

Hypoxia-inducible Factor-1 α (HIF-1 α) Protein Diminishes Sodium Glucose Transport 1 (SGLT1) and SGLT2 Protein Expression in Renal Epithelial Tubular Cells (LLC-PK₁) under Hypoxia*

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Background: Hypoxia regulates the transport systems expression in kidney cells.

Results: Expression of SGLT1 and SGLT2 is diminished in LLC-PK₁ cells exposed to low oxygen concentrations.

Conclusion: HIF-1 α modified expression of the renal transporters SGLT1 and SGLT2 in LLC-PK₁ cells.

Significance: HIF-1 regulates the expression of glucose transport in LLC-PK₁ cells, and this mechanism may be involved in the adaptation of kidney cells under reductions conditions in pO_2 .

In this work, we demonstrated the regulation of glucose transporters by hypoxia inducible factor-1 α (HIF-1 α) activation in renal epithelial cells. LLC-PK₁ monolayers were incubated for 1, 3, 6, or 12 h with 0% or 5% O₂ or 300 μ M cobalt (CoCl₂). We evaluated the effects of hypoxia on the mRNA and protein expression of HIF-1 α and of the glucose transporters SGLT1, SGLT2, and GLUT1. The data showed an increase in HIF-1 α mRNA and protein expression under the three evaluated conditions ($p < 0.05$ versus $t = 0$). An increase in GLUT1 mRNA (12 h) and protein expression (at 3, 6, and 12 h) was observed ($p < 0.05$ versus $t = 0$). SGLT1 and SGLT2 mRNA and protein expression decreased under the three evaluated conditions ($p < 0.05$ versus $t = 0$). In conclusion, our results suggest a clear decrease in the expression of the glucose transporters SGLT1 and SGLT2 under hypoxic conditions which implies a possible correlation with increased expression of HIF-1 α .

HIF-1 α is then phosphorylated by AKT1 and translocated to the nucleus, where it binds directly to HIF-1 β to form the HIF-1 dimer (4). The dimer is coupled to a hypoxia response element in a DNA promoter region that facilitates the transcription of a wide variety of genes involved in the regulation of cell survival such as those involved in iron metabolism (5), intracellular glucose homeostasis (6), metabolism, and nucleoside transport (7). Besides the wide range of genes whose transcription is induced by HIF-1, this protein is also crucial in cellular adaptation to stress.

There is evidence of high level HIF-1 expression among mammals suffering from diverse pathological conditions such as cancer (8) and diabetes (9). HIF-1 expression has been found to be increased in human malignancies such as prostate, cervical, renal cell, colon, lung, brain, and breast cancer. Tumor development as well as angiogenesis and patient mortality have been correlated with increased expression of HIF-1 (10). However, Rosenberger *et al.* found that the development of diabetic nephropathy is accompanied by chronic hypoxia and tubulointerstitial fibrosis, conditions that are associated with an increased expression of HIF-1 α in diabetic kidneys (9). Since Takiyama *et al.* proposed in 2011 that HIF-1 may have cytoprotective properties, it is not quite clear whether HIF-1 has a beneficial role or can only be a marker for disease progression (11).

Clinical studies have shown that renal glucose metabolism is modified in diabetic, hypertensive, and atherosclerotic patients (12, 13). These diseases frequently lead to renal ischemia and acute and/or chronic renal failure, conditions characterized by low tissue perfusion that results in hypoxia (12). Evidence confirms that HIF-1 is expressed in renal tissue in experimental conditions (14). High glucose in renal tissues induces oxidative stress (15) and glucose transport alterations (9, 16) and greatly decreases intracellular ATP concentrations (17) as well as the expression of enzymes involved in oxidative pathways (18), resulting in HIF-1 α stability and consequently more HIF-1 production.

Hypoxia-inducible factor 1 α (HIF-1 α)³ expression is a key response to low oxygen partial pressure among mammalian cells (1). HIF-1 α has a short half-life (of approximately 5–8 min) in normoxia (2) because it is hydroxylated by prolyl hydroxylase, bound to von Hippel-Lindau protein and then degraded by the E3 ubiquitin-ligase complex (3). By contrast, hypoxia promotes a decrease in prolyl hydroxylase activity;

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³ The abbreviations used are: HIF-1 α , hypoxia-inducible factor 1 α ; ABZ, albendazole; GLUT, glucose transporter; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; SGLT, sodium glucose transport.

Under chemical hypoxia induced with cobalt chloride (CoCl₂) or dimethylxalylglycine, the rodent kidney expresses HIF-1 α specifically in the renal cortex and inner and outer medulla. This HIF-1 α expression is accompanied by the co-detection of HIF-1-regulated proteins, such as vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT1) (16, 19, 20). Also, in streptozotocin-induced diabetic rats, high levels of HIF-1 α were found in the outer medullary region, including the medullary thick ascending limb (9). In kidney cells, HIF-1 regulates glucose transport via GLUT1 and GLUT3, and glucose metabolism mediated by enzymes such as fructose-2, 6-biphosphatase and hexokinase 2, resulting in a high rate of glucose turnover and increased oxidative metabolism (21). Also, cytokines such as IL-1 β in human proximal tubule cells induce HIF-1 and VEGF activities, as evidenced by measuring their mRNA and protein expression, as well as the increased expression of genes that are up-regulated by HIF (22). In addition, results from our laboratory have shown that cytokines and high glucose concentration regulate the expression of SGLT2 (23), which suggests that a common HIF-1-mediated pathway is shared by high glucose, cytokine effects, and hypoxia in the proximal tubule.

To evaluate a possible role of HIF-1 α protein in renal damage/protection mechanisms under low oxygen partial pressure we performed a hypoxia model where renal epithelial cells were exposed to 0 and 5% O₂. We propose that HIF-1 α regulates glucose transporter expression in the cultured epithelial renal cell line LLC-PK₁ subjected to hypoxia.

EXPERIMENTAL PROCEDURES

LLC-PK₁ Cell Culture Method—LLC-PK₁ cells are a well differentiated epithelial cell line derived from porcine proximal tubule cells; this strain has been widely used as a model system for studying regulation of glucose transport and the regulation of the HIF-1. The cells form a confluent monolayer with characteristic tight junctions and microvillus and express several proximal tubule marker enzymes (24). The main feature from this tubular segment, the distal portion of the proximal tubule, is a well characterized unidirectional transepithelial transport that includes glucose, phosphate, and amino acids. Salt and water transport from apical to basolateral surface induce the formation of typical blisters or domes easily detected by light microscopy (23–25). Noteworthy, this cell line expresses HIF-1 in response to inflammatory molecules and is used to demonstrate regulation in the expression of this protein (26)

LLC-PK₁ cells (CRL-2190, ATCC) from the 124th passage were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) plus Ham's F-12 (GIBCO) medium in a 1:1 (v/v) mixture, supplemented with 10% (v/v) fetal bovine serum (FBS; GIBCO), 2 mM L-glutamine, 15 mM HEPES, 100 units/ml penicillin, and 100 μ g/ml streptomycin, pH 7.4. Cells were incubated in an atmosphere of 5% CO₂ and 95% humidity at 37 °C until monolayers were formed. When monolayers were confluent, a 0.125% trypsin and 0.5 mM EDTA solution was added, and the cells were incubated for 10 min. Cells were then harvested and suspended in fresh medium for subculture in either 25-cm² (1.0 \times 10⁶ cells) or 75-cm² (2.5 \times 10⁶ cells) polystyrene flasks (Costar, Corning).

In Vitro Hypoxia Assays—LLC-PK₁ cells were seeded at a density of 2.5 \times 10⁶ cells in 75-cm² and 1 \times 10⁶ cells in 25-cm² flasks and grown for 72 h at 37 °C to attain at least 80% confluence. The flasks were then placed in a sealed modular hypoxic chamber (Billups Rothenberg, Del Mar, CA) flushed with different oxygen (O₂) percentages. Mixture 0% O₂ was composed of 95% N₂ and 5% CO₂ (Infra). Mixture 5% O₂ was 5% O₂, 5% CO₂, and 90% N₂ (Infra). Next, the chamber was placed in an incubator at 37 °C for 1, 3, 6, and 12 h without O₂ or with a 5% O₂ mixture. Chemical hypoxia was induced with different concentrations of CoCl₂ at different times. The effect of CoCl₂ concentration on LLC-PK₁ cells was evaluated by exposing the cells to 0–500 μ M CoCl₂ for 12 h. The effect of time of exposure was assessed with 300 μ M CoCl₂ for 0, 1, 3, 6, and 12 h. Cells not exposed to hypoxia were run in parallel as controls. The cells in 75-cm² flasks were used to determine protein expression, and those in 25-cm² flasks were used to determine mRNA levels.

