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Hypoxia Induces ZEB2 in Podocytes: Implications in the Pathogenesis of Proteinuria

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Abbreviations used: GFB- glomerular filtration barrier; GFR- Glomerular filtration rate; ESRD- End-stage renal disease, CKD- Chronic kidney disease; HIF1 α - Hypoxia-inducible factor-1alpha; ZEB2- Zinc finger E-box binding homeobox 2; NAT- Natural antisense transcript; HRE- Hypoxia response element.

ABSTRACT

The glomerular filtration barrier (GFB) plays a critical role in ensuing protein free urine. The integrity of the GFB is compromised during hypoxia that prevails during extreme physiological conditions. However, the mechanism by which glomerular permselectivity is compromised during hypoxia remains enigmatic. Rats exposed to hypoxia showed a decreased glomerular filtration rate, podocyte foot-processes effacement, and proteinuria. Accumulation of HIF1 α in podocytes resulted in elevated expression of ZEB2 and decreased expression of E- and P-cadherin. We also demonstrated that HIF1 α binds to HRE localized in the ZEB2 promoter. Furthermore, HIF1 α also induced the expression of ZEB2-natural antisense transcript, which is known to increase the efficiency of ZEB2 translation. Ectopic expression of ZEB2 induced loss of E- and P-cadherin and is associated with enhanced motility of podocytes during hypoxic conditions. ZEB2 knockdown abrogated hypoxia-induced decrease in podocyte permselectivity. This study suggests that hypoxia leads to activation of HIF1 α /ZEB2 axis, resulting in podocyte injury and poor renal outcome.

INTRODUCTION

Oxygen is a vital element and supports most of the metabolic events in higher organisms. The continuous supply of adequate levels of oxygen is crucial for normal functioning of the human body. However, human physiology is challenged with extreme environmental conditions resulting in hypoxia, a state of deficiency of oxygen in the blood and tissues. Hypoxia affects the homeostasis and functioning of various organs including kidneys. Kidneys possess low-resistance microvasculature that is exposed to both high volume and continuous perfusion. The kidneys have a high oxygen demand, so as to facilitate energy dependent basic renal functions such as active salt absorption (1). The constraints of low oxygen supply, dictated by both renal architecture and high oxygen demand, coalesce to make the kidney vulnerable to physiologic, environmental and pathological stresses that cause hypoxia. The limitation in renal tissue oxygen supply during chronic hypoxia exposes the kidneys to hypoxia-induced adaptations and has long been recognized as an important factor in the pathogenesis of acute renal injury (2-6).

The vertebrate kidneys play an essential role in the maintenance of homeostasis via its role in the filtration of plasma and the regulation of water, electrolyte, and acid-base balance. The function of the kidneys is to ensure almost protein free ultra-filtrated urine. The presence of proteins in the urine (proteinuria) is an indicator of renal injury, particularly aberrations in the glomerular filtration barrier (GFB). The crucial components of GFB include the glomerular capillary endothelium, basement membrane and glomerular podocytes (7). Albuminuria is an index of adverse renal outcome that can be assessed by measuring albumin levels in urine. Microalbuminuria describes levels of urine albumin ranging from 30 to 300 mg/24 hours, and macroalbuminuria describes a urinary albumin excretion of ≥ 300 mg/24 hours. The condition of macroalbuminuria often progresses to overt proteinuria and even to end-stage renal disease (ESRD) and is driven by an array of interlinked risk factors such as anemia, atherosclerosis, hyperglycemia, hypertension, repeated episodes of acute kidney injury, smoking, stroke and sleep apnea etc. It has been well established that all these factors subject the kidneys to hypoxia (8-10). It was proposed that chronic hypoxia is the common pathway that leads to the ESRD (11) and progression of chronic renal disease (2,5,10-13).

Population-based studies demonstrated that approximately 10 - 20% of adults have proteinuria in their lifetime (14,15). The incidence of ESRD has already reached epidemic proportions in many countries. Furthermore, the rates of ESRD among the elderly are disproportionately high. Considering the magnitude of the population with proteinuria and association of proteinuria with the clinical conditions wherein hypoxia prevails, it is important to understand the underlying mechanism of renal dysfunction to develop preventive and therapeutic strategies. Our understanding of the physiological and cellular events that transduce hypoxia-mediated events to adverse glomerular function is very limiting. In the present study, we investigated the mechanism of renal dysfunction following chronic hypoxia in rats and found that hypoxia results in elevated expression of hypoxia-inducible factor-1 (HIF1 α) in the glomerular region. Elevated HIF1 α in turn induced zinc finger E-box binding homeobox 2 (ZEB2) expression, both by directly interacting with the ZEB2 promoter and by increasing expression of a ZEB2-Natural Antisense Transcript (ZEB2-NAT). Ectopic expression of ZEB2 induced loss of E- and P-cadherin and was associated with enhanced migration of cells. Finally, hypoxia ablated the podocyte permselectivity, whereas loss of ZEB2 knockdown abrogated this effect. Elevated expression of ZEB2 is implicated in podocyte epithelial-mesenchymal transition (EMT) and foot-process effacement during chronic hypoxia. We conclude that hyperactivity of the HIF1 α -ZEB2 axis is one of the mechanisms by which acute systemic hypoxia provokes poor renal outcome.

