The Role of HIF-1α in Transcriptional Regulation of the Proximal Tubular Epithelial Cell Response to Hypoxia*

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Epithelial cells of the kidney represent a primary target for hypoxic injury in ischemic acute renal failure (ARF); however, the underlying transcriptional mechanism(s) remain undefined. In this study, human proximal tubular epithelial cells (HK-2) exposed to hypoxia in vitro demonstrated a non-lethal but dysfunctional phenotype, closely reflective of the epithelial pathobiology of ARF. HK-2 cells exposed to hypoxia demonstrated increased paracellular permeability, decreased proliferation, loss of tight junctional integrity, and significant actin disassembly in the absence of cell death. Microarray analysis of transcriptomic changes underlying this response identified a distinct cohort of 48 genes with a closely shared hypoxia-dependent expression profile. Within this hypoxia-sensitive cluster were genes identified previously as hypoxia-inducible factor-1 (HIF-1)-dependent (e.g. vascular endothelial growth factor and adrenomedullin) as well as genes not previously known to be hypoxia-responsive (e.g. stanniocalcin 2). In hypoxia, HIF-1 bound to evolutionarily conserved hypoxia-response elements (HRE) in the promoters of these genes as well as to the HRE consensus motif. A further subset of these genes, not associated with transcriptional regulation by HIF-1, was also present, suggesting alternative HIF-1-independent pathways. Overexpression of HIF-1α in normoxia induced the expression of a significant number of the hypoxia-dependent genes; however, it did not induce the pathophysiologic epithelial response. In summary, hypoxia-elicted alterations in renal proximal tubular epithelial cells in vitro closely resemble the epithelial pathobiology of ARF. Our data indicate that although this event may rely heavily on HIF-1-dependent gene transcription, it is likely that separate hypoxia-dependent transcriptional regulators also play a role.

Epithelial cells represent a primary target for hypoxia-elicted injury in a diverse array of ischemic conditions (1, 3, 6). In such pathologies, non-lethal epithelial dysfunction is typified by increased paracellular permeability and unregulated movement of molecules between compartments normally separated by the tightly controlled epithelial barrier (2, 4, 6). Hypoxia has been demonstrated to alter the expression of a number of individual genes that contribute to epithelial cell injury (1, 5). Furthermore, hypoxia has also been demonstrated to inhibit cellular proliferation (7). Conversely, pre-exposure of cells to hypoxia/reoxygenation induces genes associated with ischemic pre-conditioning, which protects cells against subsequent ischemic insult (8). The global transcriptional mechanism(s) underlying these events are not fully understood. Here we have taken an unbiased approach using oligonucleotide microarrays to define the transcriptomic response induced by hypoxia in an epithelial model of non-lethal proximal tubular cell dysfunction.

Transcriptional responses to hypoxia can be adaptive or inflammatory (1). Adaptive responses are controlled primarily through the nuclear accumulation of the heterodimeric hypoxia-inducible factor (HIF-1), which is composed of α and β subunits. In hypoxia, decreased oxygen-dependent HIF-1α proline hydroxylation at Pro-402 and Pro-564 (and subsequent ubiquitination/degradation) leads to HIF-1α stabilization (9, 10). HIF-1α dimerizes with HIF-1β to form HIF-1. Accumulated HIF-1α translocates to the nucleus where abrogation of oxygen-dependent asparagine (Asp-803) hydroxylation of HIF-1α leads to HIF-1α transactivation (11). HIF-1 binds to the hypoxia-response element (HRE) and regulates the expression of a number of adaptive genes coding for angiogenic and glycolytic and other proteins (e.g. erythropoietin and inducible nitric-oxide synthase), which support tissue survival in hypoxia (12).

A distinct tissue specific inflammatory response has been described in intestinal epithelial cells in response to hypoxia which occurs temporally downstream of the adaptive response. Such events are mediated through the activation of NF-κB and co-incidental degradation of CREB (5, 13). These events result in the induction of pro-inflammatory genes, such as tumor necrosis factor-α, which may contribute to ongoing disease processes (2). A common event in the regulation of hypoxia-sensitive transcriptional regulators is the targeted degradation of proteins through the ubiquitin/proteasomal pathway (13, 14).

In our study, the hypoxic renal proximal tubular epithelial phenotype in vitro closely resembled the epithelial pathobiology of ARF. Furthermore, although the vast majority of genes remained unaltered, a specific cluster of 48 genes with a closely shared expression profile was induced in hypoxia. These hypoxia-responsive genes could be separated into HIF-1-dependent and -independent groups. HIF-1 bound to HRE motifs in the promoters of a number of these genes in hypoxia. In

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The abbreviations used are: HIF-1, hypoxia-inducible factor-1; ARF, acute renal failure; VEGF, vascular endothelial growth factor; HRE, hypoxia-response elements; CREB, cyclic AMP-response element-binding protein; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assays.

