VEGF Production by Primary Human Renal Proximal Tubular Cells: Requirement of HIF-1, PI3-Kinase and MAPKK-1 Signaling

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Key Words

Hypoxia-inducible factor 1 • Interleukin 1 • Vascular endothelial growth factor • Phosphatidylinositol 3-kinase • ERK1/2 (p42/44 mitogen activated protein kinase) • Renal tubules

Abstract

Renal proximal tubular epithelial cells (PTEC) respond to hypoxia exposure or interleukin-1 β (IL-1 β) treatment with increased vascular endothelial growth factor (VEGF) production. With respect to O₂ deprivation, the hypoxia-inducible factor 1 α / β (HIF-1) is the most important transcription factor driving VEGF mRNA expression. HIF-1 is also activated by IL-1 β and may thus be involved in the stimulation of VEGF production by this cytokine. However, the molecular mechanisms of HIF-1 dependent VEGF synthesis are poorly understood. Herein, human PTEC in primary culture were challenged by hypoxic incubation and/or IL-1 β treatment in absence or presence of specific phosphatidylinositol 3-kinase (PI3K) or mitogen activated protein kinase kinase-1 (MAPKK-1) inhibitors for assay of VEGF protein, VEGF mRNA and detection of HIF-1 α by Western Blotting, EMSA and fluorescence microscopy. In addition, the activities

of PI3K and MAPKK-1 were studied following hypoxia and IL-1 β treatment of the cultures. The study shows that PI3K but not MAPKK-1 inhibition resulted in the loss of hypoxic and IL-1 β induced HIF-1 α accumulation, whereas VEGF synthesis was reduced by either intervention. Thus, PI3K signaling is required for HIF-1 α accumulation and VEGF synthesis, whereas MAPKK-1 signaling is required for VEGF synthesis only. Furthermore, hypoxia alone was sufficient to activate PI3K in PTEC in contrast to MAPKK-1, whose activity was lowered in hypoxia.

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Introduction

Vascular endothelial growth factor (VEGF) is a specific mitogen and survival factor for endothelial cells [1]. VEGF is produced intrinsically, with the degree depending on the type of tissue. Hypoxia leads to increased VEGF mRNA expression [2]. Hypoxia inducible factor-1 (HIF-1) is the most important transcription factor which regulates VEGF gene transcription during O₂ deprivation. Functional HIF-1 is composed of an α - and

a β -subunit. The α -subunit of HIF-1 possesses an O_2 -dependent degradation domain (ODD) which is target of specific HIF- α prolyl hydroxylases. In the presence of O_2 , HIF- α subunits are immediately prolyl hydroxylated and these hydroxyproline residues are target of the von Hippel-Lindau tumor suppressor protein (pVHL) which guides ubiquitin E3 ligases to polyubiquitinate HIF- α . Once polyubiquitinated, α -subunits are proteasomally degraded. In hypoxia, the HIF-1 α subunit escapes from degradation and dimerizes with the HIF-1 β subunit that is constitutively present in the nucleus (for references, see [3]). In addition, several soluble peptides have the potential to induce HIF-1 α accumulation under normoxic conditions, including tumor necrosis factor α (TNF- α) and interleukin-1 (IL-1) [4], insulin and insulin-like growth factors [5], angiotensin II [6], and thrombin [7]. The mechanism of HIF-1 induction by these mediators is still poorly understood. Recently, it has been demonstrated that TNF- α leads to the accumulation of ubiquitinated HIF-1 α [8].

The necessity of specific intracellular signaling cascades in the process of HIF-1 activation is still under debate. On the one hand it is reported, that hypoxia leads to activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in some permanent cell lines [9-11] and that inhibitors of PI3K signaling prevent HIF-1 dependent gene expression in hypoxia [11-14]. On the other hand there are two reports denying PI3K to be involved in the hypoxic stabilization of HIF-1 α and HIF-1 dependent target gene expression [15, 16]. Nevertheless, PI3K signaling has been implicated in the insulin-induced VEGF mRNA expression [12, 17]. Other investigations have assigned a role for p42/p44 mitogen-activated protein kinases (MAPK) in the interaction of the HIF-1 α / β complex with its DNA binding sites [18-21].