MATERIALS AND METHODS

Reagents and plasmids:

Antibodies for P-cadherin (#sc-1501), WT1 (#sc-192), Nephrin (#sc-376522), ZO1 (#sc-33725) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA); ZEB2 (#PA5-20980) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Actin (#4970), CD2AP (#2135) HIF1 α (#14179, #79233) N-cadherin (#4061) MMP2 (#4022), E-cadherin (#3195), MMP9 (#3852), MMP2 (#4022), VEGF (#9943), TG2 (#73612), Vimentin (#5741), β -Catenin (#8480), Histone3 (#4499), FSP1 (#13018) were procured from Cell-Signaling Technologies (Danvers, MA, USA). Podocin antibody is a gift from Rakesh Verma (University of Michigan, Ann Arbor) Secondary antibodies were

purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All primers used in this study procured from Integrated DNA Technologies (Coralville, IA, USA). RNA isolation kit was purchased from Qiagen (Hilden, Germany). The high capacity cDNA reverse transcription kit, SYBR Green Master Mix, and ECL Western blotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Fetal bovine serum, RPMI-1640 and Trizol reagent were procured from Life Technologies (Carlsbad, CA, USA). Dual-Luciferase Assay System was procured from Promega (Madison, WI, USA). E-cadherin-luciferase reporter construct (16) was a gift from Dr. Eric Fearon (University of Michigan, Ann Arbor). Hypoxia response element (HRE1&2) in the ZEB2 promoter and ZEB2 binding sites in E-cadherin promoter (E2 box1 and E2 box3) were mutated using the Quick-change site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). ZEB2 and ZEB2-NAT promoter constructs (17) were a gift from Dr. Antonio García de Herreros (IMIM, Universitat Pompeu Fabra, Barcelona). ZEB2 expression construct (18) was a gift from Dr. Anders Lund (University of Copenhagen, Denmark).

Hypoxia treatments and surgical procedures:

Wistar rats weighing 190 ± 10 g, provided by the National Center for Laboratory Animal Sciences (Hyderabad, India), were maintained at $22^{\circ}\pm 1^{\circ}$ C and relative humidity of $50\pm 5\%$ under a 12 h light/12 h dark cycle with food and water ad libitum. Rats were allowed to acclimatize to the animal facility for 1 week. Rats were randomly divided into normoxia and hypoxia cohorts (n=10). Rats in the normoxia group were maintained in a chamber with a fraction of inspired oxygen (FIO) of ~ 0.21 for 2 weeks. Hypoxia exposure to rats was performed as described earlier (19). Briefly, rats were exposed to FIO of 0.1 for 23 h (hypoxia) and FIO of 0.21 (normoxia) for 1 h each day and were maintained for 2 weeks (**Fig.1A**). Rats from both groups were transcardially perfused under anesthesia with saline. Organs were harvested and frozen immediately for further processing or processed for histopathology. The Institutional Animal Ethics Committee of the University of Hyderabad approved animal care and experimental protocols.

Estimation of Glomerular Filtration Rate and Urine parameters:

Glomerular filtration rate (GFR) was estimated in rats (n=6) following two weeks of exposure to hypoxia using a commercially available kit (#FIT-0415, BioPAL, Worcester, MA, USA). Briefly, a single intraperitoneal injection of inulin (25 mg/kg) was given followed by saphenous bleeds at 5, 10, 30, 60 and 90 minutes. Plasma was collected, and inulin concentration was estimated using functional immunoassay technology (FIT). Plasma clearance of inulin was determined by nonlinear regression using a one-phase exponential decay formula and GFR was calculated from inulin clearance as described earlier (20). Alternatively, spot urine was collected from control and experimental rats and centrifuged at 2000xg for 5 min. Urinary albumin (#COD11573) and creatinine (#COD11502) levels were estimated using commercially available assay kits (Biosystems, Barcelona, Spain).

Histological Examination and Transmission Electron Microscopy:

Renal cortical samples were fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned (4µm), stained with the periodic acid Schiff (PAS) stain and images were acquired using Olympus 5000 microscope with 40 X magnification. The glomerular tuft area was measured as described earlier (21). The extent of glomerulosclerosis index was determined in PAS-stained sections with the scoring system as described earlier (22,23). Briefly: 0 – normal glomerulus; 1 – mesangial expansion or sclerosis involving up to 25% of the glomerular tuft; 2 – sclerosis of 25 to 50%; 3 – sclerosis of 50 to 75% and/or segmental extracapillary fibrosis or proliferation; and 4 - global sclerosis >75%, global extra capillary fibrosis or complete collapse of the glomerular tuft. For transmission electron microscopic (TEM) analysis, renal cortex from control and experimental rats were fixed in 2.5% glutaraldehyde for 24 h. After washing with 1 X PBS for four times, cortical tissues were fixed in 1% osmium tetroxide for 2 h and ultrathin sections (60 nm) were cut and mounted on 200 mesh copper grids. These copper grids were stained on a Leica EM AC20 strainer with 3% aqueous uranyl acetate and 3% lead citrate solution. Images were acquired on a JEOL JEM-1400 TEM (Peabody, MA, USA) using a Gatan ultrascan CCD camera at 2K X 2K resolution and 120 KV.

Isolation of kidney glomeruli:

The glomeruli from the kidneys of control and rats exposed to hypoxia were isolated by a series of stainless sieves (24). Briefly, under aseptic conditions, decapsulated rat kidney slices were placed on a sieve with a size of 200 μm and gently grounded with a sterile syringe knob and washed with Hank's solution. The filtered kidney lysate was then allowed to pass through decreasing pore size sieve of 150 μm and as a final step; the glomeruli were then collected as a suspension on a 75 μm sieve. After incubation for 10 min, the suspensions were centrifuged for 5 min at 1000 RPM, then the supernatant was discarded, the purified glomerular precipitations were used for immunoblotting and gene expression studies.

Silver staining of urine samples:

Urine samples are collected from control and rats exposed to hypoxia and protein concentrations were estimated using the Bradford assay. Equal amounts of protein were subjected to SDS-PAGE, following which gel was placed in fixing solution (50 % methyl alcohol and 10 % glacial acetic acid) for 1 h at RT. Then, the gel was washed with 50% ethanol and sensitized with sodium thiosulfate for 1 min. After washing with distilled water, the gel was incubated with 0.2% silver nitrate solution containing 0.037 ml formaldehyde for 30 min. The gel was briefly washed and then developed in 6% sodium carbonate, containing 0.037 ml formaldehyde solution until protein bands were sufficiently dark. The reaction was terminated by placing the gel in 1% glacial acetic acid solution and visualized by Bio-Rad image analyzer.

Culturing of Podocytes and HEK293T cells:

Human podocytes were obtained from University of Bristol, Bristol, UK and were cultured as described earlier (25). Briefly, podocytes were propagated at 33°C (permissive conditions) and 5% CO₂ in RPMI-1640 medium containing 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, and 1% insulin-transferrin-selenium solution. At ~60% confluence, these cells were transferred to 37°C (non-permissive condition) tissue culture incubator with 5% CO₂ for 6 days to silence the temperature sensitive tsA58T antigen to allow podocyte differentiation. Differentiation of podocytes was achieved by a growth arrest and the upregulation of cyclin-dependent kinase inhibitors.