Effect of Albendazole on in Vitro Hypoxia Assays—Albendazole (ABZ) is a well known HIF-1 α inhibitor (27). In our model we evaluated the effect of hypoxia on the cells using this compound as an inhibitor of the expression of HIF-1 α . Cells under the same conditions for control and hypoxia monolayers were treated with ABZ at 0, 0.1, and 1 μ M concentrations before being placed in their respective environmental chamber and flushed with 0% O₂ or with added CoCl₂ (300 μ M) for 6 h. The cells were then processed as described above for protein determination.

Cell Homogenate Preparations—After incubation with the respective gas mixtures or CoCl₂, cell monolayers were placed on an ice bath and washed immediately with ice-cold phosphate-buffered saline (PBS, 138 mM NaCl, 3 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4). The monolayers were then detached with a scraper, collected in 5 ml of ice-cold PBS, and washed at 400 \times g for 5 min. Cells were concentrated by centrifugation at 600 \times g at 4 °C for 5 min, and the cell pellet was then resuspended in 500 μ l of ice-cold lysis solution (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 0.1 mM PMSF, and a mixture of protease inhibitors diluted 1:25 (v/v) (Sigma). Cell suspension was then homogenized by pipetting up and down until completely dissolved and subsequently incubated at 4 °C for 10 min. Cell debris, nuclei, and heavy organelles were removed by centrifugation at 13,000 \times g, for 10 min at 4 °C, and supernatant aliquots were taken and stored at –80 °C until use. A separate 50- μ l aliquot was taken for protein determination using the bicinchoninic acid assay.

Protein Determination—Total protein content was determined by the bicinchoninic acid assay method using a commercial kit (Pierce). Standard curves were prepared with bovine γ -globulin from 5 to 100 μ g/ml protein. Standard curves were repeated every time when new samples were determined, with each point of the curve done in triplicate. Each sample was performed in triplicate for every homogenate. Once protein was determined, the protein concentration in final samples was adjusted with lysis solution to 3 μ g/ μ l. Spectrophotometric readings were performed at λ = 570 nm in an ELx800 microplate reader (BioTek Instruments, Winooski, VT).

HIF-1 α Regulates Renal Glucose Transporters

Western Blot Assays—Western blot assays were performed immediately after protein determination; 30 μ g of total protein per sample was used for each SDS-PAGE lane, and electrophoresis was carried out for 120 min. Once finished, the lanes were transferred to nitrocellulose membranes in a semi-dry Trans-Blot Cell (Bio-Rad). The membranes were then incubated with fresh TBS-T buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5, plus 0.5% Tween 20) containing 5% bovine serum albumin, for 1 h at room temperature. Membranes were washed thoroughly in TBS-T buffer and incubated overnight at 4 °C with the corresponding primary antibody to HIF-1 α , SGLT1, SGLT2, and GLUT1 (dilution 1:500, 1:600, 1:600, and 1:500, respectively). The β -actin antibody (dilution 1:7500) was used as a control. The membranes were then washed three times with TBS-T buffer. Immunodetection was performed with the use of HRP-secondary antibody diluted 1:11,000 (anti-mouse to HIF-1 α), 1:13,000 (anti-goat to SGLT1 and SGLT2), 1:11,000 (anti-goat to GLUT1), 1:20,000 (anti-mouse to β -actin) followed by chemiluminescence detection (SuperSignal West Pico). Blots were contrasted to β -actin bands and results expressed as -fold versus the control value (100%).

mRNA Detection by Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)—The effects of hypoxia on HIF-1 α , SGLT1, SGLT2, and GLUT1 mRNA in LLC-PK₁ cells were evaluated by semiquantitative RT-PCR. Total RNA was isolated with TRIzol (Invitrogen), and cDNA was synthesized using 2 μ g of total RNA, 0.5 μ g of oligo(dT)₁₅ primers, and 200 units of MMLV reverse transcriptase at 38 °C for 60 min.

PCR amplification of 1 μ g of cDNA used 1.5 mM MgCl₂, 0.2 mM dNTP mix, 1.5 IU of *Taq* polymerase, and a 0.25 μ M concentration of each specific primer (see below). After an initial incubation at 94 °C for 3 min, 30 amplification cycles consisting of 94 °C for 45 s, 30 s at annealing temperature (see below), and a 90-s extension were followed by a final extension at 72 °C for 10 min. Annealing was at 56 °C for HIF-1 α , 57 °C for SGLT1, SGLT2, and r18S and 58 °C for GLUT1. The gene-specific primers used to selectively amplify genes were as follows: HIF-1 α , 5'-CTC-AAA-GTC-GGA-CAG-CCT-CA-3' (forward) and 5'-CCC-TGC-AGT-AGG-TTT-CTG-CT-3' (reverse); SGLT1, 5'-ATG-GAC-AGT-AGC-ACC-TTG-AGC-C-3' (forward) and 5'TAG-CCC-CAG-AGA-AGA-TGT-CTG-C-3' (reverse); SGLT2, 5'-CCA-ATA-GAG-GCA-CAG-TTG-GTG-G-3' (forward) and 5'-GCG-TAA-ATG-TTC-CAG-CCC-AGG-3' (reverse); GLUT1, 5'-AAG-TCT-CCT-TTA-CCC-ACA-TCC-3' (forward) and 5'-GAG-TGT-CCG-TGT-CTT-CTT-GAG-T-3' (reverse); 18S, 5'-CGA-CGA-CCC-ATT-CGA-ACG-TCT-3' (forward) and 5'-GCT-ATT-GGA-GCT-GGA-ATT-ACC-G-3' (reverse).

PCR products were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. Scanning densitometry in a Gel Doc XR System from Bio-Rad with Quantity-One one-dimensional Analysis Software, version 4.6.1, was used for a semiquantitative assessment of band densities.

Viability Assay—LLC-PK₁ cell viability was evaluated with the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation kit. The number of metabolically active cells was determined by the ability of cells to reduce the tetrazolium compound (MTS) and the electron coupling reagent (phenazinemethosulfate) into formazan. Monolayers grown in 96-well plates at a density

of 10⁵ cells/well were incubated for 24 h, and the medium was exchanged with serum-free medium 1 h before cells were exposed to hypoxia (0% O₂) or CoCl₂ for 0, 1, 3, 6, and 12 h. This was followed by the addition of 20 μ l/well of a mixture MTS/phenazinemethosulfate (1:20). After 1 h at 37 °C in a humidified, 5% CO₂ atmosphere, the absorbance at 490 nm was determined in a microplate reader (BioTek).

Determination of Glucose in the Media—To determine the medium glucose concentration of LLC-PK₁ cell monolayers under hypoxic conditions in a time-dependent manner, 2.5 \times 10⁵ cells/well were seeded in 24-well plates and incubated under conditions of 0% O₂ and 300 μ M CoCl₂ for 0, 1, 3, 6, and 12 h. Next, 500 μ l of culture medium was collected and clarified by centrifugation before the concentration of glucose was quantified using a glucose oxidase-peroxidase (GOD/POD) assay kit (Elitech). Briefly, 0–50 mg/ml glucose standard calibration curves were prepared as a reference, and 10 μ l of medium of each sample was incubated with the reaction assay buffer containing glucose oxidase-peroxidase enzymes at 37 °C for 15 min. Absorbances for both standard and samples were measured at 490 nm.