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normoxia, overexpression of HIF-1α was sufficient to drive transcriptional events; however, it did not mimic the pathophysiological phenotype implicating a role for coincidental HIF-1-independent mechanisms in the induction of a pathophysiological phenotype in proximal tubular epithelial cells in response to hypoxia.

MATERIALS AND METHODS

Cell Culture—Human proximal tubular epithelial cells (HK-2; American Tissue Type Culture Collection) (15) were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 4 µg/ml triiodo-L-thyronine, 10 ng/ml epidermal growth factor, 50 units/ml penicillin/streptomycin, and 10% (v/v) fetal calf serum.

In brief, cells were cultured on polycarbonate-permeable inserts (m pore size, 6.5-mm diameter; Costar Corp., Cambridge, MA). Epithelial paracellular permeability was assessed as described previously (16). Briefly, for analysis of F-actin arrangement, cells were fixed with 4% paraformaldehyde for 24 h at 4 °C, post-fixed in 1% (w/v) osmium tetroxide in phosphate-buffered saline, and dehydrated through ascending grades of ethanol, for 24 h at 4 °C, then embedded in EPON resin at 60 °C overnight.

Following treatment, apical to basolateral permeability of a FITC-dextran flux was calculated by linear regression using GraphPad Prism 2.01 software.

Proliferation Assay—Cell proliferation was assessed by measurement of [3H]thymidine uptake. In brief, cells were incubated in medium containing 0.25 µCi/ml [3H]thymidine (PerkinElmer Life Sciences) for 4 h at 37 °C. Cells were washed 4 times in ice-cold phosphate-buffered saline and solubilized overnight with 2% SDS, and radioactivity was determined by liquid scintillation counting (Beckman Instruments). Results are expressed as disintegrations/min as a percentage of control.

Morphologic Analysis—Fluorescence staining was carried out as described previously (16). Briefly, for analysis of F-actin arrangement, cells were stained using FITC-conjugated phalloidin (1:40 dilution; Molecular Probes, Leiden, The Netherlands). Staining was visualized using a Zeiss Axiosvert 200 inverted fluorescence microscope. For light and electron microscopy, cells were fixed in 2.5% (v/v) glutaraldehyde for 24 h at 4 °C, post-fixed in 1% (v/v) osmium tetroxide in phosphate-buffered saline, and dehydrated through ascending grades of ethanol, 70, 90, and 100%. Cell preparations were then treated with propylene oxide for 30 min before embedding in EPON resin. Ultrathin sections (70 nm) were collected on diamond knives, stained with uranyl acetate and lead citrate and examined using a JEOL 200FX transmission electron microscope.

Transepithelial Dextran Paracellular Permeability Assay—Transepithelial paracellular permeability was assessed as described previously (17). In brief, cells were cultured on polycarbonate-permeable inserts (3-µm pore size, 6.5-mm diameter; Costar Corp., Cambridge, MA). Following treatment, apical to basolateral permeability of a FITC-labeled dextran (70 kDa; 25 mg/ml; Molecular Probes, Leiden, Netherlands) was measured as basolateral fluorescence at intervals up to 60 min using a Citifluor multwell fluorescence plate reader. Paracellular dextran flux was calculated by linear regression using GraphPad Prism 2.01 software.

Cell Viability Assays—To evaluate necrosis, lactate dehydrogenase (LDH) activity was measured in cell culture medium and cell lysates using a quantitative biochemical assay according to the manufacturer’s instructions (Roche Diagnostics). Necrosis was assessed as LDH activity released into the medium as a percentage of total LDH (cellular + extracellular).

Apoptotic cells were detected by staining for externalized phosphatidylserine using FITC-conjugated annexin V (Roche Diagnostics). Distinction from necrotic cells was determined by staining for propidium iodide. Briefly, cells were trypsinized and incubated with annexin V-FITC and propidium iodide for 30 min at 4 °C and analyzed for fluorescence staining using a Coulter EPIC elite flow cytometer.