Cells used for experimentation were from passages 10 to 16. HEK293T cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin and incubated at 37°C and 5% CO₂. Differentiated podocytes and HEK293T cells were exposed to hypoxia using a modular gas chamber inside CO₂ incubator where along with carbon dioxide; nitrogen gas was supplied to reduce the oxygen levels to 1% for 24 h.

Immunoblotting:

Glomerular lysates from kidney cortex, human podocytes, and HEK293T cells were processed for immunoblotting. An equal amount of protein from control and experimental samples was electrophoresed through 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane by electroblotting. The nitrocellulose membrane was then blocked in 5% non-fat milk for 1 h, and subsequently probed with the indicated primary (1:1000 dilution) and secondary antibodies (1:10,000 dilution). Blots were developed using the ECL Western blotting substrate.

Quantitative Real-time PCR analysis:

Total RNA from glomeruli, human podocytes, and HEK293T cells was extracted using Trizol reagent and, isolated using RNA isolation kit (Qiagen, Hilden, Germany). RT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories). mRNA expression levels were normalized using the expression of β -actin and data was analyzed using the comparative threshold cycle method.

Immunofluorescence analysis:

Cells cultured on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% TritonX100 and blocked with 3% BSA in 1 X PBS. Cells were incubated with primary antibodies for HIF1 α , (1:100) and ZEB2 (1:100). HIF1 α and ZEB2 were visualized using Cy5 Horse anti-mouse IgG (1:200) and DyLight594 anti-rabbit IgG (1:200) respectively (Vector labs). Prolong gold antifade with DAPI is used to mount the slides (Thermo Fisher Scientific). Images were acquired using a confocal microscope

(ZEISS) at 63X magnification. Similarly, expression of HIF1 α and ZEB2 in glomerular sections of experimental rats was analyzed using a trinocular microscope (Leica).

Cell migration assay:

Both differentiated human podocytes and HEK293T cells were seeded in 6 well plates and grown under normal growth conditions to reach 95% confluence. When the cells are confluent, media was aspirated, and a scratch was made in a sweeping motion using a 10 μ l sterile pipette tip. Cells were washed with pre-warmed media to remove debris from damaged cells. Images of cells were taken following exposure to hypoxia at regular time points to monitor the rate of cell migration into the wound field. The area covered by cells is quantified using Image J software (NIH, USA) and represented as the % area coverage (Cell Area/ROI Area X 100).

shRNA-mediated ZEB2 knockdown in podocytes:

ZEB2 expression in human podocytes was knockdown as described earlier (26). Lentiviral particles expressing unique ZEB2 shRNA sequences and scrambled shRNA were purchased from Sigma-Aldrich. Podocytes were cultured in a 6-well plate under growth permissive conditions for 16 h. The cells were then pretreated with 6 μ g/ ml polybrene and infected with indicated lentiviral particles. Next day the medium was replaced with fresh medium and shRNA-infected cells were selected with 1 μ g/ ml puromycin. Puromycin-resistant cells were induced to differentiate under non-permissive conditions, and ZEB2 expression was verified in the differentiated cells using qRT-PCR and immunoblotting.

Albumin Influx Assay:

Podocyte permselectivity was assessed using paracellular albumin influx assay as described previously (26). Corning transwell collagen-coated 3.0 μ m pore polytetrafluoroethylene (PTFE) membrane inserts (#CLS3494, Sigma) were used in this study. Podocytes were seeded on the transwell insert and placed in a 12 well tissue culture plate, during which transwell insert serves as upper chamber and cell culture well serve as a bottom chamber (Fig 8A). Podocytes were cultured under non-permissive conditions for 6 days as detailed above in the methods section. These

differentiated cells were exposed to hypoxia for 24 h. Subsequently, the cells were washed twice with 1 X PBS containing CaCl_2 and MgCl_2 to preserve cadherin junctions. Two ml of RPMI -1640 medium with 40 mg/ml BSA was placed in the bottom chamber, and 300 μl of RPMI - 1640 medium (without BSA) was placed in the upper chamber. The cells were incubated at 37°C, aliquots of medium were collected from the upper chamber at regular intervals and albumin concentration was measured from these aliquots.

Transient transfection and luciferase assay:

HEK293T cells were transfected with a promoter-reporter construct (ZEB2/E-cadherin) and internal control expressing the Renilla luciferase, pRL-TK (Promega). 293T cells were transfected using Xfect polymer (Takara) as per manufacturer's instructions. After 48 h of transfection, cells were washed twice with 1 X PBS and harvested in 100 μl of passive lysis buffer. After a brief freeze-thaw cycle, the insoluble debris was removed by centrifugation at 12,000xg (4°C for 5 min), and 20 μl of supernatant was used for luciferase assay. The activity of the co-transfected renilla luciferase reporter plasmid was used to normalize transfection efficiency.

ChIP assay:

ChIP assay was performed as described previously (27). HEK293T (2.5×10^6) cells either naïve or exposed to hypoxia were taken for ChIP assay. Cross-linked chromatin from HEK293T cells was sonicated using Covaris S220 Focused-ultrasonicator. The whole cell extract was pre-cleared using 60 μl of Protein A/G PLUS-agarose beads and incubated overnight with anti-HIF1 α and control IgG antibodies. 1 μg of anti-HIF1 α antibody was used for 100 μg of total cell lysate. Protein G agarose beads (Millipore, Saint Charles, MO, USA) pre-blocked with salmon sperm DNA (60 μl) was used for each immunoprecipitation. 10% of chromatin from each sample was removed before immunoprecipitation and used as input for PCR amplification. Both immunoprecipitated and input DNA were purified with PCR-purification kit (Qiagen, Valencia, CA, USA). Quantitative RT-PCR was performed on this DNA to identify the amount of target sequence. HRE region of the VEGFA promoter was used as a positive

control, while the primers that do not amplify the HRE region of the ZEB2 promoter was used as a negative control.

