High glucose transactivates the EGF receptor and up-regulates serum glucocorticoid kinase in the proximal tubule

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High glucose transactivates the EGF receptor and up-regulates serum glucocorticoid kinase in the proximal tubule.

Background. Serum glucocorticoid regulated kinase (SGK-1) is induced in the kidney in diabetes mellitus. However, its role in the proximal tubule is unclear. This study determined the expression and functional role of SGK-1 in PTCs in high glucose conditions. As the epidermal growth factor (EGF) receptor is activated by both EGF and other factors implicated in diabetic nephropathy, the relationship of SGK-1 with EGFR activity was assessed.

Methods. mRNA and protein expression of SGK-1 and NHE3 were measured in human PTCs exposed to 5 mmol/L (control) and 25 mmol/L (high) glucose. The effects of SGK-1 on cell growth, apoptosis, and progression through the cell cycle and NHE3 mRNA were examined following overexpression of SGK-1 in PTCs. The role of EGFR activation in observed changes was assessed by phospho-EGFR expression, and response to the EGFR blocker PKI166. SGK-1 expression was then assessed in vivo in a model of streptozotocin-induced diabetes mellitus type 2.

Results. A total of 25 mmol/L glucose and EGF (10 ng/mL) increased SGK-1 mRNA (P < 0.005 and P < 0.002, respectively) and protein (both P < 0.02), expression. High glucose and overexpression of SGK-1 increased NHE3 mRNA (P < 0.05) and EGFR phosphorylation (P < 0.01), which were reversed by PKI166. SGK-1 overexpression increased PTC growth (P < 0.0001), progression through the cell cycle (P < 0.001), and increased NHE3 mRNA (P < 0.01), which were all reversed with PKI166. Overexpression of SGK-1 also protected against apoptosis induced in the PTCs (P < 0.0001). Up-regulation of tubular SGK-1 mRNA in diabetes mellitus was confirmed in vivo. Oral treatment with PKI166 attenuated this increase by 51%. No EGF protein was detectable in PTCs, suggestive of phosphorylation of the EGFR by high glucose and downstream induction of SGK-1.

Conclusion. The effects of high glucose on PTC proliferation, reduced apoptosis and increased NHE3 mRNA levels are mediated by EGFR-dependent up-regulation of SGK-1.

Key words: SGK, high glucose, proximal tubule, EGFR.

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Serum glucocorticoid regulated kinase (SGK) is a novel member of the serine/threonine kinase gene family that was initially identified as an immediate early gene under acute transcriptional control by serum and glucocorticoids [1]. It is now recognized to be activated at both the transcriptional and posttranslational levels by a number of extracellular signals. As a result of its wide distribution and regulation by diverse stimuli, it is thought that SGK-1 is an important focal point for the convergence of intracellular cross-talk pathways that results in changes in proliferation and apoptosis [2]. The exact mechanisms, however, by which SGK-1 is activated, and in turn activates downstream targets, remain in many cases unresolved.

In the kidney, in situ hybridisation has demonstrated SGK-1 mRNA in glomeruli and the distal tubules. However, no signal above background has been previously detected in the proximal tubule (PTC) [3, 4]. The levels of SGK-1 mRNA in the glomeruli does not change in the presence of glucocorticoids. Hence the majority of research into SGK-1 in the kidney has focussed on the distal tubules. In the distal tubules SGK-1 is thought to be a key mediator of aldosterone induced sodium reabsorption by the epithelial sodium channel ENaC. This involves alterations in the ubiquitination status of the ENaC subunits via a mechanism that may involve SGK phosphorylation of Nedd4-2 [5–8]. Importantly, elevated levels of SGK-1 transcripts have been observed in the kidneys of both animal models of diabetes mellitus and in humans with diabetes mellitus [9]. Indeed the activity of SGK-1 is known to be regulated by many of the factors integral to the diabetic state, including osmotic stress [10]. The induction of SGK-1 has been shown to confer a survival advantage to cells exposed to osmotic stress by inhibiting the apoptotic response [11–14] and promoting proliferation [15]. These findings together with the observation that SGK-1 is induced by the profibrotic cytokine transforming growth factor-β1 (TGF-β1) [16], suggested a pivotal role for SGK-1 in the genesis of diabetic nephropathy and a mechanism to explain increased...
sodium retention frequently associated with diabetic nephropathy. To date, SGK-1 protein has not been reported in the PTC of animal [3, 4], or more important, human kidneys [10]. However, the PTC forms the bulk of the volume of the human kidney and is involved in the majority of renal sodium transport. Furthermore, the activity of the prime transporter involved in the apical reabsorption of sodium by the proximal tubule, Na\(^+\)-H\(^+\) exchanger isoform 3 (NHE3), has recently been shown to be increased by SGK-1 [17] acting via the Na\(^+\)-H\(^+\) regulatory factor isoform-2 (NHERF-2) [18, 19].

