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LIM kinase function and renal growth: Potential role for LIM kinases in fetal programming of kidney development

Alexander J. Sparrow[a], Dylan Sweetman[b], Simon J.M. Welham[c,*]

[a] Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, West Wing, John Radcliffe Hospital, Oxford OX3 9DU, UK
[b] Zoetis VMRD GTR, Livestock Wellness and Performance, 333 Portage Street, Kalamazoo, MI 49007, USA
[c] School of Biosciences, University of Nottingham, Nottingham, UK

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ABSTRACT

Aims: Maternal dietary restriction during pregnancy impairs nephron development and results in offspring with fewer nephrons. Cell turnover in the early developing kidney is altered by exposure to maternal dietary restriction and may be regulated by the LIM-kinase family of enzymes. We set out to establish whether disturbance of LIM-kinase activity might play a role in the impairment of nephron formation.

Main methods: E12.5 metanephric kidneys and HK2 cells were grown in culture with the pharmacological LIM-kinase inhibitor BMS5. Organs were injected with DiI, imaged and cell numbers measured over 48 h to assess growth. Cells undergoing mitosis were visualised by pH3 labelling.

Key findings: Growth of cultured kidneys reduced to 83% of controls after exposure to BMS5 and final cell number to 25% of control levels after 48 h. Whilst control and BMS5 treated organs showed cells undergoing mitosis (100 ± 11 cells/field vs 113 ± 18 cells/field respectively) the proportion in anaphase was considerably diminished with BMS5 treatment (7.8 ± 0.8% vs 0.8 ± 0.6% respectively; $P < 0.01$). This was consistent with effects on HK2 cells highlighting a severe impact of BMS5 on formation of the mitotic spindle and centriole positioning. DiI labelled cells migrated in 100% of control cultures vs 0% BMS5 treated organs. The number of nephrogenic precursor cells appeared depleted in whole organs and formation of new nephrons was blocked by exposure to BMS5.

Significance: Pharmacological blockade of LIM-kinase function in the early developing kidney results in failure of renal development. This is likely due to prevention of dividing cells from completion of mitosis with their resultant loss.

1. Introduction

Maternal undernutrition during gestation results in developmental changes which predispose individuals to various non-communicable diseases of adulthood including coronary heart disease, hypertension [1,2] and type 2 Diabetes mellitus [3,4]. Furthermore, individuals who were growth impaired during fetal life have a 70% greater risk of developing chronic kidney disease [5]. The underlying causes are still to be fully established, but evidence points to developmental changes in organ systems which result in their limited functional capacity at maturity. Kidney development has been shown to be adversely impacted by maternal undernutrition during gestation with the result that offspring possess fewer nephrons than controls [6–11]. A reduced nephron complement has been strongly associated with a predisposition towards hypertension, coronary heart disease and renal disease [12–14] and animal studies have provided experimental support for these observations [6,15,16]. Rats supplied a low protein diet during pregnancy have elevated levels of apoptosis early in renal development which appears to play a significant role in the reduction of the final nephron number [9]. It has been speculated that this may lead to a loss of nephron precursors and is a potential mechanism by which nephron complement is diminished.

Numerous studies have examined the process of maternal macronutrient restriction mediated impairment of kidney development, however there remains no clearly defined molecular pathway by which this occurs. We have previously observed differential regulation of the enzyme Cofilin 1 (*Cfl1*; [17]) in early developing kidneys following exposure to a maternal dietary protein restriction. The actin severing activity of Cofilin1 is regulated by the Lin11, Isl-1 and Mec-3 (LIM) domain containing kinases [18–20] and members of these, notably LIMK1, are robustly expressed in the early developing kidney [21]. In addition to their role in regulating Cofilin1 function, it has been shown

* Corresponding author at: Nutritional Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Sutton Bonington, Leicestershire LE12 5RD, UK.
E-mail address: simon.welham@nottingham.ac.uk (S.J.M. Welham).

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that the LIM kinases play a critical role in mitosis. Inhibition of these enzymes using siRNA or a pharmacological agent (*N*-(5-(1-(2,6-dichlorophenyl)-3-(difluoromethyl)-1H–pyrazol-5-yl)thiazol-2-yl) cyclopropanecarboxamide; BMS5) prevents cells from progressing through anaphase and they subsequently die [22,23].

Because of the presence of LIMK1 in developing kidneys along with one of its functional targets (Cofilin1), which is repressed by maternal dietary protein restriction, we set out to determine if there is a role for the LIM kinases in mediating kidney development.

2. Materials and methods

2.1. Organ culture

All animal work was approved by the University of Nottingham Animal Welfare Ethical Review Board and was carried out in accordance with Home Office Guidance on the operation of the Animals (Scientific Procedures) Act (Great Britain Home Office, 2000). E11.5-E12.5 ICR mouse kidneys were dissected and kidneys were cultured for either 24 h or 48 h in DMEM/F12 (Sigma, Dorset, UK), supplemented with Penicillin (100 units/ml; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), streptomycin (100 μg/ml; Invitrogen), recombinant human insulin (10 mg/l; Sigma), human transferrin (5.5 mg/l; Sigma), and sodium selenite (5 μg/l; Sigma), and grown on Millicell cell culture plate inserts (pore size 0.4 μm; Millipore, Watford, Hertfordshire, UK) [24,25]. The media of the experimental group was supplemented with 50 μM BMS5 (Synkinase, Pudong, Shanghai, China).

