin-induced apoptosis. High dose BMP-7 reduced TGF-β induced p38 production and significantly reduced TGF-β induced Fn secretion at 48 hours.

Here we have shown a novel pathway of low dose BMP-7 which results in p38 activation with upstream being MKK3/6 and downstream being MAPKAPK-2 which may be involved in modulation of inflammatory responses such as activation of inflammatory cytokines IL-1, IL-6, IL-8 and TNF. We have also demonstrated high dose BMP-7 can modulate TGF-β induced p38 fibrotic outcomes. Our results suggests high dose of BMP-7 that activates Smad 1 to be PHYSIOLOGICAL and low dose that activates p38 to be PATHALOGICAL. The full interaction of the above signalling pathways remains to be classified.

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