In addition to its key role in the reabsorption of sodium, the PTC has also been demonstrated pathologically to be involved in the initial development of diabetic nephropathy. In response to high glucose the PTC undergoes an initial hyperplastic phase followed by a prolonged hypertrophic phase. These growth effects are due to changes in the circulating levels and paracrine influences of various cytokines and growth factors such as TGF-β1, angiotensin II (Ang II) and possibly less well appreciated, epidermal growth factor (EGF). EGF is a key proliferative growth factor that is up-regulated in the kidneys of animals with experimentally induced diabetes mellitus [20], where it has been shown it can promote cell growth and regulate sodium transport via NHE3 [21].

In addition to its role in binding EGF, the EGF receptor (EGFR) serves a pivotal role as a central transducer of heterologous signaling systems as a result of transactivation [22]. This transactivation of EGFR by diverse stimuli such as G protein–coupled receptors, cytokines, or cellular stress provides a mechanism for the EGFR to integrate these extracellular signals and act as a relay station to the transcriptional machinery. Both Ang II [23, 24] and aldosterone [25] are known to transactivate the EGFR and importantly, high glucose has recently been shown to transactivate EGFR [26].

The current study was therefore undertaken to determine the presence of SGK-1 in the PTC and to investigate the interactions between high glucose, EGF, and SGK-1 in the PTC in the development of human diabetic nephropathy.

METHODS

Primary culture of human PTCs

Segments of macroscopically and histologically normal renal cortex were obtained under aseptic conditions from patients undergoing nephrectomy for small (<6 cm) tumors. Patients were accepted for inclusion into the study if there was no history of renal or systemic disease known to be associated with tubulointerstitial pathology. Written informed consent was obtained from each patient prior to surgery and ethical approval for the study was obtained from the Royal North Shore Hospital Human Research Ethics Committee. The methods for primary culture of human PTCs are described in detail elsewhere [27]. In brief, tubular fragments were derived from segments of renal cortex by collagenase digestion and isolated by centrifugation in 45% Percoll (Pharmacia, Uppsala, Sweden). The PTCs were resuspended in serum-free hormonally defined media consisting of 1:1 (vol:vol) mixture of Dulbecco’s modified Eagle’s medium and Hams F-12 media (DMEM/F-12) (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA), supplemented with 5 mg/mL human transferrin, 5 mg/mL bovine insulin, 0.05 mmol/L hydrocortisone, 50 mmol/L prostaglandin E1, 50 nmol/L selenium, 5 pmol/L tri-iodothyronine (all from Sigma Chemical Co., St. Louis, MO, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.292 mg/mL L-glutamine (PSG) (Invitrogen, New York, NY, USA). At confluence the cells were harvested using Dispase (Integrated Sciences, Sydney, Australia) and stored in liquid nitrogen. When required, the cells were thawed and after reaching confluence were harvested and subcultured as below.

Cell culture

PTCs were cultured in DMEM/F-12, supplemented with the growth factors described above and maintained at 37°C and 5% CO₂. The effect of high glucose on SGK-1 and NHE3 mRNA expression and the mechanisms whereby these alterations occurred were studied. Specifically the role of EGF and its receptor phosphorylation on SGK-1 induced growth and function were studied.