Materials—DMEM, Ham's F-12 medium, and FBS were obtained from Invitrogen. SuperSignal West Pico reagents and detection system were obtained from Pierce Thermo Fisher. Molecular weight markers and electrophoresis reagents were purchased from Bio-Rad. Antibodies to HIF-1 α , SGLT1, SGLT2, and GLUT1 were purchased from Santa Cruz Biotechnology. Monoclonal anti- β -actin, polyclonal anti-goat-HRP, TNF α , and other chemicals and materials were from Sigma-Aldrich.

Data Analysis—All values are expressed as the mean \pm S.D. Protein and mRNA expression values were evaluated by one-way analysis of variance followed by post hoc Dunnett and Bonferroni tests, using SigmaStat software v11.2. (Systat Software). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Time Course of Effect of Hypoxia, 0 and 5% O₂, on HIF-1 α mRNA and Protein Expression—LLC-PK₁ cells monolayers exposed to 5% O₂ showed increased HIF-1 α mRNA expression at 6 and 12 h compared with that at $t = 0$ ($p < 0.05$) (Fig. 1A). Incubation of cells in 0% O₂ (Fig. 1B) showed a significant increase in HIF-1 α mRNA expression at 3, 6, and 12 h compared with $t = 0$ ($p < 0.05$). No effect was observed for 1 h (data not shown). HIF-1 α protein increased nearly 10 times with a 1-h exposure to 5% O₂ compared with $t = 0$ ($p < 0.05$). The effect was sustained at 3, 6, and 12 h versus $t = 0$ h ($p < 0.05$) (Fig. 1C), whereas no differences were observed in these results compared with 1 h. Fig. 1D shows a similar augmentation in HIF-1 α protein at 1, 3, 6, and 12 h ($p < 0.05$ versus $t = 0$); however, at 12 h, the protein values increased compared with 1 h ($p < 0.05$).

Effect of CoCl₂ on HIF-1 α mRNA and Protein Expression—Fig. 2A shows a concentration-dependent effect on HIF-1 α mRNA expression when cells were incubated with different CoCl₂ concentrations for 12 h. HIF-1 α mRNA increased with 300 and 500 μ M CoCl₂ compared with control ($p < 0.05$); 50 and 100 μ M CoCl₂ did not produce any effect on HIF-1 α mRNA expression (data not shown). HIF-1 α protein increased in a

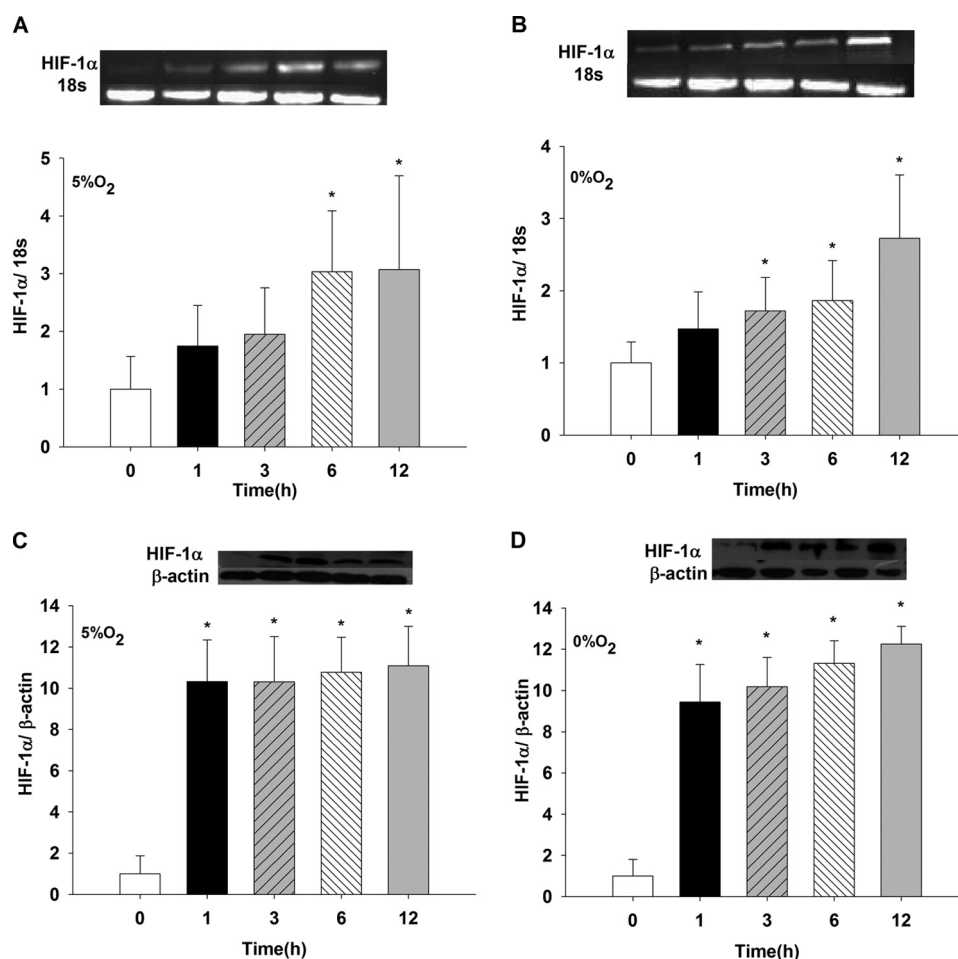


FIGURE 1. Induction of mRNA and protein expression of HIF-1 α in LLC-PK₁ cells under conditions of low oxygen (5% O₂ and 0% O₂). LLC-PK₁ cells were exposed to hypoxia, 5% O₂ (A and C) and 0% O₂ (B and D), for 1, 3, 6, and 12 h. mRNA HIF-1 α was determined by PCR and HIF-1 α protein by Western blotting (*, $p < 0.05$ versus $t = 0$ h). Values are means \pm S.D. (error bars) for six independent experiments, each in duplicate.

dose-dependent manner with increasing CoCl₂ concentrations (Fig. 2C) for 12 h ($p < 0.05$ for 50, 100, 300, and 500 μ M CoCl₂ versus control). Thus, we chose 300 μ M CoCl₂ in further experiments. In addition, as a positive control, two TNF α concentrations, 500 and 1000 ng/ml, significantly induced HIF-1 α protein expression compared with control values ($p < 0.05$). The increase of HIF-1 α protein induced with 1000 ng/ml TNF α was similar to the one obtained with 300 and 500 μ M CoCl₂ (data not shown and Fig. 2C). Incubation with 300 μ M CoCl₂ (Fig. 2B) increased HIF-1 α mRNA expression only at 6 and 12 h versus $t = 0$ ($p < 0.05$). In the presence of 300 μ M CoCl₂, protein expression was also time-dependent (Fig. 2D); between 1 and 6 h, there was almost a 9-fold increase (1 and 3 h versus $t = 0$ h, $p < 0.05$), and between 6 and 12 h, protein levels increased 10–12-fold ($p < 0.05$ versus $t = 0$).

Effect of Hypoxia, 0 and 5% O₂, on SGLT1 and SGLT2 mRNA and Protein Expression—SGLT1 mRNA decreased when cells were exposed to either 0% or 5% O₂ compared with $t = 0$ for 3, 6, and 12 h ($p < 0.05$ versus $t = 0$ h with 5% O₂) and for 1, 3, 6, and 12 h versus $t = 0$ h with 0% of O₂ ($p < 0.05$) (Fig. 3, A and B, respectively). No differences were found when the two O₂ concentrations were compared at their respective times (data not shown, $n = 6$).

SGLT1 protein decreased when cells were exposed to either 0% or 5% O₂ compared with $t = 0$ for 6 and 12 h versus 0 h with 5% O₂ and for 1, 3, 6, and 12 h versus $t = 0$ h with 0% O₂ ($p < 0.05$) (Fig. 3, C and D, respectively). No differences were found when the two O₂ concentrations were compared at their respective times (data not shown, $n = 6$).

Fig. 4A shows the effect of 5% O₂ on LLC-PK₁ SGLT2 mRNA. A 60% reduction in mRNA was observed as of 1-h incubation, the effect persisted until or up to 12 h (1, 3, 6 and 12 h versus $t = 0$, $p < 0.05$). Incubation with 0% O₂ showed a time-dependent decreasing pattern at 3, 6, and 12 h compared with $t = 0$ ($p < 0.05$) (Fig. 4B), where no change was observed for 1-h incubation (data not shown).

