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Epac1-Mediated, High Glucose–Induced Renal Proximal Tubular Cells Hypertrophy via the Akt/p21 Pathway

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The mechanisms involved in tubular hypertrophy in diabetic nephropathy are unclear. We investigated the role of exchange protein activated by cAMP 1 (Epac1), which activates Rap-family G proteins in cellular hypertrophy. Epac1 is expressed in heart, renal tubules, and in the HK-2 cell line. In diabetic mice, increased Epac1 expression was observed, and under high glucose ambience (HGA), HK-2 cells also exhibited increased Epac1 expression. We isolated a 1614-bp DNA fragment upstream of the initiation codon of *Epac1* gene, inclusive of glucose response elements (GREs). HK-2 or COS7 cells transfected with the Epac1 promoter revealed a dose-dependent increase in its activity under HGA. Mutations in GRE motifs resulted in decreased promoter activity. HK-2 cells exhibited a hypertrophic response and increased protein synthesis under HGA, which was reduced by Epac1-siRNA or -mutants, whereas the use of a protein kinase A inhibitor had minimal effect. Epac1 transfection led to cellular hypertrophy and increased protein synthesis, which was accentuated by HGA. HGA increased the proportion of cells in the G0/G1 cell-cycle phase, and the expression of pAkt and the cyclin-dependent kinase inhibitors p21 and p27 was increased while the activity of cyclin-dependent kinase 4 decreased. These effects were reversed following transfection of cells with Epac1-siRNA or -mutants. These data suggest that HGA increases GRE-dependent Epac1 transcription, leading to cell cycle arrest and instigation of cellular hypertrophy. (*Am J Pathol* 2011, 179:1706–1718; DOI: 10.1016/j.ajpath.2011.06.035)

Exchange protein directly activated by cAMP (Epac1) is a novel cAMP-activated guanine nucleotide exchange factor (GEF) for Ras-like GTPases, such as Rap1,^{1,2}

which cycle between an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state. GEFs, such as Epac1, catalyze the exchange of GDP for the more abundant GTP, and thus activate Rap1-GTP binding protein.³ The Rap1 regulates diverse pivotal cellular processes, including cell survival, proliferation, differentiation, hypertrophy, intracellular vesicular trafficking, cytoskeletal rearrangement, cell cycle events, and glucose transport.^{1–4} Although Epac1 participates in gene transcription, insulin secretion, and ion transport,¹ more recent studies have suggested Epac proteins may regulate the development of cardiac hypertrophy.⁵ Although, a related cAMP–protein kinase A (PKA) pathway modulates a number of different physiological and pathological processes, including regulation of a cell cycle, ion transport, cellular proliferation, and extracellular matrix expression in normal kidney and in various chronic kidney diseases,^{6,7} the role of Epac1 in renal pathophysiology has been delineated to a limited extent, regulating intracellular Ca²⁺ mobilization and apical exocytotic insertion of AQP2 in inner medullary collecting ducts (IMCD).⁸ However, there is no available literature report describing the role of Epac1 in the progression of diabetic nephropathy.

Diabetic nephropathy is now recognized as the most common cause of end-stage renal disease and accounts for 30% to 40% of all patients requiring renal replacement therapy, and hyperglycemia is implicated as a major factor in its pathogenesis.⁹ A number of pathophysiologic mechanisms linking hyperglycemia to the development of nephropathy have been proposed and defined regarding glomerular pathobiology.^{10–15} The well-known characteristic structural features of renal pathology include glomerular hypertrophy, mesangial cell proliferation, podocytes loss, glomerular basement membrane thickening, and amassing of extracellular matrix in the mesangium.^{9,16} Recent studies over the last decade have

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also linked hyperglycemia to the pathobiology of the tubulointerstitium, and injury to the latter has been known to also correlate with the degree of compromise in renal functions.^{17,18} The tubulointerstitial pathology includes tubular hypertrophy, thickening and reduplication of the tubular basement membrane and ensuing tubulointerstitial fibrosis, leading ultimately to progressive decline in renal dysfunctions.^{9,16} A large array of genes that are directly related to the glomerular pathobiology has been implicated in the pathogenesis of diabetic nephropathy.^{10–15} Some of these may be relevant to the pathobiology of tubulointerstitium as well. By subtractive hybridization, a handful of genes have been identified that may be relevant to the pathobiology of tubulointerstitium in diabetic nephropathy,^{19,20} among them the target of Epac1, Rap1b G-protein.²¹ But which of these genes are relevant to the tubular hypertrophy in early stages of diabetic nephropathy? Having delineated the role Rap1b in the pathogenesis of diabetic nephropathy²¹ and the literature information suggesting the role Epac1 in cardiac myocyte hypertrophy,^{22,23} modulated via β -adrenergic receptors in a protein kinase A (PKA)-independent fashion,²⁴ studies were initiated to explore the relevance of Epac1 in cellular hypertrophy of tubules in diabetic nephropathy, using *in vivo* and *in vitro* approaches.

Materials and Methods

Animal Model System

A diabetic state was induced in 10-week-old CD1 mice (Harlan Co., Indianapolis, IN) by an injection of streptozotocin, STZ (200 mg/kg body wt; Sigma Chemical, St. Louis, MO). After 1 week, the mice with blood glucose levels >250 mg/dl were selected for various studies. The mice were sacrificed 8 weeks after the induction of diabetes. All of the animal procedures used in this study were approved by the Animal Care and Use Committee of Northwestern University.

Cell Culture Systems

Various cell lines used in this investigation included the followings: HepG2 (human hepatocellular carcinoma), mouse glomerular podocyte, mouse and rat mesangial cells, HK-2 (human proximal tubular cell), rat proximal tubular cell, mIMCD-3 (mouse inner medullary collecting duct cell), LLCPK1 (porcine renal tubular cell), and COS7 (African monkey kidney cell). They were purchased from American Type Culture Collections (Manassas, VA). The cell lines were maintained in media and culture conditions recommended by the vendor, and used for various studies.