Statistical Analysis:

SPSS software was used to apply Mann-Whitney and Kruskal-Wallis nonparametric tests to analyze statistical differences between the distributions of two or multiple independent samples, respectively. $P \leq 0.05$ were considered as statistically significant.

RESULTS

Chronic hypoxia alters glomerular function *in vivo*:

In order to assess the impact of chronic hypoxia on renal function, we estimated GFR *in vivo* (Fig 1B). Rats exposed to hypoxia displayed reduced GFR (~29%) compared with control rats (2.42 Vs. 3.45 ml/min/Kg body weight). To confirm the adverse effect of hypoxia on renal function, we also assessed urinary albumin to creatinine ratio. Rats subjected to hypoxia showed ~9 fold increase in albumin excretion compared with control rats (Fig 1C). Furthermore, urinary fractions collected from rats exposed to hypoxia had a significant amount of protein as assessed by SDS-PAGE and visualized by silver staining (Fig 1D). We measured the extent of glomerulosclerosis in PAS-stained glomeruli and found that increased Bowman's space, mild and diffuse glomerulosclerosis (Fig 1E&F) and reduced glomerular tuft area (Fig 1G) in rats exposed to hypoxia. Average glomerular tuft area in rats exposed to hypoxia was $1.8 \mu\text{m}^2$ compared with $2.25 \mu\text{m}^2$ in control rats. All these data suggest altered glomerular architecture and increased glomerular permeability as evidenced by proteinuria in rats exposed to hypoxia.

Chronic hypoxia-induced podocyte injury *in vivo*:

To understand the causative factors for proteinuria in rats exposed to hypoxia, we next investigated whether there are any differences in podocyte count and podocyte structure, using immunochemistry and TEM techniques, respectively. We stained glomerular sections for WT1, a putative marker for podocytes in order to study the effect

of hypoxia on quantification of podocytes (Fig 2A). We noticed ~39% decrease in podocytes number in experimental rats. When compare with podocyte count of 23 ± 4 per glomeruli in control rats, rats exposed to hypoxia had a count of 14 ± 5 (Fig 2B). To ascertain the podocyte morphology, we performed TEM analysis and observed foot-processes effacement and disruption of slit-diaphragm from rats exposed to hypoxia (Fig 2C). TEM images also revealed that there are no noticeable changes in glomerular basement membrane and endothelium between control and experimental rats (Fig 2C). All these data suggest podocyte injury in rats exposed to hypoxia.

Chronic hypoxia-induced glomerular ZEB2 expression:

Since, we observed significant morphological and functional changes in the glomerular region, particularly in podocytes of rats exposed to hypoxia, we would like to understand the molecular basis for the observed pathological changes. HIF1 α is a major transcription factor that regulates the adaptive response to hypoxia. Earlier studies suggest that activation of HIF1 α is associated with progressive glomerulonephritis, epithelial to mesenchymal transition (EMT), fibrosis and renal failure (28). Considering the significance of HIF1 α under hypoxic conditions, we investigated HIF1 α expression in the glomerular region of rats exposed to chronic hypoxia. As expected, we observed elevated expression of HIF1 α in the glomerular region of rats exposed to hypoxia (Fig 3A&B). Further, induction of HIF1 α is correlated with the up-regulation of its downstream targets including Tissue Transglutaminase (TG2), VEGFA, MMP2, and MMP9 and decreased expression of podocyte-specific proteins nephrin and podocin (Fig 3A&B). In addition to these canonical HIF1 α targets, we have also analyzed the expression of ZEB2 in the glomerular region of the kidney, since ZEB2 gets upregulated during podocyte injury and elevated ZEB2 expression is associated with podocyte depletion (26,29). Interestingly, we observed an elevated ZEB2 expression in glomerular extracts from rats subjected to hypoxia, as measured at both protein (Fig 3A) and mRNA (Fig 3B) levels. Immunofluorescence data confirms an elevated expression of both HIF1 α and ZEB2 in glomerular regions exposed to hypoxia (Fig 3C). The data suggest that elevated expression of glomerular HIF1 α during hypoxia is associated with increased ZEB2 expression in proteinuric rats.

Expression of ZEB2 and ZEB2-NAT is associated with EMT of human podocytes:

ZEB2, which was increased in glomerular extracts of hypoxic rats, is chosen for further analysis owing to its established role in EMT of glomerular podocytes and altering podocyte permeability (26,30). ZEB2 is also involved in the development of the kidneys (31). ZEB2 genomic locus also expresses ZEB2-NAT in antisense manner and is known to bind complementary region on ZEB2 mRNA and mask the factors that would otherwise induce splicing (17,32). Thus, binding of ZEB2-NAT to ZEB2 mRNA prevents its splicing and augments ZEB2 mRNA translation (26). We found elevated expression of ZEB2-NAT in addition to ZEB2 mRNA in glomerular extracts of rats exposed to hypoxia (Fig 4A), indicating dual regulation of ZEB2 mRNA under hypoxic conditions; both at transcriptional (pre-mRNA) and translational (post-mRNA) levels. Upon confirming the elevated expression of ZEB2 in hypoxic rats, we assessed the expression of ZEB2 target genes and EMT markers: E-cadherin, a predominant epithelial marker; P-cadherin and ZO-1, constituents of cell-cell junctions; N-cadherin, vimentin, and β -catenin are mesenchymal markers. ZO-1, E- and P-cadherin were down-regulated, whereas N-cadherin, vimentin, and β -catenin were up-regulated in rat glomerular extracts in response to hypoxia (Fig 4A&B). To validate the observations found in rats also holds true in humans, we analyzed the expression of the same set of genes in Nakagawa Chronic Kidney Disease (CKD) dataset available at Nephroseq (<https://nephroseq.org>). This analysis revealed co-expression of HIF1 α , ZEB2, β -catenin in Chronic Kidney Disease dataset (Fig 4C). It should be noted that elevated expression of HIF1 α is also associated with loss of nephrin and podocin in CKD patients (Fig 4C). Thus, human renal genome-wide gene expression analysis also suggests a direct association of elevated HIF1 α levels with loss of podocyte-specific gene expression.