SGLT2 protein decreased when cells were exposed to either 0% or 5% O₂ compared with $t = 0$ (black bars) for 6 and 12 h versus 0 h with 5% O₂ and for 3, 6, and 12 h versus 0 h with 0% O₂ ($p < 0.05$) (Fig. 4, C and D, respectively). No differences were found when the two O₂ concentrations were compared (data not shown, $n = 6$).

Effect of CoCl₂ on SGLT1 and SGLT2 mRNA and Protein Expression—Fig. 5, A and B, shows that SGLT1 and SGLT2 mRNA decreased with exposure to 300 and 500 μ M CoCl₂ for 12 h compared with controls ($p < 0.05$ versus control, $n = 6$).

HIF-1 α Regulates Renal Glucose Transporters

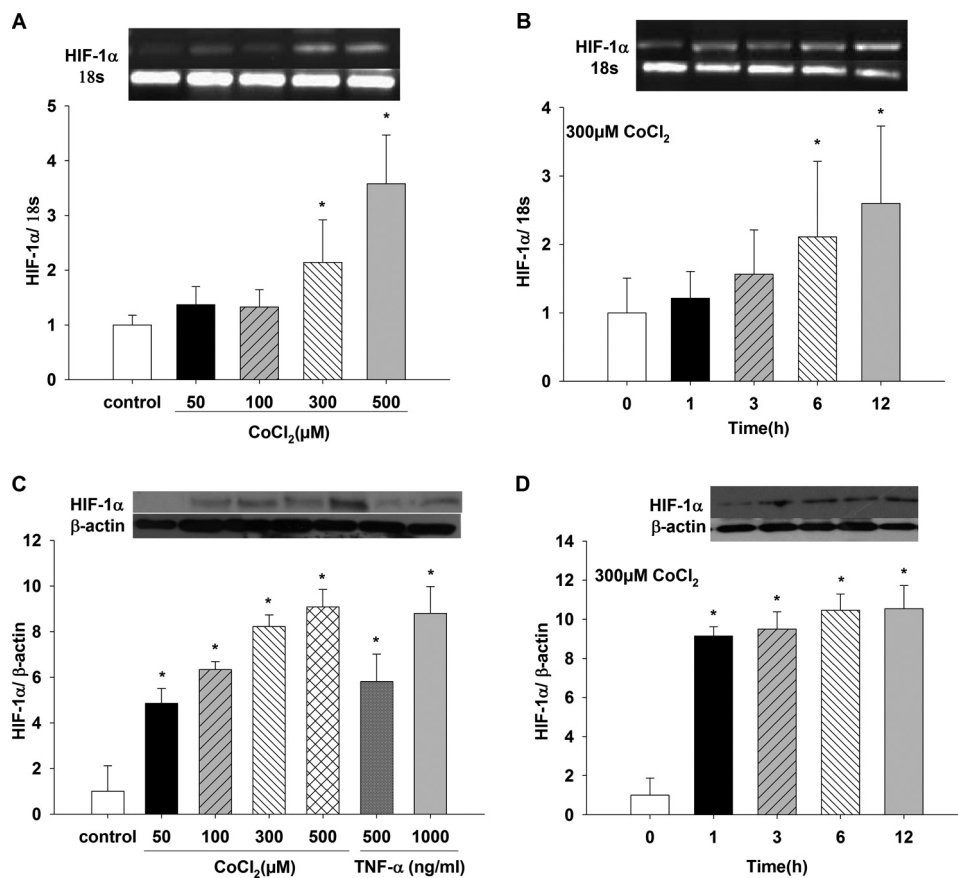


FIGURE 2. Induction of mRNA and protein expression of HIF-1 α in LLC-PK₁ cells under chemical hypoxia with CoCl₂. LLC-PK₁ cells were treated for 12 h with different CoCl₂ concentrations (50, 100, 300, and 500 μ M). **A** and **C**, changes in HIF-1 α mRNA (**A**) and protein (**C**) expression were determined by PCR and Western blotting, respectively (*, $p < 0.05$ versus control). **B** and **D**, changes in HIF-1 α mRNA (**B**) and protein (**D**) expression in LLC-PK₁ cells incubated with 300 μ M CoCl₂ for 1, 3, 6, and 12 h were assessed by PCR and Western blotting, respectively (*, $p < 0.05$ versus $t = 0$ h). Values are means \pm S.D. (error bars) for six independent experiments, each in duplicate.

Fig. 5C shows that SGLT1 protein expression decreased in a dose-dependent manner when cells were incubated with different CoCl₂ concentrations for 12 h (100, 300, and 500 μ M CoCl₂ versus control, $p < 0.05$, $n = 6$), whereas SGLT2 protein decreased with 300 and 500 μ M CoCl₂ for 12 h compared with control ($p < 0.05$, $n = 6$) (Fig. 5D).

Effect of Hypoxia, 0 and 5% O₂, on GLUT1 mRNA and Protein Expression—When the GLUT1 transporter was evaluated under similar conditions as were SGLT1 and SGLT2, mRNA increased 2-fold at 5 and 0% O₂ for 12 h, compared with $t = 0$ ($p < 0.05$, $n = 6$) (Fig. 6A and B). At 1, 3, and 6 h, values did not differ from $t = 0$ (data not shown, $n = 6$). GLUT1 protein expression showed a time-dependent course at 5% O₂, with significant high values observed at 3, 6, and 12 h when contrasted with $t = 0$ ($p < 0.05$, $n = 6$) (Fig. 6C). At 0% O₂, significant high values were obtained for 6 and 12 h versus $t = 0$ (Fig. 6D). No significant values were observed for 1 and 3 h (data not shown, $n = 6$).

Effect of CoCl₂ on GLUT1 mRNA and Protein Expression—CoCl₂ concentrations of 0, 50, 100, 300, and 500 μ M were tested on GLUT1 mRNA and protein expression. mRNA was increased at 300 and 500 μ M compared with control ($p < 0.05$, $n = 6$); 50 and 100 μ M CoCl₂ did not change mRNA values (data not shown, $n = 6$) (Fig. 7A). Protein expression was increased at 300 and 500 μ M CoCl₂ contrasted with control ($p < 0.05$, $n = 6$);

50 and 100 μ M CoCl₂ did not show significant changes compared with control (data not shown, $n = 6$) (Fig. 7B).

Effect of ABZ, Hypoxia, and CoCl₂ on HIF-1 α and GLUT1—ABZ, 0.1 and 1.0 μ M, inhibited HIF-1 α protein expression when cell monolayers were incubated with 0% O₂ for 6 h (Fig. 8A). Both concentrations were different compared with the observed increase in HIF-1 α at 0% O₂ ($p < 0.05$, $n = 6$). HIF-1 α was augmented 7-fold compared with control value incubated at 20% O₂ for 6 h ($p < 0.05$, $n = 6$). HIF-1 α Western blot assays showed a decrease in protein expression at 1.0 μ M ABZ and 300 μ M CoCl₂ compared with the increase in HIF-1 α at 300 μ M CoCl₂ ($p < 0.05$, $n = 6$) (Fig. 8B); 0.1 μ M CoCl₂ did not show a significant effect (data not shown, $n = 6$) (Fig. 8B). ABZ at 1.0 μ M significantly decreased GLUT1 protein expression (Fig. 8C) when incubated at 0% O₂ for 6 h and compared with the augmented GLUT1 expression under the same conditions. ABZ at 0.1 and 1.0 μ M in the presence of 300 μ M CoCl₂ inhibited the increased GLUT1 protein expression in the presence of 300 μ M CoCl₂ without ABZ ($p < 0.05$, $n = 6$) (Fig. 8D).