In Vivo Morphological Studies

Immunohistochemical and *in situ* hybridization studies were performed to assess the spatiotemporal expression of Epac1 in kidneys of normal and diabetic mice. *In situ* hybridization was performed as previously de-

scribed.²⁵ Briefly, 1- to 2-mm-thick kidney tissue slices were dehydrated in graded series of ethanol and embedded in paraffin. Then 4- μ m thick sections were prepared and mounted on HCl-treated and Vectabond-coated slides (Vector Labs, Burlingame, CA). The sections were hybridized with Epac1 digoxigenin (DIG)-labeled RNA sense and antisense probes prepared by using *in vitro* transcription system (Roche Diagnostics, Indianapolis, IN). For preparation of probes, a 407 bp DNA fragment of Epac1 gene was generated by PCR using 5'-CGAGCAGGAGCACAGCACCTACATCTG-3' (sense) and 5'-TCACTTCTCTCACCGAGGCCGTCACCG-3' (antisense) primers. The DIG-probes were then subjected to limited alkaline hydrolysis to obtain polynucleotide fragments with a size range of 100 to 150 bp. After hybridization, the slides were successively washed with 2 $\times$, 1 $\times$, and 0.5 $\times$ standard saline citrate in the presence of 1 mmol/L dithiothreitol. The tissue sections were blocked with 3% bovine serum albumin and incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase. They were then developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Diagnostics).

For immunohistochemical studies avidin-biotin complex method (Vector Labs) was used. The 4- μ m thick kidney sections were deparaffinized and hydrated in graded series of decreasing concentrations of ethanol. They were then successively incubated with rabbit anti-Epac1 (Abcam, Cambridge, MA), biotinylated anti-rabbit IgG, and streptavidin conjugated with horseradish peroxidase (HRP) in between brief washes of PBS. The sections were then treated with SIGMAFAST 3',3'-diamino-benzidine solution for 3 to 5 minutes at 22°C to develop the HRP reaction product. The sections were counterstained with hematoxylin, coverslip mounted, and examined.

In Vivo Gene Expression Studies

Northern and Western blot analyses were performed to assess the Epac1 expression in control and diabetic animals. For Northern analysis, total RNA was prepared from mice kidneys and other tissues by the acid guanidinium isothiocyanate-phenol-chloroform extraction method.²⁶ About 20 μ g of total RNAs extracted from kidneys of diabetic and control mice was glyoxylated, subjected to 1% agarose gel electrophoresis, and then capillary-transferred to Hybond N⁺ nylon membrane (GE Health care Bioscience Corp., Piscataway, NJ). After cross-linking of RNA to the membrane, prehybridization and hybridization of various membrane blots were performed with various α -[³²P]-dCTP labeled (1×10^6 cpm/mL) cDNA probes of Epac1 cDNA fragment described above.²⁵ Following the preparation of autoradiograms, the membrane blots were stripped by boiling in 0.1% SDS buffer for 2 minutes and re-probed with radiolabeled β -actin cDNA probe.

For Western blot analysis, tissue lysates were prepared by homogenizing control and diabetic kidneys in ice-cold extraction buffer (10 mmol/L HEPES/1.5 mmol/L

MgCl₂, 10 mmol/L KCl, 0.5 mmol/L DTT, 1 mmol/L PMSF, pH 7.4). The homogenate was centrifuged at 10,000 × g for 30 minutes at 4°C, and the supernatant was saved. The protein concentration in the supernatant was adjusted to 2 mg/mL. Equal amounts (~20 μg) of protein (control versus diabetic) were loaded onto the gel-wells and subjected to 10% SDS/PAGE under reducing conditions. The gel proteins were electroblotted onto a nitrocellulose membrane. The membrane was immersed in a blocking solution containing 5% nonfat milk in Tris-buffered saline-T (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween-20) followed by successive 60-minute incubations with Epac1 antibody and goat anti-rabbit IgG conjugated with HRP at 37°C. The membrane was washed three times with Tris-buffered saline-T and autoradiogram developed using the SuperSignal West Pico Chemiluminescent kit (Thermo Scientific, Rockford, IL).

In Vitro Gene Expression Studies

First, RT-PCR analyses were performed to assess the Epac1 expression in various liver and kidney cell lines. The primers used for various mammalian species were as follows. Epac1-Human: 5'-TTCATGAGGGAAACACACA-3' (sense), 5'-CCTTCAGCTGCTGGACATAA-3' (antisense), (product size, 246 bp); Epac1-Mouse: 5'-CTGCTGCTCAAAGACGTGAC-3', (sense) 5'-GACTGCTCAGAACACGTG GA-3' (antisense) (product size, 218 bp); Epac1-Rat: 5'-TTCATGAAGGGAACACACA-3' (sense), 5'-GCTCGGAACATGTGGAGA TT-3' (antisense) (product size, 189 bp). The proximal tubular cell line, HK-2, was used for further studies because the morphological studies indicated a relatively high expression in the renal proximal tubules. The HK-2 cells were maintained in a defined medium [3:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium containing 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), HEPES (14 mmol/L)] at 37°C. After achieving 80% confluency, the medium was changed to FBS-free DMEM. Varying concentrations of D-glucose (5 to 30 mmol/L) was added and cell culture maintained for 48 to 72 hours. L-glucose served as a control. The cells were then processed for Northern and Western blot analyses as described above.

Epac1 Gene Promoter Analyses

To identify the minimal promoter region and to understand the mechanism(s) of glucose-induced up-regulation of Epac1, the GreatEscApe SEAP system (Clontech, Mountain View, CA) was used. First, a ~1.6 kb DNA fragment upstream of 5' flanking region of Epac1 cDNA was generated by PCR and cloned into pCR11 vector (Invitrogen, Carlsbad, CA). This DNA fragment was used as a template to generate four deletion constructs (–1 to –1547, –1 to –1163, –1 to –931, and –1 to –529). For generation of the constructs, four sense primers and one antisense primer were synthesized (Integrated DNA Technologies, Skokie, IL). Their sequences were as follows: Epac-G1-SE (5'-CGAAGCTTGAAGCTCTGCAACAAATCCG-3'), Epac-G2-SE (5'-CGCTGAAGCTTCCCTAGTTTCC-

TTGTTTG-3'), Epac-G3-SE (5'-CTCTGAAGCTTTAAAGCTCGTGCCTGCTC-3'), Epac-G4-SE (5'-CGCTGAAGC TTCGGAGTCAGGGCCAAAGAA-3') and Epac-G1-AS (5'-GCAGCCTCGAGGAACACTAGCTGGTAAGAACAGCA-3'). HindIII (AAG CTT) and XhoI (CTC GAG) sites (italicized) were included in the sense and antisense primers, respectively. Using these primers and pCR11/Epac1 DNA plasmid as a template, various PCR products were generated and subcloned into XhoI- and HindIII-digested pSEAP2-Enhancer plasmid vector (Clontech, Mountain View, CA) and sequenced.

The minimal promoter activity of Epac1 gene was measured 48 hours following transfection of various cell lines (HK-2, LLC-PK1 and COS7) with plasmids containing different fragments of the promoter using a GreatEscApe SEAP fluorescence detection kit (Clontech, Mountain View, CA), as previously described.²⁷ For transfection, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent kit was used. The activities of various deletion constructs were expressed as the percentages of the activity in the deletion construct with the highest promoter activity, which was designated as being 100%.