Gene Microarray and Bioinformatic Analysis—RNA isolation, cDNA synthesis, in vitro transcription, and microarray analysis were carried out as described previously (13, 18). Briefly, total RNA was isolated using phenol/chloroform extraction and subsequently purified through an RNeasy Mini Column (Qiagen, Valencia, CA); cDNA was synthesized from total RNA using Superscript Choice kit (Invitrogen). Biotin-labeled cRNA prepared from template cDNAs was fragmented and hybridized to HG U95A arrays (Affymetrix, Santa Clara, CA). Arrays were then washed and fluorescently labeled before being scanned using a confocal scanner. Microarray Suite 5.0 software (Affymetrix) was used to test for the abundance of each gene from the average difference of fluorescent intensities. Calculated signal/log ratios for each gene at all time points of hypoxia were compared with normoxia, and those genes with a value greater than 1 or less than −1 (2-fold difference) for at least 1 time point were selected for further cluster analysis. Signal/log ratio data for selected genes were normalized and subjected to k-means clustering (k = 60) using ClusterTM and TreeviewTM software (19) (www.rana.lbl.gov). Approximately 5000 bp of sequence upstream from the NCBI predicted mRNA start site was selected for further analysis for common TF binding motifs between species. These sequences were then aligned using Clustal X1.81 (20) (www.matfys.kvl.dk/bioinformatik/exercise6.html) before analysis for potential transcription factor regulatory binding site matrices using MatInspectorTM software (21) (www.genomatix.de).

Reverse Transcription PCR—Confirmation of selected gene expression was assessed by RT-PCR from cDNA prepared from total cell RNA using random primers as described previously (5). Primers (obtained from Sigma-Genosys) for selected genes are listed as follows: VEGF, CGCAGAATCTATCAGGAAT and AGAAGCAGGTCTGGTAGAT; AK3, AGGGGAGGGGTTGTCTCTCTTAAT and ATGTTCTCCCGCAAGAAGTG- TG; PPP1R3C, AGGCAACATGGACAGGAGCT and AGGAGACAGCTTGGAATGG; AM, GCCGCAAGCATCTACTATTAC and CCCGGCAAGACTTACACTCTC; STC2, AGGAAGAGTGGGAAAGGAGGA and GTAAGGCGCATGCATTACA; GLUT III, CAGGGTTGTGATATGGTCCC and CCAAGAAGGAGAGAGAGAGAGGA; p57 (Kip2), TTCCGCGTCTCC-TTCCTC and TGGCCATCTTGTCGCCCTAGCT; MUC1, ATCCTGATGCTTGGTCTGT and ACCCTGAGTGTGAGTTCGAC and AGGAGAGGGAAGGCGCT; p27 (KIF1), ACCCCATTGTACCTGGCCAC and ATCGGATTCTTGGCTTCCAC; CYCLIN G2, CTCGGCAAGCATGTATCTGTTT and CCGCTG- CTGGAGAGGTTG; GADD153, TGAAGACGCTTGTAGGAG and CCAATGTGTCTGCTTGGTG; glucocorticoid receptor (NR3C1), CCT- AAGAGCTCTTGAGAGGC and GCCAAGCTTGCCCTCTAT; HIF-1α, CCTCAAGTGCACTTCTCA and CCGCTGAGTAGCTCTCTG- CT; and 18S, GTGAAGAGGATTTGCTGCTGT and CGGCTGAGGCT- GATAGTAG, cDNA was amplified by an initial incubation at 94 °C for 3 min followed by 25–35 cycles of 94 °C for 30 s, 55–58 °C for 45 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were separated by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining.

HIF-1a cDNA Binding—Specific to HRE motifs from various genes was determined by electrophoretic mobility shift assay. Briefly, radiolabeled double-stranded DNA oligonucleotides containing evolutionarily conserved HRE sequences for VEGF (5'-TGCATACGTGGGTTCCACAGAGG-3'), adrenomedullin (5'-GACAAACACGGTGCTCAGAG-3'), GADD153 (5'-GGAAACAGTGAGCGTGCACACGT-3'), and MUC1 (5'-CATCAGAGGTTCTCAGCTGAGG-3') were incubated with lysates from normoxic and hypoxic HK-2 cells. DNA-protein interactions were documented by EMSA as described previously (17). To investigate HIF-1 binding to the consensus HRE sequence, we utilized the TransAM HIF-1 kit according to manufacturer’s instructions (Active Motif).

HIF-1α Overexpression—Constructs expressing HIF-1α (wild type and double mutant) were a gift from Thilo Hagen, UCL, London, UK. HK-2 cells stably transfected with empty vector, as well as 5 clones resistant to 600 µg/ml G418 were used. All clones were analyzed for HIF-1α protein levels using Western blots and as a positive control were exposed to 1% CO2. Clones with the most HIF-1α were selected and termed p57Δ (wild type) and p57Δa (double mutant). Transfected cells were stressed in hypoxia and returned to control levels upon reoxy- genation (n = 3; p < 0.01: Fig. 1A) as determined by [3H]thymidine uptake assay. The impact of hypoxia on cellular morphology was investigated by light, fluorescence, and electron microscopy (Fig. 1B). Hypoxia elicited an increase in intercellular spacing (top panels), which was associated with a disruption of the actin cytoskeleton (middle panels). Electron microscopy (lower panels) further revealed dramatically decreased numbers of intercellular junctions (arrowheads) be-
tween adjacent cells. Although cells remained viable (non-necrotic and non-apoptotic) throughout hypoxic exposure (0–48 h; Fig. 1C), paracellular permeability to a 70-kDa FITC-labeled dextran molecule was significantly increased by 36 h (n = 4; p < 0.05; Fig. 1C). This non-lethal dysfunction accurately reflects the proximal tubular epithelial pathobiology of acute renal failure.