We have reported that human proximal tubular epithelial cells (PTEC) in primary culture respond to hypoxia exposure with nuclear HIF-1 α accumulation, increased HIF-1 DNA binding and greater VEGF production. Furthermore, treatment of PTEC with the proinflammatory cytokine IL-1 β results in HIF-1 activation and increased VEGF synthesis in normoxic PTEC [22]. Herein we investigated the involvement of the PI3K and the MAPK pathways in VEGF production and HIF-1 activation in response to hypoxia and IL-1 β treatment in primary cultures of human PTEC.

Materials and Methods

Tubular cell cultures

Human proximal tubular epithelial cells (PTEC) were cultured as described previously [22]. In brief, normal renal tissue was obtained from kidneys removed for renal cell carcinoma. Cells were grown from 1 mm³ pieces of renal cortex not involved by disease on a matrix of fetal bovine serum (FBS; Biochrom KG Seromed, Berlin, Germany). PTEC for maintenance and experiments were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM)/HAM's F-12 medium in a 1 : 1 ratio (Biochrom). The medium was supplemented with insulin (5 μ g/mL), transferrin (5 μ g/mL), selenium (5 ng/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL; all from Sigma, Deisenhofen, Germany). Passages 2 to 6 were used for study. 24 h before the experiments were started, cells were placed in RPMI 1640 without FBS.

Effects of hypoxia were studied in nonconfluent cultures placed in a pO₂-controlled incubator (Hereaus, Hanau, Germany) with 20% O₂ or 3% O₂, 5% CO₂, and balanced N₂ in a humidified atmosphere at 37°C for periods of 2 h, 4 h, 6 h or 24 h as specified. IL-1 β was from by Ciba-Geigy (Basel, Switzerland). LY 294002 and PD 98059 (Calbiochem, Bad Soden, Germany) were dissolved in dimethylsulfoxide (DMSO, 10 mM) and stored in aliquots at -40°C. Prior to the experiments, cultures were pre-incubated with LY 294002 for 10 to 30 min and with PD 98059 for 30 to 60 min as specified. The final DMSO concentration was 5‰ in all cultures, including the controls.

The drugs were tested for cytotoxic effects by means of the colorimetric tetrazolium salt/formazan method, which is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) to purple formazan in living cells. Confluent PTEC cultures were handled for these studies in 96-well dishes as described [23].

Assay of VEGF and soluble VEGF receptors in culture media

VEGF_{121/165} in the culture media was measured by commercial enzyme-linked immunoassay (ELISA, Quantikine; R&D Systems, Minneapolis, MN, USA). The intra- and inter-assay coefficients of variance were < 5.0% and < 7.5%, and the lower detection limit was 9 pg/mL. Commercial ELISA systems were also applied to detect soluble VEGF R-1/Flt 1 (lower detection limit 200 pg/mL; Biomol, Hamburg, Germany) and VEGF R-2/KDR (lower detection limit 10 pg/mL; R&D Systems). The rates of the production of the proteins in the supernatants were measured in duplicates per sample and related to cellular protein as a measure of culture density. To this aim, the cultures were rinsed with phosphate-buffered saline (PBS) before lysis-buffer was added (25 mM Tris, 1 mM DTT, 1 mM EDTA, 15% glycerol, 1% Triton X-100; pH 7.8). Cellular protein was measured by the Bradford method using bovine serum albumin (BSA) as standard. The results are given as the mean and standard deviation (SD). Tukey-Kramer multiple comparisons test was applied to estimate the significance of differences ($P < 0.05$).