The promoter region of Epac1 contains two glucose responsive element (GRE) (CACGTG) sites located at –1112 to –1106 (GRE1) and –479 to –473 (GRE2), flanking the open reading frame of *Epac1* gene. To assess whether the GRE are functional, COS7 cells were exposed to different concentrations of D-glucose (5 to 35 mmol/L) and then transfected with Epac1 gene promoter plasmid containing largest DNA fragment (ie, deletion construct # 1 [DC1]).²⁷ To confirm GRE's role in the transcription of Epac1 expression under high glucose ambience, GRE1 and GRE2 were modified (GRE1: CACGTG to CAGCTG; GRE2: CACGTG to CAAGTG), using site-directed mutagenesis kit following vendor's instructions (Stratagene, La Jolla, CA). They were then used for the SEAP activity analysis, as described above following their subcloning into the pSEAP2-Enhancer plasmid vector.

Role of Epac1 in Cellular Hypertrophy under High Glucose Ambience

Two human Epac1 mammalian expression vector plasmid constructs were a kind gift of Dr. Johannes Bos (University Medical Centre, Utrecht, The Netherlands). The first one included full-length *Epac1* cDNA, and it was designated as pMT2-HA-Epac1. The second one lacked the first 322 amino acids, such that the cAMP binding site is deleted, and it was designated as pMT2-HA-Epac1ΔcAMP or Epac1 mutant1 (Epac1-M1). Another Epac1 construct was generated by inserting a stop codon after the glutamate residue at position 614 using site-directed mutagenesis kit (Stratagene), such that the guanine exchange factor (GEF) domain is deleted, and it was designated as pMT2-HA-Epac1ΔGEF or Epac1 mutant2 (Epac1-M2). A cell-permeable cAMP analog, 8-CPT-2-O-Me-cAMP (8-cAMP) that selectively binds to Epac1 and triggers Epac1 signaling was purchased from Sigma Chemicals. Epac1 siRNA, that has been shown to knock down the Epac1 signaling²⁸ and siRNA transfection

tion transmessenger kit were obtained from Qiagen company (Invitrogen, Carlsbad, CA).

The HK-2 cells were maintained in a defined medium at 37°C. After achieving 80% confluency, the culture was replaced with fresh defined medium containing 1% fetal bovine serum. The cells were exposed to 5 to 30 mmol/L D-glucose for 48 hours. In various experiments the cells were either pretreated with cAMP analog or H89, PKA inhibitor, or transfected with Epac1 or its mutants, Epac1-M1 and Epac1-M2, or Epac1 siRNA. The cells were then processed for [³H] leucine incorporation, measurement of protein and DNA contents and their ratio, and also for confocal microscopy to assess the morphology. Three controls were included for these experiments, which included cells subjected to low glucose (5 mmol/L) ambience, and cells transfected either with scrambled oligo or empty pMT2-HA vector.

For protein synthesis, reflective of cellular hypertrophy, the treated cells were pulsed with [³H] leucine (10 μ Ci/mL) for 12 hours. The cells were harvested and washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS). After counting the cells with a hemocytometer, a cell pellet was prepared by sedimentation in an Eppendorf microfuge (Eppendorf AG, Hamburg, Germany) for 1 minute. The pellet was dissolved in 1 N NaOH for 30 minutes with intermittent vortexing, followed by another centrifugation for 1 minute. Then 10% TCA was added to the supernatant and it was kept at 4°C for 10 minutes. Finally, TCA-precipitated, incorporated radioactivity was determined by liquid scintillation counting and expressed as 1 $\times$ 10⁶ cpm/10³ cells.

For protein and DNA measurements, HK-2 cells were maintained in 12-well culture plates. At the termination of the experiment, the cells from each well were lysed with 100 μ l of PBS containing 0.003% digitonin and 10 ng/mL of ribonuclease A for 10 minutes at 22°C, followed by addition of 50 μ l of 0.8 M urea. For protein assay a 20 μ l aliquot of the lysate was mixed with 200 μ l of nano-orange reagent from NanoOrange protein quantitation kit (Invitrogen). The samples were then transferred to microplates and readings recorded with a fluorometer set at 485 nm (excitation) and 590 nm (capture) wavelengths. The readings were then read against bovine serum albumin standards, following vendor's instructions. Similarly, another 20 μ l aliquot was used for measuring DNA concentration by using a Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen). The absorbance in the samples was recorded at 260 nm. The values were read against dsDNA standards and DNA concentration determined. Finally, protein/DNA ratio was calculated for six samples for each experimental variable.

To measure the cell surface area, HK-2 cells were stained with rhodamine phalloidin (1:50 dilution) for 20 minutes to visualize F-actin. They were then counterstained with 300 nmol/L DAPI at 22°C for 1 minute and briefly washed with PBS. After placing a drop of Antifade solution (Invitrogen) the cells were coverslip mounted. Confocal microscopy was performed with an LSM 510 META laser scanning microscope (Zeiss, Thornwood, NY). The following wavelengths were used for excitation: 488 nm (green), 543 nm (red), and 405 nm (blue). LSM 510 software was used to

measure the cell surface area. The data were derived from at least ten randomly selected fields from six separate experiments.

Role of Epac1 on Cell Cycle Proteins under High Glucose Ambience

The HK-2 cells were maintained in 100 mm culture dishes as described above. They were then exposed to 5 to 30 mmol/L D-glucose for 72 hours. Various experimental manipulations included the pretreatment with cAMP analog, or transfection with Epac1 or its mutant, Epac1-M1, or Epac1 siRNA. The cells were processed for fluorescence-activated cell sorter (FACS) and Western blot analyses, and measurement of CDK4 kinase activity.

For FACS analyses, the cells were treated with 0.05% trypsin in PBS buffer containing 0.05% EDTA. Following which, single cell suspension (2 $\times$ 10⁶ cells/mL) in PBS buffer was prepared and fixed with 70% ethanol for 1 hour at 4°C. The fixed cells were washed with PBS and stained with propidium iodide (PI, 50 μ g/mL) buffer containing, 500 μ g/mL DNase-free RNase (500 μ g/mL) and 0.1% Triton X-100 for 3 hours at 4°C. The samples were then analyzed by BD FACS Canto II flow Cytometer (BD Biosciences, San Jose, CA).