Hypoxia induces HIF1 α and ZEB2 expression in podocytes *in vitro*:

Glomerular podocytes are instrumental to ensure glomerular permselectivity (7). It was reported that podocytes undergo EMT and such a glomerular injury with reduced podocyte count was associated with proteinuria (29,33). In order to investigate whether the observed proteinuria and decreased podocyte count in rats exposed to hypoxia were owing to podocyte injury, we then investigated the effect of hypoxia on human

immortalized podocytes. As anticipated, upon exposure to hypoxia, we noticed the accumulation of HIF1 α in podocytes (Fig 5A&B). In addition, we also observed elevated expression of ZEB2 and N-cadherin along with decreased expression of E- and P-cadherins (Fig 5A&B). We observed induction of ZEB2-NAT in podocytes exposed to hypoxia (Fig 5B), similar to the observations made in rat glomerular extracts. Next, we investigated if there is any co-localization of HIF1 α and ZEB2 in the podocytes upon exposure to hypoxia (Fig 5C). In podocytes cultured under normoxia, we observed very low levels of HIF1 α . In cells exposed to hypoxia HIF1 α levels were elevated and predominantly found in the nucleus (Fig 5C). ZEB2 was also increased in podocytes during hypoxic conditions (Fig 5C). Together, these data obtained from podocytes (Fig 5C) and glomerular sections (Fig 3C) suggest that both HIF1 α and ZEB2 are localized to glomerular podocytes during hypoxic conditions. Elevated expression of ZEB2 in podocytes was associated with cadherin switch as evidenced by attenuation of epithelial cadherins (E-and P-cadherins) and acquisition of mesenchymal cadherin (N-cadherin) (Fig 5A&B). In order to ascertain the elevated expression of ZEB2 during hypoxia ensures cadherin switch, we assessed cadherin expression in HEK293T cells that ectopically express ZEB2 (Fig 5D). Ectopic expression of ZEB2 resulted in the loss of E- and P-cadherin and increase in N-cadherin (Fig 5D). Cadherin switch that is associated with EMT imparts enhanced migratory ability to cells (34). Therefore, we next assessed the migratory properties of cells naïve to hypoxia or exposed to hypoxia. Both, podocytes and HEK293T cells exposed to hypoxia showed enhanced migratory properties as assessed by wound healing assay (Fig 6A-C), a typical feature of cells that had undergone a phenotypic switch from epithelial to mesenchymal state. In order to verify, whether the enhanced migratory properties of these cells are indeed due to elevated ZEB2 expression, we assessed migratory properties of HEK293T cells that ectopically express ZEB2. Cells overexpressing ZEB2 (Fig 6D) showed enhanced migratory properties upon exposure to hypoxia (Fig 6E). Taken together these data suggest the existence of a strong positive correlation of HIF1 α and ZEB2 expression in the podocytes during hypoxia and induction of ZEB2 ensures podocytes acquire mesenchymal phenotype.

HIF1 α increases ZEB2 and ZEB2-NAT promoter activity:

To investigate the effect of HIF1 α on ZEB2 promoter activity, we transiently transfected HEK293T cells with ZEB2 promoter-luciferase constructs and measured luciferase activity in the cell lysates with and without hypoxia treatment. We observed an increase in ZEB2 promoter activity in cells exposed to hypoxia (Fig 7A). Similarly, ZEB2-NAT promoter activity was also increased with hypoxia (Fig 7A). To verify the specificity of the effect of HIF1 α on ZEB2 promoter activity, we tested the effect of hypoxia on the promoter of an unrelated gene, growth hormone receptor (GHR). Hypoxia had no effect on GHR promoter activity (Fig. 7A).

HIF1 α binds to hypoxia response element (HRE) and activates the transcriptional activity of target genes. HRE possess two core sequences; 5'-CACGT-3' and 5'-GCGTG-3' (35,36). HREs are located at +104/+108, and +176/+180 region in 5'UTR of ZEB2 gene (Fig. 7B). To ascertain the occupancy of HIF1 α on the ZEB2 genomic loci, we engineered ZEB2-promoter luciferase construct with a mutation in the HREs that abrogate HIF1 α binding to ZEB2 promoter and tested the effect of hypoxia on the activity of this mutant luciferase reporter construct. Site-directed mutations in HREs of ZEB2 promoter failed to elicit ZEB2 promoter-reporter activity in cells exposed to hypoxia (Fig 7B). These results suggest that functional HREs present on the ZEB2 promoter are essential for ZEB2 expression under hypoxic conditions.

In order to assess the elevated expression of ZEB2 during hypoxia is due to HIF1 α occupancy on the ZEB2 promoter, we next performed chromatin immunoprecipitation (ChIP) assay. ChIP assay confirmed that HIF1 α occupies ZEB2 promoter *in vivo* at the HRE's in response to Hypoxia (Fig 7C). The specificity of these associations was verified by demonstrating a lack of binding to a region adjacent to HRE's in the ZEB2 promoter. We utilized the interaction of HIF1 α with the promoter region of VEGFA, which serves as a positive control (37) for the experimental conditions as VEGFA promoter has HRE (Fig 7C). As we observed that ZEB2-NAT was also expressed under hypoxic condition, we performed promoter analysis of ZEB2-NAT for the presence of HREs. We observed HRE sequence ('GCGTG') at two regions of ZEB2-NAT promoter +1145/+1149 and +2945/+2949. Together, this data suggests that

HIF1 α elicits ZEB2 protein accumulation by inducing both ZEB2 mRNA and ZEB2-NAT expression wherein ZEB2-NAT augments the expression of ZEB2 mRNA.