Global Gene Expression in Hypoxia—We investigated the transcriptomic response, which underlies the induction of the hypoxia-elicited phenotype in HK-2 cells. The Affymetrix human U95A microarray system was utilized to profile changes in global gene expression in hypoxia (18). This approach allows analysis of expression of 12,558 gene transcripts simultaneously. Analysis of pooled mRNA from independent experiments (n = 4) exposed to increasing periods of hypoxia (with or without reoxygenation) revealed expression of 7693 (61.1%) genes in at least one time point investigated (Affymetrix suite 5.0 analysis software). Approximately 12.5% (1571) of analyzed genes were differentially regulated in hypoxia or hypoxia/reoxygenation, using a cut-off of 2-fold change (signal/log ratio >1 or < −1) in expression between any two exposures. Following normalization of expression values, k-means clustering allowed distinct cohorts of genes with shared expression profiles in a time course of hypoxia and reoxygenation to be identified (Fig. 2A). Such cohorts include genes that are up- (red) or down-regulated (green) in hypoxia. Within this cohort, we identified a cluster of genes with a tightly shared hypoxia-dependent expression profile (outlined in yellow in Fig. 2A). Comparison of all genes expressed in a temporal manner in hypoxia reveals that the numbers of both up- and down-regulated gene numbers increase quantitatively with time (Fig. 2B) indicating that a number of the late genes may be activated secondarily to early gene expression.

Hypoxia-dependent Gene Expression—Prominent among the total cohort of hypoxia-induced genes was a tightly regulated cluster of 48 genes that demonstrated time-dependent up-regulation in response to hypoxia and a distinct down-regulation upon reoxygenation. Importantly, these genes were up-regulated at early time points of hypoxia indicating that they were within the early wave of hypoxia-responsive genes rather than being secondarily induced. Genes within this cluster were separated according to proposed functionality, and an exhaustive review of the literature was carried out to determine whether individual genes within the cluster had been associated previously with hypoxia. Furthermore, the role of HIF-1α in the regulation of these genes was reviewed. Table I demonstrates that a significant number of hypoxia-dependent genes had previously documented HIF-1α dependence (35%). These include well defined HIF-1α-dependent genes such as vascular endothelial growth factor (VEGF), adenylate kinase 3 (AK3), and adrenomedullin (30, 37, 41). The detection of increased expression of these genes indicates the success of this experiment in identifying a physiologically relevant cluster of hypoxia-responsive genes. Notably, for a number of hypoxia-dependent genes (e.g., VEGF, MXI-1, and KIP-2), two individual identifiers were present within this cluster (data not shown). Because

Fig. 1. Hypoxia-induced non-lethal renal epithelial dysfunction. A, [3H]thymidine uptake was used as an indicator of cell proliferation in HK-2 cells. Hypoxia (1% O2; 36 h) inhibited cellular proliferation (n = 3; p < 0.01) in a manner that was reversed upon reoxygenation. B, the impact of hypoxia on cell morphology was investigated by toluidine blue staining (top panels), F-actin staining (middle panels), and electron microscopy (lower panels). Hypoxia elicited increased intercellular spacing, F-actin disassembly, and decreased cell-cell junctions (arrowheads). C, transepithelial flux of 70-kDa FITC-labeled dextran was used to measure epithelial permeability in response to hypoxia in HK-2 cells. Hypoxia significantly increased paracellular permeability (n = 4; p < 0.05; blue) in a manner that did not involve necrosis (green) or apoptosis (red) measured by LDH release and annexin V staining, respectively.
We identified a subset of genes of physiologic interest from this cluster and confirmed their hypoxic expression patterns by RT-PCR analysis. These genes were separated into groups previously documented to be HIF-1α-dependent and those not previously documented to be HIF-1α-dependent (Fig. 3A). Consistently, we were able to confirm hypoxia-dependent expression of these genes at the mRNA level by RT-PCR analysis (Fig. 3B).