The expression of various cell cycle proteins was evaluated by Western blot analyses. The immunoblots were prepared from extracts of HK-2 cells subjected to various treatments as described above. They were probed with the following antibodies: anti-Epac1, anti -AKT, anti-phospho-AKT, anti -p21, anti -p27, and anti- β -actin (Cell Signaling Technologies, Danvers, MA). For measuring the kinase activity of CDK4, cellular extracts from HK-2 cells that had undergone various treatments were prepared, and protein concentration in the extracts was determined, and adjusted to 1 mg/mL. A 50 μ g of the aliquot was saved from each of the variable as an input sample for immunoblot analysis. Another aliquot of exactly equal amount (50 μ g) of the protein extract was immunoprecipitated with 5 μ g of anti-CDK4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 hours and then incubated with protein-A Sepharose beads for 12 hours at 4°C with gentle orbital shaking. After a gentle wash of the immunoprecipitate with PBS, the kinase reaction was performed using retinoblastoma protein (pRb) as a substrate, as described previously.²⁹ Briefly, immune complexes were resuspended in 35 μ l of kinase buffer containing 1 μ g of glutathione S-transferase retinoblastoma (GST-pRb), 10 μ Ci [γ -³²P]ATP (MP Radiochemicals, Solon, OH), 50 mmol/L Tris-HCl, 10 mmol/L MgCl₂, and 1 mmol/L DTT. The reaction mixture was incubated at 30°C for 30 minutes. The Sepharose beads were pelleted by a brief centrifugation. Then 25 μ l of 1 $\times$ SDS sample buffer was added to the supernatant. After boiling for 2 to 3 minutes, the sample was subjected to 10% SDS-PAGE. The gels were stained with Coomassie Blue to assess evenness of protein loading. The gels were then dried and autoradiograms prepared, followed by relative band intensity densitometric analyses.

The integrated density of each band from six blots was quantified with ImageJ analysis software (National Institutes of Health, Bethesda, MD). Data were quantified as the ratio of the luminescence of the given protein to that of the internal control. Data are expressed as mean $\pm$ SD. Unpaired Student's *t*-tests were performed for statistical comparisons. For all tests, a *P* < 0.05 was considered significant.

Results

Both *in vivo* and *in vitro* studies were performed to assess the expression of Epac1, its modulation by high glucose ambience and signaling pathways involved that affect the cell cycle proteins leading ultimately to cellular hypertrophy.

Epac1 Expression Analysis in Various Tissues

Distribution of Epac1 in various tissues of mouse was assessed by Northern blot analyses. A ~4.0 Kb transcript, corresponding to the Epac1 mRNA, was seen in the kidney and heart tissues only (Figure 1A, arrowhead). No mRNA transcript was seen in liver, lung, spleen, pancreas, muscle, and ovarian tissues. Because the integrity of the mRNA, as assessed by distinct visualization of 28S and 18S bands, was preserved following methylene staining of the transfer blot, this suggested that the absence of Epac1 in other tissues is not related to the degradation of mRNA (Figure 1B). Spatial localization of Epac1 gene within the kidney parenchyma was assessed by *in situ* hybridization analyses. Epac1 was found to be predominantly expressed in the renal cortex (Figure 1C). Cortical tubules had a high expression of Epac1, whereas it was quite low in the glomerular compartment (Figure 1C, arrows). Epac1 expression was also observed in the renal medullary tubules, including the collecting ducts, although the intensity of the signal was relatively low (Figure 1D). The kidney sections hybridized with Epac1 sense probe revealed no detectable signal either in the cortex or medulla (Figures 1E and 1F).

Epac1 Expression Analysis in Kidneys of Diabetic Mice

Both Epac1 gene and protein analyses were performed on kidneys of mice with hyperglycemia induced with the administration of streptozotocin (STZ). By *in situ* hybridization, an increase in the hybridization signal confined to the cortical tubules was observed, whereas a minimal increase in the signal intensity was observed in the glomerular compartment (Figure 2, C versus A, arrows). Similarly, immunohistochemical staining of the kidney tissues from control and diabetic mice revealed a notable increase in the Epac1 protein expression in the cortical tubules, whereas very little expression was observed in the glomeruli (Figure 2, D versus B, arrows). The tubules were somewhat larger and prominent in kidneys of mice with diabetes (Figure 2, D versus B). By Northern blot analysis a progressive increase in the signal density of the ~4.0 Kb transcripts, in proportion to the degree of

Figure 1. Gene expression of *Epac1* in various tissues. Northern blot analyses revealed a ~4.0 Kb transcript, corresponding to the *Epac1* mRNA, in the kidney and heart tissues only (A, arrowhead). Integrity of the mRNA in various tissues was preserved because methylene staining of the transfer blot revealed distinct 28S and 18S bands (B). *In situ* hybridization analyses revealed *Epac1* gene expression mainly confined to the cortical tubules (C), although it was quite low in the glomerular compartment (arrows). A relatively low *Epac1* expression was observed in the renal medullary tubules, including the collecting ducts (D). The kidney sections hybridized with *Epac1* sense probe revealed no detectable signal either in the cortex or medulla (E and F). *Epac1*, exchange protein activated by cAMP.

hyperglycemia (100 to 350 mg/dl), was observed (Figure 2E, arrowhead). By Western blot analyses, a distinct *Epac1* band of ~90 kDa was observed. Similar to the *Epac1* gene expression, an increase in the *Epac1* protein expression in proportion to the degree of hyperglycemia, was observed (Figure 2F, arrowhead). No significant change in the β -actin gene or protein expression was observed (Figure 2, G and H, arrows).

Epac1 Expression Analysis in Various Cell Lines and Modulation by High Glucose Ambience

RT-PCR analyses of mouse kidney tissue suggested that the *Epac1* expression was relatively high in the cortex ver-

Figure 2. Exchange protein activated by cAMP (Epac1) expression analyses in kidneys of mice with streptozotocin (STZ)-induced diabetes. *In situ* hybridization revealed a notable increase in the reddish-purple signal confined to the cortical tubules of diabetic mice (C versus A), whereas a minimal increase in the signal intensity was observed in the glomerular compartment (arrows). Similarly, immunohistochemical (IHC) staining demonstrated a notable increase in the Epac1 protein expression in the cortical tubules from kidneys of diabetic mice, although very little expression was observed in the glomeruli (D versus B). The tubules were somewhat larger and prominent in kidneys of mice with diabetes (D versus B). Northern blot analysis revealed a progressive increase in the signal density of the ~4.0 Kb transcripts, in proportion to the degree of hyperglycemia (100–350 mg/dl) (E, arrowhead). Western blot analyses revealed a distinct Epac1 band of ~90 kDa, and its intensity proportionately increased with degree of hyperglycemia (F, arrowhead). No significant change in the β -actin gene or protein expression was observed (G and H, arrows).

sus medulla because the 218-bp band intensity was slightly denser in the cortical fraction (Figure 3A, last 2 lanes). Then, the expression of Epac1 was examined in various kidney and liver cell lines derived from various mammalian species (human, mouse, and rat) by RT-PCR analyses. The analyses suggested that all of the kidney cell lines (including podocytes, mesangial, tubular, and collecting ducts) express Epac1 (Figure 3A), although, the size of the PCR product generated varied. The size of the generated PCR products for human (HK2), rat (mesangial and tubular) and mouse (podocytes, mesangial and collecting ducts) were 289, 189 and 218 bp, respectively. The liver cell line (HepG2) did not yield any expression for Epac1 gene (Figure 3A, lane 2), suggesting that Epac1 may have a biological significance in the pathobiology of the kidney.