ZEB2 knockdown prevents HIF1 α -dependent decrease in E-cadherin in podocytes:

We next investigated whether ZEB2 plays an essential role in the HIF1 α -mediated podocyte injury. For this, we utilized podocytes in which ZEB2 expression was knocked down using shRNA-mediated approach. After having confirmed the efficacy and specificity of knockdown (Fig 7D), these cells were exposed to hypoxia, and the expression of E-cadherin was measured by immunoblotting. In podocytes transfected with lentivirus expressing ZEB2 shRNA, exposure to hypoxia failed to elicit the decrease in E-cadherin expression (Fig 7E). These results suggest that ZEB2 is essential for transducing the effect of HIF1 α on E-cadherin expression in the podocytes. Further, we investigated the temporal association of HIF1 α -induced ZEB2 on E-cadherin expression using E-cadherin promoter construct. E-cadherin promoter has two E2-boxes (E2-box1: 'CAGGTG' & E2-box3: 'CACCTG') wherein ZEB2 interacts and transduces the repressive action on E-cadherin expression. The specific effect of HIF1 α -induced ZEB2 on E-cadherin expression was analyzed using E-cadherin (E-cad) construct in which ZEB2 binding sites (E2 boxes) were mutated (E-cad-E2M). While hypoxia treatment decreased the activity of E-cad WT promoter activity, it had no effect on E-cad-E2M (Fig 7F), suggesting that the observed loss of E-cadherin expression in hypoxia conditions is due to ZEB2 overexpression.

Essential role of ZEB2 in eliciting the effect of HIF1 α on the permeability of podocytes:

To assess the functional consequence of hypoxia on podocyte function, we next examined the podocyte permeability employing paracellular albumin influx assay. This assay measures the rate of albumin flux across the podocyte monolayer plated on semipermeable PTFE membrane inserts (Fig 8A). After 24h of exposure to hypoxia, albumin was placed in the bottom chamber of transwell and the influx of albumin across the podocyte monolayer was measured at 1h and 4h intervals. Increased albumin influx was noticed across the podocyte monolayer that was exposed to hypoxia (Fig 8B).

These results revealed a hypoxia-dependent increase in albumin influx across the podocyte monolayer. To delineate the role of HIF1 α on the permeability of the podocyte monolayer, we next investigated the effect of HIF1 α in podocytes in which ZEB2 expression was knocked down. Knocking down of ZEB2 expression resulted in blunting the ability of hypoxia to elicit an increase in the albumin influx across podocyte monolayer (Fig 8B). These results suggest that ZEB2 is necessary for transducing the effect of hypoxia on podocyte permeability. All these data confirm that ZEB2 is the key player in eliciting the proteinuric effect of elevated HIF1 α expression in the podocytes during hypoxic conditions, wherein hypoxia induces HIF1 α expression, which recognizes and binds to HREs on the ZEB2 promoter and transactivates its expression. ZEB2, in turn, regulates the expression of EMT genes, and repression of slit-diaphragm components resulting in increased permeability.

DISCUSSION

Prevalence of glomerular dysfunction is prominent in clinical conditions and adverse environmental conditions where hypoxia prevails. Nevertheless, the pathological events that connect hypoxia to glomerular dysfunction remain largely unknown. We took up the current investigation to understand the pathophysiological basis of the effects of chronic hypoxia on glomerular podocytes. Our study revealed that in rats exposed to chronic hypoxia, HIF1 α is elevated in glomerular regions. Our results indicate a novel target of HIF1 α is the induction of ZEB2. This increase in ZEB2 expression is due to the up-regulation of ZEB2 mRNA and the induction of NAT of the ZEB2 gene. We demonstrated that HIF1 α -dependent induction of ZEB2 in the podocytes results in decreased expression of P- and E-cadherin. P-cadherin is a component of the podocyte slit-diaphragm (38), and E-cadherin anchors epithelial junctions and participates in EMT (39). Our studies also establish that accumulation of HIF1 α increases permeability of a podocyte monolayer, and this effect is a consequence of HIF1 α -dependent induction of ZEB2. A hyperactive HIF1 α -ZEB2 axis is implicated in the altered podocyte biology, both by initiating EMT and altered permeability to the slit-diaphragm (Fig 8C); and our results provide a molecular basis for the role of hypoxia in the pathogenesis of glomerular diseases.

The hypoxic condition results in decreased arterial oxygen pressure that could affect multiple organs including the kidney. Arterial oxygen pressure is maintained at relatively stable levels by a unique and complex functional interplay between renal blood flow, GFR, oxygen consumption, and arteriovenous oxygen shunting (40,41). The fragility of this interplay makes the kidney vulnerable to hypoxic injury (40). Renal cells have evolved a variety of molecular mechanisms to adapt to hypoxic conditions that prevail during development, under extreme physiological and pathological conditions. HIF1 α is a predominant transcription factor involved in the control of hypoxia and oxidative stress responses. It is noteworthy that intra-renal hypoxia is a proposed pathway to renal dysfunction in conditions such as diabetes and hypertension (42). Proteinuria is a predominant manifestation of glomerular dysfunction and it refers to the alterations particularly in GFB that otherwise offers permselectivity and prevents loss of protein in the urine. Therefore, we investigated the effect of hypoxia in renal cortex, the region where glomeruli are concentrated, and in podocytes that are instrumental to ensure permselectivity. It is interesting to note that HIF1 α levels have been reported to be elevated in many organs including liver, brain, and kidney in response to hypoxia and influence target gene expression (43). Similarly, in our model HIF1 α levels were significantly upregulated in the kidney compared to liver, and brain.

Our results establish that hypoxia-induced accumulation of HIF1 α stimulates the expression of ZEB2. ZEB2, also known as Smad-interacting protein-1 (SIP1) and ZFHX1B, belongs to the ZEB protein family. ZEB2 acts as a transcriptional repressor in both C-terminal binding protein (CTBP) dependent and independent manner (44). The expression of ZEB2, a critical transcriptional factor that orchestrates EMT phenomenon is regulated by *cis*-NAT. We observed expression of ZEB2-NAT in podocytes exposed to hypoxia. Since ZEB2 has many potential targets including crucial proteins of tight junctions, adherence junctions and gap junctions (45), the increase in ZEB2 could have pleiotropic effects on the podocyte biology during hypoxia. E-cadherin is a canonical target for transcriptional repression by ZEB2. E-cadherin exists in cytoplasmic vesicles in podocytes in culture (38). The role of E-cadherin in EMT phenomenon is well established, wherein E-cadherin expression is down-regulated during the transition of cells from epithelial to mesenchymal phenotype (46). P-cadherin, another bonafide