**Evolutionary Conservation of Putative Regulatory Elements within the Promoters of Hypoxia-responsive Genes**—By having identified a cohort of hypoxia-dependent genes in renal proximal tubular epithelial cells, we separated this cluster into two sets of genes either previously demonstrated to be HIF-1-dependent or not previously associated with HIF-1. Independent of HIF-1, a number of other transcription factors including the cyclic AMP-response element-binding protein (CREB) and NF-κB have been demonstrated to control gene transcription in hypoxia. In order to identify transcriptional regulators involved in directing hypoxic gene expression in our model, we analyzed putative promoter genomic sequences from genes within the hypoxia-sensitive cluster for evolutionarily conserved HIF-1, CREB, and NF-κB response elements between species for which the relevant promoter sequences are available. Evolutionary conservation of these motifs in the same spatial orientation in the regulatory regions of genes strongly implicates a functional role in gene regulation. We obtained ~5000 bp of genomic sequence upstream from the NCBI predicted mRNA start site of each gene. In order to account for slight variation in orientation of common regulatory sequences between species, we used Clustal X1.81 software to align sequences for each gene. Sequences were then scanned for known HIF-1, CREB, and NF-κB response elements using MatInspector software. Response elements in the promoters of genes identified within the hypoxia-responsive cluster are depicted in Fig. 4 (conserved response elements are indicated with an arrowhead).

HRE consensus motifs were widely expressed in the promoters of hypoxia-dependent genes. Furthermore, evolutionarily conserved HRE motifs were identified in five of eight of the genes previously demonstrated to be HIF-1α-dependent. Interestingly, 50% of the genes not previously associated with HIF-1-dependent regulation contained conserved HREs (e.g. stanniocalcin 2). Five of the remaining seven genes lacking conserved HRE motifs contained conserved cyclic AMP-response elements, further implicating a role for CREB in HIF-1α-independent transcriptional responses to hypoxia.

**HIF-1 Binding to the Hypoxia-response Element**—We investigated the biological relevance of the high frequency of occurrence of conserved HRE sequences in the promoters of genes in the hypoxia-responsive cluster by determining HIF-1 binding to these sequences. To do this we used EMSA to examine nuclear protein binding to conserved HRE sequences in a selection of genes from the hypoxia-responsive cohort. Importantly, this selection included genes previously known to be HIF-1-dependent (e.g. VEGF) as well as those not previously identified as HIF-1-dependent (stanniocalcin 2). As demonstrated in Fig. 5A, nuclear protein bound to the conserved HRE motifs in VEGF, adrenomedullin, RTP801, and stanniocalcin 2 in a hypoxia-dependent manner.

In order to confirm that HIF-1 was binding to the HRE consensus motif in HK2 cells exposed to increasing periods of hypoxia, we used a quantitative approach to measuring HIF-1-HRE interactions which utilizes an enzyme-linked immunosorbent assay-based approach. In Fig. 5B, we demonstrate that HIF-1-HRE binding increases significantly in a time-dependent manner in HK-2 cells in hypoxia (Fig. 5B).
**HIF-1α-dependent Gene Expression**—By having demonstrated the transcriptional up-regulation of HIF-1α-dependent and -independent cohorts in hypoxia, we became interested in the functional relevance of each of these pathways in the hypoxia-elicited phenotype. To do this we obtained an expression plasmid, which allowed us to express high levels of HIF-1α in normoxia, presumably through the saturation of HIF-1α degradative pathway. Successful overexpression of wild type HIF-1α and HIF-1α mutated at Pro-564 and Pro-402 (the oxygen-dependent degradation residues) was detectable by PCR (Fig.