Modulation of Epac1 by high glucose ambience was therefore investigated by using HK-2 cells. They were exposed to various concentrations (5 to 30 mmol/L) of

D-glucose or L-glucose, the latter serving as an osmotic control. At 5 mmol/L concentration of D-glucose, a band corresponding to an ~4 Kb transcript was observed by Northern blot analyses. A dose-dependent increase in its intensity was observed with the exposure to D-glucose (Figure 3B, arrowhead). No increase in the Epac1 gene expression was observed in cells exposed to 30 mmol/L of L-glucose, and the band intensity of the transcript was similar to that seen at basal conditions at 5 mmol/L of D-glucose. Like the gene expression, the Western blot analyses revealed a dose-dependent increase in the expression of Epac1 protein (~90 kDa band) in cells exposed to high glucose ambience (Figure 3C, arrowhead). No increase in Epac1 protein expression in cells subjected high L-glucose ambience. No significant change in both the gene or protein expression of β -actin was observed subjected to high glucose ambience (Figure 3, D and E, arrows).

Modulation of Epac1 Gene Promoter Activity by High Glucose Ambience

First, four deletion constructs spanning different regions of Epac1 promoter and inclusive of various GREs were

Figure 3. Epac1 expression analyses in various cell lines and modulation by high glucose ambience. RT-PCR analyses of mouse kidney tissue revealed Epac1 expression (218 bp product) in both cortex and medulla, the latter having a slightly lower expression (A, last 2 lanes). All of the kidney tubular or glomerular cell lines, derived from human, mouse and rat tissues had Epac1 expression, although the size of the PCR product generated varied depending on the species (A, lanes 3 to 8). The human liver cell line (HepG2) did not have any expression for Epac1 gene (A, lane 2). The HK-2 cells revealed a ~4 Kb transcript (B, arrowhead) and a protein product of ~90 kDa size (C, arrowhead), by Northern and Western blot analyses, respectively; thus indicating a readily detectable expression under basal conditions of 5 mmol/L glucose. Exposure of HK-2 cells to 5 to 30 mmol/L D-glucose revealed a dose-dependent increase in the Epac1 gene and protein expression. L-glucose had no effect. Both the gene or protein expression of β -actin was unchanged (D and E, arrows). RPTC, rat proximal tubular cell.

Figure 4. Modulation of Epac1 gene promoter activity by high glucose ambience. Four deletion constructs spanning different regions of Epac1 promoter DC1 (–1 to –1547 bp), DC2 (–1 to –1163 bp), DC3 (–1 to –931 bp), and DC4 (–1 to –529 bp) were generated and subcloned in pSEAP2-Enhancer plasmid vector (panel A). The constructs were transfected into different cell lines. The highest activity was observed with the transfection of DC3, and it was designated as being 100% (panel B). The DC1 activity was about 70% that of DC3. The DC1 included both the glucose response elements (GREs) (GRE1: –1112 to –1106, and GRE2: –479 to –473), it was used for to assess the modulation of Epac1 promoter by high glucose ambience. A dose-dependent increase in the SEAP activity was observed in DC1 transfected cells subjected to high glucose ambience (5 to 30 mmol/L) (C). No significant increase in the promoter activity was observed in cells treated with 30 mmol/L L-glucose. Transfection of GRE1 or GRE2 mutant plasmids individually or simultaneously led to a 40% to 60% decrease in the promoter activity under high glucose ambience compared with the cells transfected with unmodified deletion construct, DC1, suggesting that both the GREs are functional (C). *Statistically different from the value in pSEAP1 basic vector and low 5 mM D-glucose groups.

generated. These included DC1 (–1 to –1547 bp), DC2 (–1 to –1163 bp), DC3 (–1 to –931 bp) and DC4 (–1 to –529 bp) (Figure 4A). The constructs were subcloned in pSEAP2-Enhancer plasmid vector and transfected into three different cell lines (HK-2, LLCPK1 and COS7) to assess the minimal promoter activity. The highest activity was observed with DC3, consistently in all of the lines. The activity was ~30 times higher than the baseline, and it was designated as being 100% (Figure 4B). A low activity was observed with DC2 and DC4, although it was significantly above the baseline. The DC1 activity was about 70% that of DC3 in all of the three cell lines (ie, HK-2, LLCPK1, and COS7).

Because the deletion construct 1 (DC1) included both the GREs (–1112 to –1106, GRE1; –479 to –473, GRE2) it was used to assess the modulation of Epac1 promoter by high glucose ambience. Cells transfected with DC1 were treated with various concentrations D- and L-glucose. A dose-dependent increase in the SEAP activity was observed in cells subjected to high glucose ambience (5 to 30 mmol/L) (Figure 4C). No significant increase in the promoter activity was observed in cells treated with 30 mmol/L L-glucose. To assess the specificity of the response to high glucose challenge, mutation experiments were performed. Transfection of GRE1 or GRE2 mutant plasmids individually led to a 40% to 50% decrease in the promoter activity under high glucose ambience compared with the cells transfected with unmodified DC1, suggesting that both the GREs are functional (Figure 4C). Interestingly, simultaneous transfection of both the mutants further reduced the promoter activity, thus confirming the functionality of the GREs in the Epac1 promoter.

Role of Epac1 in Cellular Hypertrophy under High Glucose Ambience

For these studies, a full-length cDNA inclusive of ORF, pMT2-HA-Epac1, two mutants, Epac1-M1, and Epac1-M2 with respective deleted cAMP binding site GEF domain were used. The control included empty pMT2-HA vector without Epac1 cDNA.