PTCs were plated at a density of 2.5 x 10^5 per well maintained in media containing DMEM/F-12, selenium/T3, transferrin, PSG in 5 mmol/L glucose (control conditions) prior to 24-hour exposure to either control conditions, 25 mmol/L glucose or 10 ng/mL EGF (Collaborative Research Inc., Bedford, MA, USA). This concentration of EGF has previously been demonstrated to exert growth and functional abnormalities in PTCs [28]. As EGF was demonstrated to increase SGK-1 (see Results section), the neutralizing effect of the EGFR inhibitors AG1478 (40 nmol/L) (Calbiochem, EMD Biosciences, La Jolla, CA, USA) and PKI166 [29], a novel EGFR tyrosine kinase inhibitor of the pyrrolo-pyrimidine class [30], on EGF and high glucose induced increased SGK-1 was assessed in cells simultaneously exposed to high glucose or EGF, in the concentrations reported above. PKI166 was synthesized in the laboratories of NIBR (Novartis Institutes for Biomedical Research, Research Oncology) and was provided by NIBR for the purpose of this study. Both PKI166 and AG1478 were similarly effective in reducing high glucose and EGF-induced increases in SGK protein. However, PKI166 was considered highly specific with the inhibition constant (IC₅₀) for the EGFR being 0.7 nmol/L [30]. Hence, this compound was used in further experiments. An initial dose response experiment of PKI166 in neutralizing the proliferative effect of EGF 10 ng/mL in control conditions was undertaken. This defined 4 μmol/L
PKI166 as the optimal dose to reverse EGF-induced proliferation, and thus was used in subsequent experiments.

**Cell count**

In order to confirm the biological activity of EGF, PTCs were grown to 90% confluence, quiesced in control conditions for 24 hours, and then exposed to 10 ng/mL EGF or a combination of 10 ng/mL EGF and 4 \( \mu \)mol/L PKI166 for 24 hours. Cells were then trypsinized and manually counted using a hemocytometer.

**SGK-1 and NHE3 competitive reverse transcription-polymerase chain reaction (RT-PCR)**

Competitive RT-PCR was performed to determine the changes in SGK-1 and NHE3 mRNA expression level induced by exposure to treatments described above. Total RNA was extracted using RNeasy Kit (Qiagen, Victoria, Australia), according to manufacturer’s instructions. RNA was reverse transcribed using the Superscript II Reverse Transcriptase kit (Invitrogen). Sense and competitive primer sequences are as detailed for both NHE3 and SGK-1 in Table 1.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Primer sense</th>
<th>Primer sequence 5′ to 3′</th>
<th>Target size</th>
<th>Competitor size</th>
</tr>
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<tr>
<td>NHE3</td>
<td>Sense</td>
<td>GTTCTTCCACCGTCATCTTCCA</td>
<td>444</td>
<td>309</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>AGGCGTGACATTTTCTTCCAG</td>
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<td></td>
<td>LAS</td>
<td>ACGCGTGACATTTTCTTCTCCAG</td>
<td></td>
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</tr>
<tr>
<td>SGK-1</td>
<td>Sense</td>
<td>GCCAATGGAGTTGGGGACAG</td>
<td>574</td>
<td>465</td>
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<tr>
<td></td>
<td>Antisense</td>
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<td></td>
<td>LAS</td>
<td>TGCCCTGGGTACCTGCAAT</td>
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</tr>
</tbody>
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LAS is long antisense primer.

PCR reactions were performed on cDNA using the sense and competitor primers and the products gel purified and quantitated. For the PCR reaction, the competitor cDNA was used at concentrations of 0.2, 0.5, 1.5, 4.6, and 13.7 fmol/L. The thermal profile consisted of denaturation at 94°C for 1 minute, annealing at 60°C for 1 1/2 minutes, and extension at 72°C for 1 1/2 minutes, for 30 cycles using Expand High Fidelity PCR System (Roche, Mannheim, Germany). The products were run on a 2% agarose gel stained with ethidium bromide and photographed. The photograph was then scanned into a computer and the relative intensities of the individual bands quantitated using NIH Image software version 1.60. It was considered that the concentration of the competitor is equivalent to the concentration of the message for the target gene.

**Western blotting**

In order to detect changes in the levels of protein for SGK-1 or NHE3 following exposure to EGF or high glucose, in the presence or absence of PKI166 or cells transfected with SGK-1 (see below), we used standard Western blotting techniques. Western blots were performed on Triton X-100 soluble fractions of cells for detection of SGK-1 and NHE3. Protein concentrations were determined using the Bio-Rad protein assay (Hercules, CA, USA). Equal amounts of protein were loaded and all samples were subjected to run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Pharmaceuticals, Bucks, UK). The membranes were blocked in 5% skim milk, and incubated with pan-SGK antibody (Cell Signaling, Beverly, MA, USA) or NHE3 antibody (Chemicon International Inc., Temulca, CA, USA) overnight at 4°C followed by incubation with antirabbit IgG (Fc) alkaline phosphatase (AP) conjugate antibody (Promega, Madison, WI, USA) for 1 hour at room temperature. Protein was detected using ECL Kit (Amersham Pharmaceuticals). The bands corresponding to SGK-1 (48.5 kD) and NHE3 (100 kD) were quantitated using NIH Image software version 1.60.