Real time RT-PCR quantification of VEGF₁₆₅ mRNA

Total RNA from PTECs were isolated after the 24 h incubation either with the GTC-method [24] or with the ABI Prism™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Darmstadt, Germany). 1 µg total RNA was reverse transcribed with Transcriptor reverse transcriptase (Roche, Mannheim, Germany) in a 25 µl assay. 5 µl cDNA were used as template in the PCR reaction. VEGF₁₆₅ cDNA was quantified in duplicate on an ABI 7000 Sequence detection system (Applied Biosystems) using a commercially available SYBR green PCR kit (Eurogentech, Seraing, Belgium). VEGF₁₆₅ isoform specific detection was achieved by a forward primer located in exon 3 and a reverse primer bridging the exon 5/7 junction. Primer sequences were VEGF₁₆₅-ex3for: 5'-CCCTGATGAGATCGAGTACATCTT-3'; and VEGF₁₆₅-ex5/7rev: 5'-AGCAAGGCCACAGGGATT-3'. For normalization the 60S ribosomal protein L28 served as housekeeping gene and was quantified in 5 µl of a 1:5000 dilution of the cDNA. Primer sequences were L28-for-48: 5'-ATGGTCGTGCGGAAGTCT-3' and L28-rev-149: 5'-TTGTAGCGGAAGGAATTGCG-3'. Relative expression levels were calculated with the $\Delta\Delta C_T$ method (normalized to L28 and related to the normoxic control). Specificity was verified by agarose gel electrophoresis and melting point curve analysis. All oligonucleotides used in this study were from MWG Biotech (Ebersberg, Germany).

Nuclear extract preparation

HIF-1 α protein levels were determined in nuclear extracts of PTEC incubated with or without IL-1 β (300 pg/mL) in a normoxic (20% O₂) or hypoxic (3% O₂) atmosphere. Cells were washed with ice-cold PBS and centrifuged at 800 x g for 5 min at 4°C. The cell pellets were washed with 4 mL ice-cold buffer A (10 mM Tris, pH 7.8, 1.5 mM MgCl₂, and 10 mM KCl), resuspended in this buffer and kept on ice for 10 min. Cell lysis was controlled by trypan blue staining. Nuclei were pelleted at 3,500 x g for 5 min at 4°C, and resuspended in 100 µl ice-cold buffer C (420 mM KCl, 20 mM Tris, pH 7.8, 1.5 mM MgCl₂, and 20% glycerol) and incubated on ice for 30 min with occasional flicking of the tubes. Just prior to use, buffers A and C were supplemented with 2 µg/mL aprotinin, 10 µg/mL leupeptin, 20 µg/mL pepstatin, 1 mM Na₃VO₄, 0.5 mM benzamidin, 2 mM levamisole, 10 mM β -glycerophosphate, 0.5 mM dithiothreitol (DTT), and 0.4 mM phenylmethylsulfonyl fluoride. Nuclei were centrifuged at 13,000 x g at 4°C for 30 min. The supernatants were stored at -80°C. Protein concentrations were determined by the Bradford method.

Western Blot analysis

For determination of immunoreactive HIF-1 α protein, nuclear extracts were subjected to Western Blot analysis as described [4]. Samples were run on sodium dodecyl sulfate/7.5% polyacrylamide gels and transferred to PVDF-membranes (Roth, Karlsruhe, Germany). Equal loading and transfer efficiency was verified by either staining with 2% Ponceau S or incubation with an anti-SP1 antibody (Santa Cruz, Heidelberg, Germany). Membranes were blocked overnight with PBS/5% fat-free skim milk and then incubated for 1.5 h at room temperature with a 1:1000 diluted monoclonal mouse antibody

against human HIF-1 α (BD Biosciences, Heidelberg, Germany). For determination of immunoreactive phosphorylated ERK1/2 proteins (pERK1/2), membranes were blocked for 3 h at room temperature and incubated over night with a 1:1000 diluted polyclonal rabbit anti-pERK1/2 antibody (Cell Signaling, Frankfurt/Main, Germany). For detection, horseradish peroxidase-linked anti-mouse or anti-rabbit IgG antibodies (1:2000, 1 h at room temperature; Santa Cruz) and enhanced chemiluminescence substrate (Amersham, Freiburg, Germany) were used.