Exposure of HK-2 cells to 30 mmol/L D-glucose (high glucose, HG) led to a 1.5- to 2-fold increase in their surface area. The increase in the cell size could be readily visualized in cells examined by confocal microscopy following staining with Rhodamine phalloidin and DAPI (Figures 5B versus 5A). This was associated with increase in *de novo* protein synthesis, as reflected by increased ³H-leucine incorporation and relative protein/DNA ratio (Figure 5, I and J). These cellular effects and increased protein synthesis were dampened by the transfection of Epac1-siRNA or Epac1 mutants in cells subjected to high glucose ambience (Figure 5, C–E, I, and J). Transfection of empty vector or scrambled oligos did not affect the cell size or protein synthesis (figure not included). To ensure that the effects on cellular hypertrophy were mediated via Epac1 and cells were either exposed to cell-permeable cAMP analog, 8-CPT-2-O-Me-cAMP (8-cAMP) or transfected with full-length Epac1 cDNA under basal low glucose (LG) conditions. Both, treatment of cells with 8-cAMP or transfection of Epac1 cDNA, induced a hypertrophic response, as reflected by the increase in the cell size (Figure 5, F and G) and increased protein synthesis and protein/DNA ratio (Figure 5, I and J) compared with the basal levels. The hypertrophic response, however, was not as high as seen in cells exposed to high glucose ambience. Because the Epac1 is insensitive to PKA modulation, the effect of PKA inhibitor, H89, on cells subjected to high glucose ambience was assessed. Only very marginal inhibition was observed on the *de novo* protein synthesis compared with the cells treated with high glucose only (Figure 5, I and J). Finally, to confirm the notion that Epac1 is absolutely insensitive to PKA modulation in the presence of high glucose, the cells were transfected with Epac1 cDNA as well as treated with PKA inhibitor, H89. The high glucose treatment in combination with Epac1 cDNA transfection resulted in a remarkable increase in the cells size (Figure 5H), as well as increase in *de novo* protein synthesis and protein/DNA ratio (Figure 5, I and J), suggesting that high glucose- or Epac1-induced hypertrophic response is independent of PKA modulation.

Figure 5. Role of Epac1 in cellular hypertrophy under high glucose ambience. A full-length cDNA, pMT2-HA-Epac1, two mutants, Epac1-M1 and Epac1-M2 with respective deleted cAMP binding site guanine exchange factor (GEF) domain were used. The control included empty pMT2-HA vector without Epac1 cDNA. Exposure of HK-2 cells to 30 mmol/L D-glucose (high glucose [HG]) led to a 1.5- to 2-fold increase in their surface area, as visualized in cells stained with Rhodamine phalloidin and DAPI (**B** versus **A**). The *de novo* protein synthesis, as reflected by the ³H-leucine incorporation and relative protein/DNA ratio, also increased (**I** and **J**). Transfection of Epac1-siRNA or Epac1 mutants in cells negated the effect of high glucose (**C-E**, **I**, and **J**). Transfection of empty vector or scrambled oligos did not affect the cell size or protein synthesis (panels not included). Treatment of cells either with 8-CPT-2-O-Me-cAMP (8-cAMP) or transfection with Epac1 cDNA under basal low glucose (LG) conditions induced a hypertrophic response (**F** and **G**) and increase in protein synthesis and protein/DNA ratio (**I** and **J**). Treatment of cells with H89, protein kinase A inhibitor, had very marginal inhibition on the protein synthesis compared with the cells treated with HG only (**I** and **J**). However, concomitant transfection of Epac1 cDNA and treatment with H89 under HG ambience resulted in a remarkable increase in the cells size (**H**), protein synthesis, and protein/DNA ratio (**I** and **J**), suggesting that HG- or Epac1-induced hypertrophic response is not modulated by protein kinase A. *Statistically different from the value in 5 mM glucose groups.

Role of Epac1 on Cell Cycle Proteins under High Glucose Ambience

To investigate the role of Epac1 in cell cycle events, the HK-2 cells were maintained in culture dishes as described above, and they were individually transfected with empty pMT2-HA vector, Epac1 cDNA, Epac1 mutant, Epac1 siRNA, scrambled oligo, or treated with 8-cAMP under basal low glucose (LG, 5 mmol/L) or high glucose (HG, 30 mmol/L) conditions.

First, the analysis of cell cycle was carried using flow cytometric methods. The HK-2 cells were serum-starved for 12 hours, and then treated with low glucose (5 mmol/L) or high glucose (30 mmol/L) in DMEM media with 0.1% sera for 72 hours. Before flow cytometric analyses the cells were stained with propidium iodide (PI), and the readings were made from six different experiments. The proportion of cells in G₀/G₁ phase under low glucose conditions were 57.6% ± 2.4%. With the high glucose treatment they significantly increased to 75.3% ± 4.6% compared with the control ($P < 0.05$) (Figure 6, B versus A). Similarly, the number cells G₀/G₁ phase increased significantly with transfection of Epac1 cDNA or activation of Epac with 8-cAMP, ie, 73.7% ±

4.4% ($P < 0.05$) and 72.9% ± 4.7% ($P < 0.05$), respectively, even under low glucose ambience (Figure 6, E and F). Increased proportion of cells in G₀/G₁ phase observed under high glucose was notably attenuated by transfection with Epac1-siRNA (62.4% ± 2.7%, $P < 0.05$, compared with HG group) or Epac1 mutant (58.9% ± 3.2%, $P < 0.05$, compared with HG group) (Figure 6, C versus D), suggesting the Epac1 may regulate the cell cycle progression under high glucose ambience, which led us to study status of cell cycle regulatory proteins.

The high glucose ambience increased the expression of phosphorylated form of pAKT, CDK inhibitors, p21 and p27: and it was associated with decreased CDK4 activity in cell transfected with empty vector (Figure 6, G-I). The increased pAKT expression was attributed to the phosphorylation of serine residue since the antibody was directed against the synthetic peptide derived from C-terminal fragment inclusive of Ser₄₇₃. These effects of high glucose ambience were negated in cells transfected with Epac1-siRNA or Epac1 mutant. Interestingly, increased expression of phosphorylated form of pAKT, CDK inhibitors, p21 and p27, and decreased CDK4 activity was observed in low glucose ambience in HK-2 cells transfected with Epac1cDNA or treated with cAMP analog,