| Accession No. | 8hrs | 16hrs | 36hrs | Reox | Hypoxia | Transcription factor (Ref) |
|---------------|------|-------|-------|------|---------|---------------------------|
| DNA synthesis, Cell Cycle | D64137 | CDKN1C p57kip2 | 1.4 | 0.2 | 1.8 | 0.6 | Yes | HIF-1α (24) |
| | U47414 | CCNG2 Cyclin G2 | 1.2 | 1.1 | 1.4 | 0.3 | Yes | HIF-1α (25) |
| | U19906 | CDKN1B p27kip1 | 1.1 | 1.2 | 2.6 | -0.2 | Yes | HIF-1α (7) |
| | S62138 | DDX11 GADD45α | 1.1 | 0.7 | 1.8 | 0.8 | Yes | N/D (26) |
| Transcriptional Factor | M10901 | NR3C1 Glucocorticoid Receptor, alpha | 2.9 | 3.3 | 3.5 | 2.1 | Yes | N/D (27) |
| | L07648 | MXI1 | 2.6 | 2.2 | 3.1 | 0.7 | N/D | |
| | X16706 | FOSL2 FOS-like antigen 2 | 2 | 1.6 | 2.5 | 1.1 | N/D | |
| | ABO04066 | BHLHB2 Dec 1 | 2 | 1.6 | 1.7 | -0.3 | N/D | |
| | AB018285 | TSGA Zinc finger protein | 1.9 | 1.5 | 1.5 | 0.3 | N/D | |
| | D15050 | TCF8 Transcription factor 8 | 1.8 | 1.5 | 1.9 | 0.2 | N/D | |
| | X64518 | NFL3 Nuclear factor, interleukin 3 | 1.6 | 1.5 | 2.1 | 1.6 | N/D | |
| | AF055993 | SAP30 | 1.4 | 1.5 | 1.2 | -0.5 | N/D | |
| | M25679 | HOXA5 Homeobox A5 | 1.2 | 1.1 | 1.4 | 0 | N/D | |
| | X51345 | JUNB | 1.2 | 0.5 | 1.1 | 0.8 | Yes | N/D (28) |
| | M74297 | HOXA4 Homeobox A4 | 1 | 0.6 | 1.3 | -0.2 | N/D | |
| Growth Factor | M12783 | PDGFB | 3.3 | 3 | 3.8 | -0.2 | Yes | VHL (29) |
| | AF022275 | VEGF | 2.3 | 1.8 | 2.3 | 0.9 | Yes | HIF-1α (30) |
| | X70340 | TGFβ | 1.1 | 1.2 | 0.6 | -0.4 | Yes | N/D (31) |
| Apoptosis | AF002697 | BNIP3 | 1.9 | 2.3 | 2.6 | 0.9 | Yes | HIF-1α (32) |
| | AA522530 | RTP801 HIF-1 responsive RTP801 | 1.6 | 1.4 | 1.6 | 0.3 | Yes | HIF-1α (33) |
| Phosphatase | N36638 | PPP1R3C | 3.6 | 3.2 | 3.6 | 0.8 | N/D | |
| | X68277 | DUSP1 PI3-kinase | 1.4 | 1 | 1.6 | 0.7 | Yes | N/D (34) |
| Transporter, Channel | M20681 | SLC2A3 GLUT3 | 3 | 2.5 | 2.9 | 1.1 | Yes | HIF-1α (35) |
| | M80244 | SLC7A5 | 1.3 | 1.4 | 1.8 | 1 | N/D | |
| | U81800 | MCT3 | 1.2 | 2.4 | 2.2 | 1.3 | N/D | |
| | K03195 | SLC2A1 GLUT1 | 1.2 | 0.8 | 1.6 | 0.7 | Yes | HIF-1α (36) |
| Signal Transduction System, Receptor | X68487 | ADORA2B Adenosine A2b receptor | 3 | 3.1 | 3.1 | 0.6 | N/D | |
| | A1950382 | PSR Phosphatidylserine receptor | 2.5 | 2.2 | 2.1 | 0.4 | N/D | |
| | M57730 | EFNA1 Ephrin-A1 | 1.3 | 1.3 | 1 | 0.7 | N/D | |
| | AF081195 | RGSP1 RAS guanyl releasing protein | 3.2 | 3.3 | 3.8 | 0.8 | Yes | HIF-1α (37) |
| Vasodilation | D14874 | AM Adrenomedullin | 4.1 | 3.7 | 5.3 | 0.6 | Yes | HIF-1α (38) |
| Collagen Synthesis | M24486 | P4H1 proline 4-hydroxylase | 1.4 | 1.7 | 2.7 | 2.4 | Yes | N/D (38) |
| | U90441 | P4H2 proline 4-hydroxylase | 0.8 | 1.8 | 2.4 | 2.3 | N/D | |
| Cell Adhesion | J05822 | MUC1 Mucin 1 | 0.5 | 1.4 | 1.8 | 1.6 | N/D | |
| Metabolism | D49818 | PFKB3 Phosphofructokinase | 3.1 | 2.3 | 3.1 | 2.2 | Yes | HIF-1α (39) |
| | Z46376 | HK2 Hexokinase 2 | 1.9 | 1.1 | 2.4 | 0.5 | Yes | HIF-1α (40) |
| | X60673 | AK3 Adenylate kinase 3 | 1.4 | 1.4 | 1.2 | 0.5 | Yes | HIF-1α (41) |
| | S81916 | PGK1 Phosphoglycerate kinase 1 | 1.2 | 1.8 | 2.8 | 2.8 | Yes | HIF-1α (42) |
| | D25228 | PFKP Phosphofructokinase, platelet | 1.1 | 1.4 | 1.8 | 2.3 | Yes | HIF-1α (42) |
| | X50236 | ALDOA Aldolase A | 0.3 | 0.7 | 1 | 0.9 | Yes | HIF-1α (42) |
| Soluble Factors/Mediators | AF098462 | STC2 Stanniocalcin 2 | 3.1 | 2.6 | 3.2 | 1.5 | N/D | |
| | AB022718 | DEPP | 2.9 | 2.2 | 3 | 0.3 | N/D | |
| | X97324 | ADGP Adipophilin | 2.2 | 2 | 2.5 | 0.5 | Yes | N/D (43) |
| | M14083 | Serpine1 PAI1 | 1 | 1.4 | 2 | 0.9 | Yes | HIF-1α (44) |
| | J02931 | F3 Tissue Factor | 1.5 | 0.7 | 1 | 0.2 | Yes | EGR1 (45) |
| Others | X79536 | HNRPA1 | 1.3 | 0.9 | 1.9 | 0.6 | N/D | |
| | U98278 | EDR2 Early development regulator 2 | 1.2 | 1 | 1.6 | 0.1 | N/D | |
| Unknowns | AA156240 ? | 1.8 | 1.5 | 2.1 | 1.2 | N/D | |
A dose-dependent increase in HIF-1-HRE binding in cells overexpressing both the wild type and mutant HIF-1α was initially demonstrated (Fig. 6B). Furthermore, overexpression of both wild type and mutant HIF-1 in normoxia was sufficient to drive transcription of HIF-1-dependent genes such as VEGF and adrenomedullin (Fig. 6C). Interestingly, a number of hypoxia-responsive genes not previously documented to be HIF-1α-dependent were up-regulated upon HIF-1α overexpression. These genes included stanniocalcin-2 as well as DDIT-3 and PPP1R3C, all of which display evolutionarily conserved HRE motifs in their promoter regions indicating that these genes represent previously unidentified targets for HIF-1-dependent up-regulation in hypoxia. A number of hypoxia-dependent genes, not up-regulated with HIF-1α overexpression (e.g. KIP-2) contained conserved CRE motifs within their promoter regions further indicating a role for CREB in transcriptional responses to hypoxia.