Western blot analysis was additionally performed as described above to determine whether high glucose and overexpression of SGK-1 induced EGF protein in PTCs, using an EGF antibody (Chemicon International, Inc.).

**EGFR phosphorylation**

The phosphorylation of the EGFR by high glucose was determined by flow cytometry after specific labeling with phospho-EGFR antibody (Cell Signaling). In brief, cultured PTCs were detached from the cell culture vessel with trypsin, washed in phosphate-buffered saline (PBS) and fixed in 0.5% formaldehyde at 1 × 10^6 cells/mL for 10 minutes at 37°C. After two washes in PBS, cells were permeabilized using 0.1% Triton X-100 then subsequently labeled with phospho-EGFR antibody for 1 hour at room temperature. Following two subsequent washes, cells were labeled with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG (BD Biosciences Pharmingen, San Diego, CA, USA) overnight. The cells were washed twice before flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Data are presented as percentage of mean fluorescent intensity (± SEM) compared to control (5 mmol/L glucose). An initial time course demonstrated
phosphorylation of the EGFR within 10 minutes that was maximal at 24 hours, persisting to 48 hours (Fig. 3D). This became the basis of the time frame of the immunoprecipitation.

**Immunoprecipitation**

To confirm phosphorylation of the EGFR in response to high glucose, standard immunoprecipitation methods were used. Briefly, 50 µg protein was used per experimental condition, made up to 500 µL in PBS. A 1:100 dilution of the phospho-EGFR antibody (Cell Signaling) was added to this protein and incubated for 2 hours at 4°C. After this time, 40 µL of EZview agarose beads (Sigma Chemical Co.) was added to each condition and incubated overnight at 4°C. The protein complexes were then washed twice in cold PBS. The samples were boiled in SDS-PAGE sample buffer for 10 minutes, then run on a gel by standard Western blot procedures as described above. An antiphosphotyrosine antibody PY20 was used to detect phosphorylated EGFR.

**In vivo experiments**

In order to determine SGK-1 expression in an in vivo model of diabetes mellitus we localized and quantitated renal SGK-1 expression in an animal model of streptozotocin-induced diabetes mellitus. This model has been previously validated for study in diabetic nephropathy [31]. SGK-1 mRNA was then assessed by in situ hybridization as previously described [31], quantitated by densitometric analysis, and expressed as cpm/unit area [32]. Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia’s Code of the Care and Use of Animals for scientific purposes and approved by St Vincent’s Hospital Animal Ethics Committee, Melbourne, Australia. Eight animals were utilized in each experimental group.

**SGK-1 transfection**

The functional effects of increased SGK-1 expression were determined by overexpressing SGK-1 in PTCs. PTCs were plated on day 1 at 1 × 10^5 cells per well. The next day, cells were transfected with hemagglutinin (HA)-tagged SGK-1, empty vector [phosphorylated cytokemegalovirus (p-CMV)], or dominant-negative SGK-1 (plasmids purchased from Dr. M. Peggie, The University of Dundee, Scotland) using the Fugene-6 transfection reagent (Roche, Indianapolis, IN, USA). Efficiency of transfection was assessed by flow cytometry after specific labeling with an HA antibody (Cell Signaling) using a protocol identical to that used for assessment of EGFR phosphorylation detailed above. This suggested a transfection efficiency of 60% to 70%. No difference was observed between cells transfected with the empty vector and dominant-negative SGK-1; therefore, control experiments contained only empty vector. Briefly, Fugene-6 and SGK-1 DNA was added to serum-free media, and incubated at room temperature for 45 minutes, the reaction was added drop wise to each well, and the cells were then incubated at 37°C, 5% CO₂ for a total time of 24 hours. Briefly, to extract protein from treated cells, the cells were washed twice with cold PBS, then lysed with 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 20 mmol/L sodium orthovanadate, and 0.5% Triton X-100 with proteinase inhibitor cocktail “Complete” (Roche Diagnostics, Mannheim, Germany) for 20 minutes, followed by centrifugation at 10,000 × g for 10 minutes at 4°C. In order to determine SGK protein expression changes in transfected cells, cell lysates were used for standard Western blot analysis, as per the methods described above.