Figure 6. Role of Epac1 in cell cycle associated events under high glucose (HG) ambience. In the absence of Epac1 transfection (empty vector) HG increased the proportion of cells in G0/G1 phase (**B** versus **A**) (75.3 ± 4.6 versus 57.6 ± 2.4 , $P < 0.05$). Similarly, the number cells in G0/G1 phase increased significantly with transfection of Epac1 cDNA or activation of Epac with 8-cAMP even under low glucose ambience (**E** and **F**) (73.7 ± 4.4 and 72.9 ± 4.7 , $P < 0.05$). The proportion of cells in G0/G1 phase under high glucose was notably decreased by transfection with Epac1-siRNA or Epac1 mutant (**C** and **D**) (62.4 ± 2.7 , 58.9 ± 3.2 , $P < 0.05$), compared with HG group (**B**). The HG ambience led to an increased expression of phosphorylated form of pAKT, CDK inhibitors, p21 and p27; and it was associated with decreased CDK4 activity in cells transfected with empty vector (**G–I**). These effects of HG ambience were negated in cells transfected with Epac1-siRNA or Epac1 mutant. Increased expression of phosphorylated form of pAKT, CDK inhibitors, p21 and p27, and decreased CDK4 activity was observed in low glucose ambience in HK-2 cells transfected with Epac1 cDNA or treated with cAMP analog, 8-cAMP (**G–I**). Overall, it seems that Epac1 regulates AKT phosphorylation, cell cycle associated proteins in a manner similar to that with HG ambience. * $P < 0.01$, ** $P < 0.05$ statistically different from the value in group of empty vector.

8-CPT-2-O-Me-cAMP (8-cAMP) (Figure 6, G–I), suggesting that Epac1 most likely regulates AKT phosphorylation, thereby expression of cyclin-dependent kinase inhibitors and CDK4 activity in a manner similar to that of high glucose ambience induced cellular hypertrophy.

Discussion

The observations made in this investigation suggest a relevance of Epac1 in the pathology of tubulointerstitium,

in particular that relates to early stages of diabetic nephropathy where tubular hypertrophy is seen as a common occurrence. Whether the changes relevant to high glucose ambience are specific to the kidney or to the tubular compartment need to be addressed because available literature information indicate ubiquitous expression of Epac1 and a restricted distribution of Epac2 in the brain and endocrine tissues by RT-PCR methods, although both of these proteins have high amino acid sequence homology and similar modes of action.²³

Moreover, by immunohistochemical techniques a widespread expression of Epac1 and Epac2 in almost all of the compartments of the kidney has been reported.³⁰ In view of such somewhat controversial information, first Epac1 gene expression was investigated by using more than one method. Northern blot analyses revealed Epac1 expression in the heart and kidney, and no expression in other organs (Figure 1). These findings are at variance with previous studies, and conceivably this may be related to the methodology used. Nevertheless, a readily detectable expression in the kidney, albeit not as heavy as in the cardiac musculature, would suggest a plausible role of Epac1 in the pathophysiology of the kidney as well. In pursuance with this notion *in situ* hybridization studies were performed to assess the Epac1 gene expression in various compartments of the kidney. The expression was mainly confined to the cortical tubules and to a lesser degree in the medullary tubules (Figure 1), suggesting that they may have some role in the pathophysiology of renal tubules. Indeed, Epac1 has been shown to modulate Na⁽⁺⁾/H⁽⁺⁾ exchanger 3 (NHE3) expressed in the brush border membrane of proximal tubules, and also to regulate UT-A1 phosphorylation to accentuate transport of urea in inner medullary collecting ducts.^{31,32} These studies suggest that Epac1 is relevant to the pathophysiology of the tubules. In light of the fact that its downstream target, Rap1b, is co-expressed and is up-regulated by hyperglycemia,²⁰ we proceeded to investigate the Epac1 expression in diabetic state. A multitude of methods, including *in situ* hybridization, immunohistochemistry, and Northern and Western blot analyses, revealed an increase in the Epac1 expression in proportion to the degree of hyperglycemia, especially in the tubular compartment (Figure 2), thus suggesting its relevance in the pathogenesis of diabetic nephropathy. In this regard, besides Epac1's downstream target, Rap1b, other small GTPase, including Rho and Ras, have also been shown to be up-regulated in renal cells subjected to high glucose ambience,^{33–36} which further strengthens the impetus to conduct the studies and elucidate the mechanisms by which Epac1 exerts its influence in the pathogenesis of diabetic nephropathy.

In vitro culture approaches were used to delineate the mechanisms relevant to tubular pathology in diabetic nephropathy. First, various cell lines were used and expression of Epac1 was investigated by RT-PCR analyses. Among the various cell lines examined, all of the tubular cell lines, ie, HK-2, rat proximal tubular cell, and mIM-DCD3, expressed Epac1; and thus, HK-2 cells that can be readily propagated were used in most of the subsequent studies. Similar to *in vivo* in kidneys of diabetic mice, a dose-dependent increase in the Epac1 gene and protein expression was observed under high D-glucose ambience (5 to 30 mmol/L) (Figure 3). The D-glucose-induced up-regulated Epac1 expression seemed to be specific and not related to osmotic or glycosylated stresses because there was no increase seen in cells treated with non-metabolizable L-glucose. These results mimic the *in vivo* observations; therefore, the HK-2 cells were considered suitable for further studies to investigate transcrip-

tional regulation of Epac1 and to delineate the signaling pathways affected.

Promoter analyses using pSEAP2-Enhancer plasmid vector containing various deletion constructs (DC) and transfected into HK-2 cells revealed highest minimal basal activity confined to DC3 (–1 to 931 bp) whereas substantial activity (~70%) was also seen in the full-length DC1 (–1 to 1547 bp) (Figure 4). Because DC1 included both the GREs, it was used to assess the effect of high glucose ambience on the promoter activity. A dose-dependent increase in the activity was observed which was significantly reduced with the mutation of the GREs. Almost identical results were seen with the transfection of other kidney cell lines [ie, LLCPK1 and COS7 (figure not included)]. Interestingly, such GREs are found in the promoters of certain metabolic enzymes, including pyruvate kinase, fatty acid synthase and S, ¹⁴ to which glucose response element binding protein or carbohydrate-responsive transcription factor bind and modulate the transcription of these genes.^{37,38} The GRE motifs (CACGTG) have been found in promoter of transforming growth factor- β 1 (TGF- β 1), a cytokine that responds to high glucose ambience and has been strongly implicated in the pathogenesis of diabetic nephropathy.³⁹ In addition to GREs, two E-Box motifs (CAGCTG) were also identified in the Epac1 promoter, and these motifs are believed to be essential for the promoter activity.⁴⁰ In our previous studies, we also observed that these E-Box motifs in the UbA52 gene that were responsive to glucose stimulation, and following their mutation the glucose responsiveness or the promoter activity was dramatically reduced.²⁷ These promoter analyses suggest that GRE and possibly also E-boxes are functional in the Epac1 gene and modulate its transcription and thereby the activity and expression of Rap1b GTPase, the latter has been previously reported to be up-regulated in diabetic nephropathy and under high glucose ambience.^{20,21,36} Beside Rap1b activation, the next issue of the pathways that are activated leading to cellular hypertrophy of the tubules under high glucose ambience was addressed.