target of ZEB2, belongs to the cadherin superfamily and is a component of the slit diaphragm apparatus. Decreased expression of P-cadherin is implicated in the pathogenesis of proteinuria and glomerulosclerosis (47,48). P-cadherin mediates cell-cell adhesion between adjacent podocyte foot processes and also serves as the basic scaffold for the slit-diaphragm. It is noteworthy that the key findings of the current study, HIF1 α -dependent increase in ZEB2 and the resultant decrease in E- and P-cadherins, were observed in both HEK cells and human podocytes. In podocytes with a ZEB2 knockdown, HIF1 α accumulation fails to provoke the loss of E-cadherin; whereas ectopically expressed ZEB2 elicited further loss of E-cadherin expression. Furthermore, elevated ZEB2 expression altered the permselectivity of podocytes to albumin. These results indicate that ZEB2 is essential for transducing the HIF1 α effects on the permeability of the podocyte monolayer. In addition to ZEB2, there are other transcription factors that elicit EMT phenomenon including ZEB1, Snail, and Slug. We observed that basal level ZEB2 expression in podocytes was relatively much higher than compared with other transcription factors that induce EMT, while ZEB1 protein expression was only minimally increased during hypoxia (data not shown).

Besides the loss of E- and P-cadherin expression, several other proteins are also deregulated during EMT of podocytes including nephrin and podocin (33). We found attenuated expression of ZO-1 and increased expression of N-cadherin, β -catenin, and vimentin in rats exposed to hypoxia. ZO-1 is a component of tight junctions, which plays a vital role in regulating permeability across the epithelium. The decreased expression of ZO-1 in podocytes exposed to hypoxia could disrupt the permeability barrier of the podocytes. The loss of ZO-1 expression in our study is concurrent with earlier studies that reported the loss of tight junction proteins under ischemic conditions (49). We have also observed the loss of podocyte-specific proteins nephrin and podocin in cells exposed to hypoxia. These two proteins are crucial components of podocyte slit-diaphragm and loss of their expression, particularly nephrin, a major determinant of glomerular vascular permeability correlates with a loss of glomerular filter integrity (50). Expression of nephrin and podocin is also decreased during EMT of podocytes (51).

The results from this study show that ZEB2 expression is induced in podocytes under hypoxic conditions. We demonstrate that ZEB2 promoter has HREs

and HIF1 α interacts with these HRE1 and HRE2 (5'-GCGTG -3' & 5'-CACGT-3'). Activation of ZEB2 gene transcription is evidenced by an increase in ZEB2 promoter activity under hypoxic conditions. It is known that expression of the ZEB2 is controlled at multiple levels, including transcriptional and post-transcriptional. Post-transcriptional regulation of ZEB2 is mediated by ZEB2-NAT, wherein ZEB2-NAT binds to a complementary region on ZEB2 mRNA. Binding of ZEB2-NAT with ZEB2 mRNA preserves Internal Ribosome Entry Site (IRES) of ZEB2 mRNA by preventing splicing of an intron in ZEB2 mRNA. ZEB2-NAT mediated control of ZEB2 expression has been described in earlier studies (17,35). Interestingly, in addition to the ZEB2 expression, we identified the induction of ZEB2-NAT in hypoxic conditions. Our study reveals that a two-level regulation of ZEB2 expression by HIF1 α under hypoxic conditions. In addition to our own observations, meta-data analysis from Nephroseq also revealed that elevated expression of HIF1 α is associated with ZEB2 overexpression in chronic kidney diseases. Hence, increased HIF1 α and ZEB2 expression is not only confined to loss of glomerular function under hypoxic conditions but is also relevant to the pathogenesis of glomerular dysfunction in chronic kidney disease.

This report establishes a mechanism for the effect of chronic hypoxia on glomerular dysfunction. A novel observation of this study is that hypoxia induces HIF1 α , which in turn increases ZEB2 expression in the podocytes, and this action of HIF1 α is mediated, in part, via increased expression of ZEB2-NAT. The HIF1 α -dependent increase in ZEB2 ensures both EMT of podocytes and podocyte foot-processes effacement. ZEB2 is critical for HIF1 α to exert its deleterious effect on glomerular function in hypoxia via changes in the epithelial phenotype and permselectivity of podocytes. We propose that the HIF1-dependent increase in ZEB2 plays a key role in transducing the podocyte injury under hypoxic conditions and that the HIF-ZEB2 axis could be a molecular basis for podocyte loss and proteinuria during hypoxic conditions that prevail during several pathological and extreme physiological conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure Legends:

Figure 1: Chronic hypoxia alters renal function. (A) Outline of animal experiment. Control rats were maintained at normoxia (24hX2 weeks) and experimental rats were exposed to hypoxia (23hX2 weeks). After 2 weeks, we assessed renal function tests and histological parameters. (B) Measurement of glomerular filtration rate (GFR) in rats exposed to hypoxia. n=6; *, p≤0.01. (C) Estimation of Albumin and creatinine levels in rats exposed to hypoxia. The ratio of Albumin/Creatinine is shown in the form of bar diagram in each condition. n=6, *, p≤0.01 (D) Urine protein analysis in rats exposed to hypoxia. Urine samples were subjected to SDS-PAGE and band intensities were visualized by silver staining as described in “materials and methods”. M- molecular weight marker (Bio-Rad#1610374), C- urine from control rat, H-urine from the rat exposed to hypoxia, and BSA was used as a standard. (E) PAS staining of glomerular regions of rats exposed to hypoxia. (F) Glomerulosclerosis index was assessed as described in the methods section and (G) Measurement of glomerular tuft area in rats exposed to hypoxia. Error bars indicate mean ± SE; n=6. *, p≤0.05.

Figure 2: Hypoxia induces podocyte injury. (A) WT1 staining of glomerulus from rats exposed to hypoxia. (B) Quantification of podocyte number in these rats. Error bars indicate mean ± SE; n=6. *, p≤0.05. (C) TEM images of podocytes from rats exposed to hypoxia. These are a representative image out of 12 sections from each condition. Podocyte foot-processes effacement and disruption of slit-diaphragm can be noticed from rats exposed to hypoxia. Significant alterations in the morphology of podocytes occurred in rats exposed to hypoxia are indicated with (*) mark.