Electrophoretic mobility shift assay (EMSA)

EMSAs were carried out essentially as described earlier [4]. Oligonucleotides containing the two HIF-1 binding sites from the transferrin promoter (sequences were: TfHBSww (sense): 5'-TTCCTGCACGTACACAAAGCGCACGTATTTC-3' and TfHBSww (antisense):

5'-GAAATACGTGCGCTTTGTGTGTACGTGCAGGAA-3') were 5'-end labeled with [γ -³²P] ATP (NEN, Köln, Germany) using T4-kinase (MBI Fermentas, St. Leon-Rot, Germany). Both strands were annealed and remaining ATP was separated by gel filtration. Binding reactions were set up on ice with 20,000 cpm of the labeled, doublestranded oligonucleotides in a total volume of 20 µl. 3 µg nuclear extract were incubated in a buffer with a final concentration of 50 mmol/L KCl, 10 mmol/L Tris, pH 7.7, 5 mmol/L DTT, 1 mmol/L EDTA, 1 mmol/L MgCl₂, 5% glycerol, 0.03% Nonidet P-40 and 400 ng salmon testes DNA. Samples were incubated over night at 4°C. For supershift analyses, the anti-HIF-1 α antibody (BD Bioscience) was added to the samples 1 h before the samples were separated by native polyacrylamide gel electrophoresis on 5% polyacrylamide gels. Gels were dried and analyzed by phosphorimaging on a BAS 1000 phosphorimager (Fuji, Düsseldorf, Germany).

Immunofluorescence analyses

For immunofluorescence analyses, cells grown on glass plates inserted in culture dishes were fixed with freshly prepared 3.7% formaldehyde in PBS (pH 7.4) for 10 min, washed with PBS, permeabilized with 1% Triton X-100 for 5 min, and rinsed with PBS. Following blockade of nonspecific binding sites by incubation with 3% BSA in PBS for 30 min, the cells were incubated for 1 h with the anti-HIF-1 α antibody diluted 1:100 in 3% BSA/PBS. HIF-1 α antibody-binding was detected by incubation with a FITC-coupled secondary anti-mouse antibody (Dako, Hamburg, Germany) diluted 1:100 with 3% BSA in PBS. After repeated washing with PBS, the slides were mounted and analyzed by fluorescence microscopy (Axioplan 2000, Zeiss, Oberkochen, Germany). Fluorescence pictures were taken with identical exposure settings.

Quantification of AKT and ERK1/2 activation

AKT and ERK1/2 activation was studied by use of commercially available assays (FACE™ AKT Chemi and FACE™ ERK1/2 cell-based ELISAs, Active Motif, Rixensart, Belgium) according to the instructions given by the manufacturer. In brief, PTEC were seeded on two separate 96-well plates and grown to ~60% confluence. PI3K or MAPKK-1 inhibitors were added 30 min before the addition of IL-1 β . Immediately after IL-

Table 1. Effects of the kinase inhibitors LY 294002 (50 μ M) and PD 98059 (40 μ M) on basal and interleukin-1 β (IL-1 β) augmented production (6 h rates) of vascular endothelial growth factor (VEGF) by normoxic and hypoxic primary cultures of proximal tubular epithelial cells (PTEC). Mean $\pm$ SE of 6 normoxic and 4 hypoxic PTEC cultures from separate kidney donors. * $P < 0.05$ vs. respective cultures without kinase inhibitor (Tukey-Kramer test).