Viability Assay—Cell viability was monitored for all the exposure times to either 0% O₂ or 300 μ M CoCl₂, and the results are shown in Table 1. No reduction in cell viability was observed for 0% O₂ at any time: 1, 3, 6, and 12 h (data not shown, versus $t = 0$ and its respective incubation time). However, 300 μ M CoCl₂ caused a reduction in cell viability after a 1-h incubation,

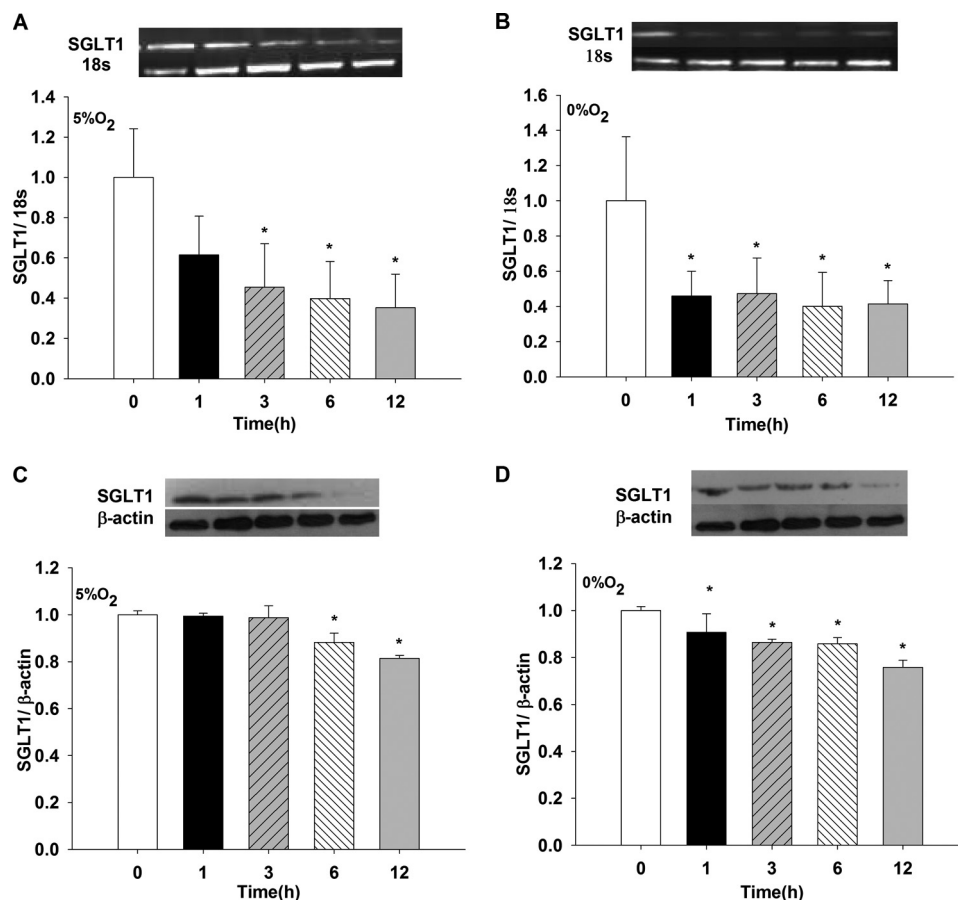


FIGURE 3. **Effects of hypoxia (5% O₂ and 0% O₂) on SGLT1 mRNA and protein expression in LLC-PK₁ cells.** LLC-PK₁ monolayers were incubated in 5% O₂ (A and C) and 0% O₂ (B and D) for 1, 3, 6, and 12 h. SGLT1 mRNA and protein were evaluated by PCR and Western blotting, respectively (*, $p < 0.05$ versus $t = 0$ h). Values are means \pm S.D. (error bars) for six independent experiments, each in duplicate.

which persisted up to 12 h ($p < 0.05$). Compared with its respective time, a significant reduction in cell viability was observed ($p < 0.05$).

Glucose Concentrations in the Medium under Hypoxia—To determine glucose concentration in the medium at different times, we measured the glucose concentration after 1, 3, 6, and 12 h (Fig. 9). Cells under control conditions showed decreased glucose concentration in the medium between 3 and 12 h ($p < 0.05$ versus $t = 0$). The effect of 300 μ M CoCl₂ at 1 h was decreased from $t = 0$ and 1 h for control and 0% O₂ values ($p < 0.05$ versus $t = 0$; $p < 0.05$ versus control 1 h and 0% O₂ 1 h). From 3 to 12 h, 0% O₂ and 300 μ M CoCl₂ significantly diminished glucose concentration compared with $t = 0$ and with their respective controls ($p < 0.05$).

DISCUSSION

HIF-1 α is considered a key molecule for many intracellular responses mediated by hypoxia in many tissues including the kidney (28). In this study, we found that HIF-1 α was increased under hypoxia in a model of epithelial tubular proximal cells in culture. These findings correlated with a decrease in the expression of renal SGLT glucose transporters. We also confirmed that GLUT1 expression is increased in these cells when HIF-1 α is stimulated by hypoxia. Similar results for GLUT1 have been reported in kidney cells, nucleus pulposus cells of the rat inter-

vertebral disk, and in mouse and human β cells from Langerhans islets (29, 30).

For this study we used two general methods to test the effects of hypoxia on cell cultures. First, CoCl₂ salts have been widely proposed as a chemical-induced hypoxia method, thus we included these assays as a positive control for the expression HIF-1 α . In addition, CoCl₂ salts have the advantage of being a rapid and inexpensive maneuver to induce concentration-dependent hypoxic states in cultured cells (27, 31).

However, to be sure that low p O₂ (0 and 5%) contributes to HIF expression we induced air hypoxia by means of modular incubator chambers. Hypoxic chambers have the advantage of not using drugs that can disrupt cell biochemistry reactions or interact with cellular organelles not directly involved in oxygen intracellular tension. Because HIF-1 α can be expressed constitutively under normoxia in some cell lines (1, 8, 10), it is critical to compare low p O₂ versus normoxic conditions (16%) to determine the basal level of HIF in these cell lines (26). Thus, in our experimental conditions we used low p O₂ (0 and 5%) because only p O₂ < 1.0% caused hypoxia in the isolated proximal tubule (32, 33).

In our results low concentrations of O₂ induced an increase in HIF-1 α protein and mRNA expression. These results were more pronounced when 0% O₂ was used. In fact, very low concentrations of O₂ (<0.5%) induce HIF-1 α expression in renal

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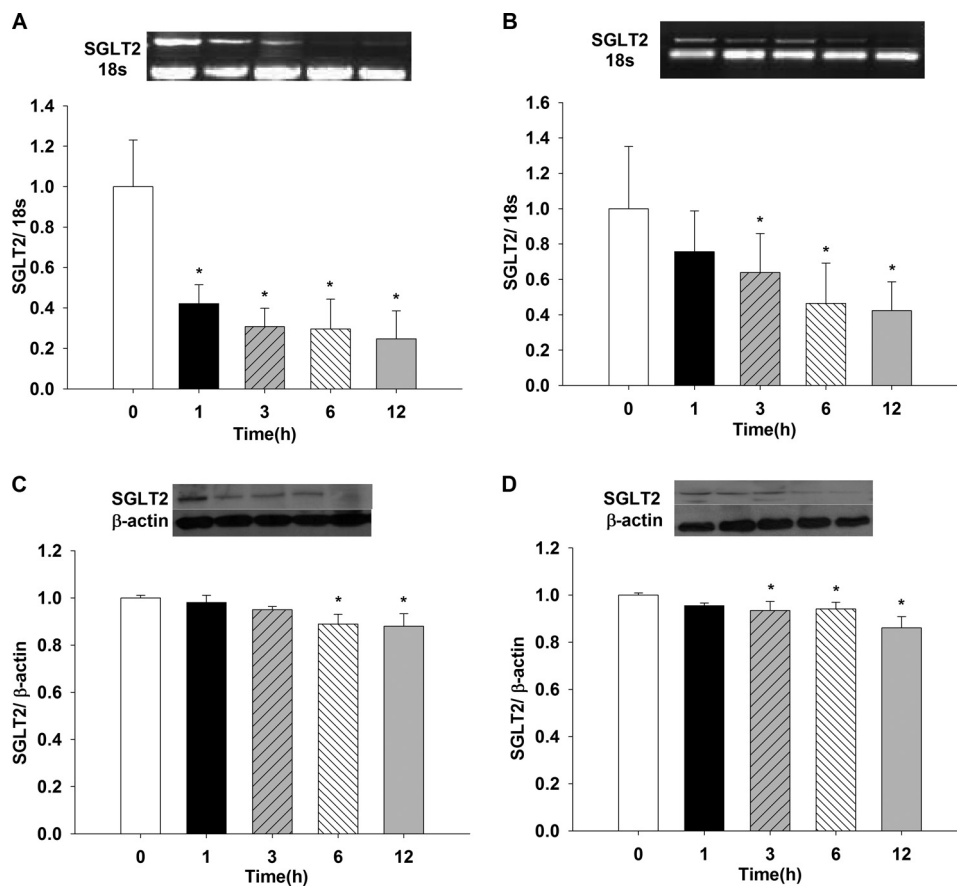