**Functional consequences of SGK-1 overexpression**

**Cell count and protein expression.** Cell count and hypertrophy were determined as detailed above. In addition [3H]-thymidine incorporation was measured. In brief, the cells were exposed to 1 µCi/well of (methyl-3H) thymidine (Amersham, Bucks, England) in the final 4 hours of each 24-hour time point. The cells were then washed four times in PBS, solubilized in 0.2 mol/L NaOH, added to scintillant (Ecolite, ICN, Costa Mesa, CA, USA) and the samples counted in a β counter (LKB Wallac, Turku, Finland). Results were expressed as [3H]-thymidine incorporation per cell as a percentage compared to control values.

**Cell cycle analysis.** PTCs were transfected as described above. Twenty-four hours after transfection, cell supernatant and cells were harvested, resuspended in 500 µL 70% ethanol to permeabilize the cell membrane. The cells were then spun down, washed with PBS twice. They were then resuspended in 400 µL PBS, 50 µL RNase (10 mg/mL), and 50 µL propidium iodide (0.5mg/mL) (all purchased from Sigma Chemical Co.). The cells were incubated in the dark at 4°C for at least 1 hour. The S phase and apoptotic population of the cell cycle were determined by flow cytometry using a FACSScan flow cytometer (Becton Dickinson). The propidium iodide fluorescence of individual nuclei and the forward and side scatter were all measured using identical instrument settings with a minimum of 20,000 events.

**Apoptosis studies.** To assess the role of SGK-1 in apoptosis, PTCs were plated and transfected as above. Twenty-four hours after transfection, apoptosis was induced by treating transfected and control cells with 150 mmol/L sodium azide [33]. Cells were then collected and processed for cell cycle analysis using the FACScan flow
cytometer as described above. Apoptosis was confirmed by flow cytometry using CaspACE FITC-VAD-FMK labeling (Promega).

**NHE3 Competitive RT-PCR.** The effect of overexpression of SGK-1 on NHE3 mRNA expression was determined by competitive RT-PCR as detailed above.

**SGK-1 induced phosphorylation of the EGFR.** The effect of overexpression of SGK-1 on EGFR phosphorylation was determined by flow cytometry after specific labeling with the phospho-EGFR as detailed above.

**Statistical analysis**

In vitro experiments were performed at least in triplicate on a minimum of three different cell culture preparations. Unless otherwise stated, results are expressed as a percentage of the control (cells grown in 5 mmol/L glucose). Statistical comparisons between groups were made by analysis of variance (ANOVA) or paired t tests where appropriate. Analyses were performed using the software package, Statview version 4.5 (Abacus Concepts Inc., Berkley, CA, USA). P values less than 0.05 were considered significant.

**RESULTS**

**EGF effects in PTCs**

A significant proliferative response was induced in PTCs exposed to 10 ng/mL EGF to 119 ± 5% of control values (P < 0.01). This response was completely reversed in the presence of the EGFR blocker PKI166 to 93.6 ± 5% of control values (P < 0.01), confirming the biologic activity of PKI166. The expression of the phospho-EGFR was increased from 50% under control conditions to 63% in the presence of 10 ng/mL EGF. This phosphorylation was attenuated in the presence of EGF and PKI166 to 52%, hence similar to control levels. These results confirmed that the EGFR is present and constitutively active in PTCs. It is further activated by EGF in human PTCs to induce a proliferative response.

**SGK-1 mRNA and protein expression following exposure to EGF and high glucose**

SGK-1 protein was expressed under basal conditions with synthesis presumed downstream of the EGFR phosphorylation as protein expression was significantly attenuated in the presence of the EGFR blocker PKI166 to 24.2 ± 6.2% (P < 0.0001). SGK-1 mRNA was significantly increased following 24-hour exposure to EGF to 216 ± 36% of control values (P < 0.002), which was completely reversed by PKI166 to control levels (115 ± 30%) (Fig. 1A). The up-regulated message was clearly translated into increased protein (174 ± 23% of control values) (P < 0.02) as demonstrated in the Western Blot and graphically represented in Figure 1B. The increase in SGK-1 protein induced by EGF returned to baseline in the presence of PKI166 to 85 ± 17% of control values (P < 0.005), confirming the regulation of SGK-1 by EGFR phosphorylation. PKI166 had no effect on SGK-1
High glucose also induced an increase in SGK-1 mRNA expression to 149 ± 13% of control values (P < 0.005) that was also completely reversed in the presence of PKI166, suggesting that the up-regulation of SGK-1 in high glucose conditions occurs through an EGF-dependent mechanism (Fig. 2A). This was confirmed at the level of protein expression as the high

protein expression under control conditions (5 mmol/L glucose).