Agents		VEGF (ng/mg cell protein)	
		Normoxia (20% O ₂)	Hypoxia (3% O ₂)
None	--	5.1 $\pm$ 0.45	6.7 $\pm$ 0.37
	+ LY	3.1 $\pm$ 0.29*	4.0 $\pm$ 0.65*
	+ PD	3.2 $\pm$ 0.49*	5.0 $\pm$ 0.53
IL-1 β (300 pg/mL)	--	6.5 $\pm$ 0.6	8.7 $\pm$ 0.7
	+ LY	3.4 $\pm$ 0.5*	4.1 $\pm$ 0.35*
	+ PD	3.6 $\pm$ 0.65*	5.5 $\pm$ 0.65*

Fig. 1. Real time RT-PCR for VEGF₁₆₅ normalized to 60S ribosomal protein L28. Normoxia (20% O₂) is indicated by white bars, hypoxia (3% O₂) by gray bars. Cells were incubated for 24 h. Mean $\pm$ SE, the number of individually tested samples is given in the respective bars. * $P < 0.05$ was regarded as statistically significant (Tukey-Kramer test).

1 β was added the cells were exposed to different O₂ concentrations for 2 h. The cultures were fixed at the end of incubation. Total AKT (ERK1/2) and phospho-AKT (phospho-ERK1/2) were each assayed in triplicate using the anti-phospho-AKT (anti-phospho-ERK1/2) and anti-total AKT (anti-total ERK1/2) antibodies from the FACE kits. All data were corrected for cell number (determined through use of Crystal Violet).

Results

PTEC in culture produced large amounts of VEGF (Table 1). Hypoxia as well as IL-1 β treatment augmented VEGF production. To find out which intracellular signal transduction pathways are involved in these processes, cultures were preincubated with either a specific PI3K inhibitor (LY 294002) or a specific MAPKK-1 inhibitor (PD 98059). Inhibition of PI3K led to decreased VEGF concentrations in the supernatants of normoxic and hypoxic cultures both in absence or presence of IL-1 β .

MAPKK-1 inhibition reduced VEGF concentrations in normoxic and hypoxic IL-1 β treated cells. Without IL-1 β , VEGF production was reduced by PD 98059 only under normoxic conditions. Soluble VEGF receptors were not detectable in the media of normoxically or hypoxically maintained PTEC (24 h incubation periods) from 3 different kidney donors, providing evidence that free VEGF was measured by ELISA. The alterations in VEGF protein levels were accompanied in general by parallel alterations in VEGF₁₆₅ mRNA levels, which were quantified by RT-real time PCR (Fig. 1). However, the magnitudes of the effects exerted by LY 294002 or PD 98059 on VEGF₁₆₅ mRNA were somewhat different from that observed in protein levels, probably due to the different incubation periods of the two experiments. Nevertheless, changes in VEGF protein formation seemed to be based on altered mRNA amounts and only to a minor extent based on modulated translation efficiencies.

Fig. 2. A: Dose-dependent inhibition of nuclear HIF-1 α accumulation by LY 294002. HIF-1 α immunoblot of hypoxic PTEC (3% O₂) and normoxic PTEC (20% O₂) treated with IL-1 β (300 pg/mL) for 4 h and increasing doses of LY 294002. B: No inhibition of HIF-1 α accumulation by PD 98059. HIF-1 α immunoblot of normoxic PTEC (20% O₂) treated with IL-1 β (300 pg/mL) and hypoxic PTEC (3% O₂), incubated with increasing doses of PD 98059 for 4 h. Decreasing amounts of pERK1 and pERK2 demonstrate the inhibitory effect of PD 98059 on MAPKK-1. As loading control SP1 immunoblotting of the same membrane was performed.

Cytotoxicity assays by means of the MTT test were carried out on PTEC from two different patients with IL-1 β at 300 pg/mL, LY 294002 up to 100 μ M and PD 98059 up to 40 μ M (n = 8, each). None of the compounds was cytotoxic under the experimental conditions (6 h treatment; data not shown).