BMS5 has been shown to selectively inhibit both LIMK1 and LIMK2 without cytotoxic effects or any effect on tubulin or Testis-Specific Kinase 1 in interphase cells [26]. Some inhibition of Protein Kinase AMP-Activated Catalytic Subunit Alpha 1 (PRKAA1) and Fibroblast Growth Factor Receptor 1 (FGFR1) has also been observed [26].

2.2. DiI injections

Cultured kidneys were injected at their periphery using a micro injector (FemtoJet, Eppendorf, Stevenage, UK) with a borosilicate glass capillary needle (GC100TF-15 - Harvard Apparatus, Cambridge, UK). Each target site within the kidney was injected with 5 μL DiI - Fluorescent lipophilic membrane dye (1 mg/ml in ethanol, diluted 1:1 in 0.3 M sucrose in Phosphate buffered saline (PBS); C-7001, CellTracker™ CM-Di I, Molecular Probes, Thermo Fisher Scientific). Each kidney was injected at 3 separate sites and grown for a further 24 h.

2.3. Organ cell count

Kidneys were dissociated in 0.25% trypsin/EDTA (Sigma) for 8 min at 37 °C, after which 10% fetal bovine serum (FBS, Sigma) diluted in PBS was added. Dissociated cells were counted using a haemocytometer (Paul Marienfeld GmbH & Co. KG, 97922 Lauda-Königshofen, Germany).

2.4. Whole mount immunofluorescence

Kidneys were fixed in 4% paraformaldehyde (PFA, Sigma) in PBS for 20 min, washed in PBS, transferred to tubes containing 0.2% Triton X-100 (Sigma) in PBS and incubated overnight at 4 °C on a tube roller to permeabilise the tissue. Kidneys were then washed three times in PBS and incubated with primary antibodies (diluted in PBS) overnight at 4 °C on the tube roller. Kidneys were washed in PBS and incubated with secondary antibodies (diluted in PBS) overnight at 4 °C on the tube roller. Kidneys were finally washed again in PBS (3 times) and mounted on microscope slides (pre-prepared with a ring of hardened DPX to preserve the 3D structure of the kidney) in Vectashield Hardset (Vector Laboratories, Peterborough, UK). Primary antibodies: Anti-Calbindin-D-28K (1/100, C9848, Sigma-Aldrich), Anti-Six2 (1/100, 11562-1, Proteintech, Manchester, UK), anti-Histone H3 (phospho S10; 1/500, ab5176, AbCam, Cambridge, UK). Secondary antibodies: Anti-Mouse IgG, Texas red (1/100, T-862, Invitrogen), Anti-Rabbit IgG, FITC (1/20, F0205, Dako, Ely, Cambridgeshire, UK). For whole mount apoptosis assessment, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used. Kidneys were prepared as described above for whole mount immunofluorescence, after which the reaction was conducted according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche Diagnostics, Welwyn Garden City, Hertfordshire, UK).

2.5. Cell culture

Human proximal tubule cells (HK2) were grown and maintained in Dulbecco's Modified Eagle's Medium with 4500 mg/l glucose (Sigma), supplemented with penicillin, streptomycin and 10% FBS for 24 h. Media of experimental groups was supplemented with BMS5 at either 3 μM, 10 μM or 50 μM.

2.6. Cell immunofluorescence

Cells were fixed in 4% PFA (in PBS) for 15 min, washed three times with PBS, permeabilised by incubation in 0.1% Triton X-100 (in PBS) for 15 min, washed three times in PBS and incubated with primary antibodies (diluted in PBS) overnight at 4 °C. The following day, cells were washed three times with PBS and incubated with secondary antibodies (diluted in PBS) for 90 min at room temperature, washed in PBS and mounted in Vectashield Hardset with DAPI. Primary antibodies: Anti-Histone H3 (phospho S10) (1/500 - ab5176, AbCam), Anti-Pericentrin (1/200, ab4448, AbCam), Anti-Alpha Tubulin (1/100, T9026, Sigma-Aldrich). Secondary antibodies were the same as those used for whole mount immunofluorescence.

2.7. Cell count

Media was removed and 0.25% trypsin/EDTA was added to the wells for 4 min at 37 °C after which media containing 10% FBS was added to quench the trypsin activity. Cells were counted using a haemocytometer.

2.8. Fluorescence image acquisition

Cells and organs were imaged using a Leica DM5000 B microscope, Leica DFC420 camera and LAS v.3.8 software (Leica Microsystems (UK) Ltd., Milton Keynes, UK).

2.9. Mitosis and anaphase counting protocol

Kidneys and cells were fixed and labelled for pH3. Images were taken (cells: 4 images per coverslip; organs: 5 images per organ) at × 20 magnification and the total number of pH3 stained cells were counted in each field of view. We additionally counted the number pH3 labelled nuclei showing a clear separation between chromosomes and assigned these as being in anaphase.

2.10. Statistical analyses

All experiments had an n number of at least 3 unless otherwise specified. Data are expressed throughout as mean ± SEM. Statistical analyses of cell counts, mitosis counts and growth data were carried out using one-way ANOVA analyses with SPSS v20.0. In the event of a significant ANOVA outcome ($P < 0.05$), a Tukey's post-hoc test was performed.