To investigate the relative role for HIF-1 in the induction of the hypoxic phenotype, we measured the impact of HIF-1α overexpression on cellular proliferation in normoxia. Overexpression of HIF-1α in HeLa cells was not sufficient to mimic the decrease in cell proliferation observed in hypoxia (Fig. 6D). These data indicate that whereas HIF-1α is central to the transcriptional hypoxic response, its activation alone is unlikely to fully account for all the phenotypic features of the cellular response to hypoxia and furthermore that alternative hypoxia-sensitive transcriptional regulators such as CREB have a role to play.

**DISCUSSION**

The kidney is particularly susceptible to acute ischemic insult leading to cellular hypoxia in a number of pathophysiologic states. Proximal tubular epithelial dysfunction in the absence of cell death is a primary pathologic hallmark of ischemic acute renal failure (4). This is typified by altered epithelial proliferative responses in the proximal tubule (6). Furthermore, increased paracellular permeability leading to fluid back leak and a reduction in the glomerular filtration rate is also a feature (4). Increased inter-epithelial spacing, cytoskeletal disruption, and decreased tight junctional integrity underlie such alterations in permeability. The molecular events underlying this pathologic response remain unknown. In this study, we used a reductionist model to investigate the global transcriptional mechanisms underlying the epithelial transformation to the hypoxic phenotype. We exposed HK-2 human proximal tubular epithelial cells to increasing periods of hypoxia and determined the impact on cell phenotype and genotype. Hypoxia did not alter cell viability (apoptosis or necrosis). However, proliferation was decreased, and transcellular permeability was significantly increased. The observed decrease in proliferation is consistent with previous studies in hypoxia (7). Morphologic analysis revealed the loss of tight junctional integrity in hypoxia. The observed increase in proximal tubular epithelial permeability in hypoxia is in contrast to colonic epithelial monolayers, which demonstrate a resistance to hypoxia-induced increases in permeability (2). Likely this is due to the organ-specific induction of barrier protective factors such as intestinal trefoil factor and CD73 in the colon which compose part of the organ-specific adaptive response to hypoxia (17, 46). Thus, exposure of proximal tubular epithelial cells to hypoxia induces an epithelial phenotype closely reflective of that seen in ARF.

We next investigated the global gene expression profile that underlies the transformation of HK-2 cells to the hypoxic phenotype. Although the vast majority of genes (87.5%) expressed remained unaltered in hypoxia or reoxygenation, 12.5% demonstrated differential expression for at least one time point.
These data compare favorably with other microarray studies where 2–12% of all genes investigated were altered in various disease models (21–23). Within this subset existed a specific cohort of 48 genes with a closely shared expression profile, which were induced at all time points of hypoxia and repressed upon reoxygenation. We thus considered this cohort to be hypoxia-responsive.

Within the cohort of hypoxia-responsive genes, a significant number have been described previously to be induced in hypoxia in a HIF-1-dependent manner. These genes included VEGF and adrenomedullin, prototypic HIF-1-dependent genes. The expression of such classically defined hypoxia-specific genes in a relatively small cohort (48 of 12,258 genes investigated) indicates that the microarray experiment represents a physiologically relevant gene expression profile.