VEGF gene expression is stimulated by the transcription factor HIF-1. Therefore, changes in normoxically stabilized HIF-1 α protein levels after IL-1 β stimulation and PI3K or MAPKK-1 inhibition were studied. Western Blot analyses of nuclear extracts from normoxic PTEC revealed accumulation of HIF-1 α upon IL-1 β treatment (Fig. 2A/B). This accumulation was prevented by PI3K inhibition (Fig. 2A) but not by

Fig. 3. Electrophoretic mobility shift assay with nuclear extracts from PTEC for the detection of HIF-1 DNA-binding. Cells were incubated in normoxia (20% O₂) or hypoxia (3% O₂) for 4 h with or without IL-1 β . LY 294002 (50 μ M) treatment prevented HIF-1 DNA-binding in normoxia and hypoxia, whereas PD 98059 (40 μ M) did not. Signal intensities of the left (normoxic) and right (hypoxic) part of the figure are not directly comparable since the specific activities of the radiolabeled probes were different. Incubation with an anti-HIF-1 α -antibody confirmed specificity.

MAPKK-1 inhibition (Fig. 2B). Furthermore the effect of LY 294002 on hypoxically and IL-1 β induced nuclear HIF-1 α accumulation was dose dependent. In contrast, no dose dependency was observed with the MAPKK-1 inhibitor PD 98059 in respect to HIF-1 α amounts. The efficiency of MAPKK-1 inhibition was demonstrated by Western Blots with antibodies against phosphorylated ERK1 and ERK2 (Fig. 2B). Equal loading was verified by reprobing with an anti SP1 antibody.

Fig. 4. Immunofluorescence study of HIF-1 α in single PTEC. Cells were maintained under normoxic (20% O₂) or hypoxic (3% O₂) conditions in absence or presence of IL-1 β (300 pg/mL) and LY 294002 (50 μ M) for 4 h.

To demonstrate functional consequences of PI3K or MAPKK-1 inhibition, electrophoretic mobility shift assays were carried out. Even in normoxia slight HIF-1 DNA-binding is detectable which is a common phenomenon that is probably due to iron and/or ascorbate depletion during culture [25]. The normoxic HIF-1 DNA binding can further be increased after stimulation with IL-1 β (Fig. 3). Pretreatment with LY 294002 prevented HIF-1 DNA-binding either in normoxia with IL-1 β or hypoxia, whereas PD 98059 had only a minor effect. Specificity was demonstrated by supershift experiments where anti-HIF-1 α antibodies were added to the binding reactions.

When immunofluorescence techniques were applied, the inhibitory effect of LY 294002 could be visualized in single PTEC grown on coverslips (Fig. 4). Interestingly, IL-1 β induced HIF-1 α is not strictly captured in the nucleus, instead a distinct portion of fluorescence is detectable in the cytoplasm.

Since PI3K activity was required for HIF-1 α accumulation and VEGF production, and MAPKK-1 activity was necessary for VEGF production, the question arose whether hypoxia and IL-1 β activate PI3K and MAPKK-1. We studied as readout for PI3K activity the phosphorylation of the downstream kinase AKT and as readout for MAPKK-1 activity the phosphorylation of ERK1/2. The total amount of AKT remained unchanged on exposure to hypoxia or treatment with IL-1 β and the

PI3K inhibitor LY 294002 (Fig. 5). However, there was a clear increase in the proportion of phosphorylated AKT when the cells were challenged with hypoxia. This increase was blocked by LY 294002. Note that there was a basal level of phosphorylated AKT even under normoxic control conditions which was reduced by preincubation with LY 294002. IL-1 β had only a moderate additional effect on the level of phosphorylated AKT.