FIGURE 4. **Effects of hypoxia (5% O₂ and 0% O₂) on SGLT2 mRNA and protein expression in LLC-PK₁ cells.** LLC-PK₁ monolayers were incubated in 5% O₂ (A and C) and 0% O₂ (B and D) for 1, 3, 6, and 12 h. SGLT2 mRNA and protein were evaluated by PCR and Western blotting, respectively (*, $p < 0.05$ versus $t = 0$ h). Values are means \pm S.D. (error bars) for six independent experiments, each in duplicate.

tissues (34). With 0% O₂, mRNA expression showed a time-dependent pattern, whereas protein HIF-1 α increased maximally after 1 h, and the response was maintained for 12 h. In our work, maximal time-dependent responses for protein expression from 1 to 12 h at 0 and 5% O₂ can be explained by the inhibition of the HIF-1 α protein degradation process normally stimulated by low oxygen concentrations (35). The results for mRNA did not correlate with HIF-1 α protein expression; one possible explanation is that basal activities of hydroxylases and von Hippel-Lindau initially inactivate protein expression, until the mRNA rate of synthesis surpasses the pool reserves of von Hippel-Lindau and associated proteins. We also suggest that other factors (for example TNF α and IL-1 β) may contribute to this mechanism. These cytokines are present in this renal cell line; in pathological conditions such as is the case with inflammatory processes in human proximal tubular epithelial cells and in retinal cells, HIF-1 α is increased (22, 23, 36).

To confirm that hypoxia is responsible for the induction of HIF-1 α protein we performed experiments in the presence of CoCl₂. The use of CoCl₂ has been well documented as a model of chemical hypoxia in *in vitro* and *in vivo* models (27). Our results indicated that CoCl₂ had similar effects on mRNA and protein expression as 0 and 5% O₂. Evidence indicates that the use of CoCl₂ modified HIF-1 α in the kidney of rats (16, 19). Sandau *et al.* demonstrated that TNF α in LLC-PK₁ cells induced HIF-1 α protein expression (26). In our model, 500

and 1000 ng/ml TNF α confirmed an increased HIF-1 α protein expression, and such experiments suggest that other factors present in the medium and released by hypoxia may also induce changes in HIF-1 α protein expression (IL-1 β , TNF α , angiotensin II, and endothelin-1). Thus, taking these results together, it is clear that hypoxia increased HIF-1 α mRNA and protein expression in our model directly or mediated by other factors.

We and others have proposed that renal glucose transporters responsible for the recovery of the sugar from the tubular proximal fluid may be regulated by high glucose concentrations and cytokines. In this sense, Ohta *et al.* (24) demonstrated a decreased expression of SGLT1 and GLUT1 mRNA in LLC-PK₁ cells exposed to high concentrations of glucose (25 mM). Furthermore, we recently reported that SGLT2 mRNA expression increased under high glucose (25 mM). These effects are also attained with IL-6 (10 pg/ml) and TNF α (10 pg/ml) on SGLT1 mRNA expression and SGLT2 mRNA and protein expression (23). Thus, it is likely that HIF-1 α is involved in the changes in renal glucose transporters shown by hypoxia, high glucose, and cytokines.

Epithelial glucose transporters may undergo changes at the transcriptional level as a result of high glucose concentrations. Specifically, the mRNA expression of GLUT1 and SGLT1 decreased when LLC-PK₁ cells were incubated with 25 mM D-glucose (25). Our results showed that both chemi-

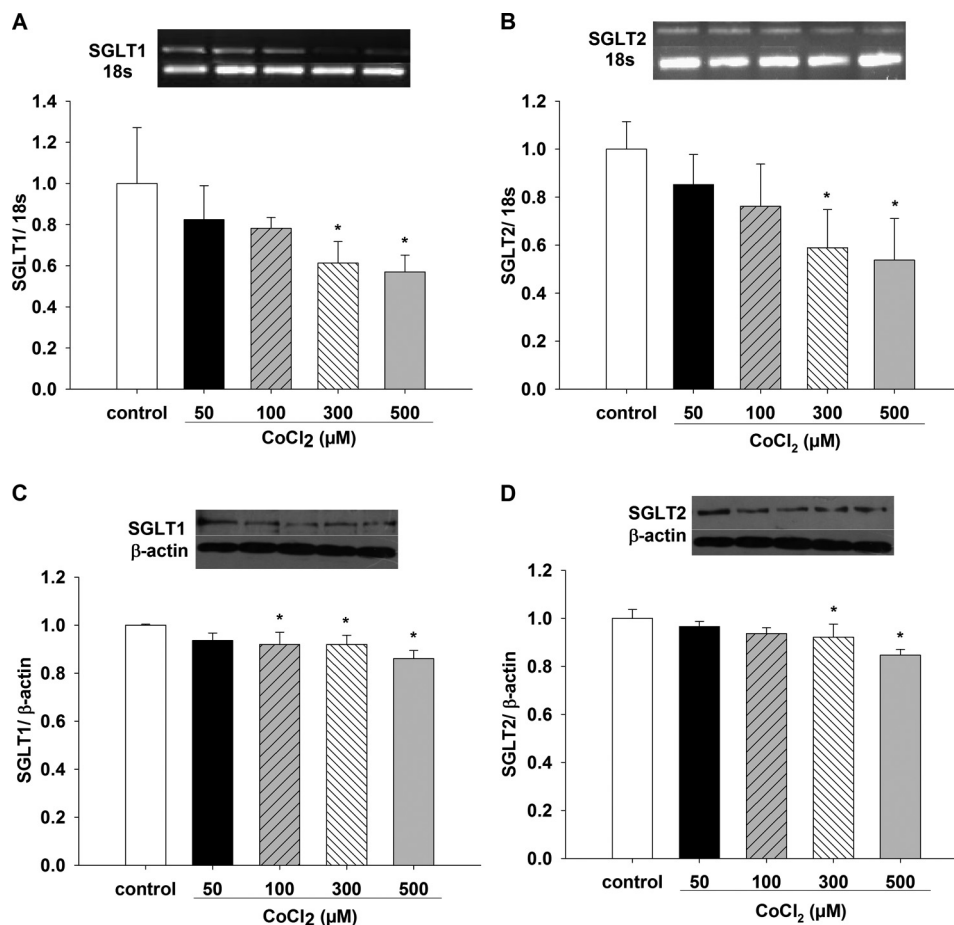


FIGURE 5. **Effects of chemical hypoxia with CoCl₂ on SGLT1 and SGLT2 mRNA and protein expression in LLC-PK₁ cells.** Changes in SGLT1 and SGLT2 expression in LLC-PK₁ cells treated for 12 h with different CoCl₂ concentrations (50, 100, 300, and 500 μM) were evaluated for mRNA (A and B) by PCR and for protein (C and D) by Western blotting (*, $p < 0.05$ versus control). Values are means \pm S.D. (error bars) for six independent experiments, each in duplicate.

cal and low O₂ hypoxia decreases mRNA and protein expression of SGLT1 and SGLT2. These results are similar to those obtained by Kles and Tappenden (37) in a model of rat jejunum epithelium hypoxia. Glucose transport was measured for 1 h in rat jejunum perfused with a solution containing mannitol, glucose, or glutamine. Under these conditions, glucose uptake was reduced, whereas glutamine transport remained unchanged. Brush border membranes obtained from these preparations showed a significant decrease in SGLT1 mRNA under hypoxic conditions, but protein expression was not modified. These data indicate that the activity and mRNA expression level of SGLT1 are regulated by hypoxia, without changes in protein expression.

