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Inhibitors of oxygen sensing prolyl hydroxylases regulate nuclear localization of the transcription factors Smad2 and YAP/TAZ involved in CTGF synthesis

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ABSTRACT

Pharmacological inhibition of oxygen sensing prolyl hydroxylase domain enzymes (PHDs) has been shown to preserve renal structure and function in various models of kidney disease. Since transforming growth factor β-1 (TGFβ-1) is one of the major mediators of kidney injury, we investigated if inhibition of PHDs with subsequent stabilization of hypoxia inducible transcription factors (HIF) might interfere with TGFβ-1 signaling with special emphasis on its target gene connective tissue growth factor (CTGF).

Overnight incubation of human renal tubular cells, primary cells and cell lines, with the PDH inhibitor DMOG increased Smad3 expression, but barely affected Smad2. Both Smads were translocated into the nucleus upon activation of the cells with TGFβ-1. Interestingly, Smad3 nuclear localization was enhanced upon pretreatment of the cells with DMOG for several hours, whereas nuclear Smad2 was reduced. This differential localization was independent of Smad2/3 phosphorylation. Reduced nuclear Smad2 correlated with impaired CTGF secretion in DMOG-treated cells and transient downregulation of Smad2 interfered with TGFβ-1-induced CTGF synthesis. Furthermore, YAP was confirmed as indispensable transcription factor involved in CTGF synthesis. Nuclear localization of YAP and TAZ was reduced in DMOG-treated cells.

Our data thus provide evidence for DMOG-mediated reduction of CTGF expression by regulating the nuclear localization of the transcription factors Smad2, YAP and TAZ. Prolonged inhibition of PHDs was necessary to achieve alterations in cellular localization suggesting an indirect HIF-mediated effect. This mechanism might be extended to other transcription factors and target genes, and may thus represent a novel mechanism of negative regulation of gene expression by PHD inhibition.

1. Introduction

Prolyl hydroxylase domain enzymes (PHDs) are ferrous iron- and 2-oxoglutarate-dependent oxygenases which are sensitive to oxygen tension [1]. In preclinical studies pharmacological inhibition of PHDs has been shown to protect against organ injury in mice and rats [2–4]. Inhibition of PHDs, by reduced oxygen tension or small molecules, leads to stabilization of hypoxia inducible transcription factors (HIFs), which activate gene expression of multiple proteins involved in cell protection [5]. Thus far, HIF binding to HIF responsive elements (HRE) has been linked to enhanced mRNA synthesis [6]. Thus, proteins the synthesis of which is reduced upon treatment with PHD inhibitors or upon exposure to hypoxia most likely do not represent direct targets of HIF. Upregulation of repressors, modification of histones, or modulation of mRNA processing and translation are potential mechanisms which may contribute to negative regulation of protein expression by HIFs. Recently, HIF-induced non-coding RNAs were described as novel modulators of HIF signaling [7]. In many instances, however, the basis of negative regulation by HIF remains mainly unsolved.

In our earlier studies we observed down-regulation of the expression of the pro-fibrotic protein connective tissue growth factor (CTGF, also named CCN2) in human tubular epithelial cells either exposed to low oxygen tension or treated with the PHD inhibitor DMOG [8,9]. Kinetic data indicated that this down regulation was an indirect effect induced by HIF stabilization: albeit CTGF mRNA has a short half life of about 1.5 h [10], down regulation was only observed after more than 6 h [8,9]. Therefore, a direct effect of HIFs on the transcriptional machinery driving CTGF mRNA expression was unlikely. Moreover, regulation of CTGF proved to be cell type-specific as stabilization of HIF by inhibition of PHDs leads to increased synthesis of CTGF in endothelial cells

Abbreviations: CTGF, connective tissue growth factor; DMOG, dimethyloxalyl glycine; HIF, hypoxia inducible transcription factor; HRE, HIF responsive element; PHD, prolyl hydroxylase domain enzymes; TAZ, transcription co-activator with PDZ-binding motif; TGFβ, transforming growth factor β; YAP, Yes associated protein.

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[11,12]. In the context of kidney injury, CTGF was increased in HIF-1 knock out mice supporting the relevance of HIF-mediated interference with CTGF synthesis [13].

There is increasing evidence that epithelial cells lining the renal tubules are actively involved in tubulointerstitial injury [14]. Being exposed to urinary components as well as interstitial proteins these cells secrete inflammatory and profibrotic proteins. Transforming growth factor β-1 (TGFβ-1) is one of the major drivers of fibrotic diseases also targeting renal tubular epithelial cells [15]. TGFβ-1-mediated activation of Smads and MAK kinases leads to the secretion of profibrotic factors among them CTGF [16]. Treatment with DMOG proved to be protective in several animal models of renal injury [17–20]. However, the impact of pharmacological HIF stabilization on TGFβ-1 signaling is poorly understood. Therefore, it was the aim of the present study to investigate the impact of PHD inhibition on TGFβ signaling with special emphasis on the regulation of CTGF.

Regulatory Smads, Smad2 and Smad3, are immediately phosphorylated and activated upon binding of TGFβ-1 to its receptor [21]. The rapid phosphorylation is followed by a comparatively slow translocation to the nucleus which reaches a maximum after about 45 min and lasts for several hours providing the basis for the kinetics of target protein induction [22]. Within the nucleus activated Smads interact with other DNA-associated transcription factors. YAP (Yes associated protein) and TAZ (transcription co-activator with PDZ-binding motif) have been shown to form complexes with activated Smad2/3 [23] YAP/TAZ themselves shuttle between the nuclear and cytosolic compartment and may thus be binding partners of Smads in both compartments [24,25]. CTGF is one of the genes which are regulated by both, TGFβ signaling and YAP/TAZ signaling. Complex formation of YAP, TEAD4, Smad3 and p300 was detected in the promoter region of CTGF [26,27]. HIF and TAZ were shown to interact at the CTGF promoter in tumor cells which upregulate CTGF upon exposure to hypoxia [24,28]. Therefore it was of particular interest to analyze a potential role of YAP/TAZ in a cellular system, where HIF activation leads to downregulation of CTGF rather than to upregulation.