Fig. 2. Effect of high glucose on serum glucocorticoid regulated kinase-1 (SGK-1). (A) SGK-1 mRNA expression in control (5 mmol/L) and high (25 mmol/L) glucose ± PKI166 (4 μmol/L). *P < 0.005 vs. control. (B) Western Blot of proximal tubule cell (PTC) SGK-1 protein expression and graphic representation of Western blot of PTC SGK-1 protein expression in control (5 mmol/L), high (25 mmol/L) glucose, and 25 mmol/L glucose + PKI166 (4 μmol/L). *P < 0.02 vs. control.

Fig. 3. Effect of serum glucocorticoid regulated kinase (SGK) and 25 mmol/L glucose on epidermal growth factor receptor (EGFR) phosphorylation. (A) Flow cytometry. Increased phosphorylation of the EGFR in proximal tubule cell (PTC) exposed to high (25 mmol/L) glucose in the presence and absence of PKI166 (4 μmol/L). *P < 0.01 vs. control. (B) Immunoprecipitation and subsequent Western blotting confirming increased phosphorylation of the EGFR in high glucose (P < 0.01 vs. 5 mmol/L glucose) and attenuation in the presence of PKI166 (4 μmol/L). (C) Flow cytometry. Increased phosphorylation of the EGFR expression in PTC transfected with SGK-1 in the presence and absence of PKI166 (4 μmol/L). *P < 0.01 vs. control cells [transfected with phosphorylated cytomegalovirus (p-CMV) vector]. (D) Time course for phospho-EGFR. Cells were treated with high glucose (25 mmol/L) showing phosphorylation of the EGFR within 10 minutes that was maximal at 24 hours, persisting to 48 hours.
glucose induced up-regulation of SGK-1 mRNA was translated into protein (180 ± 21%) of control values (P < 0.02) as shown in the representative Western Blot and represented graphically in Figure 2B.

**EGF protein expression and EGFR phosphorylation following exposure to EGF, high glucose, and overexpression of SGK-1**

Flow cytometry and immunoprecipitation with subsequent Western blotting of the phospho-EGFR demonstrated constitutive phosphorylation of the EGFR under control conditions which could be attenuated to 75.5 ± 13.5% (P < 0.05) of control in the presence of EGFR blockade using PKI166. An increase in the phospho-EGFR was observed after exposure to high glucose to 124 ± 6% of control values (P < 0.01) (Fig. 3A), which was confirmed by immunoprecipitation and subsequent Western blotting (Fig. 3B) (112.5% ± 2.12 of control) (P < 0.01). An increase in the phospho EGFR was also observed in cells overexpressing SGK-1 protein to 145 ± 2% (P < 0.01) of the levels of EGFR phosphorylation observed under control conditions (Fig. 3C). The level of phosphorylation of the EGFR in the presence of high glucose and overexpression of SGK-1 was attenuated in the presence of PKI166 to control values (Fig. 3A and C). EGF protein was not detected by Western blotting in PTCs under control or high glucose conditions which is consistent with prior studies [34]. Hence, these results suggest that high glucose induced SGK-1 can activate the EGFR, independent of local up-regulation of EGF protein, potentially amplifying the functional response.

**NHE3 mRNA and protein expression in response to EGF and high glucose**

As illustrated in Figure 4A, NHE3 mRNA was significantly increased by EGF to 168 ± 16% (P < 0.05) and high glucose to 140 ± 15% (P < 0.05) of control values. The EGF and high glucose–induced increases in NHE3 mRNA were reversed in the presence of PKI166 (Fig. 4). PKI166 also reduced the NHE3 mRNA and protein expression observed under control conditions to 33 ± 1% (P < 0.002) and 72 ± 10% (P < 0.05) of control values suggesting that EGF is involved in the proximal tubular reabsorption of sodium through NHE3 under control conditions.
SGK-1 expression in in vivo models of diabetes mellitus

Diabetic rats gained significantly less body weight (343.8 ± 4 g) than their respective controls (442.3 ± 2 g) ($P < 0.05$). All diabetic rats were similarly hyperglycemic, while systolic blood pressure was not different between control (131.3 ± 2.7 mm Hg) or diabetic rats (139.2 ± 5 mm Hg).