In contrast to its effect on AKT, hypoxia led to slightly elevated amounts of total ERK1/2. Stimulation with IL-1 β , preincubation with PD 98059 or a combination of both had no additional effect on total ERK1/2. The total amount of phosphorylated ERK1/2 remained unchanged in hypoxia, but in relation to the elevated total ERK1/2 phosphorylated ERK1/2 was reduced (Fig. 6). Preincubation with PD 98059 reduced phosphorylated ERK1/2 irrespective of the oxygen concentration or stimulation with IL-1 β .

Discussion

This report shows that the PI3K/AKT pathway is involved in the stabilization of HIF-1 α in human PTEC which is of particular interest since primary cells were used for this study. Treatment of the cultures with the specific PI3K inhibitor LY 294002 prevented the accumulation of HIF-1 α both in hypoxic PTEC and in

Fig. 5. Cell based ELISA study of AKT. Cells were maintained under normoxic (20% O₂, white bars) or hypoxic (3% O₂, gray bars) conditions in absence or presence of IL-1β (300 pg/mL) and LY 294002 (50 μM) for 2 h. Total AKT and phosphorylated AKT were detected in rapidly fixed cells by antibodies specific for AKT or phospho-AKT, subsequent incubation with a HRP-conjugated secondary antibody and chemiluminescence substrate. Light units were corrected for Crystal Violet staining. Top: total AKT; middle: p-AKT and bottom: p-AKT/AKT ratio. The negative controls (- prim antibody) were treated like positive controls but the primary antibody was omitted in the protocol. Mean + SE, n = 3.

Fig. 6. Cell based ELISA study of ERK1/2. Cells were maintained under normoxic (20% O₂, white bars) hypoxic (3% O₂, gray bars) conditions in absence or presence of IL-1β (300 pg/mL) and PD 98059 (40 μM) for 2 h. Total ERK1/2 and phosphorylated ERK1/2 were detected in rapidly fixed cells by antibodies specific for ERK1/2 or phospho-ERK1/2, subsequent incubation with a HRP-conjugated secondary antibody and colorimetric detection. Absorbance was corrected for Crystal Violet staining. Top: total ERK1/2; middle: p-ERK1/2 and bottom: p-ERK1/2 / ERK1/2 ratio. Mean + SE, n = 3.

normoxic PTEC challenged with IL-1 β . Previous studies utilized tumor cells with their potential mutational backgrounds. Oncogenic transformation is associated with the gain of growth-promoting oncoproteins and loss of function of growth-inhibiting tumor suppressor proteins. Both types of changes involve the PI3K signaling pathway [26]. The genes of the PI3K targets AKT I and AKT II are amplified in most types of human cancer. In addition, the tumor suppressor PTEN, an antagonist of PI3K, is often lost [26]. The occurrence of fundamental tumor cell type-specific responses has been also demonstrated by transient transfections resulting in constitutively active AKT which increases HIF-1 dependent transcription in glioblastoma cells, but not in hepatoma cells [16]. However, the PI3K pathway seems to participate in the hypoxic response in primary tubular epithelial cells.

HIF-1 α accumulation was not decreased in PTEC treated with the MAPKK-1 inhibitor PD 98059. In tumor cells, too, PD 98059 is without effect on the stabilization or the DNA-binding ability of HIF-1 α and HIF-1, respectively, while the trans-activation ability of HIF-1 is reduced [27]. The interaction of HIF with its co-activator p300 is disrupted on blockade of the MAPK kinase pathway. Phosphorylation of p300 or other proteins by MAPK or downstream kinases may be required for the interaction between p300 and the HIF-1 α terminal trans-activation domain [28]. If such an action is needed in primary cells too, this mechanism would provide a good explanation for our finding of reduced VEGF production on MAPKK-1 inhibition.