As an in vitro model system related to human tubulointerstitial diseases we used primary tubular epithelial cells obtained from healthy parts of tumor nephrectomies of adult patients. Within this study, we describe DMOG-mediated regulation of the nuclear localization of specific transcription factors, namely Smad2 and YAP/TAZ, as a novel regulatory principle underlying negative regulation of gene expression by PHD inhibition.

2. Materials and methods

2.1. Materials

DMEM/Ham's F12 medium was purchased from Biochrom AG (Berlin, Germany), DMEM medium and Hank's BSS from PAA Laboratories (Coelbe, Germany), insulin-transferrin-selenium supplement from Gibco (Karlsruhe, Germany), fetal calf serum (FCS) from PAN Biotech (Aidenbach, Germany), triiodothyronine from Fluka (Buchs, Switzerland), hydrocortisone from Sigma (Munich, Germany), epidermal growth factor from PeproTech (Hamburg, Germany), TGF-β1 from tebu-bio (Offenbach, Germany), and dimethyloxalyl glycine (DMOG) from Cayman Chemical.

2.2. Cell culture

Human primary tubular epithelial cells (hPTEC) were isolated from renal cortical tissues collected from healthy parts of tumor nephrectomies [16]. Isolation of human cells was approved by the local ethics committee and written consent was obtained from all donors.

In brief, human renal cortex tissue was minced and digested with collagenase type II (Gibco) and DNase I grade II (Roche Diagnostics) for 60 min. Cell suspension was sieved through 100 μm and 70 μm meshes (Cell Strainer, Falcon). After a washing step with HBSS, cells were seeded in epithelial cell selective medium (DMEM/Ham's F12 medium with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, insulin-transferrin-selenium supplement, 10 ng/ml epidermal growth factor, 36 ng/ml hydrocortisone and 4 pg/ml triiodothyronine) in the presence of 0.5% FCS. After 1–2 days, medium was replaced by FCS-free medium. Cells were subcultured by application of trypsin. For experiments, hPTEC were seeded in medium containing 2.5% FCS to facilitate cell attachment. After 24 h, medium was replaced by FCS-free epithelial cell selective medium. Cells of proximal and distal tubular origin were separated by their differential adherence to cell culture plastic. Trypsinization for 3 min resulted in a culture enriched in proximal cells (about 60% N-cadherin positive cells), while the remaining cells were over 90% E-cadherin positive representing cells of distal tubular origin. Data of both cell types were combined when no significant difference was detected between cell populations. Distal hPTEC were polarized by culture on permeable transwell inserts (Millicell PCF, Millipore) as described previously [16]. In all experiments, hPTEC at passages 1–3 were used.

HKC-8 cells were kindly provided by L. Racusen (Baltimore, MD) [29]. Cells were recloned by limited dilution and cultured as described previously [9]. HK-2 cells were obtained from the ATCC and cultured as described previously [9].

2.3. Cell fractionation

Separation of nuclear and cytosolic fractions was performed essentially as described before [11], by lysing of the cells in buffer containing 10 mM Hepes, pH 7.9, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.66% NP-40 and protease inhibitors. After centrifugation (5 min, 13,000 rpm) the washed nuclear pellet was resuspended in buffer containing 10 mM Hepes, pH 7.9, 1 mM EDTA, 400 mM NaCl, 1 mM DTT and protease inhibitors. After centrifugation (5 min, 13,000 rpm) the supernatant contained soluble nuclear proteins. An example of nuclear extraction is shown as Supplementary Fig. 1A. Proteins detected in nuclear fractions by Western blotting were related to Lamin A/C as nuclear marker protein.

2.4. Western blot analysis

Proteins in cell culture supernatants were precipitated with 80% ethanol. SDS polyacrylamide gel electrophoresis was performed by standard techniques and Western blot analyses were performed essentially as described before [9] using the following antibodies: CTGF (SC-14939), YAP-TAZ (SC-101199), vinculin (SC-5573), and Smad 2/3 (SC-376928) from Santa Cruz; phospho(Ser 465/467) Smad2 (#3108), phospho(Ser 423/425) Smad3 (#9520), phospho(Ser 127) YAP (#13008), (ERK1/2 (#9107), and Lamin A/C (#2032) from Cell Signaling. The immunoreactive bands were quantified by densitometry using the luminescent image analyzer (LAS-1000 Image Analyzer, Fujifilm, Berlin, Germany) and AIDA 4.15 image analyzer software (Raytest, Berlin, Germany) or Odyssey infrared imaging system (Li-Cor, Biosciences). For quantification purposes the intensity of specific bands was related to the intensity of control bands. Lamin A/C was used for nuclear preparations, vinculin for homogenates or cytosolic preparations. To compare different blots, fold induction was calculated as indicated in the legends. All data are presented as mean ± SD of n different experiments with cells obtained from at least 3 different donors.

2.5. Real time RT-PCR

RNA expression of CTGF was analyzed as described previously [9] using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Relative RNA was calculated using the delta–delta ct method.