SGK-1 gene expression was significantly increased in the kidneys of diabetic rats compared to vehicle treated control rats ($P < 0.005$) (Fig. 5). The oral administration of PKI166 for 3 weeks in diabetic rats resulted in a 51% reduction in SGK-1 gene expression in the kidney ($P < 0.001$) (Fig. 5). These results were confirmed by in situ hybridization (Fig. 6), where an up-regulation of SGK-1 mRNA expression was clearly demonstrated in the kidneys of diabetic animals and reversed with PKI166.

Assessment of the functional consequences of increased SGK-1 expression

The functional consequences of SGK-1 are illustrated in Figure 7. Overexpression of SGK-1 increased cell proliferation as indicated by the increase in $[^{3}H]$-thymidine incorporation to 181 ± 6% of control values ($P < 0.01$) (Fig. 7A). The proliferative response was supported by the flow cytometry (Fig. 7B) demonstrating an acceleration through the cell cycle with an increase in the number of cells in the S phase of the cycle to 203 ± 1% of that observed under control conditions ($P < 0.0001$). As expected, exposure to sodium azide induced a marked apoptotic response with 33.6% of cells in the pre-G1 phase of the cell cycle (Fig. 7C). This was reduced to 10.66% in cells overexpressing SGK-1, demonstrating that SGK-1 exerts a specific anti-apoptotic effect in the PTC. The increase in cells in the S phase of the cell cycle following SGK-1 overexpression was again confirmed in these experiments, being 198% of the control cells treated with sodium azide (Fig. 7C). This data was confirmed by flow cytometry using CaspACE staining (data not shown). Over-expression of SGK-1 in PTCs increased NHE3 mRNA to 245 ± 28% of control values ($P < 0.01$) (Fig. 7D).

DISCUSSION

This study clearly demonstrates that SGK-1 is expressed in the human PTC and is physiologically active. It elucidates the key role that SGK-1 plays in mediating the increased proliferation, reduced apoptosis, and increased transcription of NHE3 under hyperglycemic conditions, which have been previously attributed to an increase in EGF action in the kidney [20]. It specifically shows that high glucose increases the expression of SGK-1 which is blocked by the EGFR blocker, PKI166. The effects of high glucose are mimicked by the overexpression of SGK-

Fig. 5. Serum glucocorticoid regulated kinase-1 (SGK-1) expression in in vivo models of diabetes mellitus. (A) Pseudocolorized autoradiographs of SGK-1 gene expression in the kidney of control (panel i), control + PKI166 (panel ii), diabetes (panel iii), and diabetes + PKI166 (panel iv). Cortical SGK-1 mRNA was reduced with PKI166 in both control and diabetic rat kidneys. Red is intense expression; blue green is low level of expression of SGK-1. (*) $P < 0.005$ for diabetic animals vs. control animals; (#) $P < 0.001$ for diabetic animals vs. diabetic animals treated with PKI166.

1. Both result in an increase in tubular cell proliferation [34] and NHE3 expression [35]. Furthermore, it is important to note that increased tubular cell number not only involves an increase in proliferation, but also a reduction in the rates of apoptosis. This current study highlights the antiapoptotic property of SGK-1 in this renal cell system. A novel finding in this study is that in both PTCs in culture and in an in vivo model of diabetic nephropathy, the effects of glucose are highly dependent on the activation of the EGFR. We show that blocking the EGFR reverses the increase in SGK in response to high glucose and abolishes the high glucose–induced changes
in PTC cell number and NHE3 mRNA expression. These results have implications for the development of hypertension and the development of diabetic nephropathy.

Prior studies have suggested that SGK-1 is up-regulated in both animal models and patients with diabetes mellitus [9, 10]. In vitro studies in mouse 3T3 fibroblasts exposed to 30 mmol/L D-glucose have demonstrated a twofold increase in the expression of SGK mRNA within 8 hours [10]. An additional 55% increase in mRNA for SGK occurred in endothelial cells within 2 hours of exposure to 50 mmol/L glucose, an effect attributed to be due to hyperosmolality [10], which is
consistent with recent data suggesting that SGK-1 plays a significant role in the regulation of cellular osmotic stress and the subsequent regulation of cell volume [10]. However, to date, no studies have examined SGK-1 in PTC, which undergo increased proliferation and transport in association with diabetes.