A number of the hypoxia-responsive genes expressed may have roles to play in mediating the induction of the hypoxic phenotype. For example, decreased proliferation may be associated with increased expression of the cyclin-dependent kinase inhibitors KIP-1 and KIP-2. Furthermore, down-regulated genes in hypoxia included proliferating cell nuclear antigen and thymidine kinase, present normally in proliferating cells (data not shown). Interestingly, two isoforms of prolyl 4-hydroxylase, P4HA1 and P4HA2, were up-regulated in hypoxia. P4HA1 has been demonstrated as the enzyme responsible for hydroxylation of proline 564 of HIF-1α (9, 10), and therefore up-regulation of these enzymes in hypoxia indicates a self-regulatory role in hypoxia signaling.

HIF-1 is a master regulator of cellular transcriptional responses to hypoxia. This is demonstrated by the high number of HIF-1-induced genes present in the hypoxia-inducible cohort. However, a significant number of genes with no previous association with HIF-1α were also up-regulated indicating that either previously unidentified functional HREs exist in the promoters of these genes or alternatively other HIF-1-independent pathways exist. Moreover, growing evidence indicates that the specific regulation of promoter-dependent gene expression depends on the spatial organization of modules of responsive elements rather than single binding sites (47). For these reasons, we became interested in the relative role of HIF-1α in the induction of the hypoxic phenotype in renal proximal tubular epithelial cells. To investigate this, we overexpressed HIF-1α in normoxia. HIF-1α overexpression was sufficient to result in the expression of HIF-1-dependent genes. This was
surprising, as previous work has demonstrated a hypoxia-de-
pendent transactivation of HIF-1α necessary for transcrip-
tional activity. However, work from other groups has demon-
strated that HIF-1α overexpression itself is sufficient to drive
gene transcription (48). Furthermore, HIF-1-dependent tran-
scriptional activity occurs in response to a number of inflam-
matory stimuli in the absence of hypoxia (49, 50).

Some of the hypoxia-sensitive genes, which had not been
demonstrated previously to be HIF-1α-dependent, were induced
with HIF-1α overexpression. An example of such a gene is
stanniocalcin 2, a regulator of intracellular calcium gradients.
Promoter analysis of stanniocalcin 2 revealed the presence of
an HRE consensus motif. Further promoter alignment studies
revealed evolutionary conservation of this motif between
mouse, rat, and human genes. This strongly supports a physi-
ologic role for this motif in the hypoxia responsiveness of the
stanniocalcin 2 gene. The biological relevance of this putative
HRE was confirmed by EMSA demonstrating a hypoxia-de-
pendent binding of HIF-1α to this sequence in a time-dependent
manner. Similar evolutionarily conserved HRE sequences are
present within the promoter regions of DDIT3 and PPP1R3C
(Fig. 4). Conversely, with overexpression of HIF-1α we did not
observe alterations in some genes including AK3 and cyclin G2,
previously defined as HIF-1-dependent in hypoxia. This indi-
cates that HIF-1α may be necessary but not sufficient to drive
transcription of these genes.

Analysis of the promoter regions from hypoxia-responsive
genes revealed the presence of conserved HIF-1, CREB, and
NF-κB sequences between genes indicating a likely functional
role in regulating gene expression in hypoxia. Interestingly,
conserved HIF-1-binding motifs identified in VEGF, ad-
renomedullin, and RTP801 sequences are the exact binding
sites demonstrated previously to direct expression of these
genes in hypoxia (30, 33, 37). This demonstrates the accuracy
of this method for recognition of functional specific response ele-
ments and implicates a functional role for conserved sequences
for HIF-1α observed in STC2, PPP1R3C, and DDIT3. Con-
served CREB and NF-κB binding motifs observed in other
genes may also have a functional role.

Using the cell proliferative response as a model, we investi-
gated the impact of HIF-1α overexpression on the cellular
phenotype. HIF-1α overexpression alone in the absence of hy-
oxia was not sufficient to drive a decrease in the proliferative
rate. These data indicate that although HIF-1α is clearly im-
portant in the induction of significant gene expression in re-
sponse to hypoxia, other independent pathways likely contrib-
ute to the overall hypoxic response. Our group and others have
demonstrated that a number of transcriptional regulators dem-
strate hypoxic inducibility. Such factors include but are not
restricted to CREB and NF-κB. Interestingly, CREB has been
demonstrated previously to interact with HIF-1 at the level of
the promoter (51). Consequently, we hypothesize that the prox-
imal tubular epithelial response to hypoxia, a primary event in the pathophysiology of ARF, is mediated through HIF-1-dependent and -independent transcriptional processes. A greater understanding of the transcriptional regulators involved and how they interact will lead to the development of potential therapeutic targets in ARF.

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