With respect to cells of renal origin, other investigators have shown that NO-donators increase HIF-1 α levels in cultures of the porcine renal tubular LLC-PK₁ [29] and green monkey kidney COS-1 cells [30]. In LLC-PK₁ cells the induction of HIF-1 DNA-binding by added S-nitrosoglutathione is decreased following PI3K inhibition [31]. The PI3K inhibitor wortmannin has been shown to prevent the activation of pAKT by hypoxia in the canine kidney epithelial cell line ATW [11]. Despite the fact that IL-1 β was not able to exert a statistical significant induction of pAKT in our experiments (as shown in the pAKT/AKT ratio in Fig. 5), LY 294002 prevented IL-1 β induced HIF-1 α accumulation and HIF-1 DNA-binding. Even moderate activation of PI3K by IL-1 β can probably influence HIF-1 α production or stability, indicating PI3K activity to be necessary but surely not sufficient for HIF-1 α accumulation. Taken together, there is convincing evidence to assume that PI3K is involved in the hypoxic, the IL-1 β and the NO induced accumulation of HIF-1 α in tubular cells. Eventually,

however, the mechanism by which IL-1 β causes HIF-1 α accumulation under normoxic conditions in human PTEC and other tissues is still not well understood. It is also noteworthy that IL-1 β does not induce HIF-1 α accumulation in LLC-PK₁ cells [32]. Importantly, a recent study has shown that TNF- α increases the translation of HIF-1 α resulting in an accumulation of HIF-1 α in its ubiquitinated form in LLC-PK₁ cells [8]. Because TNF- α increases HIF-1 DNA-binding and upregulates specific HIF-1 responsive genes in human hepatoma cells [4, 32] it is assumed that the ubiquitinated form of HIF-1 α is indeed transcriptionally active [8]. The observation of HIF-1 α in both cytosol and nucleus of normoxic IL-1 β treated PTEC (Fig. 4) suggests that IL-1 β , like TNF- α , may stimulate HIF-1 α translation resulting in the accumulation of large amounts of ubiquitinated HIF-1 α , with proteasomal degradation becoming the limiting process.

Human PTEC in primary culture secreted significant amounts of VEGF. The rate of VEGF production was significantly reduced on treatment with either the PI3K inhibitor LY 294002 or the MAPKK-1 inhibitor PD 98059. The function of VEGF derived from proximal tubular cells *in vivo* is an actual matter of debate. PCR, Western Blotting and immunohistochemistry all show that VEGF mRNA and protein expression is relatively low in normal human renal parenchyma [33, 34]. PTEC in culture secrete VEGF in equal amounts to the basolateral and the apical side [22]. Non-polarized secretion *in vivo* would correspond to the occurrence of VEGF in both interstitial and tubular fluids. In the interstitium VEGF may promote inflammation by increasing vascular permeability and attraction of leukocytes. Grone [35] has proposed that VEGF may elevate decreased renal blood flow and foster the regeneration of damaged endothelia in patients suffering from acute vascular obstruction. The administration of VEGF has proven to be beneficial for recovery in thrombotic microangiopathy [36], glomerulonephritis [37] and progressive renal failure following partial nephrectomy [38]. Kitamoto et al. [39] have studied the relationship between urinary VEGF and renal function in patients suffering from chronic renal failure. While VEGF excretion in urine increases as renal function decreases, VEGF excretion is independent of the serum level of VEGF, indicating that urinary VEGF derives from the kidney. Apparently, there is increased VEGF production in residual nephrons possibly caused by hypoxia and inflammation. A recent study indicates that both VEGF receptor types (R-1 and R-2) are expressed in normal rat renal tubules *in vivo* and that

VEGF protects rat tubular cells of the line NRK-52E from oxidative death [40]. From these findings it has been concluded that VEGF plays a role as an anti-apoptotic and proliferation-inducing growth factor on renal tubular epithelial cells [40]. We have detected VEGF R-1 or

R-2 mRNAs by RT-PCR only occasionally in PTEC cultured from different human kidney donors (unpublished observation). Thus, further evidence is required in favor of the concept that VEGF acts as a survival or growth factor for proximal tubules in humans.

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