It has been reported that a high concentration of filtered glucose with consequential hyperactivity of Na/glucose co-transporter or Na/H exchanger may be responsible for the renal/tubular cell hypertrophy, possibly via angiotensin II (ANG II)-induced pathways.^{41,42} However, a direct connection between ANG II and Epac1 in the pathogenesis of diabetic nephropathy is unknown. The only information available in the literature relates to the transgenic mice over-expressing inducible cAMP repressor (ICER 1 γ). These mice have sustained high levels of glucose due to low synthesis of insulin by the pancreatic β -cells. Although, accentuated glomerular changes were noted in these mice following streptozotocin administration, tubular hypertrophy was not observed.⁴³ Other conceivable mechanisms that have been implicated in the pathogenesis of tubular hypertrophy include perturbation in the intracellular calcium and activation of TGF- β 1.^{44,45} Besides ANG II, the activation of TGF- β 1 has been known to occur by PKC α ,⁴⁶ reactive oxygen species,⁴⁷ and thrombospondin.⁴⁸ Here, it is worth mentioning here that Epac1 interactions with

TGF- β 1 receptor are known, but they are independent of cAMP binding domain of Epac1, and thus it is likely that Epac1 utilizes a different pathway in the induction of tubular hypertrophy.⁴⁹ In line with these above observations, studies were performed to assess if high glucose ambience could directly exert its effect via the cAMP-stimulated GEF, Epac1, and thereon modulate the downstream events leading to tubular hypertrophy. The high glucose induced a hypertrophic response in HK-2 cells as well, along with prominence of cytoskeletal organization, boosting of *de novo* protein synthesis, as assessed by confocal microscopy, and increased ³H-leucine incorporation and protein/DNA ratio (Figure 5). The hypertrophic response was substantially blunted with the transfection of Epac1-siRNA or Epac1 mutants lacking cAMP binding and GEF domains. Similar blunting effects of Epac1-siRNA have been reported in β -adrenergic receptor-induced hypertrophy in cardiomyocytes,²⁴ suggesting that Epac1 may also play a role in the tubular hypertrophy as well. To directly assess the hypertrophic effect of Epac1 the cells were either treated with cAMP analog, 8-CPT-2-O-Me-cAMP (8-pCPT-2) or transfected with Epac1 cDNA. Even under low glucose ambience this yielded a significant hypertrophic response. Interestingly, transfection of cells with full length Epac1 cDNA under high glucose ambience led to a marked hypertrophic response that was insensitive to PKA inhibitor, H89; thus confirming the role of cAMP-responsive Epac1 in cellular hypertrophy at least under *in vitro* high glucose conditions (Figure 5). Other mechanisms by which high glucose could induce cellular hypertrophy that have been reported in the literature include the activation of a prohypertrophic signaling pathway, which involves the Ca²⁺ sensitive phosphatase, calcineurin, its primary downstream effector (nuclear factor of activated T-cells),²² and ANG II-induced phosphorylation of cAMP-responsive element binding protein.⁵⁰ Besides hypertrophy, ANG II has also been shown to modulate cell cycle events that are also known to be heavily influenced by high glucose ambience.^{51,52} Having established the role of Epac1 in HK-2 cellular hypertrophy which would explain the relatively large size of the renal cortical tubules with increased expression of Epac1 in kidneys of diabetic mice (Figure 2D), the pathways that may be affected downstream of Epac1 were delineated, especially those related to cell cycle events.

A large number of studies indicate that the cyclin-dependent kinase and its inhibitors p21^{Cip1} and p27^{Kip1} are central to the pathogenesis of diabetic nephropathy, in particular, when it relates to tubular hypertrophy.^{47,51,53,54} The more recent reports also indicate that high glucose via JAK2-STAT1/STAT3 and Raf-1/MAPK pathways enhances the expression of p27^{Kip1} and p21^{Waf1/Cip1}, which apparently leads to cell cycle arrest in G0/G1 phase and increased expression of extracellular matrix proteins, such as fibronectin and type IV collagen, and cellular hypertrophy of LLC-PK1 cells.⁵⁴ Along these lines, an increased proportion of the HK-2 cells in G0/G1 phase was observed when subjected to high glucose ambience (Figure 6). With the transfection of Epac1-siRNA or Epac1-mutant the proportion of cells in G0/G1

decreased, and it approximated to the basal levels. The effect of high glucose could be mimicked with the treatment of cells with cAMP analog, 8-pCPT-2, or transfection of Epac1 cDNA in low glucose ambience (Figure 6), thus suggesting that the events related to hypertrophic response and G0/G1 cell cycle arrest may be interlinked.

Cell cycle progression is tightly regulated by a family of cyclin-dependent kinases and their inhibitors, such as p21^{Waf1/Cip1}, through the activation/phosphorylation of Akt to promote cellular growth.⁵⁵ Also, various studies suggest that Akt plays a critical role in the induction of cellular hypertrophy in high glucose ambience, and these events are initiated by phosphoinositide-3 kinase (PI-3K).^{54,56,57} In addition, Akt induces transcriptional activity by modulating TGF- β 1/Smad pathway that plays an important role in high glucose renal cell hypertrophy by increasing the activity of p^{21Cip1} and p^{27Kip1}, while blocking that of the CDK4.^{53,58–61} With respect to cardiomyocyte hypertrophy, the cAMP not only activates Epac1 but also induces Akt phosphorylation both at Thr₃₀₈ and Ser₄₇₃ residues in a dose-dependent manner, suggesting that these two events are interlinked.^{24,62} In the current investigation, we made similar observations for the events that were initiated by high glucose ambience. An increased expression of phosphorylated form of Akt, and of P21 and P27 was observed, and it was associated with decrease in the CDK4 activity under high glucose ambience (Figure 6). These effects were reversed by the transfection of either the Epac-siRNA or Epac-mutant. Interestingly, effect on the expression of pAkt, P21 and p27, and activity of CDK4 could be mimicked by the transfection of Epac1 cDNA or treatment of HK-2 cells with cAMP analog, 8-pCPT-2, under low glucose ambience (Figure 6), suggesting that the pathways induced by high glucose ambience may be similar to those seen in cardiac hypertrophy following cAMP stimulation.^{22–24}

In conclusion, a new role for the cAMP-sensitive Epac1 is described in this investigation, whereby high glucose-induced increased transcription and translation of Epac1 leads to Akt phosphorylation and modulation of cell cycle events culminating in the cellular hypertrophy of the renal tubules.

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