Figure 3: Elevated expression of HIF1α in rat glomerular extracts. Expression of HIF1α and its targets in rat glomerular extracts is shown as analyzed by immunoblotting (A) and qRT-PCR (B). Densitometric analysis of western blots (n=3-4) was normalized to β-actin expression and is shown below each blot (A). Error bars (B) indicate mean ± SE; n=4. *, p≤0.05. (C) Immunofluorescence analysis of HIF1α and ZEB2 in the rat glomeruli. Images were captured at 60X using a Leica trinocular microscope.

Figure 4: Elevated expression of EMT markers in rat glomerular extracts. qRT-PCR (A) and immunoblotting (B) showing the expression of ZEB2, ZEB2-NAT and EMT markers. Error bars (A) indicate mean ± SE; n=4. *, p≤0.05. (B) Densitometric analysis of ZEB2 targets normalized for β-actin expression is shown below each blot (n=3-4). (C) Nephroseq (The Regents of The University of Michigan, Ann Arbor) analysis comparing HIF1α, β-catenin, ZEB2, nephrin and podocin expression levels from patients with chronic kidney disease (CKD) vs. control. Expression of HIF1α (2.62 fold), β-catenin (1.70 fold) and ZEB2 (2.7 fold) are increased; nephrin and podocin expression is decreased several-fold among patients with CKD.

Figure 5: Hypoxia induces HIF1α, ZEB2 and EMT markers in human podocytes. Immunoblotting (A) and qRT-PCR (B) analysis of HIF1α, ZEB2, and its targets in human podocytes exposed to hypoxia. (A) Densitometric analysis of protein expression was normalized for β-actin expression and is shown below each blot (n=3-4). (B) The data

presented as mean \pm SE; n=4-6. *, p < 0.05. (C) Immunofluorescence images showing elevated expression and co-localization of HIF1 α and ZEB2 in podocytes exposed to hypoxia. Images were acquired using ZEISS confocal microscope. (D) qRT-PCR analysis of cadherins in HEK293T cells that ectopically express ZEB2, mean \pm SE; n=4-6. *, p \leq 0.05.

Figure 6: Elevated expression of ZEB2 induces enhanced migration of podocytes. Wound healing assay showing the enhanced migration of human podocytes (A) and HEK293T cells (B) exposed to hypoxia for varying periods of time. (C) Cell migration is monitored and presented as % area covered. (D) Western blot analysis of ZEB2 in HEK293T cells overexpressing ZEB2 and quantification of band intensities. (E) Wound healing assay of HEK293T cells (control and ZEB2 overexpressing) and cell migration is monitored and presented as % area covered. The data (C-E) presented as mean \pm SE; n=4-6. *, p \leq 0.05.

Figure 7: Hypoxia induces HIF1 α -ZEB2 axis. (A) Luciferase assay in HEK293T cells transiently transfected with indicated constructs and exposed to hypoxia for 24 h. Renilla luciferase was used as an internal control to normalize transfection efficiency. (B) *Left panel* - Schematic representation of ZEB2 5'UTR showing localization of hypoxia response elements (HREs) and point mutations in HREs. *Right panel* - HEK293T cells were transiently transfected with ZEB2 promoter-luciferase (ZEB2-WT) or ZEB2 promoter-luciferase construct with point mutations in the both HREs (ZEB2-H2M) and exposed to hypoxia for 24 h. Renilla luciferase is used to normalize transfection efficiency. The normalized luciferase activity for each individual construct exposed to normoxia is designated as 1 fold. (C) ChIP analysis with chromatin fractions from HEK293T cells exposed to hypoxia was performed as described. Input DNA and DNA from each of the immunoprecipitated samples were PCR amplified for HRE in both ZEB2 promoter and VEGFA promoter. (D) Western blot analysis of ZEB2 showing the efficiency of ZEB2 shRNA. (E) Western blot analysis showing E-cadherin expression in human podocytes transduced with a lentiviral construct expressing ZEB2 shRNA with and without hypoxia. (F) *Left panel* - Schematic representation of E-cadherin promoter region showing localization of E2-box1 and E2-box3 and point mutations in both E2-boxes. *Right panel* - HEK293T cells were transiently transfected with E-cadherin promoter-luciferase (E-cad-WT) or E-cadherin promoter-luciferase construct with point mutations in the both E2-box1 and 3 (E-cad-E2M) and exposed to hypoxia for 24 h. Renilla luciferase is used to normalize transfection efficiency. The normalized luciferase activity for each individual construct exposed to normoxia is designated as 1 fold. The data (A, B, C and F) presented as mean \pm SE; n=4-6. *, p \leq 0.05.

Figure 8: Activation of HIF1 α -ZEB2 axis alters podocyte permselectivity. (A) A simplified schematic representation of the paracellular permeability assay adopted to assess the filtration barrier function of podocyte monolayer. Podocyte monolayer on collagen-coated transwell filters was allowed to differentiate as described in method section and exposed to hypoxia for 24 h. Albumin was placed in the bottom chamber

and its migration to the upper chamber was determined at respective intervals. (B) Quantification of albumin influx across podocyte monolayer. Albumin influx is determined at 1 and 4 h following the 24 h exposure to hypoxia. Podocytes transduced with a lentiviral construct expressing scrambled RNA were considered as a control. The data presented as mean \pm SE; n=4-6. *, p< 0.05. (C) Proposed model for hypoxia-mediated podocyte injury. Upon exposure to hypoxia, HIF1 α accumulates, which in turn occupies ZEB2 promoter and drives its expression. ZEB2, in turn, suppresses both P- and E-cadherins to induce EMT. Decreased E-cadherin expression is implicated in EMT that results in podocyte dehiscence and loss. These changes in P- and E-cadherin expression will manifest in effacement of podocyte foot-processes and proteinuria in pathological states of overactivity of HIF1 α -ZEB2 axis in the kidney (CL- capillary lumen; US- urinary space; G-glomerular basement membrane; E-endothelium).