The previously demonstrated association between diabetic nephropathy [20], EGF-induced alterations in tubular growth and transport [21], and the recent demonstration that the EGFR can be transactivated by multiple pathways [22] has been further explored in the present study. The distribution of EGF protein has been previously confirmed to be absent in the PTC and largely expressed in the distal segments of the kidney, where it is increased in the early stages of experimental diabetes [20]. These findings are consistent with the findings of the present study where no detectable EGF protein was present in the cultured PTCs under control or high glucose conditions. In contrast, the EGFR is expressed widely in the PTC and well known to mediate growth responses in the PTC. However, it is clear that the EGFR can be activated independent of EGF itself. In particular aldosterone, one of the hormones originally recognized as playing a key role in SGK-induced transport is now recognized to mediate effects on sodium transporters in the PTC through EGF-dependent mechanisms [25]. The results of the present study in vitro and in vivo studies are complementary as both consistently demonstrate that high glucose increases SGK-1 and that inhibition of the EGFR tyrosine kinase phosphorylation attenuates this increase. This implies that glucose stimulates the phosphorylation of the EGFR which in turn increases SGK-1. We have clearly shown that SGK-1 attenuates pro-apoptotic stimuli while stimulating progression through the cell cycle. Therefore the mitogenic and anti-apoptotic functions of EGF may be mediated by SGK-1.

The critical role of the EGFR in up-regulation of SGK-1 has not previously been considered. Indeed, one prior study investigating SGK expression in 3T3 cells reported that cytokines responsible for a sustained activation of the extracellular-related kinase (ERK) mitogen activated protein (MAP) kinase pathway induced SGK more strongly than EGF, which only induced a transient activation of ERK MAP kinase [35]. Clearly our results differ, and suggest that factors inherent in the diabetic state, specifically high glucose as shown in this study, induce SGK-1, which is completely reversed in the presence of the highly specific EGFR blocker PKI166. Although we did not investigate the ERK MAP kinase pathway in the present study, we have previously demonstrated in an alternative model of human endothelial cells that both the ERK pathway and the p38 MAP kinase pathways were...
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up-regulated over a 72-hour period of high glucose exposure [36]. As SGK-1 induction has also been reported to be induced by a p38 MAP kinase–dependent mechanism it is possible that several upstream mediators induced by high glucose, including osmolality [10], may influence the SGK pathways to a variable extent in different cell types and models.

To define the role of SGK-1 we overexpressed SGK-1 in human PTCs and demonstrated that it conferred a growth promoting effect and protected against proapoptotic stimuli, as has previously been demonstrated in other model systems [12, 14]. Our results demonstrating that SGK-1 overexpression also induced EGFR phosphorylation are also in keeping with the previous observation that phosphorylation of the EGFR induces accelerated progression through the cell cycle [37] and protects cells from apoptosis. This has also been shown to be via a MAPK kinase–dependent pathway and independent of protein kinase C, which is also known to be up-regulated in the diabetic kidney. From our studies it is likely that a key function of SGK-1 is to modify sodium transport in the proximal tubule. We have clearly demonstrated an up-regulation of NHE3 mRNA and protein, which in all prior studies undertaken in our laboratory this has translated into an increase in NHE activity [38]. To date enhanced sodium transport has been most widely characterised in the distal tubule with SGK-1 known to induce sodium reabsorption through the epithelial sodium channel ENaC, by influencing key regulatory proteins such as Nedd4 [5–8]. However, it has recently been recognized that SGK-1 may regulate sodium reabsorption through enhanced sodium hydrogen exchange (NHE3) activity. It has been suggested that these transporters are activated by SGK-1 as a volume regulatory response due to the alterations in cell volume inherent in exposure to high glucose [39]. It has more recently been shown that SGK-1 plays a key role in regulating the glucocorticoid-induced up-regulation of NHE3 activity by interacting with the postsynaptic density protein (PDZ) domains of NHERF2, which functions to anchor NHE3 to ezrin in the formation of localized signaling complexes at the plasma membrane [17, 40]. In addition to the studies that show that SGK-1 can modulate the function of SGK-1 [17], we now show that increased SGK-1 itself can up-regulate the expression of NHE3 mRNA mediated through an EGFR-dependent mechanism. This may well contribute to the well recognized increase in sodium reabsorption in the PTC in diabetic nephropathy.

CONCLUSION

Our findings suggest that high glucose activates the EGFR in a nonligand-dependent manner, which then up-regulates SGK-1. Hence, the abnormalities in tubular cell growth known to occur in diabetic nephropathy and
the increased NHE3 expression and tubular sodium reabsorption known to be responsible for the observed hyper tension are likely to occur through an EGFR-dependent mechanism. These results suggest that SGK-1, or alternatively phospho-EGFR may be potential novel targets that may independently impact on the development of diabetic nephropathy.

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