Evidence has shown a clear increase in renal GLUT1 during hypoxia mediated by HIF-1. In human tubular epithelial cells, GLUT1 mRNA has been found to be increased under 1% O₂ hypoxia (38). However, renal HIF-1 increases the expression of the GLUT1 gene, currently used as a control to test the effects of HIF-1 α . Our results clearly confirm an increase in mRNA and protein expression of GLUT1, which correlates with the changes in HIF-1 α mRNA and protein. To explore further whether the effects were caused by this protein, we tested the response to hypoxia in the presence of albendazole, a compound reportedly known for its ability to inhibit the effect of HIF-1 α and VEGF in human ovarian cancer cells (27). Conse-

quently, a decrease in HIF-1 α and GLUT1 expression under hypoxia was confirmed with 1 μM albendazole in our model.

No changes in viability were observed under 0% O₂ hypoxia at all incubation times. However, 300 μM CoCl₂, the usual concentration used to induce HIF-1 α expression, caused a reduction in cell viability to 50% at 12 h; these changes were noted with 1-, 3-, and 6-h incubations. Because CoCl₂ has been widely used as a model to induce chemical hypoxia, results obtained with this compound must be analyzed with care, as other dose-related mechanisms are linked to CoCl₂ (39).

Cobalt salts can induce chemical hypoxia by two mechanisms of action. The first and best known of them is the inhibition of prolyl hydroxylases by binding to a HIF-1 α oxygen-dependent degradation domain (40); the second mechanism involves the inhibition of von Hippel-Lindau protein binding to HIF-1 α (31). The latter may account for the actions of cobalt in chemical hypoxia at low doses; however, there are scarce published reports describing this chemical interaction. In this sense, cobalt salts mitigate the development of diabetic nephropathy, where they decrease proteinuria and tubulointerstitial fibrosis in a type 2 diabetes model in rats (16). Matsumoto *et al.* showed that cobalt administration in a single dose activates protector genes in the kidneys before exposure to ischemia by activating HIF-1 and up-regulating HO-1, EPO, GLUT1, and VEGF mRNA expression (19).

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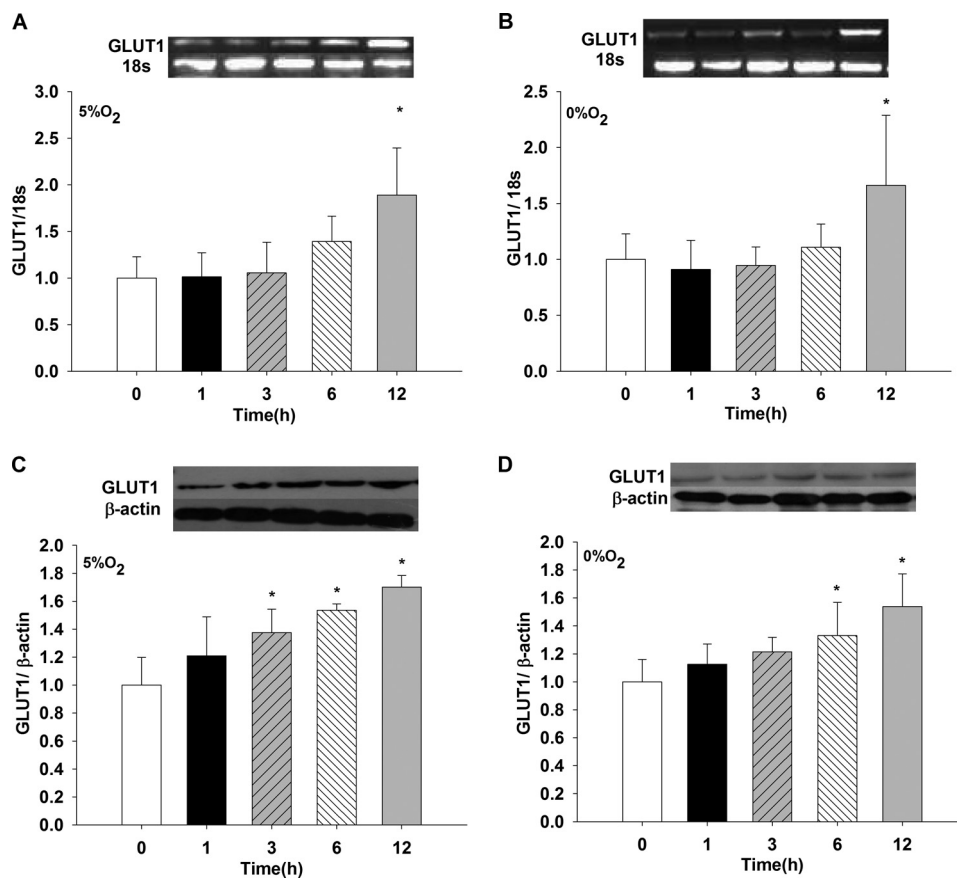


FIGURE 6. **Effects of hypoxia (5% O₂ and 0% O₂) on GLUT1 mRNA and protein expression in LLC-PK₁ cells.** LLC-PK₁ monolayers were incubated in 5% O₂ (A and C) and 0% O₂ (B and D) for 1, 3, 6, and 12 h. GLUT1 mRNA and protein were determined by PCR and Western blotting, respectively (*, $p < 0.05$ versus $t = 0$ h). Values are means \pm S.D. (error bars) for six independent experiments, each in duplicate.

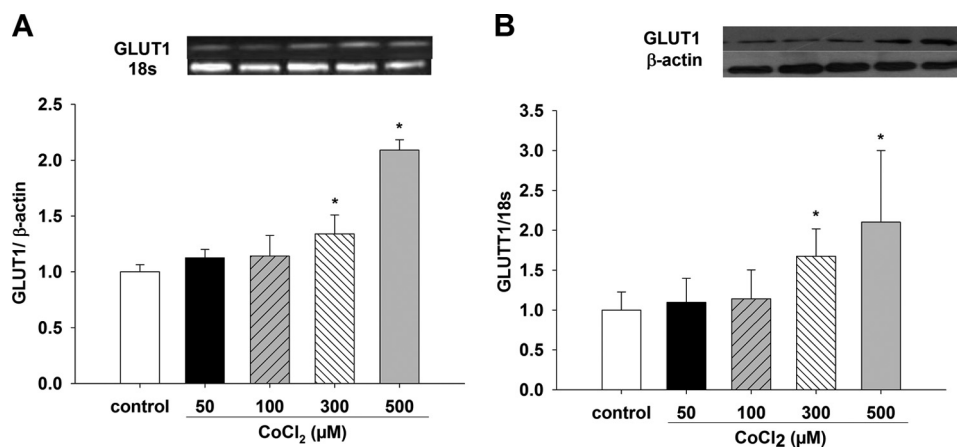


FIGURE 7. **Effects of chemical hypoxia with CoCl₂ on GLUT1 mRNA and protein expression in LLC-PK₁ cells.** Changes in GLUT1 mRNA and protein expression in LLC-PK₁ cells were evaluated under conditions of chemical hypoxia for 12 h with different CoCl₂ concentrations (50, 100, 300, and 500 μM). GLUT1 mRNA (A) and protein (B) were determined by PCR and Western blotting, respectively (*, $p < 0.05$ versus control). Values are means \pm S.D. (error bars) for six independent experiments, each in duplicate.

In addition, cobalt at high doses has genotoxic and carcinogenic effects in *in vivo* and *in vitro* models, due to resulting DNA damage and interference with DNA repair mechanisms (39, 41). This accounts for the higher cell viability with 0–5% O₂ hypoxia in a sealed modular hypoxic chamber compared with the use of CoCl₂.

Renal blood flow rate represents one fifth of the cardiac output, and compared with other organs, O₂ consumption is high

under basal conditions (42). When Na⁺ reabsorption mechanisms are activated under experimental observations, an increased O₂ consumption is normally observed in the kidney (43). However, intravenous perfusion of amino acids in healthy men has not shown to change the O₂ basal levels in the kidney (44). Thus, the kidney maintains a relatively low and constant O₂ uptake in both basal and stimulated conditions, with exception to the sodium reabsorption rate. However, a 1:7 ratio is

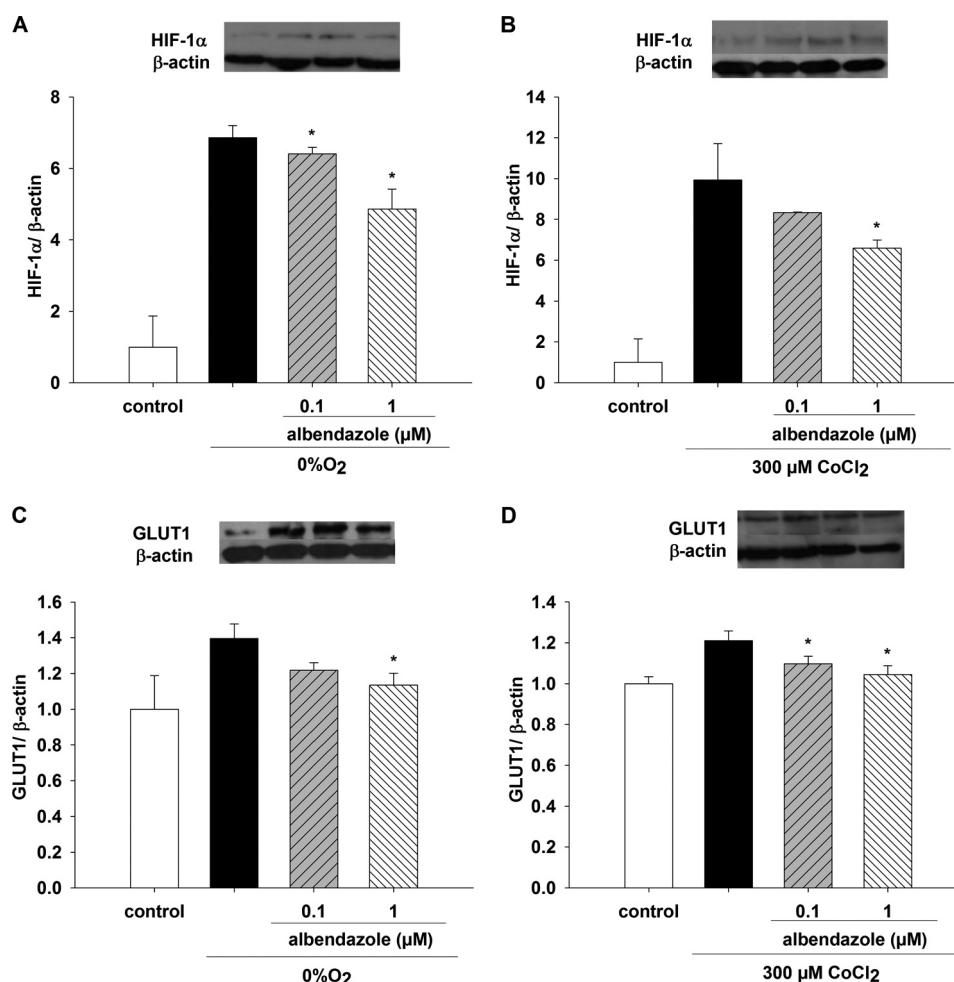


FIGURE 8. **Effect of ABZ on HIF-1 α and GLUT1 protein expression under hypoxia in LLC-PK₁ cells.** LLC-PK₁ monolayers were preincubated for 1 h with 0.1 and 1 μ M ABZ. Changes in HIF-1 α and GLUT1 expression were evaluated under conditions of 0% O₂ (A and C) or 300 μ M CoCl₂ (B and D) for 6 h. HIF-1 α (A and B) and GLUT1 proteins (C and D) were determined by Western blotting (*, $p < 0.05$ versus 0% O₂ or 300 μ M CoCl₂, in absence of ABZ). Values are means \pm S.D. (error bars) for six independent experiments, each in duplicate.

TABLE 1
Viability assay in LLC-PK₁ cells

LLC-PK₁ cell viability was evaluated under hypoxia conditions (0% O₂ and 300 μ M CoCl₂) for 0, 1, 3, 6, and 12 h. Values are means \pm S.D. for three independent experiments done in triplicate.

Hypoxia conditions	Time (h)					<i>p</i>
	0	1	3	6	12	
Control	100 \pm 17.54	100 \pm 20.40	100 \pm 9.71	100 \pm 8.40	100 \pm 27.00	NS ^a <0.05 vs. respective time
Oxygen (0%)	100 \pm 16.96	93.12 \pm 16.48	104.51 \pm 16.77	101.14 \pm 8.83	95.15 \pm 7.71	
CoCl ₂ (300 μ M)	100 \pm 17.68	68.06 \pm 6.61	77.92 \pm 22.56	65.59 \pm 13.28	50.27 \pm 11.51	

^a NS, not significant.

maintained in O₂ consumption when comparing renal inner medulla to renal cortex. As this low p O₂ is characteristic of renal proximal tubule epithelium (45), hypoxia mechanisms are activated only when very low O₂ concentrations are achieved. In this sense, minimal reductions in p O₂ may occur under diabetic nephropathy and glomerulosclerosis, inducing the activation of intracellular signaling that leads to the up-regulation of HIF-1 α , especially in the proximal tubule, and of those transporters involved in the glycolytic pathways. Our results support the hypothesis that renal transporters may be modified under hypoxia; however, it must be noted that glucose transport has not been evaluated. As such, we cannot rule out changes in sugar transport kinetics (V_{max} and K_m). Nev-

ertheless, there is evidence that tubular necrosis in acute and chronic renal failure may decrease or limit O₂ diffusion into the renal proximal tubule epithelia, inducing critical metabolic changes in the proximal epithelium as a homeostatic recovery response (46).

In conclusion, we have demonstrated HIF-1 α mediated changes in mRNA and protein expression of the renal transporters SGLT1 and SGLT2 in LLC-PK₁ cell monolayers under low O₂ concentrations similar to those prevailing in the renal cortex. These results may explain renal epithelium cellular adaptation mechanisms to maintain intracellular glucose homeostasis *in vitro*. In addition, we propose that future research regarding the use of CoCl₂ should be ana-

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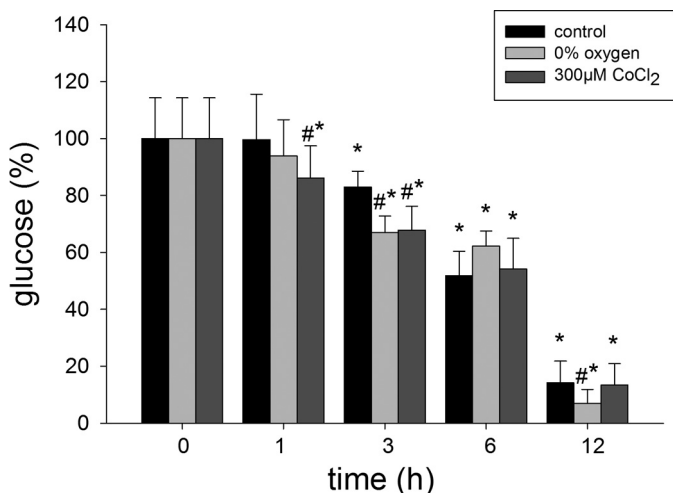


FIGURE 9. Effects of hypoxia (0% O₂) and chemical hypoxia (CoCl₂) on glucose concentrations in the medium LLC-PK₁ cell cultures. LLC-PK₁ monolayers were incubated in the presence of 0% O₂ or 300 μM CoCl₂ for 0, 1, 3, 6, and 12 h. A 500-μl aliquot of culture medium was collected, and the concentration of glucose was quantified (*, $p < 0.05$ versus $t = 0$; #, $p < 0.05$ versus control at all time points). Values are means \pm S.D. (error bars) for three independent experiments, each done in triplicate.

lyzed carefully because of its deleterious effects on cell viability.

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